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Kinetic Studies on Rat Brain Monoamine Oxidase

Z. HUSZTI

Department of Pharmacology, Research Institute for Pharmaceutical Chemistry, Budapest, Hungary

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SUMMARY

Enzyme-substrate and enzyme-inhibitor interactions were investigated for monoamine oxidase, using dialyzed rat brain homogenates. The apparent and "true" Michaelis constants for tryptamine, 5-hydroxytryptamine, tyramine, norepinephrine, and 3,4-dimethoxyphenylethylamine were determined. The influence of pH on substrate concentration-velocity curves and on apparent Michaelis constants was studied in detail. The mechanism of inhibition of monoamine oxidase by 1-*m*-aminophenyl-2-cyclopropylaminoethanol (AB-15) was also examined.

Results indicate the following characteristics of substrate and inhibitor interactions with monoamine oxidase. (a) Ionized groups are involved in the binding of different amines. (b) Multiple binding sites may exist on the enzyme; however, substrate binding at some sites may lead to the formation of inactive complexes. (c) Different ionizing groups affect the binding of different amines. (d) AB-15 binds to the enzyme reversibly and inhibits activity both *in vitro* and *in vivo*. The potency and type of inhibition depend on the amine used as substrate.

INTRODUCTION

Since the initial demonstration of the enzymatic oxidation of tyramine (1), dopamine, and norepinephrine (2), the properties and localization of monoamine oxidase have been studied in detail (3–7). Despite intensive investigations into the properties of rat brain monoamine oxidase [O_2 oxidoreductase (deaminating), EC 1.4.3.4] (8, 9), there is insufficient information about the mechanism of its interaction with substrates. This is due partly to difficulties encountered in the purification of this enzyme. However, kinetic data obtained from experiments on tissue preparations, when carefully controlled and properly interpreted, can yield useful information.

The present studies, performed on dialyzed rat brain homogenates, are a continuation of efforts to obtain more information on enzyme-substrate and enzyme-inhibitor complexes of monoamine oxidase. The enzyme-substrate interaction studies were carried out with tryptamine, 5-hydroxytryptamine, tyramine, norepinephrine, and a metabolite of dopamine, 3,4-dimethoxyphenylethylamine (10), as substrates. Inhibition studies were conducted with a "substrate-specific" inhibitor of monoamine oxidase, 1-*m*-aminophenyl-2-cyclopropylaminoethanol (11).

MATERIALS AND METHODS

The oxidation of tryptamine, 5-hydroxytryptamine, and norepinephrine was measured according to Lagnado and Sourkes (12), using nitro blue tetrazolium chloride as hydrogen acceptor. Assay mixtures routinely contained 0.1–100.0 μmoles of tryptamine, 5-hydroxytryptamine, or norepinephrine; 0.3 ml of brain homogenate (equivalent to 6 mg of protein and dialyzed against 0.001 M

phosphate buffer, pH 7.6, at 4° for 24 hr); 1.5 μmoles of nitro blue tetrazolium chloride; 20 μmoles of sodium sulfate; 200 μmoles of sodium phosphate buffer, pH 7.6; and varied amounts of AB-15[1] in a total volume of 3 ml. Samples were incubated for 15 min at 38°. Oxidation was stopped by adding 5 ml of ice-cold 1-butanol (previously washed with 0.1 N NaOH, 0.1 N HCl, and distilled water), and after vigorous shaking the mixture was allowed to stand at 4° for at least 30 min. The two phases were separated by centrifugation at 4°, and the absorbance of the organic phase was measured at 540 nm.

Standard curves were determined according to Nachlas *et al.* (13), with NADH as reducing agent. Nitro blue tetrazolium solution (1 ml containing 0.125, 0.250, 0.375, 0.500, or 0.750 mM) was then added to the reaction mixture, which contained 10 μmoles of NADH, 20 μmoles of sodium sulfate (instead of phenazine methosulfate), dialyzed brain homogenate (6–7 mg of protein), and 200 μmoles of sodium phosphate buffer, pH 7.6, in a volume of 2 ml. Each mixture was incubated at 38° for 30 min. Diformazan, formed during the reaction, was extracted into ice-cold 1-butanol, and the absorbance at 540 nm was measured. A plot of absorbance against nitro blue tetrazolium concentration served as a calibration curve. One micromole of reduced nitro blue tetrazolium was taken to correspond to 1 μmole of aldehyde formed or 1 μmole of substrate oxidized. An increase of 0.1 absorbance unit at 540 nm was judged equivalent to 75 nmoles of enzyme activity.

No "lag period" was observed with various concentrations of tryptamine and 5-hydroxytryptamine as substrates when the rate of oxidation was determined at 5, 10, 15, 30, and 60 min. Also, under the above conditions, the reaction rate was linear for 60 min at norepinephrine concentrations between 0.1 and 3 mM. With other substrates, a 10–20-min "lag period" occurred, as described by Lagnado and Sourkes (12) and Weissbach *et al.* (14).

[1] The abbreviations used are: AB-15, 1-*m*-aminophenyl-2-cyclopropylaminoethanol dihydrochloride; DMPEA, 3,4-dimethoxyphenylethylamine.

The rate of enzymatic oxidation, Q, is expressed as micromoles of substrate oxidized per hour per milligram of protein. For the substrates 5-hydroxytryptamine and tryptamine, the Q values obtained by the above procedure (0.356 ± 0.015 for 5-hydroxytryptamine and 0.300 ± 0.012 for tryptamine) agreed well with those obtained by oxygen consumption measurements using the Warburg method (0.320 ± 0.061 and 0.290 ± 0.058, respectively) and also the technique of Udenfriend and Cooper (15) (Q = 0.330 ± 0.019 for 5-hydroxytryptamine). For norepinephrine the observed reaction rate (0.100 ± 0.011) was somewhat lower than the values obtained by the oxygen consumption measurements (0.140 ± 0.020).

The enzymatic oxidation of norepinephrine was also determined by following its disappearance by the fluorometric procedure of Shore and Olin (16).

The oxidation of tyramine was measured according to Udenfriend *et al.* (17), and the oxidation of DMPEA, by the method of Kapeller-Adler (18). The latter method was modified slightly by using DMPEA instead of histamine and increasing the amount of the indigo disulfonate 4-fold.

In the presence of 5-hydroxytryptamine the concentration of tyramine could not be determined by the method of Udenfriend *et al.* because of interference from artifactual products of 5-hydroxytryptamine. To determine 5-hydroxytryptamine and tyramine simultaneously, the colored solutions containing the products of both substrates were shaken with 5 ml of a 1:1 mixture of 1-butanol and heptane that had been washed sequentially with 0.1 N NaOH, 0.1 N HCl, and distilled water. The two phases were separated by centrifugation, and the absorbance of the nonpolar and aqueous phases determined at 450 and 540 nm, respectively. The nonpolar phase contained the product of tyramine, and the aqueous phase, that of 5-hydroxytryptamine. Standard curves for both compounds were almost identical with that obtained by the method of Udenfriend *et al.*

Enzyme inhibition studies *in vitro* were carried out by adding AB-15 to the reaction

mixtures to achieve inhibitor concentrations between 10^{-7} and 10^{-3} M.

1-*m*-Aminophenyl-2-cyclopropylaminoethanol was synthesized by Dr. A. Hajós of this Institute and was subjected to complete analysis to confirm its identity. The white, crystalline compound, as the dihydrochloride salt, was dissolved in water, and the solution was adjusted to pH 7. Inhibitor solutions were always freshly prepared and incubated with the homogenate or the mitochondrial preparation for 15 min before the enzymatic reaction. Inhibition produced by AB-15 was greatest after 15 min of prior incubation. After incubation of the enzyme for 5, 10, and 15 min with 10^{-5} M AB-15, inhibition of the 5-hydroxytryptamine oxidation rate was 48%, 66%, and 77%, respectively. Longer incubation periods of 30 and 60 min did not yield further inhibition. AB-15 did not interfere with analytical measurements of the enzyme activity.

For inhibition studies *in vivo*, AB-15 was given orally to 160–180-g male rats 16 hr prior to decapitation. Brain tissue was homogenized in 2 volumes of 0.25 M sucrose at 4°, using a Biomix cell disintegrator. The homogenates were dialyzed against 0.001 M sodium phosphate buffer, pH 7, for 24 hr. The protein content of the homogenates was determined according to Palladin (19).

Mitochondria were prepared by the method of Løvtrup and Zellander (20). Brain tissue was homogenized in 0.44 M sucrose containing 0.001 M EDTA. The solution was adjusted to pH 7 with the addition of 0.1 M Na_2HPO_4. After washing in sucrose, followed by 0.01 M sodium phosphate buffer, pH 7.6, the mitochondria were suspended in 0.01 M sodium phosphate buffer, pH 7.6. The protein content was adjusted to 8–10 mg/ml, and samples were frozen at −20°.

RESULTS

Enzyme-Substrate Interactions

Effect of pH on velocities and Michaelis constants. The apparent maximal reaction rate, $\tilde{V}$ and Michaelis-Menten constant, $\tilde{K}_m$, were determined from a graphical and statistical analysis (21) of Lineweaver-Burk plots (22). The pH dependence of these quantities is illustrated in Figs. 1 and 2, respectively. Direct determination of the apparent dissociation constant of the enzyme-substrate complex (K_s) was not possible; however, it will be assumed that $K_s = K_m$. According to Dixon (23), on plots of $p\tilde{K}_m$ as a function of pH, the abscissa value corresponding to the intersection of lines with slopes of unity and zero will yield pK_e and pK_s, the negative logarithms of the respective enzyme and substrate dissociation constants. In Fig. 1 the estimated values are: tryptamine, 7.2 (slopes +1, 0); 5-

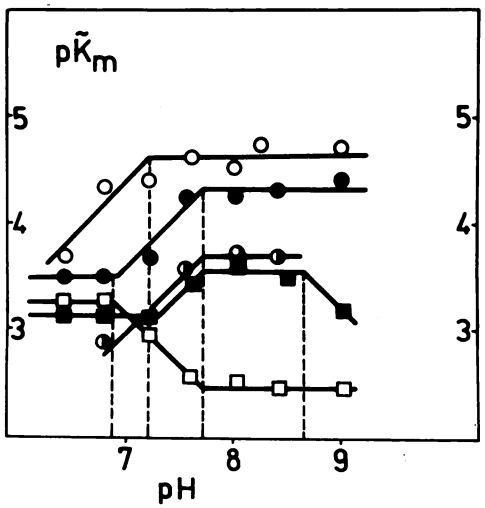

FIG. 1. *Effect of pH on apparent Michaelis constants, $\tilde{K}_m$, determined from oxidation of different amines*

○, tryptamine; ●, 5-hydroxytryptamine; ◑, DMPEA; ■, norepinephrine; □, tyramine. The rates of enzymatic oxidation of 5-hydroxytryptamine, tryptamine, and norepinephrine were measured by the Lagnado-Sourkes method (12). The reaction mixtures were incubated for 15 min with 5-hydroxytryptamine and tryptamine as substrates, and for 60 min with norepinephrine as substrate. The reactions were linear during this period. The rate of DMPEA oxidation was measured by the modified Kapeller-Adler method. The mixtures were incubated for 60 min, during which time the rates were linear. The tyramine rate measurements were carried out according to Udenfriend *et al.* (17). The mixtures were incubated for 30 min, and the rates were linear during this period. Each point represents the mean of five different graphical measurements. The standard errors of the determinations were within the range of ±5%.

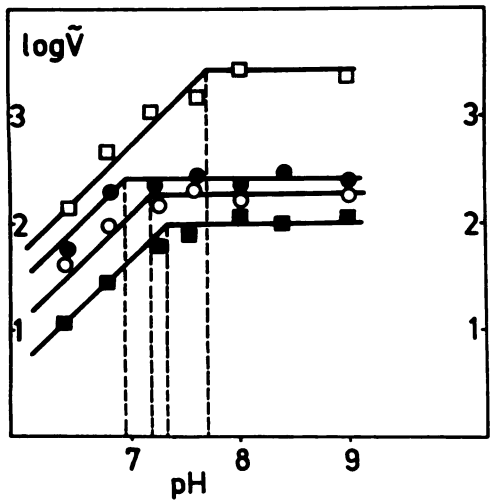

FIG. 2. *Effect of pH on apparent maximal velocities, $\tilde{V}$*

The $\tilde{V}$ values were defined as micromoles of substrate oxidized per milligram of protein per hour. □, tyramine; ■, norepinephrine; ○, tryptamine; ●, 5-hydroxytryptamine. The maximal velocities were obtained from Lineweaver-Burk plots as described in the text. Each point represents the mean of five different measurements. The standard errors of the determinations were within the range of ±5%.

hydroxytryptamine, 7.7 (slopes +1, 0); DMPEA, 7.7 (slopes +1, 0); norepinephrine, 7.7 (slopes +1, 0) and 8.7 (slopes 0, −1); tyramine, 6.9 (slopes 0, −1).

The ionizing groups with pK 7.2 and 7.7 must form a positive charge (or lose a negative charge) below pH 7.2 or 7.7 to give a slope of +1 on the $p\tilde{K}_m$–pH curves. The groups with pK 6.9 for tyramine and 8.7 for norepinephrine must gain a negative charge (or lose a poroton) above pH 6.9 or 8.7 to give a slope of −1. Since the measured dissociation constants (pK_{a2}) for the amine substrates (24, 25) (Table 1) do not correspond with those predicted above, the pK values derived from the kinetic study must represent ionizing groups on the enzyme rather than on the substrate.

An upward bend in the $p\tilde{K}_m$–pH curves, representing ionization on the enzyme-substrate complex, yields pK_{es} values of 6.9, 7.3, and 7.7 for complexes of the enzyme with 5-hydroxytryptamine, norepinephrine, and tyramine, respectively.

TABLE 1

Dissociation constants and "true" Michaelis constants of different amines

The "true" Michaelis constants were calculated as described in the text.

Amines	pK_{a2}	K_m
		$M \times 10^{-4}$
5-Hydroxytryptamine	10.0[a]	0.6 ± 0.02
Tryptamine	10.2[a]	0.2 ± 0.04
Tyramine	10.8[b]	5.0 ± 0.05
Norepinephrine	9.8[b]	3.2 ± 0.08
3,4-Dimethoxyphenyl-ethylamine	9.7	2.0 ± 0.04

[a] Data from Lewis (24).
[b] Data from Perrin (25).

The "true" Michaelis constants (23), which are independent of pH, are derived from these $p\tilde{K}_m$–pH plots, using the following equation (26):

$$\tilde{K}_m = \frac{f_e f_s}{f_{es}} K_m$$

where f_e, f_s, and f_{es} are the pH functions of E, S, and ES (23). Over the pH range studied the Dixon expressions give constant values for K_m only if the pH dependence for the substrate is neglected and the equation is applied individually to each curve. For 5-hydroxytryptamine and tryptamine,

$$p\tilde{K}_m = pK_m - \log\left(1 + \frac{H^+}{K_e}\right) + \log\left(1 + \frac{H^+}{K_{es}}\right)$$

where K_e and K_{es} are the dissociation constants of the enzyme and enzyme-substrate complex. For tyramine,

$$p\tilde{K}_m = pK_m - \log\left(1 + \frac{K_e}{H^+}\right) + \log\left(1 + \frac{K_{es}}{H^+}\right)$$

where K_e and K_{es} have the same meaning as above, but the enzyme is in the protonated form.

In the case of norephinephrine, two ioniz-

ing groups on the enzyme are required; thus

$$pK_m = pK_m - \log\left(1 + \frac{H^+}{K_e'}\right) - \log\left(1 + \frac{K_e''}{H^+}\right) + \log\left(1 + \frac{H^+}{K_{es}}\right)$$

where Ke' and Ke'' are dissociation constants. Figure 3 and Table 1 show the values for K_m obtained experimentally over the pH ranges studied.

Effect of pH on substrate concentration-activity curves. At relatively low pH the effect of substrate concentration on initial velocity obeys the Michaelis-Menten equation (Fig. 4), while at high pH the reaction slows as the substrate concentration increases in the case of tryptamine, norepinephrine and DMPEA, 5-Hydroxytryptamine exhibits Michaelis-Menten kinetics at both low and high pH values (Fig. 5).

These results indicate that inhibition is dependent on substrate affinity at various pH values (Table 2), and suggest additional binding of the substrate at a site on the enzyme that is not involved in the active complexes (27).

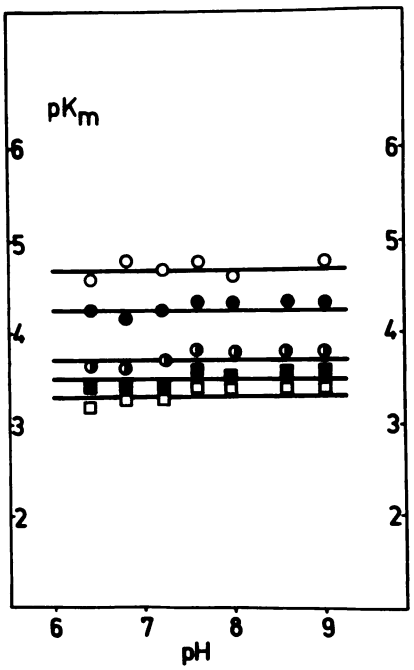

FIG. 3. *Effect of pH on "true" Michaelis constants, K_m*

○, tryptamine; ●, 5-hydroxytryptamine; ◑, DMPEA; ■, norepinephrine; □, tyramine. The "true" Michaelis constants were calculated as described in the text.

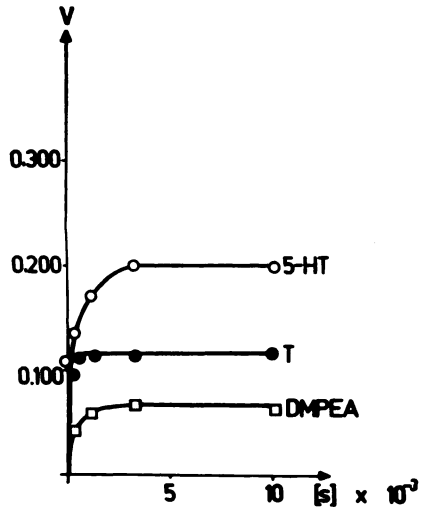

FIG. 4. *Effect of substrate concentration on rate of enzymatic oxidation of tryptamine (T), 5-hydroxytryptamine (5-HT), and 3,4-dimethoxyphenylethylamine (DMPEA) at pH 6.8*

v = micromoles of substrate transformed per milligram of protein per hour.

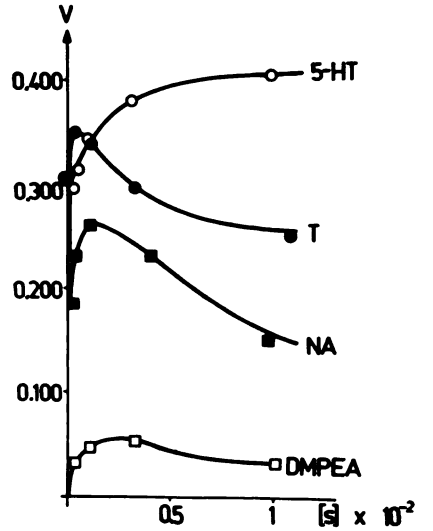

FIG. 5. *Effect of substrate concentration on rate of enzymatic oxidation of tryptamine (T), 5-hydroxytryptamine (5-HT), norepinephrine (NA), and 3,4-dimethoxyphenylethylamine (DMPEA) at pH 8.2*

v = micromoles of substrate transformed per milligram of protein per hour.

Measurements with mixed substrates. The mixed-substrate method (28) was used to determine whether oxidation took place on the same or different active sites. Rates of oxidation of equimolar mixtures of 5-hydroxytryptamine and tryptamine, 5-hydroxytryptamine and tyramine, and 5-hydroxytryptamine and norepinephrine were compared with those determined for these amines singly (Table 3). 5-Hydroxytryptamine plus tryptamine yielded the same reaction rate as that of tryptamine alone. The reaction with 5-hydroxytryptamine plus tyramine was slower than the sum of the rates observed with these amines separately, because each reaction was inhibited by the presence of the other substrate. 5-Hydroxytryptamine plus norepinephrine yielded only the sum of the reactions rates that each substrate would have produced separately.

Enzyme-Inhibitor Interactions

AB-15, a non-hydrazine inhibitor of monoamine oxidase, was selected for the enzyme-inhibitor studies. Its structure is shown in Fig. 6.

Mechanism of inhibition; reactivation of inhibited enzyme by washing. The extent of reversibility of the inhibition produced by AB-15 was examined to determine the type

TABLE 2

Apparent Michaelis constants for tryptamine, norepinephrine, and 3,4-dimethoxyphenylethylamine

The statistical values were calculated by Wilkinson's method (21).

Amine	$\tilde{K}_m$			
	pH 6.8		pH 8.2	
	Graphical	Statistical	Graphical	Statistical
	M	*M*	*M*	*M*
Tryptamine	4.7×10^{-5}	$4.0 \pm 0.0 \times 10^{-5}$	2.0×10^{-5}	$1.0 \pm 0.2 \times 10^{-5}$
Norepinephrine	8.1×10^{-4}	$9.0 \pm 0.3 \times 10^{-4}$	3.0×10^{-4}	$2.9 \pm 0.5 \times 10^{-4}$
DMPEA	1.5×10^{-3}	$1.4 \pm 0.2 \times 10^{-3}$	1.6×10^{-4}	$2.1 \pm 0.3 \times 10^{-4}$

TABLE 3

Mixed-substrate kinetics

The two substrates were added simultaneously to the reaction mixture. Oxidation rates were measured according to Udenfriend *et al.* (15, 17) for 5-hydroxytryptamine and tyramine, and according to Lagnado and Sourkes (12) for tryptamine. Norepinephrine determinations were performed fluorometrically (16). For the simultaneous reactions of 5-hydroxytryptamine and tyramine, the methods of Udenfriend *et al.* were modified as described in the text. The amounts of tryptamine oxidized in the "simultaneous" reactions were calculated from the total amount of diformazan formed.

Substrates (0.6 mM each)	Individual reactions		Simultaneous reactions (A + B)		Simultaneous reactions (A + B)			
	A	B	A	B	A	B	Observed	Calculated
	μmole substrate changed/30 min				%	%	%	%
A. Tryptamine B. 5-Hydroxytryptamine	0.810	0.900	0.772	0.08	95	9	91	200
A. 5-Hydroxytryptamine B. Tyramine	0.920	0.800	0.500	0.540	54	67	121	200
A. 5-Hydroxytryptamine B. Norepinephrine	0.900	0.380	0.855	0.380	95	100	193	200

of binding in the enzyme-inhibitor complex. As direct determinations of inhibitor concentrations were not possible, enzyme activity was measured both before and after the removal of inhibitor. Portions of the mitochondrial preparation were incubated at 38° for 20 min in the presence and absence of AB-15. After incubation the monoamine oxidase activity of each preparation was assayed. To remove the inhibitor bound to the enzyme, mitochondrial preparations were washed with the original volume of 0.1 M sodium phosphate buffer; the enzymatic activity of the preparation was assayed again and compared with the previous value. This procedure was repeated until complete removal of the inhibitor was achieved (Table 4).

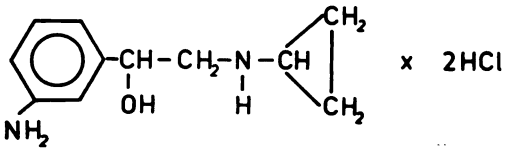

FIG. 6. *Chemical structure of AB-15*

Slow but complete reversibility of the inhibition was evident in all cases; however, slight differences in the restoration of activity were apparent when different amines were used as substrates. The inhibited enzyme required more washings (six) with tyramine as the substrate than with norepinephrine or tryptamine (once each), DMPEA (twice), or 5-hydroxytryptamine (three washings).

Kinetics of inhibition. Because the usual inhibition kinetics (in which the inhibitor is assumed to reach equilibrium with the enzyme) could be applied to AB-15, Lineweaver-Burk plots were constructed to ascertain the type of inhibition (Figs. 7–11).

Characteristic of these plots were variation of the $1/v$ intercepts with the AB-15 concentration. The diagrams of the inhibited reactions with tyramine and DMPEA, which showed an effect only on velocity, indicate noncompetitive inhibition, while those for

TABLE 4

Reversal of inhibition produced by AB-15 by removal of inhibitor

Initial concentration of AB-15	Treatment		Tyramine[a]		5-Hydroxytryptamine[b]		Enzyme activity (inhibition)					
	AB-15	No. of washings					Tryptamine[b]		Norepinephrine[b]		DMPEA[c]	
μmole/mg protein			*μmole/incubation period*									
0	−	0	0.735	(100%)	0.312	(100%)	0.315	(100%)	0.150	(100%)	0.700	(100%)
0.8	+	0	0.147	(20%)	0.094	(30%)	0.182	(58%)	0.075	(50%)	0.400	(57%)
0	−	2	0.730	(100%)	0.206	(100%)	0.243	(100%)	0.107	(100%)	0.450	(100%)
0.8	+	2	0.220	(30%)	0.108	(50%)	0.222	(92%)	0.107	(100%)	0.440	(98%)
0	−	3	0.630	(100%)	0.105	(100%)	0.165	(100%)	0.097	(100%)	0.400	(100%)
0.8	+	3	0.315	(45%)	0.105	(100%)	0.165	(100%)	0.097	(100%)	0.400	(100%)
0	−	6	0.360	(100%)								
0.8	+	6	0.360	(100%)								

[a] Oxidation rates were measured according to Udenfriend *et al.* (17), the substrate concentration was 0.4 mM, the protein content of the reaction mixture was 3 mg, and the mixtures were incubated for 60 min. The standard error of the measurements was within the range of ±5%.

[b] Oxidation rates were measured according to Lagnado and Sourkes (12), the substrate concentration was 1 mM, the protein content of the reaction mixtures was 4 mg, and the mixtures were incubated for 10 min with 5-hydroxytryptamine and tryptamine, and for 30 min with norepinephrine. The standard error of the measurement was within the range of ±3%.

[c] Oxidation rates were measured according to Kapeller-Adler (18), the substrate concentration was 10 mM, the protein content of the reaction mixture was 10 mg, and the mixtures were incubated for 60 min. The standard error of the measurements was within the range of ±5%.

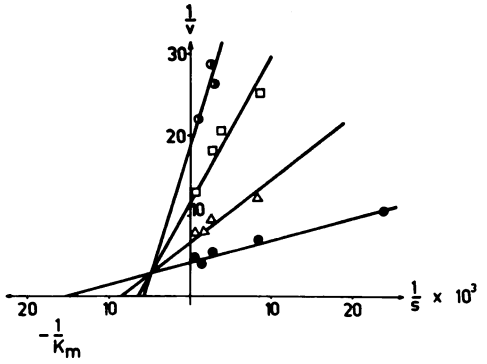

FIG. 7. *Lineweaver-Burk plot for 5-hydroxytryptamine (5-HT)*

v = micromoles of 5-hydroxytryptamine transformed per milligram of protein per hour. The inhibitor was incubated with the reaction mixture in the absence of substrate for 15 min and another 15 min after addition of the substrate. The reactions were linear during the latter 15 min. ●, 5-hydroxytryptamine; △, 3.7 × 10^{-6}M AB-15; □, 1.1 × 10^{-5}M AB-5; ◑, 3.3 × 10^{-5}M AB-15.

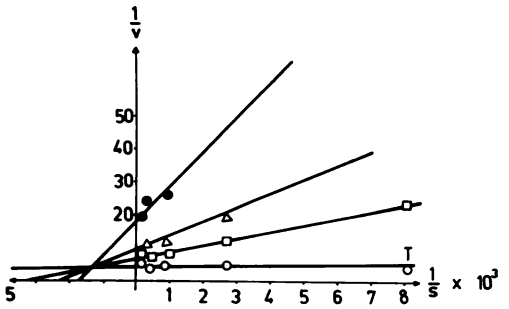

FIG. 8. *Lineweaver-Burk plot for tryptamine* (T)

v = micromoles of tryptamine transformed per milligram of protein per hour. ○, tryptamine; □, 1.1 × 10^{-3}M AB-15; △, 3.3 × 10^{-3}M AB-15; ●, 1.0 × 10^{-2}M AB-15.

5-hydroxytryptamine and tryptamine, which showed an effect on both velocity and K_m, represent a "mixed" type of inhibition, i.e., a mixture of competitive and noncompetitive effects. At low pH values the inhibition was intermediate between competitive and noncompetitive for 5-hydroxytryptamine, and was predominantly competitive for tryptamine; at pH 8.2 inhibition was competitive with both these amines.

Lineweaver-Burk plots, however, cannot provide sufficient information on the enzyme-inhibitor interaction or on the breakdown of the *EIS* complex, and therefore the inhibition was also analyzed according to Dixon (29). Reciprocals of the rates of the inhibited reactions obtained with two different substrate concentrations were plotted against the inhibitor concentrations, and the intersections of these straight lines were deter-

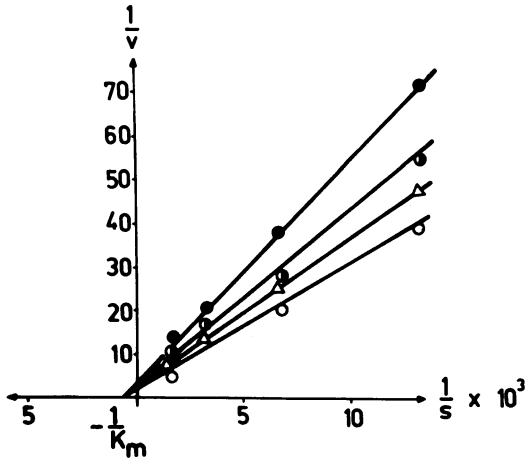

FIG. 9. *Lineweaver-Burk plot for tyramine (Tyr)*

v = micromoles of tyramine transformed per milligram of protein per hour. The inhibitor was incubated with the reaction mixture in the absence of substrate for 15 min and another 30 min after addition of the substrate. The reaction was linear during the latter 30 min. ○, tyramine; △, 1.1 × 10^{-5}M AB-15; ◑, 3.3 × 10^{-5}M AB-15; ●, 1.0 × 10^{-4}M AB-15.

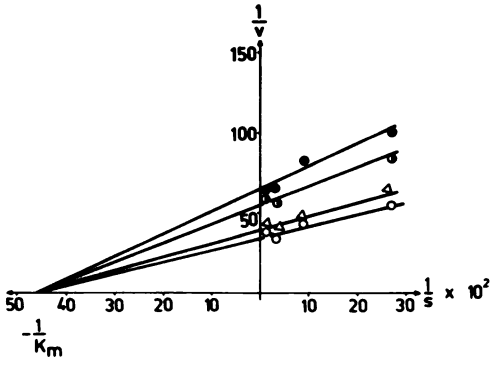

FIG. 10. *Lineweaver-Burk plot for norepinephrine (NA)*

v = micromoles of norepinephrine transformed per milligram of protein per hour. ○, norepinephrine; △, 1.1 × 10^{-4}M AB-15; ◑, 3.3 × 10^{-4}M AB-15; ●, 1.1 × 10^{-3}M AB-15.

mined. Only in the case of DMPEA were straight lines obtained. Their point of intersection represents the negative value of the apparent enzyme-inhibitor constant ($\bar{K}_i$) for AB-15 (Fig. 12).

In the other cases the lines curved, indicating that inhibition did not increase indefinitely with increasing inhibitor concentration. The designations of partially competitive and partially noncompetitive inhibitors are valid for these compounds.

Apparent constants of enzyme-inhibitor complexes. The apparent constants of the enzyme-inhibitor complexes, $\bar{K}_i$, could not be derived directly from the Dixon plots in the cases of partial inhibition, and so $\bar{K}_i$ values were determined by calculation. The modified forms of the Lineweaver-Burk equations, which were applied by Dixon in such cases (30), were used. For partially competitive inhibition,

$$\bar{K}_i = \frac{i}{K_p/K_m - 1}\left(1 - \frac{K_p}{K_m'}\right)$$

For partially noncompetitive inhibition,

$$\bar{K}_i = \frac{i}{V/V_p - 1}\left(1 - \frac{V'}{V_p}\right)$$

K_m' is the Michaelis constant, and V' is the velocity in the presence of excess inhibitor and substrate. K_p, K_m, and V_p were derived from the Lineweaver-Burk plots, and K_m' and V' were determined by extrapolating V_p and K_p to infinite inhibitor concentration.

The apparent constant of the enzyme-inhibitor complex for mixed inhibition was calculated from the point of intersection on Lineweaver-Burk plots. Table 5 shows values for the apparent K_i and inhibition index, $\{I/S\}_{0.5}$, for various amines at pH 7.6. The inhibition index was derived both from $\bar{K}_i$ and I_{50}, using the following equation (32):

$$\left\{\frac{I}{S}\right\}_{0.5} = \frac{K_i}{K_s} + \frac{K_i}{K_s}$$

where K_i and K_s are the dissociation constants of enzyme-inhibitor and enzyme-substrate complexes. The apparent K_m values were used instead of the apparent K_s values for the calculations. The I_{50} values were determined previously (11, 31).

The inhibition index values were within the same range for any one substrate; however, they varied greatly with the substrate used. The slight differences sometimes apparent between the left and right sides of the equation presumably originated from variations between K_s and K_m values.

The apparent K_m and I_{50} values for various amines as substrates are also summarized in Table 5.

Despite several measurements at various pH values, data obtained for apparent K_i values were insufficient to determine the true inhibitor constants.

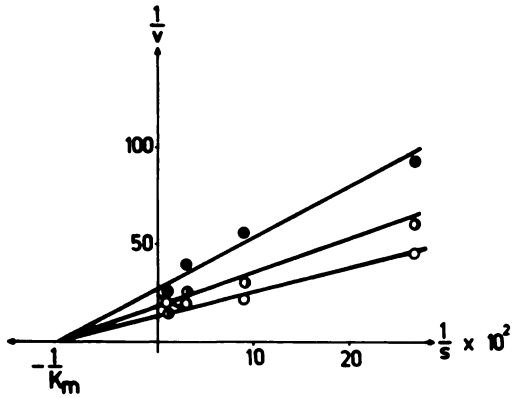

FIG. 11. *Lineweaver-Burk plot for 3,4-dimethoxyphenylethylamine (DMPEA)*

v = micromoles of DMPEA transformed per milligram of protein per hour. ○, 3,4-dimethoxyphenylethylamine; ◑, 3.3×10^{-3}M AB-15; ●, 1.0×10^{-3}M AB-15.

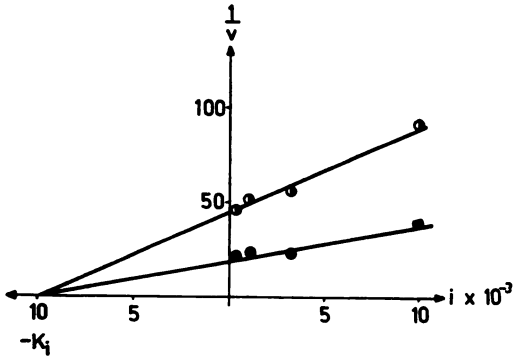

FIG. 12. *Dixon plot for 3,4-dimethoxyphenylethylamine* (DMPEA). v = micromoles of DMPEA transformed per milligram of protein per hour, ●, 3.3×10^{-6}M DMPEA; ◑, 3.7×10^{-4}M DMPEA.

Inhibition in vivo. The substrate dependence of the inhibition observed after oral administration of AB-15 was studied in detail by determining the rate of enzymatic oxidation of different amines. Either 20 μmoles or 40 μmoles of AB-15 per kilogram of body weight were administered to rats, which were killed 16 hr later. The activities of dialyzed brain homogenates of the treated animals were compared with those of the untreated ones. A comparison of activities from dialyzed brain homogenates from treated and untreated animals showed significant differences in the extent of inhibition

TABLE 5

Mechanism of inhibition, apparent dissociation constants, and inhibition index values for various amines at pH 7.6

The I_{50} values were obtained from previous experiments (11, 31). The apparent K_i values were determined as described in the text. The final substrate concentration was 1 mM, except for 5-hydroxytryptamine, which was 0.6 mM.

Substrate	Type of inhibition	K_m	$\tilde{K}_i$	I_{50}	$\{I/S\}_{0.5}$	
					Calculated from $\tilde{K}_i$	Calculated from I_{50}
		M	*M*			
5-Hydroxytryptamine	Mixed (mostly competitive)	6.0×10^{-5}	1×10^{-6}	4×10^{-6}	0.018	0.007
Tryptamine	Mixed (mostly noncompetitive)	2.4×10^{-5}	8×10^{-4}	1×10^{-2}	10.0	34.10
Tyramine	Partially noncompetitive	1.3×10^{-3}	2×10^{-5}	6×10^{-5}	0.060	0.035
Norepinephrine	Partially noncompetitive	3.7×10^{-4}	3×10^{-4}	8×10^{-4}	0.80	0.820
DMPEA	Completely noncompetitive	3.1×10^{-4}	1×10^{-2}	3×10^{-2}	30.0	42.0

TABLE 6

Substrate dependence of inhibition of rat brain monoamine oxidase in vivo by AB-15

The rates of oxidation of 5-hydroxytryptamine, tryptamine, and norepinephrine were measured by the Lagnado-Sourkes method (12). The final concentration of the substrate was 10 mM, and the protein content of the mixture was 6–7 mg. The reaction mixtures were incubated for 15 min with 5-hydroxytryptamine and tryptamine, and for 30 min with norepinephrine as substrate. The rate of DMPEA oxidation was measured by the modified Kapeller-Adler method. The final substrate concentration was 10 mM, and the protein content was 15–20 mg. Mixtures were incubated for 60 min. The tyramine rate measurements were carried out according to Udenfriend and Cooper (15), using 1.2 mM tyramine as substrate and dialyzed homogenate containing 6–7 mg of protein as the enzyme source. The mixtures were incubated for 30 min. Q represents the specific enzyme activity (micromoles per hour per milligram of protein); S, the standard error, n, the number of animals; and p, the probable error.

AB-15	n	5-Hydroxytryptamine			Tyramine			Norepinephrine			Tryptamine			DMPEA		
		Q	S	p	Q	S	p	Q	S	p	Q	S	p	Q	S	p
μmoles/kg																
0	5	0.223	±0.007		0.197	±0.016		0.133	±0.05		0.174	±0.011		0.040	±0.006	
20	5	0.159	±0.008	0.01	0.159	±0.008	0.05	0.131	±0.006	0.80	0.177	±0.003	0.80	0.037	±0.003	0.90
40	5	0.135	±0.016	0.01	0.158	±0.006	0.05	0.129	±0.009	0.70	0.171	±0.008	0.80	0.042	±0.005	0.80

when various amines were used as substrates (Table 6). The observed inhibition ought to be less than that occurring *in vivo* (11), since the use of dialysis to remove endogenous substrate would also have removed some of the bound inhibitor.

DISCUSSION

The kinetic data presented above suggest differences in the binding of various amines to the active site of the enzyme. The pH dependence of the apparent Michaelis constants varied markedly according to the substrate used. The difference in pK values for the active site on the enzyme and in the ionization states within the active site, which directly or indirectly affect the binding of substrates, can be explained by "multiple binding" within the active center. Although results obtained from the mixed-substrate studies suggest a common active site for 5-hydroxytryptamine, tryptamine, and possibly tyramine, they cannot exclude the possible existence of different active sites, especially in the case of tyramine and 5-hydroxytryptamine. For norepinephrine and 5-hydroxytryptamine the results of the mixed-substrate studies indicate different binding sites.

McEwen *et al.* (6) obtained pK_m–pH diagrams that were similar for *N*-methylbenzylamine, benzylamine, and veratrylamine, using an enzyme from human liver mitochondria. They pointed out that the graphical analyses were typical in all respects of a simple case of nonprotonated amine interacting with an electrophilic group on the enzyme to form an enzyme-substrate complex. They were unable to detect any kinetic evidence suggesting the presence of multiple monoamine oxidases. Experiments carried out on rat liver monoamine oxidase (33) resulted in similar pH profiles for 5-hydroxytryptamine and norepinephrine, yielding a pK of 6.6–6.8 for the enzyme-substrate complex and a pK of 7.2–7.4 for the enzyme, and also suggested that amines interact with a basic group on the enzyme. In contrast with these findings, Youdim *et al.* (34) showed that multiple forms of monoamine oxidase exist, and the various forms exhibit different specificities. Thus not only different ionizing groups within the active center, but the presence of multiple enzymes, may also be responsible for the marked differences in substrate-binding behavior. In light of these conflicting results, the existence of different forms of monoamine oxidase from various species and organs seems probable. Also, some of the forms may exhibit substrate-specific characteristics.

Similar conclusions could be drawn from the Michaelis constant determinations. Youdim *et al.* (34) obtained the apparent Michaelis constants for kynuramine between 24 and 83 μM at pH 7.4 for the various forms of rat brain monoamine oxidase, while Coq and Baron (33) determined the apparent K_m for rat liver monoamine oxidase as 0.5 and 2.4 mM with 5-hydroxytryptamine and norepinephrine as substrates, respectively. In the present experiments the "true" Michaelis constants with rat brain monoamine oxidase were 20 and 56 μM, respectively, for tryptamine and 5-hydroxytryptamine, and 0.23 and 0.5 mM for norepinephrine and tyramine. This supports the suggestion that the monoamine oxidases of brain and liver slightly differ in a manner reflected in their substrate dependence.

If one assumes that the Michaelis constant is equivalent to the dissociation constant of the enzyme-substrate complex, tryptamine and 5-hydroxytryptamine show high affinities while tyramine and norepinephrine display markedly lower affinities for the enzyme. However, the possibility that K_m values represent only kinetic constants should also be considered (35–37).

Another problem concerning the assay of enzyme activity should also be mentioned. Certain differences have been observed between the reaction mechanism of monoamine oxidation catalyzed by rat brain monoamine oxidase and that found with a monoamine-dehydrogenating system, using tyramine as substrate (38–41). This finding suggests that K_m values for 5-hydroxytryptamine, tryptamine, and norepinephrine, measured by the tetrazolium method, could be related, in part, to the dehydrogenating system, while that for tyramine, measured by the Udenfriend method, may be attributable to the monoamine oxidase system. In this case the

different forms of the $p\tilde{K}_m$–pH curves for tyramine and the other amines show different characteristics of the two enzyme systems. However, neither the reaction rate measurements (see MATERIALS AND METHODS) nor the inhibition studies (11) showed any discrepancies among the tetrazolium method manometric methods, or measurements of the changes in substrate concentration, except when tyramine was used as substrate.[2] Contrary to the previous studies (12, 14, 42), these findings indicate that an additional enzyme system is not involved in the dye reduction using tryptamine, 5-hydroxytryptamine, or norepinephrine as substrate, while for tyramine this possibility does exist.

AB-15 was shown to be a reversible inhibitor, and its apparent dissociation constant was substrate-dependent. Reactivation measurements also reflected differences dependent on the amine substrates. Reversal of inhibition was nearly complete after the second washing with norepinephrine, tryptamine, and 3,4-dimethoxyphenylethylamine as substrates, while with 5-hydroxytryptamine and tyramine complete enzyme reactivation could be achieved only after three or six washings.

Inhibition by AB-15 was most effective with 5-hydroxytryptamine, and least effective with tryptamine and 3,4-dimethoxyphenylethylamine. The highest and lowest values of the apparent dissociation constant for the enzyme-inhibitor complex differed by 4000-fold. The measurements *in vivo* confirmed this, showing substrate-specific differences in inhibition by AB-15 after oral administration.

The Lineweaver-Burk plots showed that the inhibitor binds to the enzyme, and not to the enzyme-substrate complex, in all cases. Thus the difference in the apparent K_i values can be explained only if one assumes different binding sites for the inhibitor. The observation that the degree of inhibition by AB-15 after dialysis was dependent on the choice of substrate adds support to this hypothesis.

The finding that at low pH values AB-15 produced mixed inhibition, while at high pH values competitive inhibition was seen with 5-hydroxytryptamine as substrate, reflects the influence of different ionizing groups on the AB-15–enzyme interaction. At lower pH AB-15 affected the ionizing group involved in the 5-hydroxytryptamine–enzyme interaction and the breakdown of the enzyme-substrate complex. At higher pH AB-15 affected only the ionizing group involved in the enzyme-substrate interaction. With tryptamine as substrate, AB-15 produced mixed inhibition at both low and high pH, although the inhibition was mostly noncompetitive at low pH and competitive at high pH.

The Dixon plots showed that inhibition increased to a definite limit at high AB-15 concentration and resulted in partial inhibition except in the case of 3,4-dimethoxyphenylethylamine, for which fully noncompetitive inhibition was apparent. The evidence for the existence of several forms of monoamine oxidase in rat brain throws new light on the partial inhibition by AB-15, since preferential affinities for individual isoenzymes must be considered. All these data support the hypothesis of multiple binding sites both for substrates and for inhibitors.

[2] Z. Huszti, unpublished observations.

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