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Inhibition of Monoamine Oxidase by Analogues of Amiloride

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SUMMARY

Amiloride analogues with nonaromatic substituents on the 5-amino group or different substituents on carbon-6 of the pyrazine ring were tested as inhibitors of monoamine oxidase A and B in rat brain homogenate. The inhibition was competitive and reversible. 5-(*N*,*N*-Tetramethylene)amiloride protected the A type in the homogenate against irreversible inhibition by clorgyline. A reciprocal relation was found to exist between inhibitory constants of 5-*N*-substituted amiloride analogues for monoamine oxidase A and the ratio of overflows of endogenous noradrenaline and 3,4-dihydroxyphenylethylene glycol from the isolated rat tail artery incubated in the presence of a 50 μM concentration of the analogue, when the tissue was exposed to 10 μM tyramine. The 5-amino group appeared to be essential for inhibition of the A but not of the B type. Bell-shaped relations between inhibitory constants of 5-(*N*-alkyl)- and 5-(*N*,*N*-dialkyl)-substituted analogues and lengths of alkyl chains were different for each type. The presence of a methyl group in the α-position of the chain increased substantially the inhibitory constant for the A type. Halogen atoms as substituents on carbon-6 increased inhibitory constants for both types of the enzyme in the sequence: I < Br < Cl < F. These findings are consistent with the existence of hydrophobic binding sites of restricted dimensions in both types of the enzyme.

Amiloride, 3,5-diamino-*N*-(diaminomethyl)-6-chloropyrazinecarboximide, is a competitive inhibitor of MAO (E.C.1.4.3.4) of the A type (MAO-A) (1, 2). The aim of this study was to determine whether this property is exhibited also by amiloride analogues, specifically those with nonaromatic substituents on the nitrogen atom of the 5-amino group, and, if so, to identify the factors that determine the structure-activity relations.

Rat brain homogenate was used to examine inhibition of the enzyme *in vitro*. Inhibition of MAO-A *in situ*, in terminal adrenergic axons in isolated rat tail artery, was assessed by measuring the ratio of overflows of endogenous NE and DPG, the main product of intraneuronal deamination of NE (3). To circumvent potential problems arising from displacement of NE from storage vesicles by highly hydrophobic analogues (2, 4), the overflows were monitored under conditions of enhanced release of NE from storage vesicles, which was induced by exogenous tyramine.

Experimental Procedures

Materials. E.J.C. contributed to the study by providing samples of amiloride analogues, except for those listed in Table 1 as **5**, **6**, **7**, **8**, **10**, **11**, **11a,11b**, **14**, **15**, **16**, **17**, **20**, **21**, **23**, **24**, and **26**, which were prepared by V.P. using a similar procedure (5). The starting material, methyl 3-amino-5,6-dichloropyrazine-2-carboxylate, was obtained from Merck Sharp & Dohme Research Laboratories or Aldrich Chemical Co. (Milwaukee, WI). Tyramine hydrochloride, 5-hydroxytryptamine creatinine sulfate, 2-phenylethylamine hydrochloride, bovine serum albumin, noradrenaline bitartrate, DPG, and 3,4-dihydroxybenzylamine hydrobromide were obtained from Sigma Chemical Co. (St. Louis, MO), amiloride hydrochloride from Merck Frosst Canada, clorgyline hydrochloride and L-deprenyl hydrochloride from Research Biochemicals, Inc., and 5-hydroxy-[*side chain*-2-^{14}C]tryptamine binoxalate, [*ethyl*-1-^{14}C]2-phenylethylamine hydrochloride, and Liquifluor from New England Nuclear (Boston, MA).

Solutions. The composition of Krebs solution was (in mM): NaCl, 115; $NaHCO_3$, 25; NaH_2PO_4, 1.2; Na_2-EDTA, 0.03; KCl, 5; $MgSO_4$, 1.2; $CaCl_2$, 1.7; D-glucose, 11. The solution was aerated with a 95% O_2/5% CO_2 gas mixture. Tyramine hydrochloride, clorgyline hydrochloride, and L-deprenyl hydrochloride were used in the form of concentrated aqueous stock solutions, and amiloride hydrochloride and its analogues in the form of concentrated (≥50 mM) stock solutions in dimethyl sulfoxide. Control experiments with the reaction mixture and bathing solution containing the maximum final concentration of dimethyl sulfoxide (0.2%, v/v) were done.

Assay of MAO activities in rat brain homogenate. Homogenate of rat forebrains (1:10, w/v) in 0.1 M sodium phosphate buffer (pH 7.4) was filtered through Miracloth and centrifuged for 15 min at 800 × *g*. The supernatant was stored at −60°. Before being used, it was diluted with the phosphate buffer, usually in a 1:7 (v/v) ratio. Two hundred microliters of diluted supernatant and 250 μl of water or aqueous solution of amiloride analogue were mixed in a test tube and the latter was placed in a 37° shaker bath. The reaction was started 10 min later by adding 50 μl of a solution of 5-hydroxytryptamine creatinine sulfate and 5-hydroxy-[*side chain*-2^{14}C]tryptamine binoxalate. Both acid-quenched and boiled blanks were used. Unless stated otherwise, the

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ABBREVIATIONS: MAO, monoamine oxidase; NE, noradrenaline; DPG, 3,4-dihydroxyphenylethylene glycol.

total initial concentration of 5-hydroxytryptamine in the reaction mixture was 0.1 mM. The reaction was terminated, usually after 10 min, by addition of 0.2 ml of 4 M HCl and immediate freezing of the reaction mixture in dry ice/ethanol. Reaction products were extracted into 7 ml of toluene/ethyl acetate (1:1; v/v). After centrifugation and freezing of the aqueous phase, the organic phase was decanted, mixed with 7 ml of a 4.2% (v/v) solution of Liquifluor in toluene, and counted. Corrections for the mean efficiency of extraction (6) were made. The reaction was found to be linear with respect to time up to 20 min. The MAO activity observed under these conditions appeared to be almost exclusively of the A type, because it was inhibited by more than 98% as a result of a prior 1-hr incubation of undiluted supernatant in the presence of 30 nM clorgyline, a selective irreversible inhibitor of MAO-A (7) (see also Table 3). Also, it was reduced by less than 2%, i.e., not significantly ($p > 0.05$; four experiments), when the supernatant had been incubated in the presence of 30 nM L-deprenyl, a selective irreversible inhibitor of MAO-B (8).

A similar procedure was used to assay the MAO-B activity. In order to suppress interfering MAO-A activity, undiluted supernatant was mixed with an equal volume of 60 nM clorgyline, incubated for 1 hr at 37°, and then diluted with 7 volumes of 0.1 M sodium phosphate buffer. The substrate was a mixture of 2-phenylethylamine hydrochloride and [*ethyl*-1-^{14}C]2-phenylethylamine hydrochloride.

Reversibility of inhibition of MAO-A. In one set of test tubes, 0.25 ml of undiluted supernatant was mixed with 0.25 ml of solution of the analogue or water. After 30 min, 1.75 ml of 0.045 M sodium phosphate buffer (pH 7.4) were added to each test tube and the contents were vortexed. The reaction was started 10 min later by combining a 0.45-ml aliquot with 0.05 ml of 1 mM 5-hydroxytryptamine substrate. In the second set, 0.25 ml of undiluted supernatant was mixed first with 1.75 ml of the buffer and then with 0.25 ml of the solution of the analogue or water. The reaction was started after 10 min by mixing a 0.45-ml aliquot with 0.05 ml of a 1 mM solution of 5-hydroxytryptamine.

Protection of MAO-A against irreversible inhibition by clorgyline. Two hundred fifty microliters of undiluted supernatant were mixed with the same volume of a solution of 5-(*N,N*-tetramethylene)amiloride or water. After 10 min, 5 μl of 6 μM clorgyline or water were added. The mixture was incubated for 1 hr at 37°, diluted with 10 ml of cold 0.045 M sodium phosphate buffer (pH 7.4), and vortexed for 2 min. A crude mitochondrial fraction was obtained by 30-min centrifugation at 12,000 × *g*. The pellet was resuspended in 10 ml of the buffer and subjected to another 30-min centrifugation at 12,000 × *g*. The final pellet was resuspended in 2 ml of the buffer, and 450-μl aliquots were used for the assay. The concentration of protein was assayed by the method of Lowry *et al.* (9), using standards prepared from bovine serum albumin.

Estimation of MAO-A activity in terminal adrenergic axons (1). Single segments weighing between 20 and 28 mg were excised from proximal ends of tail arteries of adult male rats, under sodium pentobarbitone anaesthesia (50 mg/kg, intraperitoneally), and incubated for 1 hr in aerated 37° Krebs solution. Subsequently, the segments were incubated individually in 5-ml portions of the specified solution in a shaker bath, first for 15 min in Krebs solution, then for three consecutive 15-min periods in Krebs solution supplemented with the analogue at a final concentration of 50 μM. Tyramine was present during the last period at a final concentration of 10 μM. A 4.5-ml aliquot of the incubation medium was mixed with 0.5 ml of 1 M $HClO_4$/50 nM 3,4-dihydroxybenzylamine and 0.1 ml of 0.5 M $Na_2S_2O_5$. Catechol compounds were adsorbed on alumina, eluted into 0.25 ml of 0.4 M $HClO_4$, and assayed by high performance liquid chromatography with electrochemical detection. Corrections for recoveries were made.

Results

Inhibition of MAO activities in rat brain homogenate. To ascertain that, like the parent compound (**1**), the analogues act as competitive inhibitors of MAO-A, assays were done at a constant concentration of analogue while the initial concentration of the substrate was varied. The results of the experiment with 5-(*N,N*-tetramethylene)amiloride (Fig. 1) showed that the inhibition is competitive. Additional evidence to that effect was provided by the results of similar experiments with six other representative analogues (Nos. **4**, **9**, **13**, and **18** in Table 1 and Nos. **33** and **36** in Table 2; data not shown). Assays of MAO-B activity in the presence of 50 μM amiloride, 1 μM 5-(*N*-cyclopentyl)amiloride, and 1 μM 5-(*N,N*-dipropyl)amiloride, respectively (data not shown), showed that inhibition of MAO-B by amiloride and its analogues is also competitive.

Under these conditions, the inhibition could be evaluated conveniently by assaying MAO-A activity at different concentrations of the analogue and a constant initial 0.1 mM concentration of 5-hydroxytryptamine. Inhibition of MAO-B was assessed in a similar manner, at a constant initial 2-phenylethylamine concentration of 4.1 μM. Hill plots of MAO activities, an example of which appears in Fig. 2, were linear (regression coefficients, >0.96; range of slopes, −0.95 to −1.06). Estimates of inhibitory constants, K_i, given in Tables 1 and 2 were derived using Michaelis-Menten constants of 92 μM for 5-hydroxytryptamine and 4.1 μM for 2-phenylethylamine, which were obtained in separate experiments of the kind illustrated in Fig. 1.

Reversibility of inhibition. When the reversibility of inhibition of MAO-A was tested (as described in Experimental Procedures) and 5-(*N,N*-tetramethylene)amiloride was used at final concentrations of 0.01 and 0.1 μM, mean MAO-A activities in each pair of sets (three experiments/set) were not significantly different. The same observation was made when 5-(*N*-cyclopropyl)amiloride was used at a final concentration of 0.1

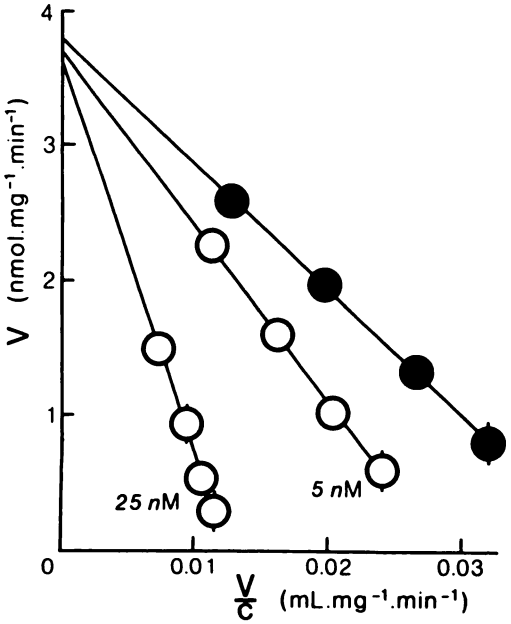

Fig. 1. Inhibition of MAO-A by 5-(*N,N*-tetramethylene)amiloride. MAO-A activity of rat brain homogenate was measured as a function of the concentration of 5-hydroxytryptamine in the absence (●) and presence (○) of indicated concentrations of 5-(*N,N*-tetramethylene)amiloride. The data in the Eadie-Hofstee plot are means of four individual observations. The limits of estimated standard deviations are indicated by *vertical lines* when they exceed the diameter of the *symbol*. The Michaelis-Menten constant, K_M, was estimated as 92 μM, the maximum velocity, V_{max}, as 3.8 nmol · min^{-1} · mg protein^{-1}, and the inhibitory constant, K_i, of the analogue as 0.010 μM. The differences in V_{max} among the three groups were not significant (the multiple range test).

TABLE 1

Inhibition of MAO-A and MAO-B in rat brain homogenate by 5-N-substituted amiloride analogues

	K_i	
	MAO-A[a]	MAO-B[b]
	μM	
1 Amiloride	2.0	40
5-(*N*-*n*-Alkyl)-substituted		
2 5-(*N*-Methyl)amiloride	1.0	22
3 5-(*N*-Ethyl)amiloride	0.16	12
4 5-(*N*-Propyl)amiloride	0.10	6.3
5 5-(*N*-Butyl)amiloride	0.16	3.9
6 5-(*N*-Pentyl)amiloride	0.25	3.0
7 5-(*N*-Hexyl)amiloride	0.32	2.5
8 5-(*N*-Octyl)amiloride	1.0	3.5
5-(*N*-Isoalkyl)-substituted		
9 5-(*N*-Isopropyl)amiloride	0.79	4.3
10 5-(*N*-Isobutyl)amiloride	0.25	3.4
11 5-(*N*-*R*,*S*-*sec*-Butyl)amiloride	1.1[c]	1.1
11a 5-(*N*-*S*-*sec*-Butyl)amiloride	0.8[c]	1.1
11b 5-(*N*-*R*-*sec*-Butyl)amiloride	1.5	1.1
12 5-(*N*-*tert*-Butyl)amiloride	1.0	2.4
5-(*N*-Cycloalkyl)-substituted		
13 5-(*N*-Cyclopropyl)amiloride	0.079	18
14 5-[*N*-(Cyclopropyl)methyl]amiloride	0.11	6.2
15 5-(*N*-Cyclobutyl)amiloride	0.25	2.3
16 5-(*N*-Cyclopentyl)amiloride	0.32	0.64
17 5-(*N*-Cyclohexyl)amiloride	0.56	0.95
5-(*N*,*N*-Dialkyl)-substituted		
18 5-(*N*,*N*-Dimethyl)amiloride	0.11	5.4
19 5-(*N*,*N*-Diethyl)amiloride	0.050	1.5
20 5-(*N*,*N*-Dipropyl)amiloride	4.0	0.79
21 5-(*N*,*N*-Dibutyl)amiloride	12.5	1.7
22 5-(*N*,*N*-Dipentyl)amiloride	50	6.7
23 5-(*N*-Methyl,*N*-ethyl)amiloride	0.040	3.3
24 5-(*N*-Methyl,*N*-propyl)amiloride	0.20	1.8
5-(*N*,*N*-Polymethylene)-substituted		
25 5-(*N*,*N*-Tetramethylene)amiloride	0.011	5.7
26 5-(*N*,*N*-Pentamethylene)amiloride	0.071	1.3
27 5-(*N*,*N*-Hexamethylene)amiloride	0.10	0.91
Other 5-*N*-substituted analogues		
28 5-[*N*-(2-Propenyl)]amiloride	0.25	11
29 5-[*N*-(2-Propynyl)]amiloride	1.6	9.6
30 5-[*N*-(2-Hydroxyethyl)]amiloride	5.0	71
31 5-(*N*-Methyl,*N*-methoxyl)amiloride	1.0	14
32 5-(*N*-Ethyl,*N*-isopropyl)amiloride	0.69	1.0

[a] MAO-A activities were assayed in quadruplicate using diluted 800 × *g* supernatants of rat brain homogenate at a constant (0.1 mM) concentration of 5-hydroxytryptamine and, typically, four different concentrations of the analogue. The inhibitory constants, K_i were derived using an independently determined value of 92 μM for the K_M of 5-hydroxytryptamine. Estimated 95% confidence limits did not exceed 82.6% or 117.9% of the indicated (mean) value of K_i.

[b] MAO-B activities were assayed in quadruplicate using diluted 800 × *g* supernatants of rat brain homogenate that had been incubated for 1 hr in the presence of 30 nM clorgyline, at a constant initial concentration of 2-phenylethylamine that was equal to its estimated K_M of 4.1 μM, and three different concentrations of the analogue. Estimated 95% confidence limits did not exceed 74.9% or 133.3% of the indicated value of K_i.

[c] The difference is significant ($p < 0.05$). The assays were done at final concentrations of the analogues equal to 10, 3.16, 1, and 0.316 μM, respectively.

TABLE 2

Inhibition of MAO-A and MAO-B in rat brain homogenate by amiloride analogues modified on carbon-6 of the pyrazine ring

	Substituent		K_i	
	carbon-6	carbon-5	MAO-A[a]	MAO-B[b]
			μM	
33	H	NH_2	1.2	200
34	F	NH_2	5.3	65
1	Cl	NH_2 (amiloride)	2.0	40
35	Br	NH_2	1.6	34
36	I	NH_2	1.2	24
37	I	$N(CH_3)_2$	0.071	3.0
38	H	H	>1000	75

[a,b] See the legend to Table 1.

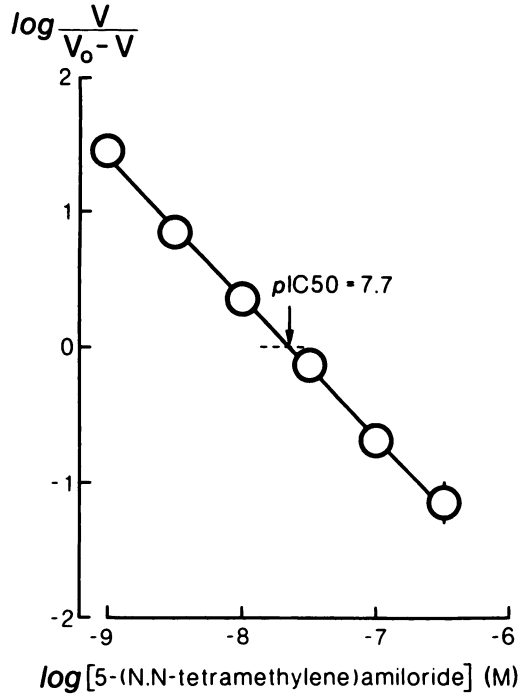

Fig. 2. Inhibition of MAO-A by 5-(*N*,*N*-tetramethylene)amiloride. The Hill plot of MAO-A activity of rat brain homogenate at constant initial 0.1 mM concentration of 5-hydroxytryptamine and indicated concentrations of 5-(*N*,*N*-tetramethylene)amiloride is shown. V_o, MAO activity in the absence of the inhibitor. The data are means of four individual observations. Using the K_M value of 92 μM (Fig. 1), the inhibitory constant, K_i of the analogue can be estimated as 0.011 μM.

μM. At a final concentration of 2 nM, clorgyline, an irreversible inhibitor of MAO-A (7), inhibited the MAO-A activity by 97.3 ± 0.5% in the first set, but only by 58.3 ± 1.6% in the second set (four experiments/set). Under these circumstances, it seemed of interest to find out whether 5-(*N*,*N*-tetramethylene)amiloride could protect MAO-A against irreversible inhibition by clorgyline. The results of that experiment are given in Table 3.

Inhibition of MAO-A activity in adrenergic axons in the isolated tail artery. When control arterial segments incubated in Krebs solution were exposed for 15 min to 10 μM tyramine, overflows of NE and DPG averaged 0.75 ± 0.04 and 10.6 ± 0.5 nmol·g^{-1}.hr^{-1} (20 experiments), respectively, yielding a mean (NE overflow)/(DPG overflow) ratio of 0.07. As illustrated in Fig. 3, a fair linear correlation (correlation coefficient = 0.94) was found to exist between the logarithm of the overflow ratio and pK_i. It was noted, however, that the overflow ratio observed in the presence of 5-(*N*-cyclopropyl)amiloride (No. **13**) was higher than expected from the pK_i of this analogue.

Discussion

Like amiloride (1, 2), the analogues act as inhibitors of MAO-A *in vitro* (Figs. 1 and 2) and *in situ* (Fig. 3). Their inhibitory potencies depend on substituents on the 5-amino group (Table 1) as well as on carbon-6 of the pyrazine ring (Table 2). With respect to the latter modification, the inhibitory constant K_i increased with increasing tendency of the substit-

TABLE 3

Protection of MAO-A by 5-(N,N-tetramethylene)amiloride against irreversible inhibition by clorgyline

Rat brain homogenate was incubated for 1 hr at 37° in the presence of the indicated concentrations of the two agents and was then diluted with 20 volumes of 0.045 M phosphate buffer. A crude mitochondrial fraction was separated by centrifugation, resuspended in the buffer, and pelleted again by centrifugation. MAO-A activity was measured by the standard procedure, in aliquots of the resuspended pellet. The initial concentrations of 5-hydroxytryptamine was 0.1 mM. The results of means of three observations ± the estimated standard deviation.

Concentration in incubated homogenate		MAO-A of the final pellet
5-(*N*,*N*-Tetramethylene)amiloride	Clorgyline	
μM	nM	$nmol \cdot mg^{-1} \cdot min^{-1}$
0	0	1.47 ± 0.03
0	30	0.015 ± 0.001
0.1	30	0.11 ± 0.02
1	30	0.64 ± 0.07
10	30	1.10 ± 0.22

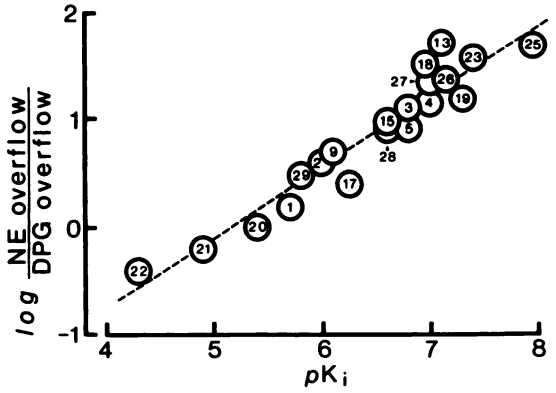

Fig. 3. Effect of 5-*N*-substituted amiloride analogues on the ratio of overflows of endogenous NE and DPG from the isolated rat tail artery. Arteries were incubated for 30 min in Krebs solution that was supplemented with the analogue in the final concentration of 50 μM. Tyramine was present during the 15-min experimental period at a final concentration of 10 μM. The logarithm of overflow ratio in control arteries incubated in analogue-free Krebs solution was −1.15. Individual analogues are identified by numbers given in Table 1. The data are means of overflow ratios in four arteries. Number of analogues tested = 20; correlation coefficient, $r = 0.94$.

uent to form a hydrogen bond or, conversely, decreasing hydrophobicity. The decreases in K_i resulting from substitution of iodine for chlorine in amiloride and 5-(*N*,*N*-dimethyl)amiloride were proportional, suggesting that the effect is independent of that of substitutions in the adjacent 5-amino group.

The large difference between the K_i values of 3-amino-*N*-(diaminomethyl)pyrazinecarboximide and 3,5-diamino-*N*-(diaminomethyl)pyrazinecarboximide (Nos. **38** and **33**; Table 2) shows that the presence of the 5-amino group is essential for inhibition of MAO-A activity. It follows that binding of amiloride and, presumably, of its analogues to the enzyme involves interaction of the nitrogen atom in the 5-amino group with the enzyme. The difference in free energy of binding, <-18 kJ·mol^{-1}, suggests a hydrogen bond.

Replacement of one or both hydrogen atoms in the 5-amino group with alkyl groups, which are effective electron donors, may lower the extreme acidity of the 5-amino group (10). Attenuation of the acidity does not, however, seem to play any major role in determining the value of K_i, for it can be brought about also by the substituent on carbon-6 of the pyrazine ring. Although the difference between the electron-withdrawing power of iodine and hydrogen in the *ortho*-position of an aromatic ring to the amino group is large [e.g., pK_a of aniline is 4.20, pK_a of 2-iodoaniline 2.60 (11)], inhibitory constants of 3,5-diaminoamino-*N*-(diaminomethyl)pyrazinecarboximide (No. **33**) and 3,5-diamino-6-iodo-*N*-(diaminomethyl)pyrazine carboximide (No. **36**) were found to be the same. Moreover, the acidity is reduced equally effectively if the substituent on the 5-amino groups is a branched alkyl group. Yet the K_i values of these analogues are quite high.

An alternative is to postulate interaction of substituents on the 5-amino group with a hydrophobic site in MAO-A, which is suggested by, among other observations, the high K_i values of relatively polar 5-[*N*-(2-hydroxyethyl)]amiloride (No. **30**) and 5-(*N*-methyl,*N*-methoxyl)amiloride (No. **31**) and the rise of K_i resulting from introduction of a double or a triple bond (Nos. **28** and **29**) (12).

The difference in pK_i between the most potent analogue, 5-(*N*,*N*-tetramethylene)amiloride, and amiloride is 2.25. If expressed in terms of the difference in the Gibbs free energy of binding, $\Delta(\Delta G^0) = -2.3\ RT\ \Delta\ pK_i$, it is equal to -13.3 kJ·mol^{-1}. The change in the free energy resulting from hydrophobic interaction of a methylene group in *n*-alkanes can be estimated as about -3.6 kJ·mol^{-1} at 37° (12). Hence, the change in free energy arising from hydrophobic interaction of four methylene groups should be about -14.4 kJ·mol^{-1}, which is comparable to the above $\Delta(\Delta G^0)$ of -13.3 kJ·mol^{-1}. Because the difference between the two values is quite small, it can be concluded, tentatively at least, that substitution of the tetramethylene chain for the two H atoms does not affect appreciably the postulated binding of the nitrogen atom of the 5-amino group to the enzyme and that binding of this analogue to the enzyme involves also all four methylene groups in the pyrrolidine ring.

The shape and dimensions of the hydrophobic site can thus be approximated, as illustrated in Fig. 4, by those of the tetramethylene chain of pyrrolidine using van der Waals radii of methylene groups. The preferred conformation of pyrrolidine and *N*-alkylpyrrolidines is the 'half-chair' (13). It can be expected that, in the absence of other constraints, the pyrrolidine ring in 5-(*N*,*N*-tetramethylene)amiloride will adopt the same conformation, with carbon 5 of the pyrazine ring in the equatorial position.

A site of this kind can accomodate readily one ethyl and one methyl group, two methyl groups, or a single propyl, ethyl, or methyl group. On the assumption that the changes in free energy, $\Delta(\Delta G^0)$, due to hydrophobic interaction of methylene and methyl groups are comparable and equal to about -3.6 kJ·mol^{-1}, K_i values of analogues with three, two, and one methylene or methyl groups interacting should be equal to or higher than 0.03, 0.11, and 0.5 μM, respectively. The fair agreement between these limits and the actual K_i values of 5-(*N*-methyl, *N*-ethyl)amiloride, 5-(*N*,*N*-dimethyl)amiloride, and, as indicated in Fig. 5, the three 5-(*N*-alkyl)amilorides provides additional support for the scheme of the site. In principle, the site could accomodate also a butyl group. The *syn*-periplanar conformation of the butyl group required for the fit is, however, fully eclipsed and its energy content is higher than of those of the preferred *anti*-periplanar and *gauche* conformations.

The minimum site width required for accommodation of the two CH_3 groups with van der Waals diameters of 4 Å (14) in 5-(*N*,*N*-diethyl)amiloride is 8 Å. However, according to the scheme in Fig. 4, the width of this part of the site is only 5.5

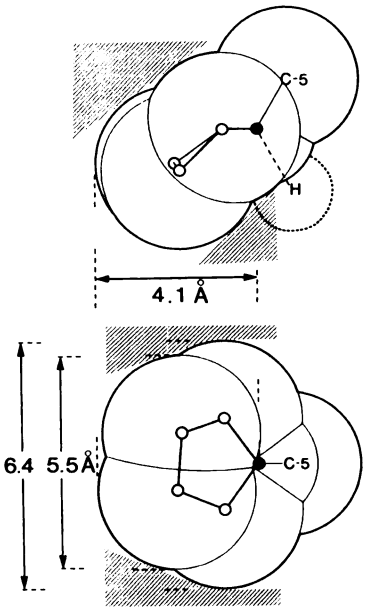

Fig. 4. Two views of the postulated hydrophobic binding site of MAO-A. The pyrrolidine ring of 5-(*N*,*N*-tetramethylene)amiloride in the half-chair conformation is shown, along with carbon-5 of the pyrazine ring. The difference in inhibitory potencies of 3,5-diamino-*N*-(diaminomethyl)pyrazinecarboximide (K_i = 1.2 μM) and 3-amino-*N*-(diaminomethyl)pyrazinecarboximide (K_i > 1000 μM), which is equivalent to $\Delta(\Delta G^0)$ of at least +16 kJ· mol^{-1}, is compatible with formation of a hydrogen bond between the N atom (*black circle*) and the enzyme. *Upper*, the view from the direction normal to the plane of the pyrazine ring; *lower*, the view from below. The van der Waals radii of methylene groups (*empty circles*) were taken as 2 Å and that of the nitrogen atom in the 5-amino group as 1.5 Å (14). The *shaded areas* in apposition to the pyrrolidine ring represent approximate boundaries of the site. The position of the H atom (*dashed circle*) in the postulated hydrogen bond is indicated in the *upper panel* only.

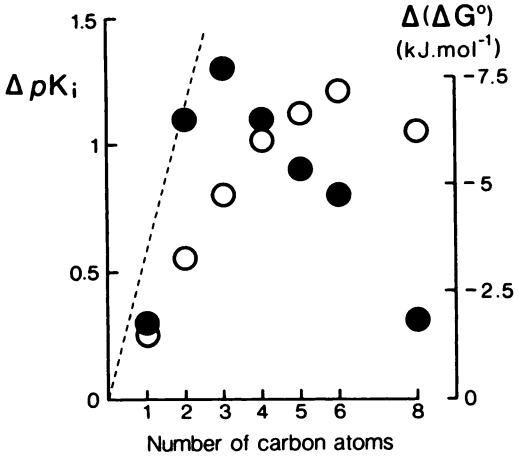

Fig. 5. Inhibitory constants K_i of 5-(*N*-alkyl)amilorides for MAO-A and MAO-B, as functions of the length of the alkyl chain. The values are the differences between pK_i values for MAO-A (●) and MAO-B (○) and the corresponding pK_i value of amiloride, 5.7 and 4.4, respectively. The *dashed line* represents the theoretical increase in pK_i due to hydrophobic binding of the alkyl chain. Its slope is approximately −3.6 kJ· mol^{-1}, or +0.6 pK_i units, per CH_2 moiety at 37°.

Å. If the site can accomodate, without major distortion, the two CH_2 groups and only one of the two methyl groups, the K_i of the analogue should be equal to or higher than 0.03 μM. The observed value was 0.05 μM. The steep rise of K_i in the 5-(*N*,*N*-dialkyl)amiloride series and the increases in K_i upon substitution of propyl for ethyl in 5-(*N*-methyl, *N*-ethyl)amiloride or insertion of additional methylene groups into the pyrrolidine ring of 5-(*N*,*N*-tetramethylene)amiloride can be interpreted in a similar manner.

Inhibitory constants of the analogues containing a methyl group in the α-position, 5-(*N*-isopropyl)amiloride and 5-(*N*-*sec*-butyl)amiloride, are much higher than those of the corresponding analogues with unbranched alkyl chains, 5-(*N*-ethyl)amiloride and 5-(*N*-propyl)amiloride, respectively. The differences are likely due to limited dimensions of the site, which cannot allow insertion of the protruding α-methyl group without substantial distortion and/or interference with the postulated bond between the nitrogen atom of the 5-amino group and the enzyme. Reduction of the volume, which results from cyclization of isopropyl to cyclopropyl or of *sec*-butyl to cyclobutyl, allows for an easier fit and it is accompanied, as apparent from the data in Table 1, by a decrease in K_i. Comparison of the K_i values of 5-(*N*-isobutyl)amiloride and 5-[*N*-(cyclopropyl)methyl]amiloride reveals that the effect of cyclization is significant even if the methyl group is in the apparently less critical β-position. The small but significant difference in K_i between the optical isomers of 5-(*N*-*sec*-butyl)amiloride suggests that the site may even exhibit modest stereoselectivity.

Because the postulated interactions do not include formation of a covalent bond, the inhibition should be readily reversible. The relation of the hydrophobic site to the active center and hydrophobic regions (15) of the enzyme remains to be determined. Concentration-dependent protection of MAO-A by 5-(*N*,*N*-tetramethylene)amiloride against irreversible inhibition by clorgyline does not necessarily imply that the two ligands compete for the same site.

In marked contrast to MAO-A, appreciable inhibition of MAO-B by 3-amino-*N*-(diaminomethyl)pyrazinecarboximide (Table 2) shows that the 5-amino group is not essential. Interaction with the enzyme involves other parts of the amiloride molecule, which may include, as suggested by the difference in K_i between 3,5-diamino-*N*-(diaminomethyl)pyrazinecarboximide (No. **33**) and 3,5-diamino-6-halogen-*N*-(diaminomethyl)pyrazinecarboximides (Nos. **1**, **34**, **35**, and **36**), even the halogen atom.

Because the inhibitory potency of amiloride is modified by substituents on the 5-amino group, it is tempting to consider, as done already by Zeller (16), that a hydrophobic binding site exists in MAO-B. The K_i minima exhibited by 5-(*N*-cyclopentyl)amiloride, 5-(*N*,*N*-dipropyl)amiloride, and 5-(*N*,*N*-hexamethylene)amiloride, respectively, as well as the relatively low K_i of analogues containing branched alkyls, suggest that the dimensions of the site must be substantially larger than those of the MAO-A site. The small cyclopropyl group may be unable to interact effectively with such a large site, and this could account for the observed high K_i of the respective analogue.

As illustrated in Fig. 5, pK_i in the 5(*N*-alkyl)amiloride series increased with increasing chain length, attaining a maximum with 5-(*N*-hexyl)amiloride. A qualitatively similar observation was made by McEwen *et al.* (17), who examined inhibition of human liver MAO by *n*-alkylamines using benzylamine as the substrate.

However, in contrast to MAO-A, Δ(ΔG0) per methylene group was only −1.2 kJ·mol^{-1}. With the exception of 5-*N*,*N*-dimethyl)amiloride (−2.6 kJ·mol^{-1}, it was always higher than

$-2.2\ kJ \cdot mol^{-1}$ and, thus, far above the theoretical value of $-3.6\ kJ \cdot mol^{-1}$. Moreover, the K_i values of 5-[*N*-(2-propenyl)]- and 5-[*N*-(2-propynyl)]amilorides were almost identical. For these reasons, it is very likely that factors other than hydrophobic binding are of importance in the interaction of substituents on the 5-amino group with the large MAO-B site.

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