Interaction of Flexible Analogs of *N*-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine and of *N*-Methyl-4-phenylpyridinium with Highly Purified Monoamine Oxidase A and B†

Matthew J. Krueger,‡ S. M. N. Efange,§ Robert H. Michelson,§ and Thomas P. Singer*,‡,∥

Department of Biochemistry and Biophysics and Division of Toxicology, Department of Pharmacy, University of California, San Francisco, California 94143, and Molecular Biology Division, Department of Veterans Affairs Medical Center, San Francisco, California 94121, and Department of Radiology and Medicinal Chemistry, University of Minnesota, Minneapolis, Minnesota 55455

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ABSTRACT: Sixteen analogs of *N*-methyl-1,2,3,6-tetrahydropyridine (MPTP) of varying degrees of flexibility have been studied as substrates of highly purified monoamine oxidases (MAO) A and B. The relative effectiveness of the various tetrahydropyridines as substrates of MAO A and B were evaluated in terms of the function turnover number/K_m, as determined by initial rate measurements. The insertion of a methylene bridge between the phenyl and tetrahydropyridine moieties of MPTP to yield *N*-methyl-4-benzyl-1,2,3,6-tetrahydropyridine, rendering the molecule more flexible, greatly enhances reactivity with MAO B, but not with MAO A, as compared with MPTP itself, in accord with data in the literature (Youngster et al., 1989a). The ethylene-bridged MPTP analog, on the other hand, is a far better substrate of both forms of MAO than is MPTP itself. The effect of molecular flexibility on the rate of oxidation of these compounds is obscured by substituents on the aromatic ring. Branching and rigidity were detrimental to the activity as substrates of both forms of MAO. Those analogs of **1** which contain small electron-withdrawing substituents in the phenyl ring were found to be more selective for MAO B, while those substituted with bulky groups were selectively oxidized by MAO A. The substrate binding site of MAO A probably contains a lipophilic pocket larger than that found in a similar site in MAO B.

N-Methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) is rapidly oxidized by monoamine oxidase (MAO) B in brain mitochondria (Chiba et al., 1984) and by pure MAO B from liver (Salach et al., 1984) and more slowly by the A form of MAO A (Salach et al., 1984) to the neurotoxic pyridinium species (*N*-methyl-4-phenylpyridinium, MPP^+), which causes parkinsonian symptoms in humans and subhuman primates (Langston et al., 1983; Burns et al., 1983). These findings created widespread interest in the bioactivation of other tetrahydro- and dihydropyridines by MAO A and B in relation to their neurotoxicity. They also called for a reexamination of the substrate specificities of these two enzymes, since they had been believed to react only slowly or not at all with tertiary amines. As a result, a large number of MPTP analogs and related compounds have been synthesized and their oxidation by the two forms of MAO has been studied in vivo, in cell cultures, in mitochondria, and by use of highly purified MAO A and B (Fuller et al., 1987; Gibb et al., 1987; Johanessen et al., 1987; Youngster et al., 1987, 1989a; Booth et al., 1989; Rollema et al., 1990; Sablin et al., 1990). MPTP and its analogs have also been found to be mechanism-based pseudo-irreversible inhibitors of MAO A and B, while their dihydropyridinium and pyridinium oxidation products proved to be potent reversible inhibitors of MAO A but not of MAO B (Salach et al., 1984; Singer et al., 1985, 1986; Tipton et al., 1986; Krueger et al., 1990; Jin et al., 1990).

Since most of the analogs studied were semirigid, with little flexibility between the aromatic and pyridine rings, Efange et al. (1990) synthesized a series of flexible MPTP analogs in which the two rings were separated by one or more carbon atoms. Their rates of oxidation in rat brain mitochondria appeared to be higher than that of the conformationally restricted MPTP. These authors also noted that pargyline, a selective inhibitor of MAO B, blocked the oxidation of some of these analogs only partially and that the oxidation of one analog was virtually insensitive to pargyline. The kinetic data reported were not derived from measurement of the initial rates of oxidation but from the accumulation of H_2O_2 in a fixed time interval (30 min). Moreover, this assay is subject to considerable uncertainty because in mitochondrial preparations H_2O_2 may be destroyed to a variable extent on prolonged incubation. A further complication is that hydrophobic compounds tend to be concentrated in the lipid layer of the mitochondria, so that the actual concentration of the substrate around MAO in the lipid environment is uncertain, resulting in artificially low K_m values and uncertainties in the turnover/K_m on which comparison of rates of oxidation of substrates is usually based. Also, rat brain mitochondria are known to contain both MAO A and B; thus, it is not clear which of the two enzymes was responsible for the activity in previous work.

In order to establish valid kinetic data for the oxidation of these compounds by the two forms of MAO, a collaborative study was undertaken by our two laboratories, using highly purified preparations of the two enzymes and a polarographic assay suitable for measuring initial rates of oxidation of MPTP

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* To whom correspondence should be addressed at the Molecular Biology Division, DVA Medical Center, 4150 Clement St., San Francisco, CA 94121.

‡ Department of Biochemistry and Biophysics, University of California, San Francisco.

§ Department of Radiology and Medicinal Chemistry, University of Minnesota.

∥ Division of Toxicology, Department of Pharmacy, University of California, San Francisco, and Molecular Biology Division, Department of Veterans Affairs Medical Center.

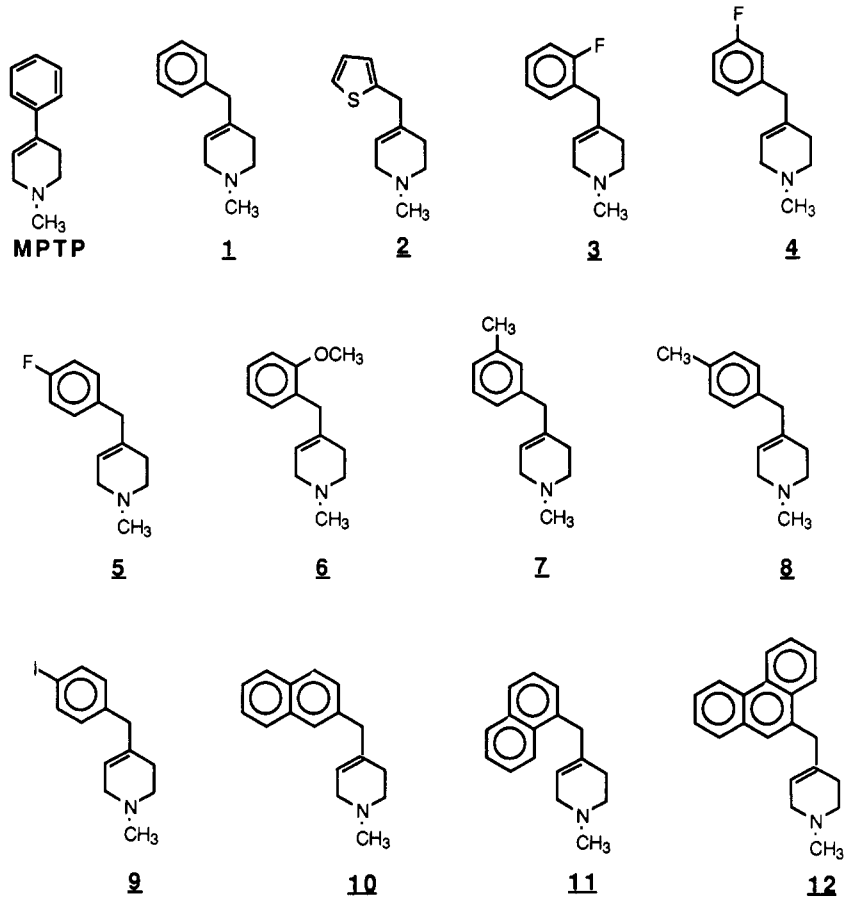

FIGURE 1: Structures of compounds in Table I.

analogs with mitochondria as well as the isolated enzymes (Youngster et al., 1989a). The results of this study are presented below. We include a brief discussion of the reasons why assays of MAO activity in membranous preparations often underestimate the rates of oxidation of various substrates as well as their K_m values.

Materials and Methods

MPTP analogs were synthesized as described (Efange et al., 1990). The pyridinium iodides (compounds **17–19**) were synthesized from the corresponding 4-(arylalkyl)pyridines by reacting the latter with iodomethane in methanol or acetone. The products were subsequently recrystallized from isopropyl alcohol or a combination of isopropyl alcohol and diethyl ether. The compounds were then characterized by NMR and mass spectrometry.

MAO A was isolated from human placenta (Weyler et al., 1985) and MAO B was isolated from beef liver by the procedure of Salach (1979), as modified by Weyler and Salach (1981). All assays were conducted at 30 °C. Initial rates of oxidation of MPTP analogs were measured polarographically in 50 μM sodium phosphate buffer, pH 7.2, containing 0.2% (w/v) Brij-35, in a 1.9-mL total volume. Following temperature equilibration, a small volume (5–20 μL) of MAO A or B was added to start the reaction. The amount of enzyme used was adjusted for each compound so as to permit accurate rate measurements within 60–120 s after initiation of the assay. Turnover numbers were calculated from V_{max}, on the basis of double-reciprocal plots, divided by the flavin content. The inhibition of MAO A and B was determined spectrophotometrically with 0.05–1 mM kynuramine and 0.1–3.3 mM benzylamine as substrates, for MAO A and B, respectively, as in previous work (Youngster et al., 1989a).

Results and Discussion

Oxidation by MAO A and B. Table I presents the rates of oxidation (turnover number at V_{max} with respect to substrates) and K_m values at 30 °C of a series of tetrahydropyridine analogs, some of which represent flexible analogs of MPTP, while others are designed to test the effect of replacement of the phenyl ring of MPTP with other ring systems. The structural formulas for the compounds are given in Figures 1 and 2. The relative effectiveness of the various tetrahydropyridines as substrates of MAO A or B were evaluated in terms of the function turnover number/K_m, as in our previous studies (Youngster et al., 1989a; Efange et al., 1990).

As evident from the data, the MPTP analog **1** is a relatively poor substrate of MAO A, compared with kynuramine, comparable to MPTP (TN/K_m = 143; Youngster et al., 1989a). In contrast, **1** is an excellent substrate of MAO B, exceeding in effectiveness both benzylamine, the standard substrate of that enzyme, and MPTP (Table I) (Youngster et al., 1989a). Replacement of the pendant phenyl group of **1** with the thienyl moiety has no significant influence on the effectiveness as a substrate of MAO A, but it substantially enhances it in the case of MAO B (**2** vs **1**). This is due to both a higher maximal rate of oxidation and a lower K_m value. Small electron-withdrawing groups, like fluorine, enhance the rate of oxidation by MAO A (compare the TN values of **3** to **5** vs **1**), but only in **3**, the 2′ substituted analog, is this reflected in the TN/K_m value, because the 3′ and 4′ substituted compounds (**4** and **5**) have significantly increased K_m values compared with that of **1**, which neutralizes the rate effect. With MAO B, **3** and **5** are as good substrates as **1**, while the 3′-F analog (**4**) shows nearly twice the TN/K_m value. The latter finding is in agreement with our previous report of the effect of fluorine substitution at 3′ in enhancing the reactivity of MPTP with

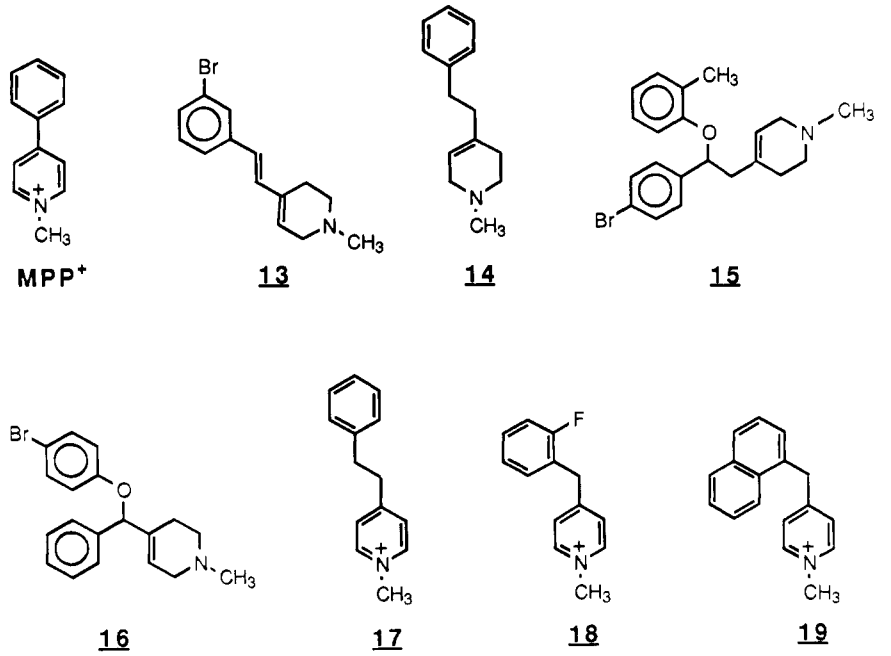

FIGURE 2: Structures of compounds in Table II.

Table I: Oxidation of Novel MPTP Analogs by Highly Purified MAO A and B

	MAO A			MAO B		
compound	TN[a]	K_m (mM)	TN/K_m	TN[a]	K_m (mM)	TN/K_m
kynuramine	146	0.17	860			
benzylamine				283	0.29	963
MPTP[b]	20	0.14	143	204	0.39	523
1	9.2	0.066	139	193	0.154	1250
2	8.4	0.048	175	337	0.102	3270
3	20.2	0.034	594	182	0.126	1440
4	30.8	0.208	148	191	0.082	2330
5	27.7	0.14	198	142	0.124	1140
6	28.8	0.0725	398	106	0.735	145
7	58.7	0.122	480	158	0.262	603
8	17.6	0.0181	973	150	0.244	615
9	76	0.042	1830	144	0.121	1190
10	121	0.036	3340	276	0.892	310
11	433	0.167	2590	89	0.847	105
12	365	0.48	759	>53[c]		
13	(113)[d]	(4)	(28)	24.6	4.16	6
14	137	0.123	1120	61	0.054	1130
15	33.6	0.462	73	16.5	1.49	11
16	19.2	0.73	26	2.3	0.068	34

[a] TN, turnover number from double-reciprocal plots at 30 °C expressed as micromoles of substrate oxidized per minute per micromoles of enzyme. [b] From Youngster et al. (1989a). [c] Activity at solubility limit. [d] Lineweaver–Burk plots are biphasic, possibly because of the presence of cis and trans isomers.

MAO B (Youngster et al., 1989a).

Lipophilic groups substituted at either the 2′, 3′, or 4′ position of the aromatic ring (**6** to **9** vs **1**) significantly increased the effectiveness as a MAO A substrate, in contrast to the MPTP series where alkyl substitution increased reactivity with MAO A only if the alkyl substitution was at 2′ (Youngster et al., 1989a). The greatest increase in activity brought about by lipophilic substitution was observed with the naphthyl-containing analogs **10** and **11** in the present work. The latter compounds are 18–24 times more reactive than 4-homo-MPTP, **1**. Their enhanced effectiveness is primarily attributed to an increase in TN. Since these two compounds may be regarded as disubstituted (4′,5′- and 5′,6′-) analogs of the parent molecule **1**, the dramatic increase in reactivity may suggest an additive effect of disubstitution on substrate activity. It may also be significant that the β-substituted naphthalene **10** binds significantly better than its corresponding α isomer **11**. This disparity may be indicative of subtleties in the topography of the substrate site of MAO A. Compound **12**, a hybrid between **10** and **11**, is a significantly better substrate than **1** but not as good as either naphthyl analog because of its high K_m. This may be indicative of the optimum bulk tolerance for this region of the substrate binding site of MAO A.

In contrast to the effect of lipophilic substitution on effectiveness as a MAO A substrate, it failed to enhance reactivity with MAO B (**6**, **7**, **8**, **10**, **11**). In fact, the fused bicyclic analogs **10** and **11** are among the poorest substrates of MAO B in this series, mostly because of the high K_m values, in sharp contrast to their rapid oxidation by MAO A. Compound **9** is an exception, since despite the lipophilic substitution it is oxidized nearly as well as **1** by MAO B.

The large disparity between the TN/K_m values of **8** and **9** with MAO A deserves comment. The difference in reactivity is in part in K_m values (0.0181 vs 0.042), which may be a reflection of preferential binding because of differences in the bulks of the *p*-I-benzyl and *p*-CH_3-benzyl groups. However, the increase in TN is greater (17.6 vs 76) and does not seem to be entirely due to differences in the van der Waals radii of the *p*-fluoro substituents but may also involve electronic effects, all the more since the TN of the *p*-fluoro-substituted **5** is also significantly higher than that of **8**, despite the smaller but still electronegative substituent. Although in terms of conventional solution chemistry inductive effects of the *p*-iodo substituent on deprotonation of the pyridine ring would not be expected because of the absence of conjugation, in the structure of the enzyme–substrate complex several possibilities exist for inductive effects, including enhanced reactivity of the overlapping flavin or stabilization of a intermediate.

Although the enhanced effectiveness as substrates (in terms of either V_{max} or K_m) by both steric and electronic influences of substituents is evident for both enzymes, the effects of such substitution on the V_{max} and K_m are variable. As suggested above, this variability may be partly due to the location and nature of the substituent. Since the steric demands of MAO B are more stringent than those of MAO A, an examination of the kinetic parameters of those analogs which appear to fit

the steric demands of both isozymes may reveal, for the low end of the bulk tolerance spectrum, the relative contributions of steric and electronic influences on substrate reactivity. For MAO B, when the substituent size is larger than methyl or iodo, steric factors are the main determinant of the reactivity of substrates. However, for methyl, iodo, or smaller substituents, electronic effects seem to play a role (compare TN/K_m values for **4** vs **7**, **5** vs **8**, and **5** vs **9**). In contrast to the effects observed with MAO B, in the case of MAO A effectiveness as a substrate of A is heavily influenced by hydrophobicity (compare **1** vs **6**, **7**, **8**; **4** vs **7**; **5** vs **8**; and **5** vs **9**), despite consistent increases in V_{max} induced by small electron-withdrawing groups (compare V_{max} values for **1** vs **3**, **4**, and **5**). Compound **9** is interesting in that it may suggest a cumulative beneficial effect of hydrophobicity and electron withdrawal (compare TN/K_m values for **5** vs **9** for MAO A). Bulky substituents at the 2′ position of MPTP have been reported to have similar effects in decreasing TN/K_m for MAO B as the alkyl chain length increases, while reactivity with MAO A increased in lengthening the alkyl substituent (Youngster et al., 1989a).

Increased flexibility afforded by an ethylene bridge (**14** vs **1**) dramatically increased the TN and, hence, the TN/K_m for MAO A, while branching (**15**, **16**) and rigidity of the molecular skeleton (**13**) lowered the effectiveness as a substrate for both MAO A and B (compare the TN/K_m of **13** in Table I with the value of 2036 reported for 3′-Br-MPTP; Youngster et al., 1989a). In contrast to 4-homo-MPTP, **1**, **14** exhibits no selectivity for either enzyme (compare TN/K_m values). This apparent loss of selectivity suggests that the conformational constraints imposed by the tetrahedral center in **1** are optimum for probing the topographies of MAO A and B. Sablin et al. (1990) have also compared a series of MPTP derivatives with the corresponding tetrahydrostilbazole derivatives with respect to V_{max} and K_m for MAO A and B and reported that the increased rigidity of the analogs leads to far lower V_{max} but increased apparent affinity for MAO B (i.e., lower K_m values). In an earlier study using rat brain mitochondria (Efange et al., 1990), the flexible **14** was shown to be significantly more reactive than the corresponding rigid tetrahydrostilbazole. Given the similarity between the dimensions of both compounds, the poor reactivity of the tetrahydrostilbazole was attributed to increased rigidity. This detrimental effect of rigidity on the reactivity of substrates led to the suggestion that the flexible **14**, in contrast to the rigid tetrahydrostilbazole, may bind MAO A or B in a form other than that depicted by its extended linear conformation.

Taken together, these observations suggest that MAO B is much less tolerant of steric bulk than MAO A. In terms of TN/K_m, MAO B appears to act preferentially on **1**, **2**, and those analogs which contain small electron-withdrawing substituents on the pendant phenyl group of **1**. On the other hand, analogs with a bulky pendant group (e.g., **10**, **11**, and **12**) are better substrates of MAO A than **1** or MPTP. Thus, one observes a crossover in selectivity of the two enzymes as the steric bulk of the pendant moiety increases from thienyl to phenanthryl. On the basis of these studies and previous reports in the literature, the substrate binding site of MAO A would appear to be larger than that of MAO B. Furthermore, the relatively high reactivity of the naphthyl analogs **10** and **11** with MAO A points to the importance of nonbonded hydrophobic interactions at the substrate binding site of MAO A; such interactions may be attributed to the presence of a hydrophobic pocket within this region of the enzyme. These conclusions are consistent with those arrived at from an earlier study by one of us of semirigid MPTP analogs (Efange & Boudreau, 1991).

Table II: Inhibition of MAO A and B by Flexible Analogs of MPP^+

compound	MAO A K_i (μM)	MAO B IC_{50}[a] (μM)
MPP^+[b]	3.0	230
17	37	12
18	29	2000
19	5.5	850

[a] Secondary plots replotted from primary double-reciprocal plots were curved. Thus, true K_i values could not be determined. IC_{50} values are at 0.1 mM benzylamine. [b] From Youngster et al. (1989a).

Several of the compounds in Figures 1 and 2 were tested for dopaminergic neurotoxicity in Swiss–Webster mice. As we have reported elsewhere (Singer et al., 1992), compounds **2**, **3**, **5**, **9**, and **11** did not show the neurotoxic effects of MPTP, since the neostriatal dopamine and DOPAC levels were normal. Since the compounds tested were very good substrates of MAO A or MAO B or both, as is the case with the benzyl analog of MPTP (Youngster et al., 1989a), which is rapidly oxidized by MAO B without being neurotoxic (Youngster et al., 1989b), Youngster et al. (1989b) suggested that a possible reason for the lack of neurotoxicity of 1-methyl-4-benzyl-1,2,3,6-tetrahydropyridine is that the pyridinium form is not the product of the 4-electron oxidation step; in fact, none of the expected pyridinium product was detected in experiments with brain mitochondria. The same possibility arises with the MPTP analogs used in this paper. It has been often stated that oxidation by MAO is necessary but not sufficient for a tetrahydropyridine to be neurotoxic. The oxidation must yield a pyridinium derivative, which is concentrated by the dopamine carrier. The positively charged oxidation product must be concentrated by the mitochondrial electrochemical gradient and be able to enter and be bound to the hydrophobic "rotenone" site on NADH–Q oxidoreductase (NADH dehydrogenase) and inhibit that enzyme. For the compounds listed in Table I, it will require synthesis of the corresponding pyridinium analogs in order to decide which of these events is responsible for the lack of neurotoxicity of the tetrahydropyridines used in this study.

Flexible MPP^+ Analogs as Inhibitors of MAO A and B. Previous studies have shown that MPP^+ and its analogs are excellent reversible competitive inhibitors of MAO A but poor inhibitors of MAO B. (Salach et al., 1984; Singer et al., 1986; Youngster et al., 1989a). It was of interest to see how the increased flexibility afforded by insertion of the methylene or ethylene bridge between the rings affects the inhibitory potency of these compounds. Table II presents the results for three of the compounds, the structures of which are given in Figure 2.

It is seen that none of the compounds tested was as good a competitive inhibitor of MAO A as MPP^+. Of the three new analogs, **19**, the α-naphthylpyridinium was the most tightly bound to MAO A, in accord with the high TN/K_m value of MAO for **11**, the parent tetrahydropyridine. In corresponding studies with MAO B, all three compounds tested gave curved secondary plots of concentration vs the slope derived from primary Lineweaver–Burk plots, in contrast to MPP^+ analogs previously tested by us. The inhibition of MAO B is therefore expressed as IC_{50} at 30 °C in the presence of 0.1 mM benzylamine in Table II. While **18** and **19** proved to be ineffective inhibitors of MAO B, as is true of most other MPP^+ analogs (Youngster et al., 1989a; Jin et al., 1990), **17** was surprisingly potent with an IC_{50} value of 12 μM. This is in accord with the low K_m value of **14**, the parent tetra-

hydropyridine for MAO B (Table I).

Unpublished collaborative studies between Bachurin's laboratory and that of the junior author have confirmed that the rigid stilbazole analogs of MPP^+ are indeed very tightly bound to MAO A, with K_i values in the nanomolar range in several instances. Given this observation, the decreased inhibitory potency of the flexible **17** (a partially hydrogenated *N*-methylstilbazolium cation) for MAO A may be attributed to the absence of unsaturation between the two rings. Such unsaturation would create an extended conjugated system and thereby permit electron delocalization throughout the molecule.

Comparison with Previously Reported Data. The data reported here using highly purified preparations of MAO A and B help explain previous observations on several of these compounds (Efange et al., 1990). Using brain mitochondria, which contain both types of MAO, it was reported that the oxidation of some compounds (e.g., MPTP, **1**) was extensively inhibited by 2.5 μM pargyline and the oxidation of several other compounds (e.g., **7**, **14**) was only partly inhibited, while the processing of **11** was essentially unaffected. At 2.5 μM concentration, pargyline would be expected to block MAO B completely but have only a small effect on MAO A. These findings agree with the relative rates of oxidation of these substrates by the two types of MAO reported in Table II.

In contrast, the V_{max}/K_m values reported earlier are more difficult to rationalize, since most of the compounds are A–B substrates and the relative concentrations of the two types of MAO in the preparation used is not known. With few exceptions (MPTP, **14**) the K_m values reported by Efange et al. (1990) are significantly lower than those reported for the individual enzymes in this paper. One reason for this may be that because of their hydrophobic nature these compounds tend to concentrate in the lipid phase of the mitochondria, so that the concentration in the immediate environment of the MAO may be considerably higher than in the surrounding solution.

Since several of the substrates used in this study are primarily MAO B substrates (**1**, **2**, MPTP) their turnover numbers relative to benzylamine with pure MAO B in Table I may be compared with the corresponding ratios of the V_{max} values reported for mitochondria, despite the presence of MAO A (Efange et al., 1990). While for compound **1** this ratio is quite different, for MPTP and **2** they are in reasonable agreement. This is surprising, considering that values derived from initial rate measurements are being compared with a single time point taken after a long incubation, during which the catalase present in contaminating peroxisomes may deplete the accumulated H_2O_2, and both reversible inhibition of MAO by the products and mechanism-based inactivation may occur (Singer et al., 1986).

Lastly, we wish to point out that the values given in Table I of this paper are not absolute but represent V_{max} with respect to the substrate at air saturation, so as to permit comparison with other data in the literature, which are in most cases based on assays conducted in air, not extrapolated to infinite oxygen concentration. It should be noted, however, that so far the oxidation of only one substrate (phenylethylamine) of either type of MAO has been reported to be greatly increased at higher O_2 concentrations (Husain et al., 1982).

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