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THE KINETICS OF MONOAMINE OXIDASE INHIBITION BY THREE 2-INDOLYLMETHYLAMINE DERIVATIVES

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Abstract—The inhibition of bovine brain mitochondrial MAO-A and MAO-B by three acetylenic and non-acetylenic derivatives of 2-indolylmethylamine, chosen among more than 100 new compounds, were studied. The non-acetylenic derivative *N*-methyl-2(5-hydroxy-1-methylindolyl)methylamine (**1**) was a weak non-selective inhibitor which was shown to act in a reversible and competitive manner towards the deamination of tyramine. The two acetylenic derivatives *N*-methyl-*N*-(2-propynyl)-2-(5-benzyloxy-1-methylindolyl)methylamine (**2**) and *N*-methyl-*N*-(2-propynyl)-2-(5-hydroxy-1-methylindolyl)methylamine (**3**) were potent MAO inhibitors, one of them non-selective (compound **2**) and the other MAO-A selective inhibitor (compound **3**). Both of them were irreversible and competitive inhibitors, compound **2** towards the deamination of tyramine and compound **3** towards the deamination of serotonin and *β*-phenylethylamine. A mechanism for the inhibition of the enzyme by both irreversible inhibitors is proposed and the inhibition parameters are determined.

Monoamine oxidases A and B (MAO-A and MAO-B, respectively) [monoamine: O_2-oxidoreductase (deaminating) (flavin-containing) EC 1.4.3.4], catalyse the oxidative deamination of monoamines to give the corresponding aldehydes. These isoenzymes are distinguished by differences in substrate preference, inhibitor specificity, tissue and cell distribution and immunological properties [1]. The efficacy of MAO-A inhibitors to treat depression [2] and of *l*-deprenyl (selegiline), a selective MAO-B inhibitor, to treat Parkinson's disease in combination with L-Dopa [3] is presently recognized.

In previous papers [4–8] we reported the synthesis and preliminary assays of MAO inhibition and selectivity for a new series of acetylenic and allenic derivatives of 2-indolylmethylamines of general formula I (Fig. 1), although we could not establish a quantitative relationship on the basis of the IC_{50} determined values for MAO-A and -B. In order to obtain information on their mechanism of action, we carried out a more detailed study of the kinetics of MAO inhibition by three of those compounds: *N*-methyl-2-(5-hydroxy-1-methylindolyl)methylamine (**1**), *N*-methyl-*N*-(2-propynyl)-2-(5-benzyloxy-1-methylindolyl)methylamine (**2**), *N*-methyl-*N*-(2-propynyl)-2-(5-hydroxy-1-methylindolyl)methylamine (**3**) (Fig. 1). These compounds were chosen among all the studied compounds (more than 100), because they showed very different inhibition behaviours and were structurally related. In this paper we report the results of such study.

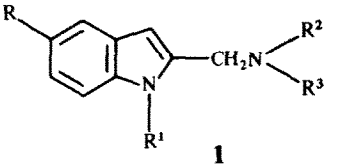

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Fig. 1. Structure of the compounds. R = H—, CH_3O—, $C_6H_5CH_2O$—, HO—; R^1 = H—, H_3C—; R^2 = H—, H_3C—; R^3 = $HC{\equiv}CCH_2$, $H_3CC{\equiv}CCH_2$—, $H_2C{=}C{=}CHCH_2$—,

Compound number	R	R^1	R^2	R^3
1	HO—	H_3C—	H_3C—	H—
2	$C_6H_5CH_2O$—	H_3C—	H_3C—	$HC{\equiv}CCH_2$
3	HO—	H_3C—	H_3C—	$HC{\equiv}CCH_2$

MATERIALS AND METHODS

Materials. The radioactively labelled substrates [^{14}C]tyramine.HCl, [^{14}C]*β*-phenylethylamine.HCl and [^{14}C]serotonin creatinine sulfate were commercially available (Amersham International, Bucks., U.K.) and so was harmine (Sigma Chemical Co., Poole, U.K.). Compounds **1** and **3** were prepared as described [8], as well as compound **2** [7]. Clorgyline and *l*-deprenyl were a gift from Mrs J. Fynn (May and Baker Ltd, Dagenham, U.K.) and Dr J. Knoll (Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary), respectively, or synthesized by us [4].

Enzyme preparation. Bovine brain mitochondria were prepared as reported [9]. The isolated mitochondria were dispersed in ice-cold 5 mM potassium phosphate buffer, pH 7.3, to give a protein concentration of 68 mg/mL as determined by the microbiuret method [10], and this suspension was stored frozen at −20° until use. The enzymatic activities of these mitochondrial preparations were 30–35% for MAO-B and 65–70% for MAO-A, as determined with tyramine as substrate and clorgyline as inhibitor.

Enzymatic assays. Enzymatic activity was determined radiochemically by the methods of Otsuka

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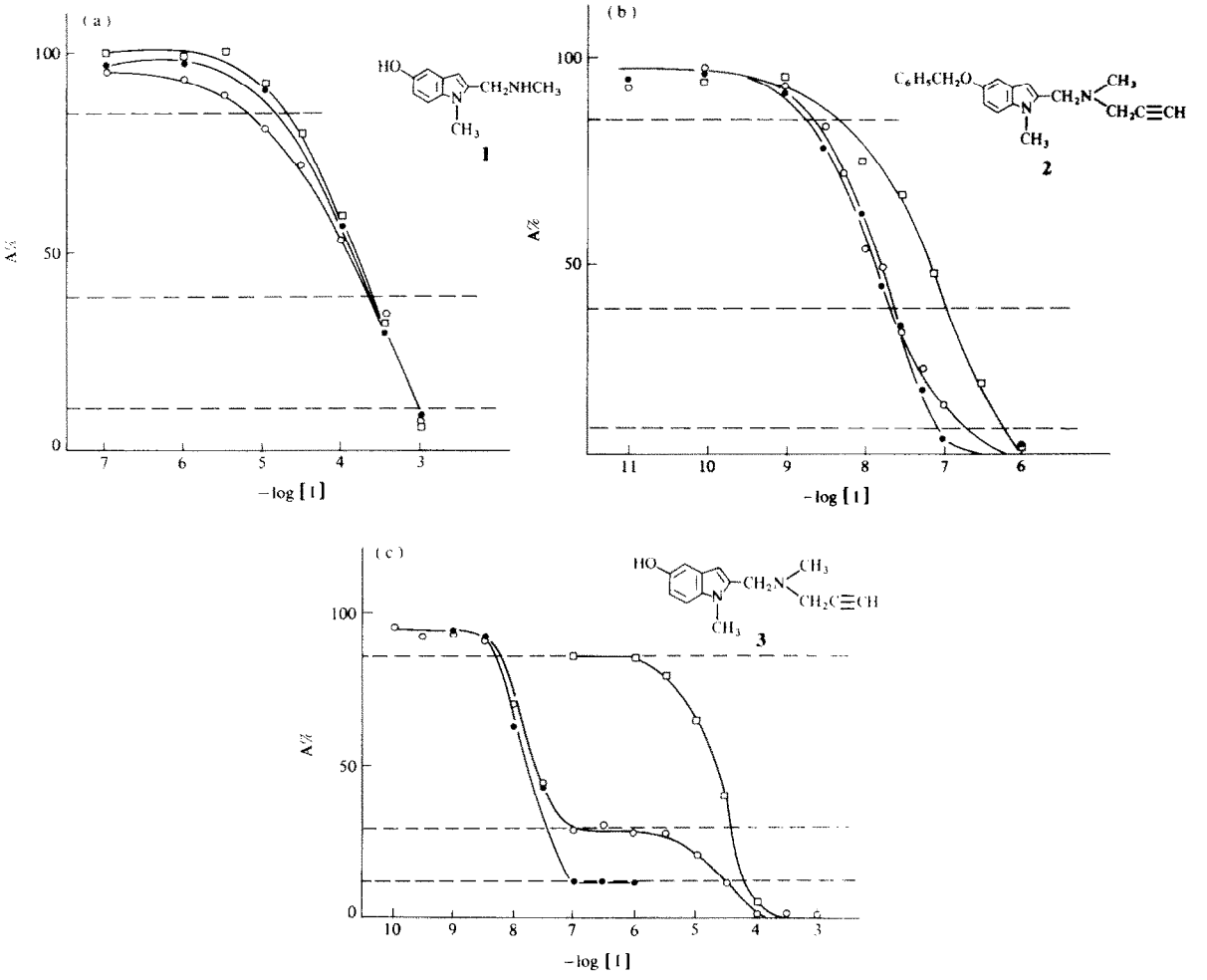

Fig. 2. Selectivity test. For each assay, bovine brain mitochondria (1.4 mg/mL of protein, 2 Tabor units/mL) were preincubated for 20 min at 37° with several inhibitor concentrations (from 0.01 nM to 1.0 mM). The remaining activity was determined in potassium phosphate buffer, pH 7.3 (0.5 mL), with (○) [^{14}C]tyramine (0.5 mM, 0.8 Ci/mol), (●) [^{14}C]serotonin (0.5 mM, 1.6 Ci/mol) or (□) [^{14}C]β-phenylethylamine (0.01 mM, 8 Ci/mol), at 37° for 20 min. The reaction was stopped by addition of 2.5 M citric acid (0.2 mL) and the products extracted into (1:1 v/v) toluene/ethyl acetate. Aliquots of these extracts were treated in the usual way and submitted for scintillation counting.

Table 1. IC_{50} values for MAO inhibition

Compound number	IC_{50} (MAO-A) (M)		IC_{50} (MAO-B) (M)	
	Tyramine	Serotonin	Tyramine	β-Phenylethylamine
1	1.4×10^{-4}	1.0×10^{-4}	1.4×10^{-4}	2.0×10^{-4}
2	1.4×10^{-8}	1.0×10^{-8}	1.4×10^{-8}	1.4×10^{-7}
3	1.3×10^{-8}	1.3×10^{-8}	2.0×10^{-5}	2.5×10^{-5}
Clorgyline	5.3×10^{-9}	5.0×10^{-9}	4.5×10^{-6}	1.0×10^{-5}
l-Deprenyl	1.3×10^{-6}	—	1.0×10^{-8}	—

IC_{50} values were determined from 8 to 16 concentrations of inhibitors in duplicate.

and Kobayashi [11] and Wurtzman and Axelrod [12] as adapted by us, with tyramine as common substrate of MAO-A and -B [6], and with serotonin as selective substrate for MAO-A and with β-phenylethylamine as selective substrate for MAO-B, by similar methods. All assays were performed at 37° in 30 mM potassium phosphate buffer, pH 7.3. For standardization of enzyme preparations, MAO-B was also estimated by Tabor's spectrophoto-metric method [13]. Inhibitors were added to the reaction

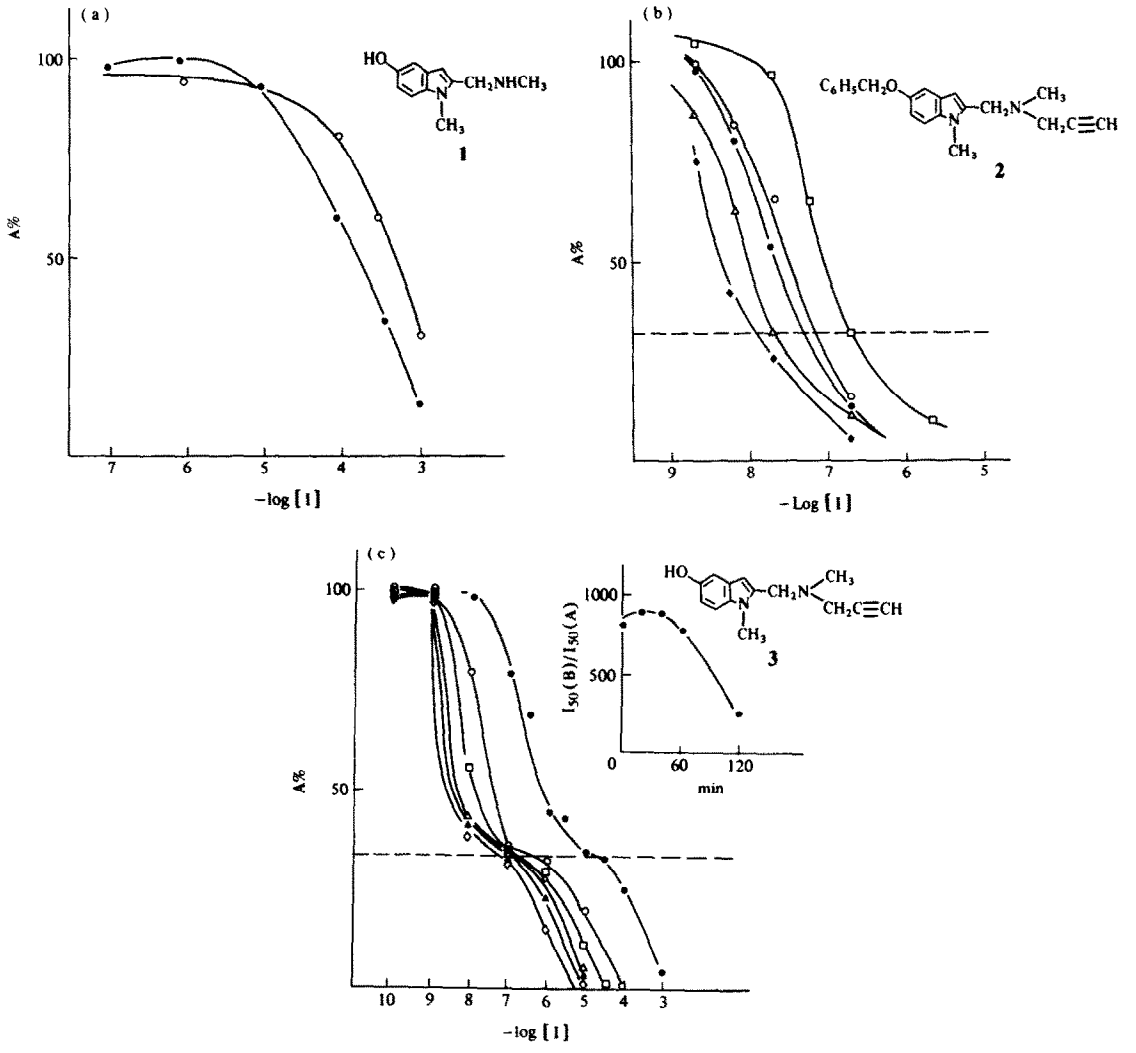

Fig. 3. Effect of preincubation time on selectivity. Assays were carried out with tyramine as substrate by the method described in Fig. 2 at the following preincubation times: (a): (○) 0; (●) 1 hr. (b): (□) 0; (○) 5 min; (●) 10 min; (△) 25 min; (◆) 1 hr. (c): (●) 0; (○) 10 min; (□) 20 min; (△) 40 min; (▲) 1 hr; (◇) 2 hr.

mixture in aqueous solution (**1** and **3**) or dimethylsulphoxide (DMSO) solution (**2**); in the latter case, it was verified that DMSO did not affect the enzymatic activity at the employed concentrations (maximum 4% v/v in assay mixture).

In time-dependent studies of inhibition by compounds **2** and **3**, brain MAO-A and -B mitochondria preparations (7 mg/mL of protein and 16 Tabor units/mL) were preincubated at 37° for 60 min with either 0.3 μM *l*-deprenyl or 0.3 μM clorgyline in order to inhibit selectively the activity of MAO-B or MAO-A, respectively [14].

All assays were carried out in duplicate or triplicate, and results are mean values from at least three independent experiments.

RESULTS AND DISCUSSION

Selectivity test

Compounds **1**, **2** and **3** were studied as inhibitors with tyramine as common substrate for MAO-A and -B, serotonin as selective MAO-A substrate and β-phenylethylamine as selective MAO-B substrate. IC_{50} values, molar concentrations of inhibitor producing 50% inhibition, were determined graphically from plots of residual activity percentage versus −log [*I*]. The residual activity percentage was calculated in relation to a sample of enzyme treated in the same conditions without inhibitor. When biphasic diagrams were obtained with tyramine as substrate, indicative of selectivity, both IC_{50} values were determined using clorgyline and *l*-deprenyl as references. Figure 2a–c shows the inhibition plots obtained for compounds **1**, **2** and **3**, respectively, and Table 1 summarizes the determined IC_{50} values for these compounds and for clorgyline and *l*-deprenyl.

These results show that compound **1** is a weak non-selective MAO inhibitor, compound **2** is a potent non-selective MAO inhibitor and compound **3** is a potent selective MAO-A inhibitor.

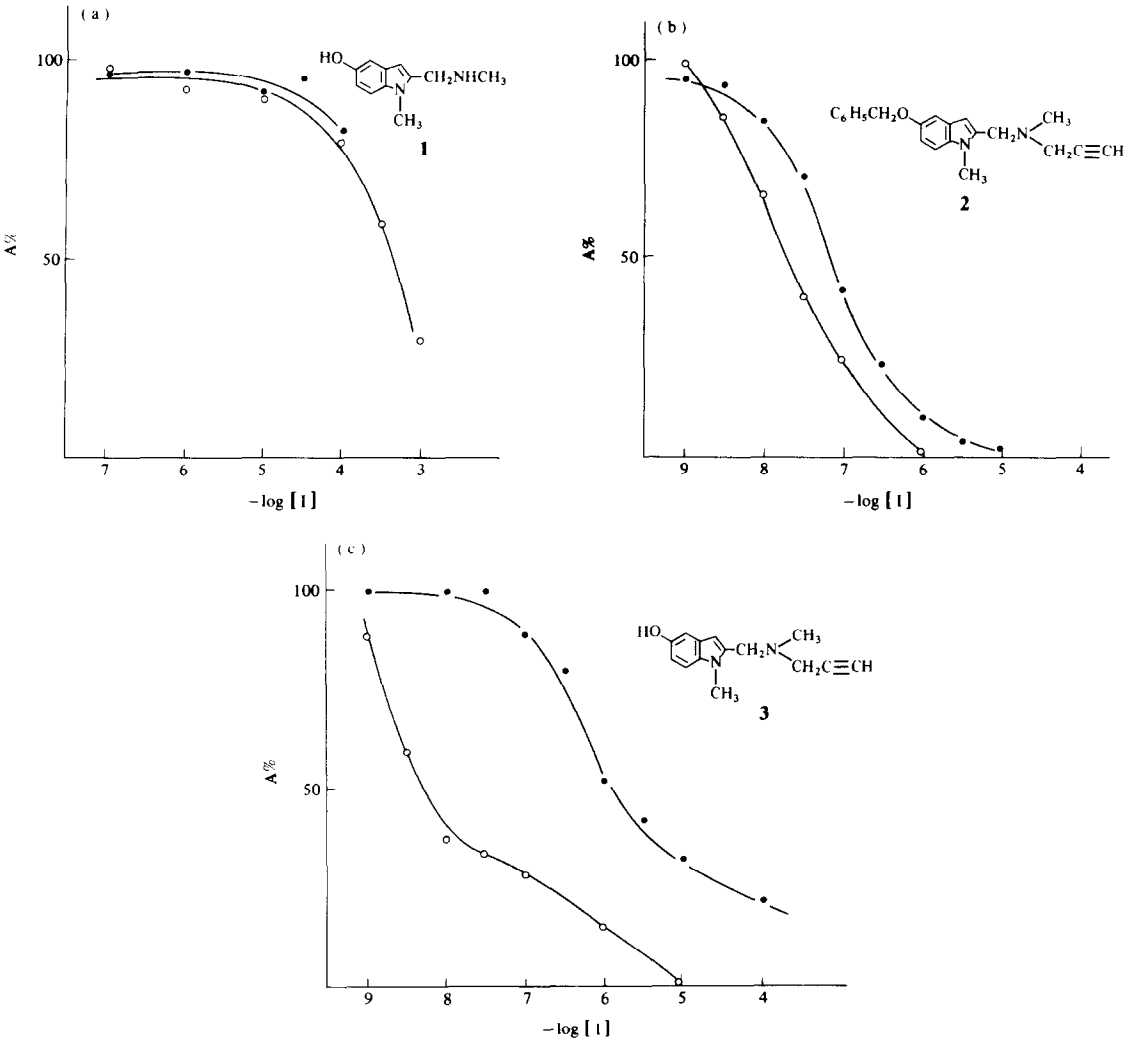

Fig. 4. Dilution test of reversibility. (●) These assays were carried out in the usual way with tyramine as substrate without preincubation of the enzyme with the inhibitor. (○) Brain mitochondrial MAO was preincubated at 37° for 1 hr (compounds **1** and **3**) or 20 min (compound **2**), with several inhibitor concentrations in a concentrated mixture (40 μL). After that, the mixture was diluted with 30 mM potassium phosphate buffer, pH 7.3, to 0.5 mL, and the residual activity was determined by the usual procedure.

Selectivity variation against preincubation time

For examining the variation on selectivity, we studied the behaviour of the compounds with tyramine as substrate at several preincubation times. Figure 3a–c shows the obtained plots for compounds **1**, **2** and **3**, respectively.

In the case of inhibition by compound **1**, its IC_{50} value was practically invariable with preincubation time, and so was the selectivity. However, inhibition by compounds **2** and **3** was time-dependent. Inhibitor **2** was non-selective at all studied times, while inhibitor **3** was always selective towards MAO-A, but this selectivity decreased over 40 min of preincubation time.

Reversibility tests

The reversibility or irreversibility of MAO inhibition was examined by two methods. The first one (sedimentation test) was carried out by incubation of a mitochondrial preparation sample with each inhibitor, at a sufficient concentration to cause more than 70% inhibition, for 30 min at 37°. The mitochondria were then sedimented by centrifugation at 26,000 *g* for 45 min and the pellet was washed twice by resuspension and centrifugation with 30 mM potassium phosphate buffer, before it was finally resuspended in that buffer and assayed. Control samples of mitochondria were carried through an identical procedure, the inhibitor solution being replaced by an equal volume of distilled water (for compounds **1** and **3**) or DMSO (for compound **2**). Failure to recover activity after the washing procedure was taken as an indication that the inhibition by compounds **2** and **3** was irreversible, whereas total recovery of the activity indicated that the inhibition by compound **1** was reversible.

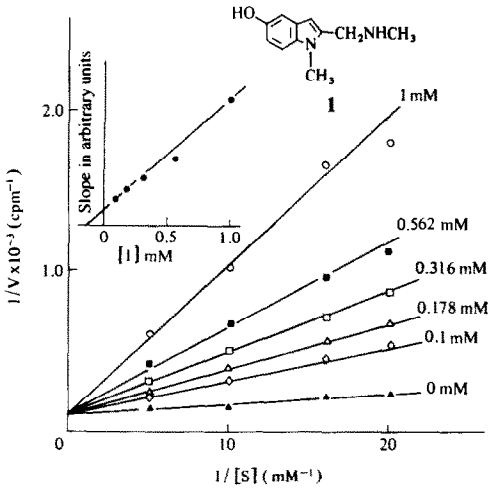

Fig. 5. Double-reciprocal and secondary plots of the inhibition of tyramine deamination by compound **1**. Mitochondria from bovine brain were incubated at 37° for 15 min with the indicated inhibitor concentrations (from 0.1 mM to 1 mM), in the presence of several concentrations of tyramine (from 0.05 mM to 0.2 mM), in 30 mM potassium phosphate buffer, pH 7.3 (0.5 mL). The enzymatic reaction was stopped by addition by 2.5 M citric acid (0.2 mL) and the samples processed in the usual way. All values are means of determinations in three mitochondrial fractions. Secondary plots of slopes (calculated by linear regression analysis) against inhibitor concentrations are shown in figure insets.

The above results were confirmed by the second method (dilution test) that was carried out by preincubation of mitochondrial preparation with several inhibitor concentrations in a concentrated mixture. After dilution (12.5-fold) of the mixture, the inhibition by compound **1** was the same as that found with this compound of the same final concentrations of enzyme and inhibitor without preincubation, whereas in the case of compounds **2** and **3**, the inhibition was less than without preincubation, as shown by Fig. 4a–c, for compounds **1**, **2** and **3**, respectively.

Inhibition patterns

Reversible inhibitor: compound **1**. Kinetic experiments indicate that compound **1** inhibited deamination of tyramine competitively (Fig. 5). The K_i value estimated from the slopes of the double reciprocal plot was 0.11 ± 0.05 mM, close to the K_m value of tyramine (0.06 mM). This result was confirmed by the Dixon method of plotting ($1/v$ vs $[I]$).

Further studies will be necessary to investigate the possible behaviour of compound **1** as MAO substrate.

Irreversible inhibitors: compounds **2** *and* **3**. MAO irreversible inhibition by propargylamine inhibitors, such as compounds **2** and **3**, is believed to occur via the preliminary formation of an intermediate reversible complex between the enzyme and the inhibitor, followed by covalent attachment of the

Table 2. K_i values for MAO inhibition by compounds **2** and **3**

Compound number	K_i (MAO-A) (μM)	K_i (MAO-B) (μM)
2	0.07 ± 0.02	0.07 ± 0.02
3	0.09 ± 0.05	100 ± 20

All values are means from at least three independent experiments.

Table 3. Protection effect by substrate

Compound number	Serotonin concentration in preincubation mixture (μM)	A (%)
2	0	0
	40	3
	400	3
3	0	13
	0.32	11
	3.2	12
	32	12

inhibitor to the flavin prosthetic group of the enzyme, according to the following scheme [15]:

$$E \underset{}{\overset{K_I}{\rightleftharpoons}} E.I \xrightarrow{k_2} E\text{–}I.$$

MAO selectivity may result from differences in the affinities of the two isoenzymes for reversible combination with the inhibitor, from differences in the irreversible rates of reaction to form the covalent complex, or from a combination of both of these factors [16].

If the second process is relatively slow ($K_I = K_i$), it may be possible to determine K_i directly by measuring the 'reversible' inhibition only [17]. We studied the inhibition by compounds **2** and **3** with the same procedures we used with compound **1**. The initial phase of their MAO inhibition was investigated by starting the reaction by addition of enzyme to the substrate/inhibitor mixture and short incubation times were used to ensure that no significant irreversible inhibition occurred during the reaction period. Details of assays conditions are shown in Fig. 6.

Compound **2** appears to be competitive towards tyramine and compound **3** appears to be competitive towards serotonin and β-phenylethylamine, with the K_i values shown in Table 2. Both of them are powerful inhibitors, compound **2** towards MAO-A and B, and compound **3** towards MAO-A but not towards MAO-B.

On the other hand, it is known that the presence of a substrate or a competitive inhibitor will protect an enzyme against the effects of an irreversible inhibitor that combines with the active site by reducing the amount of free enzyme available for combination with it [17]. Bovine brain mitochondria (16

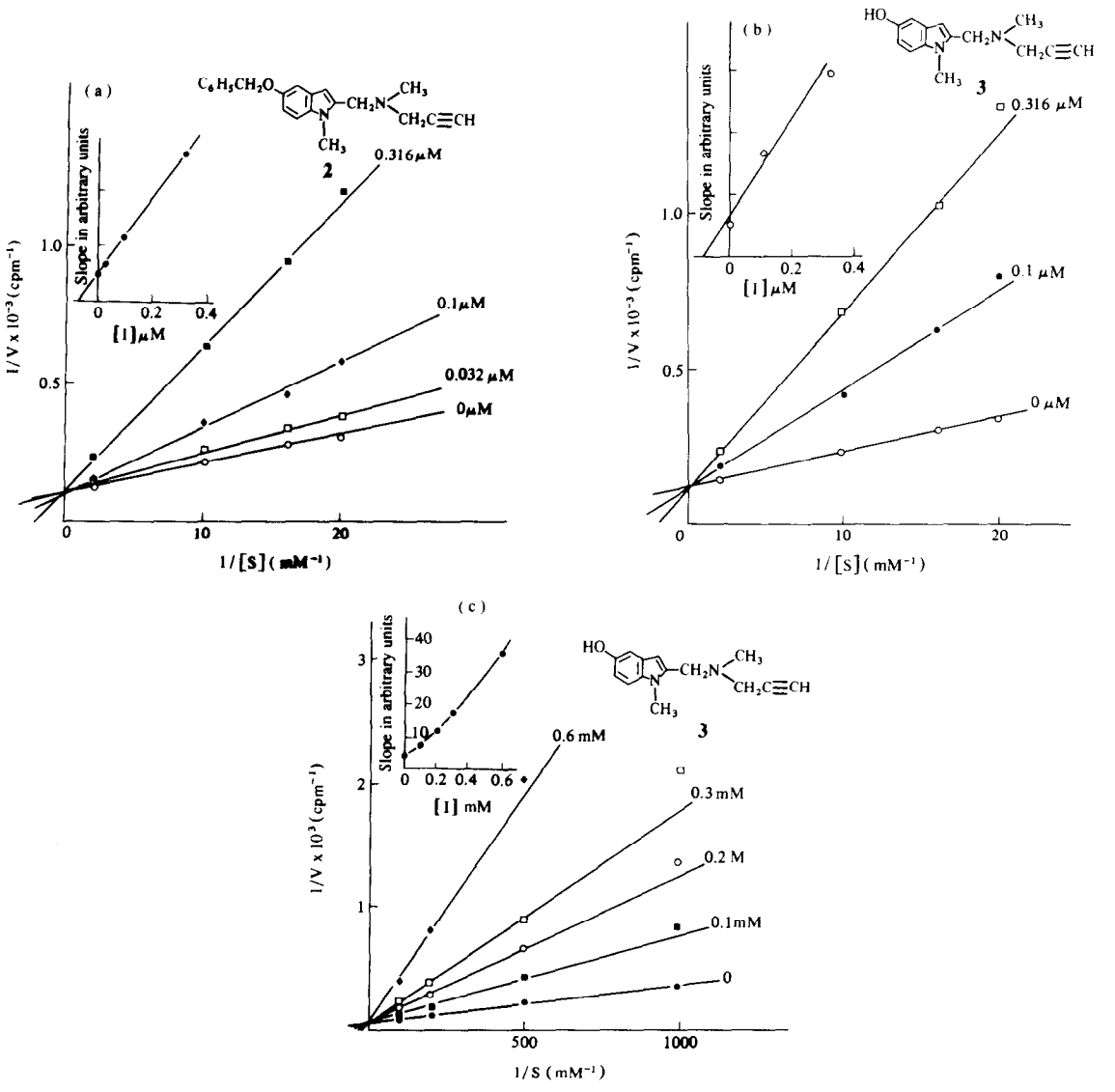

Fig. 6. Double-reciprocal and secondary plots of inhibition by compounds **2** and **3**. (a) Double-reciprocal and secondary plots of the inhibition of tyramine deamination by compound **2**. Assays were carried out by the method described in Fig. 5, with tyramine concentrations ranging from 50 μM to 0.5 mM, and inhibitor concentrations from 32 nM to 0.32 μM. (b) Double-reciprocal and secondary plots of the inhibition of serotonin deamination by compound **3**. Assays were carried out in the same way, with serotonin concentrations ranging from 50 μM to 0.5 mM, and inhibitor concentrations from 0.1 μM to 0.32 μM. (c) Double-reciprocal and secondary plots of the inhibition of β-phenylethylamine deamination by compound **3**. Assays were carried out as described above, with β-phenylethylamine concentrations ranging from 1 μM to 10 μM, and inhibitor concentrations from 0.1 mM to 0.6 mM.

Tabor units/mL, 11 mg/mL of protein) were preincubated at 37° for 15 min with several inhibitor (compounds **2** and **3**) concentrations in the presence of several serotonin or competitive reversible inhibitor (compound **1** or harmine, **4**) concentrations. The residual enzymatic activity was determined by the usual procedure with serotonin as substrate, after dilution for avoiding, as much as possible, the reversible inhibition due to compounds **1** or **4**. This residual enzymatic activity was obtained in relation to two different control samples in which serotonin or the reversible inhibitor and the irreversible inhibitor were substituted by distilled water. As shown in Tables 3 and 4 we could not observe the protection effect with serotonin under these assay conditions but there was indeed with compounds **1** and **4**. It might be that we did not use the necessary amount of serotonin in the preincubation mixture, because this was limited by the greatest concentration in the reaction mixture. These results point out that compounds **2** and **3** combine with the active site with formation of a reversible complex.

In order to determine the rate constant k_2 and to confirm the K_i values, which were obtained by the

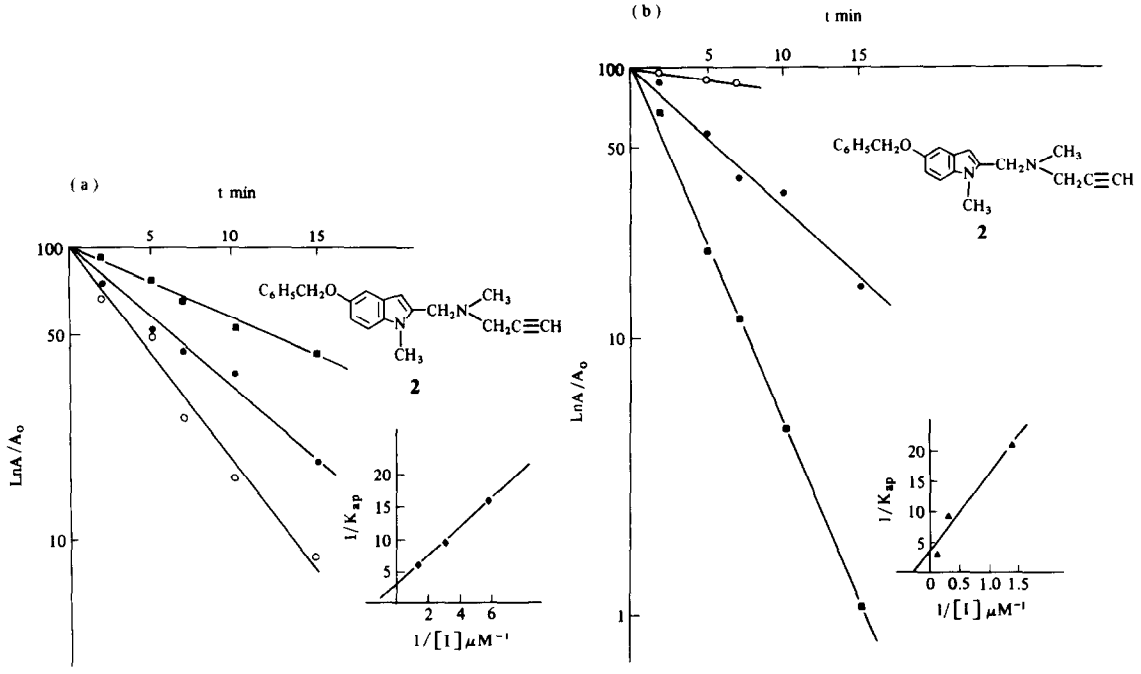

Fig. 7. Time courses of irreversible inhibition of serotonin (a) and β-phenylethylamine (b) deamination by compound **2**. Assays were carried out by preincubation at 37° of *l*-deprenyl-treated (a) or clorgyline-treated (b) mitochondrial preparation (6 mg/mL of protein) with several inhibitor concentrations in 30 mM potassium phosphate buffer, pH 7.3. At the indicated times, 20-μL aliquots were removed and the residual activity was measured, following dilution with phosphate buffer, by the usual way with serotonin (a) or β-phenylethylamine (b) as substrate. Results are means of determinations in three mitochondrial preparations. A and A_0 are activities of the enzyme preincubated respectively with and without inhibitor. Secondary plots of $1/k_{ap}$ (calculated by linear regression analysis) against $1/I$ are shown as figure insets.

Table 4. Protection effect by competitive reversible inhibitors

Compound number	$[I]_{irrev}$*		$[I]_{rev}$†	I_t (%)‡	I_{rev}§	I_{irrev}‖
2	4 μM		0	97	0	97
		1	0.45 mM	97	29	68
			0.8 mM	96	38	58
2	1 μM		0	90	0	90
		4	20 nM	90	32	58
			40 nM	88	65	23
3	0.32 μM		0	88	0	88
		1	45 μM	68	11	57
			0.45 mM	46	29	17
			0.8 mM	51	38	13
3	0.1 μM		0	28	0	28
		4	20 nM	47	32	15
			40 nM	73	65	8

* Irreversible inhibitor concentration in preincubation mixture.
† Reversible inhibitor concentration in preincubation mixture, compounds **1** or **4** (harmine).
‡ Percentage of total inhibition.
§ Percentage of inhibition by reversible inhibitor.
‖ Percentage of inhibition by irreversible inhibitor, determined by difference between ‡ and §.

study of the 'reversible inhibition', we also studied the time-courses of irreversible inhibition by compounds **2** and **3**, which were analysed by the method of Kitz and Wilson [18], according to the equation $1/k_{aP} = (K_I/k_2)\ 1/I + 1/k_2$. At first, the time-courses of irreversible inhibition of the activity towards serotonin were determined by preincubating the mitochondrial preparation (MAO-A and -B) for fixed

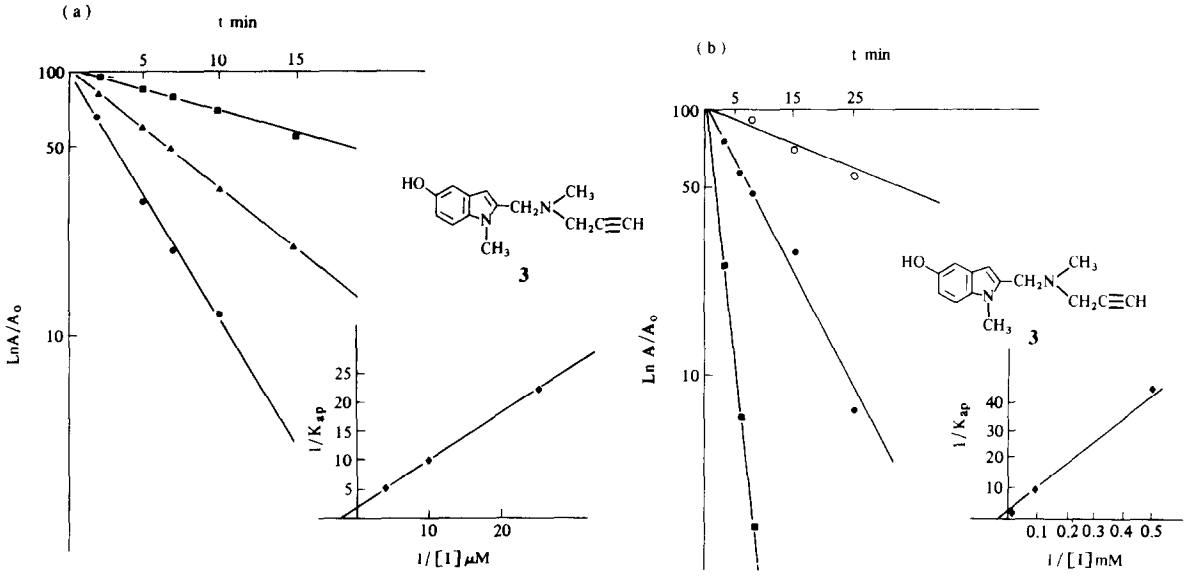

Fig. 8. Time courses of irreversible inhibition of serotonin (a) and β-phenylethylamine (b) deamination by compound **3**. Assays were carried out as described in Fig. 7.

Table 5. Inhibition parameters

Compound number	Constant*	MAO-A†	MAO-B‡
2	K_I	0.89 ± 0.04 μM	4.55 ± 0.07 μM
	k_2	0.40 ± 0.05 min^{-1}	0.40 ± 0.08 min^{-1}
	$T_{1/2}$	1.9 ± 0.2 min	1.9 ± 0.4 min
3	K_I	0.87 ± 0.05 μM	3.14 ± 0.01 mM
	k_2	1.00 ± 0.1 min^{-1}	3.40 ± 0.07 min^{-1}
	$T_{1/2}$	0.7 ± 0.1 min	0.2 ± 0.1 min

* The kinetic constants K_I and k_2 were determined from the replots of Figs 7 and 8 which follow the relationship: $1/k_{ap} = (K_I/k_2)\ 1/I + 1/k_2$, and $T_{1/2}$ values were calculated as 0.693/individual k_2 values. Data are means ± SE of determinations with three mitochondrial preparations.

† Serotonin as substrate.

‡ β-Phenylethylamine as substrate.

times at 37° in the presence of an inhibitor concentration which did not cause inhibition without preincubation, before the substrate was added and the activity determined by the usual procedure. However, the semi-logarithmic plots obtained were not linear. We tried to solve these questions by modifying enzyme and inhibitor concentrations in the preincubation mixture, to avoid the variation of inhibitor concentration during this period of time, but we also obtained non-linear plots [16, 17, 19]. At the end, we solved these questions by performing the assays using previously clorgyline-treated or *l*-deprenyl-treated mitochondrial preparations, when we studied MAO-B or MAO-A inhibition respectively [14]. In these assay conditions, the semilogarithmic plots of inhibition towards β-phenylethylamine and serotonin by compounds **2** and **3** were linear (Figs 7 and 8).

The kinetic parameters shown in Table 5 were determined from the obtained plots. The calculated K_I values were greater than the K_i values which were obtained by studying the 'reversible inhibition'. This difference may be due to several causes. It might be due to the irreversible inhibition, which perhaps was not negligible with the incubation times used in the reversible inhibition assays, since k_2 values point to a quick irreversible process. On the other hand, as recently discussed by Silverman [20], K_i and K_I values of mechanism-based enzyme inactivators can be different. These depend on the relative values of the rate constants for the sequence of events occurring from the reversible complex $E.I$ to the final covalent complex E–I.

Comparison of the values shown in Table 5 indicate that the large difference between the K_I values for the compound **3** inhibition of the activity towards serotonin and those for β-phenylethylamine, suggests that the selectivity of this inhibition may result, to a large extent, from differences in the affinity of the enzyme for reversible complex formation with

the inhibitor. However, in the case of inhibition by compound **2**, the difference between the K_I values was negligible, consistent with this inhibitor being a non-selective one.

Since compound **1** is structurally similar to compound **3**, with the exception of the reactive group ($R^3 = HC{\equiv}CCH_2$—), we thought that the first reversible complex with the active site of the enzyme may be the similar, but compound **1** is a non-selective inhibitor and compound **3** is a MAO-A selective one. For explaining this selectivity of inhibition towards MAO-A on the basis of differences between the K_i or the K_I values when serotonin or β-phenylethylamine were used as substrate, we propose the following scheme for the mechanism of inhibition by compounds **2** and **3**:

$$E + I \rightleftharpoons E.I \rightleftharpoons \ldots \rightleftharpoons E.I' \longrightarrow E\text{–}I'.$$

The pathway of inhibition involves an initial reversible combination of the enzyme and the inhibitor to give the *E.I* complex, which would be similar for compounds **1** and **3**. This process is followed by the formation of one or more different reversible complexes, taking part the acetylenic group of compounds **2** and **3**. Finally, reaction within the last non-covalent complex *E.I'* to form the covalent enzyme–inhibitor bond, which may involve several irreversible steps, to give *E–I'*. The obtained K_I values for compounds **2** and **3** are constants which include the successive equilibriums. The selectivity of inhibition by compound **3** resulted from different affinities of MAO-A and MAO-B to form the second and/or successsive reversible complexes, while in the case of compound **2**, there are no different affinities.

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