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MONOAMINE OXIDASE—II

TIME-DEPENDENT INHIBITION BY PROPARGYLAMINES

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Abstract The inhibition has been studied of monoamine oxidase from porcine brain by a number of propargylamines related to substrates or competitive inhibitors of the enzyme. All but one of the compounds were shown to be effective time-dependent inhibitors. The inactive compound was *N*-propargyl-3-phenylpiperidine, derived from 3-phenylpiperidine which had previously been found to be a good competitive inhibitor of MAO. It contrasted markedly with its 4-phenylanalogue. The effectiveness of the inhibitors against the MAO iso-enzymes of rat liver paralleled that for the porcine brain enzyme. In spite of significant variations in structure of the compounds tested, evidence for a selective action against the postulated iso-enzymes was not produced, except for clorgyline, which has previously been shown to act in this way.

DERIVATIVES of propargylamine (3-amino-1-propyne) are known to be good inhibitors of monoamine oxidase (MAO) and in general such inhibition is irreversible.[1] One of the most frequently studied of these compounds is pargyline (*N*-benzyl-*N*-methyl-propargylamine) which has found clinical application in treatment of hypertension. More recently, another propargylamine, *N*-(2,4-dichlorophenoxy-*n*-propyl)-*N*-methylpropargylamine, clorgyline, has been shown to selectively inhibit iso-enzymes of MAO from various tissues.[2–4] In spite of numerous reports on such propargylamines little is known of their mode of action. They do not apparently attack thiol groups in the enzyme,[5,6] and it is reported that pargyline interacts only with the oxidized form of MAO.[6] McEwen *et al.*[7] have shown that the same compound behaves as both an instantaneous competitive inhibitor and as a time-dependent inhibitor of MAO from human liver. This suggests that the compound is a substrate analogue, behaving as an active-site directed irreversible inhibitor.

A greater knowledge of the specificity of these inhibitors should assist in determining their mode of action and, as substrate analogues, might yield information of the active centre of the enzyme. In the present study, a number of propargylamines, some synthesized for the first time, have been assessed as inhibitors of MAO from porcine brain. They have also been investigated for selective action against iso-enzymes of MAO in a mitochondrial preparation from rat liver.

MATERIALS AND METHODS

Inhibitors

Pargyline, *N*-(2,4-dichlorobenzyl)propargylamine and *N*-(2,4-dichlorobenzyl)-*N*-methylpropargylamine were prepared essentially by a published procedure.[8,9] All the

TABLE 1. ANALYTICAL AND INFRA-RED DATA FOR PROPARGYLAMINE HYDROCHLORIDES

Propargylamine	m.p. (°C)	Analysis (%) Calc. C	H	N	Found C	H	N	IR absorption of —C≡C—H (cm^{-1})	
N-3-phenylpiperidyl-	186	71·4	7·64	5·94	71·28	7·55	5·72	2110	3205*
N-4-phenylpiperidyl-	188	71·4	7·64	5·94	71·14	7·55	6·02	2105	3160
N-γ-phenylpropyl-	169	68·7	7·63	—	68·9	7·81		2110	3250
N-δ-phenylbutyl-	159	69·8	8·05	—	69·77	8·2	—	2110	3245
N-(2,4-dichlorobenzyl)-	172	47·9	5·6	3·99	48·1	5·54	3·78	2110	3215

* The second figure in each case refers to acetylenic C—H stretching. The somewhat low and variable values are a function of either salt formation or crystal structure. i.r. Spectra of the free bases shows a shift in this band to *ca.* 3300 cm^{-1}, which is usual for acetylenes.

other compounds were sythesized by similar methods, by reacting the appropriate primary or secondary amine with propargyl bromide in ether or ethanol in presence of K_2CO_3. Separation of starting amine from the product was effected by partition between ether and strongly buffered solutions of appropriate pH, utilizing the fact that the pK_a values of the *N*-propargyl compounds were approximately two units less than their precursor amines. All compounds were converted to their HCl salts and crystallized to constant melting point and analytical purity. In every case, infra-red spectral measurements on the salts in KBr discs revealed absorption bands typical of acetylenic C—H and C≡C stretching. Analytical data for compounds previously unrecorded in the literature are in Table 1.

Clorgyline was a gift from May & Baker Ltd. Dagenham, Essex.

Monoamine oxidase

An MAO preparation from porcine brain was isolated as previously described.[10]

A mitochondrial preparation from rat liver similar to that used by Hall *et al.*[3,4] in experiments with clorgyline was isolated as follows; all operations being carried out at 2–4°. Male wistar rats were killed by a blow on the neck, exsanguinated, and their livers removed. These were blotted with tissue and weighed. They were homogenized in 5 volumes of 0·067 M phosphate buffer, pH 7·4, using a Potter–Elvehjem homogenizer of glass and Teflon. The homogenate was centrifuged at 2000 *g* for 10 min and the pellet was discarded. A further 4 volumes of phosphate buffer was added to the supernatant to give a 9:1 suspension. This preparation was used in the inhibition experiments.

Inhibition studies on MAO from porcine brain

Time-dependent inhibition. The inhibition produced by the propargylamines when incubated with the enzyme for varying times was measured in the following way. Enzyme preparation, 3 ml (0·12 units) in an unstoppered tube was diluted with 0·05 M phosphate buffer, pH 7·4 (7·5 ml), followed by addition of 0·02 M KCN (0·6 ml) in the same buffer. The tube was placed in a water bath at 30° and 0·6 ml of the inhibitor solution at appropriate concentration was added. Final concentrations were as indicated in Fig. 1. At intervals, 1·95 ml of the incubate were

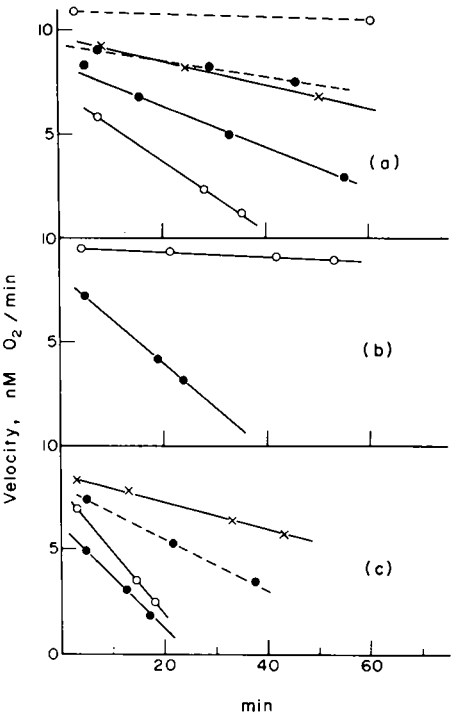

FIG. 1. Plots of pre-incubation time against residual MAO activity for a number of propargylamines at pH 7·4 and 30°. The propargylamines, and concentrations used were as follows. (a) *N*-benzyl (●——●), *N*-β-phenylethyl (○——○), *N*-γ-phenylpropyl (×——×), *N*-δ-phenylbutyl (●- -●), all 10^{-6} M. (b) -*N*-3-phenylpiperidyl (○——○), *N*-4-phenylpiperidyl (●——●), both 10^{-5} M. (c) -*N*-2,4-dichlorobenzyl (○——○), *N*-benzyl-*N*-methyl ●- - -●), both 10^{-7} M; *N*-2,4-dichlorobenzyl-*N*-methyl (●- - - -●), 10^{-8} M; *N*-2,4-dichlorophenoxypropyl-*N*-methyl (×——×), 10^{-6} M. The loss of enzyme activity in absence of inhibitor is shown in (a) (○- - -○).

transferred to an oxygen electrode and residual enzyme activity was measured as previously described,[10] after adding 50 μl of a 60 mM solution of tyramine hydrochloride in 0·05 M phosphate buffer. A graph of residual activity against time was plotted for each inhibitor (Fig. 1).

pI vs I plots for pargyline and clorgyline. The effects of inhibitor concentration on enzyme activity were measured at pH 7·4 and 30°, using either tyramine hydrochloride or serotonin oxalate as substrate. The assay method was as described in Part I, using the oxygen electrode. Each inhibitor, at final concentrations of from 10^{-3} to 10^{-10}, was pre-incubated with enzyme for 5 min in the electrode at 30° prior to addition of substrate. Residual enzyme activity was measured for clorgyline using either tyramine or serotonin, 1·25 mM, as substrate and for pargyline using tyramine, 1·25 mM. The results were used to plot curves of percentage inhibition against pI (the negative logarithm of inhibitor concentration).

Inhibition experiments on the rat liver preparation

Studies of the inhibition by the various propargylamines of the MAO iso-enzymes from rat liver were carried out using a radio-assay method similar to that described by Hall *et al.*[3,4] in their work with clorgyline.

Preparation of [^{14}C]*tyramine solutions.* [1-^{14}C]Tyramine-hydrochloride, sp. act. 44 mCi/mM (Radio-chemical Centre, Amersham) was dissolved in 0·05 M phosphate buffer, pH 7·4, to give a concentration of 1 μCi/ml (0·0227 μmoles/ml).

It was diluted with an equal volume of unlabelled tyramine hydrochloride solution, concn 2·25 mM, in the same buffer. The final tyramine concentration was thus almost 1·14 mM and the ratio of labelled to unlabelled amine was 1 : 100.

Incubation and assay. Five ml samples of the tissue preparation, in duplicate, were incubated in air at 20° with 1 ml of a solution of inhibitor in the same buffer. Final concentrations of inhibitor in the series of experiments ranged from 10^{-3} to 10^{-10} M. Control samples, in which 1 ml of buffer replaced the inhibitor solution, were similarly incubated. Blanks, without added MAO preparation, were also carried through the procedure. After 15 min, 1 ml samples were transferred to tubes containing 1 ml of the tyramine solution at 30°. These were then shaken in air at this temperature for 15 min, when the reaction was stopped by adding 2 N HCl (0·5 ml). Metabolites of tyramine were extracted by shaking vigourously for 1 min with ethyl acetate (4 ml). After briefly centrifuging to separate the layers, 2 ml of the upper phase was added to 15 ml of Bray's scintillation fluid and radioactivity was counted in a Packard Tri-Carb liquid scintillation spectrometer, gain setting 12 per cent, window setting 50–1000.

A value for MAO activity at each concentration of inhibitor was calculated by dividing the number of counts/min in inhibited samples by the number of counts/min in control samples, after subtraction of blank values. These figures were then converted to percentage inhibition and plotted against pI. The assay is linear with respect to time over the 15 min period.

RESULTS

Results of time-dependent inhibition of MAO from porcine brain (Fig. 1) show that minor structural variations result in large changes in rates of inactivation, though it should be noted that concentrations differ, having been chosen to achieve measurable rates. In those cases where comparison is possible, there is no clear correlation between the rate of inactivation and the efficiency of corresponding (non-propynylated) substrates or competitive inhibitors. (See Part I of this series.) Figure 2 shows that clorgyline behaves towards MAO from porcine brain as if it were a single enzyme. In this test, as in others, pargyline appears to be the better inhibitor.

Wide variations in activity of the propargylamines were evident in the experiments using mitochondria from rat liver (Figs. 3–5). In spite of the wide variety of structural types, none of the compounds, other than clorgyline, exhibited a selective action against the postulated iso-enzymes in this tissue. The plateau region in the curve for clorgyline (Fig. 4) is quite unmistakeable and was always apparent, even under conditions somewhat different from those reported here. The general trend in inhibitory potency of the various compounds is similar to that observed in time-dependent experiments on MAO from porcine brain (Fig. 1).

DISCUSSION

Hellerman and Erwin[6] have shown that substrate protects MAO from the action of pargyline. It is also reported that this compound is an instantaneous competitive

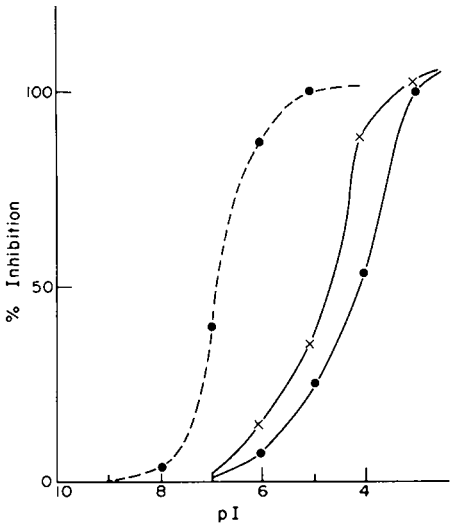

FIG. 2. Effect of a fixed pre-incubation time of differing concentrations of clorgyline (●· ·●) and pargyline (●– –●) on oxidation of tyramine, and the effects of the former on oxidation of serotonin (× —— ×). Substrate concentrations were 1·25 mM. Temp. 30°, pH 7·4.

inhibitor of MAO from human liver.[7] In view of these findings, and the fact that pargyline is a simple substrate analogue, it seems reasonable to assume that such compounds interact initially with MAO in a manner similar to that for substrates, and that the reactive species is the free base. Indeed, McEwen *et al.*[7] have shown that the effect of pH on the instantaneous competitive action of pargyline is consistent with nucleophilic attack upon the enzyme. Interpretation of inhibitory effects of such compounds must therefore take account of their pK_a values.

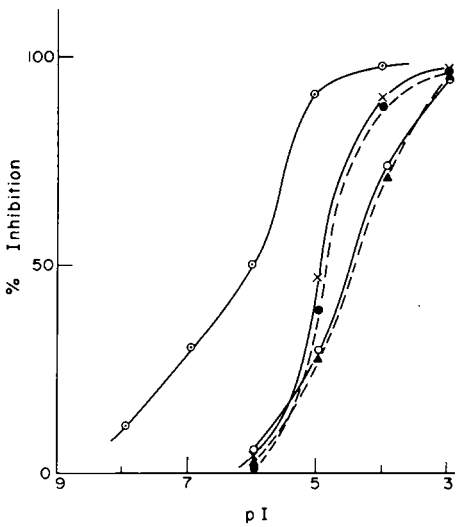

FIG. 3. Curves of percentage inhibition against pI for some propargylamines using an MAO preparation from rat liver. Compounds are as follows: pargyline (⊙——⊙); *N*-propargylbenzylamine (▲– –▲); *N*-propargyl-β-phenylethylamine (○——○); *N*-propargylphenylpropylamine (●– –●) and *N*-propargylphenylbutylamine (× ·—· ×). Temp. 30°, pH 7·4. Enzyme activity was measured as described in text, using ^{14}C-labelled tyramine.

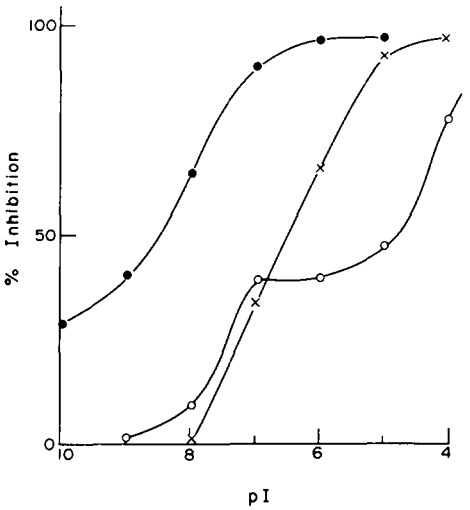

FIG. 4. As Fig. 3. Compounds are: clorgyline (O—·-·-O); *N*-propargyl-2,4-dichlorobenzylamine (× ·—·—×); *N*-methyl-*N*-propargyl-2,4-dichlorobenzylamine (●-·—·—●).

Time-dependent studies on MAO from porcine brain reveal interesting differences among the various inhibitors (Fig. 1). The order of effectiveness of the four phenyalkyl compounds (Fig. 1a) is not as might be anticipated from a knowledge of the efficiency of the respective non-propynylated substances (see Part I of this series). The K_m values of phenylethyl and phenylbutylamines are approximately equal but, as Fig. 1 shows, the propargyl derivative of the latter inactivates the enzyme much more slowly than the corresponding phenylethylamine derivative. However, assuming that the unprotonated inhibitor attacks the enzyme, then the effective concentrations of the four homologues of Fig. 1a are very different, since their pK_a values at 30° range

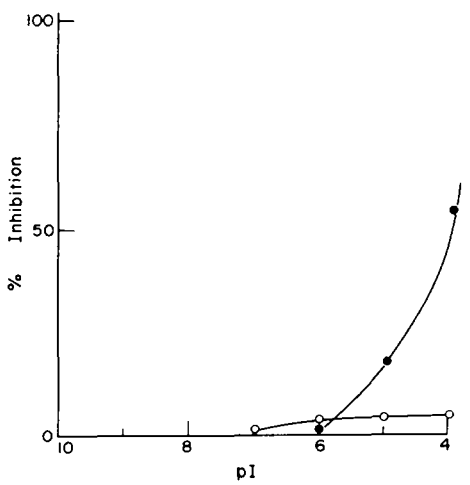

FIG. 5. As Fig. 3. Compounds are: *N*-propargyl-3-phenyl-piperidine (O— -O); *N*-propargyl-4-phenylpiperidine (●——●).

from 7·12 for the benzylamine derivative to 8·12 for phenylbutylamine derivative.* The high potency of the chlorinated compounds may be to some extent a reflection of their pK_a values also, since these are weaker bases by about one pK_a unit than their non-halogenated congeners, and at pH 7·4 exist almost entirely as unprotonated bases.

Extrapolation of the curves of Fig. 1 to zero time shows that they cross the axis at different points, which might indicate a very rapid initial rate of inactivation. More likely is that the residual inhibition at zero time is due to a time-independent effect, possibly reflecting the instantaneous competitive inhibition reported by McEwen *et al.*[7] for pargyline. If so, there is a clear correlation between this parameter and the rate of inactivation of MAO by the various inhibitors.

It has been reported by Hall *et al.*[3] that clorgyline does not exhibit a time-dependent inhibition with MAO. Further, it has been suggested that substrate potentiates the action of this inhibitor.[11] The present work indicates clearly the time-dependent nature of the inhibition by clorgyline of the enzyme from porcine brain and confirms a similar finding by Tipton.[12] These discrepancies may be due to differences in the ratio of enzyme to inhibitor since, at low ratios, inactivation might be expected to be more rapid than when there is relatively less inhibitor present.

The most striking feature of the time-dependent inhibitions is the marked contrast between the *N*-propargyl derivatives of 3- and 4-phenylpiperidine (Fig. 1). Whereas the latter inactivates the enzyme readily, the former is almost without effect. This is the more surprising when it is recalled that 3-phenylpiperidine was shown to be a better competitive inhibitor than 4-phenylpiperidine (see Part I of this series). A consideration of the stereochemistry of the two inhibitors seems pertinent. Assuming that the substituents in the heterocyclic ring of these compounds both adopt equatorial positions, with the ring in the chair form, then the propargyl moiety in the 4-phenyl compound lies roughly on a line joining the two rings. On the other hand, in the 3-phenyl analogue, the propargyl group protrudes considerably beyond the area containing the two rings. Rotation of the hetero-ring about the bond joining it to the benzene ring results, in the latter case, in the sweeping out of a considerably larger volume than would be the case for the 4-phenyl derivative. Hence it seems likely that for 3-phenyl *N*-propargylpiperidine there exists a steric hindrance to its approach to the appropriate site on the enzyme, because of an inability to conform to a suitable configuration. This again lends support to the hypothesis already advanced (see Part I) that the ability of aralkylamines to bind to MAO depends critically upon their adopting a configuration in which ring and side chain are suitably juxtaposed. The constraints in the present instance are almost certainly due to the reduced flexibility of the hetero-ring system compared to analogous open-chain compounds, since all of the latter were good inhibitors of the enzyme.

The inhibitory studies carried out on the preparation from rat liver gave results which paralleled those of porcine brain, in so far as the order of efficacy of the various inhibitors was essentially the same. Of particular interest is that *N*-propargyl-3-phenylpiperidine is almost totally ineffective as an inhibitor of the iso-enzyme complex, confirming its lack of activity against the porcine brain enzyme. These findings suggest that whatever differences there may be between monoamine oxidases

* C. H. Williams and J. Lawson, unpublished results.

from different tissues, or even from the same tissue, they are in some respects remarkably similar. In spite of the varied structures of the compounds tested though, only clorgyline exhibited a plateau region in the curve of percentage inhibition against inhibitor concentration (Fig. 4), indicative of selective inhibition.[2]

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