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The kinetics of inhibition of type-B monoamine oxidase by clorgyline, pargyline and (−)-deprenyl

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The irreversible inhibition of monoamine oxidase (MAO, EC 1.4. 3.4) by propargylamine inhibitors, such as clorgyline, is believed to occur via the preliminary formation of an intermediate Michaelis-like reversible complex between the enzyme and the inhibitor, followed by covalent attachment of the inhibitor to the flavin prosthetic group of the enzyme (i.e. it can be represented as $E + I \rightleftharpoons EI \rightarrow EI^*$) (reviewed by Fowler et al 1981). However, direct evidence for the existence of such an intermediate for the more widely used propargylamines is sparse, and no reliable measurement has been reported for the dissociation constant of any of the postulated reversible complexes. Irreversible enzyme inhibition via the formation of such an intermediate reversible complex has been widely verified for cholinesterase inhibitors and the kinetics of inhibition are well understood (Tipton 1980). Provided the inhibitor is stable and present at a much higher concentration than the enzyme, the time dependence for conversion of the enzyme into the irreversibly modified form (EI^*) at a constant inhibitor concentration is first order [i.e. $\ln E_o - \ln (E_o - EI^*) = k_1 t$, where E_o is the enzyme activity at $t = 0$]. If the inhibitor concentration (I) is much lower than the dissociation constant (K_i) for the reversible EI complex, or if EI^* is produced by direct combination of the inhibitor with the enzyme without any intermediate reversible complex being produced, then the first order rate constant (k_1) will be directly related to the inhibitor concentration (i.e. $k_1 = k_2 I$, where k_2 is the second order rate constant). However, if I is comparable in magnitude to K_i, then k_1 is related to I by the more complicated expression $k_1 = k/(1 + I/K_i)$ where k is the rate constant for the conversion of EI into EI^*. A plot of $1/k_1$ against $1/I$ would still be linear, but would no longer pass through the origin. The numerical values of k and K_i can be obtained from the reciprocals of the intercepts of this plot on the $1/k_1$ and $1/I$ axes. This technique is most easily applied when the conversion of the reversible complex into the irreversible one is relatively slow. This appeared to be the case for the inhibition of benzylamine oxidation by clorgyline (Egashira et al 1976), and it is shown below that the kinetics of this reaction can be satisfactorily interpreted on the above basis, and be used to derive values for k and K_i.

Rat liver mitochondria were isolated by differential centrifugation and treated with Tergitol NPX to give a homogeneous solubilized enzyme preparation (Green & El Hait 1980). MAO activity was measured by continuous recording of the increase in absorbance at 250 nm which occurs when benzylamine is oxidized to benzaldehyde. Aqueous clorgyline (1 ml of 0·15 to 1·2 mM) was added to the solubilized enzyme (2 ml) and 0·1 M sodium phosphate buffer (pH 7·4, 27 ml) incubated at 30 °C. At suitable time intervals (normally 1, 10, 20, 30 and 40 min after addition of inhibitor) a 3 ml sample was transferred to a spectrophotometer cell containing aqueous benzylamine hydrochloride (0·1 ml of 0·1 M) thermostatted at 30 °C. The increase in absorbance was measured for 5 min against a similar blank without the benzylamine using an SP8-100 double beam continuous recording spectrophotometer on a scale range of 0 to 0·2 absorbance units. The final concentration of benzylamine in this assay mixture (3·2 mM) is over 20 times the K_m value (130 μM, see below) and is sufficient to suppress any significant further conversion of the enzyme into the irreversibly inhibited form during the assay itself. Plots of the logarithm of the residual enzyme activity ($E_o - EI^*$) against the time of incubation between inhibitor and enzyme were linear, and first order rate constants (k_1) were calculated directly from the slopes. These constants were corrected for the very slow spontaneous denaturation of the enzyme which occurs in the absence of inhibitor (k_1 0·0013 min^{-1}). Fig. 1 shows that a plot of $1/k_1$ against $1/I$ is linear. The intercepts on the $1/k_1$ and $1/I$ axes give values of 0·06 min^{-1} for k and 12 μM for K_i.

Similar experiments were attempted with pargyline and (−)-deprenyl, but, with these more potent inhibitors of B-type MAO, the formation of the irreversibly inhibited enzyme (EI^*) was much faster than with clorgyline and the technique was modified to shorten the enzyme-inhibitor contact time. Instead of adding inhibitor to a large volume of enzyme and buffer which was then sampled at 10 min

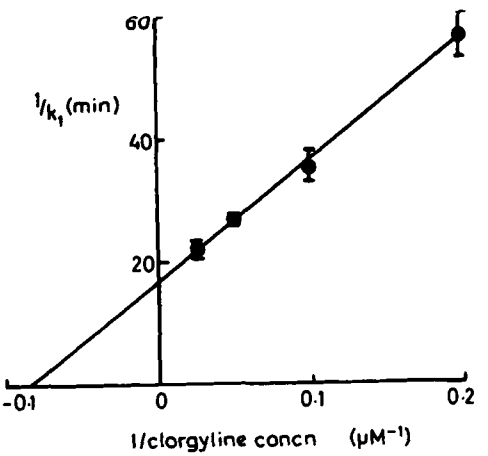

FIG. 1. Plot of $1/k_1$ against $1/I$ for inhibition of benzylamine oxidation by clorgyline. Each point is the mean (± s.e.m.) of four determinations. Ordinate: 1/first order rate constant in min^{-1}, abscissa: 1/clorgyline concentration in μM.

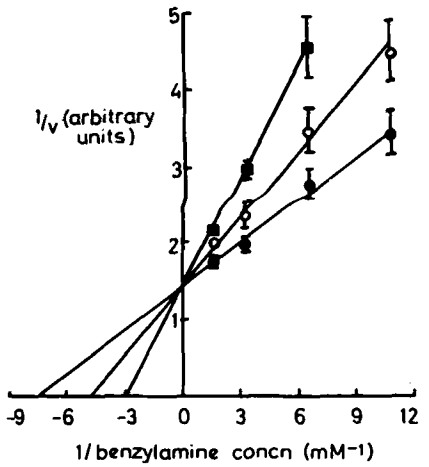

FIG. 2. Lineweaver-Burk plots for the initial phase of inhibition of benzylamine oxidation by clorgyline: (●) 0 μM; (○) 10 μM; (■) 20 μM. Each point is the mean (± s.e.m.) of four or more assays. For clarity, error bars are omitted on two of the points. Ordinate: 1/enzyme activity in arbitrary units; abscissa: 1/benzylamine concentration in mM.

intervals for enzyme assay, repeated assays were carried out whereby inhibitor (0·1 ml) was added to buffer (2·7 ml) and enzyme (0·2 ml) already in the spectrophotometer cell. One to 10 min later, 0·1 M benzylamine (0·1 ml) was added to stop any further reaction between inhibitor and enzyme and to measure the residual enzyme activity. Over these shorter reaction times, the logarithm of the residual enzyme activity was still found to be a linear function of the time of contact between inhibitor and enzyme before adding substrate, but when the inhibitor concentration was increased it was not possible to demonstrate a clear deviation from simple second order kinetics even at the highest concentrations of inhibitor which still gave accurately measurable k_1 values. For pargyline at 0·05 to 0·4 μM, k_2 was 7×10^5 litre mol^{-1} min^{-1}, and for (−)-deprenyl at 0·05 to 0·2 μM, k_2 was 2×10^6 litre mol^{-1} min^{-1}. These results do not exclude the possibility that inhibition of B-type MAO by pargyline and (−)-deprenyl also proceeds via an intermediate reversible complex, but if it does, then the value of k must be greater than 1 min^{-1} and the value of K_i must be appreciably higher than the highest concentration of inhibitor used. An alternative possibility is that the rate constant (k) for the covalent binding step is so much greater than that for the dissociation of the reversible EI complex back into E and I that the rate controlling step at low inhibitor concentration becomes the initial association of E and I to give the reversible complex EI. This would also result in the rate of formation of EI* being second order. There is evidence (Fowler & Callingham 1978) that this may be the situation for inhibition by clorgyline of 5-HT deamination. These authors cite a value of 2×10^6 litre mol^{-1} min^{-1} for the second order rate constant in this context, which is the same as the value obtained here for the second order rate constant for the inhibition by (−)-deprenyl of benzylamine deamination.

The low value obtained for k with clorgyline as an inhibitor of benzylamine oxidation means that even at very high clorgyline concentrations, the half-life for the appearance of EI* will be nearly 12 min. Thus, if the *clorgyline and the substrate are added to the enzyme* together, and the assay time is short, then what is observed will be predominantly the reversible phase of the inhibitory process. This has been exploited to obtain an independent determination of K_i by measuring the initial level of inhibition observed when the solubilized enzyme (0·2 ml) was added to buffer (2·8 ml), benzylamine (0·1 ml, 3–20 mM) and water (0·1 ml) or clorgyline (0·1 ml) already present in the spectrophotometer cell, essentially as described by Maycock et al (1976) for inhibition of MAO by the much weaker propargylamine inhibitor, dimethylaminopropyne. The results are shown in Fig. 2 in the form of a Lineweaver-Burk plot. The lines in the presence and absence of clorgyline intersect approximately on the 1/v axis, and are consistent with inhibition being largely competitive and reversible. The K_m value for benzylamine is 130 μM and the K_i value calculated from the alteration in the slope produced by clorgyline is 16·7 μM (at 10 μM clorgyline) and 13·5 μM (at 20 μM clorgyline), both being in fair agreement with the K_i value obtained from Fig.1. Maycock et al (1976) also noted that the apparent K_i value for dimethylaminopropyne fell slightly as the inhibitor concentration was raised. This effect probably results from the fact that even in the earliest phase of the reaction there is no true equilibrium established.

Although with clorgyline as inhibitor and benzylamine as substrate the two different types of experiment gave consistent results for K_i, it is important to appreciate that the second type of experiment does not in itself constitute evidence that a reversible enzyme-inhibitor complex is actually formed. Even if inhibition occurred by direct combination of the inhibitor with the enzyme to form a covalent bond (i.e. it could be represented as $E + I \rightarrow EI^*$), the simultaneous presence of the substrate will reduce the rate of this reaction by sequestering part of the free enzyme in the form of enzyme-substrate complex in which the active centre of the enzyme is inaccessible to the inhibitor (Tipton 1980). Thus the substrate and inhibitor will compete for the enzyme active centre, and when 1/v is plotted against 1/S in the presence and absence of inhibitor the resulting lines will resemble those obtained for a competitive reversible inhibitor. However, if the increase in slope in the presence of the inhibitor is used to calculate an apparent K_i value for a non-existent complex, this will obviously give totally misleading results. McEwen et al (1969) showed that the time-dependent inhibition of human liver mitochondrial MAO by pargyline (0·02 to 0·1 μM) with benzylamine as substrate at 25 °C obeyed strict second order kinetics with a rate constant of $2{\cdot}2 \times 10^5$ litre mol^{-1} min^{-1}. Allowing for the difference in temperature, this value is in fair agreement with the value of 7×10^5

litre mol^{-1} min^{-1} derived above for rat liver MAO. However these same authors also studied the initial level of inhibition produced when substrate and inhibitor were present together, and reported that inhibition appeared to be competitive with a K_i value (derived from a Lineweaver-Burk plot) of 0·11 μM. This value cannot be correct, otherwise they would have observed a marked deviation from second order kinetics in the time dependent inhibition experiments at a pargyline concentration of 0·1 μM, consequently this experiment cannot be construed as valid evidence for the existence of a reversible MAO-pargyline complex.

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Reduced accumulation in brain of orally ingested β-phenethylamine after inhibition of type A monoamine oxidase in the rat

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Monoamine oxidase (MAO, E.C. 1.4.3.4) has been found to exist in at least two forms (A and B), which exhibit different specificities for their substrates and inhibitors (Johnston 1968) MAO-A is specifically inhibited by low doses of clorgyline, and preferentially deaminates 5-hydroxytryptamine (5-HT). MAO-B is only weakly inhibited by clorgyline, and preferentially deaminates β-phenethylamine (PEA) (Houslay & Tipton 1974). In view of the renewed interest in MAO inhibitors as a treatment for depression (Quitkin et al 1979), attempts have been made to develop specific inhibitors of the two types of MAO, in the expectation that such inhibitors might be effective antidepressants while possessing fewer side effects than the non-specific MAO inhibitors (Fuller 1978). Some preliminary evidence suggests that specific inhibitors of type A MAO might be clinically active antidepressants (Lipper et al 1979). We have recently reported on the biochemical properties of MD780515 (3-[4-(3-cyanophenylmethoxy)-phenyl]-5-(methoxymethyl)-2-oxazolidinone), a new, specific and reversible inhibitor of MAO-A (Strolin Benedetti et al 1979, 1980). The inhibition constant (K_i) values [K_i slope = $K_{i(s)}$ and K_i intercept = $K_{i(i)}$] have been determined with rat brain homogenates using 5-HT and PEA as substrates (Kan & Strolin Benedetti 1981). The ratio of the K_i values [($K_{i(s)}$ 5-HT/$K_{i(s)}$ PEA = 0·026 and $K_{i(i)}$ 5-HT/$K_{i(i)}$ PEA = 0·116] showed that MD780515 perferentially inhibited type A MAO. As part of the program devised to determine the profile of MD780515, we have studied its effect on PEA metabolism in vivo. It has been shown that the ingestion of PEA can produce migraine in sensitive subjects (Sandler et al 1974), and any potentiation of this effect of ingested PEA would represent an undesirable side effect of treatment with MAO inhibitors. This consideration led us to compare the effects of MD780515 and other MAO inhibitors on the accumulation in rat brain of orally administered PEA.

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Methods

Male Sprague Dawley rats (Charles River, CD, France), 175–200 g, were fasted for 18 h then administered the solvent or one of the following MAO inhibitors (all doses refer to the base): clorgyline hydrochloride (10–20 mg kg^{-1} orally, 1 and 5 mg kg^{-1} i.v.), MD780515 (5–20 mg kg^{-1} orally), toloxatone (100 mg kg^{-1} orally), harmaline hydrochloride (30 mg kg^{-1} i.p.), Lilly 51641 (*N*-[2-(*o*-chlorphenoxy)ethyl]cyclopropylamine HCl) hydrochloride (10 mg kg^{-1} orally), (±)-deprenyl hydrochloride (10–20 mg kg^{-1} orally, 1 and 5 mg kg^{-1} i.v.), pargyline hydrochloride (10 mg kg^{-1} orally) or tranylcypromine hydrochloride (5 mg kg^{-1} orally). Thirty minutes later the rats received 0·2 mg kg^{-1} orally PEA hydrochloride (containing 10 μCi kg^{-1} [^{14}C] PEA hydrochloride, NEN). Fifteen minutes later the rats were killed and their brains were removed. Unchanged PEA was isolated and quantified essentially as described by Fuller & Roush (1972). Briefly, the rat brain was homogenized in 6 ml of 0·4 M perchloric acid and centrifuged at 18 000 *g* for 10 min. Supernatant, 2 ml, was brought to pH 11 with 250 μl of 5 M sodium hydroxide, and shaken for 15 min with 6 ml of toluene and enough sodium chloride (800 mg) to saturate the aqueous phase. After centrifugation, 4 ml of the toluene fraction was taken for radioactivity determination by liquid scintillation counting. The extraction recovery of PEA was 92–96%, whereas the deaminated metabolite phenylacetic acid was not extracted under these conditions.