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A NEW APPROACH TO THE ASSESSMENT OF THE POTENCY OF REVERSIBLE MONOAMINE OXIDASE INHIBITORS *IN VIVO*, AND ITS APPLICATION TO (+)-AMPHETAMINE, *p*-METHOXYAMPHETAMINE AND HARMALINE

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Abstract—A new kinetic formulation for the interaction between reversible and irreversible enzyme inhibitors has been used as a basis for determining enzyme active centre occupancy by reversible enzyme inhibitors *in vivo*. This formulation has been validated *in vitro* by comparing the monoamine oxidase active centre occupancy by (+)-amphetamine calculated from its ability to reduce inhibition by phenelzine with that calculated from its direct inhibitory action on the enzyme. From studies on the protection afforded against inhibition of mouse brain monoamine oxidase by phenelzine *in vivo*, I_{50} values of 5, 0.5 and 0.1 mg·kg^{-1} have been obtained for the inhibition of this enzyme by (+)-amphetamine, *p*-methoxyamphetamine and harmaline. The protection afforded by amphetamine and *p*-methoxyamphetamine against inhibition by pheniprazine, clorgyline and tranylcypromine has also been studied.

The extent to which monoamine oxidase [amine:oxygen oxidoreductase (deaminating) (flavin containing) EC 1.4.3.4] (MAO) is inhibited *in vivo* by irreversible inhibitors, such as the hydrazines or propargylamines, is readily measurable. Animals are injected with the inhibitor, and after a suitable time the appropriate tissue is excised and homogenized, and substrate is then added to the homogenate for enzyme assay *in vitro*. However, if this procedure is adopted with reversible inhibitors, such as the amphetamines or harmala alkaloids, it will underestimate the true degree of inhibition [1]. With reversible inhibitors, this type of experiment does not measure the degree of inhibition in the original tissue but the degree of inhibition produced by the concentration of inhibitor ultimately reached in the *in vitro* assay mixture. This concentration will normally be much lower than that which was originally present in the tissue owing to the extensive dilution which occurs during preparation of the homogenate and during the subsequent addition of substrate and other reagents required for the assay.

An alternative way of showing that reversible inhibitors combine with the active centres of MAO *in vivo* depends on demonstrating that they can reduce the long-lasting inhibition produced by an irreversible inhibitor if they are injected shortly before it [2, 3]. This technique has been used successfully with a variety of reversible and irreversible inhibitors [1–11], but it has not previously been exploited to provide quantitative information on the extent of inhibition which would be produced by the reversible inhibitor when given on its own. In this paper, we present a new kinetic formulation of the interaction between reversible and irreversible inhibitors. This formulation enables the level of active centre occupancy by the reversible inhibitor to be calculated from the extent to which it decreases the level of inhibition by the irreversible inhibitor without requiring any knowledge of the local concentration of the irreversible inhibitor. The validity of this kinetic formulation has been verified under *in vitro* conditions by showing that the level of MAO active centre occupancy by (+)-amphetamine calculated from the extent to which it diminishes the level of inhibition by phenelzine, pheniprazine or tranylcypromine is in good agreement with that calculated from the extent to which it inhibits MAO when measured directly. The technique has then been used to determine the potencies of (+)-amphetamine, *p*-methoxyamphetamine and harmaline as inhibitors of mouse brain MAO *in vivo* from the extent to which they protect the enzyme against inhibition by phenelzine. The relevance to this technique of the choice of irreversible inhibitor and of the relative persistence in the tissues of both the reversible and the irreversible inhibitor is also considered.

MATERIALS AND METHODS

Chemicals. [1-^{14}C] Serotonin creatinine sulphate (5-HT) was obtained from the Radiochemical Centre, Amersham, U.K. *p*-Methoxyamphetamine hydrochloride was synthesized from *p*-methoxybenzaldehyde [11]. Other MAO inhibitors were obtained as gifts or by purchase as follows: (+)-amphetamine sulphate, tranylcypromine sulphate and pheniprazine hydrochloride (Smith, Kline & French), clorgyline hydrochloride (May & Baker), harmaline hydrochloride (Sigma Chem. Co.), pargyline hydrochloride (Abbott), and phenelzine hydrogen sulphate (Fluka AG). Inhibitor doses are expressed throughout in terms of the salt, not the

free base. All other chemicals were obtained from British Drug Houses Ltd., Poole, U.K., or Koch-Light Laboratories, Colnbrook, U.K.

Animals and enzyme preparations. Brain mitochondria from male Holtzman rats (200–250 g) were isolated by differential centrifugation [12]. Male CBA strain mice were used in all the *in vivo* experiments. They were killed by cervical dislocation and the brains were immediately homogenized in 9 vol. ice-cold 0.1 M sodium phosphate buffer (pH 7.4) using an Ultra Turrax TP 18/2 homogenizer.

MAO assay. MAO activity in rat brain mitochondria or in mouse brain homogenate was assayed using minor modifications [11] of the radiochemical method of Otsuka and Kobayashi [13] with 5-HT as substrate.

Protection experiments in vitro. Appropriate concentrations of (+)-amphetamine (dissolved in 0.34 ml of 0.1 M phosphate buffer, pH 7.4) were added to rat brain mitochondria suspended in phosphate buffer (0.96 ml equivalent to 50 mg tissue) and 10 mM EDTA (0.2 ml) and shaken in air at 37°. After 5 min, the irreversible inhibitor (0.2 ml) was added, and the mixture was shaken for a further 15 min. Unlabelled 5-HT (10 mM, 0.2 ml) was then added, followed immediately by [^{14}C] 5-HT (0.23 mM, 0.1 ml). After a further 30 min, the reaction was stopped by adding 2 M citric acid (0.4 ml). The remaining steps in the assay were then as previously described [11, 13]. In the direct inhibition experiments by (+)-amphetamine at low substrate concentrations, the irreversible inhibitor and the unlabelled 5-HT were replaced by buffer and the concentration of mitochondria was reduced by a factor of 10.

Protection experiments in vivo. These were carried out as described in detail elsewhere [10, 11]. In brief, pairs of mice were injected subcutaneously with the reversible inhibitor 15 min before subcutaneous injection of the irreversible inhibitor. They were then killed 24 hr later for assay of their brain MAO activity with [^{14}C] 5-HT as substrate.

KINETIC THEORY

Kinetic analyses of the protective effect of various kinds of reversible ligand against irreversible enzyme inhibition have been presented by several workers [14–16]. However, these analyses have been confined to the *in vitro* situation in which the concentration of the irreversible inhibitor remains constant. The novelty of the approach given below lies in the demonstration that the protection can be expressed quantitatively in such a manner as to be independent of the irreversible inhibitor, and consequently it can be applied to reversible inhibitors *in vivo* as well as *in vitro*.

Inhibition of enzymes by irreversible inhibitors normally results from the formation of a stable covalent bond between the inhibitor and the active centre of the enzyme by a reaction which can be most simply represented as $E + I \rightarrow X$. The degree of inhibition is determined by the fraction of the enzyme converted into the irreversibly modified form X.

If the concentrations of E, I and X are represented by e, i and x, then the rate formation of X is given by

$$dx/dt = kei. \quad (1)$$

The concentration of the free inhibitor will normally be a time-dependent function of the administered dose or initial concentration (i_o), but provided the inhibitor is present in large excess over the enzyme, it will be independent of the concentration of free enzyme.

$$\text{Thus } dx/dt = ke\,[f(i_o, t)]. \quad (2)$$

If the concentration of enzyme is e_o at time 0, and e_t at time t, then equation 2 can be partly integrated to give:

$$\ln (e_o/e_t) = k \int_{t_0}^{t} f(i_o, t)\,dt. \quad (3)$$

If a competitive reversible inhibitor (P) is present at the same time as the irreversible inhibitor, its effect would be to render that fraction of the enzyme active centres which it occupies (α) inaccessible to the irreversible inhibitor. Thus the equation for the rate of formation of X becomes:

$$dx/dt = k(1 - \alpha)\,e\,[f(i_o, t)]. \quad (4)$$

which may also be partly integrated to give:

$$\ln (e_o/e_t)_p = k(1 - \alpha) \int_{t_0}^{t} f(i_o, t)\,dt. \quad (5)$$

In equation 5, $\ln (e_o/e_t)_p$ is the value of $\ln (e_o/e_t)$ for the irreversible inhibitor acting in the presence of the reversible inhibitor. The control enzyme activity (e_o) in this case is the activity in the presence of the reversible inhibitor alone under the same assay conditions. Since the integral terms in equations 3 and 5 are the same, they may be eliminated without them having to be evaluated, thus giving:

$$\alpha = 1 - [\ln (e_o/e_t)_p / \ln (e_o/e_t)]. \quad (6)$$

The degree of enzyme inhibition produced by a competitive, reversible inhibitor varies with the substrate concentration, but provided this is sufficiently low ($[S] \ll K_m$), the level of inhibition will be almost the same as the active centre occupancy α, as calculated above. This is likely to be the situation for MAO *in vivo* where the concentrations of free endogenous substrates, such as 5-HT or noradrenaline, are extremely low.

The following example, taken from Table 5, illustrates the use of equation 6 to calculate α. Pretreatment of mice with 0.07 mg·kg^{-1} of harmaline lowered the level of inhibition of brain MAO produced by 4 mg·kg^{-1} of phenelzine from 77 to 59 per cent. Thus, $\alpha = 1 - [\ln (100/41) / \ln (100/23)] = 0.40$, i.e. harmaline alone at this dose would produce 40 per cent inhibition of brain MAO.

To minimize the effect of small errors in the measurement of the enzyme activity on the calculated value of α, e_t must not be excessively small compared with e_o, and $(e_t)_p$ should not be too close to $(e_o)_p$. Optimum results would be achieved by choosing experimental conditions where e_t was not less than 20 per cent of e_o, and $(e_t)_p$ was not greater than 80 per cent of $(e_o)_p$.

An obvious requirement for the success of the method is accurate assessment of the residual fraction of uninhibited enzyme at time t. When the two types of inhibitor are incubated with the enzyme *in vitro*, this can be achieved simply by addition of a high concentration of substrate ($[S] \gg K_m$) at time t. This will prevent any further combination of the irreversible inhibitor with the enzyme and enable the residual uninhibited enzyme to be assayed directly. However, in *in vivo* experiments if any free irreversible inhibitor remains in a tissue at the time an animal is killed it can continue to react with any available uninhibited enzyme up until the time that the tissue is homogenized and substrate is added. Thus the measured level of inhibition may be greater by an unknown amount than that which was actually present in the tissue at the time the animal was killed. In order to apply equation (6) meaningfully *in vivo* it is necessary that all free irreversible inhibitor should have disappeared from the tissue concerned before the animal is killed, and that a constant level of MAO inhibition should have been reached. This can be achieved by not killing the animals until 24 hr after administration of the irreversible inhibitor. As shown later (Table 2), the level of inhibition by this time is normally approximately constant. However, this introduces a further problem in that the derivation of equation 6 is based on the assumption that the concentration of the reversible inhibitor remains constant, which will clearly not be true over such a long period of time. It follows that the degree of inhibition calculated for the reversible inhibitor from this type of experiment *in vivo* is not a measure of how much inhibition is present at some precisely defined time after injection, but a value which reflects the average level of inhibition during the period when both reversible and irreversible inhibitors are present together in the tissue. If the irreversible inhibitor is cleared only slowly from the tissue, this period will include the time during which the concentration of the reversible inhibitor is also declining. The measured protection, and the calculated level of inhibition by the reversible inhibitor, will thus be less than that which would be found with a more labile irreversible inhibitor. The selection of an irreversible inhibitor with a short biological half-life is thus crucial if the protection method is to give a valid estimate of the inhibitory potency of the reversible inhibitor at the time when its concentration in the tissue is at its highest level. Provided the irreversible inhibitor is sufficiently labile, the concentration of the reversible inhibitor should remain approximately constant if it is given long enough before the irreversible inhibitor to reach its maximum concentration in the tissue during the short period over which the irreversible inhibitor exerts its effect. A simulation study on the importance of the relative persistence in the tissues of the two types of inhibitor as a factor determining the level of protection is described in the Appendix.

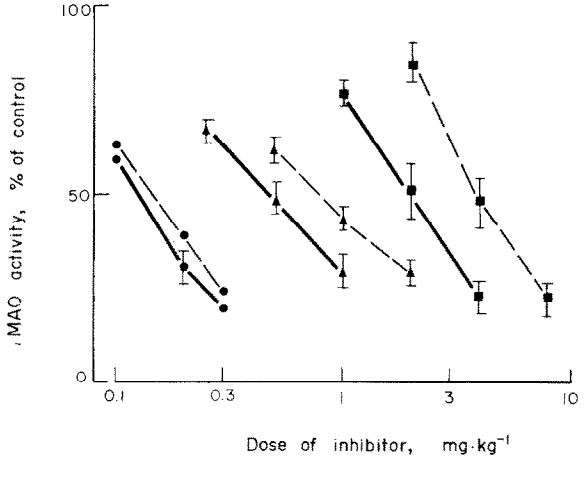

Fig. 1. Effects of 15 min pretreatment with 5 mg·kg^{-1} of (+)-amphetamine sulphate (broken lines) or with 0.9% NaCl (continuous lines) on inhibition of 5-HT oxidation by mouse brain MAO 24 hr after subcutaneous injection of clorgyline (circles), pheniprazine (triangles) or phenelzine (squares). All values are the means (± S.E.M.) from assays on at least four pairs of mice.

One other possible complication arises from the likelihood that very potent irreversible inhibitors do not combine with the enzyme active centre to form the irreversibly modified form X directly, but do so via formation of a transient Michaelis type complex, e.g.

$$E + I \rightleftharpoons EI \rightarrow X.$$

Kitz and Wilson [14] showed that the protective effect of tetraethylammonium ions on the irreversible inhibition of acetylcholinesterase *in vitro* by the methanesulphonate ester of 3-trimethylaminophenol could be interpreted on this basis. Provided the irreversible inhibitor concentration is appreciably lower than the dissociation constant of the EI complex, the derivation of equation 6 given above is still valid. However, if the irreversible inhibitor concentration is of the same order as (or greater than) this dissociation constant, the level of protection by the reversible inhibitor would be reduced due to part of the free enzyme being sequestered in the form EI. In these circumstances, application of equation 6 would underestimate the level of active centre occupancy calculated for the reversible inhibitor when present on its own, but the mathematics would become intractable if the irreversible inhibitor concentration is not constant. As shown by Kitz and Wilson [14], the existence of such an intermediate EI complex leads the relationship between ln (e_o/e_t) and inhibitor concentration to become hyperbolic at high inhibitor concentrations. From our *in vivo* data (Table 4 and Fig. 1) there is some indication that this may happen with tranylcypromine but not with phenelzine, pheniprazine or clorgyline.

RESULTS

Experiments in vitro. In these experiments the irreversible inhibitors were incubated with rat brain mitochondria at 37° for 15 min in the presence or absence of various concentrations of (+)-amphetamine. The residual MAO activity was then assayed with 1 mM 5-HT as substrate. The concentration of the irreversible inhibitor was selected so as to produce 70–80 per cent inhibition in the absence of (+)-amphetamine. Table 1 (second column) shows

Table 1. The effect of (+)-amphetamine on inhibition of rat brain mitochondrial MAO by 0.5 μM phenelzine *in vitro* and calculated MAO active centre occupancy*

(+)-Amphetamine (μM)	MAO activity (per cent of control) Amphetamine + phenelzine (e_i) (5-HT, 1 mM)	Amphetamine alone (e_o)	Calculated per cent active centre occupancy by (+)-amphetamine	Measured per cent inhibition by (+)-amphetamine (5-HT, 11.5 μM)
0	29 ± 2	100	0	0
2.5	39 ± 1	98 ± 1	26	28 ± 1
5	48 ± 1	95 ± 1	44	40 ± 1
10	52 ± 1	88 ± 1	58	55 ± 1
20	58 ± 1	79 ± 1	75	71 ± 1

* Data for measured inhibition by phenelzine or (+)-amphetamine are presented as means (± S.E.M.) from four assays and are based on the activity of control samples containing neither inhibitor. The phenelzine and enzyme were allowed to interact for 15 min at 37° before MAO assay with 1 mM 5-HT as substrate. The per cent active centre occupancy by (+)-amphetamine (fourth column) was derived from the mean values of e_i and e_o (second and third columns) by substitution into equation 6 (see Kinetic Theory). The final column gives the per cent inhibition by (+)-amphetamine alone measured directly at very low substrate concentration (11.5 μM 5-HT).

the inhibition produced by 0.5 μM phenelzine in the presence of 2.5–20 μM (+)-amphetamine. In the presence of 1 mM 5-HT, (+)-amphetamine alone at these concentrations had only a weak inhibitory effect on MAO (shown in the third column of Table 1). The percentage of the MAO active centres occupied by (+)-amphetamine in the absence of substrate was then calculated from these results using equation 6 (see fourth column of Table 1 and Kinetic Theory). This may be compared with the level of MAO inhibition produced by (+)-amphetamine when measured at a concentration of 5-HT (11.5 μM) very much lower than the K_m value (around 100 μM), and which is shown in the final column of Table 1.

Similar protection was afforded by (+)-amphetamine against inhibition of rat brain mitochondrial MAO by pheniprazine or tranylcypromine. Thus, in the presence of 10 μM (+)-amphetamine, the level of inhibition by 0.5 μM pheniprazine was reduced from 77 ± 1 to 49 ± 1 per cent (based on controls containing neither inhibitor), and that by 0.8 μM tranylcypromine was reduced from 77 ± 1 to 57 ± 1 per cent. The level of active centre occupancy by 10 μM (+)-amphetamine calculated from these results is 65 and 50 per cent, respectively, in fair agreement with the results for 10 μM (+)-amphetamine and phenelzine shown in Table 1. Both the calculated active centre occupancy, and the measured level of inhibition by (+)-amphetamine at either 1 mM or 11.5 μM 5-HT concentration are consistent with a K_i value for (+)-amphetamine of around 7 μM.

Experiments in vivo. The extent of MAO inhibition produced in mouse brain at various times after the subcutaneous injection of five irreversible inhibitors is shown in Table 2. With the two hydrazines, maximum inhibition was reached within 30 min, after which time the MAO level remained approximately constant for 24 hr, but with the two propargylamines the level of inhibition continued to increase slowly for several hours after injection. At low dose (1 mg·kg^{-1}) tranylcypromine appeared to behave like the hydrazines, but at a higher dose (5 mg·kg^{-1}) there appeared to be some recovery of MAO activity after 24 hr.

Figure 1 shows dose–response curves for the inhibition of brain MAO 24 hr after injection of various doses of clorgyline, pheniprazine or phenelzine into mice treated 15 min beforehand with 5 mg·kg^{-1} of (+)-amphetamine sulphate or 0.9% NaCl. Figure 2 shows similar curves obtained using clorgyline or phenelzine as the irreversible inhibitor and *p*-methoxyamphetamine as the reversible inhibitor. The MAO activities in the brains of mice 24 hr after injection of amphetamine or *p*-methoxyamphetamine alone were the same as those in the brains of control mice given 0.9% NaCl only. The apparent percentage inhibition of MAO by (+)-amphetamine or *p*-methoxyamphetamine calculated by applying

Table 2. Time course of brain MAO inhibition in mice injected subcutaneously with irreversible inhibitors*

Inhibitor	Dose (mg·kg^{-1})	Per cent inhibition after time (hr) 0.5	1	4	24
Phenelzine	4	77	77	69	77
Pheniprazine	2	90	88	87	84
Clorgyline	0.3	54	68	83	81
Pargyline	20	59	65	70	77
Tranylcypromine	1	46	44	40	49
Tranylcypromine	5	98	95	91	80

* Each result is from a single estimation on the pooled brains from two mice assayed with [^{14}C]5HT as substrate.

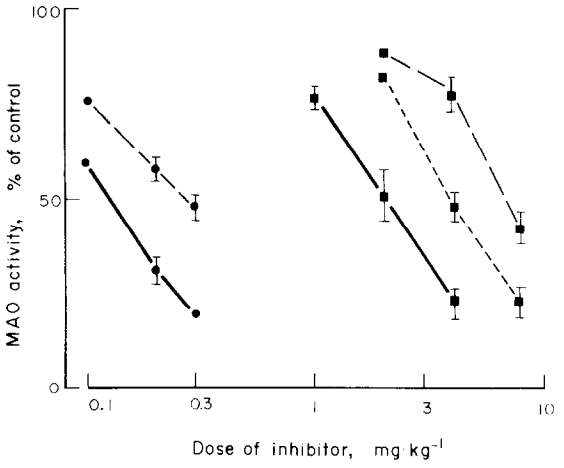

Fig. 2. Effect of 15 min pretreatment with *p*-methoxyamphetamine hydrochloride (broken lines 2.2 mg·kg^{-1}, dotted line 0.5 mg·kg^{-1}) or 0.9% NaCl (continuous lines) on inhibition of 5-HT oxidation by mouse brain MAO 24 hr after subcutaneous injection of clorgyline (circles) or phenelzine (squares). All values are the means (± S.E.M.) from assays on at least four pairs of mice.

equation 6 to the results in Figs. 1 and 2 is shown in Table 3. From the results using phenelzine as the irreversible inhibitor the doses of (+)-amphetamine and *p*-methoxyamphetamine required to cause 50 per cent inhibition of mouse brain MAO when given alone are approximately 5 and 0.5 mg·kg^{-1}, respectively.

The effect of pretreatment with *p*-methoxyamphetamine (2.2 mg·kg^{-1}) on inhibition of mouse brain MAO by tranylcypromine is shown in Table 4. Although *p*-methoxyamphetamine afforded considerable protection against inhibition by tranylcypromine, the extent of this protection (and the calculated apparent level of inhibition by *p*-methoxyamphetamine itself) fell sharply as the dose of tranylcypromine was raised. (+)-Amphetamine at 5 mg·kg^{-1} marginally lowered the degree of inhibition by 1 mg·kg^{-1} of tranylcypromine (from 44 ± 2 to 40 ± 3 per cent, corresponding to 12 per cent inhibition by (+)-amphetamine itself) but gave no measurable protection against inhibition of brain MAO in mice given 2 or 4 mg·kg^{-1} of tranylcypromine.

Table 5 shows the protection afforded by various doses of harmaline against inhibition of mouse brain MAO *in vivo* by 2 or 4 mg·kg^{-1} of phenelzine, together with the percentage inhibition by harmaline itself calculated by use of equation 6. With 2 mg·kg^{-1} of phenelzine there was more variation in the individual assays than with the higher dose, but the calculated per cent inhibition by harmaline was approximately the same at both doses. These results indicate that the dose of harmaline required to cause 50 per cent inhibition of brain MAO in mice is around 0.1 mg·kg^{-1}.

DISCUSSION

As already mentioned, no reliable direct assessment can be made of the potency of reversible enzyme inhibitors *in vivo* because of the dilution of the inhibitor which takes place when the tissue containing the enzyme is homogenized for enzyme assay. The protection technique described in this paper overcomes this dilution problem completely in that what is actually measured is irreversible inhibition, which is unaffected by dilution of the inhibited enzyme preparation.

A further complication which may arise with MAO is the existence in many tissues of multiple forms of the enzyme with differing substrate and inhibitor specificities (see Ref. 17 for a recent review). To avoid this problem, all the results reported in this paper have been obtained using 5-HT as the only substrate. 5-HT is almost exclusively oxidized by A-type MAO, for which clorgyline (at low concentration) is a specific inhibitor. All three reversible inhibitors used have been shown [11, 18, 19] to possess much higher affinity *in vitro* for the A-type enzyme than for the B-type.

The validity of the underlying kinetic theory, especially of equation 6, which is used to calculate

Table 3. Calculated per cent inhibition of mouse brain MAO *in vivo* by (+)-amphetamine sulphate or *p*-methoxyamphetamine hydrochloride as a function of the nature and dose of the irreversible inhibitor*

Irreversible inhibitor	Dose (mg·kg^{-1})	Calculated per cent inhibition by (+)Amphetamine 5 mg·kg^{-1}	*p*-Methoxyamphetamine 0.5 mg·kg^{-1}	*p*-Methoxyamphetamine 2.2 mg·kg^{-1}
Phenelzine	2	76	70	81
	4	50	50	83
Pheniprazine	0.5	33	—	—
	1	32	—	—
	0.1	12	—	50
Clorgyline	0.2	20	—	53
	0.3	11	—	54

* The percentage inhibition by (+)-amphetamine or *p*-methoxyamphetamine was calculated from the experimental data in Figs. 1 and 2 by substitution into equation 6 of the mean values of the residual MAO activity at the specified doses of irreversible inhibitor in mice pretreated with the reversible inhibitor or 0.9% NaCl.

Table 4. Effect of pretreatment with *p*-methoxyamphetamine on inhibition of mouse brain MAO *in vivo* by tranylcypromine*

Tranylcypromine sulphate ($mg \cdot kg^{-1}$)	Per cent inhibition when given 15 min after		Calculated per cent inhibition by *p*-methoxyamphetamine
	0.9% NaCl	*p*-methoxyamphetamine (2.2 $mg \cdot kg^{-1}$)	
1	44 ± 2	14 ± 4	75
2	63 ± 1	32 ± 6	61
4	76 ± 3	64 ± 1	29

* Data for inhibition by tranylcypromine are mean brain MAO activities (±S.E.M.) for assays on four pairs of mice killed 24 hr after injection of tranylcypromine. The per cent inhibition by *p*-methoxyamphetamine was calculated from the mean values for inhibition by tranylcypromine by use of equation 6 (see Kinetic Theory).

the fraction of the enzyme active centres occupied by the reversible inhibitor, has been demonstrated under *in vitro* conditions by the protection experiments summarized in Table 1. The fraction of MAO active centres occupied by concentrations of 2.5–20 μM (+)-amphetamine calculated from the protection afforded against irreversible inhibition by phenelzine is in good agreement with that obtained by direct inhibition experiments at very low substrate concentrations ($[S] \ll K_m$), where the measured level of inhibition should be approximately equal to the fraction of enzyme active centres occupied by the reversible inhibitor. Similar protection was afforded by 10 μM (+)-amphetamine against irreversible inhibition by pheniprazine or tranylcypromine *in vitro* to that found with phenelzine. The protection experiments, and the direct inhibition experiments at both low and high 5-HT concentration, are all consistent with a K_i value of about 7 μM for the interaction of (+)-amphetamine with rat brain mitochondrial MAO.

As explained in the Kinetic Theory section, for accurate assessment of the level of reversible inhibition *in vivo* by this method it is essential to choose an irreversible inhibitor with a short biological half-life. Arylalkylhydrazines are probably the most satisfactory irreversible inhibitors for this type of experiment with MAO. Although the MAO inhibition which they produce is very long-lasting, the compounds themselves disappear rapidly from intact tissues *in vivo* [6]. This is confirmed by the results in Table 2 which show that inhibition by phenelzine and pheniprazine reaches a maximum and constant level within 30 min. Inhibition by clorgyline (and pargyline) *in vivo* reaches its maximum extent much more slowly, implying that some free clorgyline remains in the brain for several hours after injection. (+)-Amphetamine and *p*-methoxyamphetamine exerted much less protection against inhibition by clorgyline than against inhibition by phenelzine or pheniprazine (Figs. 1 and 2 and Table 3). Harmala alkaloids have previously been shown [3, 6] to be more effective in protecting rat brain MAO *in vivo* from inhibition by pheniprazine than by the more persistent inhibitors iproniazid and tranylcypromine. We obtained no significant protection against inhibition by tranylcypromine using (+)-amphetamine, although appreciable protection was afforded by the more potent reversible inhibitor *p*-methoxyamphetamine (Table 4). However, the apparent level of protection exerted by *p*-methoxyamphetamine decreased markedly as the dose of tranylcypromine was raised. Although tranylcypromine is normally classed as an irreversible inhibitor of MAO, recovery of the enzyme from inhibition *in vivo* is faster than with hydrazine or propargylamine inhibitors [20, 21]. This is reflected by the results in Table 2 which show that at the higher dose of tranylcypromine the level of MAO inhibition measured after 24 hr was substantially less than that measured after shorter times. Thus, the level of inhibition measured 24 hr after tranylcypromine in the protection experiments may

Table 5. Effect of 15 min pretreatment with various doses of harmaline on the inhibition of mouse brain MAO 24 hr after subcutaneous injection of phenelzine and calculated inhibition produced by harmaline alone*

Harmaline ($mg \cdot kg^{-1}$)	% Inhibition by phenelzine		% Inhibition by harmaline calculated from	
	2 $mg \cdot kg^{-1}$ (A)	4 $mg \cdot kg^{-1}$ (B)	(A)	(B)
0	49 ± 7	77 ± 4	0	0
0.07	32 ± 9	59 ± 2	43	39
0.35	8 ± 6	9 ± 6	89	94
1.4	0 ± 5	3 ± 3	100	98

* Data for inhibition by phenelzine are mean brain MAO activities (± S.E.M.) for assays on four pairs of mice killed 24 hr after injection of phenelzine. Per cent inhibition by harmaline was calculated from the mean values for inhibition by phenelzine by use of equation 6 (see Kinetic Theory).

be lower than the true level of inhibition at the time of peak protection by *p*-methoxyamphetamine. The level of inhibition calculated for *p*-methoxyamphetamine under these circumstances would consequently be less than that actually present. As indicated earlier another possibility is that the existence of a transient reversible intermediate complex formed between MAO and tranylcypromine becomes kinetically significant at higher doses, which would also result in a fall in the level of protection. As (+)-amphetamine gave greater protection against phenelzine than against pheniprazine, phenelzine was selected as being the irreversible inhibitor of choice out of those which are readily available. We have shown elsewhere [11] that protection by (+)-amphetamine or by *p*-methoxyamphetamine of mouse brain MAO against inhibition by phenelzine *in vivo* is maximal if these drugs are given 15–30 min before the phenelzine, but declines if this interval is shortened or lengthened. Brain levels of (+)-amphetamine in rats or mice given the drug intraperitoneally are also at a maximum level between 15 and 30 min after injection [22, 23].

Even with phenelzine, there is one further complicating factor in that the apparent level of inhibition calculated for (+)-amphetamine or *p*-methoxyamphetamine by use of equation 6 was slightly greater when the dose of phenelzine was 2 mg·kg^{-1} than when it was 4 mg·kg^{-1}. This anomaly is not seen with pheniprazine or clorgyline and possibly arises from MAO itself contributing to the rapid elimination of phenelzine from the tissues *in vivo* [24]. If the level of brain MAO inhibition by the reversible inhibitor *in vivo* calculated from the extent of protection afforded against inhibition by 4 mg·kg^{-1} phenelzine can be accepted as a valid estimate of its approximate inhibitory potency at the time when its effect is maximal, then the doses of (+)-amphetamine sulphate, *p*-methoxyamphetamine hydrochloride and harmaline hydrochloride causing 50 per cent inhibition are approximately 5, 0.5 and 0.1 mg·kg^{-1}, respectively. At as little as 1 mg·kg^{-1}, inhibition by harmaline is virtually complete. The striking potency of harmaline when assessed in this way illustrates the value of this protection technique as a method for studying reversible inhibitors *in vivo*. By this technique, harmaline can be seen to be as potent an MAO inhibitor *in vivo* as clorgyline. This accords well with the exceptionally high affinity of harmaline for MAO *in vitro* [25]. Other workers have shown that harmaline inhibits rat or mouse brain MAO *in vivo* by use of conventional techniques, but they have been obliged to administer much higher doses in order to achieve comparable apparent levels of inhibition [6, 26]. *p*-Methoxyamphetamine is not as potent as harmaline but it nevertheless produces marked inhibition of brain MAO at pharmacologically active doses [11]. Although the inhibitory effect of (+)-amphetamine is weaker than that of the other two compounds, it may still be pharmacologically relevant. The behavioural effects of (+)-amphetamine in mice may be subdivided into two classes depending on the subcutaneous dose required to elicit them [27]. Group 1 effects, including exophthalmos and increased locomotor activity, had ED_{50} values in the range of 1–4 mg·kg^{-1} of (+)-amphetamine base, and it is unlikely that MAO inhibition contributes significantly to these. However, the Group 2 effects, including compulsive gnawing and other signs of the characteristic stereotyped behaviour, had ED_{50} values of 15 mg·kg^{-1} or above. At these doses, MAO inhibition would be expected to be well over 50 per cent. The behavioural effects of (+)-amphetamine appear to be mainly attributable to biogenic amines released from intraneuronal stores [28]; in so far as high levels of MAO inhibition would preserve these amines from destruction, such inhibition may well be an important factor in the production of Group 2 behavioural effects.

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APPENDIX

In the Kinetic Theory section of this paper it was assumed that although the concentration of the irreversible inhibitor might be time dependent, the concentration of the reversible inhibitor was constant. This clearly cannot be strictly true *in vivo*. The following analysis is intended to give some insight into the probable validity of equation 6 in circumstances where the concentrations of both the reversible and the irreversible inhibitor decline with time.

The fraction of enzyme active centres (α) occupied by a reversible inhibitor is given by $z/(1+z)$, where z is its concentration expressed in normalized units (i.e. $z = p/K_p$, where p is the concentration in molar units and K_p is the dissociation constant of the enzyme–inhibitor complex). Thus $(1-\alpha) = 1/(1+z)$. *In vivo*, both z and the irreversible inhibitor concentration (i) will be time-dependent functions of the initial concentrations or administered doses (z_o and i_o). Let $i = f(i_o, t)$ and $z = g(z_o, t)$. Thus in the presence of both inhibitors, the rate of formation of the irreversibly modified enzyme (X) is given by

$$dx/dt = k\, e\, f(i_o, t)/[1 + g(z_o, t)]. \quad (7)$$

Provided both f and g are independent of the enzyme concentration (i.e. both types of inhibitor are lost from the tissues largely as a result of metabolic or excretory processes not involving the enzyme being inhibited), then equation 7 may be partly integrated to give:

$$\ln(e_o/e_t)_p = k \int_o^t f(i_o, t)\, dt/[1 + g(z_o, t)]. \quad (8)$$

The concentrations of both inhibitors in the immediate vicinity of the enzyme will vary with time in a complex manner depending on the relative rates of absorption, distribution and metabolism in the animal body. However, by making assumptions about the mathematical form of the two functions f and g it is possible to use numerical integration methods to construct hypothetical dose–response curves relating the degree of enzyme inhibition to the dose of irreversible inhibitor in the presence of varied doses of the reversible inhibitor. The simplest assumption about f and g is that both inhibitors attain an instantaneous initial concentration directly related to the administered dose, and that this then declines at a first order rate, i.e. $i = i_o \exp(-k_1 t)$ and $z = z_o \exp(-k_2 t)$.

Equation 8 then becomes

$$\ln(e_o/e_t)_p = k\, i_o \int_o^t \exp(-k_1 t)\, dt/[1 + z_o \exp(-k_2 t)]. \quad (9)$$

If k_1 is given an arbitrary value of 1 time·unit^{-1}, then after 10 time units i will be vanishingly small ($<0.00005\, i_o$) and the residual enzyme activity will have reached a constant level. Thus by integrating equation 9 numerically using Simpson's rule between limits of 0 and 10 time units, e_t/e_o can be evaluated as a function of i_o for any specified values of z_o and k_2. Figures 3 a–d show per cent residual activity ($100\, e_t/e_o$) as a function of i_o (plotted on a logarithmic scale) for values of z_o of 0, 1, 3 and 9 (equivalent to 0, 50, 75 and 90 per cent active centre occupancy at zero time) and with $k_2 = 0$, 0.2, 1 or 5. When $k_2 = 5$, the lines are so close together that for clarity in Fig. 3d only the lines for $z_o = 0$ and $z_o = 9$ are included. The rate constant k has been arbitrarily put equal to 1/0.7 in order that when $z_o = 0$, approximately 50 per cent inhibition would occur with i_o equal to 1. Alteration in this value shifts the whole set of curves uniformly to the right or left, but does not alter their relative positions.

These curves may be compared with the experimental plots shown in Figs. 1 and 2, where sufficient time (24 hr) was allowed to elapse between administration of the irreversible inhibitor and killing the animals for all free inhibitor to have disappeared. In both the theoretical and experimental plots the effect of the reversible inhibitor is to shift the log dose—per cent inhibition curve for the irreversible inhibitor to the right in an approximately parallel fashion. This effect has been noted previously [7, 10, 11].

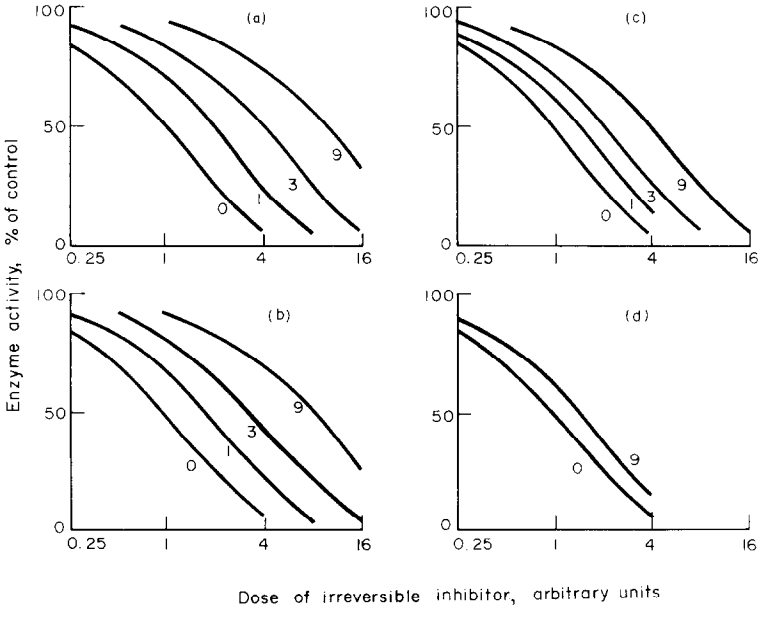

Fig. 3. Simulated plots of residual MAO activity as a function of the logarithm of the dose of irreversible inhibitor (i_0) in the presence of a labile reversible inhibitor. The dose of reversible inhibitor (z_0) is marked against each line. The rate constant for disappearance of the irreversible inhibitor (k_1) = 1; that for the disappearance of the reversible inhibitor (k_2) = 0 (a), 0.2 (b), 1(c) or 5(d). See Appendix for further details.

For any specified dose (z_0) of reversible inhibitor, the extent of the shift is very dependent on the magnitude of k_2 compared with that of k_1. If k_2 is small (Fig. 3b), the shift is almost as great as when k_2 is zero (Fig. 3a). The values of α calculated from equation 6 and the results in Fig. 3b are only slightly less than those calculated from Fig. 3a. Thus if k_2 is zero, α values corresponding to z_0 values of 1, 3 and 9 are 0.5, 0.75 and 0.90, respectively [if $i = i_0 f(t)$ the α value calculated from equation 6 will be independent of the value chosen for i_0], whereas if k_2 is 0.2, these are reduced to 0.45, 0.71 and 0.88. If k_2 is comparable with k_1 (Fig. 3c), the shift to the right is much reduced. For example, the value of z_0 must be increased from 1 to 3 to obtain the same shift to the right as found when k_2 is zero. From the experimental plots in Fig. 2, it can be seen that the dose of *p*-methoxyamphetamine must be increased about four times to achieve the same degree of protection against inhibition by clorgyline as is found against inhibition by phenelzine, suggesting that free clorgyline and *p*-methoxyamphetamine are cleared from the brain at similar rates. If k_2 is much greater than k_1 (Fig. 3d), only a small shift to the right occurs even with a dose ($z_0 = 9$) of reversible inhibitor that initially occupies 90 per cent of the enzyme active centres.

These simulated plots thus confirm the necessity for choosing a highly labile irreversible inhibitor that is rapidly cleared from the tissues concerned if the protection technique is to give a valid estimate of the inhibitory potency of the reversible inhibitor at the time when it is exerting its maximal effect.

It may also be noted from equation 5 (see Kinetic Theory) that if $i = i_0 f(t)$, an alternative method of calculating α is from the extent to which the log dose–response curve is shifted to the right (i.e. from the extent to which i_0 must be raised to exactly offset the $(1 - \alpha)$ term in equation 5. If a dose of reversible inhibitor causes an *n*-fold increase in the dose of irreversible inhibitor required to cause any specified level of inhibition, then

$$\alpha = (n - 1)/n. \quad (9)$$

Thus, the dose of reversible inhibitor which doubles the dose of irreversible inhibitor needed to cause 50 per cent inhibition (i.e. where $n = 2$) is also the dose which would itself cause 50 per cent inhibition if the reversible inhibitor were present on its own. The values of α calculated from the increase in the dose of phenelzine required to cause 50 per cent inhibition of brain MAO in mice pretreated with 5 $mg \cdot kg^{-1}$ (+)-amphetamine, 0.5 or 2.2 $mg \cdot kg^{-1}$ *p*-methoxyamphetamine or 0.07 $mg \cdot kg^{-1}$ harmaline are 0.49, 0.47, 0.74 and 0.39, respectively. These are in fair agreement with the corresponding values (0.50, 0.50, 0.83 and 0.39) calculated from applying equation 6 to the extent of inhibition produced by 4 $mg \cdot kg^{-1}$ of phenelzine. Thus, although equation 9 is less firmly grounded theoretically than equation 6, it appears to give similar values for α, and it is much simpler to use than equation 6.