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p-Methoxyamphetamine, a potent reversible inhibitor of type-A monoamine oxidase in vitro and in vivo

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p-Methoxyamphetamine is over 20 times as potent as (+)-amphetamine as an inhibitor of 5-HT oxidation by monoamine oxidase in mouse brain in vitro, with a K_i value of 0·22 μM. It is highly selective towards A-type monoamine oxidase and possesses only weak activity against the B-type enzyme (K_i value about 500 μM with benzylamine as substrate and solubilized rat liver mitochondria as enzyme source). It is 10 times more active than (+)-amphetamine in protecting mouse brain monoamine oxidase from inhibition by phenelzine in vivo. *o*-Methoxy- and *m*-methoxyamphetamines inhibit monoamine oxidase both in vitro and in vivo with potencies comparable with, or less than that of (+)-amphetamine.

p-Methoxyamphetamine is the simplest of the hallucinogenic amphetamine derivatives (Shulgin et al 1969). It causes neither the marked motor stimulation nor the stereotyped behaviour found with amphetamine itself, and it affects 5-hydroxytryptaminergic neurons much more strongly than catecholaminergic neurons (Tseng et al 1976). It is about ten times more active than amphetamine in elevating brain 5-HT and in lowering 5-hydroxyindoleacetic acid (Hitzemann et al 1971). Insertion of chloro or methyl substituents into the *para* position of amphetamine has been shown to enhance its monoamine oxidase inhibitory properties (Fuller 1966; Ross et al 1977), hence it seemed possible that the high potency of *p*-methoxyamphetamine in raising brain 5-HT concentrations could be accounted for if this compound were appreciably more active than amphetamine as an inhibitor of monoamine oxidase (MAO). In this paper it is shown that *p*-methoxyamphetamine is a highly potent and selective inhibitor of A-type MAO both in vitro and in vivo whereas the *o*- and *m*-isomers have potencies comparable with, or slightly less than that of (+)-amphetamine.

MATERIALS AND METHODS

Compounds. The methoxyamphetamines were synthesized from the corresponding commercially available methoxybenzaldehydes by condensation with nitroethane followed by reduction with lithium aluminium hydride (Bu'Lock & Harley-Mason 1951). The hydrochloride salts were characterized by chloride analysis and melting point. (+)-Amphetamine sulphate was obtained from Smith, Kline & French Laboratories and phenelzine hydrogen sulphate from Fluka AG. [^{14}C]-5-HT creatinine sulphate and [^{14}C]dopamine hydrochloride were obtained from the Radiochemical Centre, Amersham. These were diluted with aqueous solutions of the same unlabelled substrates to give a final concentration of 40 μg base containing 0·225 μCi (500 000 d min^{-1}) ml^{-1}.

Animals. Male CBA strain mice (20–30 g) were used in the in vivo experiments and as a source of brain homogenate. Mice given (+)-amphetamine were kept singly in individual boxes to minimize central excitement. Sprague-Dawley rats (200–300 g) were used as a source of brain and liver mitochondria.

Tissue and enzyme preparations. Mice were killed by cervical dislocation, and their brains were homogenized in 9 volumes of ice-cold 0·1 M sodium phosphate buffer (pH 7·4) with an Ultra-Turrax 18/2 homogenizer. Rat brain and liver mitochondria were obtained by differential centrifugation of brain or liver homogenized in 0·3 M sucrose (Green 1962). The sedimented mitochondria were suspended in phosphate buffer to give a final volume of 1 ml g^{-1} wet weight of tissue. All preparations were stored at −15 °C until required. Solubilized rat liver mitochondria were obtained by diluting the mitochondrial suspension with 9 volumes of buffer containing 1·25% Tergitol NPX (British Drug Houses). The suspension was left at room temperature (20 °C) for 10 min and was then centrifuged at 75 000 *g* for 15 min. The clear supernatant, which contained the solubilized enzyme, was decanted and kept at 0 °C. The MAO activity of this preparation declines slowly, decreasing by about 30% after 2 days, when the preparation was discarded.

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Enzyme assays. MAO activity was assayed at low substrate concentration at 30 °C and pH 7·4 essentially as described by Otsuka & Kobayashi (1964). With [^{14}C]-5-HT as substrate (final concentration 11·5 μM), the non-basic reaction products were extracted directly into anisole containing 0·6% diphenyloxazole (PPO) ready for counting, as in the original method, but with [^{14}C]dopamine, the product recovery on extraction with this solvent was poor. Consequently, with dopamine the reaction products were first extracted into 10 ml of ethyl acetate, 7 ml of which was then added to 1 ml ethanol and 7 ml of anisole containing PPO for counting. In the kinetic studies at varied 5-HT concentration, the concentration of labelled substrate was kept constant and the total concentration altered by adding various concentrations of unlabelled 5-HT immediately before the labelled substrate. The apparent MAO activity measured in terms of counts min^{-1} in the anisole extract was corrected at each substrate concentration to allow for the altered initial specific activity.

MAO activity of solubilized rat liver mitochondria with benzylamine as substrate was assayed by continuous recording on a Pye Unicam SP8-100 double beam spectrophotometer of the increase in extinction at 250 nm produced at 30 °C and pH 7·4 when 0·1 ml benzylamine (normally 10 mM) was added to 0·2 ml enzyme preparation and 2·9 ml 0·1 M sodium phosphate buffer containing the inhibitor (Gorkin et al 1963).

In vivo experiments. Pairs of male mice were given (+)-amphetamine sulphate, one of the methoxyamphetamine hydrochlorides, or 0·9% NaCl 15 min before phenelzine hydrogen sulphate. The drugs were dissolved in 0·9% NaCl and injected subcutaneously in a volume of 10 ml kg^{-1}; the phenelzine was also neutralized with sodium bicarbonate. All doses are expressed in terms of the drug salt. After 24 h, the mice were killed and the brains were removed and homogenized in 0·1 M sodium phosphate buffer (pH 7·4) for MAO assay by the radioisotopic method with 5-HT as substrate as described above. The assay mixtures contained 5 mg brain tissue in a total volume of 2 ml.

RESULTS

The oxidation of 5-HT by MAO in mouse brain homogenate was inhibited competitively by *p*-methoxyamphetamine. The level of inhibition was independent of how long the inhibitor was incubated with the enzyme before addition of substrate. Fig. 1 shows double reciprocal plots for mouse brain homogenate at 30 °C in the presence and absence of the inhibitor. The K_m value in the absence of inhibitor was 90 μM. A secondary plot of slope against inhibitor concentration was linear and gave a K_i value of 0·22 μM.

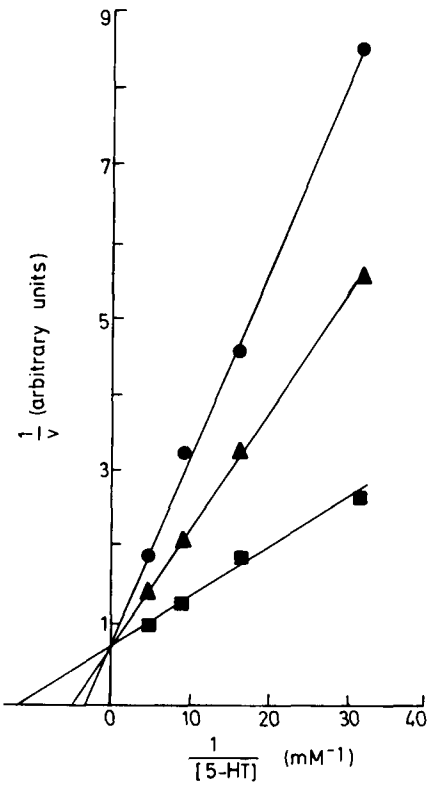

FIG. 1. Lineweaver-Burk plots for the inhibition of 5-HT oxidation at 30 °C and pH 7·4 by *p*-methoxyamphetamine: (■) 0 μM; (▲) 0·25 μM; (●) 0·5 μM. All points are the means of 4 assays with mouse brain homogenate (5 mg tissue in a total volume of 2 ml) as enzyme source.

The reversibility of inhibition was shown by incubating mouse brain homogenate for 5 min with 0·5 μM *p*-methoxyamphetamine followed by centrifugation for 10 min at 10 000 *g* to separate the particulate enzyme from the free inhibitor. On resuspension of the sediment in inhibitor-free buffer, followed by addition of 5-HT, the MAO activity was fully restored (Table 1).

The relative potencies of the three isomeric methoxyamphetamines as inhibitors of 5-HT oxidation were compared with that of (+)-amphetamine by incubation of a range of inhibitor concentrations with mouse brain homogenate at low substrate concentration (11·5 μM). Plots of 100/(% residual enzyme activity) (i.e. v_o/v_i) against inhibitor concen-

Table 1. Reversible inhibition of mouse brain MAO by *p*-methoxyamphetamine. Tubes (A) contained mouse brain homogenate (0·5 ml equivalent to 0·005 g brain tissue), phosphate buffer (pH 7·4, 0·7 ml) and *p*-methoxyamphetamine (2 μM, 0·5 ml). Tubes (B) contained the same volume of enzyme with 1·2 ml buffer and no inhibitor. Both tubes were centrifuged in order to sediment the enzyme. The sediments and supernatants were interchanged as shown in the Table. MAO activity in the resuspended sediments was assayed by the radioisotopic method as usual using [^{14}C]-5-HT as substrate. Controls were obtained by interchanging supernatants and sediments between pairs of tubes with inhibitor (similar to A) and pairs of tubes without inhibitor (similar to B). The results in parentheses are the actual results obtained in duplicate experiments.

Relative enzyme activity (% of control with no inhibitor)			
		Tests (after interchange)	
Controls		Uninhibited sediment (B) + supernatant with inhibitor (A)	Inhibited sediment (A) + supernatant without inhibitor (B)
Inhibitor absent	Inhibitor present		
100 (99, 101)	34 (32, 36)	31 (31, 32)	95 (100, 90)

tration were linear with all four compounds. That for *p*-methoxyamphetamine is shown in Fig. 2. As the substrate concentration is very much lower than the K_m value, the concentration of inhibitor causing 50% inhibition (I50), which is given by the intercept on the abscissa, is approximately equal to the K_i value. Values of K_i for the four compounds obtained in this way are included in Table 2. Similar K_i values were obtained using mitochondria from rat brain or liver as the source of MAO. Fig. 2 also shows the plot of v_o/v_i against *p*-methoxyamphetamine concentration with dopamine (13 μM) as substrate. The plot clearly falls into two sections suggesting the presence of two enzyme forms acting on this substrate, one being very sensitive to inhibition by *p*-methoxyamphetamine, and the other being very insensitive.

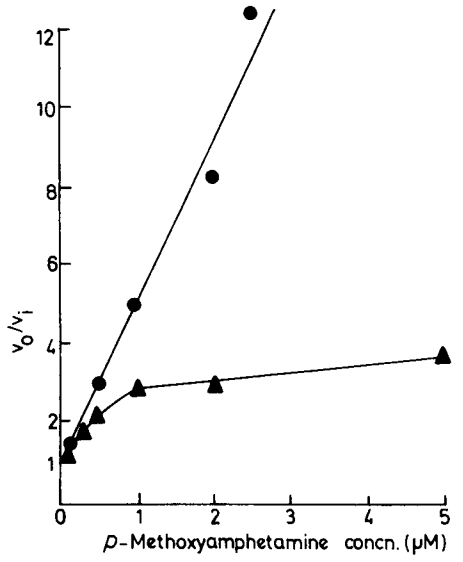

FIG. 2. Plots of v_o/v_i against inhibitor concentration for inhibition of mouse brain MAO at 30 °C and pH 7·4 by *p*-methoxyamphetamine at low 5-HT (11·5 μM) (●) or dopamine (13 μM) (▲) concentration. v_o/v_i = ratio of enzyme activity in absence and presence of inhibitor.

Table 2. I50 and K_i at 30 °C and pH 7·4 in vitro for the inhibition of MAO by (+)-amphetamine and the methoxyamphetamines.

Inhibitor	Mouse brain (5-HT) K_i (μM)	Rat liver (benzylamine) I50 (mM)	Rat liver (benzylamine) K_i (μM)
p-Methoxyamphetamine	0·2	1·5	530
(+)-Amphetamine	6	1·4	490
o-Methoxyamphetamine	9	1	350
m-Methoxyamphetamine	23	5·5	1,940

The low potency of all four compounds as inhibitors of the B form of MAO was confirmed by use of benzylamine as substrate with solubilized rat liver mitochondria as the source of enzyme. (+)-Amphetamine itself has already been shown to be a weak reversible competitive inhibitor in this system (Mantle et al 1976). These authors also showed that the inhibitory potency of amphetamine and other phenylalkylamines with benzylamine as substrate and membrane bound MAO as enzyme source was similar to that found using solubilized MAO. The concentration of benzylamine used (312 μM) is about twice the K_m value (170 μM, obtained from a double reciprocal plot), hence the K_i values were obtained from the I50 values by dividing by (1 + [S]/K_m). Both I50 and K_i values are included in Table 2. That for (+)-amphetamine is in good agreement with that (580 μM) reported by Mantle et al (1976).

Inhibitory potency against MAO in vivo was determined from the ability of these compounds to diminish the long lasting inhibition of 5-HT oxidation by mouse brain MAO produced by injection of phenelzine (Green & El Hait 1978). Plots showing the percentage residual MAO activity after 24 h as a function of the logarithm of the dose of phenelzine in mice treated 15 min beforehand with 0·5, 1·1 or 2·2 mg kg^{-1} of *p*-methoxyamphetamine hydrochloride are shown in Fig. 3. The lowest dose was

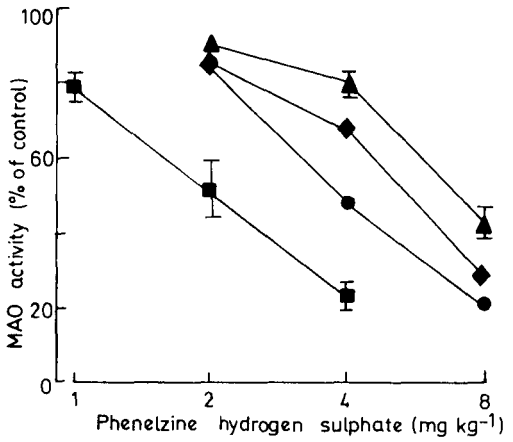

FIG. 3. Effect of pretreatment with 0·9% NaCl (■), or *p*-methoxyamphetamine hydrochloride [0·5 mg kg^{-1} (●); 1·1 mg kg^{-1} (◆); 2·2 mg kg^{-1} (▲)] on inhibition of mouse brain MAO by phenelzine in vivo. All points are the means (± s.e.m.) from assays on at least 4 pairs of mice with [^{14}C]-5-HT as substrate. For clarity, error bars are omitted on some of the points.

sufficient to double the dose of phenelzine required to produce 50% inhibition of brain MAO. The *o*- and *m*-isomers, as well as (+)-amphetamine itself, were much less effective than the *p*-isomer in this test. Table 3 shows the dose of phenelzine required to produce 50% inhibition of brain MAO after 24 h in mice pretreated with 2·2 or 5·5 mg kg^{-1} of the three methoxyamphetamines or with 2 or 5 mg kg^{-1} of (+)-amphetamine sulphate (equivalent in molar terms to 2·2 or 5·5 mg kg^{-1} of the methoxyamphetamine hydrochlorides).

The duration of action of *p*-methoxyamphetamine in vivo was assessed by measuring the level of inhibition produced by 4 mg kg^{-1} of phenelzine when given at various times after 2·2 mg kg^{-1} of *p*-methoxyamphetamine. The results are shown in Table 4. The degree of protection reaches a maximum with a pretreatment time of 15 to 30 min, and then declines. (+)-Amphetamine showed a similar time-relationship, with maximum protection being achieved if it is injected 15–30 min before the phenelzine.

Table 3. Dose of phenelzine hydrogen sulphate (mg kg^{-1}) causing 50% inhibition of brain MAO in mice pretreated with the amphetamines.

Compound	Dose of amphetamine derivative (μ mol kg^{-1}) 0	5·4	10·8	27
p-Methoxyamphetamine	2·0	5·5	6·8	n.t.
(+)-Amphetamine	2·0	n.t.	3·1	4·0
o-Methoxyamphetamine	2·0	n.t.	2·7	3·1
m-Methoxyamphetamine	2·0	n.t.	2·3	2·8

The doses of phenelzine were derived by interpolation from activity-log dose plots such as those in Fig. 3. n.t. = not tested.

Table 4. Effect of pretreatment time with *p*-methoxyamphetamine hydrochloride (2·2 mg kg^{-1}) or (+)-amphetamine sulphate (5 mg kg^{-1}) on extent of inhibition of mouse brain MAO by phenelzine hydrogen sulphate (4 mg kg^{-1}).

Compound	Pretreatment time (h) 0	0·25	0·5	1	2
p-Methoxyamphetamine	32 ± 2	26 ± 3	22 ± 2	42 ± 3	63 ± 3
(+)-Amphetamine	60 ± 5	50 ± 5	48 ± 2	52 ± 4	60 ± 2

Each result is the mean (± s.e.m.) % inhibition by phenelzine in 4 pairs of mice. Phenelzine alone produced 77 ± 4% inhibition.

DISCUSSION

From experiments on the inhibition of rat brain MAO in vitro by the irreversible inhibitor clorgyline, Johnston (1968) suggested that this tissue contained two major forms of MAO, the A-form which was highly sensitive to clorgyline and for which 5-HT was a specific substrate, and the B-form which was much less sensitive to clorgyline and for which benzylamine was a specific substrate. Dopamine and tyramine are oxidized by both forms. Although the molecular differences underlying this classification are still unclear and although difficulties sometimes arise in applying it to all tissues (Fowler et al 1978), it nevertheless still provides a useful pragmatic basis for interpreting the complex kinetic behaviour observed when inhibition is studied with a range of different substrates. A number of competitive reversible inhibitors, including harmaline (Fuller 1968) and amphetamine (Mantle et al 1976) have also been shown to possess moderate selectivity for the A-form of MAO. *p*-Methoxyamphetamine, which is over 1000 times more active in blocking 5-HT oxidation than benzylamine oxidation, must now be included in this group. The selectivity of this compound for the A-form is confirmed by the plot of the reciprocal of the residual enzyme activity against inhibitor concentration obtained with dopamine as substrate. This shows a clear division into two sections, one corresponding to a form very sensitive

to inhibition by *p*-methoxyamphetamine, the other corresponding to a form which is relatively insensitive to this inhibitor.

Selectivity for the A-form of MAO is also shown by *o*- and *m*-methoxyamphetamines, as well as by amphetamine itself, but the selectivity is less pronounced than with the *p*-isomer. The four compounds show rather similar potencies with benzylamine as substrate, but *p*-methoxyamphetamine is over 20 times as active as the other three compounds with 5-HT.

The high intrinsic potency of *p*-methoxyamphetamine against 5-HT oxidation is also shown in vivo, where it is around 10 times more active than any of the other compounds in protecting mouse brain MAO against inhibition by phenelzine. There is an excellent correlation for the four compounds between MAO inhibitory potency in vitro, and ability to protect against phenelzine in vivo. If it can be assumed that the dose of reversible inhibitor required to double the dose of phenelzine needed to produce 50% inhibition in vivo is also the dose itself producing 50% inhibition (this hypothesis will be discussed further in another paper), then the I50 for inhibition of mouse brain MAO by *p*-methoxyamphetamine in vivo is about 0·5 mg kg^{-1}. This dose is only about one quarter of the I50 for phenelzine, but whereas inhibition by phenelzine persists for several days, the high level of inhibition by *p*-methoxyamphetamine is maintained only for a short time and the extent of inhibition declines rapidly after 1 h.

The relevance of MAO inhibition for the pharmacology of *p*-methoxyamphetamine has not been investigated directly, but the doses required to elicit pharmacological effects in animals are normally around 1 mg kg^{-1} upwards (Krishna Menon et al 1976). The hallucinogenic dose in man is of the same order (Shulgin et al 1969). These doses would be sufficient to produce extensive inhibition of MAO in the brain, thus the presence of MAO inhibition should be considered in any interpretation of the effects of *p*-methoxyamphetamine on 5-HT-ergic nerve function.

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