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Oxidation and enzyme-activated irreversible inhibition of rat liver monoamine oxidase-B by 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)

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The compound 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP), which produces symptoms resembling Parkinson's disease in humans, acts both as a substrate and an enzyme-activated irreversible inhibitor of the B-form of monoamine oxidase from rat liver. Analysis of the inhibitory process showed the compound to be considerably more efficient as a substrate than as an irreversible inhibitor, with about 17000 mol of product being formed per mol of enzyme inactivated. The half-time of the inhibitory process was about 22 min. With the A-form of the enzyme, the compound had a lower K_m value and a considerably lower maximum velocity than the corresponding values obtained with the B-form. Under the conditions used in the present work the inhibition of the A-form of the enzyme was largely reversible.

INTRODUCTION

MPTP produces a condition resembling Parkinson's disease in humans and some experimental animals (see Burns *et al.*, 1983; Langston *et al.*, 1983, 1984*a*). The selective destruction of dopaminergic neurons that results from administration of MPTP can be prevented by pretreatment with selective inhibitors of the B-form of monoamine oxidase [monoamine:oxygen oxidoreductase (deaminating) (flavin containing) (EC 1.4.3.4)] (Cohen *et al.*, 1984; Heikkila *et al.*, 1984; Langston *et al.*, 1984*b*; Melamed & Youdim, 1985; Rainbow *et al.*, 1985).

The action of monoamine oxidase-B appears to involve conversion of MPTP to its corresponding dihydropyridine derivative, MPDP, which is then converted by a process that appears to be non-enzymic (Buckman & Eiduson, 1985) to MPP^+, which is believed to be the active neurotoxin (Chiba *et al.*, 1984; Fritz *et al.*, 1985; Javitch & Snyder, 1985; Markey *et al.*, 1984).

A number of studies have indicated that MPTP is an inhibitor as well as a substrate for monoamine oxidase-B (Fuller & Hemrick-Luecke, 1985; Kinemuchi *et al.*, 1985; Salach *et al.*, 1984; Singer *et al.*, 1986). This paper reports the results of a study on the mechanisms of these effects.

MATERIALS AND METHODS

Rat liver mitochondria were prepared and stored as previously described (Fowler & Tipton, 1981). Samples were preincubated for 60 min at 37 °C with either 0.3 μM-(−)-deprenyl or 0.3 μM-clorgyline in order to inhibit selectively the activity of the B or A forms of monoamine oxidase, respectively. Previous studies have shown such treatments to inactivate one of the two forms of the enzyme without significantly affecting the activity of the other (Fowler & Tipton, 1981). This was confirmed in the present study by assaying the activities of the treated preparations towards 100 μM-5-hydroxytryptamine and 10 μM-2-phenethylamine, which have been shown to be specific substrates for the A and B forms of monoamine oxidase respectively, at these concentrations (Tipton *et al.*, 1982). Mitochondria from human placenta were prepared in a similar way and stored at −20 °C until use.

The concentrations of the B-form of monoamine oxidase in rat liver mitochondria were determined from the binding of ^{3}H-labelled pargyline (Parkinson & Callingham, 1980; O'Carroll *et al.*, 1984) to be 5.4 pmol/mg of protein. Protein concentration was determined by the method of Markwell *et al.* (1978) with bovine serum albumin as the standard.

Activities towards 2-phenethylamine and 5-hydroxytryptamine were determined radiochemically by a modification (Tipton, 1985) of the method of Otsuka & Kobayashi (1964) at 37 °C in 100 mM-potassium phosphate buffer, pH 7.2. The oxidation of MPTP was followed at 37 °C spectrophotometrically at 340 nm (Fritz *et al.*, 1985). The reaction mixture contained, in a total volume of 2.8 ml, 100 mM-potassium phosphate buffer, pH 7.2, the enzyme-containing preparation and MPTP. The amount of oxidation product, presumed to be MPDP, was calculated assuming an absorption coefficient of 13000 $M^{-1} \cdot cm^{-1}$ at 340 nm (Fritz *et al.*, 1985). Activity with benzylamine as the substrate was determined under the same conditions by following the change in absorbance at 250 nm (Tabor *et al.*, 1954).

Radioactively labelled substrates were obtained from Amersham International and ^{3}H-labelled pargyline from New England Nuclear. MPTP was from Aldrich Chemical Co. Clorgyline and (−)-deprenyl were kind gifts from Professor J. Knoll, Semmelweis University School of Medicine, Budapest, Hungary, and May & Baker, Dagenham, Essex, U.K.

Abbreviations used: MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; MPDP, 1-methyl-4-phenyl-2,3-dihydropyridine; MPP^+, 1-methyl-4-phenylpyridinium ion.

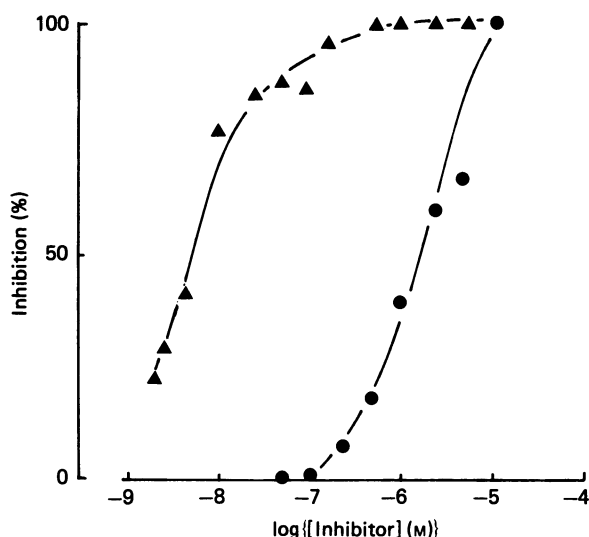


Fig. 1. Inhibition of the activities of monoamine oxidase towards MPTP by clorgyline and (−)-deprenyl

The enzyme samples were incubated with the indicated concentration of clorgyline (●) or (−)-deprenyl (▲) for 60 min at 37 °C before the activity towards 1 mM-MPTP was assayed as described in the text. Results are expressed as mean values of triplicate determinations of the activities relative to control samples that had been preincubated under the same conditions in the absence of inhibitor before assay.

Table 1. Kinetic parameters for MPTP as a substrate for monoamine oxidase

Activities of the two forms of monoamine oxidase were determined after preincubation of the preparations with either 0.3 μM-clorgyline to inhibit the activity of monoamine oxidase-A or 0.3 μM-(−)-deprenyl to inhibit the activity of monoamine oxidase-B. Results shown are the mean values ± S.E.M. from at least three determinations.

Enzyme form	Source	K_m (μM)	V (nmol · min^{-1} · mg^{-1})
A	Rat liver	12 ± 4	0.44 ± 0.12
A	Human placenta	36 ± 5	1.7 ± 0.4
B	Rat liver	153 ± 14	4.5 ± 0.7

RESULTS

The initial rates of oxidation of MPTP by monoamine oxidase-A and -B were found to be proportional to the concentrations of the enzyme-containing samples. When samples were pretreated for 60 min at 37 °C with 0.3 μM-clorgyline plus 0.3 μM-(−)-deprenyl no activity could be detected. In rat liver mitochondria the activity of monoamine oxidase-A towards this substrate was considerably lower than that of the B form, as shown by the inhibition of these activities by the selective inhibitors clorgyline and (−)-deprenyl (Fig. 1). In order to be sure that the observed activity was not due to a very small residual component of the B-form of the enzyme in the clorgyline-treated rat liver mitochondrial prepara-

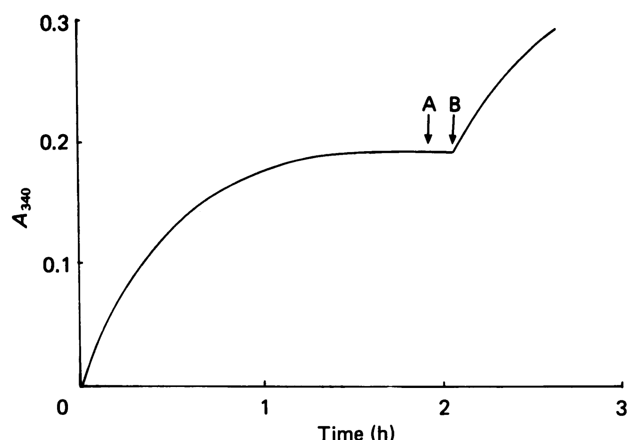


Fig. 2. Time course of the oxidation of 1.0 mM-MPTP by monoamine oxidase-B from rat liver mitochondria

At the point indicated by A, a further sample of MPTP was added to raise the final concentration by 1.0 mM and at the point indicated by B, a further sample of monoamine oxidase-B preparation was added.

tion, mitochondria from human placenta, which have been shown only to contain monoamine oxidase-A (Powell & Craig, 1977; Salach & Detmer, 1979), were also assayed for activity towards this substrate.

Initial rate determinations at a series of MPTP concentrations showed Michaelis–Menten kinetics to be obeyed and the kinetic constants determined from these studies are shown in Table 1.

The time-courses of MPTP oxidation by monoamine oxidase-B were non-linear as shown in Fig. 2. The oxidation of 100 μM-benzylamine, monitored at 250 nm, was found to proceed linearly under similar conditions. After the change in absorbance at 340 nm had ceased, activity could not be restored by the addition of a further portion of MPTP (Fig. 2), indicating that the reaction had not ceased because of substrate depletion or the establishment of the equilibrium of a reversible reaction. Neither could any activity be detected when the wavelength was changed to 250 nm and 100 μM-benzylamine was added (result not shown). The reaction could, however, be restarted by the addition of a further sample of the monoamine oxidase preparation (Fig. 2).

The effects of MPTP concentration on the activities of monoamine oxidase-A and -B towards 5-hydroxytryptamine and 2-phenethylamine, respectively, are shown in Fig. 3. The compound can be seen to be a more potent inhibitor of the A-form of the enzyme, and the degree of inhibition was significantly increased by enzyme–inhibitor preincubation. The inhibition of monoamine oxidase-B showed a more marked time-dependence.

The reversibility of the inhibition was investigated by dilution of samples that had been preincubated with MPTP at concentrations of up to 5 mM for 3 h at 37 °C (see Waldmeier *et al.*, 1983). Persistence of the inhibition of monoamine oxidase-B indicated that it was essentially irreversible under these conditions. When samples were incubated with 0.1 mM-MPTP for 2 h at 37 °C and then diluted 10-fold with 100 mM-potassium phosphate buffer, pH 7.2, the inhibition, compared with a control incubated in the absence of MPTP and similarly diluted, was 50 ± 2% (mean ± S.E.M., $n = 4$) and this value was unchanged over a 3 h period of subsequent incubation at

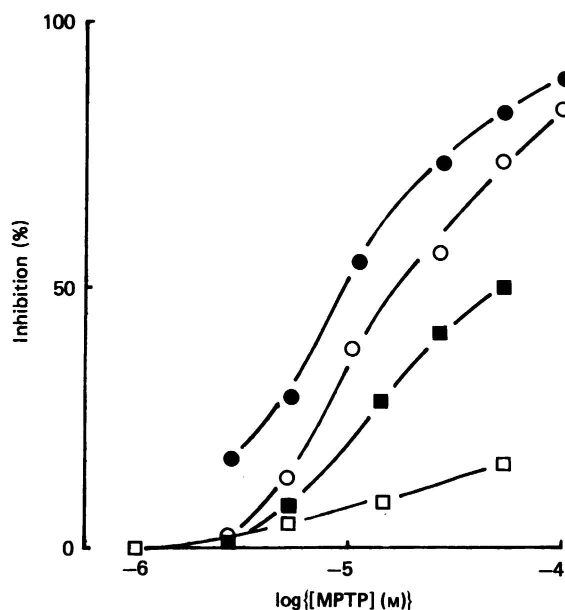
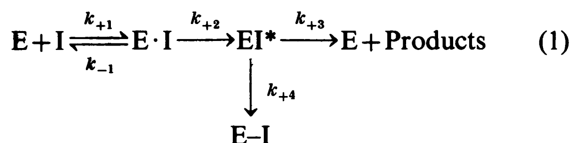


Fig. 3. Inhibition of the activites of monoamine oxidase towards 5-hydroxytryptamine and 2-phenethylamine by MPTP

The activities of the enzyme-containing preparations were determined in the presence of the indicated concentrations of MPTP with 20 μM-2-phenethylamine (□,■) or 100 μM-5-hydroxytryptamine (○,●). The open symbols represent the activities determined without preincubation of enzyme with MPTP, whereas in those represented by the closed symbols the enzyme/MPTP mixture was preincubated for 60 min at 37 °C before assay. Results are shown as mean values of triplicate determinations of the activities of control samples treated in the same way but in the absence of inhibitor.

37 °C or a 7 h storage period at 0 °C. In contrast, the dilution experiments indicated only a small persistence (less than 10%) of inhibition of monoamine oxidase-A, indicating any irreversible component of the inhibition of that form of the enzyme to be small under the conditions used.

The progress curves for the inhibition of monoamine oxidase-B by MPTP would be consistent with this compound acting as both a substrate and a time-dependent irreversible inhibitor of the enzyme. Such a process may be represented by the reaction scheme:



$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} E\cdot I \xrightarrow{k_{+2}} EI^* \xrightarrow{k_{+3}} E + \text{Products} \quad (1)$$
$$EI^* \xrightarrow{k_{+4}} E\text{–}I$$

in which the reaction of the enzyme (E) with MPTP (I) results in the initial formation of a non-covalent complex (E·I) which is subsequently converted to an activated intermediate (EI*) that can then either break down to give products and free enzyme or react to give rise to the irreversibly inhibited species (E–I). The kinetic behaviour of such systems has been considered by Waley (1980, 1985) and by Tatsunami *et al.* (1981).

The partition ratio (r), which represents the number of mol of product formed per mol of enzyme inhibited, was calculated from the amount of product formed at

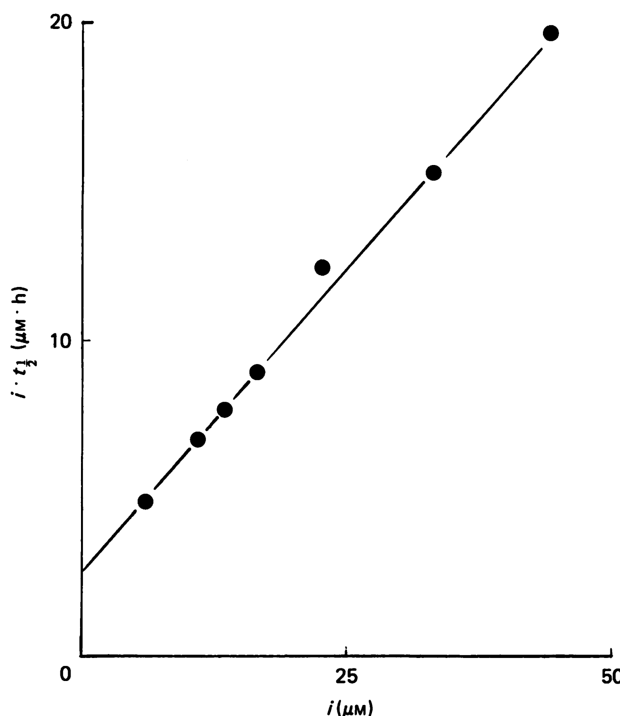


Fig. 4. Half-time plot for the mechanism-based inhibition of monoamine oxidase-B by MPTP

complete inactivation (P_∞) for six different enzyme-inhibitor ratios by using the relationship (Waley, 1980):

$$r = k_{+3}/k_{+4} = P_\infty/e \quad (2)$$

where e represents the total enzyme concentration.

A series of reaction progress curves was determined at different MPTP concentrations but with the enzyme: MPTP ratio being held constant. The half-life for inhibition ($t_{\frac{1}{2}}$) was determined from each of these curves and a graph of the product of the half-life and the initial inhibitor concentration (i) against the initial inhibitor concentration is shown in Fig. 4. From this the apparent K_m value for the reaction (K') and the inactivation constant ($k_{in.}$) were determined from the relationship (Waley, 1985):

$$i \cdot t_{\frac{1}{2}} = \frac{\ln(2-M)}{1-M} \cdot \frac{K'}{k_{in.}} + \frac{\ln 2}{k_{in.}} \cdot i \quad (3)$$

where

$$M = (1 + k_{+3}/k_{+4})e/i \quad (4)$$

$$k_{in.} = k_{+2}k_{+4}/(k_{+2}+k_{+3}+k_{+4}) \quad (5)$$

and

$$K' = \frac{k_{-1}+k_{+2}}{k_{+1}} \cdot \frac{k_{+3}+k_{+4}}{k_{+2}+k_{+3}+k_{+4}} \quad (6)$$

The half-life of the inhibitory reaction at excess concentrations of MPTP ($t_{\frac{1}{2}}$) was calculated from the relationship:

$$t_{\frac{1}{2}} = 0.693/k_{in.} \quad (7)$$

The values determined for these constants are shown in Table 2.

DISCUSSION

The mechanism whereby MPTP acts as both a substrate and an inhibitor of monoamine oxidase-B is similar to that previously shown to describe the

Table 2. Kinetic parameters for the interactions of rat liver monoamine oxidase-B with MPTP according to eqn. (1)

Values were determined by the procedures of Waley (1980, 1985) as described in the text. Each value represents the mean ± S.E.M. for at least four replicates.

Constant	Definition	Value
K'	Eqn. (6)	6.19 ± 0.36 μM
$k_{in.}$	Eqn. (5)	0.032 ± 0.002 min^{-1}
r	Eqn. (2)	17100 ± 930
$t_{\frac{1}{2}}$	Eqn. (7)	21.6 ± 1.4 min

inhibition of this form of the enzyme by an oxazolidinone derivative (Tipton *et al.*, 1983). MPTP functions as a much better substrate for the enzyme than as a mechanism-based inhibitor, as shown by the partition ratio (Table 2).

The definition of irreversibility of inhibition used in this study is an operational term that indicates that activity is not recovered during the time-scale of the experiments. No significant time-dependent recovery of activity was observed over a 3 h period at 37 °C after dilution to decrease the free inhibitor concentration. These results cannot, however, exclude an extremely slow recovery of activity either because of instability of a covalent enzyme–inhibitor adduct or slow dissociation of a tightly but non-covalently bound inhibitor (see Tipton & Fowler, 1984). Further work on the nature of the inhibited species would be necessary to resolve this question.

MPTP is a much less effective substrate for the A-form of monoamine oxidase and its inhibition of that form of the enzyme is substantially reversible under the conditions used in the present work. Salach *et al.* (1984) have reported time-dependent inhibition of monoamine oxidase-A by MPTP, after an initial phase of activation (see also Singer *et al.*, 1986). However, Kinemuchi *et al.* (1985) attributed the time dependence of the inhibition of that form of the enzyme to the accumulation of MPP^+ which they showed to be a more potent inhibitor than MPTP. These workers, and others, also showed the inhibition of the A-form of the enzyme to be reversible whereas that of the B-form was not reversed by dialysis (Kinemuchi *et al.*, 1985; Fuller & Hemrick-Luecke, 1985). It is possible that this difference in the behaviour of the A-form of the enzyme may be related to the degree of purity of the enzyme, since highly purified preparations were used in the studies where susbstantial irreversibility was obtained (Salach *et al.*, 1985; Singer *et al.*, 1986). Irreversible binding to monoamine oxidase-B would also be consistent with data indicating that enzyme to correspond to the binding sites for MPTP in brain (Boccheta *et al.*, 1985; Rainbow *et al.*, 1985).

It is unlikely that the irreversible inhibition of monoamine oxidase-B is important in the neurotoxicity of MPTP, since treatment with monoamine oxidase inhibitors does not result in selective destruction of dopaminergic nerve tracts or in the appearance of Parkinson's disease-like symptoms. Indeed irreversible inhibitors of monoamine oxidase-B are effective in protecting against the effects of MPTP (Cohen *et al.*, 1984; Heikkila *et al.*, 1984; Langston *et al.*, 1984*b*; Melamed & Youdim, 1985; Rainbow *et al.*, 1985). MPTP is also a substrate for monoamine oxidase-A, although with a lower maximum velocity than that shown by the B-form of the enzyme (see also Salach *et al.*, 1984). However, selective inhibitors of the A-form of monoamine oxidase are ineffective in protecting against the neurotoxic effects of MPTP (Heikkila *et al.*, 1984). The product of the oxidation of MPTP by either form of the enzyme has been reported to be the same (Salach *et al.*, 1984) but it is possible that the slower rate of its production by the A-form or differences in the sites of MPTP metabolism by the two forms of the enzyme (see Javitch & Snyder, 1984; Fuller & Hemrick-Luecke, 1985; Sundström & Jonsson, 1985) may account for the differences in the activities of the two forms of monoamine oxidase in this respect.

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