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SHORT COMMUNICATIONS

Inhibition of monoamine oxidase by *d*-methamphetamine

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Because of the great spread of amphetamine abuse in Europe and the U.S.A. and its unique pharmacological effect on the central nervous system, much interest has been centered around the biochemical mechanisms of its action [1]. Amphetamine has been shown to be an inhibitor of monoamine oxidase [amine: oxygen oxidoreductase (deaminating, flavin-containing); EC 1.4.3.4] (MAO) *in vitro* [2–4] and *in vivo* [5]. In Japan, however, *d*-methamphetamine (MA) is much more common than amphetamine and is now causing serious social problems. Although, many years ago, MA was briefly described to be an MAO inhibitor [6, 7], to our knowledge, its detailed action has never been determined. Since mitochondrial MAO is believed to exist in many tissues in two functional forms called type A and type B [8–10], in the present investigation we have studied the inhibition of type A and type B MAO by MA.

A crude mitochondrial fraction was isolated from whole brains of male Sprague–Dawley rats, weighing 100–200 g, as described previously [11]; a suspension of it was used as an enzyme source. For studies on MAO pretreated with clorgyline or deprenyl, the enzyme was incubated with 10^{-7} M clorgyline or 10^{-7} M deprenyl at 37° for 30 min and centrifuged at 18,000 *g* for 10 min. The resulting pellet was suspended in 30 ml of a 0.25 M sucrose solution and recentrifuged at 18,000 *g* for 10 min. This procedure was repeated once to wash the enzyme completely. The resulting pellet was suspended in a small amount of the sucrose solution and used as an enzyme source.

MA–HCl was purchased from the Dai-Nippon-Seiyaku Co., Osaka, Japan. Clorgyline, a selective inhibitor of type A MAO [8], was supplied by May & Baker Ltd., Dagenham, U.K. Deprenyl, a selective inhibitor of type B MAO [12], was donated by Prof. J Knoll, Department of Pharmacology, Semmelweis University of Medicine, Budapest, Hungary.

MAO activity toward 5-hydroxytryptamine (5-HT) was determined by a slight modification of a new photometric assay [13], as described previously [14]. In this method, the hydrogen peroxide formed in reaction with MAO is measured photometrically by converting 2′,7′-dichlorofluorescin to 2′,7′-dichlorofluorescein in the presence of peroxidase. The assays were carried out at 37° and pH 7.4 for 10 or 15 min. MAO activity toward tyramine and β-phenylethylamine (PEA) was measured fluorometrically by a slight modification of the methods of Guilbault *et al.* [15] and Snyder and Hendley [16]. The details of the procedure were described in our previous paper [11]. The assays were carried out at 37° and pH 7.4 for 30 min. Under the conditions used, all MAO assays were confirmed to be linear with time of incubation.

For kinetic analysis, the MAO activity at five or six different substrate concentrations was measured in duplicate over the concentration range of 200–800 μM for 5-HT, 75.0–400 μM for tyramine and 5.0–40.0 μM for PEA. The respective K_m and K_i values were determined graphically from Lineweaver–Burk plots.

For inhibition studies, MA was dissolved in distilled water, added to the assay mixture without substrate, and preincubated at 37° for 10 min. Seven different concentrations of MA were employed over the range of 10^{-6}–10^{-3} M. It was confirmed that MA did not interfere with the photometry or the fluorometry when hydrogen peroxide was added directly.

Protein was determined by a slight modification [17] of the conventional biuret method using bovine serum albumin as a standard.

The inhibition of MAO by various concentrations of MA was studied using 5-HT, tyramine and PEA as substrates. As can be seen in Fig. 1, MAO was hardly affected by 10^{-6} M MA and was almost completely inhibited by a 10^{-3} M concentration of the inhibitor for all substrates. The sensitivity of the MAO activity toward 5-HT was significantly ($P<0.01$, when the difference was more than 10 per cent) higher than that toward PEA; the sensitivity of the activity toward tyramine appeared intermediate. Fifty per cent inhibition was achieved at 4.6×10^{-5} M MA for 5-HT, at 6.0×10^{-5} M for tyramine and at 1.2×10^{-4} M for PEA.

Type A MAO was obtained by pretreatment of the enzyme with 10^{-2} M deprenyl, a selective inhibitor of type B MAO [12]; type B MAO was also obtained by pretreatment of the enzyme with 10^{-7} M clorgyline, a selective inhibitor of type A MAO [8], as described earlier. Type A MAO was specifically inhibited by 10^{-7} M clorgyline and type B enzyme specifically by 10^{-7} M deprenyl. These enzyme preparations were subjected to inhibition by various concentrations of MA, using tyramine as substrate. As illustrated in Fig. 2, the sensitivity of the deprenyl-treated enzyme (type A MAO) to MA was higher ($P<0.01$, when the difference was more than 10 per cent) than

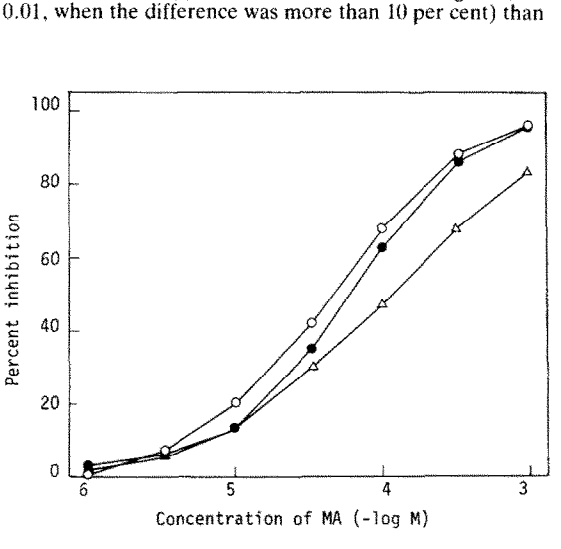

Fig. 1. Effect of various concentrations of MA on MAO activity toward 5-HT (○——○), tyramine (●——●) and PEA (△——△), using rat brain mitochondria as an enzyme source. The concentrations of 5-HT, tyramine and PEA were 100, 100 and 10 μM, respectively. Each point represents the mean of four determinations of a single enzyme source prepared from the pooled brains of seven rats. For no point was the S.D. > 5 per cent of the mean.

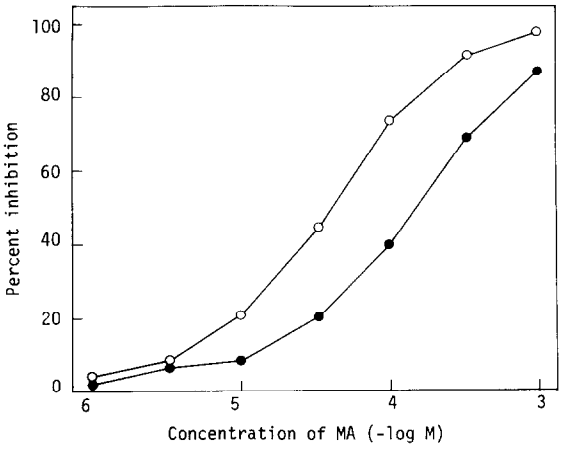

Fig. 2. Effect of various concentrations of MA on the activity of rat brain mitochondrial MAO pretreated with 10^{-7} M clorgyline (●——●) or 10^{-7} M deprenyl (○——○), using tyramine as substrate. The concentration of the substrate was 100 μM. Each point represents the mean obtained from four determinations of a single enzyme source prepared from the pooled brains of seven rats. For no point was the S.D. > 5 per cent of the mean. The procedure for the treatment is described in the text.

that of the clorgyline-treated enzyme (type B MAO). Fifty per cent inhibition was achieved at 4.1×10^{-5} M MA for the deprenyl-treated enzyme and at 1.5×10^{-4} M for the clorgyline-treated enzyme.

Lineweaver–Burk plots for MAO were carried out in the absence and in the presence of MA using the three substrates. The inhibition of rat brain mitochondrial MAO by the drug was fully competitive for all substrates. The respective K_m and K_i values are presented in Table 1. The K_i value with 5-HT was much lower than that of the corresponding K_m value, while the value with PEA was higher than the corresponding K_m value.

To further study kinetically the inhibition of MAO by MA, rat brain mitochondrial MAO pretreated with 10^{-7} M clorgyline or 10^{-7} M deprenyl was analyzed by Lineweaver–Burk plots, using tyramine as substrate. The inhibition by MA was also fully competitive (reversible) for both enzyme preparations. The K_i value for the deprenyl-treated enzyme (type A MAO) was much lower than that for the clorgyline-treated enzyme (type A MAO) to MA was higher ($P <$ K_m values were taken into account (Table 1).

In the present communication, we have studied MAO inhibition by MA with respect to the type A and type B classification. Although it is not clear if each type of MAO represents a different protein, it is unequivocally accepted that both types of MAO are demonstrable both *in vitro* and *in vivo* [18, 19] in their substrate specificity and inhibitor sensitivity. Type A MAO has been shown to be active with 5-HT and norepinephrine as substrates, and to be sensitive to inhibition by a low concentration of clorgyline. Type B MAO has been shown to be active with PEA and benzylamine, and to be sensitive to inhibition by a low concentration of deprenyl. Some substrates, such as tyramine, tryptamine and dopamine, are oxidized by either type of MAO.

We demonstrated substrate-selective MAO inhibition by MA; MAO activity toward 5-HT, a type A substrate, was more sensitive to MA than that toward PEA, a type B substrate (Fig. 1). In addition, it was observed that the activity of MAO pretreated with deprenyl (type A MAO) was more sensitive to the drug than that of MAO pretreated with clorgyline (type B MAO) (Fig. 2). These data show that MA is a more potent inhibitor of type A MAO than of type B MAO. This was also evidenced by the K_i values using different substrates and the clorgyline- and the deprenyl-treated enzyme (Table 1). Amphetamine is also more selective toward type A than type B [3, 4]. Deprenyl, which is very close to MA in chemical structure, is, in contrast, a potent type B inhibitor.

MA, like other amphetamines, may enhance the release of catecholamines such as norepinephrine and dopamine and inhibit the reuptake of the neurotransmitters in the brain [1]. Both norepinephrine and dopamine were reported to be metabolized by type A MAO in their neurons *in vivo* [20]. Therefore, the type A MAO inhibition by MA may play some role *in vivo* in protecting the catecholamines from destruction in the synaptic cleft, and thus may serve to prolong its pharmacological or toxic effects.

In summary, the present study shows that MA is a competitive (reversible) MAO inhibitor that is more potent for type A MAO than for type B MAO.

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Table 1. Substrate and inhibitor constants for rat brain mitochondrial MAO*

Substrate	Pretreatment of MAO	K_m (μM)	K_i for MA (μM)
5-HT	Untreated	606	55.5
Tyramine	Untreated	156	30.5
PEA	Untreated	21.3	70.8
Tyramine	Clorgyline-treated†	356	119
Tyramine	Deprenyl-treated†	100	19.2

* Each kinetic constant was determined graphically from Lineweaver-Burk plots using five or six concentrations assayed in duplicate of a single enzyme source prepared from the pooled brains of seven rats. The assay methods are described in the text.

† The procedure of the treatment is given in the text.

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Effect of drug vehicles on *N*-demethylase activity in isolated hepatocytes

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The use of isolated hepatocytes to study *in vitro* metabolism of drugs and other xenobiotics has become an important technique during the past 10 years. A problem often encountered in such studies is preparation of concentrated stock solutions of poorly soluble substrates for subsequent addition to incubation mixtures. It is often necessary to dissolve substrates in an organic solvent and subsequently add small amounts of this solution directly to hepatocyte incubations or to dry an aliquot of solution in the incubation vessel prior to cell addition. If the solvent is added directly to hepatocyte suspensions, it may, by several mechanisms, modify drug metabolism in the preparation. On the other hand, when poorly water soluble substrates are dried onto glass incubation vials, the rate of dissolution in the incubation mixture may be variable, resulting in poor reproducibility and unreliable estimates of actual substrate concentrations. Typical vehicles used in drug metabolism studies with hepatocytes include: ethanol [1–3], acetone [3–6] and dimethylsulfoxide [1, 7, 8].

Although *N*-demethylation can be quantitated in microsomal suspensions by monitoring formaldehyde formation from water soluble substrates such as ethylmorphine HCl, similar studies in hepatocyte suspensions are virtually impossible. When formaldehyde is formed in hepatocyte suspensions, it is further metabolized to formate and ultimately CO_2, making quantitation difficult [9]. Moreover, after dealkylation of many substrates, the dealkylated metabolite is further conjugated with sulfate or glucuronic acid in the hepatocyte system, again making quantitation difficult [5]. In order to overcome these problems, we have recently studied several substrates for *N*-demethylation that can be utilized in isolated hepatocyte suspensions. We have found that *N*,*N*′-dimethylphenobarbital (DMPB) is an excellent substrate since it is metabolized almost exclusively to *N*-methylphenobarbital (MPB) with little subsequent formation of phenobarbital or other metabolites.* Moreover, use of [^{14}C]DMPB allows one to accurately monitor formation of [^{14}C]MPB and not have to rely on formaldehyde measurements. Since DMPB is only slightly soluble in water, it was necessary to make concentrated stock solutions in organic solvents for subsequent addition to hepatocyte suspensions. Although organic solvents are often used to add substrates to microsomal suspensions and their effects on metabolism have been reported, it became apparent that extrapolation of microsomal data to the isolated hepatocyte system was not valid. We found during the development of the DMPB *N*-demethylase assay that the rate of the reaction in hepatocytes was highly dependent on the solvent used to dissolve DMPB. This report summarizes our initial observations and describes a subsequent study designed to quantitate vehicle effects on *N*-demethylase activity in isolated hepatocytes.

Male Wistar rats (225–300 g), obtained from Hilltop Labs, Scottdale, PA, were maintained on Purina Lab Chow and water *ad lib.* and were used throughout the experiments. Animals were fasted for 24 hr before being killed. Hepatocytes were isolated essentially as described by Berry and Friend [10], incorporating modifications introduced by Seglen [11]. After cannulation of the portal vein, the liver was perfused with calcium-free Krebs–Henseleit bicarbonate buffer (pH 7.5) for approximately 10 min. After hepatectomy, the liver was placed in a perfusion apparatus (MRA Corp., Boston, MA) and perfused for another 10–15 min with a recirculating solution of Sigma Type IV collagenase (90 units/ml) in Krebs–Henseleit buffer. After disruption of the liver capsule, cells were suspended in ice-cold Krebs–Henseleit buffer, successively filtered through gauze and weighted silk, and washed twice. Hepatocytes were finally resuspended to a final concentration of approximately 40 mg cells/ml in 'fortified' Krebs–Henseleit buffer containing 5.5 mM glucose, 2% bovine serum albumin, and

* R. Abraham, M. Sauers, M. Zemaitis and J. Alvin, manuscript submitted for publication.