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Pyridoxamine as inhibitor of the blood plasma benzylamine oxidase and other copper-containing amine oxidases

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Pyridoxamine inhibits rabbit and pig plasma benzylamine oxidase (BAO), the diamine oxidase of pig kidney and the lysyloxidase of pig aorta in-vitro. In-vivo in the rabbit, the inhibitory activity of pyridoxamine on plasma BAO is antagonized by an increase in the level of this enzyme that is dependent on an increase in rate of synthesis, there being no variation in the degradation rate constant.

Pyridoxamine has been described as a histaminase inhibitor by Angelakos & Loew (1957). Since then no further studies have been reported on its effect on copper-containing amine oxidases. This large class of enzymes deaminate primary amines and are clearly distinct from the mitochondrial monoamine oxidases. They are widespread in nature and probably contain copper and pyridoxal-phosphate.

In mammals this class includes diamine oxidase (DAO) or histaminase, blood plasma benzylamine oxidase (BAO) and spermine oxidase, the lysyloxidase of connective tissue (LAO) and probably the so called semicarbazide sensitive amine oxidases (SSAO) (Buffoni 1983). We have therefore further investigated the effects of pyridoxamine on some of these oxidases which belong to the class E.C.1.4.3.6.

Materials and methods

Enzyme preparation. Benzylamine oxidase (BAO) from pig plasma was purified according to Buffoni & Blaschko (1964).

Benzylamine oxidase (BAO) from rabbit was assayed in rabbit plasma, obtained by centrifugation of fresh blood collected by intracardiac puncture. Blood was first centrifuged for 15 min at 700*g* and then for 10 min at 39 000*g* to remove platelets. Diamine oxidase (DAO) of the pig kidney was obtained from Sigma Chemical Co. (St Louis, MO. USA); 0·25 U mg^{-1}). Lysyloxidase (LAO) from pig aorta was purified to step 3 of the method described by Buffoni & Raimondi (1981). Rat liver mitochondria, prepared according to Schneider & Hogeboon (1950), were used as a source of monoamine oxidase (E.C.1.4.3.4.) (MAO). Glutamate-oxolacetate-transaminase (GOT) of pig heart was obtained from Boehringer-Mannheim (Munich, W. Germany; 10 mg ml^{-1}).

Enzyme assay. BAO was assayed either using [^{14}C]benzylamine as substrate as described by Buffoni & Ignesti (1975), or by spectrophotometric assay (Tabor et al 1954). DAO was assayed using [^{14}C]putrescine as substrate as described by Kusche et al (1973). LAO was assayed using [^{3}H]elastin (0·125 mg ml^{-1}) as described by Melet et al (1977). MAO were assayed using [^{14}C]β-phenylethylamine as substrate as described by Buffoni et al (1977). GOT was assayed as described by Bergmeyer & Bernt (1974). Quenching was measured by channel ratio.

Pyridoxamine oxidation. The oxidation of pyridoxamine was measured by H_2O_2 production according to Lehman et al (1974).

Purification of [^{3}H]elastin. [^{3}H]Elastin was prepared according to Pinnell & Martin (1968) and purified according to Sandberg et al (1969). 0·135 ± 0·014 nmol of [^{3}H]lysine were incorporated for 1 mg of elastin, 3 ± 0·44 μC mg^{-1}, and the solution of elastin contained 0·55 ± 0·12 mg of protein ml^{-1} (mean ± s.e., 5 preparations).

Protein determination. Protein was determined either according to Lowry et al (1951) or Waddell (1956).

Blood collection. Blood was collected from white male New Zealand rabbits (2 kg) by intracardiac puncture using a heparinized syringe (Vistar, 500 U ml^{-1}).

Reagents and equipment. DL-[6-^{3}H](N)lysine (25 Ci $mmol^{-1}$) was obtained from New England Nuclear, Boston, Mass., USA; [7-^{14}C]benzylamine hydrochloride (12·5 mCi $mmol^{-1}$) was obtained from ICN Pharmaceuticals, Irvin, Calif., USA; 1,[4-^{14}C]-tetramethylenediamine dihydrochloride (104·6 mCi $mmol^{-1}$); β-phenethylamine hydrochloride (58·1 mCi $mmol^{-1}$) from Radiochemical Centre, Amersham, UK; bovine serum albumin was from Worthington Biochemical Co., New Jersey USA. All other reagents were analytical grade chemicals. A Packard liquid scintillation spectrometer was used for the determination of radioactivity. The efficiency for ^{14}C was 91·6% ± 0·3 (100 determinations) and for ^{3}H was 40% ± 0·5 (100 determinations, mean ± s.e.).

Results

Pyridoxamine oxidation by various amine oxidases. Pyridoxamine up to 10^{-3} M was not oxidized by BAO or DAO, however it was a very weak substrate of LAO

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and MAO. The H_2O_2 production was 4 and 6·6 nmol ml^{-1} of enzyme solution h^{-1}, respectively for LAO and MAO.

Pyridoxamine as inhibitor of various amine oxidases in-vitro. Pyridoxamine was preincubated for 30 min at 6 different concentrations ranging from 10^{-7} to 10^{-2} M with the various enzymes before the addition of the substrates at saturating concentrations. The resulting IC50 values are reported in Table 1. The high substrate concentration of β-phenylethylamine (1·7 mM) used to assay for MAO in rat liver is likely to involve a contribution of both MAO-A and MAO-B.

GOT was not inhibited by pyridoxamine up to 10^{-2} M concentration.

Table 1. IC50 (M) pyridoxamine on different enzymes. The assays were carried out at 37 °C in air, the final substrate concentrations were [^{14}C]benzylamine 1·7 mM; [^{14}C]β-phenylethylamine 1·7 mM; [^{14}C]putrescine 1 mM [^{3}H]elastin (0·125 mg ml^{-1}). The IC50 of a α-aminoguanidine on rabbit plasma BAO was 2·5 ± 0·49 × 10^{-6} M (4 concn inhibition curves). The inhibitors were always preincubated 30 min with the enzymes. The inhibition is constant to 120 min of preincubation.

Rabbit plasma BAO	Pig plasma BAO	Pig kidney DAO	Pig aorta LAO	Rat liver MAO	Pig heart GOT
1·2 ± 0·32 ×10^{-4}	4·1 ± 0·3 ×10^{-5}	1·1 ± 0·7 ×10^{-3}	2·4 ± 0·7 ×10^{-4}	3 ×10^{-3}	1 ×10^{-2}

(Mean ± s.e. of 4 concn-inhibition curves derived from 4 different preparations of each enzyme.)

Pyridoxamine as inhibitor of BAO in-vitro. The inhibition of BAO by pyridoxamine after 30–120 min of preincubation was irreversible, and was unchanged by dialysis (Table 2). Pyridoxal-phosphate was not able to remove the inhibition of pyridoxamine (Table 3).

After a short preincubation (15 min) the inhibition of BAO by pyridoxamine was uncompetitive (Fig. 1), indicating that pyridoxamine competes for benzylamine and seems to increase the rate of benzylamine oxidation by a mechanism needing further investigation.

Pyridoxamine as inhibitor of BAO in vivo. The in-vivo activity of pyridoxamine was tested on rabbit plasma benzylamine oxidase in-vivo. The drug was injected intravenously at a dosage that was well tolerated. Pyridoxamine inhibited rabbit plasma BAO (Fig. 2) but this effect decreased gradually with time in contrast to the observed irreversible effect of pyridoxamine in-vitro. α-Aminoguanidine, a well-known irreversible inhibitor of BAO, gave a constant inhibition over 2 h. Fig. 3 shows that both pyridoxamine and α-aminoguanidine, 2 h after the intravenous injection, inhibited rabbit plasma BAO; the degree of inhibition produced by α-aminoguanidine decreased after 24 h and reached a steady-state value with a daily treatment, whereas the inhibition by pyridoxamine was overcome. The effect of α-aminoguanidine observed in Fig. 3 was dependent on the rate of BAO degradation. The half life of BAO was 5–6 days (Fig. 4). The increase of the enzymic activity after pyridoxamine might depend on an increase in the

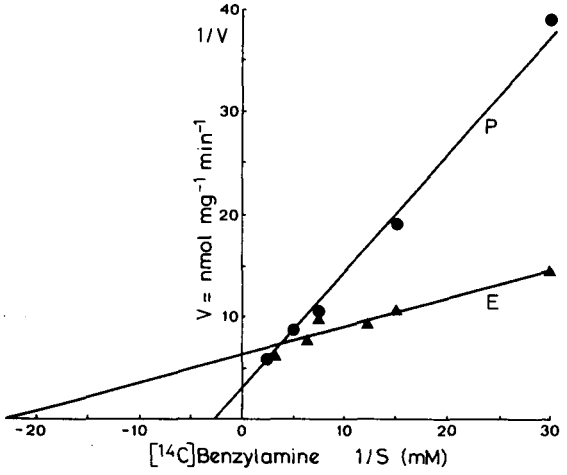

FIG. 1. Pyridoxamine is an uncompetitive inhibitor of BAO. The mixtures (E) contained 0·05 ml of enzyme solution (404 μg ml^{-1}), 2·45 ml of 0·066 M sodium phosphate buffer, pH 7·4, 0·05 ml of catalase (21 U ml^{-1}). The mixtures (P) contained 0·05 ml of enzyme solution (404 μg ml^{-1}) and 2·45 ml of 0·066 M sodium phosphate buffer, pH 7·4, containing pyridoxamine in amounts to give 10^{-5} M in the final volume. They were incubated for 15 min at 37 °C in air in a silica cell of a Perkin-Elmer spectrophotometer (1 cm light-path), then different concentrations of benzylamine hydrochloride were added and benzaldeyde production was followed at 250 nm. The final concentrations of benzylamine were: 33, 66, 131, 195, 322, 449 × 10^{-6} M.

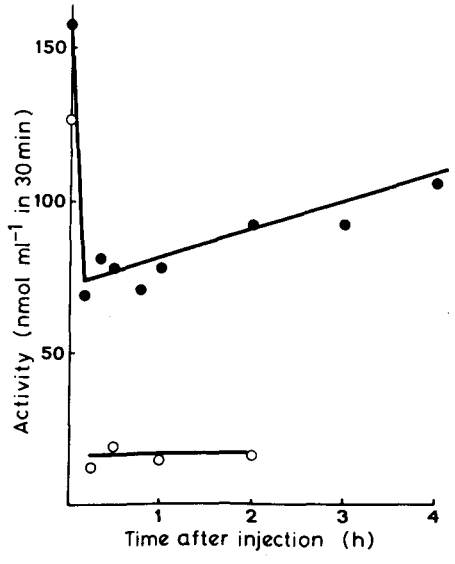

FIG. 2. Effect of pyridoxamine hydrochloride 50 mg kg^{-1} i.v. (●) and of α-aminoguanidine 100 mg kg^{-1} i.v. (○) on the rabbit plasma benzylamine oxidase. The enzymic activity was assayed with [^{14}C]benzylamine as described in Methods. The protein content of blood plasma was 60 ± 0·5 mg ml^{-1} (mean ± s.e. of 20 determinations).

synthesis rate of BAO, an increase of BAO tissue release or a decrease of the BAO degradation rate. The fact that there was an increase in the specific activity of the plasma and that the increase of enzymic activity persisted after the suspension of the treatment is consistent either with an increase in the synthesis rate or with a decrease of the degradation rate. Fig. 5 shows

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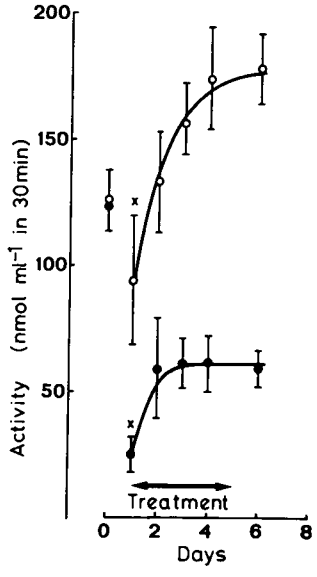

FIG. 3. Comparison between the effect of pyridoxamine (○) (50 mg kg^{-1} i.v.) and α-aminoguanidine (●) (100 mg kg^{-1} i.v.) administered daily for 5 days on the rabbit plasma benzylamine oxidase (n = 4) (mean ± s.e.). × Values obtained 2 h after the injection, the other values were obtained 24 h after the previous injection. For the enzyme assay see Fig. 2.

the end of a period of daily treatment, either with pyridoxamine or with α-aminoguanidine, was similar and indicates that pyridoxamine does not change the rate constant of enzyme degradation Protein content of the blood plasma was always measured, no variations were observed during the experimental time.

'able 2. Pyridoxamine (P) irreversible inhibitor of BAO 'om pig plasma. Activity = nmol of benzylamine oxidized)r mg of enzyme in 30 min. Pyridoxamine 10^{-4} M was reincubated 30 min with the enzyme. The assay mixtures ere dialysed for 3 days at 4 °C against 100 times their)lume of Na-phosphate buffer or 0·066 M pH 7·4 (3 ıanges), then the activities were remeasured.

	Activity	Inhibition %	% Inhibition after dialysis
ızyme	130 ± 6·9	0	0*
ızyme + P	35 ± 1·3	73	80

'Mean ± s.e. of 4 determinations.)
* The specific activity of uninhibited enzyme sample was modified by dialysis.

)le 3. Pyridoxal-phosphate (PLP) on the pyridoxamine inhibition of BAO from pig plasma.

	Activity
(A) Enzyme + PLP	43
(B) Enzyme + P	0
(C) Enzyme + P + PLP	0
(D) Enzyme	43

ctivity = nmol of benzylamine oxidized mg^{-1} of /me in 30 min.
\) The enzyme was preincubated 120 min with PLP 3 × M.
3) The enzyme was preincubated 30 min with P 3 × M.
:) The enzyme was preincubated 30 min with P 3 × M then 120 min with PLP 3 × 10^{-3} M.
)) The enzyme was preincubated in the absence of any gs.

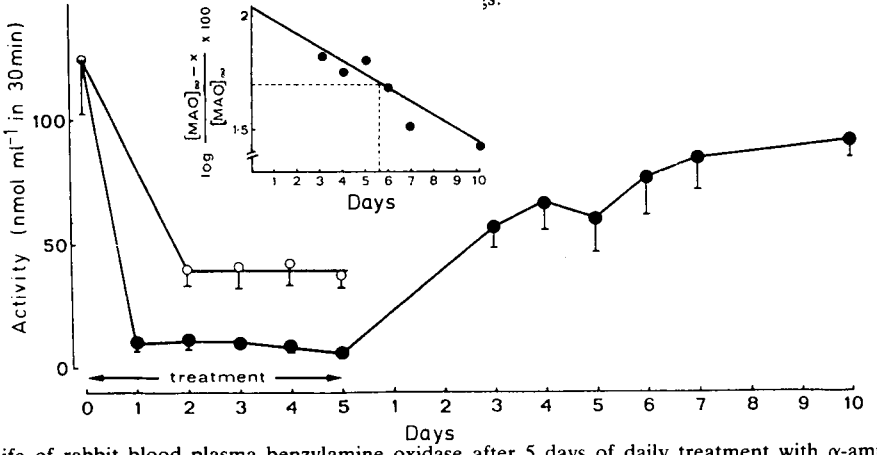

FIG. 4. half-life of rabbit blood plasma benzylamine oxidase after 5 days of daily treatment with α-aminoguanidine 100 mg kg^{-1} (i.v.). For the enzyme assay see Fig. 2 (mean ± s.e.; n = 4). (○) Values obtained 24 h after the previous injection. (●) Values obtained 2 h after the injection. The inset shows the data of the lower curve plotted for the calculation of half-life. MAO_∞ = level of blood plasma benzylamine oxidase before the treatment. x = level after the suspension of the treatment.

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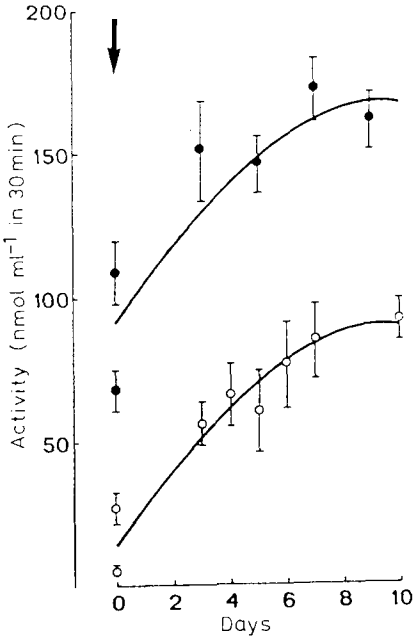

FIG. 5. Recovery of rabbit plasma benzylamine oxidase after pyridoxamine (●) and α-aminoguanidine (○) treatment. The animals were treated once a day for 5 days with α-aminoguanidine 100 mg kg^{-1} i.v. (n = 4) and for 10 days with pyridoxamine 50 mg kg^{-1} i.v. (n = 3). Normal value of activity: 123·5 ± 10 nmol of benzylamine oxidized per ml of plasma in 30 min. For enzyme assay see Fig. 2. At the arrow values before and 1 h after the last injection.

Conclusion

Pyridoxamine is an inhibitor of some copper-containing amine oxidases. It is also a very weak inhibitor of the FAD mitochondrial MAO because it is a weak substrate. Pyridoxamine shows a higher inhibition activity for the pig plasma purified BAO. In-vivo in rabbit, pyridoxamine produces a double effect: an immediate

inhibition followed by a stimulation of the rate of BAO synthesis. This latter effect further supports the presence of pyridoxal-phosphate (PLP) in this enzyme. It is known that pyridoxamine is quickly transformed in PLP (Wada & Snell 1961).

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