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Inhibition of Two Copper-Containing Enzymes, Tyrosinase and Dopamine β-Hydroxylase, by L-Mimosine

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SUMMARY

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Because of the structural similarity of L-mimosine to L-tyrosine and L-dopa and its tendency to chelate cupric ion, the influence of this compound on mammalian tyrosinase from mouse melanoma and dopamine β-hydroxylase extracted from bovine adrenal medulla was investigated *in vitro*. L-Mimosine inhibited tyrosinase competitively and reversibly, and the inhibitory effect was decreased by ferric, aluminum, or cupric ion. Dopamine β-hydroxylase was inhibited by L-mimosine, mimosinamine, and mimosinic acid, but the inhibition was uncompetitive. The results suggest that these enzymes are inhibited by different mechanisms: L-mimosine inhibits tyrosinase because of its structural similarity to the substrate, L-dopa. Dopamine β-hydroxylase is inhibited by L-mimosine, mimosinamine, and mimosinic acid because of their chelate-forming ability.

INTRODUCTION

L-Mimosine (I), β-[*N*-(3-hydroxypyridone-4)]-α-aminopropionic acid, is a toxic amino acid occurring in legumes belonging to the genera *Leucaena* and *Mimosa*, which are indigenous to tropical and subtropical areas (1-3). Many disorders, such as decreased weight gain, loss of hair, cataracts, and infertility, have been caused in experimental animals by feeding a diet containing L-mimosine (4-9). Although possible mechanisms for these toxic effects have been postulated by several investigators (10-14), the precise mechanisms remain obscure. Lin *et al.* (12) have suggested from their feeding experiment that L-mimosine may influence tyrosine metabolism. They also showed that L-mimosine was a weak inhibitor of several metal-containing enzymes such as catalase, polyphenol oxidase, alkaline phosphatase, and succinate dehydrogenase, and of vitamin B_6-requiring enzymes such as L-dopa decarboxylase and glutamate-oxaloacetate transaminase (10, 15-17). These findings seemed to us insufficient to explain all of the disorders caused by L-mimosine. Recently Mostad *et al.* (18, 19), using X-ray diffraction methods, reported that the crystal structure of L-mimosine sulfate was very similar to those of L-tyrosine and L-dopa. Tsai and Ling (20) showed that this amino acid formed a stable chelate with cupric ion as well as ferric ion.

These findings led us to investigate the effects of L-mimosine on two copper-containing enzymes concerned with L-tyrosine and L-dopa metabolism. The first was tyrosinase, the key enzyme in melanin formation from L-tyrosine or L-dopa (21). In this experiment the protective effects of several metal ions on tyrosinase inhibition by L-mimosine were examined.

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The other enzyme was dopamine β-hydroxylase, which catalyzes the conversion of dopamine to norepinephrine (22). In this experiment the effects of L-mimosine and its two metabolites, mimosinamine (II; 3-hydroxy-*N*-aminoethylpyridone-4) and mimosinic acid (III; 3-hydroxy-*N*-carboxymethylpyridone-4), were examined. Mimosinamine is a decarboxylation product of L-mimosine, and mimosinic acid is possibly formed either by oxidation of mimosinamine or by oxidative deamination of L-mimosine followed by decarboxylation. We have already shown that mimosinamine has a toxic action similar to that of L-mimosine while mimosinic acid has no such activity (14).

MATERIALS AND METHODS

Enzyme Preparations

Tyrosinase was prepared from Harding-Passey mouse melanoma according to Brown and Ward (23). The activity obtained was 5 units/mg of protein according to the method of Lerner and Bunsen (24).

Dopamine β-hydroxylase was prepared from bovine adrenal medulla by the method of Levin *et al.* (22). The crude preparation before gel filtration was sufficiently active for these experiments. Approximately 0.36 μmole of octopamine was formed from tyramine per minute per milligram of protein.

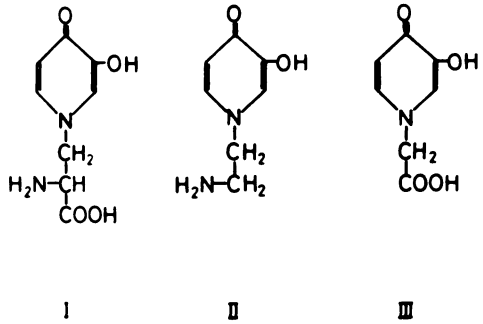
Assay of Enzyme Activity

Tyrosinase activity or, more accurately, L-dopa-oxidizing activity was determined with a Warburg respirometer by measuring oxygen consumption during melanin formation from L-dopa (24). The main compartment contained tyrosinase solution (220 μg or 190 μg of protein) and 1.0 ml of solutions of L-mimosine and/or metal ions at various concentrations, and the total volume was adjusted to 1.8 ml with buffer. One milliliter of L-dopa solution in the side arm was emptied into the main compartment after 20 min of equilibration of the solutions at 37°, and oxygen uptake was measured every 10 min after addition of the substrate. All solutions used were prepared with 0.1 M sodium phosphate buffer, pH 6.8. Additional details are provided in the legends to the figures and tables.

Dopamine β-hydroxylase activity was measured by the spectrophotometric method of Creveling *et al.* (25), with slight modification. The incubation mixture (final volume, 2.0 ml) contained 400 μmoles of acetate buffer (pH 5.5), 200 μmoles of fumarate, 20 μmoles of tyramine, 20 μmoles of *N*-ethylmaleimide, 40 μmoles of ascorbate, 1000 units of catalase, and various amounts of the compounds to be tested. The incubation mixture was incubated for 5 min at 37° in air before the reaction was started by adding 0.2 ml of enzyme solution (85 μg of protein). The reaction was stopped after 5 min by addition of 0.5 ml of 50% trichloracetic acid. A blank mixture without tyramine was incubated at the same time. The mixture was centrifuged for 10 min at 3000 rpm and the supernatant was transferred to a Dowex 50W (H^+) column. After the column was washed with water, the amines were eluted with 3 ml of 4 M NH_4OH, and the octopamine in the eluate was oxidized to *p*-hydroxybenzaldehyde with periodate. *p*-Hydroxybenzaldehyde was extracted with 4 ml of 1-butanol to avoid interference by inhibitors. The extent of extraction of *p*-hydroxybenzaldehyde with 1-butanol was about 80%.

Materials

L-Mimosine was purchased from Sigma Chemical Company. Mimosinamine and mimosinic acid were synthesized as previously described (14). L-Dopa was a gift from Sankyo Central Research Laboratories. All chemicals used, including the metallic reagents $Al_2(SO_4)_3 \cdot 18H_2O$, $BaCl_2 \cdot$



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$2H_2O$, $CaCl_2 \cdot 2H_2O$, $CuSO_4 \cdot 5H_2O$, $Fe_2(SO_4)_3(NH_4)_2SO_4 \cdot 24H_2O$, $MgSO_4 \cdot 7H_2O$, $MnCl_2 \cdot 4H_2O$, and $ZnSO_4 \cdot 7H_2O$, were of special reagent grade.

RESULTS

Studies with Tyrosinase

Neither oxygen consumption nor change in color of the incubation mixture was observed when L-mimosine at various concentrations was used as a substrate for the tyrosinase.

Inhibition of tyrosinase by L-mimosine. L-Mimosine inhibited tyrosinase at a concentration of 10 μM or more when L-dopa was the substrate. The extent of inhibition at various concentrations of L-mimosine was examined with 4 mM L-dopa (Table 1).

Although the influence of L-mimosine at concentrations lower than 10 μM was carefully examined, neither clear inhibition nor activation was observed. Lineweaver-Burk plots indicated competitive inhibition, with a K_m of 2.8 mM and a K_i of 54 μM (Fig. 1).

Effects of various metal ions on inhibition of tyrosinase by L-mimosine. When the effects of various metal ions (0.2 mM) on tyrosinase activity were examined, only ferric and cupric ions seemed to increase oxygen consumption to a degree which coincided with their L-dopa-oxidizing capability. Thus we concluded that these metal ions showed no effect on tyrosinase activity at this concentration. The nonenzymatic L-dopa-oxidizing capability of cupric or ferric ion was lowered by previous mixing of the ions with L-mimosine. Likewise

TABLE 1

Relative inhibition of tyrosinase by L-mimosine

The activity of tyrosinase was determined by comparing O_2 consumption for 60 min after the addition of substrate in the absence and presence of various amounts of L-mimosine. The final concentration of L-dopa was 4 mM.

L-Mimosine	Tyrosinase activity
mM	%
0	100
0.01	90
0.05	54
0.10	40
0.20	25
0.30	10

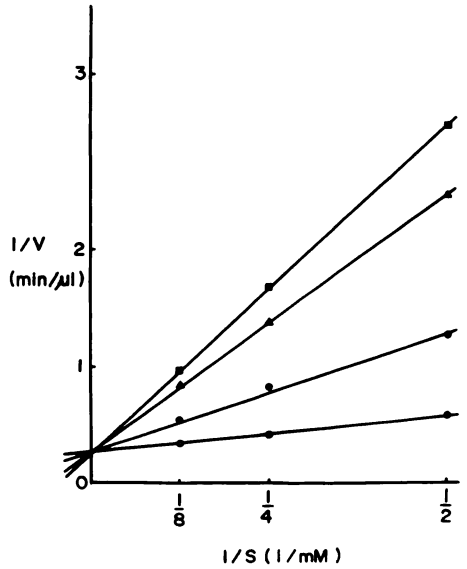

FIG. 1. *Double-reciprocal plots of velocity vs. L-dopa concentration with various amounts of L-mimosine*

Initial velocity was calculated from the rate of O_2 consumption after addition of the substrate, in the presence of the following concentrations of L-mimosine: ■——■, 0.3 mM; ▲——▲, 0.2 mM; ●——●, 0.1 mM; ○——○, none.

the inhibitory effect of L-mimosine on tyrosine was decreased by previous mixing with ferric, cupric, or aluminum ion, but not with other metal ions (Fig. 2). Similarly, when ferric or aluminum ion was added to tyrosinase which had been incubated with L-mimosine for 10 min, activity was restored (Fig. 3).

Studies with dopamine β-hydroxylase

Dopamine β-hydroxylase was inhibited 20.6%, 46.6%, and 74.5% by 0.2 mM L-mimosine, mimosinamine, and mimosinic acid, respectively. Although the structure of mimosinamine seems most similar to that of dopamine, the usual substrate for dopamine β-hydroxylase, the greatest inhibition was caused by mimosinic acid.

Kinetic examination according to the Lineweaver-Burk method revealed that these compounds inhibited the enzyme uncompetitively (Figs. 4–6), with a K_m of 1.7 mM and K_i values for L-mimosine, mimosinamine, and mimosinic acid of 180, 140, and 45 μM, respectively, calculated according to Wong (26).

DISCUSSION

The activities of two copper-containing enzymes of tyrosine metabolism, tyrosinase and dopamine β-hydroxylase, were suppressed by L-mimosine as expected, but the mode of inhibition was different in each case.

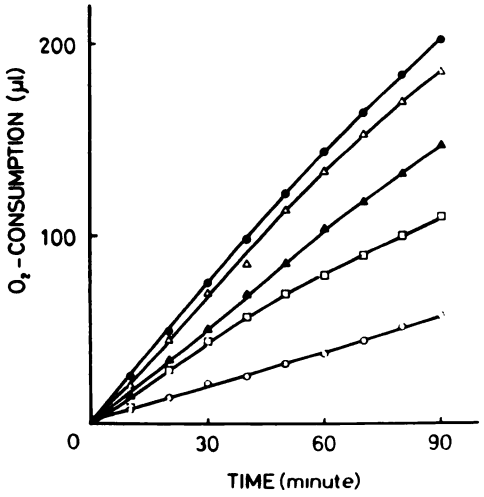

FIG. 2. *Effects of metal ions on inhibition of tyrosinase by L-mimosine*

L-Mimosine was incubated together with metal ion (0.2 mM) for 10 min before addition to the reaction mixture with L-dopa at a final concentration of 4 mM. △——△, L-mimosine and ferric ion; □——□, L-mimosine and cupric ion; ▲——▲, L-mimosine and aluminum ion; ○——○, L-mimosine only; ●——●, no L-mimosine or metal ion.

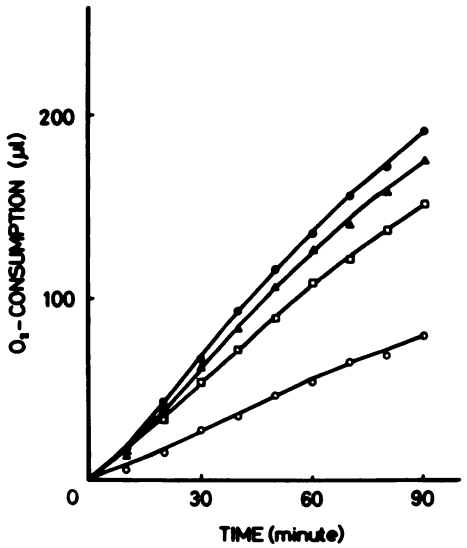

FIG. 3. *Recovery of tyrosinase activity by addition of ferric or aluminum ion*

After a 10-min incubation of tyrosinase with L-mimosine, ferric or aluminum ion was added to the mixture and O_2 consumption was measured after further addition of the substrate. △——△, L-mimosine and ferric ion; □——□, L-mimosine and aluminum ion; ○——○, L-mimosine only; ●——●, no L-mimosine or metal ion. The final concentrations of metal ions, L-mimosine, and the substrate were 0.1 mM, 0.1 mM, and 4 mM, respectively.

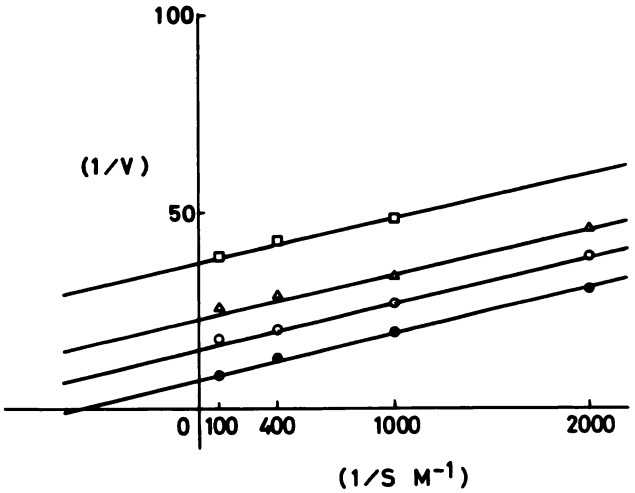

FIG. 4. *Double-reciprocal plots of velocity vs. tyramine concentration for dopamine β-hydroxylase with various amounts of L-mimosine*

Initial velocity was determined as micromoles of octopamine produced in 5 min after addition of the enzyme. The procedure is described in detail in METHODS AND MATERIALS. Concentrations of L-mimosine were: □——□, 0.8 mM; △——△, 0.4 mM; ○——○, with 0.2 mM; ●——●, none.

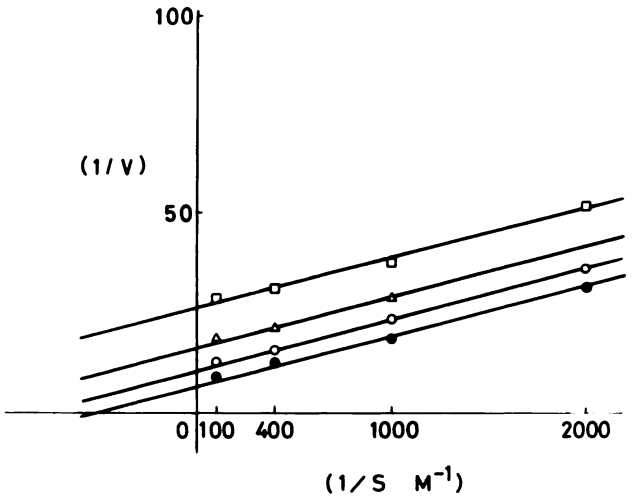

FIG. 5. *Double-reciprocal plots of velocity vs. tyramine concentration for dopamine β-hydroxylase with various amounts of mimosinamine*

Concentrations of mimosinamine were: □——□, 0.4 mM; △——△, 0.2 mM; ○——○, 0.1 mM; ●——●, none.

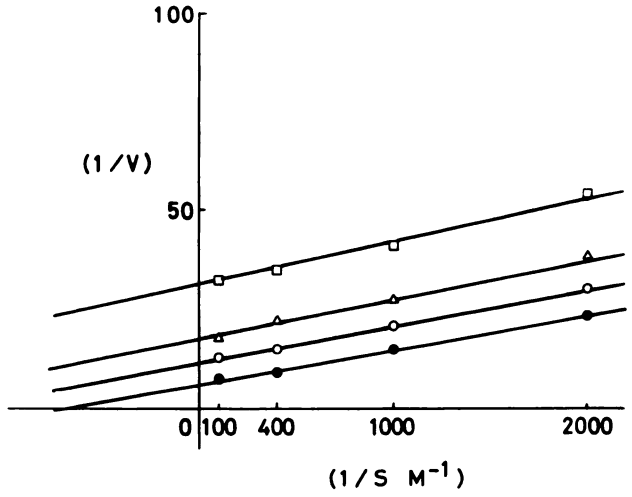

FIG. 6. *Double-reciprocal plots of velocity vs. tyramine concentration for dopamine β-hydroxylase with various amounts of mimosinic acid*

Concentrations of mimosinic acid were: □——□, 0.2 mM; △——△, 0.1 mM; ○——○, 0.05 mM; ●——●, none.

Inhibition of tyrosinase by L-mimosine was competitive, as shown using DL-4,5-dihydroxy-2-pyridylalanine (27), an isomer of L-mimosine, and the recovery of activity on addition of ferric or aluminum ion suggests that L-mimosine has affinity for the binding site and that inhibition is reversible. Previous formation of L-mimosine chelates with ferric, aluminum, or cupric ion diminished its inhibitory activity, and the extent of inhibition reversal corresponded to the order of stability constants of the chelates (20).

Dopamine β-hydroxylase was also inhibited by L-mimosine, as well as mimosinamine and mimosinic acid. This is the first evidence that L-mimosine and its metabolites affect catecholamine biosyn-

thesis. Kinetic studies indicated that all three compounds are uncompetitive inhibitors.

Many isosteres of phenethylamine are known to be either inhibitors or substrates of dopamine β-hydroxylase (25), and it has been shown that benzyloxamine is also a potent competitive inhibitior (28). However, mimosinamine was found to be neither a substrate nor a competitive inhibitor, in spite of its structural similarity to tyramine and dopamine, the usual substrates for this enzyme. In addition, the inhibition by mimosinamine was weaker than that by mimosinic acid, which is structurally less similar to tyramine and dopamine.

Our findings suggest that in the case of dopamine β-hydroxylase, the strength of chelate formation of these compounds with copper ion is more important for inhibition than structural similarity to the substrate, in contrast to the case with tyrosinase.

Since β-hydroxylation in this enzyme system is explained by the ping-pong mechanism (29), reduction of copper by ascorbate may be inhibited, as in the inhibition of dopamine β-hydroxylase by fusaric acid (30).

It has recently been reported that L-mimosine inhibits the growth of several kinds of tumors (31-33). In further studies leading to a possible therapeutic application, it will be important to clarify whether L-mimosine inhibits tyrosinase and dopamine β-hydroxylase mainly because of its structural similarity to aromatic amino acids or because of its chelate-forming ability.

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