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# INHIBITION OF PYRIDOXAL ENZYMES BY L-CANALINE

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## SUMMARY

The effect of L-canaline, a structural analogue of L-ornithine, was studied on several mammalian enzymes *in vitro*. The results obtained with three ornithine metabolizing enzymes indicated that canaline is not an effective competitor of ornithine in these reactions. However, canaline strongly inhibited the activity of all seven pyridoxal-dependent enzymes studied, including amino acid decarboxylases [ornithine decarboxylase (EC 4.1.1.17), 5-hydroxytryptophan decarboxylase (EC 4.1.1.28)], aminotransferases [ornithine–ketoacid aminotransferase (EC 2.6.1.13), tyrosine aminotransferase (EC 2.6.1.5)], ornithine transcarbamylase (EC 2.1.3.3) and plasma diamino-oxidase (EC 4.1.3.6). The reversibility of this inhibition by excess pyridoxal phosphate, as well as a strong interaction between canaline and pyridoxal phosphate in aqueous solution, support the view that canaline inhibition is due to complex formation between canaline and the pyridoxal coenzyme. L-canaline is one of the most potent inhibitors of pyridoxal enzymes. Ornithine–ketoacid aminotransferase, for example, was inhibited by 50% in the presence of $3 \cdot 10^{-6}$ M L-canaline.

## INTRODUCTION

Ureohydrolysis of canavaline, $\alpha$-amino-$\gamma$-guanidinooxybutyric acid, yields canaline, $\alpha$-amino-$\gamma$-aminooxy-butyric acid. Probably as a structural analogue, canavaline is known to interfere in a competitive manner in many reactions of arginine metabolism, such as amino acid activation[1], transamidination[2], arginase reaction[3], as well as the uptake of arginine by microorganisms[4]. Analogous studies on the relationship between canaline and ornithine are limited to the observation of KATUNUMA *et al.*[5] that canaline inhibits transamination of ornithine. A closer analysis of the mechanism of this inhibition was not reported, but the structural similarity of ornithine and canaline was alluded to.

In a search for compounds capable of interfering specifically with ornithine metabolism, we have studied the effect of canaline on various biological systems. We observed normal rates of urea synthesis in isolated perfused rat liver in the presence

of canaline[6]. On the other hand, addition of canaline to the perfusion medium effectively prevented the disappearance of ornithine from the medium. This study was designed to test whether canaline specifically influences ornithine metabolism at the enzymic level. We found that only two of three ornithine metabolizing enzymes examined, namely those requiring pyridoxal phosphate, were inhibited by canaline. Our results obtained with several other pyridoxal enzymes, as well as the interaction of canaline with pyridoxal phosphate in aqueous solution seem to suggest that the inhibition produced by canaline is due to complex formation between canaline and pyridoxal phosphate rather than to a competition with ornithine. These findings are the subject of the present report. A preliminary account of some of this work has been published[7].

## MATERIALS AND METHODS

### *Chemicals*

Anhydrous L-canaline was purchased from Sigma Chemical Corporation, St. Louis, Mo., U.S.A. On paper chromatography and paper electrophoresis it was shown to be homogeneous. Cycloserine (D-4-amino-3-isoxazolidone) was a gift of Oy Medikalis Ab, Helsinki, Finland. DL-5-Hydroxy[*me*-$^{14}$C]tryptophan and [1,4-$^{14}C_2$]-putrescine were obtained from the Radiochemical Center, Amersham, England. DL-[1-$^{14}$C]ornithine was supplied by the New England Nuclear Corporation, Boston, Mass., U.S.A. Other reagents were prepared from analytical grade chemicals.

### *Enzyme assays*

The methods used for the determination of different enzymic activities are listed in Table I. The high-speed supernatant fraction of rat liver homogenate was used for the assay of ornithine decarboxylase, tyrosine aminotransferase and 5-

TABLE I

SUMMARY OF THE STUDY OF THE EFFECTS OF L-CANALINE ON DIFFERENT ENZYMIC REACTIONS

| *Enzyme* | *EC number* | *Assay method* | *Concn. of added pyridoxal phosphate (M)* | *Concn. of L-canaline causing 50% inhibition (M)* | *Incubation system** |
|---|---|---|---|---|---|
| 1. Ornithine transcarbamylase | 2.1.3.3 | BROWN AND COHEN[11] | None | $> 2.5 \cdot 10^{-2}$ | II |
| 2. Tyrosine aminotransferase | 2.6.1.5 | DIAMONDSTONE[12] | $4 \cdot 10^{-5}$ | $4 \cdot 10^{-5}$ | I |
| 3. Ornithine–ketoacid aminotransferase | 2.6.1.13 | KATUNUMA *et al.*[5] | None | $3 \cdot 10^{-6}$ | I, P |
| 4. Ornithine decarboxylase | 4.1.1.17 | RAINA AND JÄNNE[8] | $2 \cdot 10^{-4}$ | $1.0 \cdot 10^{-4}$–$1.5 \cdot 10^{-4}$ | I, II, P |
| 5. 5-Hydroxytryptophan decarboxylase | 4.1.1.28 | SNYDER AND AXELROD[13] | $5 \cdot 10^{-4}$ | $1 \cdot 10^{-4}$ | II, P |
| 6. Diaminooxidase | 4.1.3.6 | TRYDING AND WILLERT[14] | None | $5 \cdot 10^{-5}$ | II |

* I, constant L-canaline concentration, the concentration of pyridoxal phosphate varied; II, constant pyridoxal phosphate concentration, the concentration of L-canaline varied; P, additional analyses were carried out with different preincubation systems. namely canaline + enzyme, canaline + pyridoxal phosphate, and enzyme + pyridoxal phosphate.

hydroxytryptophan decarboxylase. Rat liver homogenate was used for the determination of the activities of ornithine transcarbamylase and ornithine–ketoacid aminotransferase. Diaminooxidase activity was measured using human plasma as the source of the enzyme. The enzymic activities are expressed as per cent of control value.

*Recording of absorption spectra*

The interaction of L-canaline and cycloserine with pyridoxal phosphate was studied in 0.1 M glycylglycine buffer (pH 7.5). The absorption spectra were drawn out with a Beckman DK spectrophotometer.

## RESULTS

*Effect of L-canaline on enzymes using ornithine as substrate*

As shown in Fig. 1, the addition of canaline to the ornithine transcarbamylase

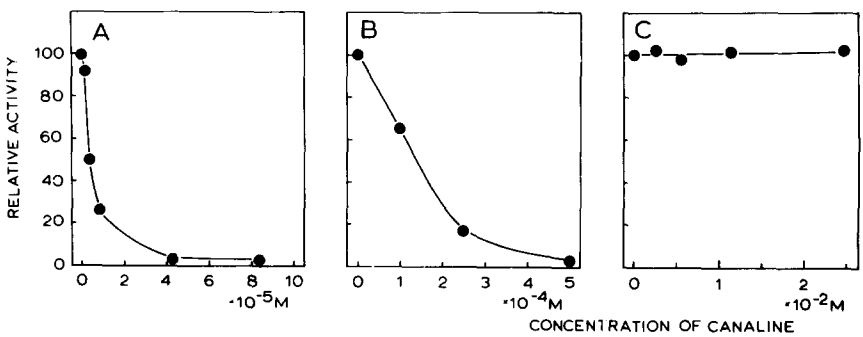


Fig. 1. Effect of L-canaline on the activities of three ornithine metabolizing enzymes. The concentration of exogenous pyridoxal phosphate in the enzymic assay media was as follows: (A) ornithine–ketoacid aminotransferase: none; (B) ornithine decarboxylase: $2.0 \cdot 10^{-4}$ M; (C) ornithine transcarbamylase: none.

assay system in concentrations up to $25 \cdot 10^{-3}$ M, *i.e.* twice the concentration of ornithine in the incubation mixture, had no effect on the observed enzymic activity. This result indicates that canaline does not compete efficiently with ornithine in this reaction. This is also in accord with our earlier observation[6] that the rate of urea synthesis was unaffected by canaline. On the other hand, both ornithine decarboxylase and ornithine–ketoacid aminotransferase were strongly inhibited by canaline (Fig. 1). The activity of ornithine–ketoacid aminotransferase, assayed without any added pyridoxal phosphate, was maximally inhibited by canaline at concentrations as low as $4 \cdot 10^{-5}$ M. About 10 times higher concentration of canaline was necessary to produce the same degree of inhibition in ornithine decarboxylase activity, which was assayed in the presence of $2 \cdot 10^{-4}$ M pyridoxal phosphate. The inhibition of ornithine decarboxylase activity by canaline could not be reversed by excess ornithine. With a constant amount of canaline in the assay mixture, ornithine decarboxylase and ornithine–ketoacid aminotransferase exhibited different patterns of reactivation by supplementary pyridoxal phosphate. As seen in Fig. 2, increasing

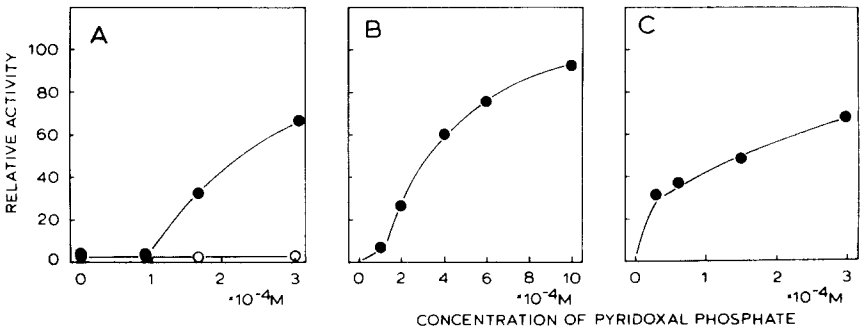


Fig. 2. Reversibility of canaline inhibition by excess pyridoxal phosphate. Assay conditions were as follows. (A) ornithine–ketoacid aminotransferase: L-canaline was present at a concentration of $7.1 \cdot 10^{-5}$ M; ○, canaline and enzyme preincubated for 5 min before the addition of pyridoxal phosphate and ornithine; ●, canaline and pyridoxal phosphate preincubated for 5 min before the addition of the enzyme. (B) Ornithine decarboxylase: L-canaline, $2.0 \cdot 10^{-4}$ M, was added simultaneously with pyridoxal phosphate before the addition of the enzyme. (C) Tyrosine aminotransferase: pyridoxal phosphate and enzyme were preincubated for 60 min before the addition of $6.3 \cdot 10^{-5}$ M L-canaline; the reaction was started 4 min later by adding the α-ketoglutarate substrate.

pyridoxal phosphate concentration in the assay system partly reversed the inhibition of ornithine decarboxylase activity. The final effect was practically independent of the order in which canaline and pyridoxal phosphate were added. When pyridoxal phosphate and canaline were added to the ornithine–ketoacid aminotransferase assay mixture, the resultant ornithine–ketoacid aminotransferase activity depended upon the sequence of additions: the inhibition of ornithine–ketoacid aminotransferase was less when canaline was preincubated with pyridoxal phosphate before the addition of the enzyme. However, once abolished by canaline, the ornithine–ketoacid aminotransferase activity could not be restored by adding exogenous pyridoxal phosphate.

*Effect of canaline on other pyridoxal phosphate-dependent enzymes*

The above results suggested that canaline might act as a general inhibitor of

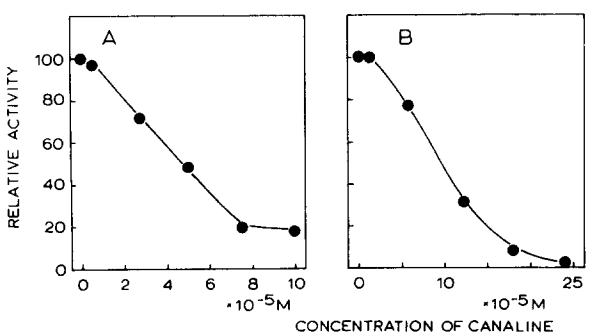


Fig. 3. Inhibition of (A) diaminooxidase and (B) 5-hydroxytryptophan decarboxylase activity by L-canaline. The concentration of exogenous pyridoxal phosphate in the assay systems were: none for diaminooxidase; $4.7 \cdot 10^{-4}$ M for 5-hydroxytryptophan decarboxylase. Canaline was added before the enzyme.

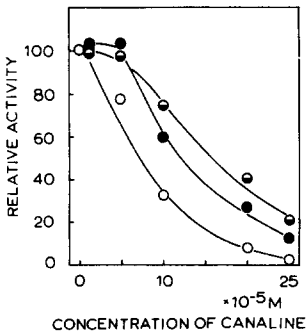


Fig. 4. The dependence of the inhibition of 5-hydroxytryptophan decarboxylase by L-canaline on preincubation conditions. ●, canaline and pyridoxal phosphate preincubated for 3 min before the addition of the enzyme; ○, canaline and enzyme preincubated for 3 min before the addition of pyridoxal phosphate and the substrate; ◒, pyridoxal phosphate and enzyme preincubated for 3 min before the addition of canaline and the substrate.

pyridoxal phosphate-dependent enzymes. Therefore, canaline was tested with some other pyridoxal enzymes, namely tyrosine aminotransferase, 5-hydroxytryptophan decarboxylase and diaminooxidase, each representing different types of pyridoxal enzyme. As Figs. 2 and 3 show, all three were inhibited by canaline. Canaline also inhibited serum aspartate and alanine aminotransferases (not tabulated). The inhibition of tyrosine aminotransferase was partly reversed by increasing concentration of pyridoxal phosphate in the assay system (Fig. 2). For 5-hydroxytryptophan decarboxylase, the resultant inhibition by canaline depended to some extent on

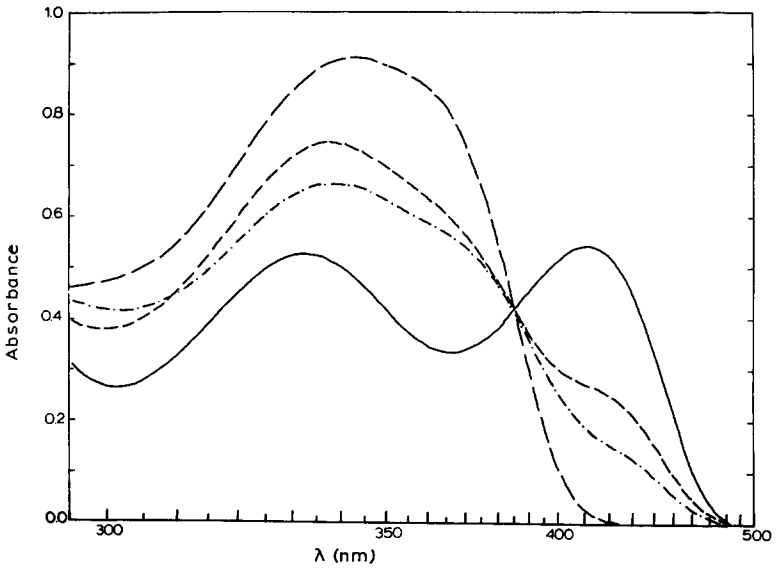


Fig. 5. Absorption spectra for pyridoxal phosphate and its mixtures with L-canaline and Cycloserine in 0.1 M glycylglycine buffer (pH 7.5). ———, 0.2 μmole pyridoxal phosphate; – – –, 0.2 μmole pyridoxal phosphate + 0.1 μmole canaline; — — —, 0.2 μmole pyridoxal phosphate + 0.2 μmole canaline; —·—, 0.2 μmole pyridoxal phosphate + 10 μmoles Cycloserine. The amounts mentioned were dissolved in 1.0 ml of the above buffer.

preincubation conditions: with a given amount of canaline, the strongest inhibitory effect was found when canaline was preincubated with the enzyme preparation (Fig. 4).

*Absorption spectra of pyridoxal phosphate and its mixtures with* L-*canaline or cycloserine*

Addition of canaline to the ornithine decarboxylase assay medium containing 0.1 M glycylglycine buffer (pH 7.5) and $2 \cdot 10^{-4}$ M pyridoxal phosphate caused the disappearance of the typical yellow colour of the latter compound. A closer analysis of the interaction of canaline and pyridoxal phosphate was performed by determining the absorption spectra for pyridoxal phosphate and for mixtures of pyridoxal phosphate and canaline. In glycylglycine buffer (pH 7.5), pyridoxal phosphate alone showed two absorption maxima, one at about 330 and the other at 410 nm (Fig. 5). The addition of a half-equimolar amount of canaline to pyridoxal phosphate solution lessened the absorption at 410 nm approximately to one half. When canaline and pyridoxal phosphate were mixed in equimolar amounts, the 410-nm peak disappeared completely. Cycloserine effected qualitatively similar but quantitatively much smaller changes in the 410-nm region than did canaline (Fig. 5).

## DISCUSSION

KATUNUMA *et al.*[5] found that canaline effectively inhibits ornithine-ketoacid aminotransferase in rat mitochondrial preparations. Canaline can also replace ornithine as an acceptor of the amido group in enzymic transamidination[2]. We found that both ornithine–ketoacid aminotransferase and ornithine decarboxylase were inhibited by canaline, whereas ornithine transcarbamylase was not. For ornithine decarboxylase, this inhibition could not be reversed by excess ornithine. Therefore, the interpretation for our finding is that the inhibition produced by canaline is not due to a structural similarity between ornithine and canaline. Both ornithine–ketoacid aminotransferase and ornithine decarboxylase have pyridoxal phosphate as coenzyme[5,8], whereas ornithine transcarbamylase has not. This leads to another interpretation, namely, that canaline-induced inhibition results from the action of canaline on the pyridoxal coenzyme. The observed differences between ornithine decarboxylase and ornithine–ketoacid aminotransferase in the type of activation and inhibition provide support for the latter interpretation. The ornithine decarboxylase activity is absolutely dependent on the presence of pyridoxal phosphate in the medium[8], whereas the activity of ornithine–ketoacid aminotransferase cannot be increased by supplementary pyridoxal phosphate. This observation suggests a higher degree of dissociation of the pyridoxal phosphate–enzyme complex for ornithine decarboxylase than for ornithine–ketoacid aminotransferase. The inhibition of ornithine decarboxylase activity was reversed by excess pyridoxal phosphate, whereas ornithine–ketoacid aminotransferase was irreversibly inactivated. This result is in accord with the differences in the dissociation constant of the pyridoxal phosphate–enzyme complexes, and thus indirectly supports the significance of formation of canaline–pyridoxal phosphate complexes as the basis of canaline-induced enzyme inhibition.

Secondly, canaline effectively inhibited all other pyridoxal phosphate dependent enzymes so far tested. Again, some differences in the action of canaline were seen

between separate enzymes, probably at least partly relating to differences in pyridoxal phosphate–enzyme dissociation constants already mentioned.

Thirdly, direct evidence on the chemical action of canaline on pyridoxal phosphate was obtained by analyses of the spectra of their aqueous solutions. A stoichiometric formation of canaline–pyridoxal phosphate complex is suggested by the abolition of the 410-nm absorption peak of pyridoxal phosphate by an equivalent amount of canaline in solution. On the basis of structural similarity of canaline with some aminooxy compounds, such as aminooxyacetic acid and 4-bromo-3-hydroxybenzoxyamine, both known as potent pyridoxal phosphate binders[9,10], an oxime formation with pyridoxal phosphate seems to be possible. On the basis of the above results we conclude that canaline probably inhibits pyridoxal phosphate-containing enzymes by its nonenzymic, irreversible and stoichiometric binding with pyridoxal phosphate.

Canaline seems to be one of the most potent inhibitors of pyridoxal enzymes and can be useful in studying their properties and mechanism of action. Further work is in progress to analyse the action of canaline on a number of other pyridoxal enzymes as well as to characterize the chemical properties of the pyridoxal phosphate–canaline complex.

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