Biochimica et Biophysica Acta, 327 (1973) 501–509

BBA 67074

ORNITHINE DECARBOXYLASE: INHIBITION BY α-HYDRAZINOORNITHINE

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(Received June 18th, 1973)

SUMMARY

α-Hydrazinoornithine is a potent inhibitor of ornithine decarboxylase (EC 4.1.1.17, L-ornithine carboxy-lyase) with K_i values for the enzyme from *Escherichia coli* and rat ventral prostate of $5 \cdot 10^{-7}$ and $2 \cdot 10^{-6}$ M, respectively. Inhibition of ornithine decarboxylase by α-hydrazinoornithine is relatively selective, since the drug is 1–2 orders of magnitude less potent in inhibiting other pyridoxal phosphate-dependent amino acid decarboxylases. Other hydrazino compounds examined are much less potent inhibitors of ornithine decarboxylase than α-hydrazinoornithine. Inhibition by α-hydrazinoornithine is reversible and competitive with respect to substrate.

INTRODUCTION

Putrescine and the polyamines spermidine and spermine are ubiquitous in nature and appear to be associated in several instances with rapid tissue growth[1]. Despite evidence that links polyamines to tissue growth, their precise site and mode of action remains unclear. In an attempt to shed more light on the biological functions of polyamines we sought to inhibit their synthesis.

Williams-Ashman and Schenone[2] have described potent inhibition of *S*-adenosylmethionine decarboxylase, an enzyme required for the biosynthesis of spermidine from putrescine and *S*-adenosylmethionine, by methylglyoxal-bis-guanylhydrazone. The activity of ornithine decarboxylase (EC 4.1.1.17, L-ornithine carboxy-lyase), the enzyme which catalyzes the decarboxylation of ornithine to yield putrescine is a rate-limiting step in the formation of the polyamines[3–6] and might therefore be a heuristic site for potential inhibitors of polyamine synthesis.

In this communication we report that α-hydrazinoornithine is a potent and relatively selective inhibitor of ornithine decarboxylase. We also describe some features of this inhibition.

METHODS

Chemicals

Tris–HCl buffer was purchased from Sigma. Pyridoxal phosphate and dithiothreitol were purchased from Calbiochem. $(NH_4)_2SO_4$ was obtained from Mann. L-Ornithine monohydrochloride was obtained from the Baker Chemical Company. α-Hydrazinohistidine was supplied by Regis. Hydrazine hydrate, methylhydrazine and 4-toluenesulfonylhydrazine were obtained from Aldrich. *N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine (RO4-4602) was a gift from Roche (Nutley, N.J.). α-Hydrazinoornithine was generously donated by Drs W. B. Skinner and J. G. Johansson of the Stanford Research Institute. Based on optical rotation data ($[\alpha]_D^{21}$ 6.56° at a concentration of 1% in water) 70% of the compound was in the L-form. DL-[1-^{14}C]Ornithine monohydrochloride (4.62 Ci/mole), DL-[1-^{14}C]glutamic acid (13.1 Ci/mole) and Omnifluor (2,5-diphenyloxazone 98%, and *p*-bis-(*o*-methylstyryl)-benzene 2%) were purchased from New England Nuclear Corporation. L-3,4-dihydroxy-[1-^{14}C]phenylalanine (9.1 Ci/mole) was purchased from Amersham/Searle.

Animals and bacteria

Adult male Sprague–Dawley rats (200–250 g) were used. *Escherichia coli* in mid-logarithmic phase was obtained in the frozen state from Grain Processing (Muscatine, Iowa).

Preparation of rat prostate ornithine decarboxylase

The enzyme was purified 3–4-fold according to the procedure of Jänne and Williams-Ashman[7] with certain modifications. Ten rats were killed by cervical fracture and their ventral prostates were rapidly dissected free of surrounding connective tissue, weighed, minced and homogenized in 10 vol. of 25 mM Tris–HCl buffer, pH 7.2, containing 2.5 mM dithiothreitol and 0.1 mM disodium EDTA. All operations were carried out at 4 °C. The homogenate was centrifuged at 50 000 × *g* for 1 h. The supernatant fluid was fractionated with solid $(NH_4)_2SO_4$ and the fraction precipitating between 20 and 50% of saturation was dissolved in 2 ml of the homogenization buffer and dialyzed against 100 vol. of the same buffer for 16 h, the dialyzing buffer being changed after the first 6 h. The dialyzed $(NH_4)_2SO_4$ fraction was divided into small aliquots and stored at —20 °C for several months without appreciable loss of activity.

Preparation of E. coli ornithine decarboxylase

The enzyme was purified 3–4-fold according to the procedure of Karpetsky and Talalay (personal communication) with certain modifications. All operations were carried out near 4 °C. 8 g of frozen *E. coli* were suspended in 40 ml of 50 mM Tris–HCl buffer, pH 7.2, containing 2.5 mM dithiothreitol. The mixture was sonicated for 15 min and then centrifuged at 50 000 × *g* for 30 min. 0.2 vol. of freshly prepared 10% streptomycin sulfate (Lilly) was added dropwise to the supernatant fluid. After occasional stirring for 30 min the solution was centrifuged for 10 min at 20 000 × *g* and the pellet discarded. The resulting supernatant fluid was fractionated with a saturated solution of $(NH_4)_2SO_4$ and the fraction precopitating between 40 and 50% of saturation was dissolved in 8 ml of the homogenization buffer and 2 ml of glycerol. This fraction was divided into small aliquots and stored at —20 °C for several months without any loss of activity.

Assay for ornithine decarboxylase activity

L-Ornithine decarboxylase activity was measured by estimating the amount of $^{14}CO_2$ released from [1-^{14}C]ornithine as previously described[8]. The standard incubation mixture contained in a final volume of 0.3 ml: 25 μmoles of Tris–HCl buffer, pH 7.2; 1.5 μmoles of dithiothreitol; 3 nmoles of pyridoxal phosphate; enzyme solution; 0.28 μCi of DL-[1-^{14}C]ornithine; an appropriate amount of unlabeled L-ornithine and the appropriate inhibitor when indicated. The incubations were carried out for 1 h at 37 °C in glass microtubes, 6 mm × 50 mm (Corning) that were placed in glass scintillation vials containing 15 ml of scintillation-trapping cocktail made up of toluene phosphor (4 g Omnifluor in 1 l of toluene), absolute ethanol, monoethanolamine and 2-methoxyethanol (30:6:2:1, by vol.). The scintillation vials were closed with plastic tops containing tightly fitting rubber stoppers. The reaction was terminated by injecting 50 μl of 5 M H_2SO_4 through the rubber stoppers into the incubation medium. The samples were maintained at 25 °C overnight before counting to ensure that all the dissolved CO_2 was released from the acidified medium into the scintillation-trapping fluid. Radioactivity was assayed in a Packard Tri-Carb liquid scintillation spectrometer at an overall counting efficiency of 33%. Blank samples, where the enzyme solution was replaced by either water or a heat-inactivated enzyme solution (heated to 95 °C for 10 min), were included in each set of determinations. Both types of blank samples yielded similar values ranging between 100–200 cpm. L-Ornithine decarboxylase activity was expressed as amount of CO_2 evolved per mg protein per h. Protein was measured by the method of Lowry *et al.*[9], with corrections made for the effect of dithiothreitol on the protein assay.

Assay for 3,4-dihydroxyphenylalanine decarboxylase and glutamic acid decarboxylase.

L-3,4-Dihydroxyphenylalanine decarboxylase and L-glutamic acid decarboxylase activities were measured in a manner similar to that of ornithine decarboxylase. The source for both enzymes was supernatant fluid obtained after homogenizing whole rat brain in 5 vol. of 50 mM Tris–HCl buffer, pH 7.2, in a motor-driven glass homogenizer and centrifugation of the homogenate at 50 000 × *g* for 1 h. The standard incubation mixture contained in a final volume of 0.3 ml: 25 μmoles of Tris–HCl buffer, pH 7.2; 1.5 μmoles of dithiothreitol; 3 nmoles of pyridoxal phosphate; the enzyme preparation and either 30 nmoles of L-3,4-dihydroxy-[1-^{14}C]phenylalanine or 60 nmoles of DL-[1-^{14}C]glutamic acid. Evolution of $^{14}CO_2$ was monitored as described for ornithine decarboxylase.

All enzyme preparations gave linear activities with respect to amount of enzyme added in the ranges used and with respect to time of incubations. The order in which the reactants were added (including the inhibitors) did not result in any measurable changes in enzyme activity or degree of inhibition. The *E.coli* and the rat prostate ornithine decarboxylase enzyme preparations exhibited an absolute requirement for the coenzyme, pyridoxal phosphate, showing negligible activity in its absence. Since many inhibitors of amino acid decarboxylases are carbonyl-trapping agents which act by reacting with pyridoxal phosphate, we determined the optimal concentration of pyridoxal phosphate in the incubation mixture (Fig. 1). For the *E.coli* enzyme the optimal coenzyme concentration was $1 \cdot 10^{-5}$ M while in the case of the rat prostate enzyme, no clear peak could be demonstrated and the minimal optimal concentration of pyridoxal phosphate was considered to be $1 \cdot 10^{-5}$ M. The concentration of the co-

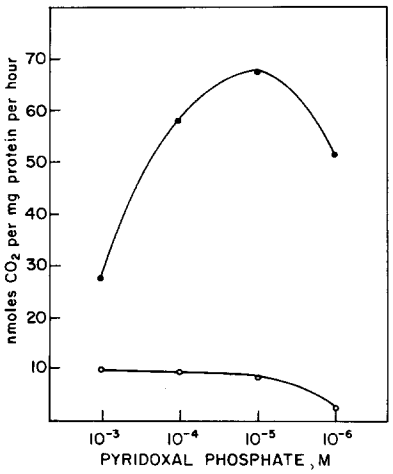

Fig. 1. Effects of pyridoxal phosphate concentration on the activity of partially purified *E. coli* and rat prostate ornithine decarboxylases. Pyridoxal phosphate in various concentrations was added to the assay system containing in a final volume of 0.3 ml the following ingredients: 30 μmoles of Tris–HCl buffer, pH 7.2; 1.5 μmoles of dithiothreitol; 60 nmoles of DL-[1-^{14}C]ornithine (0.28 μCi) and the appropriate enzyme preparation: 0.086 mg protein of the partially purified *E. coli* enzyme (●—●) or 0.15 mg protein of the partially purified rat prostate enzyme (○—○). The mixture was incubated for 1 h at 37 °C. The reaction was terminated by adding 50 μl of 5 M H_2SO_4 to the incubation mixture and the radioactivity of the evolved $^{14}CO_2$ was measured and converted to nmoles CO_2 per mg protein per h.

enzyme was therefore maintained at $1 \cdot 10^{-5}$ M throughout most of the experiments reported except when indicated.

RESULTS

α-Hydrazinoornithine inhibits *E.coli* and rat prostate ornithine decarboxylases with respective ID_{50} values of $5 \cdot 10^{-7}$ M and $5 \cdot 10^{-6}$ M, when tested with $1 \cdot 10^{-5}$ M pyridoxal phosphate and concentrations of L-ornithine equivalent to the K_m values of the 2 enzyme preparations (Fig. 2). This inhibition is greatly reduced or abolished when pyridoxal phosphate concentrations are increased 10–100-fold.

Experiments where the rate of the reaction is plotted against different enzyme concentrations in the absence and presence of 2 concentrations of α-hydrazinoornithine yield straight lines that pass through the origin but have different slopes suggesting reversibility of inhibition[10] for both the bacterial and the mammalian enzymes.

The inhibition of both enzyme preparations is competitive with respect to ornithine (Figs 3 and 4). The apparent affinity of the *E.coli* enzyme for ornithine is relatively low (K_m $1.4 \cdot 10^{-2}$ M) whereas its apparent affinity for α-hydrazinoornithine is 30 000 times higher (K_i $5 \cdot 10^{-7}$ M). The prostatic enzyme has much greater apparent affinity for ornithine than the *E.coli* enzyme but one-fourth as much affinity for α-hydrazinoornithine as the *E.coli* enzyme. (Fig. 4). Nonetheless the prostatic enzyme has 40 times as much apparent affinity for α-hydrazinoornithine as for its substrate, ornithine. The decline in prostatic enzyme activity at higher ornithine concentration suggests that the enzyme is subject to substrate inhibition.

The decline in inhibitory effects of a α-hydrazinoornithine with large concentra-

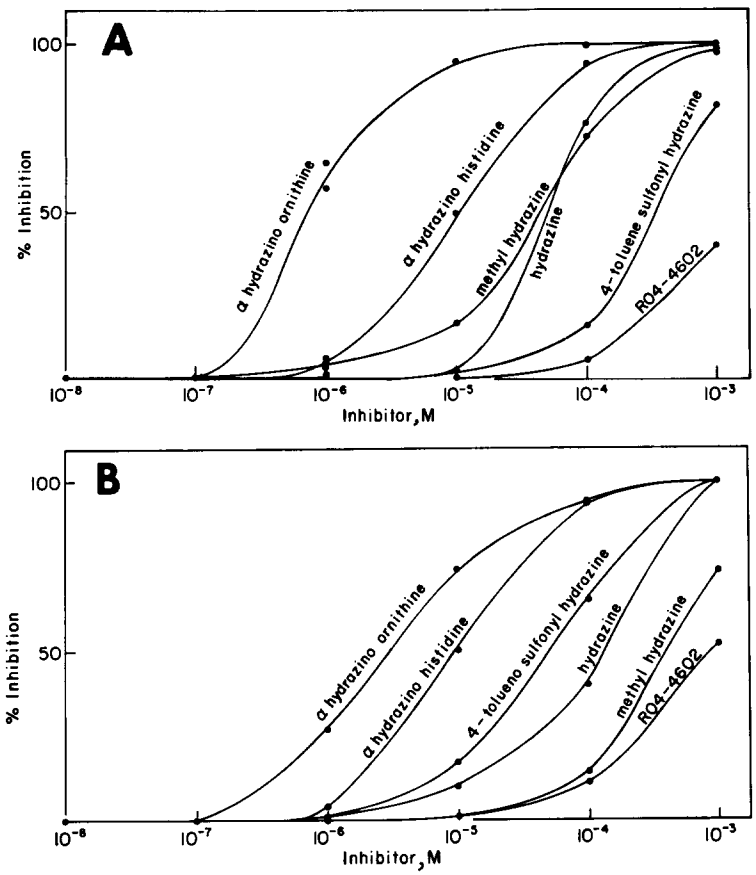

Fig. 2. Inhibition of *E. coli* ornithine decarboxylase (A) and rat prostate ornithine decarboxylase (B) as function of the molar concentration of various hydrazine compounds. Varying concentrations of the hydrazine inhibitors were added to the standard incubation mixture containing in a final volume of 0.3 ml the following ingredients: 30 μmoles of Tris–HCl buffer, pH 7.2; 1.5 μmoles of dithiothreitol; 3 nmoles of pyridoxal phosphate; 0.28 μCi of DL-[1-^{14}C]ornithine with appropriate amounts of unlabeled L-ornithine to give a final concentration of L-ornithine 10 mM in the case of *E. coli* (A) and 0.1 mM in the case of rat prostate enzyme (B) and the appropriate enzyme preparation: 0.086 mg protein of the partially purified *E. coli* enzyme (A) and 0.15 mg protein of the partially purified rat prostate enzyme (B). The mixture was incubated for 1 h at 37 °C. The reaction was terminated by adding 50 μl of 5 M H_2SO_4 to the incubation mixture and the radioactivity of the evolved $^{14}CO_2$ was measured. Percent inhibition was calculated with reference to control samples to which inhibitors were not added.

tions of pyridoxal phosphate and the fact that other ornithine analogues fail to inhibit ornithine decarboxylase[11], suggests that inhibition by α-hydrazinoornithine reflects an interaction of its hydrazine group with the carbonyl group of pyridoxal phosphate. Accordingly, we examined the effects of several other hydrazino compounds structurally unlike ornithine.

α-Hydrazinoornithine is much more potent than other hydrazines in inhibiting bacterial and mammalian ornithine decarboxylases, with ID_{50} concentrations for α-hydrazinoornithine about one order of magnitude less than those of α-hydrazinohistidine and about two orders of magnitude less than those of hydrazine and methyl-

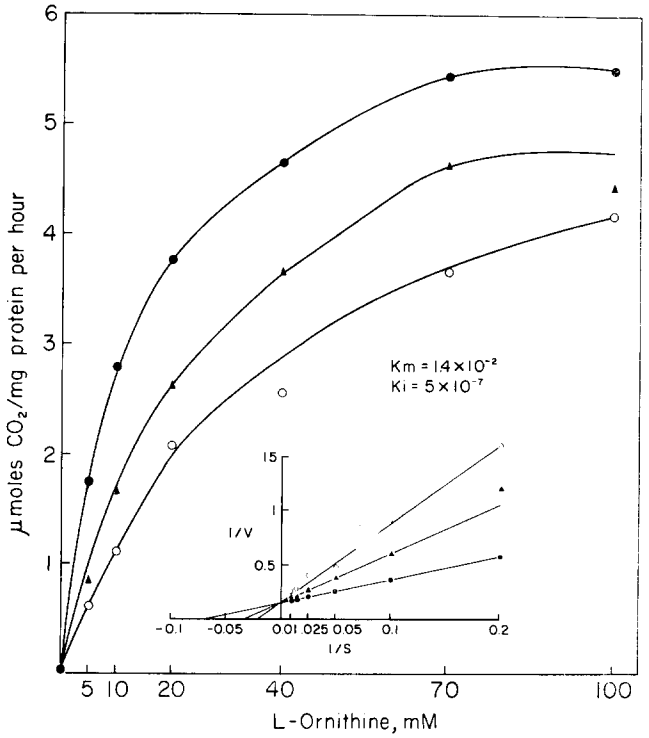

Fig. 3. Effects of substrate concentration on *E. coli* ornithine decarboxylase activity and on its inhibition by α-hydrazinoornithine. The ornithine decarboxylase activity was assayed under standard condtions (see Fig. 2) with variations in the concentration of L-ornithine. The amount of DL-[1-^{14}C]ornithine was kept constant (0.28 μCi), but the specific radioactivity was varied by addition of suitable quantities of L-ornithine. No inhibitor, ●—●; α-hydrazinoornithine: concentration $5 \cdot 10^{-7}$ M, △—△ and $1 \cdot 10^{-6}$ M, ○—○.

hydrazine (Fig. 2). *N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine (RO4-4602), a potent inhibitor of DOPA decarboxylase[12], inhibits ornithine decarboxylase only at relatively high concentrations.

Experiments according to the method of Ackermann and Potter[10] as described earlier for α-hydrazinoornithine suggest reversible inhibition by hydrazine.

Double-reciprocal analysis of varying concentrations of ornithine with saturating levels of pyridoxal phosphate and 2 concentrations of hydrazine indicate competitive inhibition by hydrazine of both bacterial and mammalian enzymes. The apparent affinities of bacterial and mammalian ornithine decarboxylases for hydrazine are respectively one-sixtieth and one-eighth of their affinities for α-hydrazinoornithine. However, the enzymes' apparent affinities for hydrazine are still much greater than for the natural substrate, ornithine. Hydrazine inhibits competitively with respect to pyridoxal phosphate. The apparent affinities of both *E.coli* and prostate enzymes for pyridoxal phosphate, however, are about 20 times their affinities for hydrazine. The K_i values of both enzymes for hydrazine are essentially the same when the concentration of ornithine is varied as when the concentration of pyridoxal phosphate is varied.

On the other hand, α-hydrazinoornithine inhibits non-competitively with respect to pyridoxal phosphate at lower concentrations of the latter (Fig. 5). At higher

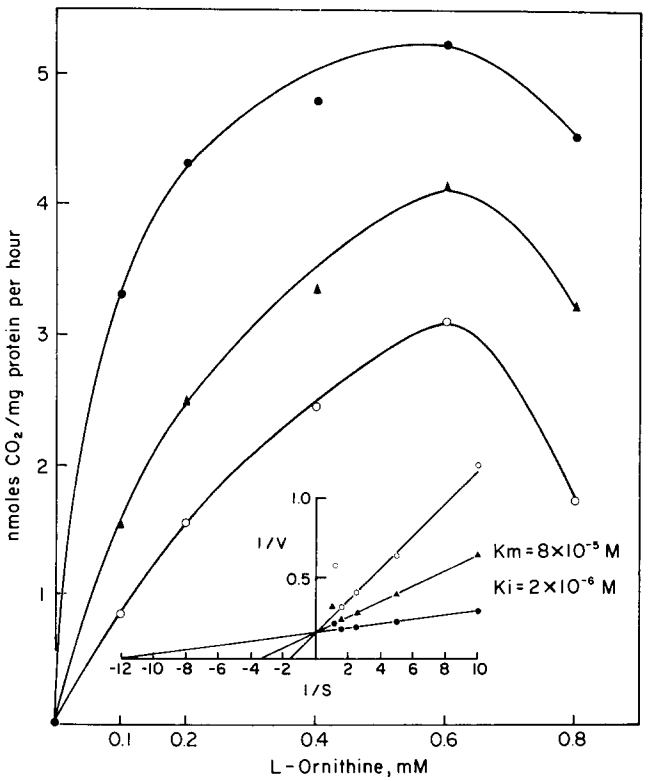

Fig. 4. Effects of substrate concentration on rat prostate ornithine decarboxylase activity and on its inhibition by α-hydrazinoornithine. The ornithine decarboxylase activity was assayed under standard conditions (see Fig. 2) with variations in the concentration of L-ornithine. The amount of DL-[1^{14}C]ornithine was kept constant (0.28 μCi), but the specific radioactivity was varied by addition of suitable quantities of L-ornithine. No inhibitor, ●—●; α-hydrazinoornithine: concentration $5 \cdot 10^{-6}$ M, ▲—▲ and $1 \cdot 10^{-5}$ M, ○—○.

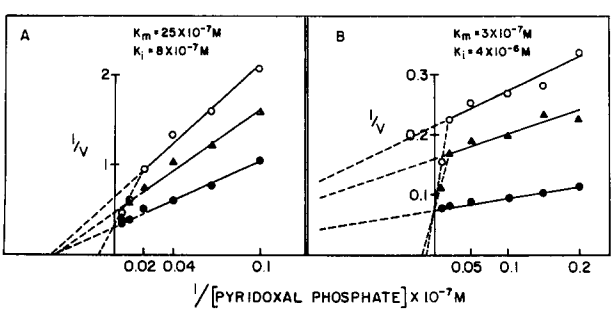

Fig. 5. Effects of pyridoxal phosphate concentration on the inhibition by two concentrations of α-hydrazinoornithine of *E. coli* (A) and rat prostate (B) ornithine decarboxylases. The enzymes activities were assayed under same conditions as described in Fig. 2, except that saturating concentrations of the substrate, L-ornithine were used ($5 \cdot 10^{-2}$ M for the *E. coli* enzyme and $5 \cdot 10^{-4}$ M for the rat prostate enzyme) with variations in the concentration of pyridoxal phosphate; α-hydrazinoornithine concentration $5 \cdot 10^{-7}$ M (▲—▲) and $8 \cdot 10^{-7}$ M (○—○) in (A) and $5 \cdot 10^{-6}$ M (▲—▲) and $7.5 \cdot 10^{-6}$ M (○—○) in (B). No inhibitor, ●—●.

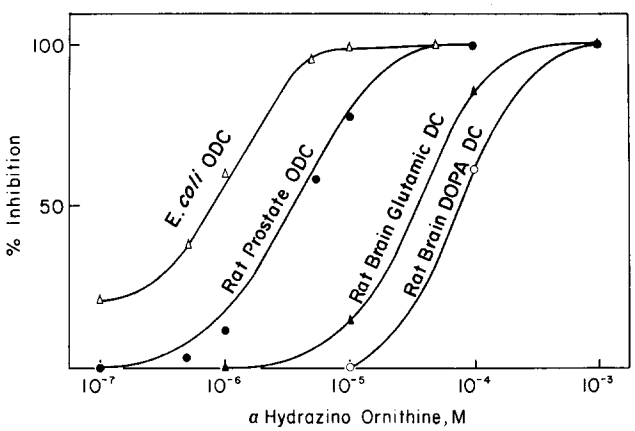

Fig. 6. Inhibitory effects of various concentrations of α-hydrazinoornithine on *E. coli* ornithine decarboxylase (△—△), rat prostate ornithine decarboxylase (●—●), rat brain glutamic acid decarboxylase (▲—▲), and rat brain 3,4-dihydroxyphenylalanine (DOPA) decarboxylase (○—○). α-Hydrazinoornithine, in various concentrations was added to the assay system containing in a final volume of 0.3 ml the following ingredients: 30 μmoles of Tris buffer, pH 7.2; 1.5 μmole of dithiothreitol; 3 nmoles of pyridoxal phosphate; the enzyme preparation indicated and the appropriate 1-^{14}C-labeled amino acid (in the case of *E. coli* ornithine decarboxylase, 2 μmoles of L-ornithine was used but for the rat prostate ornithine decarboxylase, rat brain L-glutamic acid decarboxylase and rat brain DOPA decarboxylase 30 nmoles of the appropriate L-amino acid was used). The mixture was incubated for 1 h at 37 °C and the radioactivity of the evolved $^{14}CO_2$ measured. Percent inhibition was calculated by comparing the amount of $^{14}CO_2$ evolved at that concentration of α-hydrazinoornithine to that evolved in the absence of the inhibitor.

concentrations of pyridoxal phosphate, however, inhibition is apparently competitive. The apparent affinity of the *E.coli* enzyme for α-hydrazinoornithine is 3 times its affinity for pyridoxal phosphate, while the rat prostate enzyme has greater apparent afficity for pyridoxal phosphate than for α-hydrazinoornithine.

To determine the specificity of α-hydrazinoornithine we studied its efficacy in inhibiting two other pyridoxal phosphate dependent amino acid decarboxylases: L-DOPA decarboxylase and L-glutamic acid decarboxylase. These latter decarboxylases are 1 and 2 orders of magnitude less sensitive to α-hydrazinoornithine than rat prostate and *E.coli* ornithine decarboxylases, respectively (Fig. 6). Conversely, *N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine (RO4-4602), a very potent inhibitor of L-DOPA decarboxylase, only weakly inhibits ornithine decarboxylase (Fig. 2).

DISCUSSION

The most striking finding of this study is the considerable potency of α-hydrazinoornithine as an inhibitor of bacterial and mammalian ornithine decarboxylase. This inhibition is reversible and competitive with respect to ornithine. Other hydrazino compounds are much less potent indicating specificity for α-hydrazinoornithine. Both α-hydrazinoornithine and hydrazine appear to compete with the ornithine for the carbonyl group of the holoenzyme. Similar findings have been obtained by Leinweber[13] regarding the inhibition of histidine decarboxylase by 4-bromo-3-hydroxybenzyloxyamine and several other O-substituted hydroxylamines. The selective high potency of inhibition by α-hydrazinoornithine is probably related to its structural similarity to the natural substrate, ornithine.

The fact that α-hydrazinoornithine inhibits the enzyme even with 5–20-fold excess of pyridoxal phosphate indicates that it does not produce its inhibitory effects simply by trapping pyridoxal phosphate in the incubation mixture. Furthermore, inhibition by α-hydrazinoornithine appears to be non-competitive with respect to pyrdoxal phosphate at lower concentrations. This suggests that the enzyme can simultaneously bind to α-hydrazinoornithine and pyridoxal phosphate. The apparent change in the character of the inhibition from non-competitive to competitive in the presence of high concentrations of pyridoxal phosphate may be due to the formation of a pyridoxal phosphate–α-hydrazinoornithine complex that does not bind to the enzyme but reduces the effective concentration of free α-hydrazinoornithine.

In the case of hydrazine, where inhibition is competitive with both ornithine and pyridoxal phosphate, pyridoxal phosphate–hydrazine complex may bind to the enzyme and prevent substrate binding. A pyridoxal phosphate–hydrazine complex might effectively bind to the enzyme whereas a pyridoxal phosphate–α-hydrazinoornithine complex cannot, because of its bulk and/or the charged groups present in the pyridoxal phosphate–α-hydrazinoornithine complex.

In addition to its potency, several observations suggest that α-hydrazinoornithine is a fairly specific inhibitor of the ornithine decarboxylases. α-Hydrazinoornithine is much more potent in inhibiting ornithine decarboxylase than α-hydrazinohistidine and *N*-(DL-seryl)-*N'*-(2,3,4-trihydroxybenzyl)-hydrazine (RO4-4602), potent inhibitors of histidine decarboxylase[13] and DOPA decarboxylase[12], respectively. Conversely, α-hydrazinoornithine only weakly inhibits DOPA decarboxylase and glutamic acid decarboxylase, two other pyridoxal phosphate-dependent amino acid decarboxylases.

Because of its potency and apparent specificity, α-hydrazinoornithine is of interest for studies of polyamine synthesis *in vivo*. α-Hydrazino ornithine effectively blocks the formation of putrescine in rat liver *in vivo* as well as in rat hepatoma cells in culture. (Harik, S. I., Hollenberg, M. and Snyder, S. H., unpublished).

ACKNOWLEDGEMENTS

We would like to thank Dr Karl A. Schellenberg for his advice on some of the theoretical aspects of this work. This work was supported by U.S.P.H.S. grants MH-18501, NS-07275, and DA-00266. S.I.H. was supported by the Commonwealth Fund Exchange Fellowship Program. S.H.S. is recipient of Research Scientist Development Award, MH-33128.

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