MOLECULAR PHARMACOLOGY, 10, 41–47

# Ornthine Decarboxylase Turnover Slowed by α-Hydrazino-ornithine

SAMI I. HARIK,[1] MORLEY D. HOLLENBERG,[2] AND SOLOMON H. SNYDER[3]

*Department of Pharmacology and Experimental Therapeutics and Department of Psychiatry and Behavioral Sciences, The Johns Hopkins University School of Medicine, Baltimore, Maryland 21205*

(Received July 9, 1973)

SUMMARY

HARIK, SAMI I., HOLLENBERG, MORLEY, D., AND SNYDER, SOLOMON H.: Orthinine decarboxylase turnover slowed by α-hydrazino-ornithine. *Mol. Pharmacol.* **10**, 41–47 (1974).

α-Hydrazino-ornithine is a potent and selective inhibitor of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17). When added to rat hepatoma cells at the time of dilution, α-hydrazino-ornithine elicits a dose-related increase in ornithine decarboxylase activity and a concomitant prolongation of the apparent half-life of the enzyme from 10 min to 28 min, as determined by the rate of decline of ornithine decarboxylase activity after inhibition of protein synthesis by cycloheximide. Similarly, systemic administration of α-hydrazino-ornithine to nephrectomized rats induces a dose-related enhancement of ornithine decarboxylase activity in the normal and regenerating liver, which is associated with prolongation of the apparent half-life of the enzyme. In both intact liver and hepatoma cells in culture, the decrease in the turnover rate of the enzyme can account for its increased activity.

## INTRODUCTION

Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17), which catalyzes the decarboxylation of ornithine to putrescine, is a rate-limiting enzyme in polyamine biosynthesis (1–3). Its activity is rapidly and markedly enhanced following growth stimuli such as partial hepatectomy (1), the administration of growth hormone (4, 5), and dilution of high-density cultured rat hepatoma cells with fresh medium (6). Ornithine decarboxylase turns over rapidly, with a half-life of about 11 min (7). The same short half-life of liver ornithine decarboxylase is observed both in the basal state and in regenerating liver; this observation suggests that the increase in ornithine decarboxylase after partial hepatectomy is due to new enzyme synthesis rather than to an increase in the half-life of the enzyme (7).

We have recently observed that α-hydrazino-ornithine is a potent competitive inhibitor of mammalian and bacterial ornithine decarboxylase *in vitro* (8). However, in preliminary experiments *in vivo*, when α-hydrazino-ornithine was administered to

This investigation was supported by United States Public Health Service Grants MH-18501, NS-07275, and DA-00266 and a grant from the John A. Hartford Foundation to S. H. S., and by a grant from the American Cancer Society (BC-63) to Pedro Cuatrecasas.

[1] Recipient of a Postdoctoral Fellowship from the Commonwealth Fund.

[2] Recipient of a Postdoctoral Fellowship from Medical Research Council of Canada.

[3] Recipient of Research Scientist Development Award MH-33128 from the National Institutes of Health.

rats, a marked increase in liver and prostate ornithine decarboxylase activity was observed (8). In this communication we report that α-hydrazino-ornithine enhances ornithine decarboxylase activity of rat liver, brain, and hepatoma in tissue culture by prolonging the apparent half-life of the enzyme.

## MATERIALS AND METHODS

*Chemicals.* Pyridoxal phosphate, dithiothreitol, and cycloheximide were purchased from Calbiochem. L-Ornithine was obtained from Schwarz/Mann. α-Hydrazino-ornithine was generously donated by Drs. W. B. Skinner and J. G. Johansson of the Stanford Research Institute. Materials used in the preparation of tissue culture medium were obtained from Microbiological Associates. DL-[1-$^{14}$C]Ornithine monohydrochloride (12.8 mCi/mmole) was purchased from New England Nuclear Corporation.

*Preparation of rat hepatoma cells in culture for assay of ornithine decarboxylase activity.* Rat hepatoma cells (9), generously donated by Dr. E. B. Thompson, were grown in monolayer in Eagle's minimal essential medium with Hanks' balanced salts, supplemented with 2 mM glutamine, 100 units/ml each of penicillin and streptomycin, and 10% fetal calf serum. The cultures were grown at 37° in an atmosphere of 5% $CO_2$ and air. Cells were subcultured by scraping and diluting with fresh culture medium to a density of about $3 \times 10^5$ cells/ml. A saturation density of about $2 \times 10^7$ cells/flask (75 cm$^2$ area) was reached in 3 days. Experiments were initiated by scraping and diluting cells at or near the saturation density with freshly prepared medium to $2 \times 10^5$ cells/ml (total volume, 50 ml). α-Hydrazino-ornithine and/or cycloheximide was dissolved in a small volume of the growth medium and added to the culture medium at the appropriate times. To assay ornithine decarboxylase activity, about $7.5 \times 10^6$ cells were harvested by scraping and centrifugation at $800 \times g$ for 5 min. Centrifugation and all subsequent operations were performed at or near 4° unless stated otherwise. Cells were washed twice with 10 ml of phosphate-buffered 0.9% NaCl, and the pellet was then suspended in 0.5 ml of 0.05 M Tris-HCl buffer, pH 7.2, containing 2.5 mM dithiothreitol, 10 μM pyridoxal phosphate, and 0.1 mM EDTA. Cells were disrupted by freezing and thawing, and the resultant suspension was centrifuged at $50,000 \times g$ for 30 min. The supernatant fluid was dialyzed against the same buffer for 14 hr, a treatment which completely removes α-hydrazino-ornithine from tissue preparations (10).

*Preparation of rat liver for assay of ornithine decarboxylase activity.* Male Sprague-Dawley rats (110–140 g) were subjected to bilateral nephrectomy under light ether anesthesia through a posterior approach. In some rats partial hepatectomy was performed immediately following nephrectomy according to the method of Higgins and Anderson (11). The rats were allowed to recover for 4 hr before α-hydrazino-ornithine (dissolved in 0.25 ml of 0.9% NaCl) was injected subcutaneously. Immediately after decapitation, liver tissue was removed and frozen on Dry Ice. All subsequent operations were performed near 4°. Liver samples were weighed and homogenized in 9 volumes of ice-cold buffer solution (0.05 M Tris-HCl buffer, pH 7.2, containing 2.5 mM dithiothreitol, 10 μM pyridoxal phosphate, and 0.1 mM EDTA) in motor-driven glass homogenizers. Homogenates were centrifuged at $50,000 \times g$ for 30 min, and the supernatant fluid was dialyzed against 100 volumes of buffer for 14 hr.

*Preparation of rat brain for assay of ornithine decarboxylase activity.* Male rats (160–180 g) were lightly anesthetized with ether, and 35 μg of α-hydrazino-ornithine HCl dissolved in 15 μl of 0.9% NaCl were infused into the left lateral ventricle, utilizing standard stereotaxic procedures. Control animals received 15 μl of 0.9% NaCl. Four hours after the injection rats were decapitated and whole brain homogenates were prepared as described above for the liver.

*Assay for ornithine decarboxylase activity.* Ornithine decarboxylase activity was determined by estimating the amount of $^{14}CO_2$ released from [1-$^{14}$C]ornithine. The incubation mixture contained, in a final volume of 110 μl, 5 μmoles of Tris-HCl buffer (pH 7.2),

0.25 μmole of dithiothreitol, 1 nmole of pyridoxal phosphate, 30 nmoles of DL-[1-$^{14}$C]ornithine (0.4 μCi), 15 nmoles of unlabeled L-ornithine, and the enzyme solution. The final concentration of ornithine, 0.3 mM, is about 10 times the $K_m$ for the mammalian enzyme (10). The reaction was started by the addition of the substrate, and the reaction mixture was incubated for 1 hr at 37°. Blank samples, in which the enzyme solution was inactivated by heating at 95° for 10 min, were included in each set of determinations. The evolved $^{14}CO_2$ was estimated as previously described (12). Ornithine decarboxylase activity was expressed as moles of $CO_2$ evolved per milligram of protein per hour, which under the conditions used was previously determined to be stoichiometric with putrescine formation (1). Protein was measured by the method of Lowry *et al.* (13), with corrections made for the effect of dithiothreitol present in the reaction buffer. All enzyme preparations yielded activities which were linear with respect to protein concentrations in the ranges used and with respect to time of incubation.

## RESULTS

*Effect of α-hydrazino-ornithine on ornithine decarboxylase activity in rat hepatoma cells in culture.* Ornithine decarboxylase activity in high-density-culture cells is extremely low. However, dilution of cells with fresh medium causes a marked increase in ornithine decarboxylase, which reaches its peak 4–6 hr after dilution and declines thereafter (6) (Fig. 1). Addition of α-hydrazino-ornithine to the culture medium at the time of dilution augments the increase of ornithine decarboxylase activity above that obtained by dilution alone (Figs. 1 and 2). In these experiments demonstrating enhanced ornithine decarboxylase activity, tissue preparations were dialyzed to remove α-hydrazino-ornithine, which would have inhibited the enzyme. This enhancement of ornithine decarboxylase activity by α-hydrazino-ornithine is dose-related. The effect is minimal at 10 μM and increases rapidly at higher concentrations (Fig. 2). At 500 μM α-hydrazino-ornithine the enzyme activity 4 hr after dilution of high-density cells is consistently 3

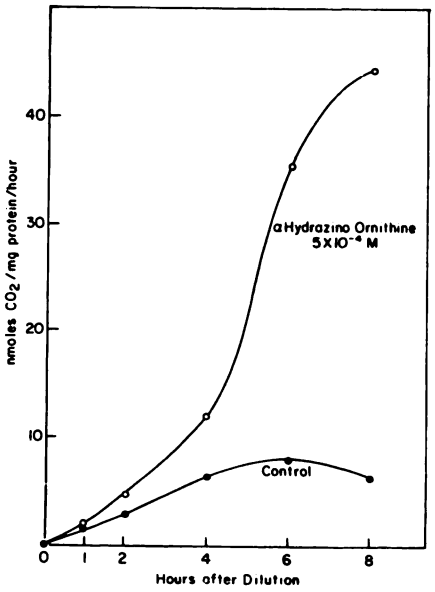


FIG. 1. *Effect of culture dilution on ornithine decarboxylase activity in rat hepatoma cells incubated in the absence and presence of α-hydrazino-ornithine*

Rat hepatoma cells at a concentration of 8 × $10^5$ cells/ml were diluted at zero time to 1.6 × $10^5$ cells/ml with fresh medium and incubated in the presence of 500 μM α-hydrazino-ornithine (○——○) and in its absence (●——●). At intervals after dilution the cells were harvested and assayed for ornithine decarboxylase activity as described under MATERIALS AND METHODS. Undiluted cells incubated with or without α-hydrazino-ornithine had a very low ornithine decarboxylase activity, similar to levels obtained at zero time (0.06 nmole/mg of protein per hour).

times that of control preparations. In contrast, when added to undiluted high-density cells, α-hydrazino-ornithine, in concentrations ranging from 10 μM to 1 mM, does not induce any increase in ornithine decarboxylase levels above that ordinarily measured in undiluted preparations. The concentration of α-hydrazino-ornithine required to elicit half the apparent maximal increase of ornithine decarboxylase activity is about 50 times the $K_i$ value for the mammalian enzyme (10).

The half-life of ornithine decarboxylase was determined after high-density cells were diluted with fresh medium in both the presence and absence of α-hydrazino-ornithine (500 μM). Four hours after dilution, cyclo-

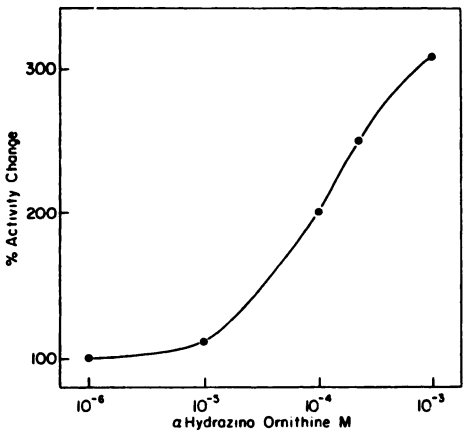


FIG. 2. *Effect of various concentrations of α-hydrazino-ornithine in culture medium on increase in ornithine decarboxylase activity in rat hepatoma cells*

High-density rat hepatoma cells ($1 \times 10^6$ cells/ml) were diluted with fresh medium to $2 \times 10^5$ cells/ml and incubated in the presence of varied concentrations of α-hydrazino-ornithine. Four hours after dilution the cells were harvested and assayed for ornithine decarboxylase activity as described under MATERIALS AND METHODS.

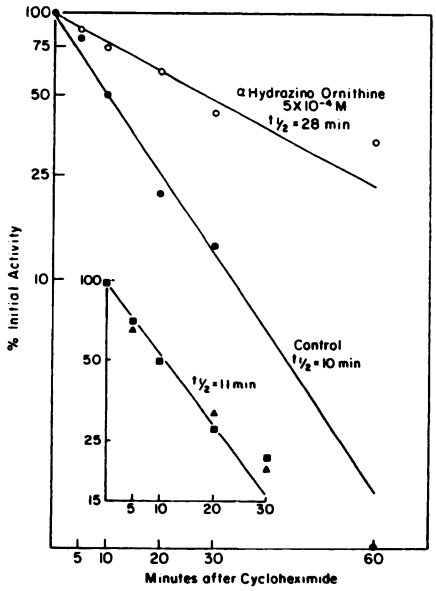


FIG. 3. *Effect of α-hydrazino-ornithine on apparent half-life of ornithine decarboxylase in rat hepatoma cells in culture*

High-density rat hepatoma cells at a concentration of $9 \times 10^5$ cells/ml were diluted to $1.8 \times 10^5$ cells/ml with fresh medium and incubated in the presence of 500 μM α-hydrazino-ornithine (○——○) and in its absence (●——●), initial enzyme activities being 3.1 and 1.0 nmoles of $CO_2$ per milligram of protein per hour, respectively. Four hours after dilution, cycloheximide was added to a final concentration of 50 μg/ml, and the rate of decline of ornithine decarboxylase activity in cell extracts was followed. The inset depicts independent experiments showing the rate of decline of ornithine decarboxylase activity following the addition of cycloheximide (50 μg/ml) to cultures 2 hr (■——■) and 8 hr (▲——▲) after dilution, with initial enzyme activities of 2.5 and 3.2 nmoles of $CO_2$ per milligram of protein per hour, respectively.

heximide (50 μg/ml) was added to determine the subsequent decay of ornithine decarboxylase activity. Inhibitors of protein synthesis block amino acid incorporation into protein of hepatoma tissue culture cells within 5 min after addition (14). In the concentrations employed, cycloheximide reduces protein synthesis of these cells by more than 95% (15). In the absence of α-hydrazino-ornithine, ornithine decarboxylase activity declines to 50% of the initial level in 10 min and is undetectable 60 min after the addition of cycloheximide, indicating a half-life of about 10 min. In the presence of α-hydrazino-ornithine, the decline in enzyme activity is much slower, with 74% and 34% of initial activity remaining 10 and 60 min after cycloheximide, respectively, indicating a half-life of about 28 min (Fig. 3). Experiments were performed to study the half-life of ornithine decarboxylase at various times after dilution of high-density cells. The rate of decline in enzyme level after cycloheximide was essentially the same when tested 2, 4, and 8 hr after dilution (Fig. 3), suggesting that the half-life of the enzyme does not change during the process of induction induced by dilution of high-density cells.

*Effect of α-hydrazino-ornithine in vivo on ornithine decarboxylase activity in rat liver.* In preliminary experiments α-hydrazino-ornithine (200 mg/kg), injected either subcutaneously or intraperitoneally, caused a small, variable increase in liver ornithine decarboxylase levels in 2–6 hr. In view of the small amounts of α-hydrazino-ornithine available, bilateral nephrectomy was per-

formed on rats prior to its administration, to achieve higher tissue concentrations with lower doses of the drug. In the nephrectomized rats, subcutaneous administration of α-hydrazino-ornithine (50–100 mg/kg) leads to a 3–4-fold dose-related increase in the level of liver ornithine decarboxylase as early as 2 hr (Fig. 4A). There is no detectable increase in the level of ornithine decarboxylase in the brains of these rats. Two hours after the subcutaneous administration of 50 mg/kg of α-hydrazino-ornithine the half-life of the enzyme was determined in liver of nephrectomized rats as well as in nephrectomized control rats, by monitoring the decline of its activity after cycloheximide administration (50 mg/kg) (Fig. 5). In control rats the half-life is 11 min, whereas in the α-hydrazino-ornithine-treated rats the half-life of the enzyme is prolonged to 29 min. An increased level of hepatic ornithine decarboxylase and the prolongation of its half-life by α-hydrazino-ornithine have also been observed in the regenerating rat liver 4 hr after partial hepatectomy. Two hours after the administration of 50 mg/kg of α-hydrazino-ornithine (i.e., 6 hr after partial hepatectomy) the regenerating liver enzyme level is 8.4 ± 0.7 nmoles of $CO_2$ per milligram of protein per hour, compared to 4.6 ± 0.8 nmoles/mg/hr in control rats ($n$ = 3). In agreement with the results obtained in the nonregenerating liver, the half-life of ornithine decarboxylase in the re-

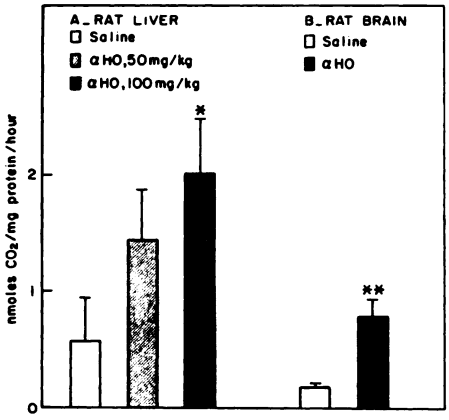


FIG. 4. *Effect of α-hydrazino-ornithine on ornithine decarboxylase activity in rat liver (A) and rat brain (B)*

A. NaCl (0.9%; saline) and α-hydrazino-ornithine (α HO) in doses of 50 and 100 mg/kg were administered subcutaneously to groups of four rats 4 hr after bilateral nephrectomy, and ornithine decarboxylase activity was assayed in liver tissue 2 hr later as described under MATERIALS AND METHODS.

B. NaCl (0.9%) and α-hydrazino-ornithine (35 μg dissolved in 15 μl of 0.9% NaCl) were injected into the lateral cerebral ventricle of two groups of four rats each, and the ornithine decarboxylase activity was assayed in brain tissue 4 hr later as described under MATERIALS AND METHODS. Vertical bars show the magnitude of one standard error on the mean value for each group of rats. Values differ from NaCl-treated controls.

* $p < 0.05$.

** $p < 0.01$.

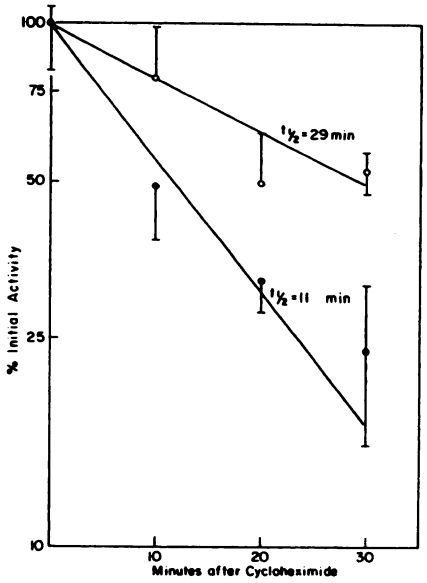


FIG. 5. *Effect of α-hydrazino-ornithine on apparent half-life of ornithine decarboxylase in rat liver*

NaCl (0.9%) or α-hydrazino-ornithine was administered subcutaneously to rats 4 hr after bilateral nephrectomy. Two hours later 50 mg/kg of cycloheximide were injected intraperitoneally. The rate of subsequent decline in liver ornithine decarboxylase activity is expressed as a percentage of the initial activity in both groups of rats (●——●, 0.9% NaCl; ○——○, α-hydrazino-ornithine, 50 mg/kg). Each point represents the mean for four rats ± the standard error, which is represented by the magnitude of the bar on either side of the mean. Slopes were estimated visually.

generating liver of α-hydrazino-ornithine-treated rats is 26 min, compared to 12 min in regenerating control liver. Thus the increase in the level of hepatic ornithine decarboxylase and the prolongation of the half-life of the enzyme by α-hydrazino-ornithine appear to be similar in regenerating and nonregenerating livers.

*Effect of α-hydrazino-ornithine on ornithine decarboxylase activity in rat brain.* The level of ornithine decarboxylase is very low in the brains of postnatal rats. Whereas large doses (200 mg/kg) of systemically administered α-hydrazino-ornithine have no effect on brain ornithine decarboxylase levels, small doses (35 μg/rat brain) injected into a lateral cerebral ventricle induce a marked increase in brain ornithine decarboxylase activity over that of NaCl-treated controls (Fig. 4B). Doses in excess of 35 μg cause seizures. Since hydrazines are well known to provoke seizures in animals, the seizures following α-hydrazino-ornithine may not be specific and may be related to its binding of pyridoxal phosphate, as reported for other hydrazines (10).

## DISCUSSION

In the present study ornithine decarboxylase activity was enhanced by α-hydrazino-ornithine both in hepatoma cells in culture and in the intact rat. In the tissue culture system, dilution of high-density rat hepatoma cells increases enzyme activity, as has been previously reported (6). This increase is presumably due to an increased rate of synthesis, since it can be completely blocked by cycloheximide (6). Moreover, in our experiments, the apparent half-life of ornithine decarboxylase remained constant throughout the period when enzyme activity was increasing. These results differ some what from those of Hogan *et al.* (16), who found changes in the apparent half-life of the enzyme at varying intervals following dilution. It is possible that the discrepancy between our results and those of Hogan *et al.* (16) is related to differences in culture technique, since they used cells in suspension, while we employed monolayers.

α-Hydrazino-ornithine (500 μM) evokes about a 3-fold increase in ornithine decarboxylase activity in hepatoma cells and also prolongs the apparent half-life of the enzyme activity in undiluted cells. Assuming that steady-state conditions obtained in these experiments, the similarity in the extent of change of half-life and of enzyme activity corresponds to predictions from theoretical models of enzyme increases dependent on enzyme stabilization (17). This suggests that the enhanced ornithine decarboxylase activity evoked by α-hydrazino-ornithine is attributable predominantly to a prolongation of the apparent half-life of the enzyme, although we cannot completely rule out a role for new protein synthesis. It must be borne in mind that measurements of enzyme activity do not necessarily reflect the amount of enzyme protein, so that direct measurements of ornithine decarboxylase turnover await the development of specific assays for this enzyme protein.

In intact rats α-hydrazino-ornithine also increases ornithine decarboxylase activity and prolongs its apparent half-life in the liver. After partial hepatectomy enzyme activity is increased 1.8-fold by α-hydrazino-ornithine and the apparent half-life is prolonged 2.2-fold, while in nonregenerating liver both the enzyme activity and apparent half-life increase 2.5-fold. Thus it appears that in the intact animal, as in hepatoma cells in culture, the α-hydrazino-ornithine-elicited increase in ornithine decarboxylase activity is associated primarily with prolongation of the apparent half-life of the enzyme. It is tempting to propose that α-hydrazino-ornithine prolongs the apparent half-life of ornithine decarboxylase via a specific interaction with the enzyme protein. Other authors have reported enhancement of enzyme activity produced by inhibitors of various enzymes, apparently mediated by protection of the enzymes from proteolytic degradation. Examples include the enhancement of dihydrofolate reductase by amethopterin (18–20) and of *S*-adenosylmethionine decarboxylase by methylglyoxal bis(guanylhydrazone) (21, 22). However, nonessential amino acids, which presumably do not interact specifically with ornithine decarboxylase, have also been observed to prolong its apparent half-life in phytohemagglutinin-

stimulated human lymphocytes (23) and in hepatoma cells grown in suspension (16). The exact mechanism whereby α-hydrazino-ornithine prolongs the apparent half-life of ornithine decarboxylase may therefore be complex and remains to be determined. It is of importance in this regard that ornithine itself does not enhance hepatic ornithine decarboxylase after large doses *in vivo*.[4]

The apparent half-life of ornithine decarboxylase is the same in regenerating liver as in normal liver, as observed previously (7), indicating that the marked enhancement of enzyme activity following partial hepatectomy is more likely due to an increased rate of enzyme synthesis than to any alteration in enzyme turnover. Interestingly, the apparent half-life of ornithine decaroxylase is the same in rat liver as in hepatoma cells in tissue culture.

Ornithine decarboxylase activity of rat brain is extremely low and almost undetectable under normal conditions. Intraventricular administration of α-hydrazino-ornithine produces a marked increase in brain ornithine decarboxylase activity. The failure of the brain enzyme to respond to the systemic administration of α-hydrazino-ornithine probably reflects the inability of the drug to pass the blood-brain barrier.

Putrescine levels of hepatoma cells are lowered 4-fold by α-hydrazino-ornithine, indicating that net putrescine synthesis is depressed by the drug even though ornithine decarboxylase is increased.[4] Similarly, in intact, nephrectomized rats, α-hydrazino-ornithine lowers hepatic putrescine levels, and intraventricular administration of the drug lowers brain putrescine levels.[4] In these situations tissue levels of the inhibitor apparently suffice to block putrescine formation even by the enhanced levels of ornithine decarboxylase.

ACKNOWLEDGMENTS

The technical assistance of Donald P. Kramer and Lydia Hernaez is gratefully acknowledged.

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