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# DECREASE OF POLYAMINE LEVELS IN RAT TISSUES BY 5-HEXYNE-1,4-DIAMINE, AN ENZYME-ACTIVATED IRREVERSIBLE INHIBITOR OF ORNITHINE DECARBOXYLASE

CHARLES DANZIN, MICHEL J. JUNG, BRIAN W. METCALF, JEFF GROVE and PATRICK CASARA
Centre de Recherche Merrell International, 16, rue d'Ankara, 67084-Strasbourg Cedex, France

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**Abstract**—1. 5-Hexyne-1,4-diamine (RMI 71696), an enzyme-activated irreversible inhibitor of ornithine decarboxylase (EC 4.1.1.17) *in vitro*, causes a rapid, long-lasting, dose-dependent decrease of ornithine decarboxylase activity in prostate and to a lesser extent in thymus and testis of rats when injected intraperitoneally. 2. 5-Hexyne-1,4-diamine activates *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) *in vitro*, analogously to putrescine. 3. Three doses of 100 mg/kg 5-hexyne-1, 4-diamine given to rats during a 24 hr period markedly decrease putrescine concentrations in prostate, thymus and testis and spermidine concentrations in prostate.

Ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17) catalyses the first and probably rate-limiting step in the biosynthesis of the polyamines putrescine, spermidine and spermine in animal tissues [1, 2]. Since polyamines have been implicated in the regulation of cell growth [2, 3], attempts have been made to inhibit ornithine decarboxylase in order to clarify their roles.

Competitive inhibition of ornithine decarboxylase [4] and repression of ornithine decarboxylase activity by analogues of putrescine in organs where the enzyme is inducible [5, 6] have been studied. However, to our knowledge, it has not been possible to decrease basal levels of spermidine and spermine in a living animal [5, 7].

We have recently shown that 5-hexyne-1,4-diamine (RMI 71696) is an irreversible inhibitor of rat liver ornithine decarboxylase *in vitro*, needing enzyme-catalysed transformation to inactivate this enzyme [8]. We now report that in rats 5-hexyne-1,4-diamine reduces ornithine decarboxylase activity in a dose-dependent and long-lasting manner in ventral prostate, testis and thymus, three organs known to have high resting levels of ornithine decarboxylase activity [9–11]. This ornithine decarboxylase inhibition results in a lowering of putrescine and spermidine levels.

## MATERIALS AND METHODS

*Chemicals.* D,L[1-$^{14}$C]Ornithine (58 mCi/m-mole) and *S*-adenosyl-L-[carboxyl-$^{14}$C]methionine (60 mCi/m-mole) were purchased from the Radiochemical Centre, Amersham; L-ornithine, pyridoxal phosphate, ammonium sulfate and sucrose were obtained from Merck, Darmstadt; EDTA, tetrasodium, dihydrate was obtained from Calbiochem, La Jolla, CA, U.S.A.; *S*-adenoysl-L-methionine, putrescine dihydrochloride and dithiothreitol were obtained from the Sigma Chemical Co., St Louis, MO, U.S.A. Methylglyoxal-bis-(guanylhydrazone), 2HCl, monohydrate was obtained from Aldrich-Europe. Scintillators were purchased from Beckman Instruments, Inc., Fullerton, CA 92634, U.S.A. 5-hexyne-1,4-diamine was synthetized in our laboratories [8].

*Animals.* Male rats of the Sprague–Dawley strain (180–200 g body wt), purchased from Charles River, France, were given food and water *ad libitum* under a constant 12 hr light–12 hr dark lighting schedule. Animals were killed by decapitation at about the same time of day to minimize effects due to diurnal fluctuations. Drugs, dissolved in 0.9% saline, were injected intraperitoneally. Rats given saline served as controls.

*Assay of ornithine decarboxylase activity* (in vivo). After decapitation at various times following administration of the drug, the ventral prostate, testis and thymus were excised rapidly and immediately processed. The tissues were homogenized with three volumes of 30mM sodium phosphate buffer, pH 7.1, containing 0.1 mM EDTA, 0.25 M sucrose, 0.1 mM pyridoxal–phosphate and 5 mM dithiothreitol. Ornithine decarboxylase activities were determined on a 5000 *g* supernatant of testis or prostate homogenate and on a whole thymus homogenate, as thymus ornithine decarboxylase is particulate, [11] essentially as described by Ono *et al.* [12].

*Assay of time-dependent inhibition of ornithine decarboxylase* (in vitro). Ventral prostate or testis were excised from ten rats and pooled. Ornithine decarboxylase from these two organs was purified using steps 1 and 2 (105,000 *g* centrifugation and ammonium sulfate precipitation) of the method described by Jänne and Williams-Ashman [13]. For the thymus however, the crude pooled homogenate of ten thymuses in four volumes of homogenization buffer was used as the enzyme source. Assays of time-dependent inhibition of ornithine decarboxylase and measurements of the kinetic constants of the inhibition were performed essentially as described previously [8].

*Assay of S-adenosyl-L-methionine decarboxylase* (in vitro). *S*-adenosyl-L-methionine decarboxylase (EC 4.1.1.50) was prepared as described by Pegg [14] from the livers of rats which had been injected with methyl-

glyoxal-bis-(guanyl hydrazone)2 HCl. The purification steps were steps 1 and 2 (105,000 *g* centrifugation and $[NH_4]_2 SO_4$ precipitation) used by this author. The enzymic activity was measured as described by Pegg and Williams-Ashman [15].

*Determination of radioactivity.* Radioactivity was assayed with a Packard Tri-Carb Liquid Scintillation Spectrometer-model 3390. The counting efficiency for $^{14}C$ was in the range 78–80 per cent. Suitable corrections were applied for background counts.

*Determination of the concentrations of putrescine and polyamines in rat organs.* Ventral prostate, testis and thymus were homogenized in 9 vol. 0.2 M perchloric acid. These homogenates were centrifuged at 3000 *g* in an Eppendorf Micro Centrifuge for two minutes and the supernatants were filtered through a Millipore membrane (0.22 $\mu$m). The filtrates were used for determination of the amounts of putrescine, spermidine and spermine with a Durrum D-500 (Durrum Instruments Corp.) amino acid analyzer using the method of Marton *et al.* [16] as modified by Bartos *et al.* [17].

## RESULTS AND DISCUSSION

As shown in Figure 1, maximum inhibition of ornithine decarboxylase activities occurred 1 to 4 hr after treatment in the three organs studied. Activities returned to control values in testis and thymus within 24 hr. The decrease in ornithine decarboxylase activity was significantly greater in prostate than in thymus and testis at all times. 24 hours after a single injection of 5-hexyne-1,4-diamine, prostatic ornithine decarboxylase activity was only 30 per cent of the control value. Figure 2 shows that the inhibition of ornithine decarboxylase activity was dose-dependent in the three organs and confirms the preferential inhibition of this enzyme in the prostate.

The kinetic constants for time-dependent inhibition of partially purified ornithine decarboxylases from prostate and testis were similar (See Table 1). Thymus ornithine decarboxylase is known to be membrane-bound [11], so that it was not surprising to find different kinetic constants for this enzyme. The organ-selective effect of 5-hexyne 1,4-diamine cannot be adequately explained by different susceptibilities of the enzymes to inhibition by this compound. Other factors are the pharmacokinetics of the drug, and the turnover of ornithine decarboxylase which is not necessarily the same in the three organs. Since it has been shown that ornithine can protect ornithine decarboxylase against the action of the inhibitor, the size of ornithine pools may also be important in this respect [8].

Injections of putrescine and diamine analogues are able to decrease ornithine decarboxylase activity induced in rat liver by a mechanism involving induction of a macromolecular inhibitor (or "antizyme") [18] and repression of ornithine decarboxylase synthesis [5]. In order to test if 5-hexyne-1,4-diamine, which is a putrescine analogue, might cause a decrease in ornithine decarboxylase activity by this mechanism in the three organs studied, putrescine was injected intraperitoneally at doses sufficient to repress the increase of ornithine decarboxylase activity in regenerating rat liver [5] or in ovaries of rats treated with human chorionic gonadotrophin [6]. This treatment had no signif-

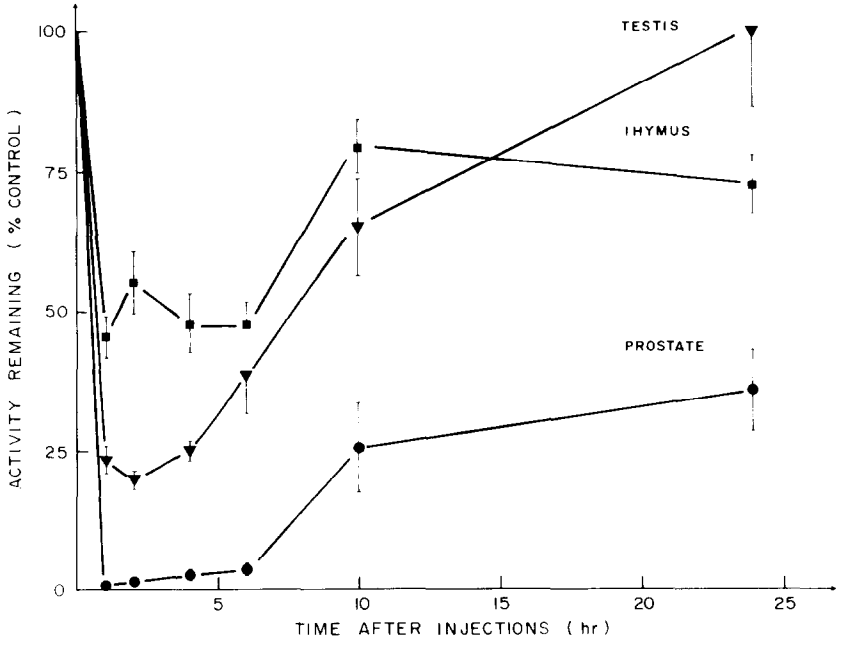


Fig. 1. Effect of a single dose of 5-hexyne-1,4-diamine on ventral prostate, testis and thymus ornithine decarboxylase activities. Rats were injected intraperitoneally at time 0 with 5-hexyne-1,4-diamine (100 mg/kg of body weight). At given intervals, the animals were killed and ornithine decarboxylase activities were measured as described in Materials and Methods in prostate (●), thymus (■) and testis(▼). The enzyme activities are expressed as per cent of control (nmol/hr/g of wet tissue). Each value is the mean ±S.E.M. of five animals. Ornithine decarboxylase activities of control animals are given in Table 2.

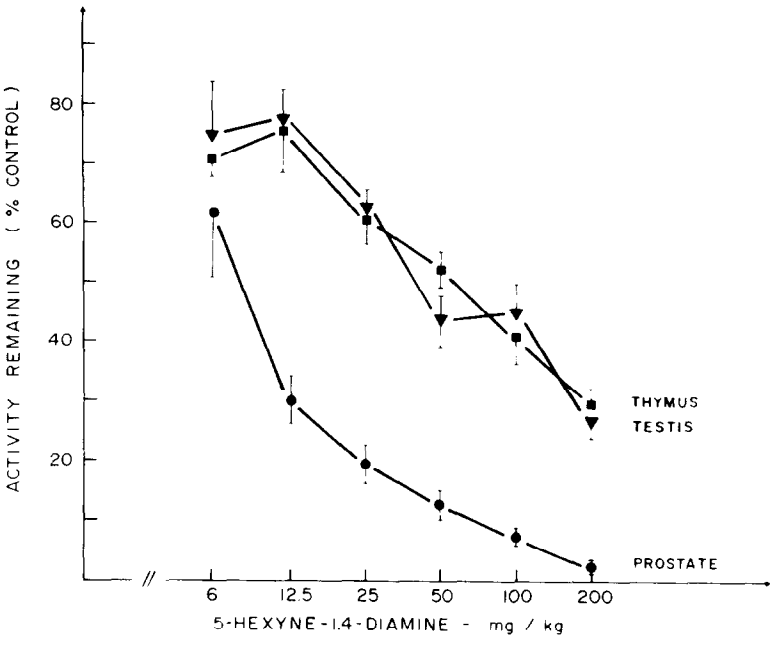


Fig. 2. Dose–effect relationship between a single dose of 5-hexyne-1,4-diamine and ventral prostate, testis and thymus ornithine decarboxylase activities. Rats were injected intraperitoneally with a single dose of 5-hexyne-1,4-diamine ranging from 6 to 200 mg/kg of body weight. Five hours after injection the animals were killed and ornithine decarboxylase activities were measured as described in Materials and Methods in prostate (●), thymus (■) and testis (▼). The enzyme activities are expressed as per cent of control (nmol/hr/g of wet tissue). Each value is the mean ± S.E.M. of five animals. Ornithine decarboxylase activities of control animals are given in Table 2.

Table 1. Kinetics constants of 5-hexyne-1, 4-diamine for ventral prostate, testis and thymus ornithine decarboxylase

| | Prostate | Testis | Thymus |
|---|---|---|---|
| $K_I$ μM | 0.35 ± 0.07 | 0.56 ± 0.09 | 2.6 ± 1.2 |
| $t_{1/2}$, min | 14.6 ± 0.9 | 12.7 ± 0.8 | 9.9 ± 1.7 |

$K_I$ (apparent dissociation constant), $t_{1/2}$ (half life of ornithine decarboxylase activity *in vitro* at infinite concentration of inhibitor) were calculated according to the method of Kitz and Wilson [26]. This method was applied to ornithine decarboxylase essentially as described previously [8].

icant effect on prostate and testis ornithine decarboxylase activities (Table 2). This suggests that the effect of 5-hexyne-1,4-diamine was probably due to a direct inhibition of ornithine decarboxylase activity in these two organs as expected from *in vitro* studies. In thymus, however, ornithine decarboxylase activity was decreased in a dose-dependent manner by putrescine in agreement with a previous finding of Kay and Lindsay [19] on stimulated lymphocytes in culture.

Since putrescine is an *in vitro* and *in vivo* activator of *S*-adenosyl methionine decarboxylase [15, 20] the putrescine-like effects of 5-hexyne-1,4-diamine on this enzyme were investigated. It was found that 5-hexyne-1,4-diamine is an activator of *S*-adenosyl-methionine decarboxylase similar to putrescine. At 2 mM concentration, it produces 70 per cent of the activation seen with the same concentration of putrescine (Fig. 3). A similar activation has been reported for trans-1,4-diaminobutene, a competitive inhibitor of ornithine decarboxylase *in vitro* [21].

Polyamine levels were measured after three 100 mg/kg doses of 5-hexyne-1,4-diamine, during a 24 hr period. Putrescine levels were significantly decreased in all three organs studied (Table 3). However, a signifi-

Table 2. Effects of a single does of putrescine on ventral prostate, testis and thymus ornithine decarboxylase activities

| | Controls | Putrescine 100 mg/kg | Putrescine 400 mg/kg |
|---|---|---|---|
| Prostate | 263 ± 45 | 364 ± 56 | 335 ± 68 |
| Testis | 35.3 ± 0.9 | 36.8 ± 1.6 | 37.3 ± 1.7 |
| Thymus | 63 ± 2 | 29.0 ± 1.8* | 21.6 ± 1.2* |

Rats were injected intraperitoneally with putrescine or saline (controls) 5 hr before death. The ornithine decarboxylase activities are expressed as nmol/hr/g of wet tissue (± S.E.M.). There were five animals in each group. The significance of the differences (as compared with the group receiving no putrescine) was calculated by Student's *t* test: *P $^{3}$M). Values shown are

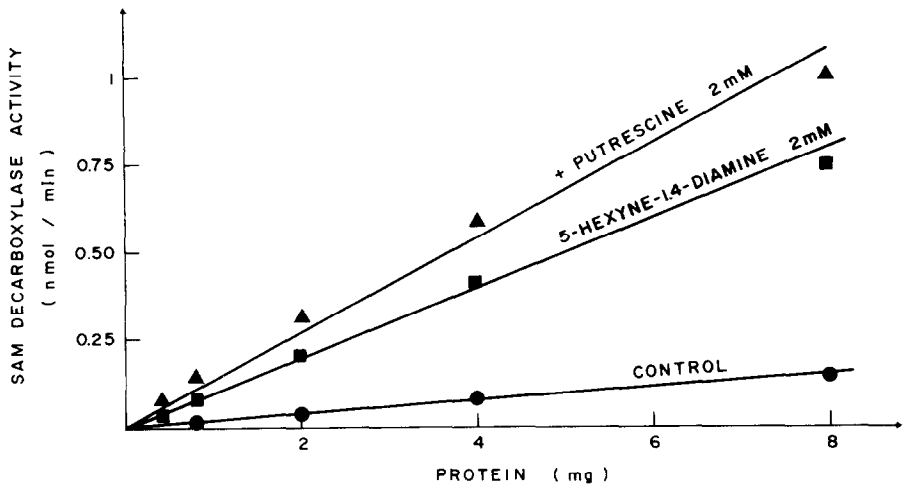


Fig. 3. *In vitro* activation of rat liver *S*-adenosyl methionine decarboxylase by 5-hexyne-1,4-diamine. The assay medium contained 100 μmol of sodium phosphate buffer pH 7.1, 0.2 μmol of *S* adenosyl L-[carboxy-$^{14}$C]methionine (0.1 μCi) and 2 μmol of either putrescine (▲) or 5-hexyne-1,4-diamine (■). The same medium without putrescine or 5-hexyne-1,4-diamine was used as control (●). Enzyme preparation and determination of its activity are described in Materials and Methods.

Table 3. Effects of three repeated doses of 5-hexyne-1, 4-diamine on polyamine levels of rat ventral prostate, testis and thymus

| | Putrescine | | Spermidine | | Spermine | |
|---|---|---|---|---|---|---|
| | C | T | C | T | C | T |
| Prostate | 406 ± 35 | 92 ± 32* | 8298 ± 486 | 4414 ± 775* | 3500 ± 177 | 3628 ± 299 |
| Testis | 25.5 ± 1.7 | 5.9 ± 3.2* | 305 ± 6 | 290 ± 12 | 585 ± 16 | 587 ± 25 |
| Thymus | 164 ± 8 | 82 ± 3* | 2496 ± 58 | 2665 ± 42 | 850 ± 18 | 760 ± 37 |

Rats were injected intraperitoneally with 5-hexyne-1,4-diamine 100 mg/kg of body weight (T), or saline (C) successively 30, 18 and 6 hr before death. Polyamine levels were determined as described in Materials and Methods and are expressed in nmol/g of wet tissue (± S.E.M.). There were five animals in each group. The significance of the differences (as compared with the group receiving no 5-hexyne-1,4-diamine) was calculated by Student's *t* test: *P < 0.005.

cant decrease of spermidine level was obtained only in the ventral prostate, once again confirming the organ-selective action of 5-hexyne-1,4-diamine. No effect on the weight of the three organs was found after this treatment.

Thus 5-hexyne-1,4-diamine, an enzyme-activated irreversible inhibitor of ornithine decarboxylase which is very potent *in vitro*, also inhibits this enzyme effectively *in vivo*. For the first time, a significant decrease of resting spermidine levels has been observed *in vivo* with an ornithine decarboxylase inhibitor. Since the half-life of spermidine and spermine in the rat seems long compared to the duration of our experiment [1, 22, 23], a more prolonged treatment with 5-hexyne-1,4-diamine could have resulted in a more marked decrease of the levels of these polyamines. However, a high single dose of this drug causes sedation, ataxia, lacrimation, piloerection, and repeated doses can produce a marked loss of body weight and ultimately death. In the accompanying paper [24], we show that these effects are most probably not due to the decrease of polyamine levels but to the formation of 4-aminohex-5-ynoic acid, a known enzyme-activated inhibitor of 4-aminobutyrate: 2-oxoglutarate aminotransferase (EC 2.6.1.19) of L-glutamate-1-carboxy-lyase (EC 4.1.1.15) [25].

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