# INHIBITION OF ORNITHINE DECARBOXYLASE BY DIAMINES IN REGENERATING RAT LIVER

## Evidence for direct action on the accumulation of the enzyme protein

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## 1. Introduction

The regulation of mammalian ornithine decarboxylase (EC 4.1.1.17) in vivo by natural polyamines (putrescine, spermidine and spermine) as well as by structurally related but unphysiologic amines (1,3-diaminopropane) appears to involve several distinctly different mechanisms [1–5]. The prevention of ornithine decarboxylase induction by putrescine and diaminopropane [2,6] in rat liver immediately after partial hepatectomy resembles the action achieved with inhibitors of RNA synthesis [6] whereas, during later periods of liver regeneration, the administration of the diamines results in an extremely swift decay of ornithine decarboxylase activity, suggesting a direct translational inhibition [2,5,6].

Another mechanism, fundamentally different from the concept of 'classic repression', has been recently advanced by E. S. Canellakis et al. [7,8]. According to their observations, it appears that the administration of amines in vivo [8] or in vitro in cell cultures [7] results in an appearance of macromolecular inhibitors of ornithine decarboxylase. The formation of such nondialyzable inhibitors after application of putrescine in H-35 hepatoma cells has been confirmed by Clark and Fuller [9]. However, they were unable to detect similar inhibitors in normal or transformed Swiss 3T3 cells or cultured human fibroblasts after exposure of the cells to putrescine [9]. We have likewise been unable to obtain any convincing evidence that the swift decay of ornithine decarboxylase in regenerating rat liver after injection of diamines would be based upon a formation of macromolecular inhibitors [6]. Extending this approach, we have further explored the mechanism of action of diamines on ornithine decarboxylase in regenerating rat liver. A single injection of putrescine or diaminopropane resulted in a marked inhibition (50–75%) of ornithine decarboxylase activity already 20 min after the injection with little evidence for either dialyzable or nondialyzable inhibitors. The rapid decrease in ornithine decarboxylase activity after the diamines was, however, accompanied by similar decrease in the amount of immunoreactive enzyme protein, as titrated with the aid of specific antibody to rat liver ornithine decarboxylase. Although longer periods of treatment with diaminopropane (resulting in complete disappearance of ornithine decarboxylase activity), but not with putrescine, appeared to give rise to the formation of nondialyzable inhibitors of the enzyme, we would like to suggest that the primary effect exerted by the amines on ornithine decarboxylase in regenerating rat liver is based upon a direct inhibition of the enzyme synthesis.

## 2. Materials and methods

### 2.1. *Animals*

Sprague-Dawley male rats, 95–110 g, were used in all experiments. Partial hepatectomy was performed under light ether anaesthesia by the method of Higgins and Anderson [10].

2.2. *Chemicals*

D,L-[1-$^{14}$C]Ornithine (specific radioactivity 59 mCi/mmol) was bought from the Radiochemical Centre (Amersham). Cycloheximide (Acti-Dione) was purchased from Nutritional Biochemicals (Cleveland) and 1,3-diaminopropane from Fluka AG (Buchs, SG). Putrescine was product of Calbiochem (San Diego).

2.3. *Analytical methods*

Ornithine decarboxylase activity was assayed by the method of Jänne and Williams-Ashman [11]. One unit of ornithine decarboxylase is defined as the amount of enzyme catalyzing the formation of 1 pmol $CO_2$/30 min. Protein was measured by the method of Lowry et al. [12] using bovine serum albumin as the standard.

2.4. *Partial purification of ornithine decarboxylase and immunization of rabbits*

Ornithine decarboxylase was purified partially from livers of thioacetamide-treated rats essentially as described by Hölttä [13]. Enzyme preparations with specific activity of 400–800 nmol/mg protein/30 min (representing 250–500-fold purification as compared with the enzyme activity in thioacetamide-stimulated liver) were used for the immunizations. Antiserum to rat liver ornithine decarboxylase was produced in female rabbits by injection of the enzyme preparation (0.66 mg protein) in complete Freund's adjuvant. The primary injection was followed by 3 booster injections (0.23 mg or 0.06 mg protein) every 2 weeks without adjuvant. The rabbits were bled 3 months after the first immunization. Control rabbits received Freund's adjuvant only.

On immunodiffusion the antiserum showed two precipitation lines when tested against cytosol fraction from regenerating rat liver. To obtain a monospecific antibody the antiserum was absorbed with liver extracts (1 vol. extract, 4 vol. antiserum) obtained from rats injected with cycloheximide (0.8 mg/100 g body wt) 60 min and 30 min before killing. The latter treatment was sufficient to cause a complete disappearance of ornithine decarboxylase activity in normal rat liver. Absorption was carried out at 4°C for 12 h and any precipitate was removed by centrifugation at 105 000 × *g* for 30 min. After the absorption only one weak precipitation line was seen on immunodiffusion.

2.5. *Immunotitration of ornithine decarboxylase*

The amount of immunoreactive ornithine decarboxylase was titrated with constant amount of absorbed antiserum by adding increasing amounts of the enzyme. Antiserum and dialyzed liver extracts were allowed to stand for 3 h at 4°C whereafter the residual ornithine decarboxylase activity was measured under standard incubation conditions.

## 3. Results

A single injection of 1,3-diaminopropane (100 μmol/100 g body wt) 20 min before the death of the rats resulted in more than 75% inhibition of ornithine decarboxylase activity, while the inhibition produced by putrescine (100 μmol/100 g) and cycloheximide (0.8 mg/100 g) was somewhat less (about 50–35%) (table 1). Three subsequent injections of diaminopropane every 2 h caused complete disappearance of ornithine decarboxylase activity (table 1). Similar treatment of the rats with putrescine likewise inhibited the enzyme activity by more than 80% (table 1).

In order to find out whether the observed diamine inhibition was due to some dialyzable or macromolecular inhibitors of ornithine decarboxylase, various combination experiments were carried out. Mixing of undialyzed extracts from regenerating liver (untreated animals) with similar extracts obtained from unoperated animals, or from rats receiving diamines, revealed that only in case of subchronic diaminopropane treatment the combination of this extract with that of normal regenerating liver resulted in a substantial inhibition of ornithine decarboxylase activity in the latter extract (table 1). The slight and varying inhibition, caused by other combinations (including extracts from unoperated animals) usually disappeared or markedly diminished after dialysis against 25 mM Tris–HCl buffer (pH 7.1) containing 0.1 mM EDTA, 5 mM dithiothreitol, 50 mM KCl and 0.05 mM pyridoxal-5′-phosphate [7–9] (table 1). The only exception was the inhibition produced by liver extracts obtained from rats treated with diaminopropane for 6 h. This inhibition only slightly diminished after dialysis (table 1). It should be emphasized, however, that no such inhibition

Table 1
Effect of diamines and cycloheximide on ornithine decarboxylase activity in regenerating rat liver

| Source of liver extracts | | | Undialyzed | | | Dialyzed | | |
|---|---|---|---|---|---|---|---|---|
| | | | Activity observed | Expected | Change (± %) | Activity observed | Expected | Change (± %) |
| Unoperated | | (A) | 114 | – | | 114 | – | |
| Partially hepatectomized | | (B) | 1380 | – | | 1079 | – | |
| + diaminopropane | (20 min) | (C) | 304 | – | | 289 | – | |
| + putrescine | (20 min) | (D) | 585 | – | | 533 | – | |
| + cycloheximide | (20 min) | (E) | 632 | – | | 533 | – | |
| + diaminopropane | ( 6 h) | (F) | 0 | – | | 15 | – | |
| + putrescine | ( 6 h) | (G) | 204 | – | | 252 | – | |
| B + A | | | 1232 | 1494 | (–11.5%) | 1155 | 1193 | (– 3.2%) |
| B + B | | | 2492 | 2760 | (–10.7%) | 2161 | 2158 | (+ 0.1%) |
| B + C | | | 1469 | 1684 | (–12.8%) | 1376 | 1368 | (+ 0.6%) |
| B + D | | | 1804 | 1965 | (– 8.2%) | 1661 | 1612 | (+ 3.0%) |
| B + E | | | 1851 | 2012 | (– 8.1%) | 1698 | 1612 | (+ 5.3%) |
| B + F | | | 623 | 1380 | (–54.9%) | 747 | 1094 | (–41.8%) |
| B + G | | | 1377 | 1584 | (–13.1%) | 1356 | 1331 | (+ 1.9%) |

The rats were partially hepatectomized 29 h before death and received 100 μmol 1,3-diaminopropane (C) or putrescine (D) or 0.8 mg cycloheximide (E)/100 g body wt 20 min before death. Two groups of rats received injections of either diaminopropane (F) or putrescine (G) starting at 6 h and repeated at 4 h and 2 h before death. The various liver extracts were assayed for ornithine decarboxylase before dialysis or after overnight dialysis against 350 vol 25 mM Tris–HCl buffer (pH 7.1) containing 50 mM KCl, 5 mM dithiothreitol, 0.1 mM EDTA and 0.05 mM pyridoxal-5'-phosphate. Undialyzed and dialyzed cytosol fraction obtained from partially hepatectomized rats receiving no treatment (B) were combined with equal amounts of cytosol obtained from unoperated animals (A) and from rats treated with diamines or cycloheximide (C–G). Ornithine decarboxylase activity is expressed as pmol $CO_2$/75 μl liver cytosol. Livers from 3 rats were pooled for each group.

arised when the liver extracts from rats treated with putrescine for 6 h (still containing some ornithine decarboxylase activity) were combined with the extracts from regenerating rat liver from untreated animals (table 1). Even the inhibition resulted from longer diaminopropane treatment was not consistent, since some other experiments revealed little evidence for macromolecular inhibitors after prolonged exposure of the rats to the amine (results not shown).

It thus appeared that the rapid decay of ornithine decarboxylase activity after the injection of putrescine or diaminopropane was due neither to dialyzable nor to macromolecular inhibitors formed in response to the diamine administration.

We next injected rats with diaminopropane, putrescine or cycloheximide and killed the animals 20 min after the injection. The inhibition of ornithine decarboxylase activity in this particular experiment was about 70% after diaminopropane, 55% after putrescine and 50% after cycloheximide (results not shown). The liver extracts were then extensively dialyzed against 25 mM Tris–HCl buffer (pH 7.1) containing 5 mM dithiothreitol and 0.1 mM EDTA and all the extracts were diluted with the buffer to obtain approx. equal ornithine decarboxylase activities per ml. Increasing amounts of the liver extracts were then added to constant amount of antiserum to ornithine decarboxylase. Figure 1 illustrates that the inhibition of ornithine decarboxylase activity by the antiserum was constant, irrespective of the source of the extracts. The increase in the residual activity, upon increasing the amount of the enzyme, occurred in a similar way in every preparation. This indicates that the decrease in the enzyme activity following the injection of the diamines or cycloheximide was accompanied by a similar decrease in the amount of immunoreactive ornithine decarboxylase. However, the possibility that the combination of the macromolecular inhibitor with ornithine decarboxylase prevented the recognition of the enzyme by the

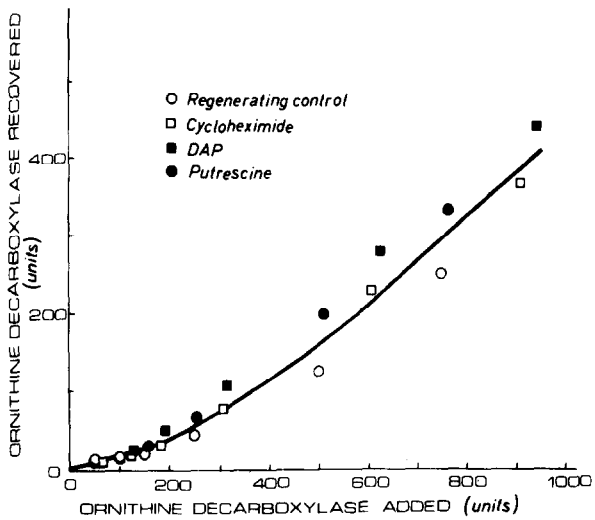


Fig.1. Immunotitration of ornithine decarboxylase with anti-ornithine decarboxylase serum. Increasing amounts of dialyzed liver extracts obtained from rats partially hepatectomized 24 h earlier and receiving no treatment (regenerating control) or cycloheximide (0.8 mg/100 g), diaminopropane (DAP; 100 μmol/100 g) or putrescine (100 μmol/100 g) 20 min before killing were added to constant amounts (8.8 mg protein) of absorbed anti-ornithine decarboxylase serum. The mixtures of the liver extracts and antiserum were allowed to stand for 3 h whereafter residual ornithine decarboxylase activity was measured.

antiserum still remained, and could, in fact, explain the apparent decrease in the amount of immunoreactive protein. To investigate this possibility we treated partially hepatectomized rats with diaminopropane for 6 h resulting in a complete disappearance of ornithine decarboxylase activity with concomitant appearance of non-dialyzable inhibitors. When the cytosol fraction from diaminopropane treated animals was mixed with equal volume of extracts obtained from partially hepatectomized rats without any amine treatment, a marked inhibition (more than 50%) of ornithine decarboxylase activity in the latter extract was found.

We then titrated the amount of immunoreactive ornithine decarboxylase with the antibody in liver extracts of partially hepatectomized rats in the absence or presence of similar extracts from diaminopropane-treated rats containing nondialyzable inhibitors of the enzyme. As shown in table 2, the marked inhibition of ornithine decarboxylase activity of regenerating rat liver brought about the combination of the cytosol fraction from diaminopropane-treated animals apparently was not associated with a similar decrease in the amount of immunoreactive protein. This is clearly shown by the fact that the inhibition of ornithine decarboxylase activity by the

Table 2
Immunotitration of ornithine decarboxylase activity from regenerating rat liver before and after combination with inhibitory cytosol fraction obtained from partially hepatectomized rats treated with diaminopropane

| A. Ornithine decarboxylase activity of regenerating liver cytosol (units) | | B. Ornithine decarboxylase activity after combination with inhibitory cytosol (units) | |
|---|---|---|---|
| Added | Recovered | Added | Recovered |
| 58 | 18 | 27 | 19 |
| 117 | 22 | 54 | 43 |
| 293 | 95 | 134 | 88 |
| 585 | 224 | 269 | 191 |
| 878 | 365 | 403 | 288 |

Rats were partially hepatectomized 31 h before killing and received no treatment, or were injected with diaminopropane (100 μmol/100 g) every 2 h starting 6 h before death. Cytosol fractions were dialyzed overnight and ornithine decarboxylase from partially hepatectomized rats without any amine treatment (A) was titrated in the presence of constant amount of antiserum as described in the legend to fig.1. Dialyzed cytosol fractions from untreated animals and those receiving diaminopropane were combined (equal vols) (B) and ornithine decarboxylase of the combination was titrated with the antiserum. Pooled cytosol fractions were obtained from 3 animals in each group.

antiserum was less pronounced in the combined extracts (containing the inhibitor) than in liver cytosol fraction from partially hepatectomized rats without any treatment. The latter phenomenon simply means that, at least under these experimental conditions, the inhibition of ornithine decarboxylase by non-dialyzable inhibitors does apparently not result in a decrease of immunoreactive protein.

## 4. Discussion

The present result support the view that diamines, such as putrescine and diaminopropane, regulate ornithine decarboxylase activity in regenerating rat liver by directly influencing the rate of enzyme protein synthesis, most likely at some post-transcriptional level. It is thus unlikely that any protein inhibitors ('antizymes' [7,8]) are primarily involved in the acute inhibition of ornithine decarboxylase. This concept is supported by the following experimental findings:

(i) Combination experiments (table 1) revealed no additional inhibition upon mixing liver extracts from partially hepatectomized rats with those obtained from rats receiving a single injection of diamines.

(ii) The decrease in the amount of immunoreactive protein after amine administration (fig.1) in a similar way as after inhibitors of protein synthesis [13] strongly suggest that the effect of amines involve changes in the synthesis of enzyme protein. In agreement with the present results, Z. N. Canellakis and Theoharides [14] found that the stimulation of ornithine decarboxylase by cyclic AMP in rat hepatoma cells could be abolished by spermine which also prevented the enhanced incorporation of radioactivity from [$^{14}$C]leucine to immunoprecipitable ornithine decarboxylase.

(iii) Finally, diaminopropane does not alter the decay rate of ornithine decarboxylase after cycloheximide [6].

We have been able to partially confirm the induction of non-dialyzable inhibitors for ornithine decarboxylase in response to diamine administration in regenerating rat liver. However, this only occurred after long diaminopropane treatment while similar treatment with putrescine did not reveal signs of such inhibitors. Even in case of diaminopropane the appearance of the inhibitor was not consistent in all experiments and seemed to require a complete disappearance of ornithine decarboxylase activity. It thus appears to us that the inhibition of ornithine decarboxylase in vivo by a variety of amines primarily involves an interference with the synthesis of the enzyme protein and might later result in a secondary appearance of macromolecular inhibitors of the enzyme, the physiologic significance of which remains to be established.

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