Acta Chemica Scandinavica B 32 (1978) 759—764

# Evidence for Several Mechanisms Involved in the Regulation of Ornithine Decarboxylase by Diamines during Rat Liver Regeneration

A. KALLIO

Department of Biochemistry, University of Helsinki, SF-00170 Helsinki 17, Finland

The regulation of ornithine decarboxylase activity in regenerating rat liver by various amines appears to require at least two amino groups since only diamines, or their derivatives, inhibited the enzyme activity *in vivo* while all monoamines tested were largely ineffective.

During very early periods of rat liver regeneration a single injection of 1,3-diaminopropane, a known potent inhibitor of ornithine decarboxylase *in vivo*, produced a profound depression of ornithine decarboxylase activity which was accompanied by a similar decrease in the apparent amount of enzyme protein as titrated with the aid of antiserum to rat liver ornithine decarboxylase. In striking contrast to early regeneration, injection of diaminopropane during later periods of liver regeneration, while equally effectively depressing the enzyme activity, did not decrease the amount of immunoreactive enzyme protein, but markedly increased it. In support of the view that the mechanism of action of diamines is different during early and late phases of liver regeneration was the finding that putrescine and diaminopropane produced a longer lasting inhibition of ornithine decarboxylase activity immediately after partial hepatectomy rather than 24 hours later.

The present results are in accordance with the idea that diamines have a direct action (transcriptional/translational?) on ornithine decarboxylase during the supposed gene activation of the enzyme while an induction of macromolecular inhibitors or antizymes to ornithine decarboxylase is a distinct feature of later periods of regeneration when the apparent gene activation has been completed.

Partial hepatectomy of the rat causes a dramatic stimulation of the activity of ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17.) (ODC), which is the rate-controlling enzyme in the synthesis of polyamines.[1–5] This rapid stimulation of the enzyme activity, reaching its first peak already at 4 h postoperatively,[4] and evidently requiring the synthesis of new enzyme protein and/or new mRNA for the enzyme,[5–9] can be prevented by natural polyamines (putrescine and spermidine)[10,11] or by 1,3-diaminopropane,[12] if administered at the time of the operation or shortly thereafter. These amines decrease ODC activity extremely rapidly also during later phases of liver regeneration possibly *via* a different mechanism than that operating immediately after partial hepatectomy.[9,10] Although the exact mechanism of the amine action is not known, it has been proposed that diamines may control the activity of ODC by directly influencing the rate of synthesis of the enzyme protein,[5,10,11,13] possibly through a repression type inhibition. It is noteworthy that the inhibition exerted by the amines is an indirect one since ODC activity is not inhibited even in the presence of high concentrations of the amines *in vitro* under normal assay conditions.[10,14,15]

A novel, fundamentally different mechanism of diamines to regulate ODC activity has been proposed by Canellakis *et al.*[16,17] They found that putrescine, the end product of the reaction catalyzed by ODC, induces the synthesis of a new kind of protein, that combines with and inhibits the action of the enzyme. The formation of this inhibitory protein (termed as ODC antizyme [16]) in response to administration of various diamines, has been confirmed by several investigators in a variety of cultured cell lines

as well as in rat liver.[9,18,19] This antizyme is reported to inhibit ODC activity by forming a tight, yet reversible, complex with the enzyme abolishing the catalytic activity.[16–18,20]

In the present paper I have tested various amines as regards their specificity to inhibit ODC activity *in vivo*. 1,3-Diaminopropane, which is one of the most potent inhibitors of the enzyme,[14,21] has been used as a model compound for experiments aimed to elucidate the mechanisms of amine action on ODC. It appears that several distinct mechanisms, including the formation of macromolecular inhibitors, are involved in the regulation of ODC activity in regenerating rat liver.

## MATERIALS AND METHODS

*Chemicals.* DL-[1-$^{14}$C]Ornithine (specific radioactivity 59 mCi/mmol) was purchased from the Radiochemical Centre (Amersham, Bucks., U.K.) and (carboxyl-$^{14}$C)*S*-adenosyl-L-methionine (specific radioactivity/60 mCi/mmol) from New England Nuclear Corp. (Dreieichenhain, West-Germany). Unlabelled *S*-adenosyl-L-methionine was synthesized by the method originally described by Cantoni *et al.*[22] and modified by Pegg and Williams-Ashman.[23] Cycloheximide (3-[2-(3,5-dimethyl-2-oxocyclohexyl)-2-hydroxyethyl]glutarimide (Acti-Dione) and 2,4-diaminobutyric acid were obtained from the Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.). Monoamines, methylenediamine, 1,2-diaminopropane, 1,3-diaminopropane, 1,7-diaminoheptane, 1,3-diamino-2-propanol, 2,3-diaminopropionic acid and 1,3-phenylenediamine were products of Fluka AG (Buchs SG, Switzerland) and putrescine was obtained from Calbiochem (San Diego, Calif., U.S.A.). 1,4-Diaminobutanone was purchased from Aldrich Chemicals (Milwaukee, Wis., U.S.A.) and 1,2-diaminoethane and 1,6-diaminohexane were from BDH Laboratory Chemicals (Poole, England). All the other chemicals were of analytical grade. Amines were neutralized before use. The purity of various amines was checked by paper electrophoresis.

*Partial hepatectomy.* Male rats of the Spraque-Dawley strain weighing 90 – 310 g (90 – 310 g in Table 1; 95 – 145 g in Table 2; 140 – 160 g in Fig. 1; 110 – 180 g in Table 3) were used. Partial hepatectomy was performed under light ether anaesthesia by the method of Higgins and Anderson [24] and all compounds were injected intraperitoneally to the animals.

*Preparation of liver extracts.* After decapitation of the rats, the livers were removed and immediately homogenized with 2 vol. of cold 25 mM Tris-HCl buffer, pH 7.4, containing 0.1 mM EDTA and 1 mM dithiotreitol. The homogenates were centrifuged at 105 000 $g_{max}$ for 30 min at 3 °C. The enzyme activities were assayed as quickly as possible using undialyzed supernatant fractions as the source of enzymes.

*Preparation of anti-ornithine decarboxylase serum.* Ornithine decarboxylase was partially purified (about 500-fold) from livers of thioacetamide treated rats and used to immunize rabbits as described earlier.[25]

*Analytical methods.* The activity of ODC was measured by the method of Jänne and Williams-Ashman [26] and that of *S*-adenosylmethionine decarboxylase as described by Jänne and Williams-Ashman.[27] The enzyme activities are expressed as (pmol of product formed)/(mg protein)(30 min). The relative amount of immunoreactive ODC was titrated with the antiserum by the method of Obenrader and Prouty.[28] In this method the amount of antiserum required for 50 % inhibition of enzyme activity ($Ab_{50}$) was determined. An increasing amount of antiserum was added to a constant amount of the enzyme and the $Ab_{50}$ value was determined graphically by the least squares method. Protein was measured by the method of Lowry *et al.*[29]

## RESULTS

*Effect of various amines on the activity and amount of immunoreactive protein of ornithine decarboxylase in 24 h regenerating liver.* A single intraperitoneal injection of various diamines (100 μmol/100 g body wt.), or their derivatives, into partially hepatectomized rats (operated 24 h earlier) 1 h before killing invariably resulted in a highly significant inhibition of ODC activity (Table 1). In contrast, a similar injection of various monoamines, with the possible exception of ethylamine, did not show any inhibitory effect on ODC activity, although variations between animals were quite large (Table 1). The extent of the inhibition by amines was uninfluenced by a overnight dialysis prior to the assay of the enzyme activity thus ruling out a direct action of the amines on the enzyme.

In order to study the effect of amines on the amount of ODC protein, partially hepatectomized rats were treated with 1,3-diaminopropane (DAP) (50 μmol/100 g), cycloheximide (0.8 mg/100 g) or butylamine (50 μmol/100 g) 30 min before killing the animals. As illustrated in Table 2, a single injection of DAP caused a rapid accumulation of catalytically inactive but immunologically reactive ODC in regener-

*Table 1.* Effect of various amines on the activity of ODC in regenerating rat liver.

| Treatment | Number of animals | ODC activity % of controls S.E.M. |
|---|---|---|
| None | (8) | 100 |
| Methylenediamine | (5) | 51 ± 12**[a] |
| 1,2-Diaminoethane | (5) | 12 ± 3*** |
| 1,3-Diaminopropane | (8) | 5 ± 2*** |
| 1,2-Diaminopropane | (5) | 22 ± 6*** |
| Putrescine (1,4-diaminobutane) | (3) | 3 ± 0.3*** |
| 1,6-Diaminohexane | (2) | 24 (15 – 34) |
| 1,7-Diaminoheptane | (2) | 24 (11 – 41) |
| 1,3-Diamino-2-propanol | (5) | 2 ± 0.5*** |
| 2,3-Diaminopropionic acid | (2) | 31 (15 – 46) |
| 1,4-Diaminobutanone | (2) | 40 (33 – 48) |
| 2,4-Diaminobutyric acid | (2) | 30 (20 – 40) |
| 1,3-Phenylenediamine | (2) | 32 (14 – 50) |
| Ethylamine | (5) | 64 ± 8*** |
| Propylamine | (7) | 87 ± 20 |
| Butylamine | (5) | 73 ± 15 |
| Pentylamine | (5) | 91 ± 16 |
| Ethanolamine | (2) | 111 (99 – 123) |

[a] The significance of the differences was: **=$p<0.01$, ***=$p<0.001$, as compared with the animals receiving no treatment.

*Table 2.* Effect of 1,3-diaminopropane, butylamine and cycloheximide on the activity and amount of immunoreactive ODC in regenerating liver.

| Treatment | Number of animals in pooled sample | ODC activity pmol/mg protein | $Ab_{50}$/ mg protein |
|---|---|---|---|
| None | 3 | 1049 | 6.4 |
| Diaminopropane | 3 | 130 | 8.4 |
| Butylamine | 3 | 143 | 4.0 |
| Cycloheximide | 3 | 142 | 3.3 |

ating liver. Similarly, 1,3-diamino-2-propanol, a derivative of 1,3-diaminopropane, produced a very rapid, approximately 3-fold, increase in the apparent amount of ODC antigen ($Ab_{50}$) value) in regenerating liver, which was associated with a disappearance of the enzyme activity (results not shown). This increase in the immunoreactive protein in the absence of enzyme activity may be due to the formation of ODC antizyme-enzyme complex, which could stabilize the enzyme against intracellular degradation.[30] In this particular experiment, where butylamine showed an unusually strong inhibition of ODC activity, its action on the amount of immunoreactive ODC resembled the effect of cycloheximide, an inhibitor of eukaryotic protein synthesis, which is known to produce a swift decay of ODC activity [7] accompanied by an only slightly slower decrease in the amount of immunoreactive ODC protein.[6,26] In thus seems likely that the inhibition of ODC activity by monoamines, if any, operates through different mechanisms than the inhibition by diamines.

*Different effects of diamines on the amount and activity of ODC in 4-h and 24-h regenerating rat liver.* The stimulation of ODC activity 4 h after partial hepatectomy could be largely prevented, as reported earlier,[10,12] by an injection of DAP or putrescine (75 μmol/100 g body wt.) given at the time of operation (Fig. 1). When these compounds were ad-

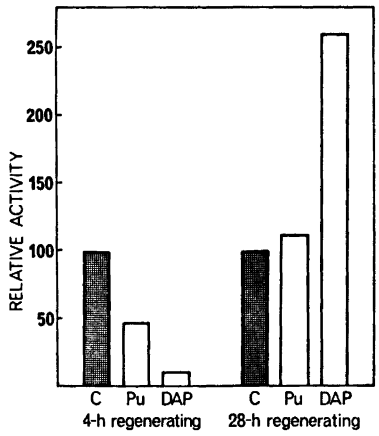


*Fig. 1.* Effect of putrescine and 1,3-diaminopropane on the activity of ODC in 4-h and 28-h regenerating rat liver. There were three to four animals in each group. C, regenerating control; Pu, putrescine; DAP, 1,3-diaminopropane.

ministrated in equivalent doses to rats, partially hepatectomized 24 h earlier, both showed a stimulatory rather than inhibitory effect on ODC activity measured 4 h later (Fig. 1.) In this experiment the activity of *S*-adenosylmethionine decarboxylase (EC 4.1.1.50), used as a reference enzyme of short half-life, was practically unaffected by the treatments (results not shown).

To further investigate the effect of diamines on ODC activity at different stages of liver regeneration, rats, operated 4 or 24 h earlier were treated with DAP (40 μmol/100 g body wt.) 30 min, 1 h or 4 h before sacrifice. During the first 4 h of liver regeneration DAP injections diminished both the activity and the apparent amount of the enzyme irrespective of the time of administration (Table 3). In 24-h regenerating liver the effect exerted by DAP was stringently dependent on the time of the injection. DAP given 4 h before killing the animals tended to increase ODC activity, as expected,[9] and it likewise increased slightly the amount of immunoreactive protein. An injection of DAP one h or 30 min before death again diminished the enzyme activity, but at the same time produced a distinct increase in the apparent amount of the antigen thus clearly differing from the effects found during the first 4 h of liver regeneration.

These experiments can be taken as evidence indicating that diamines regulate ODC activity by several mechanisms depending on the stage of liver regeneration.

## DISCUSSION

The intense stimulation of ODC in response to partial hepatectomy of the rat offers a convenient model for studies on the regulation

*Table 3.* Effect of 1,3-diaminopropane on the activity and amount of immunoreactive protein of ODC in 4-h and 24-h regenerating rat liver. The animals, partially hepatectomized 4 h or 24 h earlier, received an injection of diaminopropane (40 μmol/100 g body wt.) at the time indicated. The activity and the amount of immunoreactive protein ($Ab_{50}$) was measured as described in the text. There were three animals in each group.

| Treatment | Time of injection (prior to death) | ODC activity pmol/mg protein | $Ab_{50}$ mg protein |
|---|---|---|---|
| *Exp. 1* | | | |
| 4-h regenerating liver | – | 1644 | 11.3 |
| 4-h regenerating liver + diaminopropane | 4 h | 590 | 5.4 |
| 4-h regenerating liver + diaminopropane | 30 min | 368 | 5.8 |
| 24-h regenerating liver | – | 336 | 4.9 |
| 24-h regenerating liver + diaminopropane | 4 h | 418 | 5.4 |
| 24-h regenerating liver + diaminopropane | 30 min | 77 | 9.3 |
| *Exp. 2* | | | |
| 4-h regenerating liver | – | 460 | 7.0 |
| 4-h regenerating liver + diaminopropane | 1 h | 59 | 3.7 |
| 24-h regenerating liver | – | 312 | 4.9 |
| 24-h regenerating liver + diaminopropane | 1 h | 17 | 9.5 |

of this rapidly vanishing enzyme. The present results suggest that the extensively documented indirect inhibition of ODC by various diamines [10,11,13–15,31] may involve effects at different levels of gene expression. This is supported by the following findings: During the time of ODC gene activation, which is supposed to take place within a few hours after partial hepatectomy,[9,21] the inhibition by DAP, given at the time of operation or shortly thereafter, resulted in similar decreases both in the activity as well as in the relative amount of immunoreactive ODC. This is consistent with the concept that during the first hours of liver regeneration diamines might regulate the synthesis of ODC at the level of transcription by preventing the synthesis of new mRNA for ODC. Later, *i.e.* at the time when activation of the ODC message has been completed, diamine injections, while effectively inhibiting ODC activity, rapidly gave rise to an accumulation of catalytically inactive ODC. This accumulation of immunoreactive ODC, which apparently is based on the formation of bound macromolecular inhibitors to ODC, supports the idea that a reversible combination of these inhibitory proteins is also involved in the regulation of ODC activity at the later stages of liver regeneration.

We have reported earlier [25] that DAP and putrescine caused a decrease in the amount of immunoreactive ODC also during later periods of regeneration if these compounds were administrated 20 min prior to the death of the animals. This apparent discrepancy to the results presented in this paper may be due to the fact that a certain short lag period is required before the formation of ODC antizyme begins. Thus the initial decrease in the enzyme activity could be based on mechanisms not involving the formation of antizymes. However, the $Ab_{50}$-method to measure the amount of ODC protein used in the present experiments is more quantitative than the straightforward immunotitration used earlier.[25]

The antizyme mechanism, the physiological importance of which still remains open, might operate *in vivo* in response to alterations of intracellular putrescine concentrations, as suggested by Jefferson and Pegg.[19] In any case, this regulatory mechanism appears to be specific for diamines, physiological or non-physiological, since monoamines even when inhibitory to ornithine decarboxylase, did apparently not induce the formation of this kind of bound inhibitors as revealed by the fact that an enhanced accumulation of immunoreactive enzyme protein was not associated with the inhibition of enzyme activity by monoamines.

*Acknowledgements.* The skilfull technical assistance of Miss Merja Kärkkäinen and Mrs. Riitta Sinervirta is gratefully acknowledged. The author wishes to thank Prof. J. Jänne for his help during the work and in the preparation of the manuscript. This work received financial support from the Emil Aaltonen Foundation (Tampere, Finland).

## REFERENCES

1. Russell, D. H. and Snyder, S. H. *Proc. Natl. Acad. Sci. U.S.A. 60* (1968) 1420.
2. Raina, A. and Jänne J. *Acta Chem. Scand. 22* (1968) 2375.
3. Pegg, A. E. and Williams-Ashman, H. G. *Biochem. J. 108* (1968) 533.
4. Hölttä, E. and Jänne, J. *FEBS Lett. 23* (1972) 117.
5. Morris, D. R. and Fillingame, R. H. *Annu. Rev. Biochem. 43* (1974) 303.
6. Hölttä, E. *Biochim. Biophys. Acta 399* (1975) 420.
7. Russell, D. H. and Snyder, S. H. *Mol. Pharmacol. 5* (1969) 253.
8. Fausto, N. *Biochim. Biophys. Acta 238* (1971) 116.
9. Kallio, A., Pösö, H., Scalabrino, G. and Jänne, J. *FEBS Lett. 73* (1977) 229.
10. Jänne, J. and Hölttä, E. *Biochem. Biophys. Res. Commun. 61* (1974) 449.
11. Schrock, T. R., Oakman, N. J. and Bucher, N. L. R. *Biochim. Biophys. Acta 204* (1970) 564.
12. Pösö, H., Kallio, A., Scalabrino, G. and Jänne J. *Biochim. Biophys. Acta 497* (1977) 288.
13. Pett, D. M. and Ginsberg, H. S. *Fed. Proc. Fed. Am. Soc. Exp. Biol. 27* (1968) 615.
14. Pösö, H. and Jänne, J. *Biochem. Biophys. Res. Commun. 69* (1976) 885.
15. Kay, J. E. and Lindsay, V. J. *Biochem. J. 132* (1973) 791.
16. Heller, J. S., Fong, W. F. and Canellakis, E. S. *Proc. Natl. Acad. Sci. U.S.A. 73* (1976) 1858.
17. Fong, W. F., Heller, J. S. and Canellakis, E. S. *Biochim. Biophys. Acta 428* (1976) 456.
18. McCann, P. P., Tardif, C. and Mamount, P. S. *Biochem. Biophys. Res. Commun. 75* (1977) 948.
19. Jefferson, L. S. and Pegg, A. E. *Biochim. Biophys. Acta 484* (1977) 177.

20. Heller, J. S., Chen, K. Y., Fong, W. F., Kyradies, D. and Canellakis, E. S. *Fed. Proc. Fed. Am. Soc. Exp. Biol. 36* (1977) 799.
21. Pösö, H. *Acta Chem. Scand. B 31* (1977) 71.
22. Cantoni, G. L. and Durrell, J. *J. Biol. Chem. 255* (1957) 1033.
23. Pegg, A. E. and Williams-Ashman, H. G. *J. Biol. Chem. 244* (1969) 682.
24. Higgins, G. H. and Anderson, R. M. *Arch. Pathol. 12* (1931) 186.
25. Kallio, A., Löfman, M., Pösö, H. and Jänne, J. *FEBS Lett. 79* (1977) 195.
26. Jänne, J. and Williams-Ashman, H. G. *J. Biol. Chem. 246* (1971) 1725.
27. Jänne, J. and Williams-Ashman, H. G. *Biochem. Biophys. Res. Commun. 42* (1971) 222.
28. Obenrader, M. F. and Prouty, W. F. *J. Biol. Chem. 252* (1977) 2866.
29. Lowry, O. H., Rosebourgh, N. J., Farr, A. L. and Randall, R. J. *J. Biol. Chem. 193* (1951) 265.
30. Kallio, A., Löfman, M., Pösö, H. and Jänne, J. *Biochem. J. In press.*
31. Clark, J. L. *Biochemistry 13* (1974) 4668.

Received July 3, 1978.