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Effect of 1,3-diaminopropane on ornithine decarboxylase enzyme protein in thioacetamide-treated rat liver

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A radioimmunoassay for ornithine decarboxylase was used to study the regulation of this enzyme in rat liver. The antiserum used reacts with ornithine decarboxylase from mouse, human or rat cells. Rat liver ornithine decarboxylase enzyme activity and enzyme protein (as determined by radioimmunoassay) were measured in thioacetamide-treated rats at various times after administration of 1,3-diaminopropane. Enzyme activity declined rapidly after 1,3-diaminopropane treatment as did the amount of enzyme protein, although the disappearance of enzyme activity slightly preceded the loss of immunoreactive protein. The loss of enzyme protein after cycloheximide treatment also occurred rapidly, but was significantly slower than that seen with 1,3-diaminopropane. When 1,3-diaminopropane and cycloheximide were injected simultaneously, the rate of disappearance of enzyme activity and enzyme protein was the same as that seen with cycloheximide alone. These results show that the rapid loss in enzyme activity after 1,3-diaminopropane treatment is primarily due to a loss in enzyme protein and that protein synthesis is needed in order for 1,3-diaminopropane to exert its full effect. A macromolecular inhibitor of ornithine decarboxylase that has been termed antizyme is induced in response to 1,3-diaminopropane, but our results indicate that the loss of enzyme activity is not due to the accumulation of inactive ornithine decarboxylase–antizyme complexes. It is possible that the antizyme enhances the degradation of the enzyme protein. Control experiments demonstrated that the antiserum used would have detected any inactive antizyme–ornithine decarboxylase complexes present in liver since addition of antizyme to ornithine decarboxylase *in vitro* did not affect the amount of ornithine decarboxylase detected in our radioimmunoassay. Anti-(ornithine decarboxylase) antibodies may be useful in the purification of antizyme since the antizyme–ornithine decarboxylase complex can be immunoprecipitated, and antizyme released from the precipitate with 0.3M-NaCl.

Ornithine decarboxylase catalyses the conversion of ornithine into putrescine (Pegg & Williams-Ashman, 1968, 1981; Tabor & Tabor, 1976; McCann, 1980), which is the sole precursor of the polyamines in mammalian tissues. This enzyme has been of great interest in recent years owing to its rapid and many-fold stimulation by a number of physiological and pharmacological agents (Russell, 1980; Pegg & Williams-Ashman, 1981).

The apparent half-life of ornithine decarboxylase activity has been reported to be from 15 to 90min, which is shorter than that known for any other mammalian enzyme (Russell & Snyder, 1969; Hölttä, 1975; Prouty, 1976; Jefferson & Pegg, 1977; Seely *et al.*, 1982*a*,*b*). The most probable reason for the rapid decline in enzyme activity after the removal of a stimulating agent or inhibition of protein synthesis is that the protein turns over very rapidly and there is direct evidence to support this (Seely *et al.*, 1982*a*; Seely & Pegg, 1983). However, a number of other explanations have been forwarded concerning this rapid loss of enzyme activity, including covalent modification via phosphorylation (Atmar & Kuehn, 1981; Kuehn & Atmar, 1982) or transglutamination (Russell, 1981), alteration in the affinity of the enzyme for pyridoxal cofactor (Mitchell *et al.*, 1975, 1978, 1981), complexing with inhibitory (Canella-

kis *et al.*, 1979) or stimulatory proteins (Fujita *et al.*, 1982*a*,*b*). Although there is suggestive evidence for these theories, most of the experiments were done *in vitro*. It is, therefore, not known to what extent (if any) they contribute to the overall regulation of ornithine decarboxylase *in vivo*.

Certain diamines, particularly putrescine and 1,3-diaminopropane, have been shown to produce a rapid decrease in ornithine decarboxylase activity and the appearance of a 26000-mol.wt. inhibitory protein termed 'antizyme' (Heller *et al.*, 1976; McCann *et al.*, 1977; Pösö *et al.*, 1978; Pegg *et al.*, 1978; Canellakis *et al.*, 1979). Antizyme binds and completely inhibits activity *in vitro* but the antizyme–ornithine decarboxylase complex can be dissociated with high salt concentrations (Heller *et al.*, 1976; McCann *et al.*, 1977). It has been suggested that the rapid decrease in enzyme activity seen in liver after 1,3-diaminopropane treatment is brought about by the synthesis of antizyme and the formation of enzymically inactive antizyme–ornithine decarboxylase complexes (Heller *et al.*, 1976; Canellakis *et al.*, 1979).

Attempts to provide definite proof for this hypothesis using antibodies to quantify the amount of enzyme protein were made by Kallio *et al.* (1977, 1979). However, the results were inconclusive, giving contradictory results when the assay was carried out in different ways and, because of the low titre of the antibodies and the small amount of ornithine decarboxylase present in tissues, the protein could be measured only by immunotitration of the activity (Kallio *et al.*, 1979). Also, the specificity of the antibodies used is seriously in doubt since more recent workers have purified ornithine decarboxylase to a specific activity 50–100 times greater than that used to raise antibodies in these experiments (Persson, 1981; Seely *et al.*, 1982*c*; Kameji *et al.*, 1982). More recently, the homogeneous enzyme from mouse kidney (Seely & Pegg, 1983; Persson, 1982) or rat liver (Kameji *et al.*, 1982) has been used to provide specific antisera of much higher titre and proven monospecificity. We have set up a specific radioimmunoassay for the enzyme using such antibodies and the enzyme labelled by reaction with α-difluoromethyl[5-^{3}H]ornithine as the tracer ligand.

This radioimmunoassay was used to determine the effect of 1,3-diaminopropane treatment on the levels of ornithine decarboxylase enzyme protein in thioacetamide-treated rat liver. Our results show that the primary effect of 1,3-diaminopropane is to bring about a rapid decrease in the amount of enzyme protein. Although this does not rule out a role for antizyme in the regulation of ornithine decarboxylase, it does indicate that inactive antizyme–ornithine decarboxylase complexes do not accumulate to a large extent *in vivo*.

Materials and methods

Materials

α-Difluoromethyl[5-^{3}H]ornithine (sp. radioactivity 17.2Ci/mmol) was purchased from New England Nuclear, Boston, MA, U.S.A. Labelled ornithine decarboxylase was prepared by reacting α-difluoromethyl[5-^{3}H]ornithine with the purified mouse kidney enzyme as described by Seely & Pegg (1983). Mouse kidney ornithine decarboxylase was purified to homogeneity as described by Seely *et al.* (1982*c*) and a specific antiserum to the enzyme was raised in rabbits as described by Seely & Pegg (1983). 1,3-Diaminopropane was purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. [1-^{14}C]Ornithine (50–60Ci/mol) was purchased from Amersham Corp., Arlington Heights, IL, U.S.A. Bacterial protein A adsorbent was purchased from Miles-Yeda, Rehovot, Israel. All other reagents were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

Preparation of liver extracts

Female Sprague–Dawley rats (250–300g) were treated with thioacetamide as described by Seely *et al.* (1982*b*) and killed approx. 22h after treatment. At this time, ornithine decarboxylase activity is increased about 50-fold over control values and in the absence of additional treatment remains at this elevated value for a further 6h (Pegg *et al.*, 1978). 1,3-Diaminopropane was administered to the thioacetamide-treated rats by intraperitoneal injection at a dosage of 1mmol/kg body wt. and the animals were killed at times indicated. Cycloheximide was administered at a dosage of 5mg/kg body wt. and the animals were killed at times indicated. Livers were homogenized in 2.5vol. of 25mM-Tris/HCl, pH7.5, containing 0.1mM-EDTA, 2.5mM-dithiothreitol and 0.02% (w/v) Brij 35 (buffer A) and centrifuged at 100000*g* for 45min. The supernatants were dialysed overnight against 600vol. of buffer A containing 1mM-dithiothreitol. Ornithine decarboxylase activity and immunoreactivity measurements were done on these extracts.

Radioimmunoassay and enzyme activity measurement of ornithine decarboxylase

The radioimmunoassay for ornithine decarboxylase was done as described previously (Seely & Pegg, 1983) using α-difluoromethyl[5-^{3}H]-ornithine-labelled mouse kidney ornithine decarboxylase as tracer ligand. Approx. 0.6ml of extract containing 15–20mg of protein was used per assay. Antibody (50μl of a 1:150 dilution) was added to each sample 45min before the addition of 10000d.p.m. of tracer ligand. This 'pre-incubation' method had been shown to greatly enhance the

sensitivity of the assay (Seely & Pegg, 1983). Incubation in the presence of tracer ligand was for 30min. The immune complexes were precipitated with bacterial protein A, washed and counted for radioactivity as reported previously (Seely & Pegg, 1983). Standard curves were set up as described by Seely & Pegg (1983), except that various amounts of extract from thioacetamide-treated rat liver were used as standard; 100% binding was that amount of tracer ligand bound by the antibody in the presence of 0.6ml of a liver extract containing essentially no (<0.3ng/ml) ornithine decarboxylase. This liver extract was obtained from rats that were not treated with thioacetamide, but that received cycloheximide 60min before being killed. This extract was also used to adjust the total amount of protein in the standards to 15–20mg. Ornithine decarboxylase enzyme activity was determined by monitoring the release of $^{14}CO_2$ from (1-^{14}C)-labelled ornithine (Pegg & Williams-Ashman, 1968). Protein determinations were done by the method of Bradford (1976). One unit of ornithine decarboxylase activity is that amount that will decarboxylate 1nmol of ornithine in 30min. One unit of rat liver ornithine decarboxylase was assumed to be equivalent to 1.43ng of enzyme protein based on the fact that one unit binds 26fmol of α-difluoromethylornithine and that one molecule of α-difluoromethylornithine is bound per 55000-mol. wt. subunit (Pritchard *et al.*, 1981; Seely *et al.*, 1982*b*).

Purification of antizyme using ornithine decarboxylase antiserum

Crude liver extracts from rats treated with 1,3-diaminopropane at 4h and again at 2h before being killed were prepared as described above. Extracts (containing 30mg of protein) were incubated at 4°C with 2μl of ornithine decarboxylase antiserum for 90min in the presence of 40 units of ornithine decarboxylase. Bacterial protein A adsorbent [100μl of a 10% (w/v) suspension] was then added and the samples were incubated at 4°C for an additional 90min. The samples were centrifuged for 30s in an Eppendorf 3200 Microfuge and the precipitate was washed twice in buffer A. Antizyme was released by resuspending the immunoprecipitate in buffer A containing 0.3M-NaCl and incubating for 60min at 4°C. The samples were centrifuged and the supernatant dialysed overnight against 500vol. of buffer A, containing 10μM-pyridoxal phosphate. Antizyme activity was determined by measuring the ability of the extract to inhibit ornithine decarboxylase activity. Antizyme measurements were carried out under such conditions that ornithine decarboxylase activity was not inhibited by greater than 60%. One unit of antizyme is defined as that amount that will inhibit one unit of ornithine decarboxylase activity.

Results

The radioimmunoassay procedure described previously (Seely & Pegg, 1983), using α-difluoromethyl[5-^{3}H]ornithine-labelled mouse kidney ornithine decarboxylase as tracer ligand, can be used to determine ornithine decarboxylase from rat and human as well as mouse (Fig. 1). Both rat and human ornithine decarboxylase could be determined with roughly the same sensitivity as the mouse enzyme. This finding agrees with the recent paper of Persson (1982), who showed that antiserum raised in rabbits to mouse kidney ornithine decarboxylase inhibited both rat and mouse ornithine decarboxylase. The fact that the rat standard curve did not parallel the mouse or human curves may suggest that the antibody is recognizing different antigenic determinants on this enzyme, the significance of which is unclear at this time but the rat and mouse enzymes have been shown to have slightly different physical properties (Seely *et al.*, 1982*b*,*c*). By this method we could measure as low as 1 unit of rat liver ornithine decarboxylase protein, which is equivalent to 1.4ng of enzyme protein.

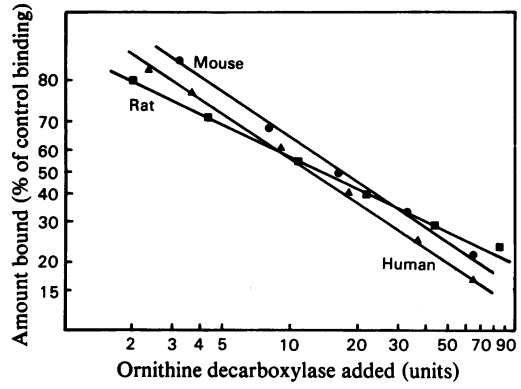

Fig. 1. *Radioimmunoassay of mammalian ornithine decarboxylase*

The assay was carried out using a tracer ligand of α-difluoromethyl[5-^{3}H]ornithine-labelled ornithine decarboxylase as described in the Materials and methods section. The results are expressed as the percentage of the amount of tracer bound in the absence of unlabelled ornithine decarboxylase from androgen-stimulated mouse kidney (●), thioacetamide-treated rat liver (■) and serum-stimulated human fibroblast (▲) ornithine decarboxylase. The results are plotted as the logit of the percentage of the tracer bound against the logarithm of the standard protein added in order to generate a linear standard curve (Hunter, 1978).

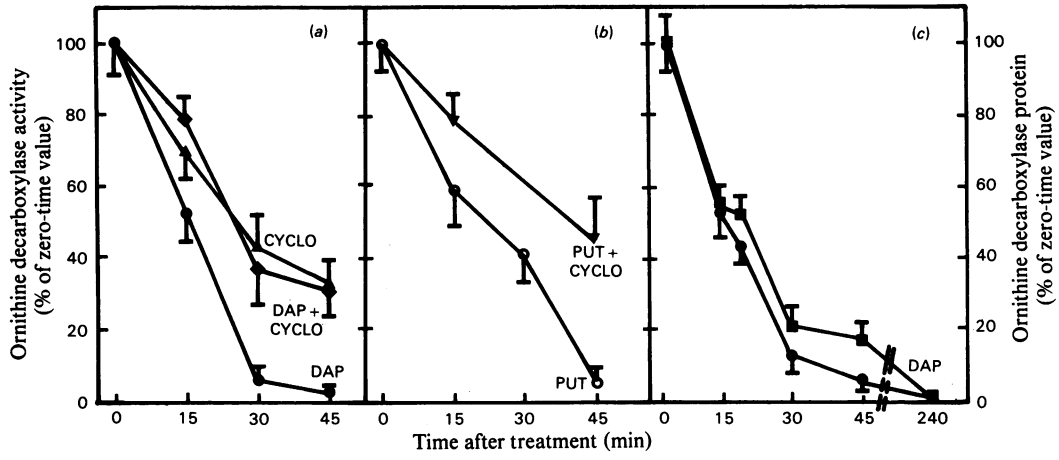

Fig. 2. *Time course for loss of ornithine decarboxylase activity after treatment with 1,3-diaminopropane (DAP), putrescine (PUT) or cycloheximide (CYCLO) and for loss of ornithine decarboxylase protein after DAP*

All animals received thioacetamide 22h before treatment with 1,3-diaminopropane (●), cycloheximide (▲), 1,3-diaminopropane plus cycloheximide (◆), putrescine (○) or putrescine plus cycloheximide (▼). Results are shown in (*a*) and (*b*) for the loss of enzyme activity and in (*c*) for the loss of enzyme activity (●) and immunoreactive protein (■). Results are means ± S.E.M. for at least five separate estimations at each time point. Results are expressed as percentages of the control activity at zero time. The activity was 0.96 unit/mg of protein and the amount of ornithine decarboxylase protein was 1.35 ng/mg of protein at this time.

When rats were treated with 1,3-diaminopropane, putrescine or cycloheximide, there was a rapid fall in the activity of ornithine decarboxylase (Figs. 2*a*–2*c*). The loss of activity was more rapid with 1,3-diaminopropane than with putrescine and the former was, therefore, chosen for further experiments. As can be seen in Fig. 2(*c*), the rate of loss of ornithine decarboxylase protein in response to 1,3-diaminopropane was similar to the rate of loss of enzyme activity. At 45 min after treatment, however, there was a somewhat greater fraction of protein remaining than of activity, which could indicate the presence of a small amount of antizyme–ornithine decarboxylase complexes. By 4h after treatment, both enzyme activity and enzyme protein had fallen below the limit of detection. Although there may be a small amount of enzymically inactive antizyme–ornithine decarboxylase complex formed at 45 min after treatment, it is important to note that the major cause of the rapid loss of enzyme activity is the loss of enzyme protein. It is not known to what extent 1,3-diaminopropane affects ornithine decarboxylase synthesis or degradation; these experiments would suggest that inhibition of synthesis is not its sole effect. The rate of loss of enzyme activity after 1,3-diaminopropane treatment was significantly greater than that seen after inhibition of protein synthesis by cycloheximide (Fig. 2*a*). This indicates that 1,3-diaminopropane is having an augmentative effect on the rate of ornithine decarboxylase breakdown. Whether this effect is mediated by

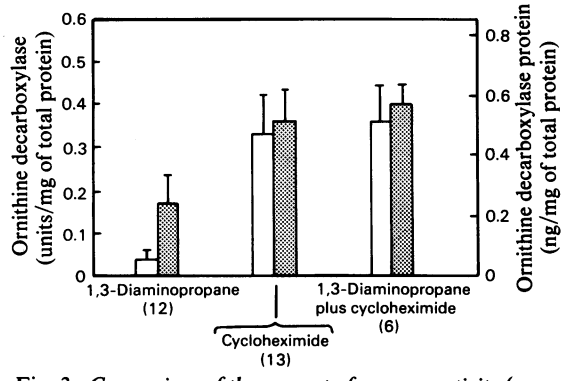

Fig. 3. *Comparison of the amount of enzyme activity (open columns) and immunoprecipitable enzyme protein (shaded columns) remaining in thioacetamide-treated rats at 45 min after injection of 1,3-diaminopropane, cycloheximide, cycloheximide or cycloheximide + 1,3-diaminopropane*

The number of samples in each group is shown in parentheses. Results shown are means ± S.E.M. (indicated by the bars). At the time of injection the ornithine decarboxylase was 1.01 unit/mg of protein and the amount of ornithine decarboxylase protein was 1.41 ng/mg of protein.

antizyme or some other factor remains to be elucidated. However, some rapidly synthesized protein appears to be involved, since when cycloheximide was administered simultaneously with 1,3-diaminopropane or with putrescine the loss of enzyme activity and protein was similar to that seen with cycloheximide alone (Figs. 2*a*, 2*b* and 3).

Table 1. *Radioimmunoassay of antizyme–ornithine decarboxylase complexes from rat liver formed in vitro*

Crude antizyme extract (prepared as described in the Materials and methods section) containing 20–25mg of protein was incubated with 13.2 units (*a*) or 6.4 units (*b*) of rat liver ornithine decarboxylase for 5min at 0–4°C. Enzyme activity measurement or radioimmunoassays were then performed as described in the Materials and methods section. Control liver extract was prepared in a way identical with the antizyme liver extract except from rats that did not receive 1,3-diaminopropane. The amount of ornithine decarboxylase activity present in this control extract was negligible. Values in parentheses are percentages of the respective control values.

	Ornithine decarboxylase activity (units/sample)	Immunoprecipitable enzyme protein (ng/sample)
(*a*) Rat enzyme + control liver extract	13.2	18.9
Rat enzyme + antizyme liver extract	4.6 (35%)	17.2 (91%)
(*b*) Rat enzyme + control liver extract	6.4	9.4
Rat enzyme + antizyme liver extract	0.7 (11%)	9.2 (97%)

Table 2. *Purification of rat liver antizyme using ornithine decarboxylase antiserum*

Crude antizyme extract (100000*g* supernatant) was incubated with ornithine decarboxylase in the presence of ornithine decarboxylase antiserum. Antizyme was released from the immunoprecipitate with 0.3M-NaCl, as described in the Materials and methods section. Antizyme was also incubated with ornithine decarboxylase antiserum in the absence of ornithine decarboxylase. Antizyme measurements were done as described in the Materials and methods section.

	Antizyme activity (units/mg)	Yield (%)
Dialysed 100000*g* supernatant	0.4	100
NaCl wash from immunoprecipitate (dialysed)	36.7	34
NaCl wash from immunoprecipitate in the absence of ornithine decarboxylase	Not detectable	0

In order to validate these results, it was necessary to show that our antibody could bind antizyme–ornithine decarboxylase complexes. To do this, we made crude (100000*g* supernatant) antizyme preparations from 1,3-diaminopropane-treated rats as described in the Materials and methods section. Various amounts of antizyme were added to partially purified rat liver ornithine decarboxylase and enzyme activity measurements and immunoassays were performed. The crude antizyme and ornithine decarboxylase were incubated together for 5min at 0–4°C before addition of antiserum to start the radioimmunoassay. Incubation for longer times at higher temperature (37°C) before the addition of antibody had no effect on the measurable amount of immunoreactivity. As can be seen in Table 1, even when antizyme was added in proportions such that only 11% of the ornithine decarboxylase activity remained, there was still no effect on the measurable amount of enzyme protein as determined by radioimmunoassay. This demonstrated that our radioimmunoassay procedure was able to detect any ornithine decarboxylase bound to antizyme in 1,3-diaminopropane-treated rat liver. In addition, we were unable to unmask additional ornithine decarboxylase immunoreactivity from samples in Fig. 3 by doing the immunoassay in the presence of 0.3M-NaCl, a condition that would dissociate any antizyme–ornithine decarboxylase complexes that may have been present (results not shown).

Since our antibody was able to precipitate antizyme–ornithine decarboxylase complexes and since antizyme can be dissociated from ornithine decarboxylase in the presence of high salt concentrations, we postulated that ornithine decarboxylase antibodies may be useful in the purification of antizyme. As shown in Table 2, when antizyme and ornithine decarboxylase were incubated with ornithine decarboxylase antiserum and precipitated with bacterial protein A, antizyme could be recovered from the immunoprecipitate by washing in 0.3M-NaCl. The specificity of this procedure is demonstrated by the fact that when ornithine decarboxylase was omitted from the incubation no antizyme was recovered from the washed precipitate. By this method nearly a 100-fold purification could be achieved with an approx. 30% yield.

Discussion

The effect of 1,3-diaminopropane on ornithine decarboxylase from rat liver is quite striking and undoubtedly complicated. Although 1,3-diaminopropane may cause direct inhibition of protein synthesis at relatively high concentrations (Kay & Benzie, 1980; Tuomi *et al.*, 1980), the rate of loss of enzyme protein after 1,3-diaminopropane treatment is greater than in animals treated with cycloheximide, indicating that the diamine leads to an increased rate of enzyme degradation. The enhanced rate of enzyme breakdown can be blocked

by cycloheximide (Fig. 3), suggesting that a rapidly synthesized protein(s), possibly antizyme, is involved in mediating the effect of the diamine ornithine decarboxylase degradation. In addition, the fractional amount of protein remaining at 45min after 1,3-diaminopropane treatment was slightly, but significantly, greater than the fraction of activity remaining. This may indicate the accumulation of a small amount of antizyme–ornithine decarboxylase complexes at this time. Fujita *et al.* (1982*b*) have recently shown that a small amount of ornithine decarboxylase activity can be regained from 1,3-diaminopropane-treated rat liver extracts by the addition of an antizyme-inhibitory protein. This observation notwithstanding, the activity recovered was still only a fraction of that seen in animals not receiving treatment. The results of Fujita *et al.* (1982*b*) agree with our observations that the primary effect of 1,3-diaminopropane is to decrease enzyme protein.

An alternative explanation for the lower rate of loss of ornithine decarboxylase in the presence of cycloheximide would be that cycloheximide slows protein degradation as well as inhibiting protein synthesis (Woodside, 1976). This is unlikely to explain our results, since direct measurements of the turnover of ornithine decarboxylase protein in mouse kidney indicated that the protein was degraded at the same rate in the presence or absence of cycloheximide (Seely *et al.*, 1982*a*).

It is conceivable that our antibody preparations are unable to recognize an altered form of ornithine decarboxylase that is formed in the diamine-treated liver. However, this is highly unlikely since the antibody does interact with the complex between antizyme and ornithine decarboxylase (Table 1), ornithine decarboxylase conjugated with α-difluoromethylornithine (Seely & Pegg, 1983) and aggregated forms of the enzyme produced on prolonged storage (J. E. Seely & A. E. Pegg, unpublished work).

Results from a number of experiments *in vitro* have suggested that ornithine decarboxylase activity may be regulated by post-translational modifications (Atmar & Kuehn, 1981; Russell, 1981) or by inhibitory or stimulatory proteins (Canellakis *et al.*, 1979; Fujita *et al.*, 1982*a*,*b*). The experiments *in vivo* reported in the present paper, however, in which both enzyme-activity and enzyme-protein measurements were made, suggest that in liver the net regulation of ornithine decarboxylase activity is primarily at the level of synthesis and degradation *de novo*. Similar results were obtained when the regulation of ornithine decarboxylase in mouse kidney was tested, but this may be a special case since enzyme levels 100–200 times greater than in other cell types exist in the male mouse kidney (Seely & Pegg, 1983). This is not to say that post-translational modification or enzyme-inhibitory proteins such as antizyme play no role in ornithine decarboxylase regulation. Rather, such enzyme modifications may be prerequisites in order for ornithine decarboxylase degradation to occur. One could speculate that the role of antizyme is to make ornithine decarboxylase available to a particular fast-acting proteolytic system. Antizyme could then be either degraded or freed to bind another ornithine decarboxylase molecule. This could explain why few (if any) antizyme–ornithine decarboxylase complexes are detected *in vivo*. Such a mechanism would not be dissimilar from the ATP-ubiquitin proteolytic system from reticulocytes (Etlinger & Goldberg, 1977; Wilkinson *et al.*, 1980) in which proteins are 'earmarked' for degradation by conjunction to the protein ubiquitin. Also, it is conceivable that our results can be explained by a sequestration of ornithine decarboxylase–antizyme complexes into some subcellular organelle since our measurements were made only on the soluble fraction from the homogenates. The purification technique for antizyme herein described may be very useful in elucidating the function of this unique protein.

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