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Polyamine-mediated turnover of ornithine decarboxylase in Chinese-hamster ovary cells

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We have used Chinese-hamster ovary (CHO) cells maintained in a chemically defined medium to study the regulation of ornithine decarboxylase (ODC) by polyamines. Cells maintained in the defined medium had no detectable putrescine, and approx. 1–3 units of ODC activity/10^6 cells, where 1 unit corresponds to 1 nmol of substrate decarboxylated in 30 min. The defined medium is ornithine-deficient, thus limiting the exogenous substrate for ODC, and subsequently decreasing intracellular polyamine accumulation. Restoration of intracellular putrescine and increased formation of spermidine by addition of exogenous ornithine or putrescine led to a marked decrease in ODC activity, which was paralleled by a decrease in a α-DL-difluoromethyl[3,4-^{3}H]ornithine (DFMO)-binding protein of M_r approx. 53000, which is precipitable with anti-ODC antibody. Calculation of DFMO binding per unit of activity showed no change in the specific activity of the enzyme. We identified [^{35}S]methionine-labelled peptides corresponding to ODC by immunoprecipitation of radiolabeled whole cell proteins. Only one protein was precipitated, of M_r approx. 53000, which co-migrated with the DFMO-binding protein. Immunoprecipitation of radiolabelled proteins from cells incubated in the presence of exogenous ornithine indicated that the observed activity decrease was not due to an inhibition of ODC protein synthesis. Analysis of immunoprecipitable ODC protein from cells that had been pulse-labelled with [^{35}S]methionine, and then treated for 5 h with 100 μM-ornithine, -putrescine or -spermidine, revealed a distinct disappearance of labelled ODC protein after restoration of intracellular polyamine pools. No detectable turnover of ODC was observed in the absence of exogenous polyamine treatment. These data support the hypothesis that ODC protein, and subsequent activity, is regulated by intracellular polyamine content through mechanisms that influence turnover of the enzyme.

INTRODUCTION

L-Ornithine decarboxylase (ODC) is the first enzyme in the polyamine-biosynthetic pathway, catalysing the conversion of ornithine into putrescine. This particular enzyme has generated wide interest, owing to its rapid fluctuations in activity in response to a variety of stimuli, and to observations that polyamines are essential for optimal cell proliferation. Another interesting feature of this enzyme is that the biological half-life is the shortest known for any mammalian enzyme (for reviews, see Heby, 1981; Pegg & McCann, 1982; Tabor & Tabor, 1984).

Definitive studies on the mechanism of regulation of ODC have been hampered by the small quantity of active enzyme present in mammalian cells, in most cases representing less than 0.0001% of the total cell protein (Pegg & McCann, 1982; Tabor & Tabor, 1984). The radiolabelled form of the enzyme-activated suicide inhibitor of ODC, DL-α-difluoromethylornithine (DFMO) (Metcalf *et al.*, 1978), has proved beneficial in measuring changes in active protein content after stimulation of enzyme activity (Pritchard *et al.*, 1981; Erwin *et al.*, 1983). These data have shown that the observed activity increases are a result of increases in the total active ODC protein pool. Polyclonal antibodies generated against purified mouse kidney ODC have also been used to analyse total ODC protein content, and these data corroborate those observations made by using [^{3}H]DFMO quantification, showing that in stimulated systems regulation is mainly due to changes in absolute amounts of active enzyme (Erwin *et al.*, 1983; Seely & Pegg, 1983).

The effect of diamines and polyamines to abolish ODC activity is well documented (Jänne & Hölttä, 1974; Pegg *et al.*, 1978; Canellakis *et al.*, 1979). The mode of this action has been postulated to be a result of polyamine-mediated conversion of the enzyme into multiple forms (Mitchell *et al.*, 1985), production of inhibitor molecules (Canellakis *et al.*, 1979), or possible post-translational modifications (Atmar & Kuehn, 1981; Russell, 1981). More recent results, however, indicate that the degradation rate of ODC is enhanced by exogenous amine addition (Persson *et al.*, 1984).

The precise study of the regulation of ODC by polyamines has been hampered in cell-culture systems, since the growth media characteristically contain undefined components in the fetal bovine serum. We have developed a medium lacking fetal bovine serum, which contains known growth factors (transferrin, $FeSO_4$, insulin) capable of maintaining Chinese-hamster ovary (CHO) cell growth rates and viability of values near those of cells growing in serum-supplemented medium. A detailed comparison of the polyamine contents, growth rates and ODC activity between CHO cells maintained in the defined medium or the medium containing serum

Abbreviations used: ODC, L-ornithine decarboxylase (EC 4.1.17); DFMO, DL-α-difluoromethylornithine; CHO cells, Chinese-hamster ovary cells

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has been described (Sertich *et al.*, 1985). The chemically defined medium lacks an exogenous ornithine supply, thus limiting polyamine accumulation, and we have used these conditions to study the regulation of ODC activity in unstimulated exponentially growing cells after restoration of intracellular polyamine content. We have used both [^{3}H]DFMO binding and immunochemical techniques to measure changes in active enzyme and protein content after the addition of exogenous substrate and end product.

MATERIALS AND METHODS

Materials

L-[1-^{14}C]Ornithine (60 mCi/mmol), L-[^{35}S]methionine (1120 Ci/mmol), α-DL-difluoromethyl[3,4-^{3}H]ornithine ([^{3}H]DFMO; 26.5 Ci/mmol) were purchased from New England Nuclear (Boston, MA, U.S.A.). A bacterial absorbant, *Staphylococcus* A (IGSORB) was purchased from The Enzyme Center (Malden, MA, U.S.A.). Tissue-culture plastic was purchased from Falcon (Oxnard, CA, U.S.A.), and culture medium was obtained from Gibco Laboratories (Grand Island, NY, U.S.A.). All other chemicals were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.).

Cell culture techniques

CHO cells were maintained in exponential growth at 37 °C in McCoy's 5A medium supplemented with insulin (10 μg/ml), transferrin (5 μg/ml), $FeSO_4$ (1.1 μg/ml), 100 units of penicillin/ml and 100 μg of streptomycin/ml, at 37 °C in air/CO_2 (9:1). Cells maintained under these conditions have a colony-forming efficiency of 60–80% and a doubling time of 16–18 h

Measurement of ODC activity

Cells were scraped from culture dishes, centrifuged at 900 *g* for 6 min, and disrupted by sonication in buffer containing 0.05 M-sodium/potassium phosphate, pH 7.2, 0.1 mM-EDTA, 1.0 mM-dithiothreitol and 20 μM-pyridoxal phosphate (ODC buffer), at a concentration of 10^7 cells/ml. A soluble fraction was obtained by centrifugation at 10000 *g* for 5 min in a Beckman Minifuge. Then 200 μl of the soluble fraction was incubated at 37 °C for 30 min in the presence of 0.15 μCi of L-[1-^{14}C]ornithine (final concn. 500 μM) and the reaction was terminated by addition of 0.5 ml of 1 M-citric acid. ODC activity was determined by the liberation of $^{14}CO_2$ during the reaction period. One unit of activity corresponds to 1 nmol of substrate decarboxylated in 30 min.

Polyamine analysis

Acid-soluble polyamine contents were determined by using the reverse-phase ion-pair h.p.l.c. procedure originally described by Seiler & Knödgen (1980) Cells were collected from exponentially growing cultures by first removing the culture medium and washing the cell monolayer twice with ice-cold phosphate-buffered saline (70 mM-NaCl, 3 mM-KCl, 8 mM-Na_2HPO_4, 1 mM-KH_2PO_4, pH 7.3). The cells were harvested into phosphate-buffered saline with a rubber policeman, and cell numbers were determined with an electronic particle counter (Coulter Electronics, Hialeah, FL, U.S.A.). The cells were disrupted by sonication, and the soluble proteins precipitated with 0.2 M-$HClO_4$. The acid-soluble fractions were obtained by centrifugation at 15000 *g* for 5 min. Polyamine contents were then determined from samples of these, by using diaminoheptane as an internal standard and by comparing the relative retention times with those of standard preparations of putrescine, spermidine and spermine.

Radiolabelled DFMO binding

To prepare radiolabelled ODC protein, soluble cell homogenates (at least 5 mg of soluble protein per reaction) were incubated with 0.28 μM-[^{3}H]DFMO in ODC buffer (500 μl) for 2 h at 37 °C. This concentration of drug and incubation time produced over 95% inactivation of activity (results not shown). Primary antiserum (the characterization of this antiserum has been previously described; Seely & Pegg, 1983) was then added (1:500 final dilution). This dilution yields 100% removal of ODC activity from supernatants after immune precipitation (results not shown). The mixture was incubated for 1 h at 20 °C, and immunoreactive proteins were collected by addition of 20 μl of 10% (v/v) suspension of formalin-fixed *Staphylococcus* A by centrifugation after 30 min at 20 °C. The pellet was washed once with 750 μl of Buffer A (1% Triton X-100, 0.5% sodium deoxycholate, 5 mM-EDTA, 250 mM-NaCl, 25 mM-Tris/HCl, pH 7.5) and proteins were solubilized into 40 μl of SDS solubilization buffer at 100 °C. Specificity of binding and quantification were determined by fractionation of labelled proteins by SDS/polyacrylamide-gel electrophoresis, by a slight modification of the method of Laemmli (1970). Bisacrylamide was replaced with *NN'*-diallyltartardiamide (Bio-Rad), so that gel slices were dissolved by the addition of 2% periodic acid. Samples for SDS/polyacrylamide-gel electrophoresis were heated at 100°C for 5 min in 2% SDS/5% β-mercaptoethanol/3% sucrose in 300 mM-Tris/HCl, pH 7.0 (SDS solubilization buffer).

Immunoprecipitation

Exponential-phase cultures (1.5×10^7 cells/reaction) were labelled with 100 μCi of [^{35}S]methionine/ml in methionine-free medium. At appropriate times after incubation, cells were harvested by scraping with a rubber policeman, disrupted in ODC buffer at 2×10^7 cells/ml, and then 50 μl of a 1:500 dilution of primary antiserum was added to 500 μl samples of the cell homogenate. Controls were incubated with non-immune serum. After 1 h at 20 °C, 3 μl of 10% suspension of formalin-fixed *Staphylococcus* A was added, and 30 min later the complexes were collected by centrifugation at 10000 *g* for 5 min. Non-specific binding was decreased by washing the pellet with 10×750 μl Buffer A. Proteins were eluted from the *Staphylococcus* A at 100 °C in 40 μl of SDS solubilization buffer and separated by SDS/polyacrylamide-gel electrophoresis.

Protein determination

Protein concentration was determined by the method of Bradford (1976), with bovine serum albumin as standard.

RESULTS

To identify proteins corresponding to ODC, we detected [^{35}S]methionine-labelled immunoreactive peptides which co-migrated with radiolabelled [^{3}H]DFMO-

bound proteins on polyacrylamide gels. It had been shown that this enzyme-activated irreversible inhibitor serves as a useful marker for ODC protein, binding specifically in a 1:1 molar ratio with the enzyme (Erwin *et al.*, 1983; Persson *et al.*, 1984). These data are shown in Fig. 1. Labelling cells in methionine-deficient medium plus 100 μCi of [^{35}S]methionine/ml, followed by immunoprecipitation with anti-ODC serum, yielded one band which was present in the homogenates treated with anti-ODC serum (lane *c*), which co-migrated with the marker band seen in lane (*a*). This protein was not present in homogenates treated without anti-ODC serum (lane *b*) and had a subunit M_r of approx. 53000, consistent for that of ODC isolated from another Chinese-hamster cell line (Choi & Scheffler, 1983).

Since diamines and polyamines were known to influence ODC activity, and CHO cells growing in the chemically defined medium had substantially depressed intracellular putrescine and spermidine pools (Sertich *et al.*, 1985; see also the next section), we determined ODC activity and [^{3}H]DFMO binding to anti-ODC-antibody-immunoprecipitable protein after restoration of intracellular polyamine pools. Restoration was accomplished by addition of 100 μM-ornithine or -putrescine to the culture medium. This concentration was chosen for study, as previous work had shown that 100 μM-ornithine led to maximal inhibition of ODC activity after a 24 h incubation interval. The data shown in Fig. 2(*a*) demonstrated that treatment with exogenous ornithine (○) or putrescine (●) (each 100 μM) led to a rapid decay in enzyme activity, with putrescine having a greater effect than ornithine. The results are plotted as a percentage of control, where the control activity represents untreated samples. Total inhibition of activity was observed after 8 h of treatment with ornithine or 6 h of treatment with putrescine. The decline in activity paralleled a decrease in [^{3}H]DFMO binding (Fig. 2*b*), implying that polyamines were not altering the enzyme catalytic activity, but that the number of active molecules was decreasing. The ratio of drug bound to activity was about 13 fmol/unit, and was consistent with that described for ODC from mouse kidney (Seely *et al.*, 1982).

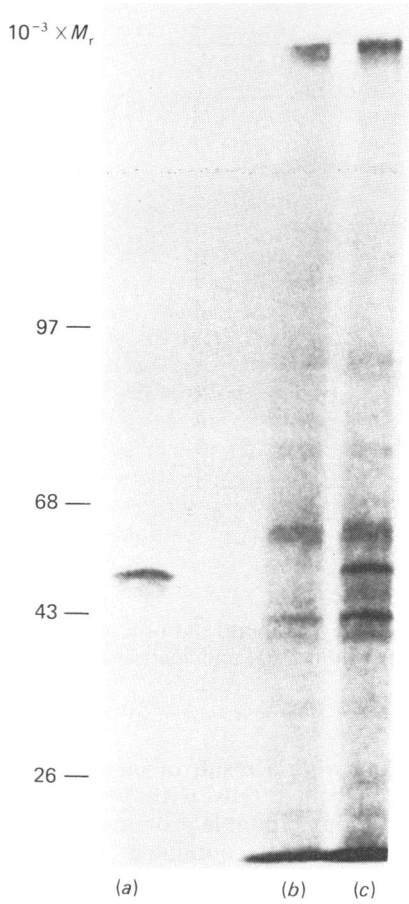

Fig. 1. Identification of radiolabelled peptides corresponding to ornithine decarboxylase from Chinese hamster ovary cells by immunoprecipitation

Exponentially growing CHO cells were labelled with 100 μCi of [^{35}S]methionine/ml for 1 h in methionine-deficient media. Cells were harvested by scraping, sonicated into ODC buffer and immunoprecipitated as describe in the Materials and methods section. Lanes: (*a*) [^{3}H]DFMO-binding proteins immunoprecipitated from whole cells homogenates; (*b*) control, incubated without the primary antiserum; (*c*) proteins precipitated by the anti-ODC serum.

The data in Fig. 3 document changes in the polyamine content of cells after treatment with the exogenous amines. Cells treated with 100 μM-ornithine (Fig. 3*a*) exhibited a steady increase in putrescine content, reaching a maximum of approx. 0.67 nmol/10^6 cells after 6 h of exposure. Spermidine content increased linearly as a function of treatment time. At 8 h, the content of intracellular spermidine was approx. 1.25 nmol/10^6 cells. Addition of exogenous putrescine (Fig. 3*b*) led to rapid intracellular accumulation of this diamine, reaching a maximum value of 1.25 nmol/10^6 cells after 2 h, and the rate of formation of spermidine was identical with that of cells treated with ornithine.

The data presented in Fig. 2 indicate that the inhibition of ODC by polyamines is mediated by an overall change in active protein content, with subsequent activity and protein amounts decreased to zero. One possible mechanism responsible would be a polyamine effect to inhibit the synthesis of ODC totally, thereby leading to a decline in ODC content as the protein is degraded. To determine whether ODC synthesis was being inhibited by polyamine restoration, we pulse-labelled cells for 30 min with [^{35}S]methionine at various times after addition of 100 μM-ornithine to the culture medium. Determination of labelling kinetics of ODC under these conditions revealed that 30 min is the minimal labelling time required to recover sufficient incorporation of radiolabel into ODC protein to yield a significant autoradiographic signal (results not shown). Anti-ODC-antibody-immunoprecipitable protein was collected immediately after each labelling period and analysed by SDS/polyacrylamide-gel electrophoresis. Fig. 4 shows that incorporation of [^{35}S]methionine into the unique immunoreactive peptide was not totally inhibited at any time of treatment, even though ODC activity was substantially depressed after 2 h of treatment. Lane (*a*) represented the untreated control sample (100% ODC activity), lane (*b*) 2 h of ornithine treatment, representing 82% of control enzyme activity, lane (*c*) 4 h treatment representing 24% of control enzyme activity, lane (*c*) 4 h treatment, representing 24% activity, lane (*d*) 6 h treatment, represent 20% activity, and lane (*e*) 8 h treatment representing 15% of control ODC activity. Lanes (*f*)–(*j*) were equivalent

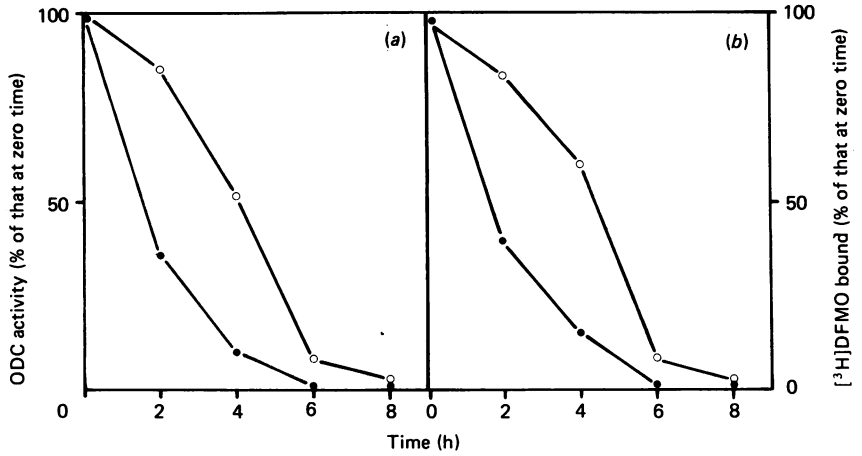

Fig. 2. ODC activity and [^{3}H]DFMO-binding-protein contents in Chinese-hamster cells after treatment with exogenous 100 μM-ornithine or putrescine

(*a*) ODC activity of exponentially growing CHO cells, maintained in defined medium, after various times of treatment with either 100 μM-ornithine (○) or 100 μM-putrescine (●). (*b*) Immunoprecipitable [^{3}H]DFMO-binding proteins in samples treated in a manner identical with those in (*a*).

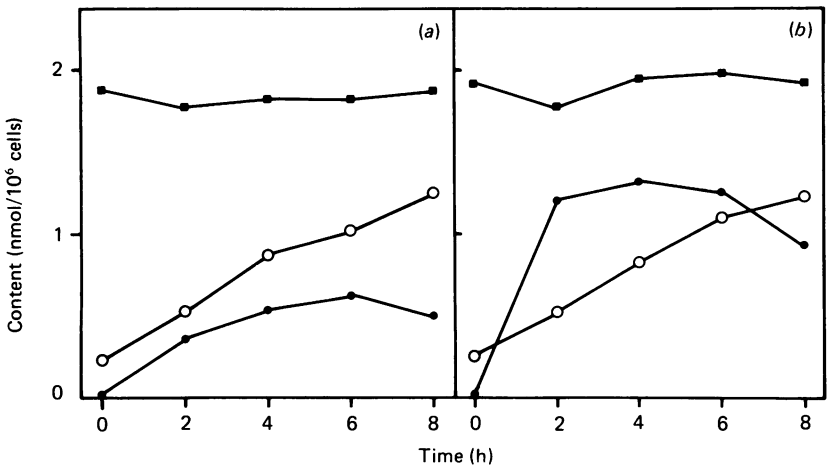

Fig. 3. Polyamine content after addition of 100 μM-ornithine or -putrescine.

Cells were treated for various times with 100 μM-ornithine or -putrescine, harvested by scraping, and processed for polyamine analyses as described in the Materials and methods section. Putrescine (●), spermidine (○) and spermine (□) contents are shown after ornithine addition (*a*) and after putrescine treatment (*b*).

amounts of cell protein treated with non-immune serum. Although there is some variance in labelled protein recovered under these conditions, this is a result of differences in total radiolabelled protein recovered for each time point. Densitometric analysis of this autoradiograph reveals less than 10% variance in the unique immunoprecipitated peptide with respect to absolute protein recovered (results not shown). These data showed that addition of exogenous ornithine did substantially alter the rate of ODC protein synthesis, although enzyme activity was decreased by up to 85%, indicating that synthesis is not being totally inhibited during the treatment period.

It had previously been shown that the 1,3-diaminopropane-induced inhibition of ODC activity in androgen-stimulated mouse kidney was due to enhanced degradation of protein (Persson *et al.*, 1984). To determine whether our observed changes in activity and DFMO binding were a result of increased degradation rates, we pre-labelled cells with [^{35}S]methionine and analysed immunoprecipitable proteins in cells after a 5 h chase with medium containing 100 μM-ornithine, -putrescine or -spermidine, and qualitatively analysed the recovery of ODC under these conditions. These data are shown in Fig. 5. The exposure of pulse-labelled ODC protein to subsequent exogenous amine led to a measurable decrease in immunoprecipitable protein, as determined by autoradiography. Treatment with no amine addition (lane *b*) showed no change in radiolabelled peptide during the 5 h chase interval. Addition of 100 μM-ornithine (lane *c*), -putrescine (lane *d*) or -spermidine (lane *e*) led to a dramatic decrease in the amount of labelled protein of M_r approx. 53000, even though equivalent total protein contents were applied to each gel lane. These data represent one example of this type of experiment, with replicate experiments all

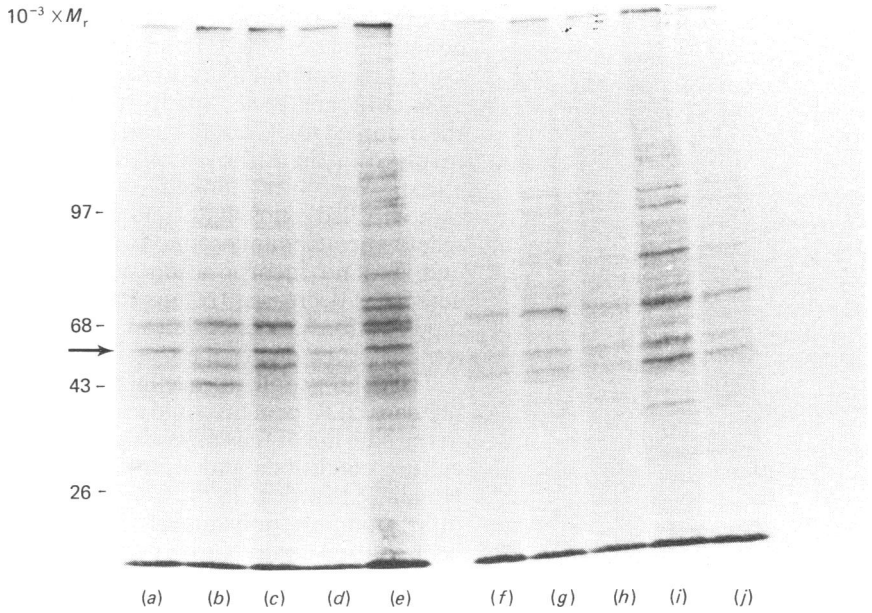

Fig. 4. Immunoprecipitation of newly synthesized proteins in the presence of exogenous 100 μM-ornithine

Ornithine (100 μM) was added to the medium of replicate CHO cultures, and cells were incubated for periods up to 8 h. At various times after ornithine addition, cultures were pulse-labelled with 100 μCi of [^{35}S]methionine/ml for 30 min in methionine-deficient media containing 100 μM-ornithine. Samples were harvested and protein was immunoprecipitated with anti-ODC serum. Lanes (*a*)–(*e*) represent newly labelled proteins immunoprecipitated from cells treated for 0, 2, 4, 6 or 8 h with ornithine. Lanes (*f*)–(*j*) are control lanes treated identically with those in lanes (*a*)–(*e*), but without the addition of the primary antiserum. Arrow points to band corresponding to ODC.

showing very low turnover of ODC in the absence of exogenous polyamine addition. These data indicated that ODC activity was mediated by intracellular polyamine content through mechanisms enhancing the turnover of the protein.

DISCUSSION

We have used immunochemical techniques to analyse the regulation of ODC under conditions of substrate limitation in exponentially growing Chinese-hamster cells. The maintenance of cells in a chemically defined medium allows the elimination of an external ornithine supply (serum ornithine concentration is approx. 4.5 μM; Baetz *et al.*, 1975), thus limiting polyamine accumulation. The cells grown in the defined medium have no detectable putrescine and lowered spermidine contents, and ODC activity is 1–3 units/10^6 cells during exponential-phase growth. The use of the chemically defined medium has allowed us to study the effects of dramatic changes in intracellular putrescine and spermidine concentrations on ODC, without using inhibitors, and should facilitate studies on polyamine regulation of ODC. Restoration of intracellular polyamine pools by addition of exogenous ornithine or putrescine leads to a rapid decay in ODC activity and [^{3}H]DFMO binding (Fig. 2). These data are similar to those reported by Persson *et al.* (1984), who have shown that total ODC protein in androgen-stimulated mouse kidney rapidly declines after treatment with 1,3-diaminopropane. The measured decline in activity after restoration of polyamine pools by ornithine treatment is not due to a total inhibition of synthesis of the protein, as had been postulated (Clark & Fuller, 1975; McCann *et al.*, 1979; Murakami *et al.*, 1985), as isolation of newly synthesized proteins by immunoprecipitation is not decreased after treatments with exogenous ornithine, which diminishes activity to less than 15% of that of untreated controls (Fig. 4). The observed effects appear to be a result of enhanced turnover of the protein, as measured by decreased recovery of immunoprecipitated pre-labelled ODC (Fig. 5) after treatment with the various naturally occurring amines. There is no detectable turnover of the protein in the absence of polyamine (compare Fig. 5, lanes *a* and *b*), and this could explain the increased ODC activity in the cells grown under these conditions (Sertich *et al.*, 1985). The determination of a basal slow rate of ODC turnover, and its relation to the polyamine-mediated turnover in these cells, remain to be determined. These data support the contention that ODC degradation is mediated by intracellular polyamines, yet specific digestion of the protein to individual amino acid has not been demonstrated. An alternative possibility to explain the decreases in recoverable protein observed in Fig. 5 is a translocation of ODC from subcellular compartments (i.e. soluble to insoluble), as has been previously suggested (Seely *et al.*, 1982; Canellakis *et al.*, 1985). Therefore, we have chosen the word 'turnover' to describe our observations until the specific mechanisms of this observation are determined.

The mechanism responsible for enhanced turnover of ODC protein under these conditions is not yet known. The appearance of an ODC-inhibitory protein in response to exogenous amine exposure has been well documented (Canellakis *et al.*, 1979). It has been proposed that this protein could participate in the turnover of ODC by targeting the protein to specific degradative pathways (Persson *et al.*, 1984), similar to the ubiquitin conjugation mechanism described by Ciechan-

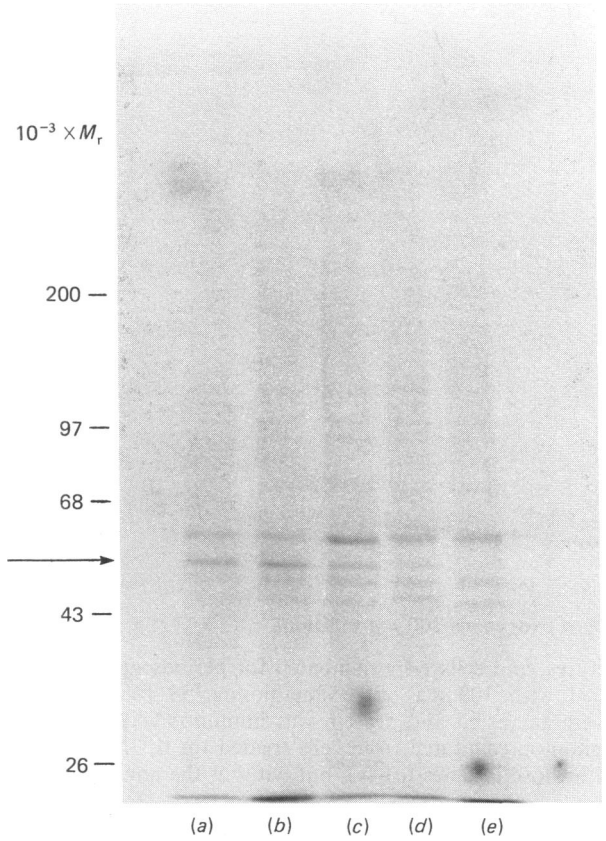

Fig. 5. Effects of 100 μM-ornithine, -putrescine or -spermidine on the turnover of ODC

Cells were labelled with 100 μCi of [^{35}S]methionine/ml for 30 min. Cultures were washed once, and medium without label, but containing 100 μM-ornithine, -putrescine or -spermidine, was added. After a 5 h incubation, cells were harvested and proteins immunoprecipitated with anti-ODC serum. Lanes: (*a*) proteins precipitated from cells immediately after the 30 min labelling period; (*b*) proteins precipitated from cells not treated with any exogenous amine during the 5 h time period; (*c*), (*d*) and (*e*) proteins precipitated from cells treated with ornithine, putrescine or spermidine respectively. Arrow points to band corresponding to ODC.

over *et al.* (1984). We have not yet determined whether the antizyme is induced under these experimental conditions, and if it is modulating the turnover of the protein. Kyriakidis *et al.* (1984) have reported that DFMO will bind to ODC–antizyme complexes, thus predicting an alteration in binding/unit activity after addition of exogenous polyamines. Our data show that the amount of ODC-antibody-immunoreactive protein capable of binding DFMO decreases in parallel with enzyme activity (Fig. 2), such that catalytic activity is not changed after restoration of intracellular polyamine content. It is possible that the complexes do not accumulate, are labile intermediates in the turnover, and therefore are not detectable under these experimental conditions. Further work is needed to determine the involvement of antizyme in polyamine-mediated turnover of ODC.

Polyamine-mediated post-translational modifications of ODC have been reported, including phosphorylation (Atmar & Kuehn, 1981), transglutaminase-mediated conjugation of polyamine (Russell, 1981), and the appearance of multiple forms of the protein (Loeb *et al.*, 1984; Mitchell *et al.*, 1985). It has been postulated that the modified ODC could be an intermediate in a pathway involved in the turnover of the protein (Mitchell *et al.*, 1985). We have looked for the incorporation of radiolabelled phosphate and putrescine into immunoreactive proteins under conditions of polyamine treatment, and find no incorporation of label into ODC as the activity is decreased (results not shown). Whether those modifications are specific to ODC from other cell types remains to be determined.

The ability of intracellular polyamine content to regulate ODC protein could explain measured alterations in the half-life of the enzyme under various growth states, culture conditions and stimuli of activity (Clark, 1974; Hogan & Murden, 1974; Chen & Canellakis, 1977). The utilization of polyamines for functions related to proliferation could release this control of ODC turnover, leading to an increase in the measured half-life of enzyme activity. The stabilization of ODC under conditions of polyamine deprivation has been previously reported (McCann *et al.*, 1977; Mamont *et al.*, 1978; Karvonen & Pösö, 1984), and our results with a cell line which has a normally low intracellular polyamine content in the absence of any inhibitor treatment support these observations. The subsequent re-accumulation of polyamines then accelerates the turnover rate of ODC protein.

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