

Comparison of ornithine decarboxylase from rat liver, rat hepatoma and mouse kidney

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Comparisons were made of ornithine decarboxylase isolated from Morris hepatoma 7777, thioacetamide-treated rat liver and androgen-stimulated mouse kidney. The enzymes from each source were purified in parallel and their size, isoelectric point, interaction with a monoclonal antibody or a monospecific rabbit antiserum to ornithine decarboxylase, and rates of inactivation *in vitro*, were studied. Mouse kidney, which is a particularly rich source of ornithine decarboxylase after androgen induction, contained two distinct forms of the enzyme which differed slightly in isoelectric point, but not in M_r . Both forms had a rapid rate of turnover, and virtually all immunoreactive ornithine decarboxylase protein was lost within 4 h after protein synthesis was inhibited. Only one form of ornithine decarboxylase was found in thioacetamide-treated rat liver and Morris hepatoma 7777. No differences between the rat liver and hepatoma ornithine decarboxylase protein were found, but the rat ornithine decarboxylase could be separated from the mouse kidney ornithine decarboxylase by two-dimensional gel electrophoresis. The rat protein was slightly smaller and had a slightly more acid isoelectric point. Studies of the inactivation of ornithine decarboxylase *in vitro* in a microsomal system [Zuretti & Gravela (1983) *Biochim. Biophys. Acta* 742, 269–277] showed that the enzymes from rat liver and hepatoma 7777 and mouse kidney were inactivated at the same rate. This inactivation was not due to degradation of the enzyme protein, but was probably related to the formation of inactive forms owing to the absence of thiol-reducing agents. Treatment with 1,3-diaminopropane, which is known to cause an increase in the rate of degradation of ornithine decarboxylase *in vivo* [Seely & Pegg (1983) *Biochem. J.* 216, 701–717] did not stimulate inactivation by microsomal extracts, indicating that this system does not correspond to the rate-limiting step of enzyme breakdown *in vivo*.

ODC catalyses the first step of polyamine biosynthesis (reviewed by Jänne *et al.*, 1978; Pegg & McCann, 1982). This enzyme is induced very rapidly and many-fold by various growth-promoting stimuli, including hormones, drugs and toxins, and during instances of compensatory tissue hypertrophy, such as occur after partial hepatectomy or unilateral nephrectomy (Jänne *et al.*, 1978; Russell, 1980; McCann, 1980).

Although numerous suggestions of post-translational modifications or regulatory proteins affecting enzyme activity have been made (Canellakis *et al.*, 1979; Russell, 1981, 1983; Atmar & Kuehn, 1981; Kuehn & Atmar, 1982; Mitchell, 1981; Mitchell *et al.*, 1982; Mitchell & Wilson, 1983), definitive evidence that these mechanisms actually occur under physiological conditions is lacking. On the contrary, it has been found that changes in enzyme activity are accompanied by parallel changes in enzyme protein, as measured by radioimmunoassay or by titration with the irreversible inhibitor DFMO (Metcalf *et al.*, 1978; Seely *et al.*, 1982*a,b,c*; Seely & Pegg, 1983*a,b*; Erwin *et al.*, 1983; Isomaa *et al.*, 1983). These results suggest that total ODC activity is regulated by variations in the amount of enzyme protein. Since activity can change very rapidly, this implies that the protein turns over very rapidly, and a

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Abbreviations used: ODC, ornithine decarboxylase (EC 4.1.1.17); DFMO, α -difluoromethylornithine; SDS, sodium dodecyl sulphate.

variety of techniques have been used to confirm the original observation of Russell & Snyder (1969) that this is indeed the case (Seely *et al.*, 1982c; Seely & Pegg, 1983b; Isomaa *et al.*, 1983; McConlogue & Coffino, 1983a; Persson *et al.*, 1984). At present, little is known of the biochemical mechanism underlying the rapid degradation of ODC protein, although systems for inactivation *in vitro* have been described (Icekson & Kaye, 1976; Pegg & Williams-Ashman, 1981; Zuretti & Gravela, 1983; Murakami *et al.*, 1984; Zuretti *et al.*, 1984). The existence of multiple forms of ODC has been suggested by several investigators, who have noted heterogeneity in the activity when fractionated by column chromatography (Obenrader & Prouty, 1977; Richards *et al.*, 1981; Mitchell & Mitchell, 1982; Pereira *et al.*, 1983). Differences in the half-lives of these forms of the enzyme have also been described (Pereira *et al.*, 1983; Mitchell & Mitchell, 1982).

ODC activity is enhanced in some neoplastic tissues (Russell & Durie, 1978). It has been suggested that the increased activity in cancer cells may be due to an increased stability of the enzyme (Bachrach, 1976; Canellakis *et al.*, 1978, 1979; Gravela *et al.*, 1983). Indeed, increases in ODC half-life have been reported in neoplastic tissue (Bachrach, 1976; Canellakis *et al.*, 1978) as well as an increased stability *in vitro* of enzyme from hepatoma cells in the presence of liver microsomes (microsomal fractions) (Gravela *et al.*, 1983). Since these differences in the stability of ODC from neoplastic tissue may be due to a novel form of the enzyme present in cancer cells, we decided to compare enzymes purified from normal (liver and kidney) and neoplastic tissues (hepatoma). In these comparisons use was made of a monospecific rabbit antiserum raised against ODC (Seely & Pegg, 1983a) and of a monoclonal antibody to ODC (Pegg *et al.*, 1984). The specific labelling of ODC protein by reaction with [5-³H]DFMO (Seely *et al.*, 1982b; Erwin *et al.*, 1983) was also used to identify the enzyme protein. The inactivation of these preparations of ODC *in vitro* was examined by using the microsome-dependent inactivation system described by Zuretti & Gravela (1983).

Experimental

Animals and treatment

Control and Morris-hepatoma-7777-bearing male rats (Buffalo strain, 250–300g) were shipped from Howard University, Washington DC, to the Milton S. Hershey Medical Center. Tumours were transplanted subcutaneously and allowed to grow for 3–4 weeks before removal. These animals were used in comparison of hepatoma with normal liver ODC. Male Wistar rats (300g) were used in all

other experiments involving rat liver microsomes. Male CD-1 mice (25–30g) were used as a source of purified ODC from kidney. The mice were treated with androgens as described by Persson *et al.* (1984). Where indicated, they also received 0.5 mCi doses of L-[³⁵S]methionine (1050 Ci/mmol; New England Nuclear) 30 min before death. Thioacetamide was administered to rats as described by Seely *et al.* (1982b), and animals were killed 19–21 h later. 1,3-Diaminopropane was administered at a dosage of 1 mmol/kg, and animals were killed after 4 h (Seely & Pegg, 1983b).

Purification of ODC

Tumour-bearing animals were killed at 3–4 weeks after inoculation. Livers from thioacetamide-treated non-tumour-bearing animals were removed in parallel and processed in the same way as the tumours. Tissues were homogenized in 2.5 vol. of buffer A, containing 25 mM-Tris/HCl, pH 7.5, 0.1 mM-EDTA and 2.5 mM-dithiothreitol, with a Polytron homogenizer, and centrifuged at 100000g for 40 min. Supernatant was fractionated by (NH₄)₂SO₄ precipitation, DEAE-cellulose chromatography and pyridoxamine-agarose chromatography as described by Seely *et al.* (1982a). Liver and tumour enzymes were purified under identical conditions and at the same time. ODC from androgen-treated mouse kidney was isolated in the same way (Seely *et al.*, 1982a). The same enzyme was also labelled with [5-³H]DFMO (sp. radioactivity 15.8 Ci/mmol) as described by Seely *et al.* (1982b).

Preparation of microsomal extracts

Rat liver microsomal extracts were prepared as described by Zuretti & Gravela (1983). Liver was homogenized in 4 vol. of phosphate-buffered saline (6.8 g of NaCl, 1.69 g of Na₂HPO₄ and 0.2 g of KH₂PO₄ in 1 litre) and centrifuged at 15000g for 15 min. In experiments where the cytosol and microsome fractions were used together, this low-speed supernatant was used as such. In experiments where only crude microsomes were used, the 15000g supernatant was centrifuged at 100000g for 40 min and the pellet (microsomes) reconstituted at the same concentration in phosphate-buffered saline. All incubations of ODC with microsomes, cytosol or a combination of both were carried out at 37°C for the times indicated.

Polyclonal- and monoclonal-antibody precipitation of ODC

Approx. 4 units of either purified liver or tumour ODC was incubated with 1, 0.1, 0.025 or 0.004 μl of antiserum (Seely & Pegg, 1983a) or a solution of a monoclonal antibody (Pegg *et al.*, 1984) in 0.2 ml of buffer B, containing 50 mM-Tris/HCl, pH 7.5,

0.1 mM-EDTA, 2.5 mM-dithiothreitol and 0.02% Brij 35 for 4 h at 0–4°C. Goat anti-mouse IgM (10 µl) was added to each sample containing the monoclonal antibody and incubation was continued overnight at 0–4°C. Then 150 µl of a 10% (w/v) suspension of bacterial protein A in buffer B was added to each sample and incubated for 2 h at 0–4°C with shaking. The pellet was removed by centrifugation at 13000g for 2 min and the supernatant analysed for ODC activity remaining. Parallel experiments were set up in which 1.0 µl of non-immune serum was used. No enzyme activity was precipitated by non-immune serum.

Polyacrylamide-gel electrophoresis and immunoblotting

Two-dimensional electrophoresis was carried out essentially as described by O'Farrell (1975). The second dimension consisted of a separation carried out in 10% polyacrylamide gels. After two-dimensional electrophoresis the enzymes were electrophoretically transferred to nitrocellulose papers as described by Towbin *et al.* (1979). Transfer was performed in 25 mM-Tris/HCl/0.19 M-glycine/0.1% SDS/20% (v/v) methanol, pH 8.3, for 3 h at 60V/gel. Remaining unbound sites on the nitrocellulose sheet were blocked by incubating the paper in 3% (w/v) gelatin in TTBS (20 mM-Tris/HCl, pH 7.5, 0.5 M-NaCl, 0.05% Tween 20) for 50 min. All incubations were performed at room temperature. The paper was then incubated overnight with the antiserum against ODC (Seely & Pegg, 1983a), diluted 1:10000 in TTBS containing 1% gelatin. After three 10 min washes in TTBS, the paper was incubated for 1 h with a 1:2000 dilution of peroxidase-labelled goat anti-rabbit IgG in TTBS containing 1% gelatin. After washing in TTBS for 3 × 10 min, the bound antibodies were detected by incubation for 20 min with 200 ml of a solution containing 4-chloronaphth-1-ol (0.5 mg/ml), 0.015% H₂O₂, 20 mM-Tris/HCl, pH 7.5, and 0.5 M-NaCl. Photographs were taken of the immunoblots within a few hours of development, as the coloured spots faded with time. The photographs were taken on Kodak Technical Pan Film 2415.

[5-³H]DFMO-labelled mouse kidney ODC was incubated with liver microsomes, and cytosol was subjected to polyacrylamide-gel electrophoresis under denaturing conditions as described by Seely *et al.* (1982a). Samples (100 µl) containing labelled enzyme and 1–2 mg of protein were heated to 100°C for 2 min in the presence of 2.5% (v/v) SDS, 5% (v/v) β-mercaptoethanol, 5 mM-dithiothreitol, 20% (v/v) glycerol and 0.001% Bromophenol Blue, and electrophoresis was done as described by Laemmli (1970) on 10%-acrylamide cylindrical

gels, with a 3%-acrylamide stacking gel, for 7 h at 3.5 mA/gel. The gels were then sliced into 2.2 mm slices for determination of radioactivity. The radiolabelled protein was eluted from the gel by shaking overnight in 1.0 ml of NCS II (New England Nuclear, Boston, MA, U.S.A.). The samples were then placed in 10 ml of toluene-based scintillation fluid (LSC 949; New England Nuclear) and counted for radioactivity.

ODC activity determination

This was done by measuring the liberation of ¹⁴CO₂ from [1-¹⁴C]ornithine (Seely *et al.*, 1983a). Incubations were either for 15 or 30 min at 37°C in the presence of 50 mM-Tris/HCl, pH 7.5, 2.5 mM-dithiothreitol, 40 µM-pyridoxal 5'-phosphate, 0.4 mM-L-ornithine and 0.25 µCi of L-[1-¹⁴C]ornithine (New England Nuclear; sp. radioactivity 57 Ci/mol). One unit of enzyme activity is defined as the amount that will produce 1 nmol of CO₂ in 30 min.

Results

Comparison of the physical and immunological properties of rat liver, hepatoma and mouse kidney ODC

The mouse kidney after androgen induction is by far the richest source of mammalian ODC, except for cells adapted to grow in the presence of ODC inhibitors (Seely *et al.*, 1982b; McConlogue & Coffino, 1983a,b). The cytosolic protein in mouse kidney was labelled by administration of [³⁵S]methionine 30 min before death, and the proteins present were fractionated by two-dimensional gel electrophoresis (Fig. 1). Two distinct but closely related spots were identified as ODC. These spots have the same *M_r* (about 55000) and slightly different pI values (around 5.4). They were identified as ODC on the following evidence. First, as shown in Fig. 1(c), treatment of the extract with an antibody to ODC removed these two spots selectively. Second, as shown in Fig. 1(b), addition of [³H]DFMO-labelled ODC to the extracts specifically enhanced these two spots. The [³H]DFMO-labelled ODC preparation alone gave only these two spots (results not shown). The binding of DFMO to ODC (Figs. 1a and 1b) caused a small increase in the isoelectric point of both of the ODC spots (Fig. 1d). This increase is similar to that reported by McConlogue & Coffino (1983a) and Persson *et al.* (1984).

The ODC protein could also be detected on two-dimensional gels by transferring the protein to nitrocellulose filters and immunoblotting with anti-ODC serum. Again, two spots were detected (Fig. 2b). After immunoblotting, the nitrocellulose filter was placed in contact with a photographic

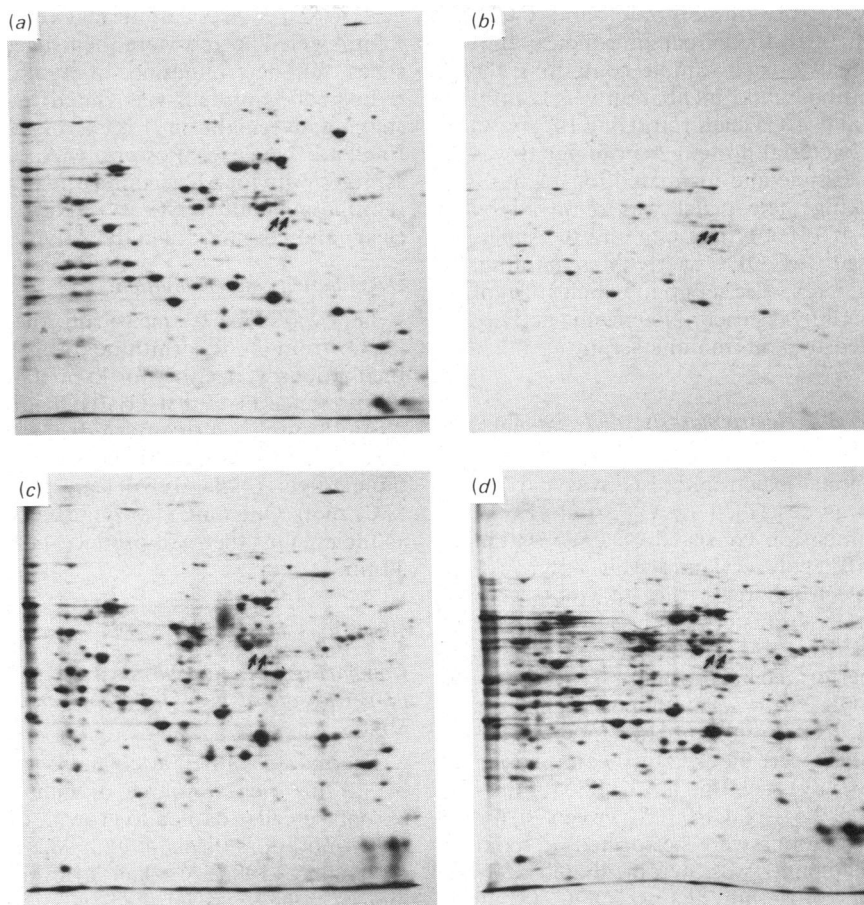


Fig. 1. Identification of mouse kidney ODC on two-dimensional gel electrophoresis

Total [^{35}S]methionine-labelled mouse kidney soluble proteins were separated as described in the Experimental section. In panel (a), the extract was allowed to react with unlabelled DFMO, and a $10\ \mu\text{l}$ extract loaded on the gel. In panel (b), $2\ \mu\text{l}$ of the same extract was used and supplemented with $8\ \mu\text{l}$ of a solution of [^3H]DFMO-labelled ODC (about 8000 c.p.m.). The two spots arrowed are the only ones enhanced by supplementation with this labelled ODC. [Note that the dye front moved further in panel (b), but that all the major spots seen in panel (a) occur in the same relative positions.] In panel (c) the extract was treated with anti-ODC serum and the precipitate removed before electrophoresis. In panel (d), a control antiserum was used. The gels were then subjected to autoradiography as described by Persson *et al.* (1984). Panels (c) and (d) were exposed to the film for 4 weeks, panel (a) for 2 weeks and panel (b) for 3 weeks. The gels are shown with the isoelectric-focusing dimension from left to right (acidic end on the right) and with the highest- M_r region at the top.

film to detect the radioactive proteins (Fig. 2a). The indicated spots correspond to those detected by immunoblotting and those identified as ODC in Fig. 1. A faint third immunoreactive spot with a more acidic pI was also observed (Figs. 2a and 2b), but this may represent a degradation product, since it was not always seen in fresh extracts. These experiments indicate that (a) the rabbit antiserum is highly specific for ODC, since no other proteins reacted with it, and (b) mouse kidney ODC exists in two (or possibly three) forms with the same M_r ,

but different pI values. No other forms amounting to more than 5% of the total ODC can be present, since it is known that the antiserum used removes all of the ODC from mouse kidney homogenates (Persson *et al.*, 1984).

ODC is a very minor component of the total soluble protein in mammalian cells. Even in the androgen-induced mouse kidney it represents only about 1 part in 10000 of the soluble protein (Seely *et al.*, 1982b), and ultracentrifuged homogenates of this tissue had ODC activities about 100–200 times

greater than that of extract from rat liver or hepatoma 7777 (Table 1). It was therefore necessary to partially purify the enzyme from these sources before its properties could be examined. A purification of 1500–5000-fold was obtained by using $(\text{NH}_4)_2\text{SO}_4$ fractionation, DEAE-cellulose chromatography and affinity chromatography on

pyridoxamine phosphate-agarose as previously described for the mouse kidney enzyme (Seely *et al.*, 1982a). Both enzymes were eluted in the same positions from these columns. The yield of enzyme was about 30–40% (Table 1), and no discrete peaks of enzyme activity that could represent different forms of the enzyme were observed and discarded. Also, mouse kidney ODC put through the same purification procedure still contained the two forms described above. These observations make it unlikely that discrete forms of ODC were lost during the purification, although this possibility cannot be entirely ruled out.

The rabbit antiserum to mouse kidney ODC completely precipitated ODC from rat liver and hepatoma (Fig. 3), and was therefore suitable for examining these proteins by the immunoblotting technique. Also, this antiserum (Fig. 3a) and a monoclonal antibody to mouse kidney ODC (Fig. 3b) had exactly the same effects on both the liver and hepatoma enzyme, indicating that the enzymes contained no differences in the sites recognized by these antibodies (Fig. 3).

Examination of the rat liver and hepatoma ODC by the immunoblotting technique indicated that each consisted of a single form of M_r about 50000 and pI of about 5.3 (Fig. 4). When the extracts from the two sources were combined, only one spot was detected, indicating that the liver and hepatoma ODC are indistinguishable on two-dimensional gel electrophoresis. It is possible that subtle differences exist in the amino acid sequence which do not change the M_r or pI, but at present there is no evidence that the hepatoma ODC is in any way different from that present in normal rat liver after induction with thioacetamide. The rat ODC is clearly distinguishable from the two forms in mouse kidney, since it has a slightly smaller M_r and slightly more acidic pI (Fig. 4).

Degradation of ODC

ODC activity is lost very rapidly *in vivo* after inhibition of protein synthesis, suggesting that the

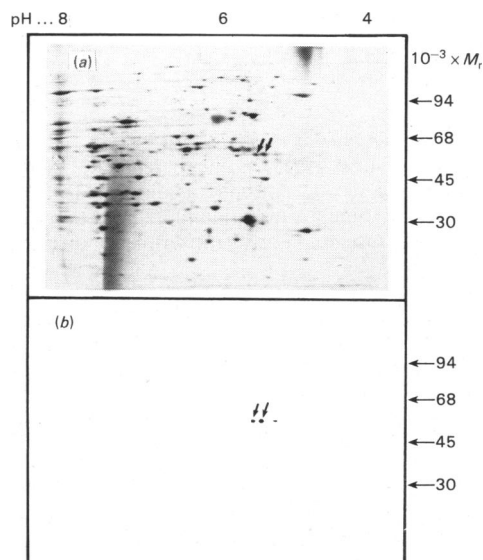


Fig. 2. Immunoblotting of mouse kidney ODC

A 30 μl sample of a homogenate prepared from mouse kidney after administration of $[^{35}\text{S}]\text{methionine}$ was subjected to two-dimensional electrophoresis as in Fig. 1. The proteins were then transferred electrophoretically to nitrocellulose paper and the spots corresponding to ODC identified by immunoblotting as described in the Experimental section (panel b). The paper was then placed in contact with LKB Ultrafilm for 4 weeks and the film developed to indicate the total proteins present (panel a). Approximate positions of M_r markers and the pH are indicated.

Table 1. Specific activities of ODC preparations from rat liver, rat hepatoma and mouse kidney
For details see the Experimental section. A unit of activity catalyses the releases of 1 nmol of $\text{CO}_2/30\text{min}$ under standard assay conditions.

Source of enzyme	Specific activity of 100000g fraction (units/mg)	Specific activity of eluate from pyridoxamine phosphate-agarose (units/mg)	Yield of enzyme after pyridoxamine phosphate-agarose (%)
Thioacetamide-induced rat liver	1.4	2100	31
Hepatoma 7777	0.7	3200	43
Androgen-induced mouse kidney	144	38400	36

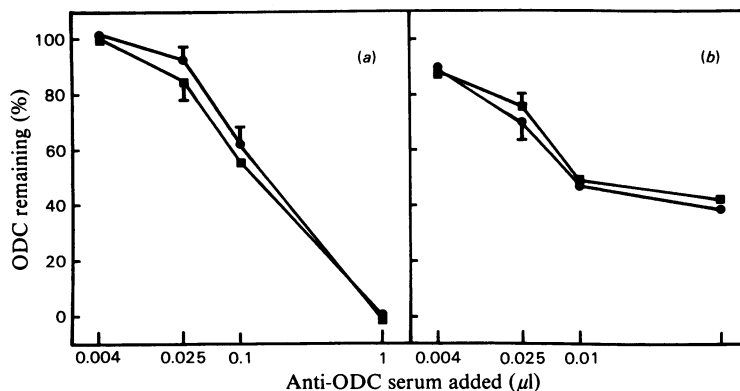


Fig. 3. Immunoprecipitation of ODC from hepatoma and rat liver

Approx. 4 units (100000g supernatant) of ODC from hepatoma (●) or liver (■) was incubated with rabbit antiserum to ODC (a) or monoclonal antibody to ODC (b), followed by a second antibody as described in the Experimental section. The amount of the original enzyme activity remaining soluble after immunoprecipitation was then determined and expressed as a percentage of that remaining after exposure to a control antiserum not directed against ODC.

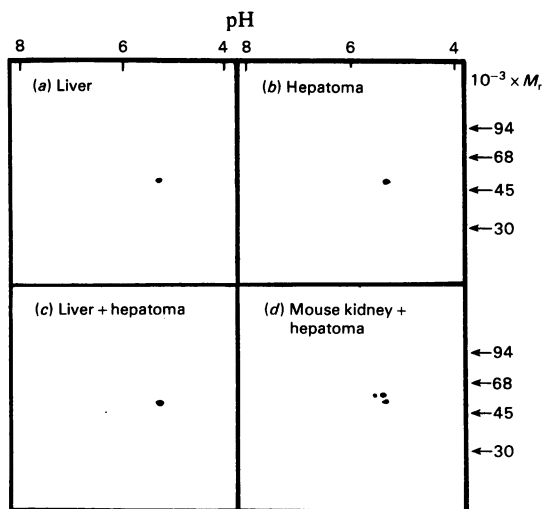


Fig. 4. Immunoblotting of rat liver, rat hepatoma and mouse kidney ODC

Samples of partially purified ODC from rat liver (a; 25 units), rat hepatoma (b, 30 units), rat liver (25 units)+rat hepatoma (30 units) (c) and rat hepatoma (30 units)+mouse kidney (40 units) (d) were subjected to two-dimensional gel electrophoresis, transferred to nitrocellulose paper and detected by immunoblotting as in Fig. 2.

tered to androgen-treated mice and, at various times later, extracts were prepared, fractionated on polyacrylamide gels in the presence of SDS, transferred to nitrocellulose paper, and ODC protein was detected by immunoblotting. There was a marked decline in the protein by 1 h, and within 4 h no ODC protein remained (Figs. 5a and 5b). [The faint band of higher M_r detected in these samples is not ODC, and is present in samples treated with a control antiserum (Fig. 5c).] When crude extracts from these samples were stored frozen at -20°C for a few days before analysis, an ODC band of lower M_r appeared (Fig. 5b). This is presumably due to proteolytic degradation (Persson *et al.*, 1984) and could be an intermediate in the breakdown of the protein *in vivo*, but this band did not accumulate after inhibition of protein synthesis and was not seen in extracts processed immediately without storage (results not shown).

In attempts to demonstrate the rapid degradation of ODC *in vitro* and to confirm suggestions that liver and hepatoma ODC differ in their turnover rates (Gravela *et al.*, 1983), we incubated ODC with microsomes and cytosol preparations as described by Zuretti & Gravela (1983). In agreement with their results, mouse kidney ODC activity was quite stable in the presence of cytosol, but was lost rapidly when microsomes were present (Table 2). Pyridoxal phosphate stabilized the enzyme against inactivation by microsomes, but addition of cytosol as well as microsomes slightly increased the rate of loss. We then tested the effect of microsomes on the liver and hepatoma ODC. As shown in Fig. 6, there was no difference between liver and hepatoma ODC in the loss of activity

protein itself turns over very rapidly (Russell, 1980; McCann, 1980; Seely *et al.*, 1982c). Direct proof that the loss of enzyme activity is due to degradation of the enzyme protein is shown in Fig. 5. In this experiment, cycloheximide was adminis-

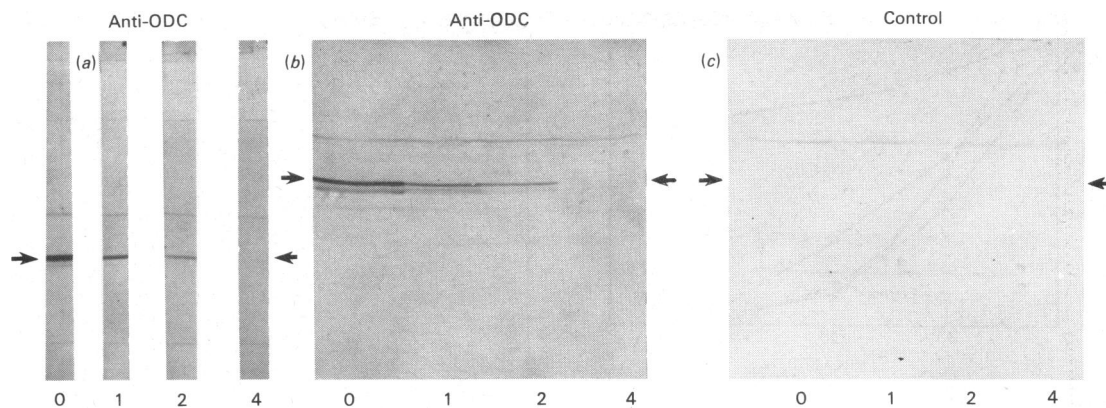


Fig. 5. Turnover of ODC protein *in vivo*

Mice were treated with androgens to induce ODC (Seely *et al.*, 1982b) and then with cycloheximide (10 mg/kg) to inhibit protein synthesis. At the times indicated (in h), kidneys were removed, homogenized, and 0.025 ml samples were separated by polyacrylamide-gel electrophoresis on 10% gels in the presence of SDS, transferred to nitrocellulose paper, and the band corresponding to ODC was detected by immunoblotting. Panel (a) shows results obtained with extracts analysed immediately; panels (b) and (c) show results for extracts analysed after storage for 2 days at -20°C . Panels (a) and (b) were developed with anti-ODC serum and panel (c) was developed with a control rabbit serum. The arrows indicate the position of a marker preparation of purified ODC. The experiment in panel (a) was carried out in a different electrophoretic cell from that shown in (b) and (c).

Table 2. Loss of ODC activity on incubation *in vitro*

Microsomes and cytosol were prepared from rat liver as described in the Experimental section and incubated with purified mouse kidney ODC (70 units per 0.35 ml total volume).

Additions	Incubation time (min)	ODC activity (units/sample)
Microsomes + cytosol	0	10.0 ± 0.5
Cytosol	60	8.7 ± 0.4
Microsomes	60	2.5 ± 0.5
Microsomes + cytosol	60	1.2 ± 0.2
Microsomes + pyridoxal phosphate	60	5.2 ± 0.6

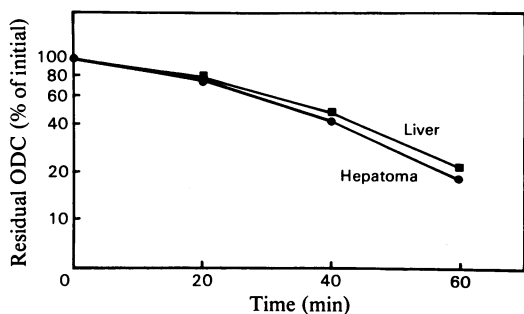


Fig. 6. Comparison of rates of loss of ODC activity from liver and hepatomas in presence of microsomes

Samples (0.06 ml) of liver (■) or hepatoma (●) partially purified ODC were incubated with 1.69 ml of a suspension of rat liver microsomes at 37°C for the times indicated. The residual ODC activity is expressed as a percentage of that present at zero time, which was 17 units/sample for the liver enzyme and 7 units/sample for the hepatoma enzyme.

when they were incubated with liver microsomes. The activity declined with a half-life of about 30 min in both cases (Fig. 6). An approximately similar decline in mouse kidney ODC activity was observed on incubation with crude kidney homogenates (Fig. 7). However, the addition of Mg^{2+} -ATP, an essential cofactor in the cell-free protein-degradation system described by Etlinger & Goldberg (1977), did not stimulate loss, and actually slightly stabilized the activity (Fig. 7). The addition of 2 mM-spermidine, -spermine, or -putrescine also had no effect on the rate of enzyme inactivation *in vitro* (results not shown).

It is well known that ODC activity is dependent on the presence of thiol reducing agents such as dithiothreitol (reviewed by Pegg & Williams-Ashman, 1981). The loss of ODC activity on incubation with microsomes under the conditions of Zuretti & Gravela (1983) in the absence of dithiothreitol may therefore be due to the absence of reducing agents. To test this possibility, ODC

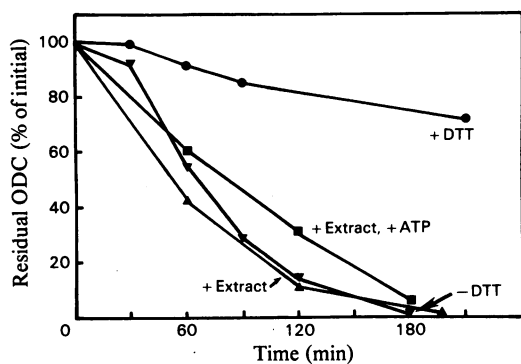


Fig. 7. Loss of ODC activity on incubation *in vitro*. Purified mouse kidney ODC (150 units) was incubated in a total volume of 0.25 ml containing 1.5 mg of bovine serum albumin + 5 mM-dithiothreitol (+ DTT, ●), or 1.5 mg of bovine serum albumin alone (- DTT, ▼), or 0.1 ml of kidney homogenate (+ Extract, ▲), or 0.1 ml of kidney homogenate + 5 mM-ATP + 2.5 mM-MgCl₂ (+ Extract, + ATP, ■). The ODC activity remaining at the time shown is expressed as a percentage of that initially present.

was incubated with bovine serum albumin in the presence and absence of 5 mM-dithiothreitol (Fig. 7). The activity was stable in the presence of dithiothreitol, but was lost in its absence almost as rapidly as in the presence of the tissue extracts. Furthermore, there was no indication of any proteolytic degradation of ODC in the experiment of Figs. 6 and 7 even when all the activity was lost. The absence of protein degradation was indicated by: (a) radioimmunoassay, as described by Seely & Pegg (1983a), which revealed no change in the immunoreactive protein (results not shown); (b) immunoblotting of the ODC protein after separation by polyacrylamide-gel electrophoresis, which showed no change in the size of the protein (results not shown); and (c) when [³H]DFMO-labelled ODC was incubated under the same conditions and then separated by polyacrylamide-gel electrophoresis and detected by autoradiography there was no decrease in the size or intensity of the labelled band (Fig. 8).

These results suggest that the loss of ODC activity in these systems is related to oxidative inactivation of the protein. Such inactivation may or may not be related to its rapid turnover *in vivo*. In order to test the physiological relevance of the loss of activity *in vitro*, extracts were prepared from rat liver after treatment with 1,3-diaminopropane. It is known that such treatment increases the rate of turnover of ODC *in vivo* and that the increase can be prevented by blocking protein synthesis at

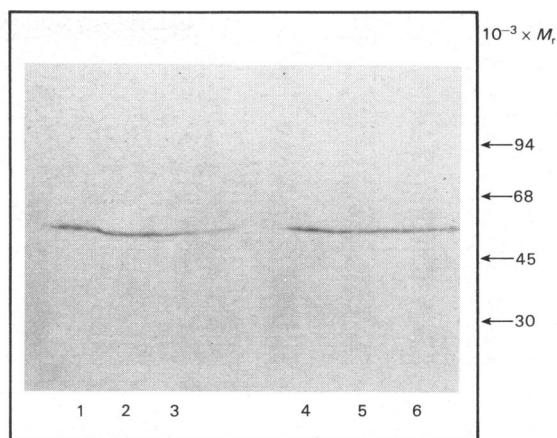


Fig. 8. Lack of degradation of ODC protein on incubation *in vitro*.

Partially purified mouse kidney ODC was labelled by reaction with [³H]DFMO, and samples equivalent to 30000 c.p.m. were incubated with bovine serum albumin (lanes 1 and 4), mouse kidney homogenate (lanes 2 and 5) and rat liver microsomes (lanes 3 and 6) as in Figs. 6 and 7. Samples were removed at zero time (lanes 1-3) and after 180 min (lanes 4-6), fractionated by SDS/10% polyacrylamide-gel electrophoresis, and the labelled protein was detected by autoradiography. Approximate positions of M_r markers are indicated.

the same time as treatment with 1,3-diaminopropane (Seely & Pegg, 1983b). As shown in Table 3, extracts from liver of rats treated with 1,3-diaminopropane and with 1,3-diaminopropane plus cycloheximide were equally potent (and not different from control untreated liver extracts) in causing the inactivation of ODC.

Discussion

Our results do not provide any support for the hypothesis that rat liver or hepatoma ODC exists in multiple forms (Richards *et al.*, 1981; Mitchell & Mitchell, 1982; Pereira *et al.*, 1983). It is possible that minor forms of ODC were lost during the partial purification procedure needed to enrich the preparations before electrophoresis and immunoblotting, but no sign of heterogeneity of the preparations was seen during this procedure and the two forms of mouse kidney ODC are preserved by the same purification. Only a single protein interacting with the antiserum to ODC was found on two-dimensional electrophoresis (Fig. 4), and all of the ODC activity in crude extracts is inhibited by the antiserum (Fig. 3). One possible explanation for the heterogeneity seen by others is

Table 3. Loss of ODC activity on incubation with liver extracts from rats treated with 1,3-diaminopropane

Samples of purified mouse kidney ODC (500 units) were incubated with rat liver cytosol + microsomes (20 mg of protein; prepared as described in the Experimental section) in a volume of 1 ml. Samples were removed at zero time, 20 min and 40 min for measurement of ODC activity, which is expressed as the percentage of that present at zero time.

Treatment of rat	Time of incubation (min)	ODC activity remaining (%)
1,3-Diaminopropane (1 mmol/kg) at 4h before death	0	100
	20	74 ± 5
	40	48 ± 7
1,3-Diaminopropane (1 mmol/kg) + cycloheximide (10 mg/kg), both at 4h before death	0	100
	20	68 ± 8
	40	44 ± 6

the presence of proteins which bind to ODC and change the chromatographic properties on DEAE-Sephrose, which was used to isolate the putative multiple forms (Richards *et al.*, 1981; Mitchell & Mitchell, 1982; Pereira *et al.*, 1983). Whether such proteins play any role in the regulation of ODC activity *in vivo* remains to be determined. Another possible artifact that can give rise to apparent multiple forms of ODC is partial proteinase degradation, since we have shown that mouse kidney ODC can readily be degraded to smaller forms, which retain enzyme activity and immunoreactivity (Persson *et al.*, 1984).

The rat liver or hepatoma ODC is very closely related to the two forms of mouse kidney ODC, but can be separated from them by polyacrylamide-gel electrophoresis (Fig. 3). This is consistent with the similar, but not identical, behaviour of the rat and mouse ODC in radioimmunoassay (Seely & Pegg, 1983b) and the slightly different catalytic centre activity (turnover number) determined by titration with DFMO (Seely *et al.*, 1982b).

The pI reported for ODC in the present paper (approx. 5.3–5.4) is markedly higher than that previously reported by us (Seely *et al.*, 1982a) and by others (Ono *et al.*, 1972; Kameji *et al.*, 1982), based on studies with the native enzyme. This discrepancy is probably due to a conformational change in the presence of high concentrations of urea (Ui, 1973), since the present value is in closer agreement with those reported by McConlogue & Coffino (1983a), Choi & Scheffler (1983) and Kitani & Fujisawa (1983) based on electrophoresis under these conditions. The acidic pI of ODC is consistent with the hypothesis that proteins with low pI values are degraded more rapidly (Dice & Goldberg, 1975).

As shown in Fig. 5, the rapid turnover of ODC activity is reflected in the complete loss of immunoreactive protein as detected by Western immunoblotting. This is consistent with data on

loss of ODC protein after inhibition of protein synthesis (Isomaa *et al.*, 1983; Seely & Pegg, 1983a,b), or in pulse-labelling experiments (Seely *et al.*, 1982c; McConlogue & Coffino, 1983a; Pegg *et al.*, 1984; Persson *et al.*, 1984). Therefore there is no doubt that the apparent rapid turnover of ODC is due to the rapid synthesis and degradation of the protein, although post-translational modifications or binding of additional proteins may facilitate the degradation (Canellakis *et al.*, 1979; Kuehn & Atmar, 1982; Seely & Pegg, 1983b). The development of an appropriate cell-free system in which this degradation of ODC protein could be studied would be a significant advance. However, ODC is a very labile enzyme, which is well known to be very readily inactivated *in vitro*, particularly in the absence of thiol reducing agents and non-ionic detergents (Pegg & Williams-Ashman, 1981; Seely *et al.*, 1982a). Whether the formation of inactive aggregates in the absence of dithiothreitol or 2-mercaptoethanol, which was first observed by Jänne & Williams-Ashman (1971), is related to the physiological process responsible for ODC breakdown is unknown at present, but the results shown in Table 3 indicate that inactivation in the system described by Zuretti & Gravela (1983) is unlikely to be a rate-limiting step in ODC degradation. Treatment with 1,3-diaminopropane is known to increase the rate of ODC degradation in rat liver (Seely & Pegg, 1983b), but microsomes prepared from rats receiving 1,3-diaminopropane were not more active in bringing about the loss of ODC activity. In fact, microsomes were no more active than was incubation in the presence of bovine serum albumin and the buffer lacking dithiothreitol (Fig. 7), and no actual degradation of the ODC protein occurred on incubation with microsomes (Fig. 8). Therefore this system seems unlikely to be a physiologically relevant model for ODC degradation. The cysteine-dependent inactivation of ODC described by Murakami *et al.*

(1984) may prove to be more valuable in this respect.

Since we were unable to find any differences between ODC from normal and malignant liver, our results do not support the hypothesis that the appearance of a new, longer-lived, form of ODC plays a role in the neoplastic state. However, one possible way in which our results could be reconciled with previous work would involve the presence of other factors in the crude ODC preparations used by Gravela *et al.* (1983). Such factors could change the sensitivity of ODC to inactivation, as suggested by Zuretti *et al.* (1984), and might regulate polyamine accumulation *in vivo*.

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