Ornithine Decarboxylase from Hepatoma Cells and a Variant Cell Line in Which the Enzyme Is More Stable*

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The half-life of ornithine decarboxylase in HMOA cells, a variant cell line derived from hepatoma tissue culture (HTC) cells, was 5 to 10 h compared to 14 min in the parental cell line. The half-lives of two other rapidly turning over proteins, S-adenosylmethionine decarboxylase and tyrosine aminotransferase, as well as the turnover of total cellular protein, were the same in the two types of cells, suggesting that other proteins did not share the alteration in turnover observed with ornithine decarboxylase. The possible existence of a mutant enzyme was investigated by purifying ornithine decarboxylase 8000-fold from HTC and HMOA cells, the key step being a pyridoxine-phosphate affinity column. The enzymes from both cell types behaved in an identical manner throughout the purification procedure, were equally thermostable, and were inhibited in a similar manner by antiserum to ornithine decarboxylase and by ornithine decarboxylase antizyme. The $K_m$ of the two purified enzymes for L-ornithine were approximately the same (0.04 mM) as were the $K_{i}$s for putrescine (0.14 mM). Studies employing [14C]difluoroornithylornithine, a radioactive irreversible inhibitor that specifically labeled ornithine decarboxylase, showed that the enzyme from both cell types had a subunit $M_r$ of 64,000. The amounts of ornithine decarboxylase in the soluble fractions of logarithmically growing HTC and HMOA cells, as determined by binding of the inhibitor, were 3.5 and 11.0 ng/mg protein, respectively. It is concluded that the increased half-life of ornithine decarboxylase in HMOA cells is not due to a more stable form of the enzyme, the alternative possibility being that a specific deactivation system for the enzyme is altered in these cells.

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EXPERIMENTAL PROCEDURES

Materials—Labeled [14C]difluoroornithylornithine (60 mCi/mmol) was synthesized by Amer sham Corp. according to the method of Metcalf et al. (15). Unlabeled α-difluoromethylornithine as well as the HTC and HMOA cell stocks were kindly provided by Dr. P. P. McCann of Merrell Research Center, Cincinnati, OH.

Maintenance of HTC and HMOA Cells in Culture—Cells were maintained in suspension (spinner) culture in Swim’s medium supplemented with 10% newborn calf serum (16). Sixteen hours prior to experimentation, the cells were diluted into fresh medium to a

otes, yeast and slime mold, enzyme activity is lost in response to polyamines. This loss is brought about in yeast by an as yet unknown post-translational modification (5), and in Physarum by the conversion of an active into an inactive form (6, 7) which may involve phosphorylation (8). The presence of a macromolecular inhibitor of the enzyme which is induced by polyamines has been observed in mammalian cells (9, 10), and ornithine decarboxylase activity can be lost as a result of transamination (11). However, there is a great deal of evidence supporting the concept that ornithine decarboxylase activity in mammalian cells is regulated primarily by the amount of enzyme present (2-4, 12). The rapid turnover of the enzyme protein provides a ready means by which the amount of enzyme can be adjusted. Therefore, understanding the regulation of turnover of this protein could offer much towards answering both how the biosynthesis of the polyamines and the degradation of a short half-life protein are controlled within the cell.

Recently, a clone of hepatoma tissue culture (HTC) cells designated HMOA has been isolated and characterized by Mamont et al. (13). These cells are able to grow normally in the presence of α-methylornithine, a competitive inhibitor of ornithine decarboxylase, whereas HTC cells cultured under the same conditions grow at a greatly reduced rate. The HMOA cells have a higher specific activity of ornithine decarboxylase as well as higher intracellular quantities of putrescine throughout the cell cycle. Overproduction of putrescine has been suggested to be responsible for making the HMOA cells more resistant to α-methylornithine than the parental cell line (13). However, of more importance for the purpose of this investigation has been the finding that the half-life of ornithine decarboxylase activity in HMOA cells is greatly increased compared to the half-life of the enzyme in HTC cells (14).

At present, there is no explanation for the prolonged half-life of ornithine decarboxylase in HMOA cells. However, there are at least two general possibilities that could account for the difference in half-lives observed in the two cell types. Firstly, ornithine decarboxylase may be structurally altered to a more stable form in the HMOA cells. Alternatively, one or more components of the system responsible for the degradation of the enzyme may not be the same in HMOA cells. The first of these two possibilities was investigated in the present study.

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density of 1.5 to 1.7 x 10^7 cells/ml and incubated at 37 °C to induce ornithine decarboxylase activity.

Enzymatic Assays—Ornithine decarboxylase activity was measured at a saturating concentration of L-[1-14C]ornithine (0.82 mM, 1.22 Ci/mmol) by the 14CO2 trapping method described by Pegg and Williams-Adamson (17). Samples were prepared for this assay by the following steps: the eluents containing enzyme activity were centrifuged (Sorvall GRC-1) at 800 x g for 5 min at room temperature. The cell pellet was washed twice in phosphate-buffered saline (0.12 M NaCl, 12 mM Na2HPO4, 1.5 mM KH2PO4) and resuspended in 1 ml of Buffer A (20 mM Tris-HCl, pH 7.5; 0.1 mM EDTA; 2.5 mM dithiothreitol); then the cells were lysed by two cycles of freeze-thawing employing liquid N2. After storage on ice for 30 min, the suspension of disrupted cells was centrifuged at 17,000 x g for 30 min at 4 °C to yield a postmitochondrial supernatant. This fraction was either assayed directly for ornithine decarboxylase activity or frozen at -20 °C for assays the following day. Each nmol of CO2 released per 30 min is equivalent to one unit of enzyme activity.

S-Adenosylmethionine decarboxylase activity was determined by 14CO2 trapping (18) in the same manner as described for ornithine decarboxylase except the concentration of S-adenosyl-l-[carboxyl-14C] methionine (17 µM, 45 Ci/mmol) was nonlimiting and the assay conditions were as before. Samples for the assays were incubated like those for the ornithine decarboxylase assay except that more cells were needed per sample (10^6 cells). Moreover, the cell pellet was resuspended in 1 ml of 50 mM NaPO4 buffer (pH 7.0), 0.1 mM EDTA, and 0.5 mM dithiothreitol.

For the assay of tyrosine aminotransferase, the procedure of Diamandoupolos and Strickland (19) was employed. In order to have sufficient tyrosine aminotransferase activity that could be followed reliably after the addition of cycloheximide, the enzyme was induced for 16 h with dexamethasone (10 ^-3 M Decadron phosphate; Merck Sharp and Dohme). Cell samples were processed as described above; only the washed cell pellet was resuspended in 0.5 ml of 0.14 M KCl and assayed immediately for enzyme activity.

Protein Determination—Protein was quantified by the Coomassie brilliant blue dye-binding method of Bradford (20) using bovine serum albumin as the protein standard. The dye was prepared according to procedure B of Veisberg et al. (21).

Measurement of Protein Synthesis in Cell Cultures—Protein synthesis was determined by measuring the incorporation of L-[3H]leucine into protein. After the cells were diluted into fresh medium and preincubated as described above, aliquots (10 ml) were incubated for 10 min at 37 °C, then L-[4,5-3H]leucine (200 µCi, 26 Ci/mmol) was added to each flask to start the period of label incorporation. After another 80 min of incubation, the cells in each flask were pelleted, washed twice with phosphate-buffered saline, and resuspended in Buffer A as outlined in the assay of ornithine decarboxylase activity, except that the samples were not centrifuged after the two cycles of freeze-thawing. Instead, 10 ml of the suspension of disrupted cells were spotted in triplicate onto filters (Grade No. 540, Whatman), the protein was precipitated with acid, and the filters were prepared for the determination of radioactivity (22).

Purification of Ornithine Decarboxylase—Highly purified ornithine decarboxylase (approximately 8000-fold purified) was prepared from HTC and HMOA cells as follows. HTC and HMOA, cytosol was prepared as described for the assay of ornithine decarboxylase activity except the cell pellet from 1 liter of cell suspension was resuspended in 5 ml of Buffer A. The postmitochondrial supernatant was frozen in liquid N2 and stored at -70 °C until sufficient material from both cell types had been collected to proceed. Subsequently, the postmitochondrial supernatants from the respective cell types were pooled and fractionated with (NH4)2SO4. Ornithine decarboxylase was then purified as described previously for the enzyme from rat liver (23). The key step in this scheme was affinity chromatography using pyridoxal phosphate linked to agarose. The affinity resin was prepared by using Affi-Gel 10 (Bio-Rad) as the agarose support (23). Brj-35 (0.03%), poloxamine (23) lauryl ether) was present in Buffer A throughout the affinity chromatography procedure. As with the rat liver enzyme (23), inclusion of detergent was found to stabilize ornithine decarboxylase activity making experiments with the purified enzyme possible. After these experiments were completed, a recent report from Kitani and Fujisawa (24) confirmed that another detergent, Tween 80, can also stabilize the activity of purified ornithine decarboxylase from rat liver.

In some studies (Figure 5a and Table II), chromatography on DEAE-cellulose was utilized from the purification procedure. Under these conditions, enzyme eluting from the affinity column was typically purified 400-fold.

Isolation of Protein Labeled with [14C]Difluoromethanamine—Protein-bound radioactivity in samples which had been incubated with radiolabeled difluoromethanamine was determined as follows. An equal volume (0.5 or 1 ml) of 1 M perchloric acid was added to samples that had been appropriately radiolabeled. To each tube, an additional 2.5 ml of 0.5 M perchloric acid was added. After a 10-min incubation on ice, the samples were placed in a boiling water bath for 10 min; the boiling step was omitted when enzyme eluting from DEAE-cellulose was precipitated. A pellet of the precipitate was obtained by centrifugation for 10 min at top speed in a TJ-6 centrifuge (Beckman Instruments). After decanting the supernatant, the pellet was resuspended in 4 ml of 0.5 M perchloric acid and the suspension was centrifuged as above. The supernatant was again decanted and the 0.5 M perchloric acid wash was repeated. The resulting pellet was washed twice with 3 ml of ethanolchloroform (3:2:1) and finally with 3 ml of ether. The dried pellet was dissolved in 0.5 ml of 0.1 M NaOH and quantitatively transferred to a 20-ml scintillation vial with 10 ml of Liquisint (National Diagnostics). Radioactivity was determined and moles of label incorporated per mg of protein were calculated.

Polyacrylamide Gel Electrophoresis—The discontinuous buffer system of Laemmli (25) was employed for the separation of proteins by polyacrylamide gel electrophoresis. Electrophoresis was performed in tube gels which consisted of 9 cm of resolving gel (7.5% acrylamide; 0.375 M Tris-HCl, pH 8.8; 0.1% sodium dodecyl sulfate) topped with 1.5 cm of stacking gel (5% acrylamide; 0.125 M Tris-HCl, pH 6.8; 0.1% sodium dodecyl sulfate). Samples were concentrated by precipitation in 17% trichloroacetic acid. A pellet of the acid-precipitable material was obtained by centrifugation at 10000 x g for 4 min in a microtuge (Brinkmann Instruments). The supernatant was carefully removed by aspiration and acetone (600 µl) was washed down the side of the tube with care not to disturb the pellet. After centrifugation at 8000 x g for 4 min, the acetone was removed by aspiration and another acetone wash was performed. The pellet was resuspended in 100 µl of solubilization buffer containing 0.125 M Tris-HCl (pH 6.8, 20% glycerol (v/v), 1% 2-mercaptoethanol, 0.1% sodium dodecyl sulfate, and 0.001% bromphenol blue). To resolubilize the samples, it was necessary to heat them in boiling water for up to 5 min. Molecular weight standards were included with each set of samples subjected to electrophoresis.

RESULTS

Measurement of the Half-lives of Rapidly Turning Over Proteins—Fig. 1A depicts the measurement of the half-life of ornithine decarboxylase activity in HTC and HMOA cells using cycloheximide to inhibit protein synthesis. Under these conditions, the half-life of enzyme activity in HMOA cells was increased almost 50-fold over that measured in HTC cells. The same results were obtained when puromycin (0.42 µg/ml), another inhibitor of protein synthesis, was tested (data not shown). These results were not due to differing effects of the inhibitors on protein synthesis in the two cell types. Since protein synthesis, measured by the incorporation of [3H]leucine, was inhibited 97% by either cycloheximide or puromycin in both cell types.

To test whether the half-lives of other proteins were prolonged in HMOA cells, the turnover of two other short-lived enzymes, S-adenosylmethionine decarboxylase and tyrosine aminotransferase, was investigated. The data demonstrate that the half-life of S-adenosylmethionine decarboxylase (Fig. 1B) was the same in both HTC and HMOA cells. Moreover, the initial specific activity of the enzyme was not different in the two cell types. When tyrosine aminotransferase activity in the hepatoma cells was induced with dexamethasone, decay of enzyme activity in the presence of cycloheximide was the same in both HTC and HMOA cells (Fig. 1C). The specific activity of the induced enzyme at t₀ was lower in the HMOA cells (HTC = 50 nmol product formed/ min x mg protein); HMOA = 12 nmol product formed). The basal activity of tyrosine aminotransferase activity 16 h after dilution into...
Ornithine Decarboxylase from Hepatoma Cells

**Fig. 1.** Half-lives of activities for ornithine decarboxylase, 
S-adenosylmethionine decarboxylase, and tyrosine aminotransferase. *A*, ornithine decarboxylase activity in whole cells after inhibition of protein synthesis. HTC and HMO\(\alpha\) cells grown to maximum density in suspension culture were diluted into fresh medium 16 h prior to the addition of cycloheximide (0.18 mM) at zero time. At the indicated time, samples (2 x 10^6 cells) were taken for the determination of ornithine decarboxylase activity and protein. Specific activity of ornithine decarboxylase at the start of experiments was 1.6 units/mg protein for HTC cells and 4.1 units/mg protein in HMO\(\alpha\) cells. Half-life of enzyme activity determined after inhibition of protein synthesis was 14 min and approximately 5 to 10 h for HTC (\(\bullet\)) and HMO\(\alpha\) (\(\Delta\)) cells, respectively. The slopes of the lines were arrived at by linear regression analysis and the half-lives were calculated from the relationship \(t_{1/2} = 0.693/\text{slope}\). Maintenance of the cell cultures, assay of enzyme activity and protein determination are described under “Experimental Procedures.” *B*, S-adenosylmethionine decarboxylase activity in whole cells after inhibition of protein synthesis. HTC and HMO\(\alpha\) cells were diluted into fresh medium 16 h before the addition of cycloheximide (0.18 mM) at zero time. Samples (9 x 10^6 or 15 x 10^6 cells) were taken for the assay of enzyme activity and protein as indicated. These procedures are described under “Experimental Procedures.” Specific activity at the start of the experiment was 0.162 units/mg protein in HTC cells and 0.144 units/mg protein in HMO\(\alpha\) cells. The half-life of enzyme activity was 34 min for both the HTC (\(\bullet\)) and HMO\(\alpha\) (\(\Delta\)) cells (calculated as described in the legend for Fig. 1A). *C*, tyrosine aminotransferase activity in whole cells after inhibition of protein synthesis. HTC and HMO\(\alpha\) cells were diluted into fresh medium containing 10^{-7} M dexamethasone 16 h prior to the start of the experiment. The cells were collected by centrifugation (900 x g, 5 min) and resuspended in medium without dexamethasone. At zero time, cycloheximide (0.18 mM) was added to the cell suspension. Samples of 3 x 10^6 cells were taken at each time point and processed for the assay of enzyme activity and protein as described under “Experimental Procedures.” The specific activity of tyrosine aminotransferase at 5, i.e., 100%, was 90 nmol of \(p\)-hydroxybenzaldehyde formed/(min x mg protein) in HTC cells and 12 nmol in HMO\(\alpha\) cells. This experiment was repeated twice in dexamethasone-induced cells and the half-life was approximately 8 h in both HTC (\(\bullet\)) and HMO\(\alpha\) (\(\Delta\)) cells (calculated as described in the legend for Fig. 1A).

Effect of Various Concentrations of Exogenous Putrescine on Ornithine Decarboxylase Activity—The effect of increasing concentrations of exogenous putrescine was tested in HTC and HMO\(\alpha\) cells in order to investigate the nature of dexamethasone regulation of ornithine decarboxylase activity in the two cell types (Fig. 3, Panel A). The HTC and HMO\(\alpha\) cells were found to differ drastically in this respect. The concentration of putrescine required to produce a 50% reduction in enzyme activity after 3 h was almost 1000 times higher in the HMO\(\alpha\) than in the HTC cells. One situation that could explain this difference would be if putrescine was taken up more slowly by the HMO\(\alpha\) cells. However, this was not the case because at 3 h the cellular content of putrescine was actually higher in the HMO\(\alpha\) cells (Fig. 3, Panel B).

Purification of Ornithine Decarboxylase from HTC and HMO\(\alpha\) Cells—In order to examine the possible existence of a variant form of ornithine decarboxylase in HMO\(\alpha\) cells, the enzyme was purified from both HTC and HMO\(\alpha\) cells. 

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**Fig. 2.** Release of \(^{3}H\)leucine from prelabeled HTC and HMO\(\alpha\) cells. Cells were diluted into fresh medium containing \(^{3}H\)leucine (16 Ci/mol, 5 \(\mu\)Ci/ml). After 16 h the cells were centrifuged (600 x g, 5 min) and the labeled medium replaced with medium containing 2 nmol leucine to prevent the reincorporation of \(^{3}H\)leucine into protein. The cells were reincubated and suspended into a second change of leucine-containing medium. The release of 10% trichloroacetic acid-soluble radioactivity was determined in the presence and absence of cycloheximide (0.18 mM). At each time point, samples (2 ml) of cell suspension were taken into tubes on ice containing 0.2 ml of 100% trichloroacetic acid. The samples were incubated on ice for 30 min, then centrifuged for 15 min at top speed in a Beckman TJ-6 centrifuge. Supernatant (1 ml) was transferred to a 20-ml scintillation vial and radioactivity determined. The radioactivity in the acid precipitate at zero time was determined as described under “Experimental Procedures.” Samples containing radiolabeled difluoromethylornithine. The figure depicts the release of acid-soluble radioactive activity in cells plus medium as a percentage of the total acid-precipitable radioactivity in the cell protein at time zero and is from one of two such experiments. Panels A and B show radioactivity released from control (\(\bullet\)) and cycloheximide-treated (\(\Delta\)) HTC and HMO\(\alpha\) cells, respectively.

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*Note: The PDF contains additional figures and references.*
Ornithine decarboxylase from both cell types behaved identically throughout all steps of the purification procedure (Table I, Fig. 4). No enzyme activity was lost during the 30–50% saturated ammonium sulfate fractionation (Table I). In fact, an increase in activity was often observed at this step, resulting probably from the removal of small molecular weight inhibitors during dialysis or ammonium sulfate fractionation. Both HTC and HMOA ornithine decarboxylase activity eluted from DEAE-cellulose at 0.15 M NaCl (Fig. 4) and from the pyridoxamine phosphate affinity column at the same position (data not shown). The overall purification procedure, in which the key step was chromatography on the pyridoxamine phosphate affinity column, yielded approximately an 8000-fold enrichment in ornithine decarboxylase activity for both the HTC and HMOA cell enzymes (Table I). The presence of Brij-35 during this step greatly improved the method of Boucek and Lembach (27) by stabilizing the enzyme; the highly purified material retained 100% enzyme activity for up to 25 days when stored at −70 °C. Brij-35 may act by preventing ornithine decarboxylase from unfolding and denaturing in solutions of very low protein concentration.

Fig. 3. Ornithine decarboxylase activity and putrescine content of intact cells after exposure to increasing concentrations of putrescine. Ornithine decarboxylase activity was stimulated by diluting cells into fresh medium 16 h before the start of the experiment. Aliquots (10 or 30 ml) of cell suspension were incubated with the indicated concentrations of putrescine for 3 h and then samples (2 × 10^6 cells) were taken for the assay of enzyme activity and protein. Data points represent the mean of two separate determinations. In one of the experiments, samples (approximately 3 × 10^6 cells) from several of these flasks were also analyzed for polyamine content. Ornithine decarboxylase activity (Panel A) and polyamine content (Panel B) for the two cell types are shown in this figure for HTC (○) and HMOA (△) cells. The assay of enzyme activity and protein are given under "Experimental Procedures." Polyamines were measured as previously described by Pegg et al. (26).

Fig. 4. DEAE-cellulose chromatography of ornithine decarboxylase from HTC and HMOA cells. The elution of HTC (Panel A) and HMOA (Panel B) ornithine decarboxylase from DEAE-cellulose is depicted here. These data are from the same purification detailed in Table II. Ornithine decarboxylase activity, ○; salt concentration, △.

### Table I

<table>
<thead>
<tr>
<th>Purification step*</th>
<th>Total activity</th>
<th>Total protein</th>
<th>Specific activity</th>
<th>Relative fold purification</th>
<th>Recovery</th>
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<td>Cytosol</td>
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*The HTC starting material was the combined cytosol extracts of 43 liters of cell suspension, while the HMOA was 22 liters.
Neutralization of Activity of the Enzyme with Antiserum and Antizyme—Often enzymes that are more stable in vivo also are more resistant to heat denaturation (29). Therefore, the thermostability of HTC and HMOA enzyme activities were tested over a wide range of temperatures (Fig. 5A). Under the conditions investigated, ornithine decarboxylase activity in cytosol extracts from both cell types was inhibited equally by antisera (Fig. 5B) prepared against the rat liver enzyme by Theoharides and Cannellakis (30) and by ornithine decarboxylase antizyme (Fig. 5C) prepared from rat liver as described by Heller et al. (9).

Labeling Ornithine Decarboxylase with [$^{14}$C]Difluoromethylornithine—Initial experiments with radiolabeled difluoromethylornithine showed that the inhibitor could be used to label specifically rat liver ornithine decarboxylase (23). Binding of the inhibitor to protein correlated with the degree of inactivation and with the amount of enzyme activity present indicating that the inhibitor would be a useful tool for answering questions about the hepatoma enzyme.

Results of studies in which the binding of radiolabeled difluoromethylornithine was used to estimate the amount of ornithine decarboxylase are presented in Table III. These

<table>
<thead>
<tr>
<th>Table II Kinetic parameters of the ornithine decarboxylase reaction</th>
<th>HTC</th>
<th>HMOA</th>
</tr>
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<tr>
<td>$V_{max}$ (pmol/mg protein)</td>
<td>430 ± 12</td>
<td>780 ± 33</td>
</tr>
<tr>
<td>$K_m$ (ornithine) (mM)</td>
<td>0.032 ± 0.006</td>
<td>0.43 ± 0.003</td>
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<tr>
<td>$K_m$ (putrescine) (mM)</td>
<td>0.13</td>
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Fig. 5. Inactivation of ornithine decarboxylase with thermal denaturation, ornithine decarboxylase antiserum, and ornithine decarboxylase antizyme. A, thermal inactivation of purified ornithine decarboxylase. Samples (200 μl) of 400-fold purified enzyme from HTC (●) and HMOA (▲) cells were subjected to 4-min incubations at the indicated temperatures and then assayed for ornithine decarboxylase activity as described under "Experimental Procedures." Activity of the 37°C sample was set at 100% and was determined to be 7.2 units/ml for the HTC preparation and 5.2 units/ml for the HMOA preparation. B, immunoinactivation of ornithine decarboxylase activity. Increasing amounts of cytosol extracts from HTC (●) and HMOA (▲) cells were incubated with either 0.1 mg of antiornithine decarboxylase immunoglobulins, normal rabbit immunoglobulins, or no immunoglobulins in a total volume of 240 μl. After 1 h of incubation at room temperature, 50 μl of IgG/Sorb (The Enzyme Center, binding capacity, 2.01 mg/ml), a commercial preparation of Protein A, was added to each tube and incubated for an additional 5 h at 4°C. A pellet of the precipitate was obtained by centrifugation at 8000 × g for 2 min in a microfuge (Brinkmann instruments) and a sample of the supernatant was assayed for ornithine decarboxylase activity. Enzyme activity added to each tube was plotted against enzyme activity remaining. The equivalence point (determined by linear regression) was 25 units/ml extract for the HTC preparation and 20 units/ml for HMOA. C, titration of ornithine decarboxylase activity with rat liver antizyme. Ornithine decarboxylase antizyme (50 μl) was added in increasing amounts to assay tubes containing 150 μl of HTC or HMOA cell cytosol. All tubes were then assayed for enzyme activity. The slopes of the lines (determined by linear regression analysis) were 0.20 and 0.18 units of activity/mg antizyme which indicated loss in ornithine decarboxylase activity per mg of antizyme preparation added to HTC (●) and HMOA (▲) cell extracts, respectively. Methods for the preparation of cytosol extracts and for the assay of enzyme activity are described under "Experimental Procedures."
data reveal the proportion of protein that was ornithine de
carboxylase in cytosol from HTC and HMOA cells in early log
phase growth. Assuming that one molecule of inhibitor was
needed to inactivate one molecule of enzyme (subunit $M_1 =
54,000$, see Fig. 6), ornithine de
carboxylase represented 3.5
ng/mg protein and 11 ng/mg protein in HTC and HMOA
cytosol, respectively. Calculations based on similar experi-
ments with rat liver ornithine de
carboxylase from thioacetamide-treated rats gave a value of 1.4 ng/mg protein (23). These results also established the relationship between en-
zyme activity and enzyme protein. As shown in Table III the ratio of enzyme specific activity between the HTC and HMOA
cells equaled the ratio of radiolabeled difluoromethyl-orni-
thine bound to protein. This result indicated that there was
more enzyme activity in HMOA cells because there was more
enzyme present.

**Polyacrylammide Gel Electrophoresis of Ornithine Decar-
boxylase**—The subunit $M_1$ of highly purified ornithine de
carboxylase from each cell type was determined in this study
using $[14C]$difluoromethylornithine as a specific label. For both
the HTC and HMOA enzyme, the major peak ($a$) of radioac-
tivity was found at $M_1 = 53,000$ to 54,000 (Fig. 6). Minor peaks
($b$ and $c$) appeared at $M_1 = 30,000$ and 67,000, respectively. Peaks $a$, $b$, and $c$ contained 64, 18, and 17% of the radioactivity
in both the HTC and HMOA gels, respectively. As shown by

![Polyacrylamide gel electrophoresis of highly purified ornithine de
carboxylase from HTC and HMOA cells. Ornithine
decarboxylase from i) HTC and iii) HMOA cells was incubated with
$[14C]$difluoromethylornithine, precipitated with trichloroacetic acid,
and subjected to electrophoresis as described in the legend for Fig. 6.
After electrophoresis, the gels were stained for protein. The direction
of migration was from top to bottom. The arrows mark the location of the major band of radioactivity and PC shows the location of the
protein carrier, cytochrome $c$, on gels i and iii. Molecular weight
standards run on a separate gel (ii) are shown for comparison. Standards are $a$, phosphorylase b, 94,000; $b$, bovine serum albumin,
67,000; $c$, ovalbumin, 43,000.]

**Fig. 6.** Radioactivity profiles of $[14C]$difluoromethylor-
ithine-labeled ornithine decarboxylase run on sodium dodecyl
sulfate-polyacrylamide gels. Highly purified ornithine de
carboxylase from HTC and HMOA cells was incubated with $[14C]$difluo-
methylornithine (0.25 $\mu$Ci/ml) and 50 $\mu$m pyridoxal phosphate for 1 h
at 37°C. The enzyme had been purified as described under
"Experimental Procedures." Approximately 200 units of activity, 17
$\mu$g of protein, were incubated in a total volume of 2 ml with the
radiolabeled drug. Enzyme activity was inhibited greater than 98%
after 1 h. Cytochrome $c$ (1 mg) was added to each sample as a protein
carrier and each sample was precipitated in 17% trichloroacetic
acid. The protein pellet was prepared by centrifugation and subjected
to electrophoresis as detailed under "Experimental Procedures." Panel
A depicts the profile of radioactivity across the gel for the HTC
decarboxylase enzyme. The same thing is shown in Panel B for the HMOA prepa-
ration. **Arrows** mark the location of the dye fronts in each gel.
Photographs of equivalent gels stained for protein are shown in Fig. 7.

**Fig. 7.** Polyacrylamide gel electrophoresis of highly purified ornithine de
carboxylase from HTC and HMOA, cells. Ornithine
decarboxylase from i) HTC and iii) HMOA cells was incubated with
$[14C]$difluoromethylornithine, precipitated with trichloroacetic acid,
and subjected to electrophoresis as described in the legend for Fig. 6.
After electrophoresis, the gels were stained for protein. The direction
of migration was from top to bottom. The arrows mark the location of the major band of radioactivity and PC shows the location of the
protein carrier, cytochrome $c$, on gels i and iii. Molecular weight
standards run on a separate gel (ii) are shown for comparison. Standards are $a$, phosphorylase b, 94,000; $b$, bovine serum albumin,
67,000; $c$, ovalbumin, 43,000.

**DISCUSSION**

Until this investigation it was not known whether the
turnover of other proteins was altered in HMOA cells. Evi-
dence presented here shows that the prolonged half-life of
ornithine de
carboxylase activity in HMOA cells is not a property
shared by the other short-lived proteins investigated in the
present study. One such protein, S-adenosylmethionine
decarboxylase, was investigated because it, like ornithine de-
carboxylase, plays a key role in polyamine biosynthesis. The data
reveal that the half-life of S-adenosylmethionine deca-
boxylase was not prolonged in HMOA cells when compared to
HTC cells. Moreover, the half-life reported here (34 min) compares well with other such measurements for S-adenosylmethionine decarboxylase; these range from 25 min to 2 h (18, 31–34). Since S-adenosylmethionine decarboxylase does not have a cofactor requirement for pyridoxal-phosphate, the turnover of tyrosine aminotransferase was also examined. Tyrosine aminotransferase is also a short half-life cytosolic protein which, like ornithine decarboxylase, requires pyridoxal phosphate for activity. The finding that the half-life of tyrosine aminotransferase was unchanged, whereas that of ornithine decarboxylase was prolonged, supports the notion that the two enzymes are being handled by different deactivation steps. The half-life measured for tyrosine aminotransferase is in good agreement with that of 4 h measured by Herskóko and Tomkins (35) in HTC cells under very similar experimental conditions.

Further support for the lack of an overall change in the degradation of short-lived proteins in HMOA cells is provided by the present study in which the degradation of general cellular protein was investigated by following the release of acid-soluble radioactivity from prelabeled protein (35). The more commonly used dual labeling method (36) could not be employed here because the requirement that the cells be in steady state could not be met. Cells had to be studied during early log phase growth because there is very little ornithine decarboxylase activity when the cells are not growing, i.e., in steady state. Schimke (37) indicated that the "average" liver protein is degraded with a half-life of 3.3 days. If this is also the case for hepatoma proteins, then label released after a 16-h prelabeling period would be primarily from shorter half-life proteins. Therefore, the data presented here show that these proteins are degraded at the same rate in both HTC and HMOA cells. It was also observed that the release of labeled leucine after 1 h was slowed in the presence of cycloheximide to an equivalent degree in both cell types. An inhibitory effect of cycloheximide or puromycin on protein degradation was noted by other investigators in HTC cells (35), in perfused heart (38) and in perfused liver (39). It was suggested that the inhibition observed with cycloheximide is due to a build-up of some intermediate of protein synthesis such as aminocyclitRNA (29).

Much indirect evidence exists to suggest that polyamines block the synthesis of ornithine decarboxylase (40–42). If that is the case, then ornithine decarboxylase activity in HMOA cells incubated in the presence of increasing concentrations of exogenous putrescine would be greater than its activity in HTC cells because of the prolonged half-life of the enzyme in the HMOA cells. Although this is exactly what was observed in the present study, when one examines these data (Fig. 3) quantitatively, another explanation is necessary for the low enzyme activity remaining at higher concentrations of putrescine. If only synthesis was being blocked, one would expect more than 50% of enzyme activity to remain in HMOA cells after 3 h. Since this clearly was not the case, another mechanism must be responsible in HMOA cells for the reduction in enzyme activity seen at high concentrations of extracellular putrescine. One possible explanation is the induction of ornithine decarboxylase antizyme, a noncompetitive inhibitor of the enzyme (9). Support for this idea was presented by McCann et al. (14) who found that higher concentrations of putrescine were required for the induction of ornithine decarboxylase antizyme in HMOA cells than are required for induction in HTC cells. Thus, it appears that at least two mechanisms are necessary to explain the regulation of ornithine decarboxylase activity by polyamines. Effects of 10^{-2} M putrescine are probably not of physiological importance, however, since cells are not normally exposed to such high concentrations of putrescine.

Several lines of investigation provided evidence that the markedly longer half-life of ornithine decarboxylase in HMOA cells was not due to the presence of a variant form of the enzyme. For example, the enzymes from both cell types behaved in an identical manner throughout the purification procedure, were equally thermostable, and were inhibited in a similar manner by antisera to ornithine decarboxylase and by ornithine decarboxylase antizyme. If an alteration in the HMOA enzyme was responsible for its longer half-life, the enzyme would have been expected to elute earlier from DEAE-cellulose since cytosol proteins with longer half-lives tend to elute from DEAE-cellulose at a lower salt concentration than those with shorter half-lives (43). The finding that ornithine decarboxylase from both cell types eluted from the affinity column at the same position indicated that the enzymes had similar affinities for pyridoxal phosphate. Further evidence for ornithine decarboxylase from HTC and HMOA cells being the same was provided by kinetic analysis with the purified enzymes. The K_{m} obtained for L-ornithine in HTC and HMOA cells was almost identical to that obtained by Weiss et al. (44) in mouse fibroblasts (0.03 mM). Other investigators have measured a slightly higher K_{m} for ornithine, approximately 0.14 mM (40–42), and some have found purified ornithine decarboxylase from HMOA to exist in two forms with different K_{m}(9, 47). When purified enzyme was stabilized with albumin, Boucek and Lembach (27) found that the K_{m} for L-ornithine was 0.066 mM, and 0.125 mM if no albumin was present. This observation suggests that the higher K_{m}s measured for ornithine were determined on enzyme that was partially denatured. Such a possibility seems likely, especially after lengthy purification procedures. In addition to the similarity of the K_{m}s the behavior of the enzymes with respect to inhibition by putrescine was identical. It should be recalled that in intact cells exogenous putrescine led to a much greater reduction in ornithine decarboxylase activity in HTC cells (see Fig. 3). Since putrescine had an equivalent effect on purified enzyme from both cell types, the diamine added to intact cells probably does not have its primary effect directly on ornithine decarboxylase.

Radiolabeled a-difluoromethylornithine has proven to be a useful tool for the study of ornithine decarboxylase. The data presented here and in a previous report (23) support the conclusion that at the end of the inactivation reaction, the inhibitor becomes covalently bound to ornithine decarboxylase. For the purposes of this investigation, [14C]-difluoromethylornithine was used to determine the subunit M, and to measure the amount of ornithine decarboxylase in HTC and HMOA cells. The subunit M, data are further evidence in support of the two enzymes being the same. The major peak of radioactivity at M, = 54,000 was observed for both the HTC and HMOA enzyme preparations. This value compares well with the subunit M, for rat liver ornithine decarboxylase (55,000) previously determined in this laboratory (23) and with the M, (55,000) measured for mouse fibroblast ornithine decarboxylase by Weiss et al. (44). In addition to the major peak of radioactivity, a lower (30,000) and a higher (67,000) M, peak appeared on both gels (see Fig. 6). These peaks were not seen in previous studies of purified ornithine decarboxylase from rat liver (23), but the hepatoma enzymes were prepared for electrophoresis using different conditions which may have caused them to dissociate in a different manner than the rat liver enzyme.

Also of significance was the use of radiolabeled a-difluoromethylornithine for the calculation of the amount of ornithine decarboxylase in cytosol extracts of the hepatoma cells. These measurements show that increased ornithine decarboxylase...
activity in HMOA cells was due to increased enzyme protein (see Table III). Furthermore, they show that the enzyme was present at very low levels in both of these cell types (0.00035% of cytosol protein in HTCl cells and 0.0011% in HMOA cells). The low amount of ornithine decarboxylase in the hepatoma cells meant that even with an 8000-fold purification of the enzyme, a homogenous preparation was not obtained as evidenced from Fig. 7. Indeed, based on the stoichiometry of the labeled inhibitor, it appears that the preparation obtained is about 5-10% of the theoretical value for homogeneous enzyme. It should be noted, however, that the specificity of the hepatoma cell ornithine decarboxylase achieved in the present experiments is higher than previous preparations (27, 47, 48) and is comparable to that observed recently for the rat liver enzyme (24).

In summary, all of the data presented here support the conclusion that the increased half-life of ornithine decarboxylase in HMOA cells is not due to a more stable form of the enzyme. The alternative explanation is that a specific deactivation system for ornithine decarboxylase activity in HMOA cells was due to increased enzyme protein.

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