Specific Induction of Ornithine Decarboxylase in 3T3 Mouse Fibroblasts by Pituitary Growth Factors: Cell Density-Dependent Biphasic Response and Alteration of Half-Life†

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ABSTRACT: Pituitary growth factors contained in a partially purified preparation of luteinizing hormone (NIH-LH-B8) markedly increased the activity of ornithine decarboxylase and stimulated cell division in both growing and density-inhibited 3T3 cells. At 6–9 hr after addition of NIH-LH-B8 to density-inhibited 3T3 cells, a 100-fold increase in the specific activity of ornithine decarboxylase activity was observed. This activity fell off rapidly to prestimulation levels by 24 hr. A 25% increase in cell number was obtained within the next 4 days. In stimulated, growing 3T3 cells, a subsequent larger peak of activity was also observed, the magnitude of which was inversely correlated with the cell density at the time of stimulation. The growth rate of stimulated, low density cells was increased within 48 hr after addition of NIH-LH-B8. Addition of inhibitors of RNA or protein synthesis at the time of stimulation by NIH-LH-B8 totally prevented increases in enzyme activity. The half-life of ornithine decarboxylase activity in density-inhibited cells was shorter and the total activity was lower than in growing cells. In addition, a transient decrease in the half-life of the enzyme occurred in cells of all densities after stimulation. Thus ornithine decarboxylase levels in 3T3 cells appear to be related to the growth rate and regulated by mechanisms affecting both synthesis and degradation. Actinomycin D, but not cordycepin, resulted in a "superinduction" of ornithine decarboxylase if added at 2–4 hr after stimulation. Stabilization of the enzyme was at least partly responsible for this increase in activity.

The diamine putrescine and the polyamines spermidine and spermine occur ubiquitously in nearly all organisms. An elevation in the concentrations of these compounds is associated with a shift from a nongrowing or slowly growing state to one of rapid proliferation in a large number of animal tissues (Williams-Ashman *et al.*, 1969, 1972; Tabor and Tabor, 1972). In these systems, and in many others in which polyamine levels have not been monitored, an earlier, rapid increase in the activity of L-ornithine decarboxylase (EC 4.1.1.17; L-ornithine carboxy-lyase) is also observed upon application of a wide variety of growth stimuli. This enzyme catalyzes the decarboxylation of ornithine to putrescine, which is the first, and probably rate limiting, step in the biosynthesis of the polyamines.

Although polyamines stabilize nucleic acids, polyribosomes, and ribosomes, and stimulate the activity *in vitro* of many of the enzymes involved in protein and RNA synthesis (Cohen, 1971; Bachrach, 1973), their distribution and function *in vivo* are not clear. However, because of the abundance of data relating polyamine increases with accelerated growth, it seems likely that polyamines are in some manner intimately involved in growth regulation.

Several laboratories have begun to examine polyamine metabolism in cultured cells. Large increases occur in the activities of ornithine decarboxylase and *S*-adenosylmethionine decarboxylase (an enzyme essential for spermidine and spermine synthesis) in short-term human lymphocyte cultures stimulated to divide by the plant lectin phytohemagglutinin (Kay and Lindsay, 1973a,b). Similar results have been obtained with established cell lines. Ornithine decarboxylase activity of hepatoma (Hogan, 1971) and KB (Pett and Ginsberg, 1968) cells increases many-fold upon dilution of stationary phase cells into fresh medium. In KB cells, a five- to tenfold increase in the rate of putrescine synthesis within 3 hr after dilution is also observed. Subconfluent, serum-starved, BHK cells respond to growth stimulation by added serum with a rapid increase in ornithine decarboxylase activity (Melvin *et al.*, 1972).

As cultured cells are maintained in an environment which is both well defined and readily controlled, it seems likely that more extensive studies with such systems will increase our understanding of polyamine functions. Thus, the activity and stability of ornithine decarboxylase in density-inhibited Swiss 3T3 mouse fibroblasts and in exponentially growing 3T3 cells of various densities were examined as the first stage in attempting to clarify the role of polyamines in cell growth. In nearly all of these initial studies, a preparation of luteinizing hormone (NIH-LH-B8) was employed to produce an increase in the rate of cell division. This preparation provided a readily available source of pituitary growth factors found to be present as contaminants. The purification of these factors from bovine pituitary glands will be the subject of a future communication.

Experimental Section

Materials. [1-^{14}C]-DL-Ornithine (specific activity 12.8 Ci/mol) was purchased from New England Nuclear. [3,4-^{3}H]-L-Leucine was obtained from Amersham/Searle. Trichodermin was the gift of Dr. Calvin McLaughlin. NIH-LH-B8, a preparation of bovine luteinizing hormone, was kindly supplied by the Endocrine Section of the National Institutes of Health. All other reagents were of analytical grade.

Cell Culture. Swiss 3T3 mouse fibroblasts were initially

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obtained from Dr. Dennis Cunningham. Diploid human fibroblasts were provided by Dr. David Kingsbury. The rat ovary cells used have been in continuous culture in our laboratory for 3 years (Clark *et al.*, 1972). Cells were cultured as described (Cunningham, 1972) in 100-mm dishes containing 10 ml of complete medium, unless otherwise indicated. Fresh cultures were initiated each month from frozen ampoules. Cultures were monitored three times during the course of these studies for mycoplasma contamination (Nordone *et al.*, 1965); none was detected.

Experimental cultures of 3T3 cells were plated at densities varying from 0.64 to 15 $\times 10^3$ cells/cm^2. NIH-LH-B8 was stored frozen in phosphate-buffered saline at 1–10 mg/ml, diluted immediately before use when necessary, and added to cultures in a volume 1% of that of the culture medium. Control cultures received only PBS.[1] The approximate cell densities at the time of addition of NIH-LH-B8 were as follows: low density exponentially growing cells, 4 $\times 10^3$ cells/cm^2; medium density exponentially growing cells, 8 $\times 10^3$ cells/cm^2; confluent (or nearly confluent) cells, 2 $\times 10^4$ cells/cm^2; and density inhibited cells, 2.3 $\times 10^4$ cells/cm^2. Cells were considered to be density inhibited if cultures had achieved confluency at least 1.5 days previously.

Preparation of Cell Extracts. Cultures were iced and rinsed three times with cold PBS. Between 0.3 and 1.0 ml of a standard buffer consisting of 25 mM Tris-HCl, 0.1 mM Na_2EDTA, and 5 mM dithiothreitol was distributed evenly over the cells. The cells were allowed to swell for a few minutes in this hypotonic buffer and were then partially disrupted and harvested by scraping with a rubber policeman. The broken-cell suspension was freeze–thawed once to complete enzyme release and centrifuged at 45,000*g* for 15 min at 4° in a Sorvall RC2-B centrifuge. The clear supernatant fluid was used for assay of ornithine decarboxylase. Extracts to be tested for other enzymatic activities were prepared in the appropriate assay buffer in an otherwise identical manner.

Ornithine Decarboxylase Assay. Ornithine decarboxylase activity was routinely assayed essentially as described (Jänne and Williams-Ashman, 1971). The assay was linear for at least 1 hr when the reaction mixture contained between 0.1 and 0.8 mg of cytosol protein. All assays were done within 40 hr of harvesting. Extracts assayed more than 2 hr after harvesting were frozen at −70° until used.

Other Assays. Histidine decarboxylase (Hakanson, 1967), glutamate decarboxylase (Haber *et al.*, 1970), and ornithine:keto acid aminotransferase (Phang *et al.*, 1973) were assayed as described by others. Tyrosine aminotransferase assays were done on "aged" supernatant solutions as described (Diamondstone, 1966). To eliminate interference from sulfhydryl compounds when present, protein was assayed by a carboxymethylation modification (Schatz, 1973) of the Lowry method. Leucine uptake and incorporation were measured essentially as described (Hershko *et al.*, 1971). Relative pool sizes of stimulated and unstimulated cultures were determined by comparing the ratios of incorporation obtained when cultures were incubated with different specific radioactivities of leucine.

Results

Properties of Ornithine Decarboxylase from 3T3 Cells. Studies on cell-free extracts demonstrated that the properties of ornithine decarboxylase from 3T3 cells were similar to those of the purified enzyme from rat ventral prostate (Jänne and Williams-Ashman, 1971), thioacetamide-treated rat liver (Ono *et al.*, 1972), and regenerating rat liver (Friedman *et al.*, 1972). The K_m for ornithine was 0.22 mM, and putrescine (K_i of 2.6) and spermine (K_i of 7.1) were weak competitive inhibitors of the enzyme. The 3T3 cell enzyme had a pH optimum of 7.0–7.2, and was quantitatively recovered in the 45,000*g* supernatant solution. The lack of activity in the pellet was not due to the presence of inhibitors as shown by mixing experiments.

The 3T3 extract rapidly lost activity if prepared or stored in the absence of the sulfhydryl compound dithiothreitol. Activity could be partially recovered by dialysis against standard assay buffer, which contained 5 mM dithiothreitol. The enzyme extract invariably lost less than 5% of its activity if stored at −70° for several weeks in the presence of 5 mM dithiothreitol. Similar observations have been made with the purified enzymes from prostate and liver, and crude extracts from these and other sources.

Stimulation of Ornithine Decarboxylase Activity and Cell Division in 3T3 Cells by Pituitary Growth Factors. Ornithine decarboxylase activity in exponentially growing low density 3T3 cells was 6–8 units[2]/mg of soluble protein.[3] As the cells grew to higher densities, this activity decreased progressively to a value of less than 0.5 unit/mg of soluble protein in density-inhibited cells. Enzyme activity also declined, ultimately to barely detectable levels, as BHK cells grew toward the stationary phase (Melvin *et al.*, 1972). Addition of partially purified preparations of pituitary growth factors to 3T3 cells of all densities resulted in a rapid and substantial increase in the specific activity of ornithine decarboxylase. Purification efforts to date utilizing bovine pituitary glands as starting material have demonstrated the presence of at least two protein factors, each of which stimulates RNA and DNA synthesis and cell division as well as ornithine decarboxylase activity (Bihler and Clark, unpublished experiments). Both factors are distinct from known pituitary hormones. These factors were initially detected in a preparation of luteinizing hormone provided by the National Institutes of Health (NIH-LH-B8). Because quantities of purified growth factors have been limited, NIH-LH-B8 was used as a source of these factors in the balance of the studies described herein.

Addition of NIH-LH-B8 to a final concentration of 10 μg/ml also produced large increases in ornithine decarboxylase activity in cultures of 3T3 cells (Figure 1) which occurred as rapidly as those seen with the purified factors. This increase was totally dependent on *de novo* RNA and protein synthesis (see below). Mixing of cell extracts from stimulated and unstimulated cells invariably yielded activities which were additive, thus ruling out the involvement of soluble activators or inhibitors of the enzyme. NIH-LH-B8 added directly to the assay was without effect.

With density-inhibited cells, a single peak of activity (a 100-fold increase over basal levels) was observed 6–9 hr after stimulation (Figure 1). This activity declined almost as rapidly as it appeared, and approached prestimulation values by 24 hr. In contrast, in exponentially growing cells which had just reached confluency, a biphasic response was

[1] Abbreviations used are: PBS, phosphate-buffered saline; DME, Dulbecco–Vogt modified Eagles medium.

[2] One unit of enzyme activity is defined as 1 nmol of CO_2 released/hr.

[3] Soluble protein in all extracts described herein was 31 ± 2.1% of the total cell protein.

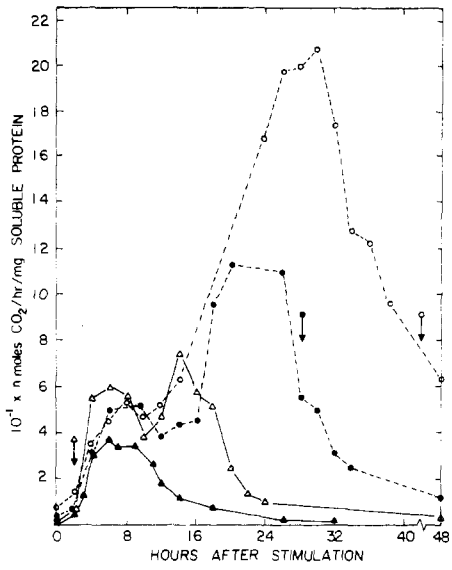

FIGURE 1: Stimulation of ornithine decarboxylase activity in cells of various densities by NIH-LH-B8. Each tracing is from a single experiment. Other experiments with cells at comparable densities gave qualitatively similar results. Arrows indicate the approximate times at which confluence was reached. Low density exponentially growing cells (O- -O); medium density exponentially growing cells (●- -●); cells at or near confluency (△—△); density-inhibited (quiescent) cells (▲—▲).

invariably observed. The second peak of activity was cell density dependent in terms of both its magnitude and time course, being of greater magnitude and occurring later the lower the cell density at the time of stimulation (Figure 1). The first peak of activity in growing cells was remarkably constant regardless of cell density and was only slightly higher than the corresponding peak of activity observed in density-inhibited cells.

The patterns of activity obtained from all time course experiments were related qualitatively only to cell densities at the time of stimulation; they were not related to the plating density, the time required to reach the desired density (manipulated in some cases by suboptimal temperature or pH),

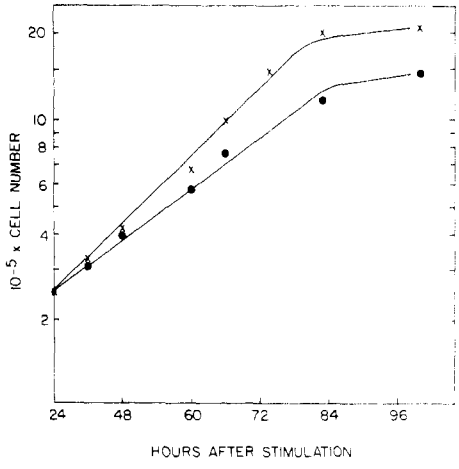

FIGURE 2: Stimulation of cell growth by NIH-LH-B8. Cells were plated at 5 × 10^4/100-mm plate and NIH-LH-B8 was added 32 hr after plating. Cell counts for experimental and control plates were identical between 0 and 24 hr after stimulation. The graph is a composite of two experiments. These growth curves have been repeated several times; stimulated cultures always grew more rapidly and reached a saturation density at least 30% greater than controls. With NIH-LH-B8 (X); without NIH-LH-B8 (●).

TABLE I: Ornithine Decarboxylase Activity in Growing Cultured Cells.[a]

Cell Type	Unstimulated	Stimulated (12 hr)	Fold Increase
3T3	4.2 ± 0.2	41.8 ± 1.1	10.0
Human fibroblasts	3.1 ± 0.3	5.5 ± 0.4	1.8
Chinese hamster ovary	<0.1	<0.1	
Hela S-3	28 ± 2	53 ± 1	1.9
Adrenal Y-1	65 ± 7	145 ± 6	1.1
Rat ovary	<0.1	<0.1	

[a] Exponentially growing cultures received either PBS (unstimulated) or NIH-LH-B8 in PBS (stimulated). Cells were harvested 12 hr later and assayed for ornithine decarboxylase. Each value for enzyme activity is the average of at least six determinations. Activity is expressed as units per milligram of soluble protein ± S.E.

or the source of plating medium (fresh or from confluent cultures).

The addition of NIH-LH-B8 to very low density growing cultures increased the growth rate within 48 hr. These cells ultimately reached a density 30–40% greater than unstimulated cells (Figure 2). Addition of NIH-LH-B8 to density-inhibited cultures produced up to a 25% increase in cell number within 4 days. However, in no stimulated culture was the total cell protein increased by more than 7% over controls. This is consistent with the results from a previous study (Clark *et al.*, 1972) in which a qualitative estimate of 3T3 cell growth, utilizing only a protein stain, revealed no significant growth stimulation by NIH-LH-B8. The small increase in cell protein observed is also in agreement with the finding that a disproportionate share of macromolecule synthesis in newly subcultured 3T3 cells occurs before cell division resumes (Meisler, 1973).

Ornithine Decarboxylase in Other Cultured Cells. Five other cell lines were assayed for ornithine decarboxylase activity during exponential growth. Activity was readily detected in three of these, including human diploid fibroblasts, and in each of these cases was augmented by NIH-LH-B8 (Table I). In all cell lines in which the enzyme was detected, activity declined markedly as the cells approached saturation densities.

Specificity of the Response in 3T3 Cells. The apparent stimulation of synthesis of ornithine decarboxylase by NIH-LH-B8 was specific with respect to total protein synthesis in 3T3 cells. Neither the rate of uptake of leucine, the size of the total leucine pool, nor the rate of incorporation of leucine into protein 4 hr or 8 hr poststimulation was substantially different from the corresponding values observed immediately before or after stimulation. (Four determinations were done for each parameter in cells of each of the four densities; the maximum change for any parameter was 6%, and was usually much less.) The stimulation was also specific with respect to other enzymatic activities, as no appreciable changes were observed in the activities of histidine decarboxylase, glutamate decarboxylase, tyrosine aminotransferase, or ornithine:keto acid aminotransferase (Table II).

Alteration in Half-Life of Ornithine Decarboxylase Activity after Stimulation. The half-life of ornithine decarboxylase activity was approximately 4 hr in seven separate

TABLE II: Specificity of Enzymic Response to Stimulation.[a]

Enzyme	Relative Specific Activity 0 hr	4 hr	8 hr	No. of Expt
Ornithine decarboxylase	1	22	27	15
Glutamate decarboxylase	1	0.8	1.1	2
Histidine decarboxylase	1	1.7	1.0	2
Ornithine:keto acid aminotransferase	1	1	0.9	3
Tyrosine aminotransferase	1	1.2	1.2	3

[a] Slightly sub-confluent 3T3 cells were stimulated with NIH-LH-B8 and duplicate cultures were harvested immediately, 4 hr, and 8 hr later in the appropriate buffer for each enzyme assay. Values for enzyme activity are averages of three determinations from each experiment, and are relative to the specific activity of the same enzyme in parallel unstimulated cultures.

experiments with unstimulated low or medium density 3T3 cells (see the legend to Figure 4 for methodology). In two experiments, values of 132 and 160 min were obtained. The reason for this variability is not clear. It may be due to minor variations in culture conditions as suggested by Kay and Lindsay in their experiments with lymphocytes (Kay and Lindsay, 1973a). The half-life progressively decreased as 3T3 cells grew to higher densities, and reached a relatively stable value of 65–70 min in density-inhibited cells.

Upon stimulation of cells at any density by NIH-LH-B8, a large transient decrease in the half-life of the enzyme was observed concomitant with the marked increase in total activity. In density-inhibited cells, the half-life decreased to about 30–35 min at 5–8 hr after stimulation, and then returned to the value of unstimulated cells by about 16 hr (Figure 3b). An even greater decrease in the half-life was observed in cells stimulated while at medium density (Figure 3a). The half-life in these cells eventually stabilized at a value typical of density-inhibited cells as this state was approached.

Exponential decay curves determined when the half-life was changing rapidly usually deviated from first-order kinetics in the direction expected. For instance, as the half-life was increasing at 14 hr after the stimulation of medium density cells, an upward deviation from linearity was observed in the semilogarithmic plot of activity *vs.* time (lower line in Figure 4).

Effect of Inhibitors of RNA or Protein Synthesis on the Increase in Ornithine Decarboxylase Activity. The stimulatory activity of NIH-LH-B8 was completely blocked in both growing and density-inhibited cells by prior or simultaneous addition of actinomycin D, cordycepin, puromycin, or trichodermin. Cordycepin inhibits the appearance of nascent mRNA in the cytoplasm (Penman *et al.*, 1970); trichodermin inhibits protein chain termination (Stafford and McLaughlin, 1973). Thus *de novo* synthesis of enzyme is apparently responsible for the increase in activity.

Addition of cordycepin to density-inhibited cells between 1 and 3 hr after stimulation, but not at 4 hr or later, diminished the subsequent enzyme activity (Figure 5). These data indicate that the cordycepin-sensitive step(s) is completed within 4 hr after stimulation. In contrast, actinomycin D added to density-inhibited cultures at 2 or 3 hr after

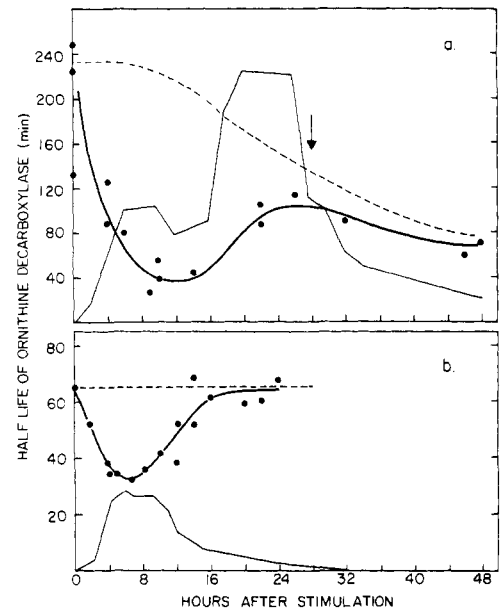

FIGURE 3: Variability of half-life. The light solid lines in each panel indicate ornithine decarboxylase activity and are identical to the corresponding tracings in Figure 1. Each point on the heavy lines represents a separate half-life determination at the indicated time after stimulation, as described in the legend to Figure 4. The dotted lines indicate the approximate half-life of the enzyme in unstimulated cells of similar density. The arrow in (a) indicates the approximate time of confluency. (a) Medium density cells; (b) density-inhibited cells.

NIH-LH-B8 resulted in an increase in enzyme activity ("superinduction") over the next several hours which was up to 80% greater than that seen in cultures not receiving actinomycin D (Figure 6). Stabilization of the enzyme accounted for at least part of this increase, as the decrease in

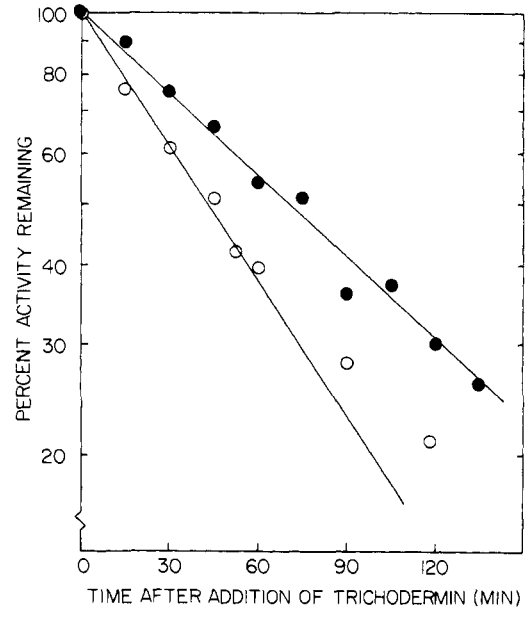

FIGURE 4: Half-life determinations 14 hr after stimulation. Trichodermin was dissolved in ethanol at a concentration of 50 mg/ml and diluted to 5 mg/ml in PBS; 25 μl was added to a series of replicate plates (final concentration 12.5 μg/ml) and individual plates were harvested at intervals thereafter and assayed for ornithine decarboxylase; 5–15 μg/ml of trichodermin inhibited protein synthesis by at least 95%. All half-lives reported in this communication were determined from a minimum of five points. Similar results were obtained when puromycin was used (not shown). Density-inhibited cells (●); medium density cells (○).

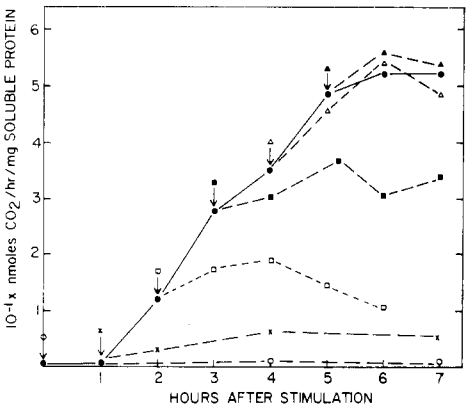

FIGURE 5: Effect of cordycepin on density-inhibited cells. NIH-LH-B8 was added to all cultures at 0 hr; 25 μl of cordycepin in PBS was then added to appropriate cultures at the times indicated by the arrows. The final concentration of cordycepin was 6.25 μg/ml. This concentration inhibited RNA synthesis by 96–100%. Duplicate plates were harvested at hourly intervals for each variable and assayed for ornithine decarboxylase. Each point is the average of two experiments, or a total of eight assays. No value deviated more than 7% from the mean. 25 μl of PBS at 0 hr (●—●); cordycepin at 0 hr (○—○); cordycepin at 1 hr (X- -X); cordycepin at 2 hr (□- -□); cordycepin at 3 hr (■- -■); cordycepin at 4 hr (△- -△); cordycepin at 5 hr (▲- -▲).

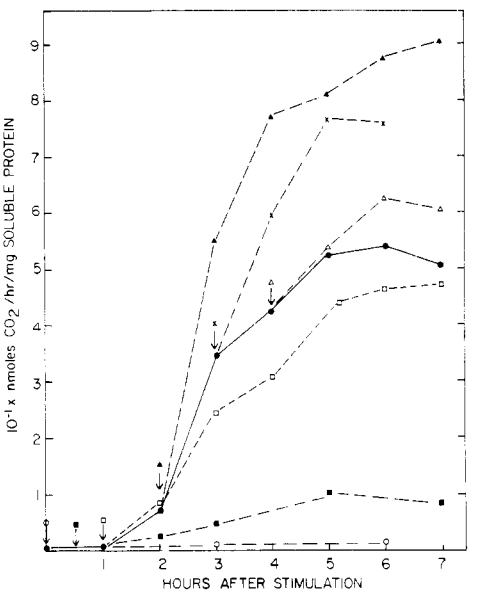

FIGURE 6: Effect of actinomycin D on density-inhibited cells. NIH-LH-B8 was added to all cultures at 0 hr; 50 μl of actinomycin D in ethanol was then added to appropriate cultures at the times indicated by the arrows. The final concentration of actinomycin D was 0.5 μg/ml. This concentration inhibited RNA synthesis by about 94%. This figure is a composite of five experiments. Single plates were harvested for each point in each experiment. All points represent an average value from at least three experiments (six assays). The maximum deviation from the mean was less than 12%. 50 μl of ethanol at 0 hr (●- -●); actinomycin D at 0 hr (○- -○); actinomycin D at 0.5 hr (■- -■); actinomycin D at 1 hr (□- -□); actinomycin D at 2 hr (▲- -▲); actinomycin D at 3 hr (X- -X); actinomycin D at 4 hr (△- -△).

the half-life normally seen upon stimulation was arrested within 30 min of actinomycin D addition. A "superinduction" of hepatic ornithine decarboxylase activity in rats treated with puromycin has recently been reported (Beck and Canellakis, 1973). Qualitatively similar results with cordycepin and actinomycin D were also obtained in the first 8 hr after stimulation of exponentially growing medium-density cells.

Discussion

The basal specific activity of ornithine decarboxylase in exponentially growing mouse 3T3 and human fibroblasts was higher by nearly an order of magnitude than for any tissue previously examined, but was comparable to that observed with exponentially growing hepatoma cells in culture (Hogan, 1971). The absolute increase in activity upon growth stimulation was much greater than has been previously observed in any stimulated system. Two other cell lines, Hela S-3 and adrenal Y-1, had basal and maximal specific activities which were about an order of magnitude greater than any previously reported values. Cells in culture are not an invariably rich source of enzyme, however, as activity could not be detected in stimulated or unstimulated Chinese hamster ovary cells or rat ovary cells (Table I). The fact that enzyme activity (when detectable) declined markedly in all cell lines as they approached the quiescent state is consistent with polyamine synthesis being of greatest importance during periods of rapid growth.

Biphasic Response of Ornithine Decarboxylase in Stimulated Exponentially Growing 3T3 Cells. A biphasic response similar to that observed in these studies (Figure 1) has also been seen in phytohemagglutinin-stimulated lymphocytes (Kay and Lindsay, 1973a), rat kidney during compensatory hypertrophy (Brandt *et al.*, 1972), and in regenerating liver (Hölttä and Jänne, 1972). The reason(s) for this bimodality is not understood. The transient decrease in the half-life of the enzyme in 3T3 cells after stimulation (Figure 3) clearly contributed to this pattern in the current study, but statistical analysis demonstrated that a biphasic response would still occur if the half-life remained constant. K_m values and heat sensitivities were identical for the enzyme activity from all peaks in Figure 1, rendering unlikely the possibility that separate enzymes were responsible for the biphasic response. The involvement of soluble activators or inhibitors was ruled out by the appropriate dialysis and mixing experiments. Two periods of transcription is also an unlikely explanation for this bimodality, as cordycepin did not prevent the appearance of the second peak of activity in 3T3 cells if administered as early as 4 hr after stimulation.

A more plausible mechanism of the biphasic response in exponentially growing cells, suggested by work both with KB cells (Pett and Ginsberg, 1968), and human lymphocytes (Kay and Lindsay, 1973b), is inhibition of translation of new enzyme by polyamines. Putrescine or spermidine added to cultures of KB cells or lymphocytes results in a rapid fall in the specific activity of ornithine decarboxylase. With lymphocytes, continued protein synthesis is essential to see this inhibitory effect, and simple feedback inhibition has been ruled out. Similar results have been obtained with 3T3 cells (Clark, unpublished experiments). Thus it may be that an early increase in polyamine concentrations in the cell temporarily inhibits further enzyme synthesis, but as utilization of polyamines for other purposes increases, this inhibition is gradually released and a second increase in translation occurs. A second peak of activity may not be observed in density-inhibited 3T3 cells because of their limited capacity to utilize polyamines in a growth response. This possibility is under investigation.

Specificity of the Response. The apparent induction of ornithine decarboxylase was specific with respect to both total protein synthesis and the activity of selected enzymes (Table II). In agreement with studies on lymphocytes (Kay and Lindsay, 1973a), no change was observed in the rate of

protein synthesis in 3T3 cells at 4 or 8 hr after stimulation. Also, the activities of histidine decarboxylase and glutamate decarboxylase did not change appreciably during this time, in accord with findings in other systems (Russell and Snyder, 1968; Raina and Jänne, 1968). The activity of ornithine: keto acid aminotransferase, the only other widely distributed ornithine enzyme, was also virtually unchanged, indicating that ornithine utilization for polyamine synthesis is preferentially augmented under these conditions. The activity of this enzyme decreases in other systems under circumstances in which ornithine decarboxylase activity is elevated (Williams-Ashman *et al.*, 1969; Ikuko *et al.*, 1974). The possibility that the apparently specific stimulation of synthesis of ornithine decarboxylase in 3T3 cells may actually be only a reflection of its relatively short half-life (Berlin and Schimke, 1965) was eliminated by demonstrating that the activity of tyrosine aminotransferase, another very short-lived enzyme, increased only slightly upon stimulation. Tyrosine aminotransferase had a half-life of 90 min in density-inhibited 3T3 cells. By all the above criteria, the synthesis of ornithine decarboxylase was specifically stimulated by NIH-LH-B8. We have not, however, established this directly by determining the actual rates of synthesis of the decarboxylase or of other enzymes.

The Half-Life of the Enzyme. The half-life of ornithine decarboxylase in unstimulated exponentially growing 3T3 cells (132–244 min) is longer than has been reported (5–90 min) for other tissues and cells (Russell and Snyder, 1969; Williams-Ashman *et al.*, 1972; Melvin *et al.*, 1972; Kay and Lindsay, 1973b; Hogan *et al.*, 1973). Although it is not apparent why the enzyme is relatively long-lived in 3T3 cells, its greater stability may partially account for the high activity observed in these cells. The decrease in the half-life of the enzyme as cells approached the density-inhibited state is the first clear demonstration of a growth- or density-dependent change in the turnover rate of a specific protein in animal cells. As 3T3 cells show no change in the average protein half-life when plated in serum containing medium and allowed to grow to the resting state (Weber, 1972), the present results indicate that regulatory mechanisms governing turnover of specific proteins must exist. A similar decrease in the half-life of the enzyme in hepatoma cells is suggested by the observations of Hogan and coworkers (Hogan *et al.*, 1973). In these cells, the half-life of the enzyme is as high as 90 min at 2 hr after subculture, whereas it is 5–13 min in stationary cultures.

The paradoxical destabilization of ornithine decarboxylase concomitant with the large increase in total activity observed upon growth stimulation of 3T3 cells by NIH-LH-B8 (Figure 3) has not previously been observed for any enzyme in any system. This putative concurrent activation of opposing mechanisms may allow the cell to finely regulate the level of ornithine decarboxylase, although such a mechanism seems wasteful. These results are apparently in contrast with those from studies on hepatoma cells and lymphocytes. Both glutamine addition to high-density cultures and serum addition to serum-starved cultures of hepatoma cells (Hogan *et al.*, 1973), as well as addition of nonessential amino acids to phytohemagglutinin-stimulated lymphocytes (Kay and Lindsay, 1973a), increase the half-life of ornithine decarboxylase. These are conditions which would be expected to stimulate growth. Possible explanations for this difference are under study.

"Superinduction" by Actinomycin D. The further increase in ornithine decarboxylase activity in stimulated cells upon addition of actinomycin D (Figure 6) is similar to observations in many diverse systems (Tomkins *et al.*, 1972) in which inhibitors of macromolecule synthesis increase the activity or concentration of specific proteins. Tomkins and coworkers have proposed that such increases are due to *de novo* protein synthesis (Tomkins *et al.*, 1969), permitted by the decay of a labile repressor of translation which is transcribed from a short-lived mRNA. His laboratory has demonstrated an increase in synthesis of tyrosine aminotransferase in liver cells after actinomycin D administration, in agreement with the above hypothesis.

However, Kenney and coworkers reported that the increased activity of tyrosine aminotransferase after actinomycin D is due to a decrease in the rate of degradation (Reel and Kenney, 1968) caused by the antibiotic. In addition, cordycepin did not lead to greater activity of the enzyme, and did not prevent "superinduction" by actinomycin D (Butcher *et al.*, 1972; Kenney *et al.*, 1973). These results are similar to those obtained in the present work with ornithine decarboxylase. Together they suggest the existence of a general "superinduction" mechanism distinct from the labile repressor model.

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