Regulation of Ornithine Decarboxylase in 3T3 Cells by Putrescine and Spermidine: Indirect Evidence for Translational Control†

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ABSTRACT: Addition of putrescine or spermidine prevents the increase in ornithine decarboxylase activity in cultures of 3T3 cells brought about by pituitary growth factors and results in a rapid, specific, and reversible reduction of enzyme activity in cultures previously stimulated by the growth factors. These effects are not due to polyamine toxicity and do not require other organic medium components. The amines apparently share a single carrier-mediated transport system in 3T3 cells. Methylglyoxal bis(guanylhydrazone), an inhibitor of spermidine synthesis from putrescine, was found to also inhibit uptake of each amine. Studies with this drug indicate that each amine is effective without further metabolism. Since ornithine decarboxylase activity decays more rapidly in the presence of each polyamine than after addition of camptothecin, the major locus of amine action appears to be in the cytoplasm. However, direct inhibition of the enzyme in vivo by assimilated amines appears to account for at most a small part of the reduction in activity, a conclusion supported by the inability to recover activity in vitro. Also, neither amine seems to act by accelerating enzyme inactivation. When amines are removed from the medium, the subsequent recovery of enzyme activity is totally prevented by trichodermin, an inhibitor of protein synthesis, but is only slightly reduced by camptothecin. It is suggested that both putrescine and spermidine reduce ornithine decarboxylase activity by selectively inhibiting translation.

Ornithine decarboxylase (EC 4.1.1.17; L-ornithine carboxy-lyase), the first enzyme in the polyamine pathway in eukaryotes, may be a critical enzyme in the control of cell growth (Williams-Ashman et al., 1972; Bachrach, 1973; Morris and Fillingame, 1974). If this is the case, close regulation of its activity would be expected. Polyamines are weak competitive inhibitors of the enzyme from some sources (Jänne and Williams-Ashman, 1971a; Clark, 1974), but polyamines in vivo do not appear to cause significant inhibition. Extensive testing of other small molecules has not revealed a potential physiological effector of the eukaryotic enzyme (Jänne and Williams-Ashman, 1971a; Ono et al., 1972, Friedman et al., 1972; Jänne and Hölttä, 1974; J. L. Clark, unpublished experiments). Thorough searches for post-translational modification mechanisms have not been conducted. However, no evidence for a phosphorylation–dephosphorylation sequence, the most common of such mechanisms, has been found (J. L. Clark, unpublished experiments; Byus and Russell, 1975). Therefore, control of the activity of existing enzyme molecules does not appear to be important.

Although direct confirmation is lacking, studies with metabolic inhibitors indicate that ornithine decarboxylase is regulated primarily at the levels of synthesis and degradation (Morris and Fillingame, 1974; Clark, 1974). Because the turnover of ornithine decarboxylase is very rapid (Morris and Fillingame, 1974; Lembach, 1974; Clark, 1974), relatively small changes in either the rate of synthesis or degradation result in rapid fluctuations in total enzyme activity. However, no mechanisms for control of ornithine decarboxylase synthesis and degradation are known.

There is considerable indirect evidence for both transcriptional and post-transcriptional control of synthesis (Fausto, 1971; Jänne and Hölttä, 1974; Kay and Lindsay, 1973; Hogan et al., 1974). In regenerating liver (Schrock et al., 1970; Jänne and Hölttä, 1974) and in cultured cells (Pett and Ginsberg, 1968; Kay and Lindsay, 1973), exogenous polyamines rapidly reduce ornithine decarboxylase activity. A direct effect on the enzyme does not appear to account for these observations (Kay and Lindsay, 1973; Jänne and Hölttä, 1974). On the basis of experiments with human lymphocytes, Kay and Lindsay (1973) proposed that spermidine, but not putrescine, modulates the synthesis of ornithine decarboxylase. We have now performed similar and additional experiments with 3T3 cells. Our results are consistent only with a specific inhibition of the translation of ornithine decarboxylase message by either putrescine or spermidine.

Experimental Section

Materials. DL-[1-^{14}C]Ornithine (7.1 Ci/mol), [1,4-^{14}C]putrescine·2HCl (19.5 Ci/mol), and [*tetramethylene*-1,4-^{14}C]spermidine·3HCl (12.4 Ci/mol) were obtained from New England Nuclear. Other radioisotopes were from ICN Pharmaceuticals. MGBG[1] was from Aldrich. Camptothecin and trichodermin were the gifts of Drs. H. B. Wood, Jr. (National Cancer Institute) and Calvin McLaughlin (University of California, Irvine), respectively. NIH-LH-B8, a preparation of bovine luteinizing hormone, was from the Endocrine Section of the National Institutes of Health.

Cell Culture. Swiss 3T3 mouse fibroblasts were initiated monthly from frozen ampoules and cultured in medium containing 10% calf serum as described (Clark, 1974), ex-

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[1] Abbreviations used are: MGBG, methylglyoxal bis(guanylhydrazone); poly(A), poly(adenylic acid).

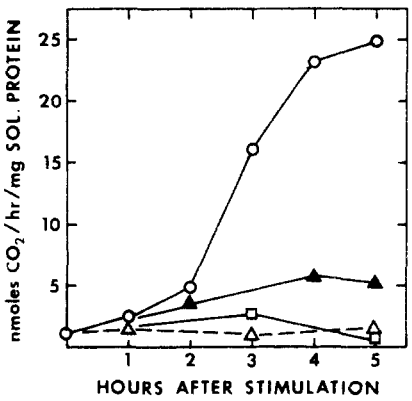

FIGURE 1: Blockage of increase in ornithine decarboxylase activity by putrescine. Putrescine was added to cultures immediately before stimulation by NIH-LH-B8. Duplicate cultures were harvested at the indicated times and assayed for enzyme activity. Unstimulated (Δ--Δ); stimulated (O—O); stimulated plus putrescine: 10^{-4} *M* (□—□); 10^{-5} *M* (▲—▲).

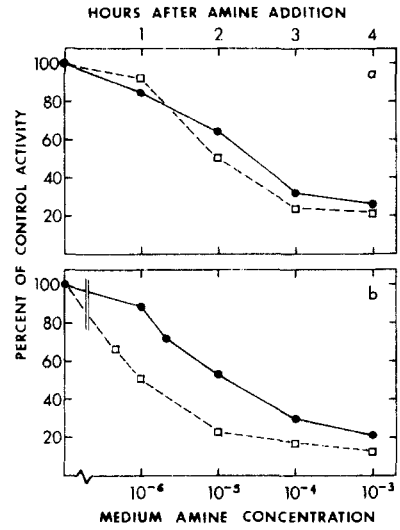

FIGURE 2: Decrease in enzyme activity after incubation with putrescine or spermidine. Duplicate cultures were harvested and assayed for enzyme activity at the indicated times after amine addition (a) or at 3 hr after amine addition (b). All values are the averages of at least eight assays from at least three experiments. No datum deviated from the mean by more than seven percentage points of the control activity. (a) 10^{-4} *M* putrescine (●—●); 10^{-5} *M* spermidine (□--□); (b) putrescine (●—●); spermidine (□--□).

cept that no antibiotics were used. Cultures were periodically monitored for mycoplasma contamination by the uridine/uracil ratio method (Schneider et al., 1974). Only mycoplasma-free cultures were used for experiments. Unless otherwise stated, the growth of subconfluent cells (approximately 8×10^3 cells/cm^2) was stimulated by addition of 10 μg/ml of NIH-LH-B8 (final concentration) 9–14 hr prior to use (Clark, 1974). The stimulatory factors in NIH-LH-B8, present as minor components, are pituitary growth factors (Bihler and Clark, unpublished experiments) which are similar or identical to fibroblast growth factor (Gospodarowicz, 1973). All agents were added in a volume of saline solution that was 1% of the culture volume; control cultures received only saline. MGBG and polyamine stock solutions were made immediately before use.

To avoid rapid oxidation of spermidine due to oxidase activity found in calf serum (Bachrach, 1973), cultures were rinsed with isotonic saline and placed in medium containing 8% horse serum and 2% fetal calf serum 24 hr before addition of spermidine. This medium change produced a small increase in ornithine decarboxylase activity, but activity returned to basal levels by 6–8 hr.

Enzyme and Protein Assays. Cell extracts were prepared and handled as described previously (Clark, 1974). At least one control culture was harvested with each experimental culture. Ornithine decarboxylase was assayed as described (Jänne and Williams-Ashman, 1971a) with the following exceptions: 0.025–0.25 μCi of DL-[1-^{14}C]ornithine was included in each assay; the reaction was stopped with 1 ml of 2 *M* citric acid; and ethanolamine–2-methoxyethanol (2:1) was used to trap released CO_2. *S*-Adenosylmethionine decarboxylase (Jänne and Williams-Ashman, 1971b) and protein (Ross and Schatz, 1973) were assayed as reported. Other enzyme assays and uridine and leucine uptake and incorporation studies were performed as before (Clark, 1974).

Determination of Intracellular Radioactive Polyamines. Cultures incubated with radioactive ornithine, putrescine, or spermidine were rinsed in situ in four successive baths of ice-cold saline. These rinsings were completed in less than 15 sec; loss of intracellular polyamines was not detectable. Cells were harvested with a rubber policeman and extracted overnight at 4° in 1.5 ml of 10% trichloroacetic acid. The acid was removed by repeated extractions with ether, 10 μg of both putrescine and spermidine were added, and the amines were partially purified by AG-50 chromatography (200–400 mesh) essentially as described (Hammond and Herbst, 1968). After desiccation, the residue was redissolved in 0.5 ml of H_2O, extracted with ether, and redesiccated. The polyamines were separated by electrophoresis (Raina et al., 1970) and stained with ninhydrin. Radioactivity was determined by scanning on a Nuclear Chicago radiochromatogram. In some cases, the identity of the labeled compounds was confirmed by dansylation (Seiler, 1970) of unstained polyamines eluted from chromatograms and separation of the dansylated derivatives by thin-layer chromatography on silica G, using cyclohexane–diethyl ether (1:9) as the developing solvent (Seiler and Wiechman, 1970). All radioactivity was found in fluorescent spots which corresponded to dansylated standards, and the distribution of radioactivity was the same as that found by scanning of paper chromatograms.

Estimate of Intracellular Fluid Volume. The intracellular fluid volume of $5–6 \times 10^7$ cells was measured as described for L cells (Runyan and Liao, 1973), except that the 3T3 cells were harvested by trypsinization to avoid premature rupture and [^{14}C]inulin was used as the radioactive tracer to preclude production of $^{14}CO_2$ by the cells.

Results

Stimulation of 3T3 cells with NIH-LH-B8 results in a rapid increase in ornithine decarboxylase activity which is totally dependent on de novo RNA and protein synthesis (Clark, 1974). If putrescine (10^{-3} *M* final concentration) is added immediately before stimulation, this increase is prevented (Figure 1). Spermidine is equally effective at lower concentrations (data not shown). Addition of either amine at any time between 2 and 16 hr after stimulation results in a progressive decline in enzyme activity relative to controls. Representative data are shown in Figure 2a. This decline is concentration dependent (Figure 2b). Undegraded amines are the effective agents in the medium, as they are not altered by the medium in these experiments. Other organic medium components are not involved, since qualitatively

Table I: Conversion of Exogenous Ornithine to Polyamines by 3T3 Cell Cultures.[a]

Incubation Period (hr after NIH-LH-B8)	Medium Amine	Intracellular Polyamines (cpm)	
		Putrescine	Spermidine
0–4	None	1041	151
	10^{-4} *M* putrescine	134	20
	10^{-5} *M* spermidine	86	27
10–13	None	1597	438
	10^{-4} *M* putrescine	512	81
	10^{-5} *M* spermidine	382	107

[a] Cultures were incubated with DL-[^{3}H]ornithine (1 μCi/ml) for the indicated period. Intracellular radioactive polyamines were extracted and determined as described in the Experimental Section. The maximum number of counts in any nonpolyamine region of the chromatograms was 26 cpm. The rate of uptake was altered less than 10% by these concentrations of polyamines. Each value is the average of three determinations.

Table II: Distribution of Radioactivity among Intracellular Polyamines from Cultures Incubated with [^{14}C]Polyamine and with or without MGBG.[a]

Medium Polyamine[b]	MGBG	Intracellular Polyamines[c]		
		Putrescine	Spermidine	Spermine
10^{-4} *M* putrescine		79	18	3
10^{-4} *M* putrescine	10 μ*M*	>99	<1	0
10^{-4} *M* putrescine	1 m*M*	100	0	0
10^{-5} *M* spermidine		3	95	2
10^{-5} *M* spermidine	10 μ*M*	2	97	1

[a] Cultures were incubated with the indicated agents for 3 hr. Intracellular radioactive polyamines were then extracted and determined as described in the Experimental Section. All values are the averages of 2–6 determinations. [b] 4 μCi/ml. [c] Expressed as percent of the total intracellular radioactivity.

similar results are obtained when cultures are transferred to phosphate-buffered saline immediately before amine addition. Each amine reduces putrescine synthesis from exogenous [^{3}H]ornithine in approximate proportion to the reduction of enzyme activity in extracts (Table I). Neither amine (10^{-4} *M* final) has a detectable effect on activity when added to cultures immediately before harvest or directly to assay mixtures.

Spermidine itself, and not a metabolite, is most likely an effective intracellular agent since 95% of the amine taken up by the cells is unchanged after 3 hr (Table II). Because medium spermidine is more effective than putrescine in reducing enzyme activity (Figure 2b), and since about 20% of the putrescine taken up by 3T3 cells is converted to spermidine during 3-hr incubations (Table II), it seemed possible that only spermidine is active within the cell and that putrescine must first be converted to spermidine. This possibility was examined by determining uptake parameters of the two amines, and by use of MGBG, a potent inhibitor of spermidine synthesis from putrescine (Williams-Ashman and Schenone, 1972).

The uptake of each amine was found to be carrier-mediated and probably energy-dependent in 3T3 cells. The kinetic parameters in stimulated cells are: putrescine, K_m = 4.6×10^{-6} *M*, V_{max} = 3.4 pmol per mg of protein per hr; spermidine, $K_m = 3 \times 10^{-7}$ *M*, V_{max} = 1.5 pmol per mg of protein per hr. Simple diffusion is evident only above 10^{-3} *M*. Uptake was not measurable at 0°, and was depressed more than 90% at 37° by addition of 2 m*M* sodium cyanide and 10 m*M* sodium iodoacetate. The polyamines compete with each other for uptake, and each has a K_i for the other nearly equal to its own K_m. Similar results are obtained for unstimulated cells, except that K_m values are slightly higher.

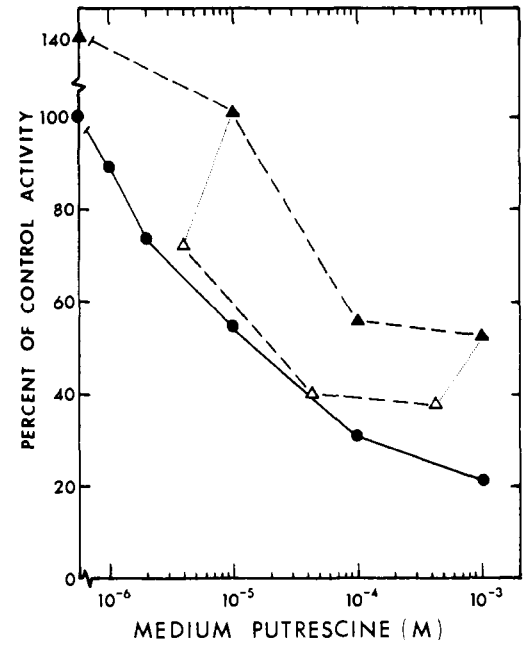

FIGURE 3: Effect of MGBG on reduction of enzyme activity by medium putrescine. Cultures were incubated for 3 hr with various concentrations of putrescine and with or without MGBG added 5–10 min prior to putrescine. All cultures were harvested and assayed for enzyme activity immediately after incubation. The data for putrescine alone are taken from Figure 2b. Each MGBG point is the average of at least eight assays in four experiments. Circles, putrescine alone; triangles, putrescine plus 10 μ*M* MGBG. Solid symbols, data corrected only for the protein content of cultures; open symbols, the above data adjusted for the effect of MGBG on putrescine uptake and on ornithine decarboxylase activity (see text).

MGBG competitively inhibits the uptake of both putrescine and spermidine, with a K_i in each case of 5 μ*M*. Simultaneous incubation with MGBG greatly increases the concentration of both putrescine (Figure 3, solid triangles) and spermidine (Figure 4, solid triangles) needed to reduce enzyme activity. 10–20 μ*M* MGBG consistently increases ornithine decarboxylase activity in 3T3 cells after 3 hr by an average of 40% (S.E.M. = 13%) in the absence of medium polyamines, similar to observations in lymphocytes (Fillingame and Morris, 1973). If it is assumed that this effect and the drug's inhibition of uptake are independent, the apparent blockage of putrescine disappears (Figure 3, open triangles) when the MGBG data are adjusted for the "direct" effect of MGBG on enzyme activity and shifted to lower putrescine concentrations (determined graphically from double reciprocal plots of inhibition data) to quantitatively take into account the inhibition of putrescine uptake. Putrescine itself is active, as none is converted to spermidine or any other metabolite in the presence of MGBG (Table II). When similar corrections are made for the spermidine data, this MGBG blockage also vanishes (Figure 4, open triangles).

The reduction of enzyme activity by medium putrescine or spermidine could be due to one or more of the following:

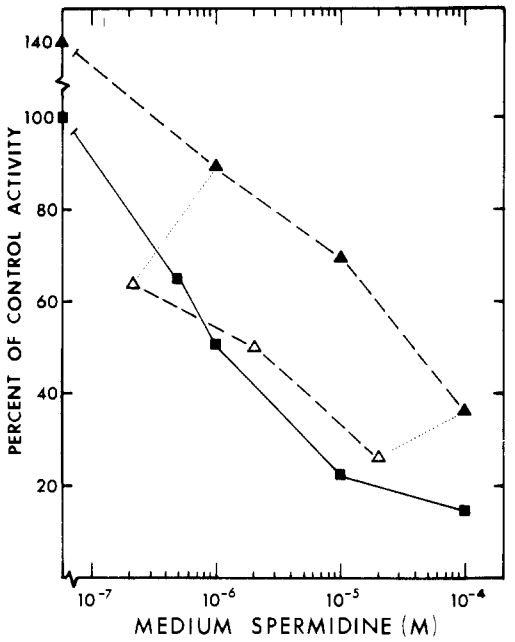

FIGURE 4: Effect of MGBG on reduction of enzyme activity by medium spermidine. Cultures were incubated for 3 hr with various concentrations of spermidine and with or without MGBG added 10 min prior to spermidine. All cultures were harvested and assayed for enzyme activity immediately after incubation. The data for spermidine alone are taken from Figure 2b. Squares, spermidine alone; triangles, spermidine plus 20 μM MGBG. Solid symbols, data corrected only for the protein content of cultures; open symbols, the above data adjusted for the effect of MGBG on spermidine uptake and on ornithine decarboxylase activity (see text).

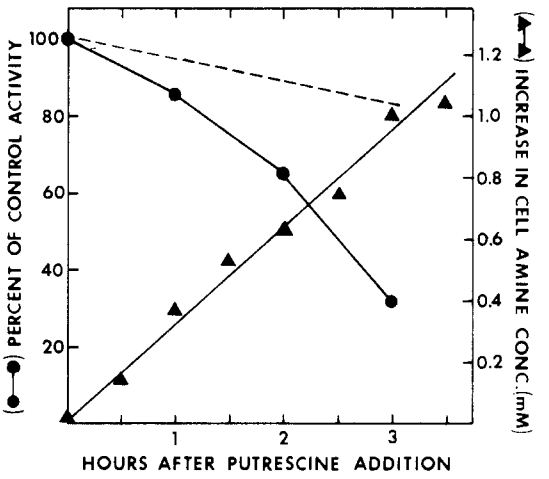

FIGURE 5: Increase in intracellular polyamine concentration due to uptake of medium putrescine. Cultures were incubated with 10^{-4} M putrescine (0.5 μCi/ml) and processed as described in the Experimental Section. Polyamines accounted for all of the intracellular radioactivity throughout the incubation period (see Table II). Increases in polyamine concentrations (▲—▲) were calculated from intracellular fluid volume and cell number determinations on replicate plates. Each concentration is derived from the average of three uptake determinations. The observed decrease in enzyme activity relative to control cultures in undialyzed extracts from cells incubated with 10^{-4} M putrescine (●—●) is taken from Figure 2a. The dotted line estimates the maximum decrease in enzyme activity which would obtain in the presence of 1.1 mM ornithine if all of the assimilated putrescine were competitively inhibiting the enzyme.

(a) nonspecific or toxic effects; (b) direct inhibition of the enzyme; (c) acceleration of enzyme degradation; (d) a decrease in the rate of enzyme synthesis.

(*a*) *Absence of Nonspecific or Toxic Effects.* The reduction of ornithine decarboxylase activity is specific, as the activities of three other enzymes, ornithine:keto acid aminotransferase; tyrosine aminotransferase; and *S*-adenosylmethionine decarboxylase, are essentially unchanged (data not shown). Also, there is no significant effect on either the uptake or incorporation of [^{3}H]uridine during 4-hr incubations (data not shown) in the presence of amine concentrations which cause large decreases in ornithine decarboxylase activity. Incorporation of [^{3}H]leucine is decreased by about 5% after 4-hr incubations with 10^{-4} M putrescine or 10^{-5} M spermidine, and by a greater degree (up to 20%) for shorter incubation periods. This may be largely due to a decrease in the synthesis of ornithine decarboxylase (J. L. Clark, in preparation). When the amine-containing medium is replaced after 4 hr with medium from control cultures, enzyme activity increases rapidly. These cultures have doubling times identical with controls (18 hr).

(*b*) *Inhibition of Enzyme Activity.* When extracts from control and polyamine-treated cultures are mixed, additive activities are observed. Exhaustive dialysis (48 hr) of extracts prepared from cultures exposed to 10^{-4} M putrescine or 10^{-5} M spermidine for 3 hr did not significantly increase activity. By these criteria, the reduction in activity is not due primarily to reversible inhibition by polyamines or other compounds.

However, since it is possible that inhibited enzyme is lost during extract preparation and is therefore not detected by these in vitro experiments, the potential inhibition of the enzyme in vivo by assimilated amines was estimated as follows. The intracellular fluid volume of 3T3 cells, their ornithine content, and the rate of accumulation of exogenous polyamine were measured. The intracellular fluid volume of 10^6 cells was 2.12 and 2.42 μl in two experiments. These values agree with earlier 3T3 cell intracellular fluid space measurements using a different method (Foster and Pardee, 1969).[2] The average ornithine content of 10^6 cells, determined by amino acid analysis, was 2.6 ± 0.3 nmol in four experiments. The average cell concentration of ornithine is thus about 1.1 mM. This value compares favorably to that of normal rat prostate (Pegg et al., 1970). The rate of net uptake of each polyamine was linear for at least 3 hr. The maximum *increase* in cell putrescine concentration that is *due to uptake*[3] is shown in Figure 5. These data, together with kinetic constants for ornithine and polyamines determined in vitro, permit an estimate of the maximum degree of competitive inhibition[4] of the enzyme which could occur in vivo due to assimilated polyamines. This putative inhibi-

[2] These workers related intracellular fluid volume to cell protein, not cell number. Using their value of approximately 1 mg of protein/10^6 exponentially growing cells, a volume of 2.3 μl/10^6 cells is determined. The cells used in the present studies also contain about 1 mg of protein/10^6 cells.

[3] *Total* putrescine concentration increases more slowly (J. L. Clark, unpublished experiments).

[4] These calculations provide the maximum inhibition for the following reasons: (a) if the enzyme is already inhibited by endogenous polyamines, any increment due to assimilated putrescine would be even less; (b) it is assumed that all assimilated putrescine is free to inhibit, whereas much is undoubtedly complexed to polyanions; (c) by the end of the 3-hr period, some of the assimilated putrescine has been converted to spermidine and spermine (Table II) which are even weaker inhibitors of the enzyme; (d) it is possible that cell ornithine is restricted primarily to the cytoplasmic compartment, rather than equally distributed throughout the cell fluid as assumed, due to the very low levels of enzymes which produce or utilize ornithine in other compartments (Raijman, 1974; Williams-Ashman et al., 1969).

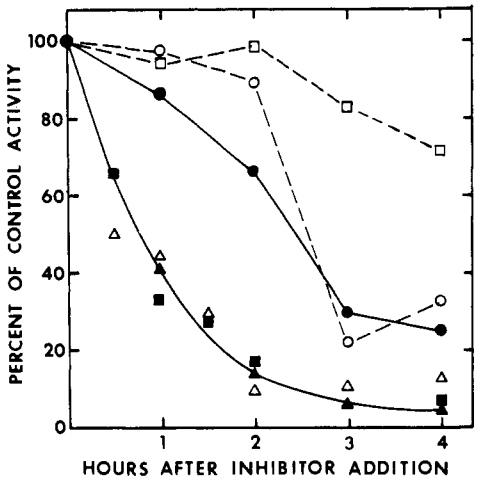

FIGURE 6: Decrease in enzyme activity in the presence of various agents. Cultures were harvested and assayed for enzyme activity at the indicated times after addition of inhibitors. 5 μg/ml of trichodermin inhibits protein synthesis in 3T3 cells by at least 95% (Clark, 1974). 10^{-4} *M* putrescine (●—●) (data from Figure 2a); 5 μg/ml of trichodermin (▲—▲); 5 μg/ml of trichodermin and 10^{-3} *M* putrescine (■—■); 5 μg/ml of trichodermin and 10^{-3} *M* spermidine (△—△); 20 μg/ml of camptothecin (□--□); 6.25 μg/ml of cordycepin (○--○).

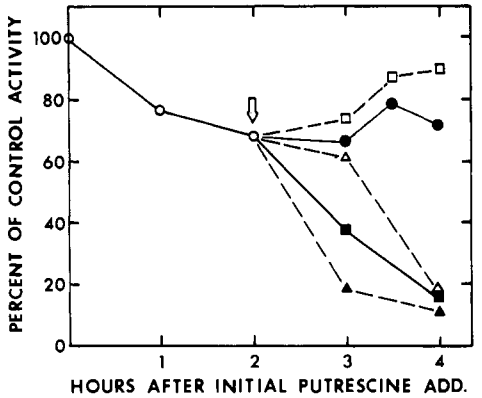

FIGURE 7: Recovery of enzyme activity after removal of putrescine. 10^{-4} *M* putrescine was added to all but control cultures at 0 hr. Duplicate putrescine-containing and control cultures were harvested at 0, 1, and 2 hr. All remaining putrescine and control cultures were rinsed with 37° saline solution at 2 hr (arrow), and placed in medium from identical cultures which had not received putrescine. 10^{-4} *M* putrescine was then re-added to some experimental cultures, while others received the agents listed below. Subsequent harvestings were at the indicated times. The data are the averages of two experiments. Putrescine for first 2 hr (○—○); no putrescine after 2 hr (□--□); putrescine re-added at 2 hr (■—■); 5 μg/ml of trichodermin at 2 hr (▲--▲); 6.25 μg/ml of cordycepin at 2 hr (△--△); 20 μg/ml of camptothecin at 2 hr (●—●).

tion by putrescine (dotted line in Figure 5) and spermidine (data not shown) is far from sufficient to explain the reduction in activity observed in the assay.

(*c*) *Apparent Lack of Acceleration of Enzyme Degradation.* Ornithine decarboxylase may be subject to product destabilization, similar to glutamine synthetase in Chinese hamster ovary cells (Milman et al., 1975) and other systems. To explore this possibility, protein synthesis was inhibited in stimulated cultures by the addition of trichodermin, and incubation was continued with and without 10^{-3} *M* polyamine for 4 hr. Enzyme activity decayed with a half-life of 45 min whether or not polyamine was present (lower curve in Figure 6); thus polyamines do not accelerate enzyme inactivation in the absence of protein synthesis.

(*d*) *Effects on Enzyme Synthesis.* On the basis of the above results, it appears that medium polyamines are reducing enzyme activity by interfering with synthesis. Such an effect could be at the level of transcription, transport of mRNA to the cytoplasm, or translation.

Cordycepin inhibits the appearance of mRNA in the cytoplasm of HeLa cells (Penman et al., 1970); camptothecin prevents the appearance of RNA larger than 5 S in the cytoplasm of HeLa cells (Abelson and Penman, 1972). Similarly, 6.25 μg/ml of cordycepin was found to inhibit the appearance of cytoplasmic poly(A) containing (messenger) RNA in 3T3 cells by at least 85%, as determined by the nitrocellulose filter method (Brawerman et al., 1972), and 20 μg/ml of camptothecin reduced the appearance of RNA larger than 5 S in the cytoplasm of 3T3 cells by greater than 90% after a 90-min incubation period as determined by sucrose density gradients (Abelson and Penman, 1972). Since enzyme activity decays more slowly in the presence of either of these inhibitors than with polyamines (Figure 6), the amines appear to act after the transcription and transport processes. The effect of cordycepin becomes more pronounced after 2 hr, while that of camptothecin remains slight for 4 hr. This may be because cordycepin directly inhibits chain elongation by about 2 hr (Penman, 1974), whereas camptothecin has no direct effect on protein synthesis for at least 3 hr (Wu et al., 1971). In contrast, trichodermin, an inhibitor of translation, reduces activity slightly more rapidly than 2×10^{-3} *M* putrescine, the maximally effective nontoxic concentration, and submaximal concentrations of each together are more effective than either separately (data not shown). When putrescine is removed after a 2-hr exposure, a rapid recovery in activity is observed (Figure 7) which is prevented by trichodermin, but only slightly diminished by camptothecin. The effect of cordycepin is similar qualitatively (Figure 7) to that observed when the drug is added without putrescine pretreatment (Figure 6). Results consistent with a partial block of translation are also obtained using these inhibitors and spermidine.

Discussion

The rapid reduction of ornithine decarboxylase activity in 3T3 cells by medium polyamines is specific, as tyrosine aminotransferase and *S*-adenosylmethionine decarboxylase activities are not markedly affected. Similar results were obtained in hepatectomized rat liver after polyamine injection (Jänne and Hölttä, 1974). These unstable enzymes are appropriate controls, because they would also be expected to show rapid changes in activity if the polyamine perturbation is general. Polyamines are effective with 3T3 cells at concentrations which are not toxic by several additional criteria; enzyme activity is similarly "inhibited" in human lymphocytes (Kay and Lindsay, 1973), with no accompanving general effects. In the present studies, it was shown that the activity reduction required neither metabolism of the polyamines nor other exogenous organic compounds.

Dialysis and mixing experiments demonstrated that the lower enzyme activity in extracts of 3T3 cultures exposed to polyamines is not due to soluble inhibitors present in excess. Similar results were obtained in the studies with liver and lymphocytes. However, as inhibited enzyme may not be recovered in extracts, these results do not preclude a greater inhibition in intact cells. The putrescine concentration in regenerating liver after injection of the amine did not rise above 1 m*M* (Jänne and Hölttä, 1974). This concentration

did not cause significant inhibition in vitro under the assay conditions employed, but may in vivo, as liver ornithine concentration appears to be no higher than 0.15 m*M* (Raijman, 1974), and is possibly much lower (Schimke, 1963). The ornithine concentration in 3T3 cells was found to be about 1.1 m*M*. Calculations using this ornithine value and kinetic constants determined in vitro at pH 7.2 and physiological salt concentration indicate that the maximum inhibition caused by assimilated putrescine in vivo is probably small compared to the activity loss observed in extracts. These kinetic parameters are probably similar to the corresponding parameters in intact cells, as the enzyme is completely soluble and intracellular pH in most eukaryotic cells is close to 7.2 (Waddell and Bates, 1969).

The polyamines do not act by accelerating degradation of the enzyme in either 3T3 cells or lymphocytes (Kay and Lindsay, 1973) when protein synthesis is inhibited. Protein turnover studies with 3T3 cells suggest that the same is probably true when protein synthesis is allowed to continue (J. L. Clark, to be published).

The polyamines appear to be interfering with synthesis of the enzyme in 3T3 cells. Because the polyamine-induced decay of activity is rapid, Jänne and Hölttä (1974) suggested that a post-transcriptional step is involved. However, messenger half-lives in higher organisms are heterogeneous (Kafatos and Gelinas, 1974), and a sizable percent may have half-lives as short as 1 hr (Puckett et al., 1975). Decay of an unstable template whose synthesis or transport to the cytoplasm is blocked would also result in a rapid decay of enzyme activity.

Actinomycin D is commonly used as an inhibitor of transcription in efforts to localize effects to either the level of transcription or translation. However, this drug "superinduces" ornithine decarboxylase in 3T3 cells stimulated with pituitary growth factors (Clark, 1974). This is in part due to a stabilization of the enzyme. Stabilization by actinomycin D may also be a factor in similar "superinductions" of the enzyme in the liver of rats injected with histidine (Fausto, 1971) and in concanavalin A stimulated lymphocytes (R. H. Fillingame, personal communication), as well as in other studies in which enzyme activity does not actually increase over controls (Fausto, 1971; Hogan et al., 1974; Kay and Lindsay, 1973). Because of this stabilization effect, the drug was considered unsuitable as a tool to distinguish among possible sites of inhibition in the present studies.

Camptothecin, which does not superinduce, was the most useful inhibitor in experiments with 3T3 cells; 20 μg/ml of this drug almost totally inhibits the appearance of large RNA in the cytoplasm of 3T3 cells, yet is much less effective in reducing enzyme activity than either polyamine. Furthermore, the recovery of enzyme activity when polyamine is removed is only slightly diminished by the drug. In contrast, trichodermin reduces activity more rapidly than each polyamine, and prevents the recovery in activity when the polyamine is removed. These results show that recovery is dependent on protein synthesis, but not on new message availability, and suggest that it is the translational process that is limiting in the presence of medium polyamine.

Each polyamine was found to be taken up by a carrier-mediated process in 3T3 cells which is inhibited competitively by MGBG.[5] The two amines appear to share the same transport system in 3T3 cells since each competes with the other for uptake with a K_i nearly equal to its K_m in the absence of the other amine, and MGBG has a K_i for each amine of about 5 μM. MGBG may also be taken up by the same system, as it and spermidine compete for uptake in mouse L1210 cells (Dave and Caballes, 1973). Putrescine is not metabolized in 3T3 cells in the presence of MGBG. Thus, if the inhibition of putrescine uptake by MGBG is taken into account, and the effect of MGBG on enzyme activity in the absence of medium polyamines is assumed to be unrelated to this inhibition, then putrescine, as well as spermidine, can reduce enzyme activity. Kay and Lindsay (1973) found that MGBG increased the requirement for putrescine by two orders of magnitude and concluded that only spermidine is active. It now appears that this result was due primarily to an inhibition of putrescine uptake by MGBG.

Medium putrescine and spermidine reduce ornithine decarboxylase activity in *E. coli* by direct inhibition of the enzyme and also by repression of the enzyme (Tabor and Tabor, 1969). In contrast, ornithine decarboxylase activity in eukaryotes appears to be controlled only by alterations in the amount of enzyme protein. In human lymphocytes (Kay and Lindsay, 1973) and in rat liver (Jänne and Hölttä, 1974), exogenous polyamines were found to apparently reduce the concentration of enzyme by a post-transcriptional mechanism. In the present work, evidence was obtained that putrescine or spermidine taken up by 3T3 cells can inhibit synthesis of ornithine decarboxylase at the level of translation. This possibility awaits confirmation by studies with cell-free systems in which immunoprecipitated enzyme is measured. Because polyamines from exogenous sources may not enter the same pool as amines synthesized within the cell (e.g., Russell et al., 1970), the physiological significance of this putative translational control currently is difficult to assess.

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[5] Any rapidly reversible binding or absorption of polyamines to 3T3 cells such as occurs for each amine with *E. coli* (Tabor and Tabor, 1966) and for fat cells exposed to spermine (Amatruda and Lockwood, 1974) would not be detected in the present studies because of the extensive rinsing employed.

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Isolation and Structure of a Cross-Linked Tripeptide from Calf Bone Collagen†

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ABSTRACT: A cross-linked tripeptide has been isolated from alkaline hydrolysates of NaB^3H_4-reduced calf bone collagen. The peptide contains dihydroxylysinonorleucine, the most abundant cross-link in bone collagen, and it has a single N-terminal proline and a single C-terminal valine. These amino acids are in peptide linkage with the cross-link, in a trans configuration with respect to the secondary amine.

The majority of the borohydride-reducible intermolecular cross-links in collagen have been characterized with regard to their covalent structures (Tanzer, 1967, 1973, Bailey, 1967). Most of these compounds reflect condensation products between the carbonyl moiety of the collagen aldehyde, α-aminoadipic δ-semialdehyde, and other amino acids in collagen. The relative abundance of each cross-link varies with the tissue source of the collagen and with the chronologic age of the animal. For example, it is generally agreed that the mineralized collagens of adult animals are particularly rich in the cross-link, dihydroxylysinonorleucine (Mechanic and Tanzer, 1970). This compound appears to be present in two different forms prior to borohydride reduction, namely the aldimine and its ketoamino rearrangement form (Eyre and Glimcher, 1973a; Tanzer, 1973). It may also contain *O*-glycosidic galactose and glucose as the disaccharide, galactosyl glucose, linked to one of the hydroxyl groups of the cross-link (Eyre and Glimcher, 1973a,b).

In the present study, we have examined which amino acids are adjacent to the dihydroxylysinonorleucine found in mineralized bone collagen. A tripeptide, prolyldihydroxylysinonorleucylvaline, has been isolated and its primary structure has been determined.

Materials and Methods

Preparative Steps. Calf tibiae were cleaned, cut into small pieces, and powdered in a liquid nitrogen cooled Spex freezer/mill. The powdered bone was demineralized with

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