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Control of Ornithine Decarboxylase Activity in α-Difluoromethylornithine-resistant L1210 Cells by Polyamines and Synthetic Analogues*

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The regulation of ornithine decarboxylase (ODC) activity by the polyamine derivatives N^1,N^8-bis(ethyl)-spermidine and N^1,N^{12}-bis(ethyl)spermine was studied using a line of L1210 cells resistant to α-difluoromethylornithine (D-R cells), which contain very high levels of ODC, and a synthetic mRNA prepared from a plasmid containing an insert corresponding to ODC mRNA adjacent to an SP6 RNA polymerase promoter. Studies in which ODC protein was labeled in the D-R cells by exposure to [^{35}S]methionine indicated that the polyamine derivatives and their physiological counterparts led to an increased rate of degradation of ODC and to a rapid reduction in ODC synthesis without affecting the content of ODC mRNA. Direct evidence that the polyamine derivatives act by inhibiting the translation of the ODC mRNA was obtained by studying their effects on the translation of ODC mRNA in reticulocyte lysates. This translation was strongly inhibited by the addition of N^1,N^8-bis(ethyl)spermidine, spermidine, N^1,N^{12}-bis(ethyl)spermine, or spermine but was not affected much by putrescine. The inhibition of the translation of ODC mRNA by either of the bis(ethyl) polyamine derivatives occurred at concentrations which stimulated total protein synthesis showing the selectivity of the reduction in ODC. The effects of polyamine derivatives and polyamines on translation of the plasmid-derived ODC mRNA were identical with those found with the D-R L1210 cell mRNA. This synthetic ODC mRNA lacks 261 bases of the 5′-leader sequences and 200 bases plus the poly(A) section from the 3′-nontranslated sequence. Therefore, these regions appear not to influence sensitivity of the ODC mRNA to inhibition of translation by polyamine derivatives.

Polyamine biosynthesis is a highly regulated process in mammalian cells (1-4). One important aspect of this regulation is the control of ornithine decarboxylase (ODC)[1] by the polyamines themselves. It is well established that the activity of this enzyme is inversely related to the cellular polyamine content (4-13). A variety of regulatory mechanisms has been suggested for the reduction in ODC activity in response to exogenous polyamines. These include post-translational modifications of the protein (13, 14), an increased degradation rate of the protein (5, 7-11), the formation of a complex with an inhibitory protein termed "antizyme" (4), and a decreased rate of synthesis of the protein (6, 7, 9-12). Recently, it has been shown that a reduction in ODC activity is brought about by exposure to the polyamine analogues N^1,N^8-bis(ethyl) spermidine and N^1,N^{12}-bis(ethyl)spermine (15-17). These analogues have considerable potential for use as anticancer agents (18, 19), and a more detailed understanding of how they act will enable more effective protocols employing them to be designed. Porter and Bergeron and colleagues (15) have shown that exposure of L1210 cells to N^1,N^8-bis(ethyl)spermidine reduces the amount of ODC protein and have suggested that it probably suppresses ODC activity in a similar manner to spermidine.

Detailed studies of the biochemical mechanism underlying this phenomenon have been hampered by the very small amount of ODC protein and mRNA present in most mammalian cells. Two approaches to circumvent this problem have been employed in the studies described in this paper. First, the regulation of ODC has been studied in a L1210 cell line which was selected for resistance to the ornithine decarboxylase inhibitor, DFMO. When grown in the absence of DFMO, these D-R L1210 cells have levels of ODC and its mRNA more than 100 times greater than of the original cells (20). Under these conditions, the D-R cells do contain greatly elevated concentrations of putrescine, but spermidine and spermine are not different from control L1210 cells (20). It was therefore possible to study the effects of exogenous polyamines and analogues on the ODC protein and mRNA in these cells. Second, mRNA from D-R cells and synthetic ODC mRNA prepared from a plasmid containing an insert corresponding to a portion of the ODC cDNA adjacent to an SP6 RNA polymerase promoter was used to study the translation of ODC protein in the presence of polyamines (21, 22). The results indicate that the degradation of ODC protein is enhanced by these polyamine derivatives and that the synthesis of ODC is diminished at the level of mRNA translation.

EXPERIMENTAL PROCEDURES

Materials—L-[1-^{14}C]ornithine (55 Ci/mol) was obtained from Du Pont-New England Nuclear. [^{35}S]Methionine (1100-1400 Ci/mmol) and [5-^{14}C]DFMO (60 Ci/mmol) were purchased from Amersham Corp. Biochemical reagents were purchased from Sigma, Promega Biotec, Madison, WI, and Bethesda Research Laboratories. DFMO was a generous gift from Dr. P. P. McCann, Merrell Dow Research Institute, Cincinnati, OH. N^1,N^8-Bis(ethyl)spermidine and N^1,N^{12}-bis(ethyl)spermine were synthesized as described (15). Plasmid pODC934, which contains an insert complementary to ODC mRNA

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[1] The abbreviations used are: ODC, ornithine decarboxylase; DFMO, α-difluoromethylornithine; D-R cells, DFMO-resistant L1210 cells.

(23), was a gift from Dr. F. G. Berger, University of South Carolina, Columbia, SC. A plasmid derived from pmODC-1 (21) which contains the ODC sequence adjacent to an SP6 RNA polymerase promoter site was a generous gift from Dr. D. Nathans and C. Kahana, Johns Hopkins University School of Medicine. Monospecific rabbit antiserum prepared to homogeneous mouse kidney ODC (24) was used to isolate the ODC protein.

Cell Culture—L1210 cells were maintained and grown in suspension culture in RPMI-1640 medium containing 10% Nu-Serum (Collaborative Research, Inc., Lexington, MA) as described by Pera *et al.* (25). The polyamines or derivatives were added to the cells in exponential growth at the concentrations indicated. Degradation of the added polyamines by oxidases present in the culture medium was prevented by the presence of 1 mM aminoguanidine.

The DFMO-resistant D-R cells were obtained by growing the cells in increasing concentrations of DFMO starting with 0.2 mM and proceeding through 0.5, 1, 5, 10, and 20 mM (20). The cells were maintained at each concentration of DFMO until their growth rate increased to that of the control cells without DFMO. The entire selection period was about 4 months. Cells were cloned by limiting dilution. The resulting cells grew at a control rate in the presence of 20 mM DFMO and are designated as D-R L1210 cells. The cells were maintained in medium containing 10 or 20 mM DFMO. Prior to use in studies in which ODC activity was measured they were washed, seeded at a density of 5×10^4 cells/ml in medium lacking DFMO, and grown for 48 h in this medium to remove DFMO.

Assay of Ornithine Decarboxylase Activity, Protein, mRNA Content, and Rate of Synthesis and Degradation—ODC activity was measured by measuring the release of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described by Seely *et al.* (26). ODC protein was determined by radioimmunoassay (24). Total protein was measured by the method of Bradford (27). The content of ODC mRNA was determined by Northern and dot blot analysis using published methods for RNA extraction by the guanidinium/hot phenol method (28) and hybridization with a cDNA probe for mouse ODC (16, 29). The ODC mRNA in the D-R cells was found to consist of a major species of about 2.2 kilobases and a faint minor species of 2.6 kilobases (20).

ODC protein was labeled by the addition of 50 μCi/ml [^{35}S]methionine to the culture medium. (It was not necessary to reduce the amount of unlabeled methionine present in the culture medium to obtain adequate incorporation of radioactivity into ODC and, since this reduction affected the rate of cell growth, it was avoided.) The synthesis of ODC was studied using a 30-min labeling period. Cells were then harvested, extracts prepared, and the ODC precipitated by the addition of 3 μl of antiserum to ODC. After 30 min at room temperature, 30 μl of 10% protein A bacterial adsorbent was added and the mixture shaken for 30 min. The precipitate was collected, washed with a buffer containing 10 mM Tris-HCl, pH 7.4, 0.1% sodium dodecyl sulfate, 0.1% Triton X-100, 2 mM EDTA, and 5 mM methionine, and then solubilized by heating in 62.5 mM Tris-HCl, pH 6.8, 2.3% sodium dodecyl sulfate, 5% 2-mercaptoethanol, and 10% glycerol, and separated on 10% gels as previously described (30). The degradation of ODC was studied by labeling the cells for 2 h and then adding the polyamines with or without 200 μM cycloheximide to block further protein synthesis. At various times later, samples were taken and the content of labeled ODC analyzed as described above.

Isolation and Translation of D-R Cell mRNA and of Plasmid-derived ODC mRNA—RNA was isolated from the L1210 and D-R cells as described by Maniatis (28) and poly(A) containing mRNA was prepared using Hybond-mAP paper (Amersham Corp.) as described in Ref. 31 with modifications according to the manufacturer's instructions. ODC mRNA was synthesized from the pmODC-1-derived plasmid and capped by incubation at 40 °C for 60 min of 5 μg of plasmid (linearized by reaction with *Bam*HI and *Pvu*II prior to reaction) in 0.05 ml containing 40 mM Tris-HCl, pH 7.5, 2 mM spermidine, 6 mM $MgCl_2$, 10 mM NaCl, 0.1 mM ATP, 0.1 mM CTP, 0.1 mM UTP, 0.01 mM GTP, and 0.1 mM m^7GpppG with 5 units of SP6 RNA polymerase (32). After 60 min, another 5 units of polymerase was added and incubation continued for a further 60 min. The DNA was then degraded by the addition of 5 units of RQ1 deoxyribonuclease (Promega Biotec) and incubation at 37 °C for 15 min. The mRNA was isolated by extraction with an equal volume of chloroform and precipitated from the aqueous phase by addition of 0.1 volume of 3 M sodium acetate and 2.5 volumes of ethanol. The solution of mRNA was analyzed to check that no spermidine remained in the mRNA pellet, and none was found. The limit of sensitivity was such that less than 10 μM spermidine could have been added to the assays from the mRNA. The mRNA was translated in a gel-filtered reticulocyte lysate system and the synthesis of ODC measured as described by Kameji and Pegg (33).

RESULTS

Effect of N^1,N^8-Bis(ethyl)spermidine and N^1,N^{12}-Bis(ethyl)-spermine on ODC in D-R L1210 Cells—Exposure of the D-R L1210 cells to N^1,N^8-bis(ethyl)spermidine or N^1,N^{12}-bis(ethyl)spermine led to a rapid fall in ODC activity. This decline, which was maximal by 6 h and amounted to an 85% reduction (Fig. 1), is similar to that seen in control L1210 cells exposed to these compounds (16, 17). A comparable decrease in ODC activity was brought about by exposure to spermidine or spermine. As shown in Table I, the bis(ethyl) analogs were about as potent in bringing about the reduction in ODC activity as the polyamines themselves. Spermine and its derivatives gave an almost maximal effect at 10 μM whereas 50 μM spermidine or N^1,N^8-bis(ethyl)spermidine was needed. The content of mRNA for ODC in the cells under these conditions was measured by densitometric scanning of dot blots and Northern blots. There was no decrease in the mRNA content over the 6-h time period when ODC activity was reduced by at least 85% (Fig. 1 and Table I). In fact, as indicated in Fig. 1, there may have been a small rise in the amount of ODC mRNA in D-R cells exposed to 50 μM N^1,N^8-bis(ethyl)spermidine or N^1,N^{12}-bis(ethyl)spermine, but this change is too small to be statistically significant.

The synthesis of ODC was measured by pulse labeling the cells with [^{35}S]methionine for 30 min. Extracts were then prepared and the ODC protein isolated by immunoprecipitation and separated by polyacrylamide gel electrophoresis. As shown in Fig. 2, there was a significant reduction in the synthesis rate of ODC in cells exposed to the polyamines or their analogs. This effect was quantitated and the results expressed as the percentage of the synthesis in control cells not exposed to polyamines (Table I). All of the polyamines tested reduced the ODC synthesis rate, but spermidine was more active than spermine and was more effective than N^1,N^8-bis(ethyl)spermidine. However, N^1,N^{12}-bis(ethyl)spermine was more potent than spermine and was actually the most

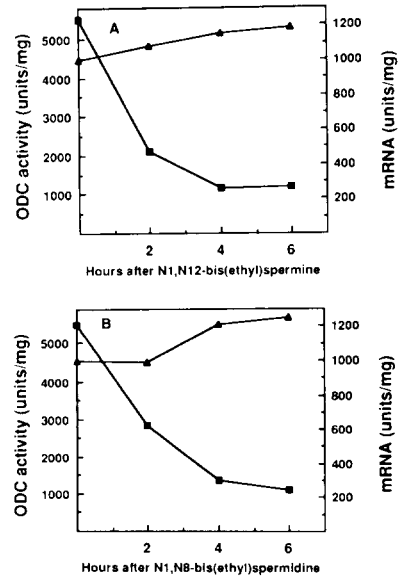

FIG. 1. **Effect of polyamines on ODC activity and mRNA content in D-R cells.** D-R cells were freed from DFMO by washing and by growth for 48 h in the absence of the drug. They were then treated with 50 μM N^1,N^{12}-bis(ethyl)spermine (*Panel A*) or N^1,N^8-bis(ethyl)spermidine (*Panel B*), and at the times indicated the activity of ODC (■) or the mRNA content (▲) was measured.

TABLE I

Effect of polyamines and analogues on ODC in D-R L1210 cells

Treatment	Ornithine decarboxylase[a]			
	Activity[b]	mRNA[c]	Synthesis rate[d]	Half-life[e]
	% control	*% control*	*% control*	*min*
Control	100	100	100	50
10 μM N^1,N^8-bis(ethyl)spermidine	45	90	85	ND[f]
50 μM N^1,N^8-bis(ethyl)spermidine	20	110	55	20
10 μM N^1,N^{12}-bis(ethyl)spermine	20	120	30	ND
50 μM N^1,N^{12}-bis(ethyl)spermine	15	115	15	15
10 μM spermidine	60	95	25	ND
50 μM spermidine	20	105	20	25
10 μM spermine	15	100	50	ND
50 μM spermine	15	115	40	15

[a] The results are the mean of several experiments which agreed within ±10% and are rounded up to the nearest unit of 5.

[b] Measured 6 h after treatment with the polyamine shown. The results are expressed as the percentage of the activity at zero time which was 5,450 units/mg protein and did not change significantly over the 6-h incubation period without addition of polyamines.

[c] Measured 4 h after addition of the polyamine shown. The results are expressed as the percentage of the activity at zero time.

[d] Measured 2 h after the addition of the polyamine at the concentration shown. Results from experiments such as Fig. 2 were quantitated by densitometric scanning of the band corresponding to ODC and are expressed as a percentage of the ODC synthesis in control cells not exposed to the polyamine derivatives.

[e] Measured by quantitating the ODC band isolated as described in Fig. 3 using cells treated with the polyamines or derivatives for 2–4 h and with cycloheximide for 0–2 h.

[f] ND, not determined.

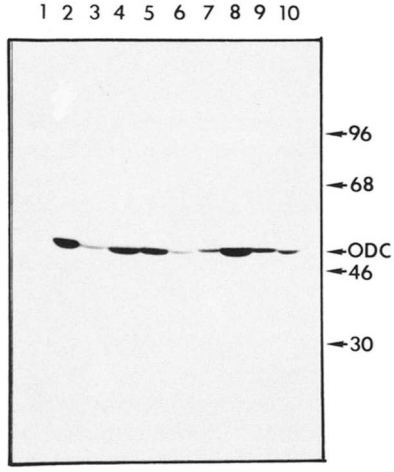

FIG. 2. **Effect of polyamines on ODC synthesis in D-R cells.** D-R cells were treated with spermidine, spermine, N^1,N^8-bis(ethyl)-spermidine or N^1,N^{12}-bis(ethyl)spermine for 2 h. The synthesis of ODC was then measured by the addition of 50 μCi/ml [^{35}S]methionine for 30 min, immunoprecipitation of the labeled ODC, and electrophoresis. The lanes from left to right represent: *1*, control antiserum precipitation; *2*, no addition; *3*, 50 μM spermidine; *4*, 50 μM N^1,N^8-bis(ethyl)spermidine; *5*, 50 μM spermine; *6*, 50 μM N^1,N^{12}-bis (ethyl)spermine; *7*, 10 μM spermidine; *8*, 10 μM N^1,N^8-bis(ethyl) spermidine; *9*, 10 μM spermine; *10*, 10 μM N^1,N^{12}-bis(ethyl)spermine.

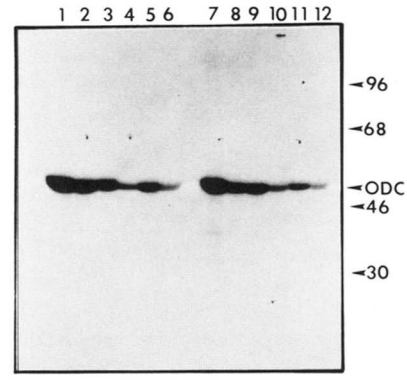

FIG. 3. **Effect of polyamines on ODC degradation in D-R cells.** The ODC in D-R cells was labeled by incubation for 2 h in medium containing 50 μCi/ml [^{35}S]methionine. After treatments as described below, the ODC protein was isolated by immunoprecipitation and electrophoresis. The *lanes* shown represent treatments as follows: *1*, control cells; *2*, 200 μM cycloheximide alone for 1 h; *3*, 50 μM N^1,N^8-bis(ethyl)spermidine for 1 h; *4*, 50 μM N^1,N^8-bis(ethyl) spermidine plus 200 μM cycloheximide for 1 h; *5*, 50 μM N^1,N^{12}-bis (ethyl)spermine for 1 h; *6*, 50 μM N^1,N^{12}-bis(ethyl)spermine plus 200 μM cycloheximide for 1 h; *7*, control cells after 2 h; *8*, 200 μM cycloheximide alone for 2 h; *9*, 50 μM N^1,N^8-bis(ethyl)spermidine for 2 h; *10*, 50 μM N^1,N^8-bis(ethyl)spermidine plus 200 μM cycloheximide for 2 h; *11*, 50 μM N^1,N^{12}-bis(ethyl)spermine for 2 h; *12*, 50 μM N^1,N^{12}-bis(ethyl)spermine plus 200 μM cycloheximide for 2 h.

effective compound in reducing ODC synthesis when tested at 50 μM (Table I).

In order to study the effects of N^1,N^8-bis(ethyl)spermidine and N^1,N^{12}-bis(ethyl)spermine on ODC degradation, the ODC in D-R L1210 cells was labeled by growing the cells in the presence of [^{35}S]methionine for 2 h. Aliquots of the cell cultures were then exposed to the polyamines or derivatives alone or combined with cycloheximide to block further protein synthesis. Extracts were prepared at various times and the amount of labeled ODC protein determined as described above. ODC protein was lost very rapidly in the cells treated with N^1,N^8-bis(ethyl)spermidine or N^1,N^{12}-bis(ethyl)spermine in the presence or absence of cycloheximide (Fig. 3). This decline in ODC protein occurred more quickly after the addition of 50 μM N^1,N^8-bis(ethyl)spermidine or N^1,N^{12}-bis(ethyl)spermine than the decline when protein synthesis was blocked by cycloheximide alone. This indicates that the degradation of ODC is enhanced in the presence of the polyamine analogs. However, this increased rate of degradation did not appear to depend on the synthesis of a new protein such as antizyme (4) since the simultaneous addition of cycloheximide and the polyamine derivatives gave an even greater rate of decline. The approximate half-life of the decline of ODC protein measured over the period from 2 to 4 h after exposure to the polyamines analogs was determined and is given in Table I. The polyamines and derivatives reduced the

half-life of ODC protein by 2–3-fold with N^1,N^{12}-bis(ethyl) spermine and spermine being slightly more active than N^1,N^8-bis(ethyl)spermidine and spermidine in this respect.

Attempts were also made to study the degradation of ODC by labeling the protein with [5-^{14}C]-α-difluoromethylornithine (26). Although insufficient radioactivity was incorporated into ODC for accurate quantitation of the protein, these results (not shown) also supported the general trend that treatment with the polyamine derivatives increased the rate of ODC breakdown.

Effect of Polyamine Derivatives on Translation of ODC mRNA—The synthesis of ODC protein was readily detected by immunoprecipitation of the ^{35}S-labeled products formed by translation of the mRNA isolated from the D-R L1210 cells in reticulocyte lysates which were depleted of endogenous polyamines by gel filtration (33). ODC amounted to about 2% of the total protein synthesis in these experiments. The effects of putrescine, spermidine, spermine, N^1,N^8-bis(ethyl) spermidine, and N^1,N^{12}-bis(ethyl)spermine on total protein synthesis and on the synthesis of ODC were examined by adding increasing amounts of these amines to the reticulocyte lysates and quantitating the band corresponding to ODC. A typical gel is shown in Fig. 4*A*. It can be seen that in addition to the ODC band having a M_r of about 53,000, there were several lower proteins of lower M_r which were also precipitated by the ODC antiserum. These bands probably correspond to initiation at internal AUG codons in the ODC mRNA. Their synthesis appeared to be affected to approximately the same extent by the addition of polyamines as the full-size ODC band.

Total protein synthesis and albumin synthesis were also measured in these experiments to determine the relative specificity of the effect of the amines toward ODC (Figs. 5 and 6). A small stimulation of total protein synthesis and of albumin synthesis was observed in response to the amines but, at higher concentrations, spermidine and spermine were inhibitory. The studies were only carried out over a range in which total protein synthesis was at least 70% of the value in the absence of added amines.

Both N^1,N^{12}-bis(ethyl)spermine (Fig. 5*A*) and N^1,N^8-bis-(ethyl)spermidine (Fig. 5*B*) selectively inhibited ODC synthesis. These compounds were somewhat less potent than their respective parent molecules in their effects on ODC (Fig. 6), but they were also less inhibitory to total protein synthesis at higher concentrations. Spermine had little effect on total protein synthesis below 0.1 mM but was quite strongly inhibitory at higher concentrations and could therefore not be tested above 0.2 mM. However, it was considerably more inhibitory toward ODC synthesis than albumin or total protein synthesis and 50% inhibition occurred with about 0.12 mM (Fig. 6*A*). Spermidine stimulated total protein synthesis slightly with a maximal effect at 0.4 mM but was strongly inhibitory toward ODC synthesis with 50% inhibition at about 0.4 mM and 80% inhibition at 1.2 mM (Fig. 6*B*). Putrescine was much less active in reducing ODC synthesis. Addition at up to 1.6 mM inhibited the synthesis of ODC to only a small extent with 25–30% inhibition at concentrations above 1 mM.

In Table II, the effects of these compounds are expressed in terms of the ratio of ODC synthesized from the D-R mRNA to total protein synthesis. When compared in this way, it is apparent that the bis(ethyl) derivatives are almost as active

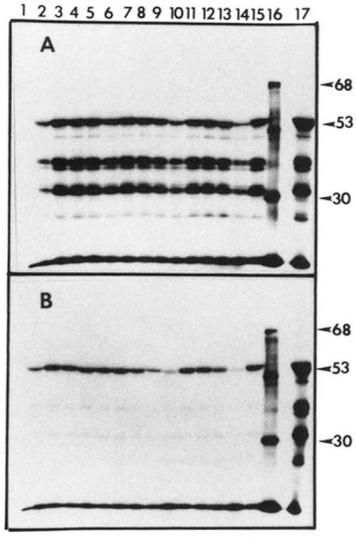

FIG. 4. **The effects of polyamines on synthesis of ODC in reticulocyte lysates supplemented with mRNA from D-R cells or with ODC mRNA made from a plasmid.** *Panel A* shows results when the reticulocyte lysate protein synthesis system was supplemented with 1 μg of mRNA from D-R cells, and *panel B* shows results when 0.2 μg of synthetic ODC mRNA prepared from a plasmid was used. In each case, *lane 1* was a control precipitated with control antiserum, *lanes 2–15* and *17* were precipitated with antiserum to ODC, and *lane 16* was a marker. *Lane 2* shows results with a nonfiltered lysate, and all other *lanes* are results for lysates freed from endogenous polyamines by gel filtration. The synthesis system contained no added polyamine (*lanes 2* and *15*) or putrescine (0.1 mM, *lane 3*; 0.2 mM, *lane 4*; 0.4 mM, *lane 5*; and 0.8 mM, *lane 6*), spermidine (0.1 mM, *lane 7*; 0.2 mM, *lane 8*; 0.4 mM, *lane 9*; and 0.8 mM, *lane 10*), or spermine (0.02 mM, *lane 11*; 0.04 mM, *lane 12*; 0.08 mM, *lane 13*; and 0.16 mM, *lane 14*). *Lane 17* shows results when a much larger amount (10 times greater) of a sample similar to *lanes 2* and *15* was used.

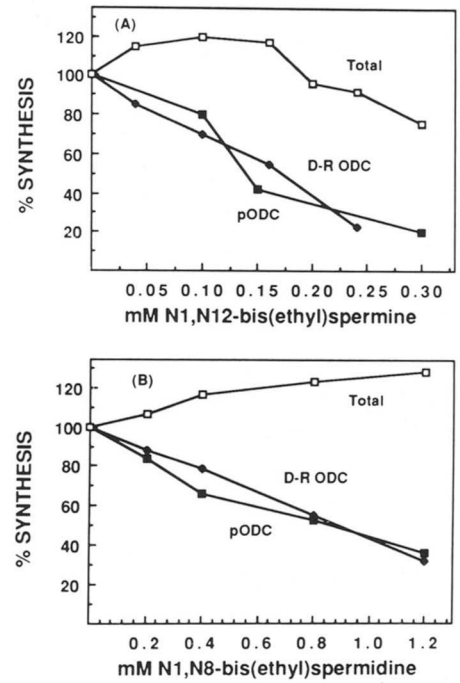

FIG. 5. **Quantitation of the effects of bis(ethyl)polyamines on the synthesis of ODC in reticulocyte lysates supplemented with mRNA from D-R cells or with ODC mRNA made from a plasmid.** The reticulocyte lysate protein synthesis system was supplemented with 1 μg of mRNA from D-R cells (*D-R ODC*, ◆) or with 1 μg of mRNA from control L1210 cells plus 0.2 μg of mRNA prepared from a plasmid (*pODC*, ■) and the amount of N^1,N^{12}-bis(ethyl) spermine (*panel A*) or N^1,N^8-bis(ethyl)spermidine shown. The amount of ODC synthesis was determined as in Fig. 4. The total protein synthesis was also determined. The value shown (*Total*, □) is from the experiment with D-R mRNA, but there was no significant difference between the results for total protein synthesis with either mRNA.

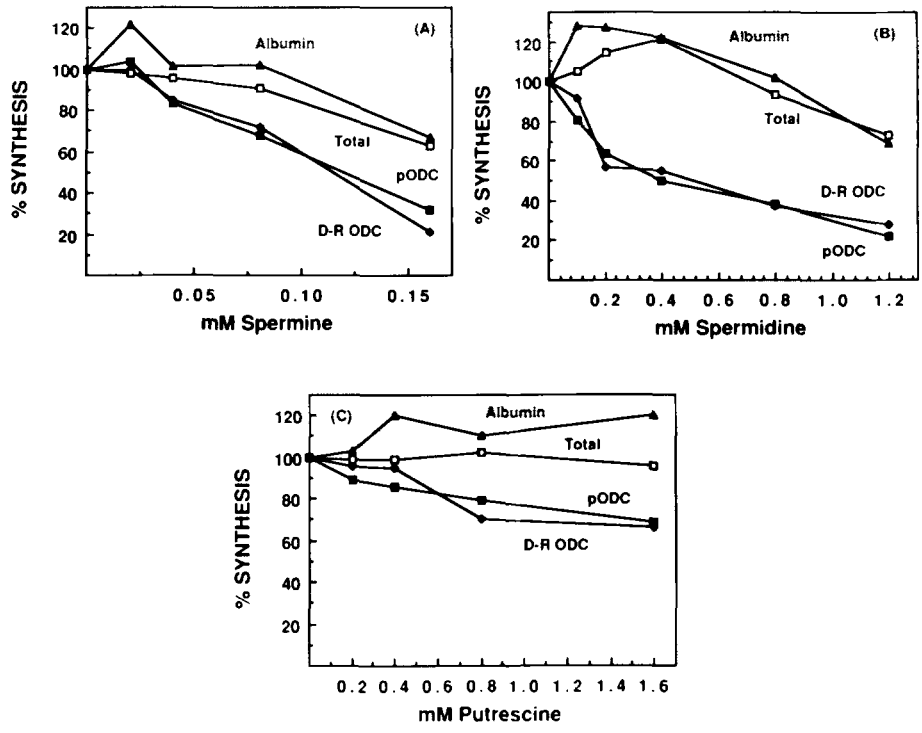

FIG. 6. **Quantitation of the effects of putrescine and polyamines on synthesis of ODC in reticulocyte lysates supplemented with mRNA from D-R cells or with ODC mRNA made from a plasmid.** The reticulocyte lysate protein synthesis system was supplemented with 1 μg of mRNA from D-R cells (*D-R ODC*, ◆) or with 1 μg of mRNA from control L1210 cells plus 0.2 μg of mRNA prepared from the plasmid (*pODC*, ■) and the amount of spermine (*panel A*) or spermidine (*panel B*) or putrescine (*panel C*) shown. All tubes also contained 0.1 μg of rat liver mRNA so that albumin synthesis (*Albumin*, ▲) could be measured (33). The amount of ODC synthesis and total protein synthesis (*Total*, □) was determined as in Fig. 5.

TABLE II

Effect of polyamines and analogues on the synthesis of ODC in vitro

All experiments were carried out with mRNA from D-R cells.

Addition/treatment	Protein synthesis in lysate			
	Total[a]	ODC[b]	ODC/total	Inhibition of ODC synthesis[c]
			× 10^2	%
None	145	1.3	0.9	55
Filtered	100	2.0	2.0	0
+0.16 mM spermine	65	0.4	0.6	70
+0.16 mM N^1,N^{12}-bis(ethyl)spermine	120	1.1	0.8	60
+0.30 mM N^1,N^{12}-bis(ethyl)spermine	75	0.4	0.5	75
+0.8 mM spermidine	95	0.7	0.7	65
0.8 mM N^1,N^8-bis(ethyl)spermidine	120	1.1	0.9	55
+1.2 mM N^1,N^8-bis(ethyl)spermidine	130	0.6	0.5	75
+1.6 mM putrescine	100	1.4	1.4	30

[a] The total protein synthesis is given as the percentage of counts/min incorporated into protein by the reticulocyte lysate which had been freed from endogenous polyamines by gel filtration. Values are the mean of at least three experiments rounded out to the nearest 5%.

[b] The ODC synthesis was measured by quantitation of the ODC band using a densitometer and expressed in relative units compared to synthesis in the control gel-filtered lysate which was set at 2 since about 2% of the radioactivity incorporated into protein was precipitated by the ODC antibody. Values are the mean of at least three experiments rounded out to the nearest 5%.

[c] The percentage inhibition of ODC synthesis was calculated from the ratio of ODC/total protein synthesis column setting the value by filtered reticulocyte lysate assayed in the absence of polyamines at 100%.

as their parent polyamines in selectively reducing the synthesis of ODC.

Experiments were also carried out using a synthetic ODC mRNA prepared from a plasmid containing a cDNA insert corresponding to a 1692-base pair fragment from the ODC mRNA inserted adjacent to an SP6 promoter (22). This mRNA contains 69 bases of the 5′-nontranslated region of the mRNA, the entire coding sequence, and 240 bases of the 3′-nontranslated sequence. The synthetic mRNA was translated considerably better by the lysates after they were freed from polyamines by gel filtration, and ODC synthesis from it was greatly reduced by addition of spermidine (Fig. 4*B*). In these respects, its translation was similar to that of the ODC mRNA present in the D-R cells. However, it differed in two ways. First, its translation was not as efficient in the lysate since 200 ng had to be added to get the same amount of ODC synthesis as from 1 μg of D-R mRNA. Analysis by dot blots indicated that about 3.6% of the D-R mRNA corresponded to ODC (20) so 1 μg is equivalent to 36 ng of ODC mRNA. This result may be due to the fact that the synthetic plasmid-derived mRNA lacks the poly(A) tail, but it could also be that the missing portion of the 5′-leader sequence actually enhances translation. Second, the lower M_r bands corresponding to ODC proteins which are described above were much less prominent when the plasmid-derived mRNA was used (compare Fig 4, *A* and *B*). The reason for this is not clear. All of the smaller bands seen in Fig. 4*A* were observed in films obtained from the plasmid mRNA when these were developed

for a longer time than the exposure shown in Fig. 4*B* or a larger amount was loaded (Fig. 4, *lane 17*), but the amounts are clearly much less than the major ODC band.

The translation of the plasmid-derived ODC mRNA when added alone to the reticulocyte lysates is obviously not directly comparable to those in which 1 μg of the D-R L1210 cell RNA containing many different mRNAs as well as that for ODC was used. Therefore, in order to study the effect of the polyamine derivatives on the translation of the synthetic ODC mRNA under comparable conditions, it was added to mRNA isolated from control L1210 cells in an amount which gave a similar extent of ODC synthesis as was found with the D-R L1210 cell mRNA. (The content of ODC mRNA in the control L1210 cells is less than 1% of that in the D-R cells and is negligible in these experiments.) As can be seen from Figs. 5 and 6, the effects of polyamine derivatives and polyamines on the plasmid-derived ODC mRNA translation were identical with those found with the D-R L1210 cell mRNA. Since the plasmid-derived ODC mRNA lacks a substantial portion (about 261 bases) of the 5′-leader sequences and 200 bases plus the poly(A) section from the 3′-nontranslated sequence, these experiments rule out these regions as contributing to the sensitivity of the ODC mRNA to inhibition of translation by polyamines.

DISCUSSION

These results provide substantial evidence in support of the proposal by Porter, Bergeron, and colleagues (16, 17) that the bis(ethyl)derivatives of the polyamines reduce ODC activity by the same mechanism as the polyamines themselves. Our results based on studies with the treated cells do not rule out the possibility that the derivatives actually act by displacing intracellular polyamines from bound sites and thus making them available for the regulation of ODC. However, this possibility is unlikely since the derivatives have long lasting effects on ODC which continue over a sufficiently long period that the cellular spermidine and spermine are depleted (17). Furthermore, our studies of the translation of mRNA *in vitro* show clearly that the bis(ethyl) derivatives alone can selectively suppress the translation of ODC mRNA.

The finding that there is a substantial decrease in the rate of synthesis of ODC in the D-R cells in response to the addition of polyamine derivatives or the polyamines themselves but that there is no change in the mRNA content is in agreement with other work in which putrescine, spermidine, or spermine were tested in a number of cell types (9–12, 15). These results demonstrate that the regulation of ODC synthesis occurs at the level of translation of the mRNA. This is also supported by the direct inhibition of the translation of ODC mRNA *in vitro* when the polyamines or their bis(ethyl) derivatives were added (Figs. 5 and 6). These findings with mRNA from the D-R L1210 cells are in agreement with previous studies showing that addition of spermidine or spermine to reticulocyte lysates inhibited the translation of ODC mRNA using mouse kidney as a source of mRNA (33). This inhibition of translation occurs over a range of spermidine and spermine concentrations which are comparable to those likely to pertain in cells under normal physiological conditions. Therefore, it may represent an important physiological regulation mechanism to maintain intracellular polyamines within a narrow range. (It should be noted that it is not clear to what extent the polyamines, particularly spermine, exist in a free form in the cell, and it is likely that the effective concentration is lowered appreciably by binding to cellular macromolecules.) Putrescine was relatively ineffective in reducing the translation of ODC mRNA *in vitro* (Fig. 6*C*), and it probably requires conversion to spermidine in order to exert any effect on ODC synthesis *in vivo*.

The translation of the plasmid-derived ODC mRNA was affected by the polyamine derivatives in exactly the same way as the D-R L1210 mRNA. This indicates that the majority of the 5′-leader sequences and a substantial part of the 3′-nontranslated regions are not involved in this regulation. It should be mentioned that Glass *et al.* (22) have previously demonstrated that this synthetic mRNA can be translated to form ODC protein in reticulocyte lysates, but they found no ODC synthesis when less than 250 ng of mRNA was added. In our experiments the synthesis of ODC was linear with mRNA added over the range from 30 to 500 ng. This may be due to the lower concentration of polyamines in the reticulocyte lysates used in our experiments since, as shown in Table II, ODC synthesis was significantly lower in the non-gel-filtered lysates.

At present, it is not known whether the inhibition of ODC translation requires the interaction of the polyamines with proteins present in the lysate or whether it results from a direct effect of the polyamines on the mRNA structure. The investigation of the mechanism of this effect should be facilitated by using N^1,N^8-bis(ethyl)spermidine or N^1,N^{12}-bis(ethyl)spermine since these derivatives act as strong inhibitors of the translation of ODC at concentrations which have little effect on total protein synthesis from L1210 cell mRNA. Furthermore, the use of the plasmid-derived mRNA in which additional changes can be engineered by various techniques including the use of site-specific mutagenesis should enable the regions of the mRNA which are of particular importance for this effect to be identified.

The increased degradation of ODC protein which is brought about by the polyamine derivatives also clearly contributes to the fall in ODC protein. The mechanism by which ODC is degraded is at present very poorly understood. It has been suggested that the degradation process may be initiated by the formation of a complex with the protein described as antizyme, which may be induced by polyamines (3–5) or by some post-translational modification initiated by polyamines (13). Since our results show that an enhanced rate of degradation was produced by the polyamine analogs in D-R L1210 cells even when protein synthesis is prevented by cycloheximide, this stimulation cannot involve the *de novo* synthesis of antizyme. A stimulation of a post-translational modification is possible, but it should be noted that, although the D-R cells contain several hundred times more ODC than the original L1210 cells, the turnover of ODC is not changed (20). It seems unlikely that the rate-limiting factor in the degradation of ODC would be increased in parallel to ODC itself in the D-R cells unless it was an integral part of the ODC protein. Rechsteiner and colleagues (34) have postulated that the PEST sequences are important in the turnover of rapidly degraded proteins, and ODC contains a strong PEST sequence. The possibility that binding of the polyamines or derivatives to this sequence facilitates the degradation warrants further investigation.

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