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The Modulation of the Induction of Ornithine Decarboxylase by Spermine, Spermidine and Diamines

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ABSTRACT Extremely low concentrations of putrescine, spermidine and spermine added to the extracellular medium of cultures of mammalian cells inhibit the induction of ornithine decarboxylase activity despite 100- to 1,000-fold greater intracellular polyamine concentrations. The diamines, 1,2-diaminoethane, 1,3-diaminopropane, 1,5-diaminopentane, 1,7-diaminoheptane, 1,10-diaminodecane, 1,12-diaminododecane also inhibit ornithine decarboxylase at all concentrations tested (greater than 10^{-6} M). In contrast, 10^{-6} M to 10^{-3} M 1,8-diaminooctane, the alkyl analog of spermidine, enhances ornithine decarboxylase activity.

The concentration of putrescine required to inhibit the activity of ornithine decarboxylase by 50% is a characteristic of each cell line; however, it varies by as much as 1,000-fold among the five cell lines we have tested (L1210 leukemic, H35 hepatoma, N18 neuroblastoma, W256 carcinosarcoma and 3T3 fibroblasts).

The antizyme to ornithine decarboxylase can be induced in all these cells by high (di)(poly)amine concentrations.

Based on these and other experiments we suggest a working hypothesis: that the polyamines regulate ornithine decarboxylase activity through two different sites that may be interrelated; a sensitive membrane-mediated site that responds to minute fluctuations of extracellular polyamine levels and a coarse site which may be intracellular or membrane associated that responds to larger fluctuations of intracellular polyamine levels. The consequences of such a control mechanism operating within the whole organism are discussed.

The physiologically occurring (di)(poly) amines, putrescine, spermidine, and spermine, inhibit the activity of ornithine decarboxylase (ODC) (E.C.4.1.1.17) in many mammalian cells in cell culture (Pett and Ginsberg, '68; Kay and Lindsay, '73; Clark and Fuller, '75; Fong et al., '76; Heller et al., '76; McCann et al., '77) and in the rat liver in vivo (Jänne and Höltta, '74; Pösö and Jänne, '76a). We have shown that they induce the synthesis of a noncompetitive protein inhibitor of ODC (Fong et al., '76; Heller et al., '76). Because of the specificity of this protein and its apparent ubiquity, we have suggested the name ODC antizyme; we furthermore proposed that the interplay between the physiological (di)-(poly)amines, the ODC antizyme and ODC could participate in a sensitive modulation of intracellular ODC activity (Heller et al., '76; Canellakis et al., '78). The induction of ODC antizyme by other cells in culture as well as in rat liver has been confirmed and extended by other laboratories (McCann et al., '77; Jefferson and Pegg, '77).

The synthetic analogs, 1,3-diaminopropane and 1,6-diaminohexane are also effective inhibitors of intracellular ODC activity (Pösö et al., '77; Pösö and Jänne, '76a; Jänne and Höltta, '74; Guha and Jänne, '77). In order to investigate the mechanism of this inhibition and to detail further the mechanism of the regulation of ODC activity we have examined the effect of spermidine and spermine and of a series of diamines, of the general structure $NH_2(CH_2)_nNH_2$ (where n = 2,3,4,7,10,12), on the *induction* of ODC activity and on the induction of the ODC antizyme. A preliminary

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report of these results has been presented (Heller et al., '77).

MATERIALS AND METHODS

L1210 cells were grown in Fischer's medium plus 10% horse serum as described previously (Tsai et al., '75). The cells were grown to the plateau phase (8-9 × 10^5 cells/ml) and then diluted with fresh medium plus 10% horse serum, to about 3 × 10^5 cells/ml. The neutralized α,ω-diaminohydrocarbons, or polyamines, were added to 15-ml portions of the diluted cells to give the final concentrations indicated in the figure legends. After a 4-hour incubation at 37°, the cells were harvested by centrifugation and washed twice with phosphate buffered saline. The cell pellets were resuspended in 0.5 ml of assay buffer (50 mM Tris-HCl, 0.1 mM EDTA, 5-mM dithiothreitol and 0.05 mM pyridoxal phosphate) pH 7.2 rapidly freeze-thawed twice and dialyzed overnight against 10 mM Tris-HCl, 50 mM KCl, 0.1 mM EDTA, 0.05 mM pyridoxal phosphate, 5 mM dithiothreitol, final pH 7.2 at 4°C. The controls lost little or no ODC activity during dialysis.

H-35 rat liver hepatoma cells were grown in Eagle's minimal essential medium supplemented with 10% fetal calf serum; ODC activity was induced by starving for serum overnight and refeeding with medium supplemented with 10% fetal calf serum as previously described (Fong et al., '76; Heller et al., '76). N-18 mouse neuroblastoma cells were grown in Dulbecco's medium supplemented with 10% fetal calf sera; ODC activity was induced with prostaglandin E_1 and isobutyl methyl xanthine as previously described (Bachrach, '75) or by starving for serum overnight and refeeding with serum.

Mouse 3T3 cells were obtained from the American Type Culture Collection (CCL. 92) and grown as described by Cunningham ('72); ODC activity was induced with serum after overnight serum starvation and harvested as described by Lembach ('74). W-256 rat carcinosarcoma cells were kindly provided by Doctor J. R. Bertino of this department and grown in Fischer's medium plus 10% horse serum. ODC activity was induced by diluting plateau phase cells, 8.0-8.5 × 10^{-5} cells/ml with fresh medium plus 10% horse serum to 2.7-2.8 × 10^{-5} cells/ml. In all these cases the (di)(poly)amines were added to the media at the time of induction of ODC activity.

The content of putrescine in the horse and fetal calf sera and in L1210 cells was analyzed by the enzymatic method described by Harik et al. ('73). No putrescine was detected in either serum. The lower limit of detectability with this assay was 4 × 10^{-7} M putrescine so that the putrescine content of the medium supplemented with 10% sera was less than 4 × 10^{-8} M. The spermidine content of L1210 cells was determined by the method of Dion and Herbst ('70). We wish to thank Doctor A. E. Pegg for a sample of yeast S-adenosylmethionine decarboxylase and Doctor E. J. Herbst for the determination of the spermidine concentrations.

The oxidation of radioactive putrescine added to 100% horse and fetal calf serum at 37°C was determined after four hours incubation. All the added radioactive putrescine was recovered intact indicating that no oxidation of putrescine had occurred. This is consistent with published reports that these sera oxidize little if any of the α, ω-diamines with a connecting chain of six carbons or less, or of spermidine, or of spermine, (Blaschko et al., '59; Blaschko and Bonney, '62; Kapellar-Adler, '70).

Enzyme reactions were carried out in 17-mm × 100-mm polystyrene culture tubes (Falcon 2057), sealed with polyethylene caps. A ½" filter paper disc (Schleicher and Schuell, Inc., New Hampshire) impregnated with 0.1 ml of NCS (Amersham-Searle) diluted 1:1 with toluene, transfixed with an 18-gauge syringe needle through the cap was present during the incubation. The reaction contained 0.15-ml of the dialyzed cell homogenate, 0.015 ml of substrate (0.088 μmol, 0.75 Ci/0.1 mole DL-1-^{14}Cornithine) and 0.01 ml of 0.5 M Tris-HCl buffer; final pH was 7.2 at 37°C. The reaction was incubated for one hour at 37°C, stopped by injecting 0.2 ml of 10% trichloroacetic acid into each reaction tube through the syringe needle. This was stoppered and incubation at 37°C was continued for an additional hour. The liberated radioactive CO_2 was determined by placing the paper disc into 8-ml of ACS (Amersham-Searle) scintillation fluid and counting in a Packard Tri-carb liquid scintillation spectrometer. The values presented in the figures represent the averages of duplicate samples of two to four experiments with standard deviations of less than 10%.

ODC antizyme activity in the cell homogenate was assayed by adding known amounts of partially purified rat liver ODC activity to portions of the homogenate and measuring the

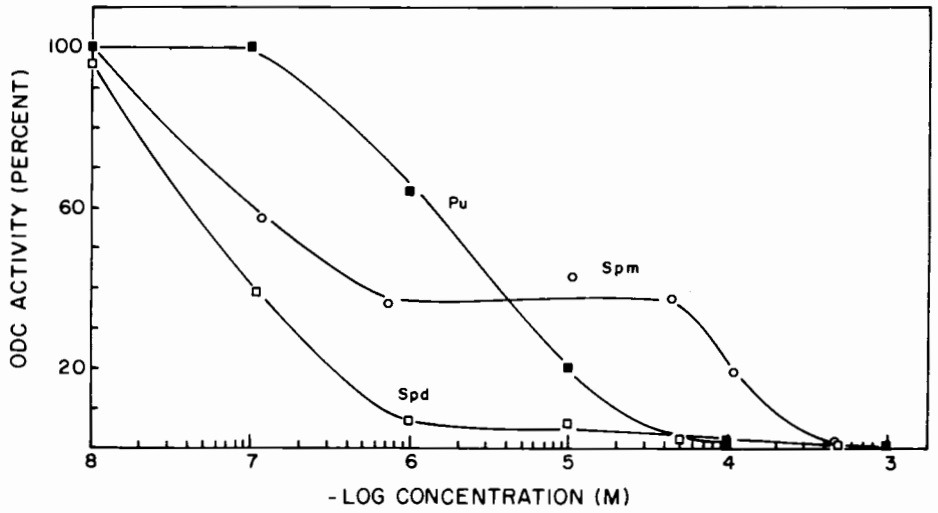

Fig. 1 The effects of varying concentrations of putrescine (■), spermidine (□) and spermine (○) added to the medium on the induction of ODC activity in L1210 cells. The 100% value for ODC activity is 26.2 nmol/mg-hr. The experimental details are as described in MATERIALS AND METHODS.

recovery of this added activity and therefore the inhibition, as previously described (Fong et al., '76; Heller et al., '76). One unit of inhibitor is defined as that amount of inhibitor which inhibits one unit of ODC activity; this latter is equivalent to 1 nmole of CO_2 released per 60 minutes.

The (di)(poly)amines (500 mg) (Aldrich Chemical Corp.) were purified on a preparative scale using 20 cm × 20 cm silica gel plates 60 F-254 (Eastman-Kodak Co.) in the following chromatographic solvents: (1,2-diaminoethane, 1,3-diaminopropane and 1,5-diaminopentane) in n-butyl alcohol: pyridine: acetic acid: water (30:20:6:24 v/v); 1,7-diaminoheptane) in n-butyl alcohol: acetic acid: water (40:10:50 v/v); spermidine and spermine in chloroform: methanol: acetic acid (55:40:5 v/v). The relevant amine bands were eluted and the amines crystallized. We were unable to resolve the impurities in spermidine and spermine with some batches of these plates for no apparent experimental reason. Putrescine was purified by vacuum distillation. Final purity of all the amines used was defined by obtaining a single ninhydrin positive and a single dansylated spot as described by Dion and Herbst ('70).

RESULTS

In each of the following experiments, the induced value of ornithine decarboxylase (ODC) activity four hours after dilution of stationary L1210 cells (MATERIALS AND METHODS), is taken as 100% while the 0% value represents no detectable ODC activity; the numerical correspondence to each 100% value is provided in the legend of each figure.

The addition of the physiologically occurring (di)(poly) amines to the medium at the time of dilution of L1210 cells affects the induction of ODC activity as shown in figure 1. Their common characteristic is to cause a precipitous fall of ODC activity at concentrations between 10^{-8} and 10^{-6} M. Spermidine, added at an external concentration as low as 10^{-8} M, causes a nominal 5% inhibition of the induction of ODC activity; this inhibition becomes accentuated as the external concentration of spermidine is raised. The presence of 10^{-7} M spermine inhibits the induction of ODC activity by 40% while 10^{-6} M putrescine is required to cause a similar inhibition.

Radioactive putrescine (10^{-6} M) added to the medium following dilution of stationary L1210 cells is concentrated ten-fold, after a 4-hour exposure, resulting in an intracellular concentration in the L1210 cells of radioactive putrescine of 10^{-5} M; radioactive spermidine added, at 10^{-7} M, under the same conditions is concentrated approximately 60-fold, to provide an intracellular concentration of 6×10^{-6} M radioactive spermidine. These increases in the intracellular concentrations of putrescine and of spermidine are well below the total extractable levels of spermidine and

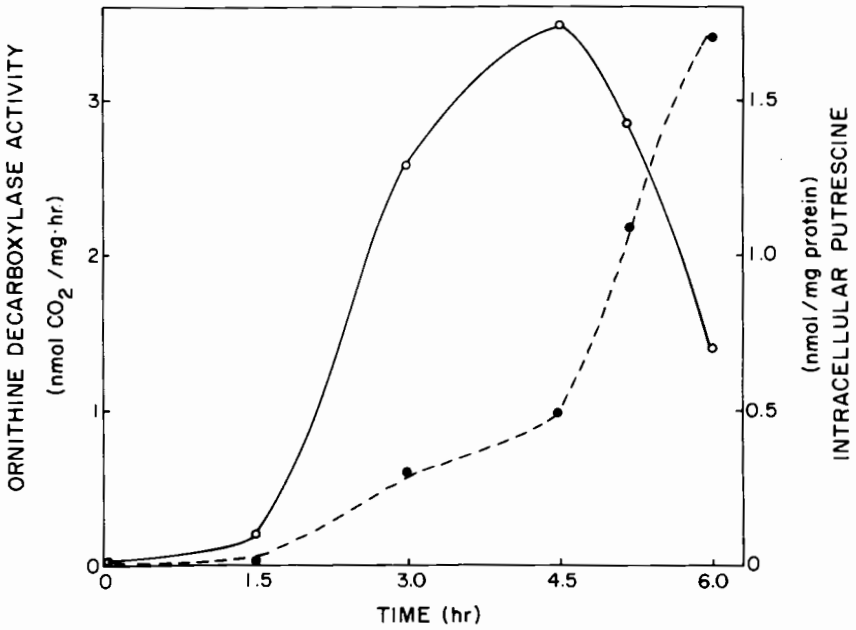

Fig. 2 Time course of the induction of ODC activity in N-18 neuroblastoma cells (○) and the appearance of intracellular putrescine (●). ODC activity was induced with 0.1 μM prostaglandin E_1 and 5 μM 3 isobutyl-1-methylxanthine as described by Bachrach ('75) and putrescine was assayed using the method of Harik et al. ('73).

putrescine contained in the L1210 cells under these conditions. These intracellular concentrations approximate 1×10^{-3} M for spermidine and 1.5×10^{-4} M for putrescine.

Consequently, the existing intracellular spermidine and putrescine levels of L1210 cells are several orders of magnitude greater than the amount that has been concentrated by the cells from the extracellular medium. Therefore, the decrease in ODC activity following the addition of minute concentrations of these amines to the medium is not the result of a substantial increase of the intracellular polyamines from polyamines in the medium.

On the other hand, a temporal relationship between alterations of the intracellular putrescine concentration and ODC activity can be detected, as shown in figure 2. When ODC activity is induced in N-18 neuroblastoma cells, the time sequence of increase in ODC activity shows the attainment of a maximum ODC activity which then decreases as the intracellular putrescine concentration increases (fig. 2). Such tandem curves have been obtained both in intact animals (Hayashi et al., '71) as well as in cell culture (Maudsley et al., '78); it appears that the decrease in ODC activity may be related to the increase in intracellular putrescine concentration.

Reexamination of figure 1 shows that a striking characteristic of the inhibition of ornithine decarboxylase activity by spermine, in contrast to that incurred by putrescine, is that over a broad range of extracellular spermine concentrations, only relatively minor fluctuations of ornithine decarboxylase activity can be detected. This effect is most prominent for spermine, and for this reason we have termed it the "spermine effect" (Canellakis et al., '78). A qualitatively similar response of ornithine decarboxylase activity occurs in response to a 500-fold range of extracellular spermidine concentrations. In this case, the plateau level of the inhibited ornithine decarboxylase activity is maintained at much lower, but substantially above zero, levels.

The "spermine effect" is also reproduced by the addition of 1,3-diaminopropane and 1,7-diaminoheptane to the diluted medium of L1210 cells. Figure 3 shows that after an initial inhibition of ornithine decarboxylase activity at low concentrations of these two diamines, (less than 10^{-5} M), there is a maintenance of a

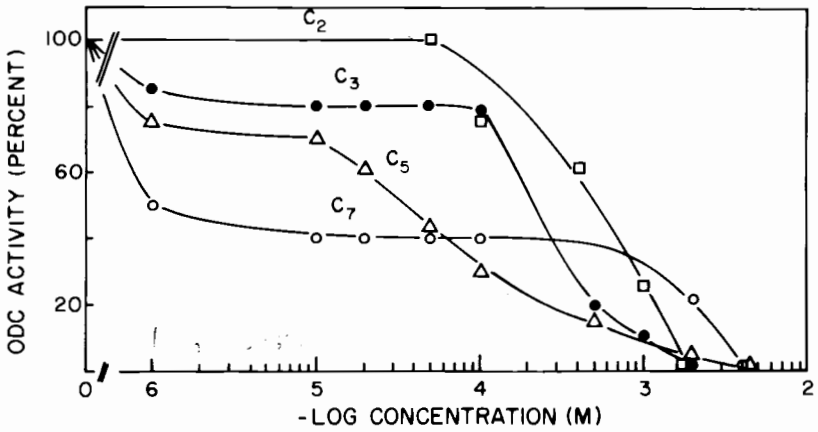

Fig. 3 The effects of varying concentrations of 1,2-diaminoethane, C_2 (□), 1,3-diaminopropane, C_3 (●), 1,5-diaminopentane, C_5 (△) and 1,7-diaminoheptane, C_7 (○) added to the medium on the induction of ODC activity in L1210 cells. The 100% value for ODC activity is 20.9 nmol/mg-hr. The experimental details are as described in MATERIALS AND METHODS.

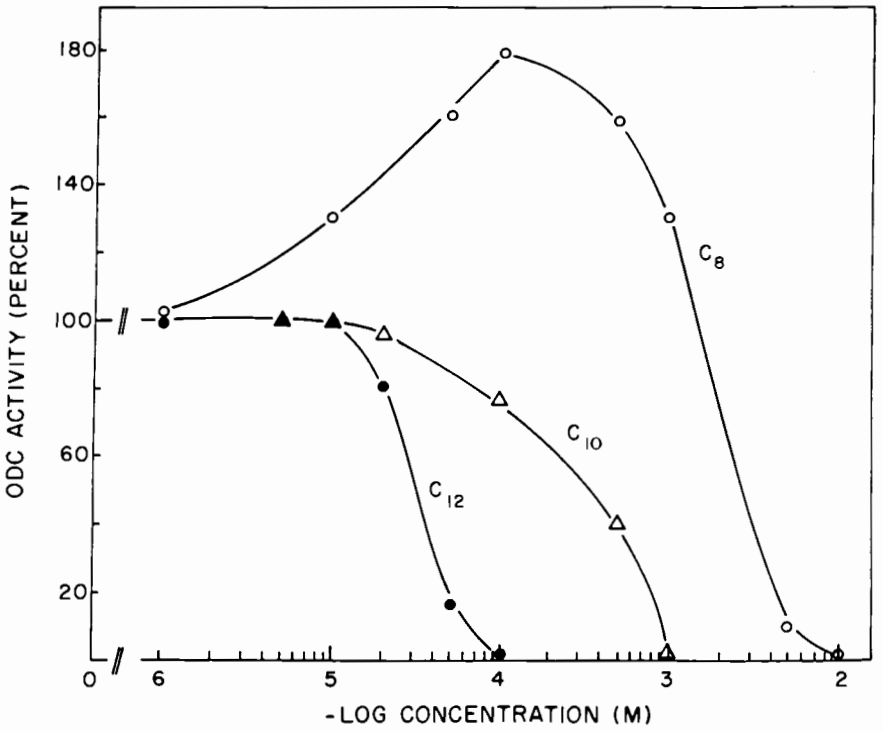

Fig. 4 The effects of 1,8-diaminooctane, C_8 (○), 1,10-diaminodecane, C_{10} (△), and 1,12-diaminododecane C_{12} (●) added to the medium on the induction of ODC activity in L1210 cells. The 100% value for ODC activity is 23.1 nmol/mg-hr. The experimental details are as described in MATERIALS AND METHODS.

constant ODC activity over a 100- to 1,000-fold range of added diamines; further increases of these two diamines result in complete inhibition of ODC activity. 1,2-diaminoethane, 1,5-diaminopentane, 1,10-diaminodecane and 1,12-diaminododecane inhibit the induction of ODC activity without eliciting the "spermine effect" (figs. 3, 4).

The enhancement of ODC activity that is elicited by concentrations of 1,8-diaminooctane over a 1,000-fold range, from 1×10^{-6} M to 1×10^{-3} M is notable (fig. 3). This observation is particularly interesting in that 1,8-diaminooctane is the alkyl analog of spermidine, and because spermidine in these concentration ranges is extremely inhibitory. Its next closest analogs that we have studied, (1,7-diaminoheptane and 1,10-diaminodecane) produce only inhibitory effects. The determination of the site and the mechanism of action of 1,8-diaminooctane should provide interesting information on the regulation of ornithine decarboxylase.

The detailed mechanism through which *low* concentrations of (di)(poly)amines inhibit the induction of ODC activity remains to be established. One, as yet unproven possibility, is that this occurs through the induction of ODC antizyme. On the other hand, the experimental evidence is clearly definitive that *higher* concentrations of these amines will induce the appearance of *free* ODC antizyme. The concentrations of (di)(poly)amines at which ODC antizyme is first detected in L1210 cells range from 5×10^{-4} M for spermidine to 1×10^{-2} M for 1,8-diaminooctane. The appearance of free ODC antizyme activity can be detected in the cell extracts when the added amines have produced a total inhibition of ODC activity.

For each of these diamines and for each of the cell lines described below, the free ODC antizyme was characterized: (a) as a non-competitive inhibitor of ODC; (b) by its molecular weight (Sephadex chromatography) 26,500 ± 2,000; (c) by its general properties which were comparable to those previously reported (Fong et al., '76; Heller et al., '76); i.e., heat labile, sensitive to chymotrypsin and to trypsin but not to RNase and/or to DNase. It was assayed by adding partially purified rat liver ODC to extracts of the cells and measuring the subsequent extent of inhibition of ODC as previously described (Fong et al., '76; Heller et al., '76; MATERIALS AND METHODS).

Under standardized conditions of exposure of L1210 cells to 10 mM α,ω-diamines, the amount of free ODC antizyme formed is presented in table 1. These values are presented only as evidence that the ODC antizyme is formed subsequent to the exposure of L1210 cells to the diamines and not as a measure of the relative potential of these α,ω-diamines to produce ODC antizyme. Such a quantitation will require more detailed experiments, including variable times of exposure, α,ω-diamine concentration curves, etc. Because of their insolubility, the higher, α,ω-diamines were not tested for their ability to produce ODC antizyme.

TABLE 1

ODC antizyme activity in L1210 cells after treatment with α,ω-diamines, spermidine and spermine

Compound	ODC antizyme (units)
1,2-Diaminoethane	9.1
1,3-Diaminopropane	7.3
1,4-Diaminobutane	11.1
1,5-Diaminopentane	3.9
1,7-Diaminoheptane	6.0
1,8-Diaminooctane	1.73
Spermidine	3.8
Spermine	9.2

α,ω-Diamines (10 mM), spermidine (2.5 mM), spermine (2.5 mM) were added to L1210 cells at the time of dilution of the cells. Experimental details and assay of ODC antizyme activity are described in MATERIALS AND METHODS and by Fong et al. ('76), and Heller et al. ('76). As indicated in the text, these values are not intended to provide quantitative relationships; they are presented to indicate that ODC antizyme can be induced in the presence of a variety of (di)(poly)amines.

We have detected the production of ODC antizyme in all cell lines tested so far, i.e. L1210, P388, W256, neuroblastoma N18, rat hepatoma H35 and mouse 3T3 fibroblasts. Figure 5 shows the extent of production of ODC antizyme by 3T3 cells with time of exposure to 10 mM putrescine. It is apparent that free ODC antizyme can be detected subsequent to the total inhibition of ODC activity; ODC antizyme then progressively increases in total units. It has been claimed that these cells do not produce free ODC antizyme (Clark and Fuller, '76). However, figure 5 emphasizes that unless the conditions of exposure to the inducer, i.e., time, concentration, etc., are such as to eliminate the ODC activity completely, it is patently impossible to detect the existence of the *free* form of ODC antizyme, a non-competitive inhibitor of ODC.

The inhibitory effect of increasing concentrations of putrescine on the induction of ODC activity in rat carcinosarcoma W-256, leukemia L1210, 3T3 fibroblasts, neuroblastoma N-18 and hepatoma H-35 cells is shown in figure

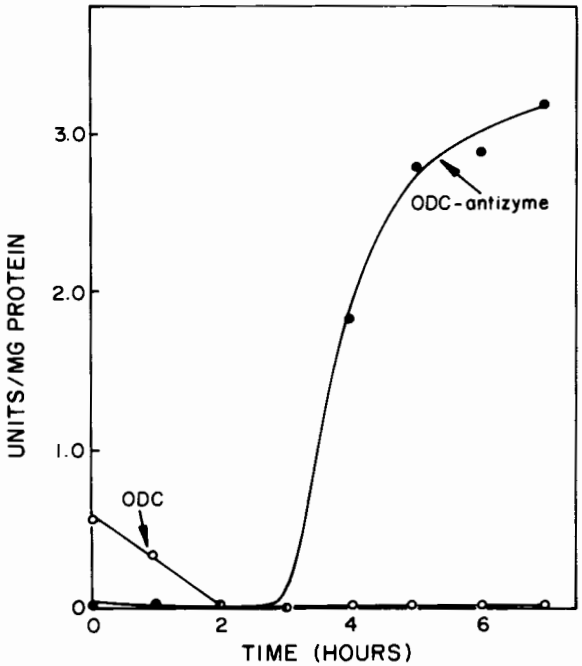

Fig. 5 Time course of the appearance of ODC antizyme activity following the exposure of 3T3 cells to 10 mM putrescine. 3T3 cells were starved for serum overnight; at time zero the medium was replaced with medium supplemented with 10% fetal calf sera plus 10 mM putrescine. At the times indicated, cells were harvested with trypsin as described by Lembach ('74) and the cell pellet rinsed twice with phosphate buffered saline. The pellet was freeze thawed with 0.5-ml assay buffer and the homogenate centrifuged at 9,000 rpm for ten minutes. ODC activity and ODC antizyme activity were assayed as in MATERIALS AND METHODS and as described by Fong et al. ('76) and Heller et al. ('76). One unit of ODC activity equals 1 nmol of CO_2 released per hour and one unit of ODC antizyme activity corresponds to the inhibition of one unit of ODC activity.

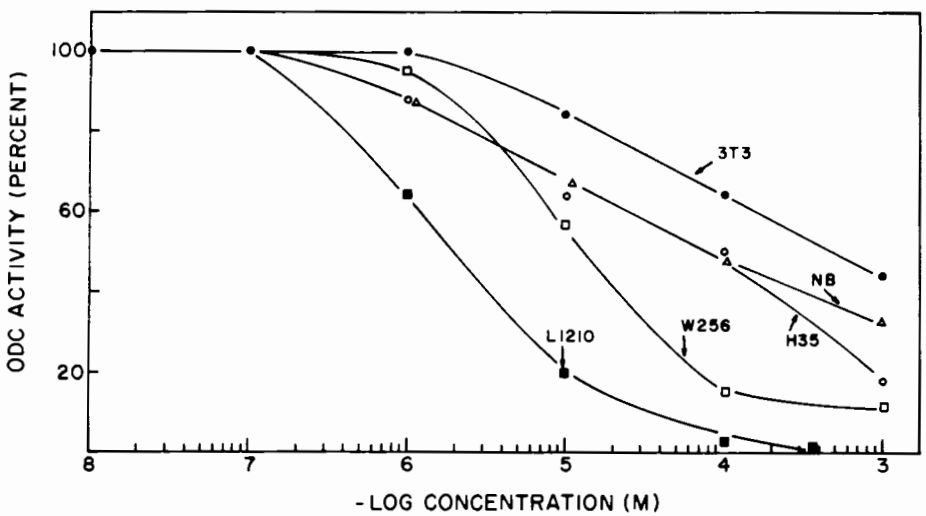

Fig. 6 The effect of varying concentrations of putrescine on the induction of ODC in L1210 (■), W-256 (□), N-18 neuroblastoma (△), 3T3 (●) and H-35 (○) cells. The 100% values for ODC activity are L1210 - 25.4 nmol/mg-hr; W-256 - 14.1 nmol/mg-hr; N-18 neuroblastoma - 22.5 nmol/mg-hr; 3T3 - 14.3 nmol/mg-hr; H-35 - 4.5 nmol/mg-hr. The experimental details are as described in MATERIALS AND METHODS.

6. These results emphasize that there is a 1,000-fold latitude in the putrescine concentrations necessary to produce 50% inhibition of the induction of ODC activity in these five different cells lines; most sensitive to inhibition are the L1210 cells while the 3T3 fibroblasts proved to be the most resistant.

DISCUSSION

These results suggest that a cellular homeostatic mechanism for the control of ODC activity exists in cells. This is emphasized by the sensitivity of the induction of ODC activity to minute variations of extracellular concentrations of spermidine in the face of high intracellular concentrations of spermidine. This apparent contradiction could be accomodated for by assuming that different cellular sites exist for the control of ODC activity.

Some experimental evidence exists for the presence of membrane associated sites that affect ODC activity. Chen et al. ('75) have shown that agents known to affect the membrane via the cytoskeleton (colchicine, cytochalasin, vinblastine), inhibit the induction of ODC; rabbit antisera prepared against L1210 plasma membranes inhibit the induction of ODC (K. Y. Chen and E. S. Canellakis, unpublished results); furthermore, Quash et al. ('76) have reported that putrescine may be associated with sites on the surface of embryonic cells.

Based on these results, we suggest as a working hypothesis that there exist two regulatory mechanisms for the control of ODC activity. At low levels of extracellular polyamines, the induction of ODC activity can be inhibited through sensitive membrane-mediated sites. This decrease in ODC activity results in a decreased rate of putrescine synthesis; this decreased rate of putrescine synthesis may elicit an increase in the activity of ODC. The existence of such a compensatory mechanism would explain the maintenance of a constant level of ODC activity over a range of inhibitory extracellular spermine and spermidine concentrations; we have referred to this buffering region as "the spermine effect." When the extracellular polyamine concentrations are further increased, raising at the same time the intracellular polyamine concentrations, the second site which may be either intracellular or membrane associated becomes predominate. The cell now becomes geared toward minimizing the further synthesis of polyamines. Consequently, the trend would now be to limit any further increase in ODC activity; in fact, to inhibit the activity of ODC. It is possible that, at this time, the ODC antizyme comes into play. However, it is also possible that these mechanisms, at all times, act through the induction of ODC antizyme, and that the appearance of free ODC antizyme is only the extreme consequence of maximal titration of ODC by its non-competitive inhibitor. Such a two-site hypothesis fits both the inhibition of ODC activity by minute amounts of extracellular ODC as well as the decay of the induced ODC activity that occurs when the intracellular polyamine concentration increases.

A considerable and definitive body of literature emphasizes that ornithine decarboxylase activity fluctuates and is inducible in a number of tissues in vivo (Tabor and Tabor, '72; Morris and Fillingame, '74; Tabor and Tabor, '76; Raina and Jänne, '75; Hopkins et al., '73; Hayashi et al., '72; Yanagi et al., '75). The range of putrescine concentrations necessary for 50% inhibition of the induction of ODC activity in the various cell lines and the differential response of the L1210 cells to a variety of α,ω-diamines indicates a differential sensitivity which is probably not unique to cells in culture; it is likely that it is also represented among the normal cells of different tissues (kidney, liver, spleen, etc.). Furthermore, the experimental evidence indicates that the levels of spermidine in human blood are in the range of $1\text{-}4 \times 10^{-6}$ M (Rennert et al., '76; Lundgren et al., '76) and in the range of $1\text{-}6 \times 10^{-7}$ M in human serum (Bartos et al., '78). These two values are approximately 5 and 50 times higher than the concentration required for 50% inhibition of ODC activity in L1210 cells, our most sensitive line.

It is therefore apparent that the normal human serum levels of spermidine are such that they could tend to minimize the induction of ODC in many polyamine sensitive cells. Consequently, if normal or tumor cells synthesize excessive amounts of polyamines, there will occur an additional elevation of the serum levels of polyamines. This increase in the serum levels of polyamines may inhibit the induction of ODC activity in both the high polyamine producing cells as well as in the large mass of host tissues. As a consequence of this inhibition of ODC activity, a decreased output of polyamines by these tissues will occur.

The net level of urinary polyamines is there-

fore not simply an indication of the high level of production of polyamines by a discrete tissue; it also mirrors the ability of the large mass of host tissues to respond to the increase in serum polyamine levels, by decreasing their output of polyamines. The degree of compensation will depend on the mass of available tissue, on the differential sensitivity of ODC in various tissues and on the ability of the tissue ODC to respond to the increased serum polyamine levels.

If we accept that the results presented in this paper are not peculiar characteristics of cells in culture, but have counterparts in the cells of an organism, it becomes apparent that the urinary output cannot be a reliable index of a high polyamine producing tumor (Bachrach, '76; Durie et al., '77; Lipton et al., '76; Russell, '71; Russell et al., '71; Russell, '77; Townsend et al., '76). The urinary polyamine output will vary depending on the differential ability of an individual tissue's ornithine decarboxylase to respond to the serum polyamine levels. This would result in a differential compensation for the increased polyamine production and would therefore result in an unpredictable and variable urinary polyamine output.

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