The role of polyamine depletion and accumulation of decarboxylated *S*-adenosylmethionine in the inhibition of growth of SV-3T3 cells treated with α -difluoromethylornithine

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The effects of α -difluoromethylornithine, a specific inhibitor of ornithine decarboxylase, on cell growth rate, polyamine content and the content of decarboxylated *S*-adenosylmethionine in SV-3T3 transformed mouse fibroblasts were studied. DL- α -Difluoromethylornithine at 1 mM or higher concentrations decreased the growth rate by over 90% after 2 or more days of exposure, but the cells remained viable, although quiescent for at least 9 days. Addition of 10 μ M-spermidine or -spermine or 50 μ M-putrescine at any time throughout this period completely reversed the inhibition of growth. Treatment with α -difluoromethylornithine decreased putrescine and spermidine contents by more than 98% and that of spermine by 60%, but cells exposed to exogenous polyamines did not require complete replenishment of the polyamine pools to resume growth. In fact, a virtually normal growth rate was obtained in cells lacking putrescine, having 2% of normal spermidine content and 156% of normal spermine. These results suggest that the well-known increase in putrescine and spermidine in cells stimulated for growth is not essential for this to occur and that mammalian cells can utilize spermine as their only polyamine. A substantial reversal of the growth-inhibitory effect of α -difluoromethylornithine was produced by a number of polyamines not normally found in mammalian cells, including the spermidine analogues aminopropylcadaverine and *sym*-homospermidine, which were partially converted into their respective spermine analogues by addition of an aminopropyl group within the cell. The spermine analogue *sym*-norspermine was also effective, but the maximal growth rate produced by these unphysiological polyamines was only 60–70% of that produced by the normal polyamines. These results indicate that spermidine and spermine have the optimal length for activation of the cellular processes critically dependent on polyamines and should help in identifying these processes. Exposure to α -difluoromethylornithine leads to an enormous rise in the concentration of decarboxylated *S*-adenosylmethionine, which reached a peak at 530-fold after 3 days of exposure and steadily declined to 140-fold after 11 days. This increase was abolished by addition of exogenous polyamines, which rapidly decreased the activity of *S*-adenosylmethionine decarboxylase. The increase in decarboxylated *S*-adenosylmethionine is unlikely to be solely responsible for the decrease in growth rate, since decarboxylated *S*-adenosylmethionine content was decreased to the same extent by spermine, *sym*-norspermidine and *sym*-homospermidine, which produce 97%, 16% and 60% of the control growth rate, respectively. However, the change in the content of this nucleoside may contribute to the effects of α -difluoromethylornithine in these cells.

Abbreviations used: DFMO, α -difluoromethylornithine; AdoMet, *S*-adenosyl-L-methionine.

There is convincing evidence that polyamines are needed for a normal rate of growth of eukaryotic cells (reviewed by Morris & Harada, 1980; Heby, 1981; Heby & Jänne, 1981; Pegg & McCann, 1982). Much of this evidence has been obtained by the use of inhibitors of polyamine biosynthesis, which also inhibit growth. Such inhibitors, if they are highly specific, provide important tools with which to study the function of polyamines. The most valuable inhibitor currently available is DFMO, which is an irreversible, enzyme-activated, inhibitor of ornithine decarboxylase (Metcalf *et al.*, 1978). In an important series of experiments, Mamont and colleagues have shown that exposure of HTC and mouse L1210 cells to DFMO depletes spermidine and putrescine contents and decreases cell growth unless exogenous putrescine or polyamines are provided (Mamont *et al.*, 1978a,b, 1980). In general terms, this observation has been confirmed by others using a variety of cultured cell types (Pegg *et al.*, 1981a,b, Hölttä *et al.*, 1981; Luk *et al.*, 1981, 1982; Seidenfeld *et al.*, 1981; Medrano *et al.*, 1983; Porter & Bergeron, 1983). However, the extent of inhibition of growth reported in those studies was quite variable, and cytotoxicity was seen in one series of studies (Luk *et al.*, 1981, 1982), but not in others. More recently, a second consequence of exposure to DFMO has been described. The content of decarboxylated AdoMet was increased several-hundred-fold in cells treated with DFMO for up to 3 days (Mamont *et al.*, 1982; Pegg *et al.*, 1982a). In the present experiments, a detailed study of the changes in polyamine content, decarboxylated AdoMet content, cell growth and viability was carried out over an extended period of exposure to DFMO. The abilities of various polyamines to reverse the effects of DFMO on these parameters were tested. It was found that, although a variety of polyamines not normally present in mammalian cells could stimulate cell growth and decrease decarboxylated AdoMet content, only the naturally occurring spermidine and spermine completely reversed the growth-inhibitory effects of DFMO.

Materials and methods

Materials

DFMO was a gift from Merrell Dow Research Center, Cincinnati, OH, U.S.A. Decarboxylated AdoMet was kindly provided by Dr. K. Samejima, Tokyo Biochemical Research Institute, Tokyo, Japan. *sym*-Homospermidine (1,9-diamino-5-azanonane) and *N*-(3-aminopropyl)cadaverine (1,9-diamino-5-azanonane) were generously provided by Dr. T. Oshima, Institute of Life Sciences, Machida, Tokyo, Japan, and by Dr. R. J. Bergeron, University of Florida, Gainesville, FL,

U.S.A. *sym*-Norspermidine (3,3'-diaminodipropylamine) and *sym*-norspermine [*NN'*-bis(3-aminopropyl)propane-1,3-diamine] were purchased from Eastman Organic Chemicals, Rochester, NY, U.S.A. Other polyamines and diamines were from Sigma Chemical Co., St. Louis, MO, U.S.A. The compounds were checked for purity and repurified to ensure that they contained less than 0.1% of any other polyamine, as previously described (Pegg *et al.*, 1981b).

Cell culture and determination of polyamines and decarboxylated AdoMet

Simian-virus-40-transformed Swiss-mouse embryo fibroblasts (SV-3T3 cells) were maintained and seeded at 4×10^5 cells per 10cm-diameter dish as previously described (Pegg *et al.*, 1981b, 1982a). Cell number (Pegg *et al.*, 1981a), polyamine content (measured with an amino acid analyser, with fluorescence detection as described by Pegg *et al.*, 1981b) and decarboxylated AdoMet content (Pegg *et al.*, 1982a) were determined as previously described.

Assay of AdoMet decarboxylase

Cell extracts were prepared by freeze-thawing in 0.5ml of 25mM-sodium phosphate buffer, pH7.0, containing 0.1mM-EDTA and 2.5mM-dithiothreitol (Bethell *et al.*, 1982). After centrifugation at 17000g for 30min in a Sorvall SR-5 refrigerated centrifuge at 2°C, samples of the supernatant was used for assay of AdoMet decarboxylase. The final assay medium contained 2.5mM-putrescine, 0.4mM-S-adenosyl-L-[carboxy- ^{14}C]methionine (2Ci/mol), 50mM-sodium phosphate, pH7.0, 1mM-dithiothreitol and 0.05mM-EDTA. After incubation at 37°C for 30min, release of $^{14}\text{CO}_2$ was determined as previously described (Bethell *et al.*, 1982). One unit of activity represents 1nmol of CO_2 released/30min. Results were expressed as units/mg of protein added. Protein was determined by the method of Bradford (1976).

Results

Effect of DFMO on cell growth

The presence of DFMO greatly decreased the rate of growth of SV-3T3 cells (Fig. 1). The extent of inhibition depended on the time at which the measurements were made, since DFMO had no effect on growth over the first 12h (results not shown). (Fig. 1 gives results for cell number only after 18h of growth and later times. The lack of effect of DFMO over the first few hours is therefore not evident from Fig. 1.) After this time, the cells in DFMO grew very slowly, whereas the control cells had a doubling time of about 9.5h. There-

Fig. 1. Effect of DFMO and polyamines on growth of SV-3T3 cells

SV-3T3 cells were seeded at an initial density of 4×10^5 cells/dish in the presence or absence of DFMO and polyamines. The number of cells present at the time shown was determined. Results in (a) show data for control cells (■), cells in presence of 0.1 mM-DFMO (◆), 1.0 mM-DFMO (▲) and 5 mM-DFMO (●). At 4 days 10 μM -spermidine (SPD) was added to some dishes (▼), or at 5 days, 10 μM -spermine (SPM) was added (○). In (b), all dishes contained the polyamines themselves in the absence of DFMO did not affect cell growth.

fore, by 2 days of culture the inhibition of cell growth was more than 90% if measured in terms of the number of cells present. As shown in Fig. 1, 1 mM-DFMO was sufficient to produce this inhibition; no further effect was produced by increasing the DFMO concentration to 5 mM, but 0.1 mM was less effective. The cells grown in the presence of DFMO remained viable for at least 9 days. If 50 μM -putrescine was added at zero time, 3, 7 or 9 days after addition of DFMO, the cells resumed a normal growth rate (Fig. 1b), indicating that the cells remained viable although quiescent. Complete reversal of the inhibitory effects of DFMO on cell growth could also be obtained by the addition of 10 μM -spermidine or -spermine (Fig. 1a). Several other di- and poly-amines were also tested for the abilities to reverse the growth inhibition by DFMO. Typical results are shown in Table 1. None of the other compounds examined was as effective as putrescine, spermidine or spermine, although many produced a significant reversal. Of the diamines tested, 1,3-diaminopropane was only slightly active even at 500 μM , whereas 1,5-diaminopentane at this concentration gave 63% of the control rate. It is likely that the diamines, including putrescine, were active by virtue of their conversion into higher polyamines, since *sym*-norspermidine [which is formed from 1,3-diaminopropane (Pegg *et al.*, 1981b)] was also very poorly active, whereas aminopropylcadaverine [which is formed from 1,5-diaminopentane (Pegg *et al.*, 1981b)] also gave 64% of the control rate. Although data are shown in Table 1 for only one or two con-

Table 1. Effect of various diamines and polyamines on growth of SV-3T3 cells in the presence of DFMO

The compounds were added together with 5 mM-DFMO and cells were grown as in Fig. 1. The percentage of control growth of the cells is calculated from the number present after 2 days minus the initial number, divided by the number of cells in dishes not receiving DFMO minus the initial number, $\times 100$. Results are means $\pm$ S.D. of four or more estimations or the mean of three estimations.

Treatment	Growth (% of control)
Control	100 $\pm$ 4
DFMO alone	12 $\pm$ 3
DFMO + 50 μM -1,3-diaminopropane	21
DFMO + 500 μM -1,3-diaminopropane	32 $\pm$ 6
DFMO + 50 μM -putrescine	103 $\pm$ 7
DFMO + 50 μM -1,5-diaminopentane	32
DFMO + 500 μM -1,5-diaminopentane	63 $\pm$ 12
DFMO + 50 μM -1,6-diaminohexane*	29
DFMO + 10 μM - <i>sym</i> -norspermidine*	16 $\pm$ 3
DFMO + 10 μM -spermidine	99 $\pm$ 5
DFMO + 10 μM - <i>sym</i> -homospermidine	61 $\pm$ 8
DFMO + 10 μM -aminopropylcadaverine (<i>unsym</i> -homospermidine)	64 $\pm$ 7
DFMO + 10 μM - <i>sym</i> -norspermine	68 $\pm$ 11
DFMO + 10 μM -spermine	94 $\pm$ 5

* Toxic at higher concentrations.

centrations of the amines, all were tested over a range of concentrations, and no greater effects were observed at other concentrations. A maximal stimulation of growth occurred with exposure to

10 μM of the spermidine analogues, and higher or lower concentrations were less effective, even though the higher concentrations were not obviously cytotoxic. In contrast with the ineffectiveness of *sym*-norspermidine, *sym*-norspermine had an effect at least as good as any of the other unnatural polyamines tested.

Changes in concentrations of polyamines and decarboxylated AdoMet

Exposure to DFMO for 56h decreased putrescine content to below detectable values, decreased spermidine by more than 98% and made a small decrease (about 40%) in spermine content (Table 2).

At the same time, decarboxylated AdoMet increased by more than 400-fold. On continued exposure to DFMO, there were no further changes in the polyamine contents (results not shown), but decarboxylated AdoMet content reached a peak at 3 days (> 500-fold increase) and then declined steadily over the next 8 days (Fig. 2a). However, even at the last time point observed, the concentration of this nucleoside was more than 100 times that in control cells. The rise and fall in decarboxylated AdoMet content is clearly linked to the activity of AdoMet decarboxylase, which reached a peak at 2 days in the presence of DFMO and then declined slowly to about 50% of maximum at 11

Table 2. Effect of various concentrations of putrescine on growth, polyamine content and decarboxylated AdoMet content of SV-3T3 cells exposed to 5mM-DFMO

The experiment was carried out as described in Table 1, except that cells were grown for 56h.

Addition	$10^{-6} \times$ Cell number	Growth (% of control)*	Content of substance shown (fmol/cell)			
			Decarboxylated AdoMet	Putrescine	Spermidine	Spermine
None	13.7	100	0.001	0.38	2.95	1.28
DFMO	3.9	11	0.440	<0.03	0.03	0.60
DFMO + 100 μM -putrescine	14.5	106	<0.001	0.55	2.83	1.12
DFMO + 50 μM -putrescine	14.3	104	<0.001	0.65	3.41	1.33
DFMO + 25 μM -putrescine	14.0	102	<0.001	0.33	2.71	1.28
DFMO + 10 μM -putrescine	13.8	100	<0.001	0.12	3.10	1.47
DFMO + 7.5 μM -putrescine	11.2	81	0.070	<0.03	0.64	1.81
DFMO + 5 μM -putrescine	9.8	71	0.150	<0.03	0.13	1.69

* Calculated as described in Table 1.

Fig. 2. Effect of DFMO on AdoMet decarboxylase activity and content of decarboxylated AdoMet in SV-3T3 cells. Cells were grown for the time shown in 5mM-DFMO, and the content of decarboxylated AdoMet (a) or the activity of AdoMet decarboxylase assayed *in vitro* in the presence of a saturating concentration of putrescine (b) was measured.

days (Fig. 2b). The increase in enzyme activity over control values was only 8–12-fold at most. The much greater increase in decarboxylated AdoMet is due to the absence of putrescine and spermidine to serve as acceptors of the aminopropyltransferase reactions with decarboxylated AdoMet as substrate.

Addition of exogenous putrescine at concentrations of 25–100 μ M completely prevented the changes in polyamine and decarboxylated AdoMet contents brought about by DFMO and gave a normal growth rate (Table 2). When only 10 μ M-putrescine was provided, the cells grew normally with normal polyamine content, although putrescine contents were only 32% of normal. Lower exogenous concentrations of putrescine did not restore the growth rate entirely to normal, but produced substantial reversal without restoring spermidine content or completely decreasing decarboxylated AdoMet, although spermine values were above control (Table 2).

The polyamine and decarboxylated AdoMet contents of cells exposed to DFMO and various polyamines are shown in Table 3. Spermidine restored contents of polyamine and decarboxylated AdoMet to normal, but no putrescine was present in these cells. These results indicate that putrescine itself is not required for cell growth, except as a precursor of the polyamines. Spermine also restored growth to almost normal, and these cells contained only trace amounts of spermidine, but had somewhat elevated contents of spermine (156% of control). However, the decarboxylated AdoMet of the cells exposed to spermine was greatly decreased. These results and those in Table 2 (last line) indicate that SV-3T3 cells can grow at a normal rate with spermine as virtually the only intracellular polyamine, but do not rule out the possibility that the growth inhibition by DFMO is due to the accumulation of decarboxylated AdoMet.

The other substances which partially restored the cell growth rate were *sym*-norspermine, which is a spermine analogue and accumulated in the cell to a substantial extent, and *sym*-homospermidine and aminopropylcadaverine (*unsym*-homospermidine). These latter compounds are known to be substrates for mammalian spermine synthase *in vitro* (Pegg *et al.*, 1981b) and the cells exposed to them contained measurable concentrations of the spermine analogues (Table 3). All of these exogenous polyamines caused a substantial fall in decarboxylated AdoMet contents, and this could relate to the stimulation of cell growth. However, *sym*-norspermidine also greatly decreased decarboxylated AdoMet, but did not appreciably increase the growth rate.

The mechanism by which the decrease in decarboxylated AdoMet was brought about was studied

Table 3. Effect of polyamines on growth, polyamine and decarboxylated AdoMet content of SV-3T3 cells exposed to 5 mM-DFMO. The experiment was carried out as in Table 1, except that cells were grown for 2 days.

Addition	10 ⁶ × Cell number	Growth (% of control)*	Content of substance shown (fmol/cell)				
			Decarboxylated AdoMet	Putrescine	Spermidine	Spermine	Others
None	11.2	100	0.001	0.31	2.89	1.24	None
5 mM-DFMO	1.8	13	0.397	<0.03	0.04	0.78	None
5 mM-DFMO + 10 μ M- <i>sym</i> -norspermidine	2.1	16	0.010	<0.03	<0.03	0.48	4.8 <i>sym</i> -norspermidine + 0.2 <i>sym</i> -norspermine
5 mM-DFMO + 10 μ M-aminopropylcadaverine	7.3	64	0.012	<0.03	<0.03	0.57	5.1 aminopropylcadaverine + 0.4 bis(aminopropyl)cadaverine
5 mM-DFMO + 10 μ M- <i>sym</i> -homospermidine	6.9	60	0.015	<0.03	<0.03	0.58	5.5 <i>sym</i> -homospermidine + 0.9 aminopropyl- <i>sym</i> -homospermidine
5 mM-DFMO + 10 μ M-spermidine	11.1	99	0.002	<0.03	3.25	1.51	None
5 mM-DFMO + 10 μ M- <i>sym</i> -norspermine	7.8	68	0.011	<0.03	<0.03	0.15	0.2 <i>sym</i> -norspermidine + 6.9 <i>sym</i> -norspermine
5 mM-DFMO + 10 μ M-spermine	10.5	93	0.009	<0.03	0.05	1.93	None

* Calculated as described in Table 1.

by measuring the content of this nucleoside and the activity of AdoMet decarboxylase at various times after addition of the amines. These studies were carried out starting at day 3 and at day 7 after exposure of the cells to DFMO, since these times represent the peak decarboxylase activity and the start of the plateau of decarboxylase activity, re-

spectively. As shown in Fig. 3(b) (results for day 3) and Table 4 (results for day 7), all of the polyamines tested produced a substantial fall in the decarboxylase activity, which was complete by 24 h. Spermine was the most potent compound, decreasing the activity by more than 95%; spermidine produced a 80–90% decrease and *sym*-homospermi-

Fig. 3. Effect of polyamines on AdoMet decarboxylase activity and content of decarboxylated AdoMet in DFMO-treated SV-3T3 cells

Cells were grown for 3 days in the presence of 5 mM-DFMO and then they were exposed to 50 μM-putrescine (PUT, ◆), 10 μM-spermidine (SPD, ■), 10 μM-spermine (SPM, ▲), 10 μM-*sym*-homospermidine (HSPD, ▼) or no polyamines (●). Cells were then grown for the time shown, and the content of decarboxylated AdoMet (a) and the activity of AdoMet decarboxylase (b) were measured. AdoMet decarboxylase activity was measured as in Fig. 2, and results are given as the percentage of the activity in the cells after 3 days of exposure to DFMO, which was 5.6 units/mg of protein.

Table 4. Effect of polyamines on AdoMet decarboxylase activity and content of decarboxylated AdoMet in SV-3T3 cells treated with DFMO for 7 days

After growth for 7 days in 5 mM-DFMO, cells were treated with the addition of the polyamine shown and measurements of decarboxylated AdoMet and AdoMet decarboxylase were made 24 h and 48 h later. The 48 h AdoMet decarboxylase activities were not significantly different from the 24 h values. The AdoMet decarboxylase activity at 7 days was 2.69 units/mg, and values in parentheses are the percentages of this value remaining in the cells at 24 h.

Addition	Decarboxylated AdoMet (fmol/cell)		AdoMet decarboxylase (units/mg) at 24 h
	at 24 h	at 48 h	
None (at 7 days)	0.275	0.225	2.61 (96%)
10 μM-spermine	0.018	<0.01	0.06 (2%)
10 μM-spermidine	0.016	<0.01	0.29 (11%)
50 μM-putrescine	0.019	<0.01	0.31 (12%)
10 μM- <i>sym</i> -norspermidine	0.021	<0.01	0.08 (3%)
10 μM- <i>sym</i> -homospermidine	0.169	0.015	0.48 (18%)
10 μM-aminopropylcadaverine	0.190	0.019	0.53 (20%)

dine and aminopropylcadaverine were somewhat less effective. Putrescine produced the same decrease of AdoMet decarboxylase activity as did spermidine, but a longer time was needed for the full decrease to become apparent. This is consistent with the effect being mediated via the conversion of putrescine into spermidine. In the experiments carried out with cells exposed to DFMO for 3 days, all of the compounds tested decreased the decarboxylated AdoMet content by more than 95% within 24h, although spermidine had the most rapid effect and spermine the slowest (Fig. 3a). In the experiment starting after 7 days, *sym*-homospermidine and aminopropylcadaverine were clearly less effective than the other polyamines tested, but all compounds examined decreased decarboxylated AdoMet by more than 90% by 48h (Table 4).

Effects of sym-homospermidine on cell growth and contents of polyamine and decarboxylated AdoMet

Although the homospermidine analogues were not as effective as spermidine in restoring a normal growth rate to DFMO-treated cells, they produced a substantial increase in growth. The changes in cellular polyamines were therefore studied and compared with growth rates in cells exposed to *sym*-homospermidine at various concentrations. As shown in Fig. 4(e), 1 μM -*sym*-homospermidine increased the growth rate of DFMO-treated cells from 2–5% of the controls to about 35%. Increasing the *sym*-homospermidine concentration to 10 μM increased growth to 60% of control, but higher concentrations were less effective. *sym*-Homospermidine accumulated in the cells (Fig. 4b) and was converted into its spermine analogue to a small extent (Fig. 4c). The content of spermine in the cells fell somewhat (Fig. 4d), probably owing to competitive inhibition of spermine synthase by the *sym*-homospermidine. The content of decarboxylated AdoMet fell greatly and was at the limit of sensitivity within 48h (Fig. 4a). These results suggest that the rise in decarboxylated AdoMet cannot be solely responsible for the inhibition of growth by DFMO in these cells, and that *sym*-homospermidine or its aminopropyl derivative can partially, but not completely, substitute for the normal polyamine requirement. The reason that growth is better with 10 μM -*sym*-homospermidine than with 100 or 250 μM is not readily apparent, since by 48h these concentrations had similar effects on contents of spermine, decarboxylated AdoMet and aminopropyl-homospermine, and there was only a slight difference in the content of *sym*-homospermidine itself (Fig. 4). It is possible that at the higher concentrations a toxic metabolite of *sym*-homospermidine accumulates to sufficient concentrations to exert a growth-inhibitory effect. How-

Fig. 4. Effect of *sym*-homospermidine on growth (e), spermine content (d), aminopropyl-*sym*-homospermidine content (c), *sym*-homospermidine content (b) and decarboxylated AdoMet content (a) of SV-3T3 cells treated with DFMO

The cells were exposed to 5 mM-DFMO for 3 days and then the concentration shown of *sym*-homospermidine was added. Results are shown for 24h (●), 48h (■) and 72h (▲) after this addition. The growth was calculated as in Table 1, except that the control value was obtained by measuring the growth of cells to which 50 μM -putrescine was added after they had been exposed to 5 mM-DFMO for 3 days.

ever, we have maintained SV-3T3 cells in a medium containing DFMO and 10 μM -*sym*-homospermidine for up to 3 weeks without any change in the growth rate or viability (results not shown), so it is clear that the cells do not produce such a compound under these conditions.

Discussion

In SV-3T3 cells the inhibition of ornithine decarboxylase activity by DFMO leads to a profound inhibition of growth without affecting viability. In part, the difference between our results, in which SV-3T3-cell growth was inhibited by more than 90%, and those of some others, e.g. Porter &

Bergeron (1983), who obtained only a 45% inhibition with L1210 cells, is likely to be due to the difference in the number of cell doublings occurring before measurement. The SV-3T3 cells do not achieve the maximal inhibition of growth until they have gone through two doublings in the presence of DFMO. A period of growth equivalent to at least 6 times the normal cell-doubling time is needed to observe the full effects of the inhibitor. Similar conclusions were reached by Mamont *et al.* (1978a,b, 1980) and by Rudkin *et al.* (1984), who also observed substantial reversal of the inhibition of growth if exogenous putrescine, spermidine or spermine was provided. Although the experiments do not eliminate the possibility that putrescine itself could support growth even if its conversion into the higher polyamines was prevented, it is clear from Table 2 that growth in the presence of low concentrations of putrescine is due to its acting as a precursor of spermine. Similarly, although spermidine itself may support growth, the experiments with exogenous spermine indicate that a normal growth rate can be obtained in cells which have slightly elevated spermine content, but very little spermidine (Table 3). This finding, which is in agreement with studies on HTC cells by Mamont *et al.* (1980) and Rudkin *et al.* (1984), is quite surprising, for several reasons: (a) the DFMO-inhibited cells, which are growth-arrested, have 60% of the normal spermine content, (b) the well-known increase in cellular polyamine contents in response to growth-promoting stimuli usually involves a rapid increase in spermidine (Heby & Jänne, 1981; Heby, 1981; Pegg & McCann, 1982), and (c) work with other inhibitors has implicated spermidine as the critical polyamine (Heby & Jänne, 1981; Heby, 1981; Pegg *et al.*, 1982b). It is possible that there is compartmentation of spermine in mammalian cells and that exogenous spermine is more readily available to a key site than the endogenously synthesized spermine. This could also explain why exogenous spermine rapidly represses AdoMet decarboxylase (Fig. 3b; Alhonen-Hongisto, 1980; Mamont *et al.*, 1981), but an increased concentration of spermine produced by intracellular synthesis in response to drug administration does not decrease AdoMet decarboxylase activity (Pegg *et al.*, 1982b).

It is known that spermine can be converted into spermidine via the acetylase/oxidase pathway (Seiler *et al.*, 1981; Pegg *et al.*, 1981c), and it is quite likely that this occurs in the DFMO-treated cells when spermine is provided. However, the resulting spermidine is then converted back into spermine by spermidine synthase. This reaction is favoured by the very high content of decarboxylated AdoMet in the cell and accounts for part of the decrease in decarboxylated AdoMet. The

increase in decarboxylated AdoMet that occurs in response to DFMO is due to the absence of putrescine and spermidine to serve as aminopropyltransferase acceptors and to the increase in AdoMet decarboxylase activity. It should be noted that at physiological pH AdoMet decarboxylase is activated about 10-fold by putrescine (Williams-Ashman & Pegg, 1981). The difference in AdoMet decarboxylase activity between extracts from DFMO-treated and control cells shown in Fig. 2(b) is greater than would actually pertain in the cell, since the control cell activity would be activated by putrescine, which is absent from the DFMO-treated cells. As previously reported, addition of exogenous polyamines (Alhonen-Hongisto, 1980; Mamont *et al.*, 1981; Pösö & Pegg, 1981), decreases AdoMet decarboxylase activity (Fig. 3b and Table 4) and provides an aminopropyltransferase substrate. These two factors combine to decrease the content of decarboxylated AdoMet. Although putrescine and spermidine are the preferred substrates of the aminopropyltransferases, all of the other polyamines tested are also weak substrates (Pegg *et al.*, 1981b), except for spermine, which, as described above, could generate spermidine. The fact that spermidine decreases decarboxylated AdoMet content most rapidly is consistent with this interpretation, since spermidine is a good aminopropyltransferase substrate and a good repressor of AdoMet decarboxylase. *sym*-Homospermidine and aminopropylcadaverine are as active as spermidine in repressing AdoMet decarboxylase (Fig. 3b and Table 4), but are much less active as aminopropyltransferase substrates (Pegg *et al.*, 1981b), and these polyamines take longer to decrease the decarboxylated AdoMet content.

The accumulation of decarboxylated AdoMet implies that polyamine synthesis is the normal metabolic fate of most, if not all, of this compound. The marked decrease in decarboxylated AdoMet between 3 and 11 days after exposure to DFMO may be indicative of another pathway of degradation or excretion of this nucleoside, but part of the decrease is clearly due to the decline in AdoMet decarboxylase activity (Fig. 2b). The reason for this decline is unknown, but it is possible that AdoMet decarboxylase is repressed not only by polyamines, but also by decarboxylated AdoMet itself. At present the stereochemistry of the accumulated decarboxylated AdoMet is unknown. It has been shown that the normal *SS* stereoisomer of AdoMet that is formed by methionine adenosyltransferase slowly changes into an equilibrium mixture of the *RS* and *SS* isomers (Wu *et al.*, 1983; Hoffman, 1984). Under normal circumstances, AdoMet turns over very rapidly in the cell and the unnatural isomer has little chance to accumulate. If a similar interconversion occurs with decarboxylated Ado-

Met, the *RS* stereoisomer may build up with time in cells treated with DFMO.

The accumulation of decarboxylated AdoMet may have a significant inhibitory action on cell growth in SV-3T3 cells. All of the substances which promoted growth in the DFMO-treated cells also decreased AdoMet decarboxylase activity to normal or near-normal values. Mamont *et al.* (1982) have reported that decarboxylated AdoMet is not responsible for the growth inhibition of DFMO in HTC cells, because 1,3,6-triaminohexane could reverse the decrease in growth rate without decreasing decarboxylated AdoMet. In our experiments, this triamine did not support continued growth of SV-3T3 cells, although an initial increase is obtained for about 2 days (K. McGovern & A. E. Pegg, unpublished work), and further work is needed to test whether this difference is related to the cell type or the experimental protocol. Whether or not decarboxylated AdoMet contributes to the inhibition of growth by DFMO, it is clearly not solely responsible for this inhibition, since *sym*-norspermidine, which barely affected growth, *sym*-homospermidine, which increased to 60% of control, and spermine, which increased growth to 97% of control, had similar effects in decreasing decarboxylated AdoMet content.

Our results indicate that polyamines with slightly different numbers of carbon atoms from the physiological spermidine and spermine can stimulate cell growth, but that the growth rate in the presence of such compounds is not restored completely to control values. This suggests that the distance between the positively charged amino groups is important for the full activity of the polyamines and that the optimal distribution has a C₃ and a C₄ unit in the same molecule. This strict specificity should help in ascertaining which of the many processes that are affected by polyamines *in vitro* are actually essential for growth *in vivo*. It is interesting that Linderoth & Morris (1983) report that *sym*-homospermidine and aminopropylcadaverine could promote growth of a spermidine auxotroph of *Escherichia coli*, but were not as effective as spermidine itself. Our results are entirely consistent with this. Our results differ slightly from those of Porter & Bergeron (1983) and Mamont *et al.* (1980), who found that closely related non-physiological polyamines restored growth rate to that produced by spermidine. However, this may be due to a difference in the experimental protocols, since their studies were carried out for only a short period of time. This decreases the sensitivity of the analysis and also may conceal differences in response to polyamines of some cellular processes.

Finally, SV-3T3 cells exposed to DFMO resemble HTC, L1210 and 9L cells in entering a quiescent phase without loss of viability. It is

possible that the residual spermine in these cells permits the cell to remain in a viable, although quiescent, state and that a more potent inhibitor than DFMO or combination of inhibitors may be more cytotoxic. Indeed, there is evidence that this may be the case (Pegg *et al.*, 1982b). It has been reported that human small-cell lung carcinoma cells lose viability in the presence of DFMO (Luk *et al.*, 1982), but the reason why these cells, which were grown as multicellular aggregates, are so sensitive is not known.

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