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Effects of *S*-Adenosyl-1,8-diamino-3-thiooctane on Polyamine Metabolism†

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ABSTRACT: Exposure of mammalian cells (transformed mouse fibroblasts or rat hepatoma cells) to *S*-adenosyl-1,8-diamino-3-thiooctane produced profound changes in the intracellular polyamine content. Putrescine was increased and spermidine was decreased, consistent with the inhibition of spermidine synthase by this compound, which is a potent and specific "transition-state analogue inhibitor" of the isolated enzyme in vitro. The spermine content of the cells was increased by exposure to this drug presumably since spermine synthase was able to use a greater proportion of the available decarboxylated *S*-adenosylmethionine when spermidine synthase was inhibited. The decarboxylated *S*-adenosylmethionine content rose substantially because the activity of *S*-adenosylmethionine decarboxylase was increased in response to the decline in spermidine. These results indicate that *S*-adenosyl-1,8-diamino-3-thiooctane is taken up by mammalian cells and is an effective inhibitor of spermidine synthase in vivo and that *S*-adenosylmethionine decarboxylase is regulated by the content of spermidine, but not of spermine. The growth of SV-3T3 cells was substantially reduced in the presence of *S*-adenosyl-1,8-diamino-3-thiooctane at concentrations of 50 μM or greater. Such inhibition was reversed by the addition of spermidine but not by putrescine. When SV-3T3 cells were exposed to 5 mM α-(difluoromethyl)ornithine and 50 μM *S*-adenosyl-1,8-diamino-3-thiooctane, the content of all polyamines was reduced. Putrescine and spermidine declined by more than 90% and spermine by 80%. Such cells grew very slowly unless spermidine was added.

Polyamines are synthesized in mammalian cells from ornithine and AdoMet[1] by the actions of four key enzymes: ornithine decarboxylase, AdoMet decarboxylase, spermidine synthase, and spermine synthase. The two decarboxylases provide putrescine and decarboxylated AdoMet. Spermidine synthase catalyzes the transfer of an aminopropyl group from decarboxylated AdoMet to putrescine forming spermidine and 5′-(methylthio)adenosine. Spermine synthase transfers a second aminopropyl group from another molecule of decarboxylated AdoMet to spermidine forming spermine and 5′-(methylthio)adenosine (Tabor & Tabor, 1976; Jänne et al., 1978; Pegg & Williams-Ashman, 1981; Williams-Ashman & Pegg, 1981). The function(s) of polyamines in cellular physiology is (are) still not well understood, but evidence from experiments in which the polyamine content was depleted by exposure to inhibitors of biosynthetic enzymes or by mutations affecting these enzymes suggest that they are essential for normal growth (Tabor & Tabor, 1976; Jänne et al., 1978; Cohn et al., 1978, 1980; Morris, 1981; Tabor et al., 1981). Although mutations affecting the activity of each of the key biosynthetic enzymes have been produced in yeast (Cohn et al., 1978, 1980), such mutants are not available in mammalian cells except for CHO cell lines lacking ornithine decarboxylase (Steglich & Scheffler, 1982; Pohjanpelto et al., 1981). Studies of the roles of polyamines in mammalian cell physiology have, therefore, mainly been carried out by using inhibitors (Jänne et al., 1978; Heby & Jänne, 1981; McCann et al., 1981; Koch-Weser et al., 1981; Heby, 1981; Porter et al., 1981).

Many attempts to synthesize potent and specific inhibitors have been made, and some useful inhibitors have resulted that may have valuable pharmacological actions (McCann et al., 1981; Koch-Weser et al., 1981; Porter et al., 1981). However, all of the inhibitors presently available have disadvantages, and at present, only certain inhibitors of ornithine and AdoMet decarboxylases have effects on cellular metabolism that are reversed by addition of polyamines and thus pass a first test

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[1] Abbreviations: AdoDATO, *S*-adenosyl-1,8-diamino-3-thiooctane; AdoMet, *S*-adenosylmethionine; DFMO, α-(difluoromethyl)ornithine.

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for specificity of the inhibitor. Even this criterion is not sufficient for methylglyoxal bis(guanylhydrazone) and various congeners of this compound that inhibit AdoMet decarboxylase (Corti et al., 1974; Pegg, 1978; Porter et al., 1981). Although the effects of these drugs are reversed by spermidine or spermine, the transport of the drug into the cell is accomplished by an active transport system that also acts on polyamines (Mandel & Flintoff, 1978; Porter et al., 1981). Furthermore, these drugs are small strongly basic aliphatic molecules that resemble the polyamines, and their effects could be reversed by polyamines because of their displacement by polyamines from critical binding sites. Finally, these compounds also inhibit diamine oxidase (Hölttä et al., 1973; Pegg & McGill, 1978). Therefore, it is difficult to prove that their effects on cellular metabolism are due to interference with polyamine synthesis.

Many inhibitors of ornithine decarboxylase have been described (Jänne et al., 1978; Stevens & Stevens, 1980; Heby & Jänne, 1981), including some potent irreversible enzyme-activated inhibitors (Metcalf et al., 1978; Danzin et al., 1981). The most studied of these has been α-(difluoromethyl)-ornithine, and many workers have confirmed the original reports of Mamont et al. (1978a,b) that this compound brings about extensive depletion of putrescine and spermidine in mammalian cells and that such cells grow at a greatly reduced rate. Moreover, there is evidence that the pharmacological and growth inhibitory effects of this drug are due to depletion of polyamines since the effects are reversed by exposure to spermidine or putrescine (Mamont et al., 1978a, 1981a; McCann et al., 1981; Koch-Weser et al., 1981; Pegg et al., 1981; Hölttä et al., 1981). However, even with this compound certain experiments are not possible. This inhibitor effectively depletes cells of putrescine and spermidine but has little effect on spermine content presumably because the residual ornithine decarboxylase activity is sufficient to maintain spermine levels (Mamont et al., 1978a, 1981a). A further problem is that ornithine decarboxylase turns over very rapidly (Jänne et al., 1978; Pegg & Williams-Ashman, 1981) and a high inhibitor concentration must be maintained constantly to prevent the accumulation of active enzyme by resynthesis. Finally, the inhibitor cannot be used to deplete cells of spermidine without also reducing putrescine.

A better understanding of the roles of polyamines in the cell would obviously be facilitated if the pathway could be blocked at various points, allowing selective accumulation of putrescine, spermidine, or spermine. For these reasons, we have attempted to exploit inhibitors of the aminopropyl transferases spermidine synthase and spermine synthase. A number of compounds known to inhibit the enzymes in vitro have been tested for the ability to influence polyamine levels of cultured cells, and some, including 5′-(methylthio)tubercidin, had significant effects (Hibasami et al., 1980a; Pegg et al., 1981; Pankaskie et al., 1981). However, these compounds may have had other influences on the cell because their inhibition of growth could not be reversed by provision of exogenous spermidine (Pegg et al., 1981; Pankaskie et al., 1981).

Recently, we have demonstrated that compounds designed as transition-state analogues (Wolfenden, 1976) are very potent inhibitors in vitro of spermidine synthase (Tang et al., 1980). Very powerful inhibition of spermidine synthase, but no effect on spermine synthase or 5′-(methylthio)adenosine phosphorylase, was produced by AdoDATO (Tang et al., 1980; Pegg & Coward, 1981). The corresponding methylsulfonium salt was also inhibitory, but less potent. When the corresponding deamino analogues or the non-adenosine fragment (i.e., 1,8-diamino-3-octanol) was tested, no inhibitory effect was seen. However, it was not known whether compounds such as AdoDATO could enter the cell and influence polyamine metabolism in vivo. In the present paper, we describe the effects of these compounds on the polyamine content, on the content of decarboxylated AdoMet, and on the activities of the enzymes in the polyamine biosynthetic pathway in cultured fibroblasts and hepatoma cells. The results are discussed in relation to the regulation of polyamine biosynthesis and the function of polyamines.

Experimental Procedures

Chemicals. L-[1-^{14}C]Ornithine (53 mCi/mmol), [*carboxyl*-^{14}C]AdoMet (54 mCi/mmol), and [*methyl*-^{14}C]AdoMet (50 mCi/mmol) were obtained from New England Nuclear, Boston, MA. Unlabeled and methyl-^{14}C-labeled decarboxylated AdoMets were prepared by using bacterial AdoMet decarboxylase (Hibasami et al., 1980a). AdoDATO and related compounds were synthesized by the method described by Tang et al. (1981). Other biochemical reagents were obtained from Sigma Chemical Co., St. Louis, MO.

Cell Culture. Simian virus 40 transformed 3T3 fibroblasts were maintained and cultured in Dulbecco's modified medium with 3% donor horse serum and 2% fetal calf serum as previously described (Bethell & Pegg, 1979; Pegg et al., 1981). In some experiments, 1 mM aminoguanidine was added to the culture medium to reduce amine oxidase activity. This did not affect the polyamine levels in the cells, the rate of growth, nor the response to AdoDATO. Rat HTC hepatoma cells were grown in suspension (spinner) culture in Swim's 77 medium supplemented with 10% newborn calf serum as described by Mamont et al. (1978a). The cell number was determined by counting in a Coulter counter with at least four samples for each point.

Polyamine Determination. Cells for measurement of polyamine content were harvested as previously described for fibroblasts (Pegg et al., 1981) or HTC cells (Mamont et al., 1978a). Aliquots of the deproteinized extracts were then analyzed as described by Seidenfeld & Marton (1979) by using an amino acid analyzer fitted with fluorescence detection. In some experiments, extracts prepared from the medium were assayed for polyamine content. No polyamines were found except in those experiments in which extracellular polyamines were added when the content found agreed with that added. These results indicate that polyamine excretion from the cells was negligible.

Determination of Decarboxylated AdoMet. This was determined after separation by high-performance liquid chromatography and quantitated by the absorbance at 260 nm (Pegg et al., 1982). The cell samples were processed as previously described (Pegg et al., 1982) with the following addition, which reduced the background and allowed for simple concentration of the samples. The cell extracts were prepared by homogenization in acid as before, but prior to chromatography the samples were brought to a concentration of 0.25 M in ammonium acetate (pH 8.8) and applied to a small column (bed volume 0.1 mL; prepared in a plastic pipet tip) of Affi-Gel 601 boronate affinity resin (Bio-Rad Laboratories, Richmond, CA). The columns were washed with 0.5 mL of 0.25 M ammonium acetate, and the decarboxylated AdoMet was then eluted with 1 mL of 0.1 M formic acid. An aliquot of this eluate was then applied directly to the chromatography column.

Enzyme Assays. Ornithine decarboxylase and AdoMet decarboxylase activities were assayed by measuring the release of $^{14}CO_2$ from the appropriate carboxyl-labeled substrate

Table I: Effect of AdoDATO on Polyamine Content, Decarboxylated AdoMet Content, and Cell Number in SV-3T3 Cells[a]

concn of AdoDATO (μM)	cell no./dish (×10⁻⁶)	putrescine (fmol/cell)	spermidine (fmol/cell)	spermine (fmol/cell)	decarboxylated AdoMet (amol/cell)
0	9.3 ± 0.7	0.34 ± 0.08	3.11 ± 0.36	1.26 ± 0.13	0.9 ± 0.5
1	9.2 ± 0.5	0.59 ± 0.04	1.54 ± 0.21	1.93 ± 0.26	7.8 ± 2.1
5	8.7 ± 0.4	0.75 ± 0.11	1.01 ± 0.13	2.46 ± 0.30	13 ± 4
10	8.3 ± 0.5	0.95 ± 0.18	0.90 ± 0.18	2.94 ± 0.29	21 ± 4
25	7.8 ± 0.7	1.17 ± 0.19	0.82 ± 0.21	3.18 ± 0.27	32 ± 5
50	7.4 ± 0.7	0.74 ± 0.26	0.43 ± 0.19	2.82 ± 0.30	68 ± 14
100	3.4 ± 0.9	0.49 ± 0.16	0.36 ± 0.10	3.03 ± 0.64	89 ± 21
175	2.8 ± 1.0	0.42 ± 0.08	0.29 ± 0.11	2.54 ± 0.12	NM[b]
250	0.7 ± 0.3	0.13 ± 0.05	0.09 ± 0.04	2.22 ± 0.58	NM

[a] SV-3T3 cells were seeded at 0.4 × 10⁶ cells/dish and grown for 3 days in the presence of the AdoDATO concentration shown. Results are given as mean ± SD for 4–10 estimations. [b] NM, not measured.

(Bethell & Pegg, 1979; Pegg et al., 1982). Spermidine and spermine synthase activities were determined by following the production of 5′-([*methyl*-^{14}C]methylthio)adenosine from decarboxylated [*methyl*-^{14}C]AdoMet in the presence of the appropriate acceptor (putrescine or spermidine) of the aminopropyl group (Hibasami & Pegg, 1978; Hibasami et al., 1980a). In order to remove any AdoDATO or polyamine from the extracts used for the assay of aminopropyl transferase activities, we dialyzed the extracts overnight against 1000 volumes of 0.1 M sodium phosphate, pH 7.5, 1 mM dithiothreitol, and 0.1 mM EDTA. The extracts for the decarboxylase assays were not dialyzed routinely, but checks on a number of samples indicated that dialysis did not affect the activity. All results of enzyme assays were obtained from experiments in which the activity was proportional to both the time of incubation and the amount of protein added. Results were expressed as nanomoles of product per milligram of protein added per 30-min incubation. Protein was determined by the method of Bradford (1976).

Results

When transformed mouse fibroblasts (SV3T3 cells) were grown for 3 days in the presence of concentrations of AdoDATO from 1 to 250 μM, the results were as shown in Table I. Even 1 μM AdoDATO had a significant effect on polyamine levels, increasing putrescine and spermine by more than 50% and reducing spermidine by 50%. Increasing the concentration of AdoDATO up to 25 μM produced a dose-dependent increase in both putrescine and spermine and a dose-dependent decrease in spermidine. With 25 μM AdoDATO, putrescine was increased 3.4-fold, spermine was increased 2.5-fold, and spermidine fell by 75%. Increasing the concentration of AdoDATO up to 250 μM continued to depress the spermidine level even further, but putrescine and spermine also fell from the peak seen at 25 μM AdoDATO. Concentrations of AdoDATO below 50 μM had only a small (<20%) effect on growth rate as indicated by the cell number, but the higher concentrations were strongly inhibitory.

In addition to the effects on polyamine levels, AdoDATO had a very striking action on the content of decarboxylated AdoMet in these cells. As previously reported (Pegg et al., 1982), the normal value for this nucleoside in these cells was less than 1 amol/cell and was difficult to measure precisely, but even 1 μM produced about an 8-fold increase, and there was a dose-dependent increase in this nucleoside that increased about 90-fold in the presence of 100 μM AdoDATO (Table I).

In order to test the effects of prolonged exposure to AdoDATO on polyamine levels in these fibroblasts, we replated the cells every 3 days in medium containing 50 μM AdoDATO or control medium (Figure 1). The cell growth rate was reduced by a constant value of about 30% over a 12-day exposure, and polyamine levels were also constant at about 15% of control values for spermidine and 250% of control values for spermine. Putrescine decreased slightly from its peak 2-fold increase but remained substantially above control values.

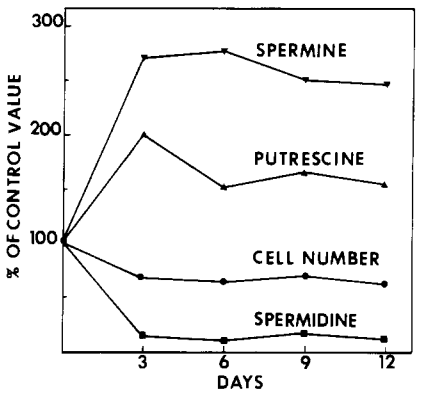

FIGURE 1: Effect of prolonged exposure to AdoDATO on polyamine content of SV-3T3 cells. The cells were replated every third day at a density of 400 000 cells/dish in either fresh complete medium or fresh complete medium plus 50 μM AdoDATO. The content of putrescine (▲), spermidine (■), and spermine (▼) per cell was measured as in Table I and expressed as a percentage of that present in the control cells not exposed to AdoDATO. The cell number (●) in the dishes having AdoDATO was also expressed as a percentage of that in the controls.

These effects of AdoDATO on polyamine levels were not peculiar to virally transformed fibroblasts. Essentially similar results were obtained with 3T3 cells and with rat HTC hepatoma cells grown in suspension culture (results not shown). Exposure of HTC cells to 100 μM AdoDATO reduced spermidine content by about 90%, increased spermine to values almost twice those in controls, and produced an increase in putrescine that peaked at 24 h at 2.4-fold and then declined slightly to 1.8-fold at 72 h. When the AdoDATO concentration was reduced to 10 μM, the changes observed at 24 h were similar but less marked and the effect decreased with time. This may indicate decomposition of the AdoDATO or a reduced response at later times. The rate of cell growth was not affected in the HTC cells by 10 μM AdoDATO, and the cell number was only reduced by 14% after 3 days in the presence of 100 μM.

In order to test the specificity of the changes observed in response to AdoDATO in SV3T3 cells, we exposed the cells to related nucleosides, including the methylsulfonium derivative of AdoDATO, which also inhibits spermidine synthase, and the corresponding deamino derivatives, which do not (Tang et al., 1980). The results are shown in Table II. The methylsulfonium salt derivative also led to an increase in putrescine and spermine and a decrease in spermidine but produced smaller changes than AdoDATO itself at the same

Table II: Effect of Compounds Related to AdoDATO on Polyamine Content and Cell Growth in SV-3T3 Cells[a]

compound added	cell no./dish ($\times 10^{-6}$)	putrescine (fmol/cell)	spermidine (fmol/cell)	spermine (fmol/cell)
none	8.1	0.43	3.09	1.14
AdoDATO	7.3	1.35	0.75	3.28
S-adenosyl-3-thiooctane	4.6	0.33	2.56	1.19
S-adenosyl-1,8-diamino-3-(methylthio)octane	7.9	0.99	2.19	1.80
S-adenosyl-3-(methylthio)octane	8.0	0.41	3.22	1.11

[a] The compounds indicated were added to culture dishes of SV-3T3 cells at a concentration of 25 μM. The cells were seeded at 0.4×10^6 cells/dish and grown for 3 days. Results are the mean of three separate experiments that agreed within ±15%.

Table III: Polyamine Content and Cell Number in SV-3T3 Cells Exposed to AdoDATO or DFMO and Polyamines[a]

additions	cell no./dish ($\times 10^{-6}$)	putrescine (fmol/cell)	spermidine (fmol/cell)	spermine (fmol/cell)
none	7.7 ± 0.8	0.41 ± 0.03	3.37 ± 0.20	1.22 ± 0.27
AdoDATO	2.8 ± 0.2	0.73 ± 0.09	0.36 ± 0.13	3.81 ± 0.27
AdoDATO plus spermidine (10 μM)	7.2 ± 1.1	0.06 ± 0.01	2.84 ± 0.11	1.99 ± 0.23
AdoDATO plus putrescine (10 μM)	1.8 ± 0.3	0.22 ± 0.02	0.32 ± 0.08	3.42 ± 0.59
AdoDATO plus putrescine (50 μM)	1.6 ± 0.4	0.71	0.56	3.43
spermidine (10 μM)	7.1 ± 0.6	0.09 ± 0.01	4.19 ± 0.17	1.62 ± 0.24
putrescine (10 μM)	7.9 ± 0.7	0.37 ± 0.04	2.89 ± 0.23	1.15 ± 0.17
putrescine (50 μM)	7.8 ± 0.2	0.47	3.28	1.67
DFMO	1.4 ± 0.4	<0.03	0.10 ± 0.05	0.87 ± 0.12
DFMO plus putrescine (10 μM)	8.3 ± 0.9	0.12	3.10 ± 0.62	1.47 ± 0.15
DFMO plus spermidine (10 μM)	7.3 ± 1.1	<0.03	3.42 ± 0.07	1.39 ± 0.17

[a] All dishes were seeded with 400 000 cells and grown for 3 days. The medium contained 1 mM aminoguanidine in all experiments. AdoDATO was added at 100 μM and DFMO at 5 mM. Results are given ±SD for six estimations or as the mean of three estimations.

concentration. This is consistent with the in vitro results showing that this compound is a less potent inhibitor of spermidine synthase (Tang et al., 1980) but is likely to also reflect a lower rate of uptake of the sulfonium derivative. The deamino derivatives had no significant effect on polyamine levels, but *S*-adenosyl-3-thiooctane was quite inhibitory to the growth of the SV3T3 cells, reducing the cell number by 43% (Table II). Higher concentrations were even more strongly inhibitory and led to cell death. Virtually all the cells were killed by exposure to 100 μM *S*-adenosyl-3-thiooctane for 24 h. In contrast, although 100 μM AdoDATO reduced the rate of cell growth by 70%, the effect was readily reversible either by changing the medium to remove the drug (results not shown) or by adding spermidine (see below).

The inhibition of the growth of SV-3T3 cells by 100 μM AdoDATO could be completely prevented if 5 μM spermidine was provided, and even 1 μM spermidine increased the growth rate to 86% of that of controls. Putrescine did not prevent the inhibition of growth and, in fact, seemed to increase the inhibition (Table III). It is possible that the reversal of the effects of AdoDATO by spermidine is due to interference with the uptake of the drug, but this appears unlikely since 1,8-diamino-3-octanol, which is more closely related to AdoDATO, had no effect when added at 10–50 μM concentrations (results not shown).

Table III shows the cell numbers and the levels of polyamines in cells exposed to 100 μM AdoDATO and spermidine or putrescine. Results are also given for cells exposed to α-(difluoromethyl)ornithine (DFMO) with and without these amines for comparison. Addition of 10 μM spermidine in the presence of 100 μM AdoDATO restored the cellular spermidine to 85% of the control value and decreased both putrescine and spermine, although the latter remained higher than that in the control cells. The reduction of putrescine to very low levels was also seen in cells treated with spermidine alone and is probably due to the reduction in ornithine decarboxylase produced by exposure to exogenous spermidine (Canellakis et al., 1979; Jänne et al., 1978; Bethell & Pegg, 1979; Heby & Jänne, 1981). The provision of putrescine to cells treated with AdoDATO did not lead to the restoration of normal spermidine levels. As a control for this experiment, it can be seen that provision of either putrescine or spermidine was able to restore the normal cellular content of spermidine in cells exposed to DFMO (Table III). These results, therefore, indicate that the cells exposed to AdoDATO are inhibited at the spermidine synthase step and that when normal spermidine levels are restored, a normal rate of cell growth occurs.

It is well documented that inhibitors of ornithine and AdoMet decarboxylases produce striking changes in the activities of the enzyme in the polyamine biosynthetic pathway when these are measured after dialysis to remove the inhibitors (Pegg et al., 1973; Fillingame & Morris, 1973; Harik et al., 1974; McCann et al., 1977; Pegg, 1979; Alhonen-Hongisto, 1980; Mamont et al., 1981b; Pegg et al., 1982). Therefore, the activities of these enzymes were measured in cells exposed to AdoDATO (Figures 2 and 3). Exposure for 3 days to either AdoDATO or DFMO did not affect the activity of spermine synthase or spermidine synthase in dialyzed extracts prepared from SV-3T3 cells (Figure 2) or HTC cells (Figure 3). Exposure to AdoDATO produced a significant (i.e., 2–3-fold) increase in ornithine decarboxylase in SV-3T3 cells at all times and in HTC cells at 1 day but not at other times. However, as shown in Figures 2 and 3, the activity of this enzyme in control cells shows a considerable fluctuation of more than 6-fold during the period tested. The apparently elevated ornithine decarboxylase activity may be a secondary phenomenon due to the change in growth rate produced by AdoDATO.

Table IV: Effect of Combined Exposure to DFMO and AdoDATO on Polyamine Content in SV-3T3 Cells[a]

addition	days before harvest	cell no./dish ($\times 10^{-6}$)	putrescine (fmol/cell)	spermidine (fmol/cell)	spermine (fmol/cell)
none	3	13.5 ± 1.5	0.39 ± 0.07	2.90 ± 0.34	1.10 ± 0.19
DFMO	7	1.8 ± 0.1	<0.03	0.05 ± 0.02	0.79 ± 0.11
DFMO plus AdoDATO	7	2.1 ± 0.3	<0.03	0.03 ± 0.01	0.25 ± 0.04
DFMO plus AdoDATO plus putrescine	3	10.7	0.91	0.42	2.43
DFMO plus putrescine	3	13.4	0.31	3.02	1.11

[a] The dishes were seeded with 400 000 cells and incubated for the period shown in the presence of 5 mM DFMO, 50 μM AdoDATO, and 25 μM putrescine as shown. Results are shown as mean ±SD for five or six estimations or as the mean for three estimations.

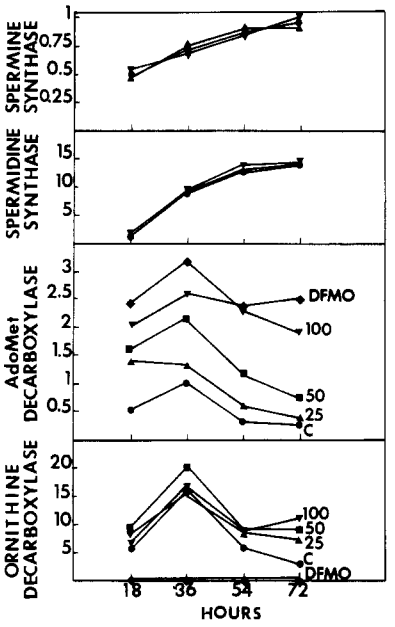

FIGURE 2: Effect of AdoDATO and DFMO on polyamine biosynthetic enzymes in SV-3T3 cells. The cells were seeded and cultured as in Table I with the addition of 5 mM DFMO (♦), 100 (▼), 50 (■), or 25 (▲) μM AdoDATO, or no addition (●). At the times shown, the cells were harvested, and the activities or ornithine decarboxylase, AdoMet decarboxylase, spermidine synthase, and spermine synthase were measured, by using extracts freed from the inhibitors by dialysis. The enzyme activities are given as nanomoles of product produced per milligram of protein per 30 min. The results for the synthases were so close together that only selected points are shown.

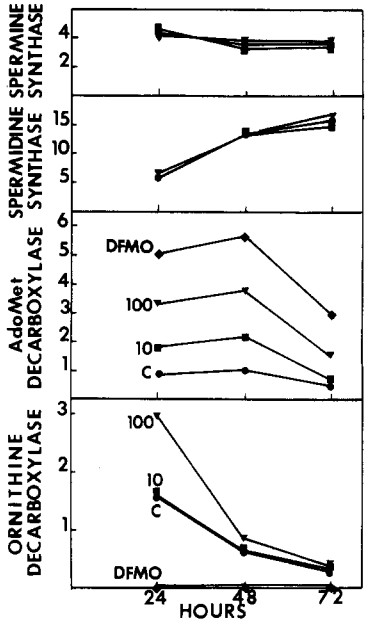

FIGURE 3: Effect of AdoDATO and DFMO on polyamine biosynthetic enzymes in HTC cells. The cells were seeded and grown in suspension culture as described in Figure 2 with no addition (●), 5 mM DFMO (♦), 10 μM AdoDATO (■), or 100 μM AdoDATO (▼). At the times shown, aliquots were taken for the assay of ornithine decarboxylase, AdoMet decarboxylase, spermidine synthase, and spermine synthase as in Figure 2. No significant effect on the synthase activities was seen, and only selected points where they were not directly overlapping are shown.

The most striking and significant change in enzyme activities produced by exposure to AdoDATO was in AdoMet decarboxylase. This enzyme was increased by 5–8-fold in both cell types exposed to 100 μM AdoDATO (Figures 2 and 3). Lower concentrations of AdoDATO produced smaller but still significant increases in activity. The increase produced by AdoDATO was slightly smaller than that produced by exposure to DFMO in HTC cells (Figure 3) and the same as that produced by DFMO in SV-3T3 fibroblasts (Figure 2).

It has not yet been possible to completely deplete mammalian cells of polyamines by exposure to inhibitors. As indicated in Table III, although DFMO produced decreases of more than 95% in putrescine and spermidine, there was only a small decrease of 30% in spermine. Similar results have been obtained by others in a variety of cells in that spermine levels were affected only slightly or even increased in response to DFMO (Mamont et al., 1978a,b, 1981a; Seidenfeld & Marton, 1979; Alhonen-Hongisto, 1980; Heby & Jänne, 1981; Hölttä et al., 1981; Pegg et al., 1981). We, therefore, tested whether the combination of AdoDATO and DFMO would produce a more significant depletion in spermine. As shown in Table IV, the addition of 50 μM AdoDATO in the presence of 5 mM DFMO did lead to a greater decline in spermine than that with DFMO alone. After growth in 5 mM DFMO for 7 days, the spermine content was 72% of that in control fibroblasts grown for 3 days. (The controls could not be grown for 7 days since confluence is reached in 4 days.) When AdoDATO was added as well as DFMO, the spermine content was only 23% of that in controls. The results shown in Table IV are for the addition of 50 μM AdoDATO, but no difference in polyamine content was produced when AdoDATO was added over the range of 10–200 μM in the presence of 5 mM DFMO (results not shown). When 25 μM putrescine was added to cells cultured in the presence of DFMO, a normal growth rate and polyamine content were restored (Table IV). However, when 25 μM putrescine was added to cells exposed to 50 μM AdoDATO and 5 mM DFMO, the polyamine content was very similar to that seen in cells exposed to 50 μM AdoDATO alone (compare Tables IV and I) with spermidine being only 15% of the normal level and putrescine and spermine increased by 2.3- and 2.2-fold, respectively.

In order to test the viability of cells exposed to both DFMO and AdoDATO and, therefore, maintained in a highly polyamine-depleted state, we grew cells for 7 days in the presence of these drugs and then added spermidine and measured the growth over the next 3 days. The results were compared with the increase in cell number in control dishes seeded with ap-

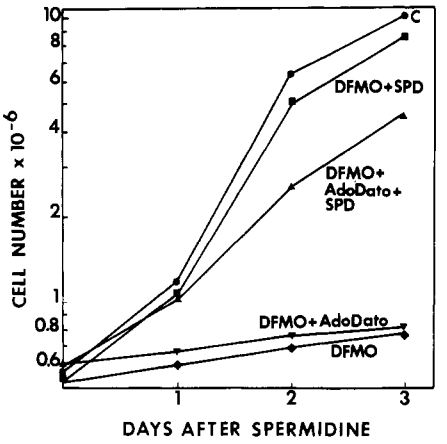

FIGURE 4: Effect of spermidine on growth of SV-3T3 cells exposed to DFMO and AdoDATO. The cells were seeded at a density of 160000/dish and grown for 7 days in the presence of 5 mM DFMO or 5 mM DFMO plus 50 μM AdoDATO. At this time, the medium was changed to either fresh medium containing 1 mM aminoguanidine, with either the drugs as before or 10 μM spermidine plus the drugs as shown. The control cells were also grown in the medium containing 1 mM aminoguanidine and 10 μM spermidine (●). Results are shown as the mean of three estimations that agreed ±15% for cells in 5 mM DFMO (♦), in 5 mM DFMO plus 10 μM spermidine (■), in 5 mM DFMO plus 50 μM AdoDATO (▼), and in 5 mM DFMO plus 50 μM AdoDATO plus 10 μM spermidine (▲).

proximately the same number of cells as were present after 7 days in the presence of the inhibitors. When cells that had been exposed to DFMO for 7 days were given spermidine, the rate of growth was increased to a value identical with that of the control. In the cells exposed to both AdoDATO and DFMO for 7 days, spermidine substantially increased the growth rate but could not restore the increase in cell number to control values (Figure 4).

Discussion

Most attempts to synthesize inhibitors of polyamine production have concentrated on ornithine and AdoMet decarboxylases, which are the earliest and rate-limiting steps in the pathway (Jänne et al., 1978; Pegg & Williams-Ashman, 1981; Williams-Ashman & Pegg, 1981). However, these enzymes turn over rapidly and are subject to complex regulatory influences that tend to overcome the effects of the inhibitors and permit rapid resumption of normal enzyme activities when the inhibitor concentration falls. The aminopropyl transferases have much longer half-lives and, although usually present in excess, are limited by the very low concentrations of decarboxylated AdoMet (Hibasami et al., 1980b; Pegg et al., 1982). Therefore, inhibitors of these enzymes, particularly those competitive with the nucleoside substrate, might prove to be effective antagonists of polyamine accumulation in vivo. In the present work, we provide further evidence that the approach of synthesizing inhibitors of these enzymes is likely to provide useful reagents for studies of the biochemistry of polyamines. The experiments with AdoDATO indicate that this compound can enter the cell and selectively suppress spermidine synthase activity, leading to a reduction in spermidine and increases in decarboxylated AdoMet, spermine, and putrescine. Significant changes in polyamine levels were produced by concentrations of AdoDATO that had little or no effect on the growth rate of the cells. Therefore, the changes were not secondary to changes in growth rate.

Exposure to AdoDATO brought about an increase in cell spermine content. This increase is likely to be due to the increased availability of decarboxylated AdoMet that can be used for maximal activity of spermine synthase whereas spermidine synthase is inhibited by the presence of AdoDATO. Therefore, the spermidine that can be made in the presence of the inhibitor is extensively converted into spermine. These results confirm the in vitro finding that AdoDATO is highly specific, inhibiting spermidine synthase (Tang et al., 1980; Pegg & Coward, 1981).

The combination of DFMO and AdoDATO brought about an additional reduction in spermine over that seen with DFMO alone. DFMO is a potent inhibitor of ornithine decarboxylase in vivo but does not deplete cells of spermine (Mamont et al., 1978b, 1981a; Heby and Jänne, 1981; Hölttä et al., 1981; Pegg et al., 1981). At least two factors contribute to the lack of reduction in cellular spermine by DFMO. First, exposure to DFMO greatly reduces the rate of cell growth, and therefore when expressed on a per cell basis, the content of spermine declines slowly because the cell number increases slowly. Secondly, a small extent of ornithine decarboxylation leading to putrescine continues even in the presence of DFMO, and this putrescine is rapidly converted into spermine (Mamont et al., 1978b; Pegg et al., 1981). AdoDATO brings about a reduction in spermine levels in the presence of DFMO by interference with this production of spermine. The cell numbers were not significantly different when exposures to DFMO or DFMO plus AdoDATO are compared (Table IV) so the effect on spermine could not be due to an increased dilution because of increased cell growth. AdoDATO is a more effective inhibitor of spermidine synthase at low putrescine concentrations (Pegg & Coward, 1981), and it is probable that it reduces the conversion of putrescine into spermidine very effectively in the DFMO-treated cells, thus preventing spermine production. However, there was no increase in putrescine corresponding to the decline in spermine of about 0.54 fmol/cell when AdoDATO was added in the presence of DFMO (Table IV). It appears that another pathway for metabolism of putrescine must, therefore, exist in these cells. Putrescine is known to be oxidized by diamine oxidase and is converted into γ-aminobutyric acid in some tissues (Seiler, 1980; Diekema et al., 1982) and to 5-hydroxy-2-pyrrolidone in rat liver (Lundgren & Fales, 1980).

Degradation of putrescine may also be responsible for the rather puzzling decrease in putrescine accumulation seen when cells were exposed to levels of AdoDATO above 25 μM. This decrease is seen over a dose range in which spermidine levels continue to fall, which is consistent with increasing inhibition of spermidine synthase by the drug (Table I), and therefore, increased levels of putrescine might be expected. It is well-known that ornithine decarboxylase activities are reduced in cells exposed to exogenous diamines or polyamines (Jänne et al., 1978; Bethell & Pegg, 1979; Canellakis et al., 1979; Heby & Jänne, 1981). AdoDATO is itself a diamine, and either AdoDATO or the increased cellular content of putrescine, spermine, and decarboxylated AdoMet could repress ornithine decarboxylase and bring about the observed changes. However, as shown in Figures 2 and 3, exposure to AdoDATO increased rather than decreased ornithine decarboxylase activity so this explanation does not appear to be correct.

Exposure to reversible inhibitors of ornithine decarboxylase (Harik et al., 1974; McCann et al., 1977) or AdoMet decarboxylase (Fillingame & Morris, 1973; Pegg et al., 1973; Pegg, 1979) brings about a paradoxical increase in the amount of the enzyme inhibited, which reduces the effectiveness of these inhibitors. This increase is due to the stabilization of these proteins, which prolongs their usually short half-lives. As shown in Figures 2 and 3, the activity of spermine synthase [which does not turn over rapidly (Jänne et al., 1978; Wil-

liams-Ashman et al., 1981)] was not increased by exposure to AdoDATO, and this inhibitor would not suffer from this disadvantage. These figures also show that AdoDATO did not bring about an increase in spermine synthase activity, confirming that the rise in spermine is, as discussed above, due to the increased availability of decarboxylated AdoMet.

The striking accumulation of decarboxylated AdoMet brought about by exposure to AdoDATO may be due partly to the reduced use of decarboxylated AdoMet by spermidine synthase. However, the results of Table I show that the total of spermidine plus spermine is not greatly decreased by AdoDATO. Hence, the total use of decarboxylated AdoMet is not reduced and may even be increased since spermine levels (which account for two molecules of this nucleoside) increase in the presence of AdoDATO. Therefore, the major factor influencing the accumulation of decarboxylated AdoMet is the 8-fold increase in AdoMet decarboxylase activity in response to AdoDATO (Figures 2 and 3). This increase was detected in in vitro assays in which saturating amounts of putrescine, which activates the reaction (Pegg, 1979; Pegg et al., 1981), were added. The content of putrescine in vivo in the AdoDATO treated cells would probably be sufficient to activate the enzyme to a maximal extent. The increase in AdoMet decarboxylase produced by AdoDATO is similar to that brought about by DFMO and is due to the decrease in intracellular spermidine that normally represses AdoMet decarboxylase activity (Mamont et al., 1981b; Pösö & Pegg, 1981).

It should be noted that there is a disagreement in the literature concerning the effect of polyamines on AdoMet decarboxylase. Mamont et al. (1981b) and others (Hopkins & Manchester, 1980; Pösö & Pegg, 1981) have suggested that this enzyme is regulated negatively by spermidine while Alhonen-Hongisto (1980) claimed that it was total of spermidine plus spermine in the cell that regulated the enzyme. Our results provide strong support for the hypothesis that spermidine alone is the important factor since the activity of AdoMet decarboxylase is increased in cells in which the decline in spermidine is balanced by an increase in spermine.

Our results are consistent with the idea that mammalian cells require certain levels of polyamines for optimal growth but can survive and grow with substantial perturbations of these levels. The results show that concentrations of AdoDATO producing 80–90% depletion of spermidine and 2–3-fold increases in spermine had only small effects on growth rate. Higher concentrations of AdoDATO did affect cell growth in a manner that was reversible by spermidine but not putrescine, suggesting that a certain level of spermidine (or spermidine/spermine ratio) is needed for normal growth. The results on cell growth shown in Tables I–III of this paper were obtained with cells that were plated in the presence of the inhibitor. It is, therefore, conceivable that the drug affected both the plating efficiency and the growth rate and AdoDATO was somewhat more inhibitory toward growth of the anchorage-dependent fibroblasts than the HTC cells that were grown in suspension culture. However, the effects of AdoDATO added 6 h after subculture were not significantly different with regard to cell number or polyamine levels than when the drug was added at the time of seeding (unpublished experiments). The experiment shown in Figure 4 indicates that SV-3T3 fibroblasts can survive quite well with very depleted polyamine levels. However, the failure of spermidine to completely restore the growth rate in the cells exposed to AdoDATO and DFMO in the experiment of Figure 4 may indicate that such depletion can lead to irreversible damage. It is unlikely that this failure indicates that these inhibitors affect pathways other than polyamine metabolism since the growth rate was exactly that of controls when spermidine was added at the same time as the inhibitors.

The long-term metabolic stability of AdoDATO remains to be established, but the present experiments suggest that compounds of this type (including the methylsulfonium salts) do penetrate into the cell and can influence polyamine metabolism there. Other compounds related to AdoMet have also been shown to influence polyamine metabolism in the cell although these effects were not reversed by added polyamines (Wang et al., 1980; Pankaskie et al., 1981; Pegg et al., 1981). The use of AdoDATO and derivatives either alone or in combination with inhibitors of ornithine and AdoMet decarboxylases may provide useful information on the roles of polyamines and the regulation of polyamine levels and could have useful chemotherapeutic potential.

Acknowledgments

We thank K. Shuttleworth for expert technical assistance and P. Gering for manuscript preparation.

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Largomycin: Preparation, Properties, and Structure†

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ABSTRACT: Largomycin has been purified to homogeneity by chromatography on hydroxylapatite whereby carbohydrate and protease impurities were removed. Largomycin is an acidic protein (p*I* 4.13, molecular weight 29 300) which forms a dimer in phosphate buffer. An N-terminal amino acid sequence analysis from the amino-terminal residue gave, for the first 32 residues, Asp-Ile-Leu-Ile-Ala-Gly-Ala-Thr-Gly-Asn-Val-Gly-Lys-Pro-Leu-Val-Glu-Gly-Leu-Leu-Ala-Ala-Gly-Lys-Pro-Val-Arg-Ala-Leu-Thr-Arg-Asn... The sequence from the carboxyl terminus was -Ala-Ala-Leu-Phe-OH with threonine, valine, and glutamic acid being released upon prolonged digestion. The same amino acid sequences were found for largomycin prepared from either the culture broth or the mycelium of *Streptomyces pluricolorescens*. The similarities extended to the other physical properties, the antimicrobial activity against *Staphylococcus aureus* and *Sarcina lutea*, and the antitumor activity against KB cells. Largomycin inhibits the biosynthesis of DNA and RNA. An iodinated derivative did not bind to KB cells. The antimicrobial activity was lost following ultraviolet irradiation, protection against which was not afforded by *p*-aminobenzoic acid.

One of more than fifty proteins reported to date with antitumor activity (Montgomery et al., 1981), largomycin is a fermentation product with antibiotic activity and antitumor activity against several tumors, including KB, P388, HeLa, Ehrlich ascites, and Sarcoma 180 (Yamaguchi et al., 1970a,b). Of three fractions isolated from the culture medium, largomycin II was purified further to a yellow, amorphous powder, with the properties of a chromoprotein. The protein was reported to contain carbohydrate and be similar to other an-

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0006-2960/82/0421-5089$01.25/0