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GROWTH OF MAMMALIAN CELLS IN THE ABSENCE OF THE ACCUMULATION OF SPERMINE

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SUMMARY. Mammalian spermine synthase activity was strongly inhibited by S-methyl-5'-methylthioadenosine. This nucleoside was readily taken up by SV-3T3 cells and blocked the synthesis of spermine by these cells. However, there was a corresponding increase in spermidine content and there was no effect on cell growth. These results indicate that S-methyl-5'-methylthioadenosine should be a useful compound to evaluate the role of spermine in mammalian cell physiology and that in at least one cell line the synthesis of spermine is not required for a normal growth rate.

The polyamines, spermidine and spermine, are synthesized in eukaryotes by the following reactions (1,2). Putrescine is formed by ornithine decarboxylase and is converted into spermidine by spermidine synthase which uses decarboxylated S-adenosylmethionine (AdoMet) as an aminopropyl donor. Spermidine is converted into spermine by the addition of a second aminopropyl group in a reaction catalyzed by spermine synthase. Despite the close similarities in the spermidine and spermine synthase reactions these steps are catalyzed by completely distinct enzymes which have been purified and shown not to have overlapping specificities (3-6). There is convincing evidence that polyamines play an essential although still unclear role in the growth of mammalian cells. When polyamine synthesis was blocked by the application of inhibitors of ornithine decarboxylase the rate of growth was greatly retarded as soon as the cellular putrescine and spermidine content was de-

Abbreviations Used: AdoMet, S-adenosylmethionine; AdoDATO, S-adenosyl-1,8-diamino-3-thiooctane; AdoS $(CH_3)_2$, S-methyl-5'-methylthioadenosine.

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pleted (2,7-10). In most of these experiments the cellular content of spermine was decreased only slightly if at all but cell growth could be restored if either exogenous spermidine or spermine was provided. These results suggest that cells require a certain minimal level of polyamines for growth and that this can be either a mixture of spermidine and spermine or predominantly spermine. A similar conclusion was reached on the basis of experiments with AdoDATO which is a specific inhibitor of spermidine synthase (11-13). AdoDATO leads to a reduction in spermidine but a compensatory increase in spermine (12).

It is not possible in experiments with either ornithine decarboxylase inhibitors or spermidine synthase inhibitors to determine whether spermidine alone can satisfy the cellular requirement for polyamine since spermine is not depleted in these studies. No selective inhibitors of spermine synthase have yet been reported. The availability of a potent spermine synthase inhibitor would allow the importance of spermine to be investigated more fully. In the present work we have studied the possibility that $AdoS^+(CH_3)_2$ could be used for this purpose.

MATERIALS AND METHODS

Materials. $AdoS^+(CH_3)_2$ was synthesized by methylation of 5'-methylthioadenosine with methyl iodide (11,13). [^{3}H-methyl]-Decarboxylated AdoMet was synthesized using bacterial AdoMet decarboxylase (14). All biochemical reagents were obtained from the Sigma Chemical Co., St. Louis, MO. Full details of the cell culture materials are given in reference 10.

Assays of aminopropyltransferases. Spermidine synthase was isolated from rat prostate (4,5) and spermine synthase from rat brain (3). The enzymes were assayed by following the production of [^{3}H]-methylthioadenosine from [^{3}H-methyl]-decarboxylated AdoMet in the presence of spermidine [for spermine synthase] or putrescine [for spermidine synthase] (14). The assay medium contained 1 nmol [^{3}H-methyl]-decarboxylated AdoMet (about 70,000 cpm), 100 nmol spermidine or putrescine, 10 µmol Na phosphate buffer, pH 7.2, 0.2 µmol 2-mercaptoethanol, the concentration of $AdoS^+(CH_3)_2$ shown and the enzyme in a total volume of 0.2 ml. After incubation for 30 min at 37°C, the reaction was halted by the addition of 1 ml of 25 mM HCl and the [^{3}H]-methylthioadenosine produced was isolated using small columns of cellulose phosphate (15) and counted. The results were expressed as the percentage of the activity found when no $AdoS^+(CH_3)_2$ was added. The amount of enzyme used was such that this value corresponded to about 7,000 cpm.

Cell culture and polyamine analysis. SV-3T3 cells were seeded with 400,000 cells and grown in Dulbecco's modified Eagle's medium with 3% horse serum/2% fetal calf serum for 2.5 days in the presence of various concentrations of $AdoS^+(CH_3)_2$ as indicated in the Figures and Tables. The cells were then harvested, extracted with 10% (w/v) trichloroacetic acid and aliquots

used for the determination of polyamine content using an amino acid analyzer with fluorescence detection (12). The cell number and protein content was determined as previously described (10).

Determination of the intracellular content of $AdoS^+(CH_3)_2$. Cell extracts were prepared as for polyamine analysis and the content of AdoMet and of $AdoS^+(CH_3)_2$ were determined using an ion-pair reversed-phase separation essentially as described by Seiler (16). The aliquots (up to 0.25 ml) were mixed with 0.45 ml of buffer A (0.1 M Na acetate, 0.01 M Na octane sulfonate, pH 4.5), centrifuged and applied to a column (Beckman Ultrasphere ODS 5 micron; 4.6 mm x 25 cm protected by a 4.6 mm x 4 cm guard column of ODS-5S from BioRad) equilibrated with a mixture of 90% buffer A and 10% buffer B (10 parts 0.2 M Na acetate, 0.01 M Na octane sulfonate, pH 4.5; 3 parts acetonitrile). The column was then eluted with a linear gradient of 90% buffer A/10% buffer B to 100% buffer B over 40 minutes at a flow rate of 1 ml/min at 35°C. The eluate was monitored at 254 nm and the amounts of AdoMet and of $AdoS^+(CH_3)_2$ were calculated from the peak heights using standard curves constructed with known amounts of the authentic compounds. The results were expressed as nmol of the nucleoside present per mg of total protein in the cell extracts. The column was washed with buffer B for a further 10 min and returned to 90% buffer A/10% buffer B over 5 min and re-equilibrated with this buffer for a further 10 min before the next sample.

RESULTS

The effect of $AdoS^+(CH_3)_2$ on isolated preparations of spermidine synthase and spermine synthase is shown in Figure 1. Spermidine synthase was not much affected by the compound showing only 36% inhibition in the presence of 1 mM $AdoS^+(CH_3)_2$. In contrast, spermine synthase was quite strongly inhibited with concentrations above 50 μM giving 80% or more inhibition. The approximate I_{50} was 8 μM. These assays were carried out using 5 μM decarboxylated AdoMet and 0.5 mM amine acceptor. These conditions approximate those existing in the cell although the decarboxylated AdoMet concentration used is slightly higher than that usually present in vivo (17-19). Although

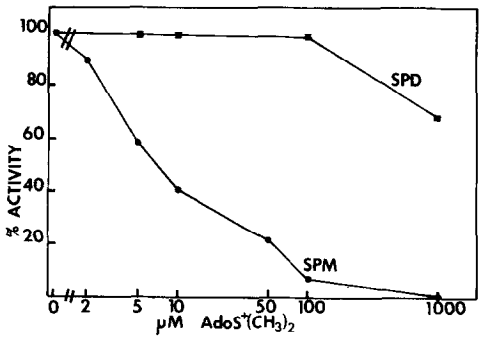

Figure 1. Inhibition of aminopropyltransferases by $AdoS^+(CH_3)_2$. Results are shown for spermidine synthase (SPD) and spermine synthase (SPM).

TABLE 1
INTRACELLULAR CONTENT OF $AdoS^+(CH_3)_2$ IN SV-3T3 CELLS EXPOSED TO THIS DRUG

Concentration of $AdoS^+(CH_3)_2$ in Medium (mM)	Intracellular content of $AdoS^+(CH_3)_2$ (nmol/mg protein)
None	0
0.05	0.26 ± .13
0.1	0.60 ± .20
0.2	0.98 ± .17
0.4	1.99 ± .19
0.7	2.9
1.0	6.5

The cells were grown in the presence of the concentration of $AdoS^+(CH_3)_2$ for 2.5 days.

a full kinetic analysis was not carried out, $AdoS^+(CH_3)_2$ was less active as an inhibitor of spermine synthase as the decarboxylated AdoMet concentration was increased (the I_{50} was about 40 μM with 20 μM decarboxylated AdoMet as a substrate). These results indicated that $AdoS^+(CH_3)_2$ might prove to be a useful inhibitor of spermine synthesis if it was taken up into mammalian cells.

This was tested by exposing SV-3T3 cells to various concentrations of $AdoS^+(CH_3)_2$ for periods of 2.5 days. The intracellular content of $AdoS^+(CH_3)_2$ was found to be approximately proportional to the amount present in the medium (Table 1). These values can be converted into approximate mM intracellular concentrations by dividing by 5.6 (assuming an approximate intracellular volume of 1.5 pl/cell and that 10^6 cells corresponds to 0.27 mg protein). This calculation indicates that the intracellular $AdoS^+(CH_3)_2$ had approximately equilibrated with the extracellular concentration. The cellular content of AdoMet was not changed by exposure to $AdoS^+(CH_3)_2$.

$AdoS^+(CH_3)_2$ had a striking inhibitory effect on the production of spermine by SV-3T3 cells (Figure 2, lower panel). Exposure to 0.05 mM $AdoS^+(CH_3)_2$ for 2.5 days reduced cellular spermine content by about 80% and with concentrations of 0.2 mM or above there was practically no increase at all in the total spermine present in the culture. The reduction of spermine brought about by the lower concentrations (0.2 mM or less) of $AdoS^+(CH_3)_2$ was accompanied by

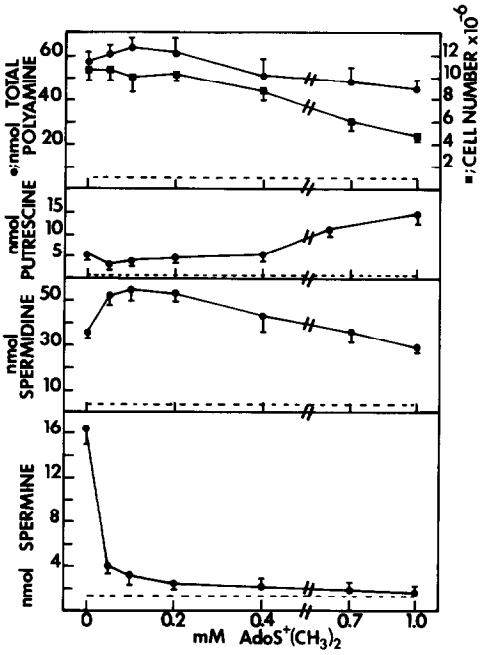

Figure 2. Effect of $AdoS^{+}(CH_3)_2$ on polyamine synthesis and growth of SV-3T3 cells. SV-3T3 cells were grown for 2.5 days in the presence of the concentration of $AdoS^{+}(CH_3)_2$ indicated. The results were expressed as the mean ± S.D. for at least 6 estimations of the total intracellular polyamine per culture (●—● , upper panel), the total spermine per culture (●—● , lower panel), the total spermidine per culture (●—● , second panel) and the total putrescine per culture (●—● , third panel). The upper panel also shows results (■—■) for the total cell number. In each panel the dotted line represents the starting value for the respective polyamine content of the cultures at the time of seeding.

an increase in spermidine and a decrease in putrescine (Figure 2, middle panels). This increase in spermidine is consistent with the block between spermidine and spermine and the fall in putrescine is probably due to the repression of ornithine decarboxylase by spermidine (20,21). There was no significant effect on the total polyamine (spermidine plus spermine) content of the cell cultures in the presence of concentrations of $AdoS^{+}(CH_3)_2$ below 0.2 mM and there was no inhibition of the cell growth rate as indicated by the increase in cell number (Figure 2, top panel).

When concentrations of above 0.4 mM $AdoS^{+}(CH_3)_2$ were used there was a significant reduction in spermidine and a rise in putrescine (Figure 2). These changes are consistent with the inhibition of AdoMet decarboxylase by the higher intracellular levels of $AdoS^{+}(CH_3)_2$. $AdoS^{+}(CH_3)_2$ has been reported

to inhibit this decarboxylase from rat prostate with a K_i of 6 μM (22) and from rat liver with a K_i of 2 μM (23). The fall in total polyamine (spermidine + spermine) at concentrations of $AdoS^+(CH_3)_2$ above 0.4 mM was accompanied by a reduction in the cellular growth rate.

DISCUSSION

Our results indicate clearly that $AdoS^+(CH_3)_2$ does enter mammalian cells relatively well even though the positively charged sulfonium salt might be expected to present a barrier to such uptake. The selective inhibition of spermine production in the presence of $AdoS^+(CH_3)_2$ should render it valuable in the investigation of the importance of spermine in mammalian cell physiology. Our results with the virally transformed mouse SV-3T3 cells suggest that spermine synthesis is not required for cell growth provided that a compensatory increase in spermidine occurs. These results raise the interesting question as to why mammalian cells contain spermine synthase if the production of spermine is not needed for growth. However, these results may not apply to all cell types. Furthermore, there is evidence that blocking polyamine synthesis with ornithine decarboxylase inhibitors influences differentiation (2,24-28). In some cases such inhibition induces differentiation and in other cases it is completely prevented. Application of $AdoS^+(CH_3)_2$ should enable experiments to be carried out to test whether spermine or spermidine is the critical polyamine for differentiation in these systems.

Inhibitors of polyamine biosynthesis appear to have a variety of promising therapeutic uses (29,30). Since the presently available compounds produce little if any depletion of spermine in many cells (3) it is possible that their effects might be enhanced if combined with a spermine synthase inhibitor such as $AdoS^+(CH_3)_2$. This compound is relatively easy to synthesize and our results indicate that it is taken up by cells. However, it has the disadvantage that its inhibitory effects towards spermine synthase may be much less pronounced in cells with elevated decarboxylated AdoMet content and such elevations occur in cells depleted of spermidine by exposure to ornithine decarboxylase inhibitors (17-20). It is possible that more

potent inhibitors of spermine synthase which do not have this drawback can be developed using the same principles as those used to construct mechanism-based inhibitors of spermidine synthase (11,12).

Inhibitors of all of the steps in the pathway for polyamine biosynthesis and interconversion are now available (2,7,13,30-32). Such inhibitors should provide additional insight into the function of polyamines and the regulation of their content in mammalian cells.

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