*Biochem. J.* (1980) **187**, 419–428
*Printed in Great Britain*



# Studies of Inhibition of Rat Spermidine Synthase and Spermine Synthase

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(*Received 17 September 1979*)

1. *S*-Adenosyl-L-methionine, *S*-adenosyl-L-homocysteine, 5′-methylthioadenosine and a number of analogues having changes in the base, sugar or amino acid portions of the molecule were tested as potential inhibitors of spermidine synthase and spermine synthase from rat ventral prostate. 2. *S*-Adenosyl-L-methionine was inhibitory to these reactions, as were other nucleosides containing a sulphonium centre. The most active of these were *S*-adenosyl-L-ethionine, *S*-adenosyl-4-methylthiobutyric acid, *S*-adenosyl-D-methionine and *S*-tubercidinylmethionine, which were all comparable in activity with *S*-adenosylmethionine itself, producing 70–98% inhibition at 1 mM concentrations. Spermine synthase was somewhat more sensitive than spermidine synthase. 3. 5′-Methylthioadenosine, 5′-ethylthioadenosine and 5′-methylthiotubercidin were all powerful inhibitors of both enzymes, giving 50% inhibition of spermine synthase at 10–15 $\mu$M and 50% inhibition of spermidine synthase at 30–45 $\mu$M. 4. *S*-Adenosyl-L-homocysteine was a weak inhibitor of spermine synthase and practically inactive against spermidine synthase. Analogues of *S*-adenosylhomocysteine lacking either the carboxy or the amino group of the amino acid portion were somewhat more active, as were derivatives in which the ribose ring had been opened by oxidation. The sulphoxide and sulphone derivatives of decarboxylated *S*-adenosyl-L-homocysteine and the sulphone of *S*-adenosyl-L-homocysteine were quite potent inhibitors and were particularly active against spermidine synthase (giving 50% inhibition at 380, 50 and 20 $\mu$M respectively). 5. These results are discussed in terms of the possible regulation of polyamine synthesis by endogenous nucleosides and the possible value of some of the inhibitory substances in experimental manipulations of polyamine concentrations. It is suggested that 5′-methylthiotubercidin and the sulphone of *S*-adenosylhomocysteine or of *S*-adenosyl-3-thiopropylamine may be particularly valuable in this respect.

Polyamine biosynthesis has been studied intensively in recent years because of many reports correlating the production of these amines with cell growth [reviewed by Tabor & Tabor (1976), Jänne *et al.* (1978) and Russell & Durie (1978)]. Considerable progress in elucidating the function of the polyamines has been made by utilizing inhibitors of their biosynthesis (Williams-Ashman *et al.*, 1976; Jänne *et al.*, 1978; Pegg, 1978; Mamont *et al.*, 1978; Seiler *et al.*, 1978; Williams-Ashman & Canellakis, 1979). All of the inhibitors presently in use act on either ornithine decarboxylase or *S*-adenosylmethionine decarboxylase. These decarboxylases have been regarded as particularly appropriate targets for the design of inhibitors because, when assayed *in vitro* under optimal conditions with saturating

The correct chemical name for *S*-adenosyl-L-homocysteine 2′,3′-acyclic derivative is 2′-{*O*-[(*R*)-hydroxymethyl(adenin-9-yl)methyl]}-3′-[*S*-(*R*)-homocysteinyl]-3′-deoxy-(*S*)-glycerol; *S*-adenosyl-L-homocysteine dialdehyde derivative is 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*(*R*)-homocysteinyl-3′-deoxy-(*S*)-glyceraldehyde; *S*-adenosyl-L-methionine dialdehyde derivative is 2′-*O*-[(*R*)-formyl(adenin-9-yl)methyl]-3′-*S*(*R*)-methioninyl-3′-deoxy-(*S*)-glyceraldehyde; the full structures of Sinefungin {5-[5′-deoxy-5′-(*C*)-adenosyl]-L-ornithine} and compound A9154C {5-[5′-deoxy-5′-(*C*)-4′,5′-didehydro-adenosyl]-L-ornithine} are given in Pugh *et al.* (1978).

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concentrations of substrates, their activity in tissue extracts is much less than that of the corresponding aminopropyltransferases responsible for spermidine and spermine synthesis (Jänne *et al.*, 1978). However, the fast turnover of the decarboxylases permits rapid increases in their concentration that can overcome even irreversible inhibitors (Mamont *et al.*, 1978; Seiler *et al.*, 1978; Pegg, 1978, 1979), and all of the inhibitors presently available have disadvantages. In particular, they are not very effective at producing substantial depletion of intracellular spermine. We have pointed out that the limiting factor in spermidine and spermine synthesis is the supply of decarboxylated *S*-adenosylmethionine (Pegg & Hibasami, 1979). The concentration of this substrate for the aminopropyltransferases is very low in the cell and these enzymes turn over relatively slowly. Therefore they may well prove to be important sites at which polyamine biosynthesis can be inhibited. Furthermore, the possibility that polyamine production in the cell might be subject to regulation by endogenous sulphur-containing nucleosides has not been considered in any detail. For these reasons, the present studies were undertaken to evaluate the inhibitory potency of *S*-adenosylmethionine, *S*-adenosylhomocysteine, 5′-methylthioadenosine and synthetic analogues of these nucleosides against rat spermidine synthase and spermine synthase. A third rationale for these studies was that several of these analogues have been shown to be potent inhibitors of various methyltransferase reactions. It is of obvious importance to determine to what extent these inhibitors interfere with polyamine synthesis, another important cellular reaction utilizing *S*-adenosylmethionine.

## Materials and Methods

### *Chemicals*

*S*-Adenosyl-L-methionine, 5′-methylthioadenosine, *S*-adenosyl-L-homocysteine and *S*-adenosyl-L-ethionine were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., and purified as described by Pegg & Williams-Ashman (1969*a*,*b*). *S*-Adenosyl-L-[*Me*-$^{14}$C]methionine (50–60mCi/mmol), [1,4-$^{14}$C]putrescine (52mCi/mmol) and [*tetramethylene*-1,4-$^{14}$C]spermidine (62mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. Decarboxylated *S*-adenosylmethionine, both unlabelled and labelled in the methyl group, was prepared by the action of *S*-adenosylmethionine decarboxylase from *Escherichia coli* and purified by chromatography on Dowex-50 ($H^+$ form) and high-voltage paper electrophoresis (Pösö *et al.*, 1976). All other biochemicals were products of the Sigma Chemical Co.

### *Preparation of inhibitors*

Except for the compounds described below, synthesis of analogues of *S*-adenosylhomocysteine have been reported earlier (Borchardt & Wu, 1974, 1975, 1976; Borchardt *et al.*, 1974, 1976*a*, 1978*a*,*b*). Analogues of *S*-adenosylmethionine were prepared as described by Borchardt *et al.* (1976*b*). 5′-Methylthiotubercidin was synthesized by the procedure of Coward *et al.* (1977). *S*-Adenosyl-3-thiopropylamine sulphoxide was prepared by oxidation of *S*-adenosyl-3-thiopropylamine with 30% (v/v) $H_2O_2$ by using a procedure similar to that for the preparation of *S*-adenosyl-L-homocysteine sulphoxide (Borchardt & Wu, 1974). *S*-Adenosyl-3-thiopropylamine sulphone was prepared by oxidation of *S*-adenosyl-3-thiopropylamine with ammonium molybdate and perchloric acid by using a procedure similar to that for the preparation of *S*-adenosyl-L-homocysteine sulfone (Borchardt & Wu, 1974).

### *Preparation of enzymes*

Ventral prostate glands were removed from large male Sprague–Dawley-strain rats (300–500g body wt.) purchased from Charles River Breeding Laboratories, Wilmington, MA, U.S.A. Extracts containing both aminopropyltransferase activities were prepared and fractionated into separate preparations of spermidine synthase and spermine synthase by treatment with $(NH_4)_2SO_4$ followed by DEAE-cellulose and Sephadex G-200 chromatography (Hannonen *et al.*, 1972; Pajula *et al.*, 1978). The enzyme preparations used in the present study represented purifications of about 110-fold (spermidine synthase) and 80-fold (spermine synthase) over the specific activity present in crude ultracentrifugal prostate extracts. The spermidine synthase preparation catalysed the formation of 55 nmol of spermidine/min per mg of protein and the spermine synthase preparation produced 6.2 nmol of spermine/min per mg of protein. These preparations were not homogeneous, but the synthases were free from each other and neither preparation contained significant amounts of *S*-adenosylmethionine decarboxylase [less than 1 pmol of $^{14}CO_2$ produced/min per mg of protein under assay conditions of Pegg (1979)] or of 5′-methylthioadenosine phosphorylase [(less than 100 pmol of methylthioribose 1-phosphate produced/min per mg of protein under assay conditions of Pegg & Williams-Ashman (1969*b*) with the method of Zappia *et al.* (1978)].

### *Assay of aminopropyltransferase activities*

Aminopropyltransferase activity was determined by measuring the production of 5′-[*Me*-$^{14}$C]methylthioadenosine from decarboxylated *S*-adenosyl[*Me*-$^{14}$C]methionine in the presence of putrescine (spermidine synthase) or of spermidine (spermine synthase) as described by Hibasami & Pegg (1978*a*). The assay medium contained 100mM-sodium phos-

phate buffer, pH 7.5, 42 μM decarboxylated *S*-adenosyl[*Me*-$^{14}$C]methionine (2 μCi/μmol), 5 mM-dithiothreitol, 0.5 mM-putrescine or 0.5 mM-spermidine, and the enzyme extract in a total volume of 0.2 ml. The results were expressed as percentages of the control (without addition of inhibitor) activity. Sufficient enzyme was added to achieve 5000–10000 c.p.m. incorporation into 5′-methylthioadenosine in 30 min incubation at 37°C.

In some experiments the labelled decarboxylated *S*-adenosylmethionine was replaced by unlabelled material and radioactive putrescine or spermidine was utilized to follow the reaction. The substrate and product were then separated by high-voltage paper electrophoresis (Hibasami & Pegg, 1978*a*).

### Results

Nucleosides were tested for their ability to inhibit spermidine synthase and spermine synthase by addition at concentrations of 0.1 and 1 mM to a standard assay medium containing 42 μM decarboxylated *S*-adenosylmethionine as the propylamine donor and 0.5 mM-putrescine or -spermidine as acceptor. Assays were incubated at 37°C for 30 min and the amount of 5′-methylthioadenosine produced was measured. Production of 5′-methylthioadenosine was entirely dependent on the addition of the putrescine acceptor when spermidine synthase was used and on the presence of the spermidine acceptor when spermine synthase was employed. The results are shown in Table 1.

Part 1 of Table 1 shows the results obtained when nucleosides containing a sulphonium centre were tested. *S*-Adenosyl-L-methionine itself was quite strongly inhibitory, giving about 90% inhibition of both enzymes at 1 mM concentrations. Spermine synthase was more sensitive than was spermidine synthase (Table 1 and Fig. 1). This inhibition was not due to the conversion of *S*-adenosyl-L-methionine into the decarboxylated derivative (which if formed could dilute the specific radioactivity of the labelled substrate) because the enzyme preparations used were free of *S*-adenosylmethionine decarboxylase activity. This was confirmed by addition to the assays of 10 μM-methylglyoxal bis(guanylhydrazone), which did not decrease the inhibition by *S*-adenosyl-L-methionine (Fig. 1). Methylglyoxal bis(guanylhydrazone) is a potent inhibitor of *S*-adenosylmethionine decarboxylase (Williams-Ashman & Schenone, 1972). Furthermore a similar inhibition of spermidine and spermine synthesis was observed when *S*-adenosyl-L-methionine was added to assay mixtures in which the reaction was measured by following the conversion of labelled putrescine or spermidine into the product (Fig. 1).

*S*-Adenosyl-D-methionine was also inhibitory to the aminopropyltransferases but less active than was

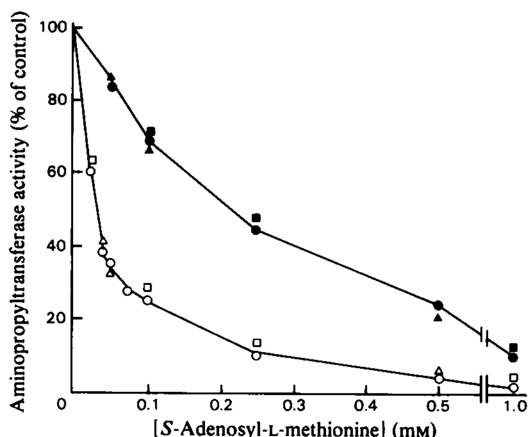


Fig. 1. *Inhibition of spermidine synthase and spermine synthase by S-adenosylmethionine*

The results are expressed as percentages of the control activity measured in the absence of *S*-adenosylmethionine. Experimental details are given in the text. Results are shown for spermidine synthase (black symbols) assayed by production of [$^{14}$C]methylthioadenosine in the presence (●) or absence (■) of methylglyoxal bis(guanylhydrazone) or by the incorporation of [$^{14}$C]putrescine into spermidine (▲). Values for spermine synthase activity (open symbols) are shown for assays of the production of [$^{14}$C]methylthioadenosine in the presence (○) or absence (□) of methylglyoxal bis(guanylhydrazone) or of the incorporation of [$^{14}$C]spermidine into spermine (△).

the L-isomer. As previously reported with less purified enzyme extracts (Hibasami & Pegg, 1978*b*), *S*-adenosyl-L-ethionine in which the methyl group attached to the sulphonium centre is replaced by an ethyl group was also an inhibitor. In the present experiments the inhibition by this compound was somewhat greater than that found before. This may be partly due to impurities present in the former preparation and partly to the more highly purified enzymes used in the present work. The replacement of methionine by *S*-methylcysteine, which has one carbon atom less, substantially diminished the inhibitory potency. However, *S*-adenosyl-4-methylthiobutyric acid, the deaminated derivative of the amino acid, was strongly inhibitory to both enzymes, showing that the amino group is not required for binding at the inhibitory site. The intact ribose moiety did appear to be needed, because oxidation of the *cis*-hydroxy groups with periodic acid, yielding *S*-adenosyl-L-methionine dialdehyde derivative, diminished inhibition.

A number of compounds related to *S*-adenosyl-L-methionine but having different bases were also

Table 1. *Inhibition of aminopropyltransferases by substrate and product analogues*

The compounds were tested by addition to the standard assay medium at the concentrations shown. Experimental details are given in the text. The percentage inhibition due to the addition is shown.

| Compound | Concn. (mM) | Inhibition (%) Spermidine synthase | Spermine synthase |
|---|---|---|---|
| Part 1. Analogues of *S*-adenosylmethionine | | | |
| *S*-Adenosyl-L-methionine | 1 | 88 | 95 |
| | 0.1 | 30 | 75 |
| *S*-Adenosyl-L-ethionine | 1 | 91 | 93 |
| | 0.1 | 36 | 51 |
| *S*-Adenosyl-S-methyl-L-cysteine | 1 | 49 | 74 |
| | 0.1 | 11 | 31 |
| *S*-Adenosyl-D-methionine | 1 | 74 | 91 |
| | 0.1 | 19 | 37 |
| *S*-Adenosyl-4-methylthiobutyric acid | 1 | 98 | 97 |
| | 0.1 | 85 | 84 |
| *S*-Adenosyl-L-methionine dialdehyde derivative | 1 | 57 | 47 |
| | 0.1 | 16 | 8 |
| *S*-3-Deaza-adenosyl-L-methionine | 1 | 48 | 61 |
| | 0.1 | 10 | 14 |
| *S*-$N^6$-Methyl-3-deaza-adenosyl-L-methionine | 1 | 31 | 1 |
| | 0.1 | 7 | 0 |
| *S*-7-Deaza-adenosyl-L-methionine (*S*-tubercidinylmethionine) | 1 | 70 | 83 |
| | 0.1 | 23 | 37 |
| *S*-Inosyl-L-methionine | 1 | 20 | 6 |
| | 0.1 | 5 | 0 |
| *S*-Uridyl-L-methionine | 1 | 5 | 2 |
| *S*-Cytidyl-L-methionine | 1 | 8 | 0 |
| Part 2. Analogues of 5′-methylthioadenosine | | | |
| 5′-Methylthioadenosine | 1 | 95 | 99 |
| | 0.1 | 82 | 98 |
| 5′-Ethylthioadenosine | 1 | 95 | 97 |
| | 0.1 | 82 | 90 |
| 5′-Methylthio-7-deaza-adenosine (5′-methylthiotubercidin) | 1 | 95 | 99 |
| | 0.1 | 64 | 91 |
| Part 3. Analogues of *S*-adenosyl-L-homocysteine | | | |
| *S*-Adenosyl-L-homocysteine | 1 | 7 | 47 |
| | 0.1 | 0 | 12 |
| *S*-Adenosyl-3-thiopropylamine (decarboxylated *S*-adenosylhomocysteine) | 1 | 55 | 65 |
| | 0.1 | 4 | 12 |
| *S*-Adenosyl-3-*N*-acetylthiopropylamine | 1 | 12 | 34 |
| | 0.1 | 0 | 8 |
| *S*-Adenosyl-4-thiobutyric acid | 1 | 41 | 72 |
| | 0.1 | 7 | 31 |
| *S*-Adenosyl-4-thiobutyrate methyl ester | 1 | 59 | 85 |
| | 0.1 | 17 | 52 |
| *S*-Adenosyl-L-cysteine | 1 | 21 | 62 |
| | 0.1 | 0 | 15 |
| *S*-Adenosyl-D-homocysteine | 1 | 0 | 5 |
| | 0.1 | 2 | 0 |
| Sinefungin | 1 | 4 | 10 |
| | 0.1 | 0 | 2 |
| Compound A9154C | 1 | 53 | 93 |
| | 0.1 | 22 | 70 |
| *S*-Adenosyl-L-homocysteine dialdehyde derivative | 1 | 31 | 40 |
| | 0.1 | 3 | 12 |
| *S*-Adenosyl-L-homocysteine 2′,3′-acyclic derivative | 1 | 41 | 58 |
| | 0.1 | 6 | 10 |
| *S*-Adenosyl-L-homocysteine sulphoxide | 1 | 11 | 24 |
| | 0.1 | 3 | 5 |
| *S*-Adenosyl-3-thiopropylamine sulphoxide (decarboxylated *S*-adenosylhomocysteine sulphoxide) | 1 | 79 | 61 |
| | 0.1 | 28 | 14 |
| *S*-Adenosyl-L-homocysteine sulphone | 1 | 99 | 84 |
| | 0.1 | 82 | 33 |
| *S*-Adenosyl-3-thiopropylamine sulphone (decarboxylated *S*-adenosyl-L-homocysteine sulphone) | 1 | 95 | 80 |
| | 0.1 | 63 | 25 |
| *S*-3-Deaza-adenosyl-L-homocysteine | 1 | 6 | 27 |
| *S*-$N^6$-Methyl-3-deaza-adenosyl-L-homocysteine | 1 | 2 | 7 |
| *S*-7-Deaza-adenosyl-L-homocysteine | 1 | 19 | 17 |
| *S*-8-Aza-adenosyl-L-homocysteine | 1 | 1 | 0 |
| *S*-2-Aza-adenosyl-L-homocysteine | 1 | 0 | 4 |
| *S*-$N^6$-Methyladenosyl-L-homocysteine | 1 | 6 | 13 |
| *S*-Guanyl-L-homocysteine | 1 | 2 | 9 |
| *S*-Inosyl-L-homocysteine | 1 | 4 | 4 |
| *S*-Uridyl-L-homocysteine | 1 | 2 | 4 |
| *S*-Cytosyl-L-homocysteine | 1 | 3 | 9 |

tested as inhibitors, but none was as active as the parent compound. Replacing the adenine by inosine, uracil or cytidine resulted in the complete loss of inhibitory activity, indicating the importance of the adenine base for binding to the enzyme. Analogues containing derivatives of adenine were inhibitory, the 3-deaza and 7-deaza derivatives showing activity approching that of *S*-adenosylmethionine itself. Addition of a methyl group at N-6 substantially diminished the inhibitory potency.

A product of both aminopropyltransferase reactions is 5′-methylthioadenosine. This product has previously been reported to be inhibitory to sper-

mine synthase (Hibasami & Pegg, 1978*b*; Pajula & Raina, 1979), and as shown in Table 1 it was a powerful inhibitor, producing virtually complete inhibition at 0.1 mM concentrations. This inhibition was greater than found in our earlier experiments in which a crude enzyme preparation was used (Hibasami & Pegg, 1978*b*). It is probable that, as suggested by Pajula & Raina (1979), this discrepancy was due to the presence of 5′-methylthioadenosine phosphorylase in the crude enzyme fractions, which could have lowered the concentrations of the added nucleoside. Spermidine synthase was also inhibited by 5′-methylthioadenosine, although not quite so potently as was spermine synthase. Previous studies did not show inhibition of spermidine synthesis by 5′-methylthioadenosine (Williams-Ashman *et al.*, 1972; Hibasami & Pegg, 1978*b*), but, as mentioned above, the enzyme preparations used contained 5′-methylthioadenosine phosphorylase. 5′-Ethylthioadenosine was practically as active as the methyl derivative in inhibiting both enzymes, and 5′-methylthiotubercidin, in which the adenine was replaced by 7-deaza-adenine, was only slightly less potent.

The remainder of Table 1 (part 3) details the effects on these reactions of *S*-adenosylhomocysteine and related compounds. Spermidine synthase was not significantly affected by *S*-adenosyl-L-homocysteine, but, in agreement with our previous report (Hibasami & Pegg, 1978*b*), spermine synthase was inhibited by 1 mM concentrations. Modification of the amino acid portion of the molecule by removal of either the carboxy or the amino group increased the inhibitory activity somewhat for both enzymes. Decarboxylated *S*-adenosylhomocysteine and the methyl ester of *S*-adenosyl-4-thiobutyric acid inhibited both enzymes by more than 50% when present at 1 mM concentrations. The removal of one carbon atom from the amino acid moiety (*S*-adenosyl-L-cysteine) did not significantly alter the inhibition of spermine synthase. The antifungal antibiotic Sinefungin, which is related to *S*-adenosylhomocysteine but has the sulphur atom replaced by $-CH(NH_2)-$ (see footnote on title page), was not inhibitory at the concentrations used, but the related compound A9145C was strongly inhibitory, particularly to spermine synthase. *S*-Adenosyl-D-homocysteine was inactive against either enzyme. Alterations to the ribose moiety of the molecule where the C-2′–C-3′ bond was cleaved, producing either the dialdehyde or the corresponding alcohol, did not affect the inhibition of spermine synthesis significantly, but these compounds were slightly more potent inhibitors of spermidine production than was *S*-adenosylhomocysteine itself.

The sulphoxide of *S*-adenosylhomocysteine was only slightly inhibitory to both enzymes, but the decarboxylated derivative was significantly more active. Surprisingly, the sulphone and its decarboxylated derivative were quite strongly inhibitory to both enzymes, but particularly active against spermidine synthesis.

Modifications to the base moiety of *S*-adenosylhomocysteine were deleterious to the inhibitory action towards spermine synthase. Only the 3-deaza- and 7-deaza-adenosine derivatives had any significant activity. Substitutions of methyl or ethyl groups at N-6 of adenosine, modifications forming 2-aza or 8-aza derivatives or replacement with other purines or pyrimidines led to the loss of all activity (Table 1).

Detailed kinetic investigations of the mode of action of the various compounds found to be strong inhibitors were not attempted because of the complex kinetics of the aminopropyltransferases. In confirmation of a previous report (Coward *et al.*, 1977), it was found that spermidine synthase was strongly inhibited at high substrate concentrations (Fig. 2). It was also found that the apparent $K_m$

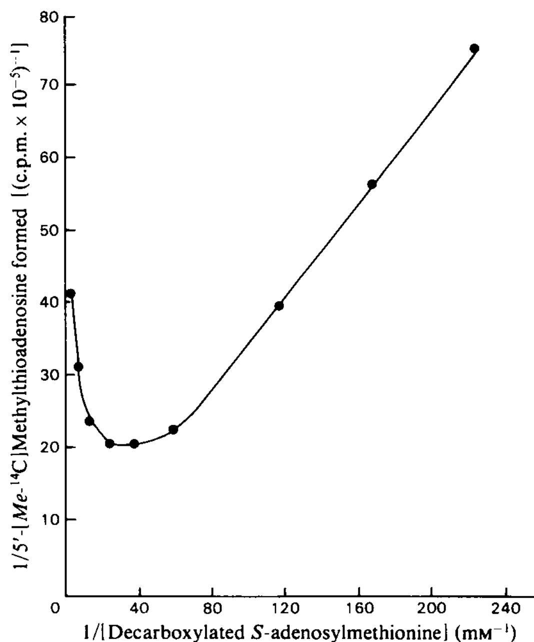


Fig. 2. *Effect of concentration of decarboxylated S-adenosylmethionine on the spermidine synthase reaction*

A double-reciprocal plot of the formation of 5′-[*Me*-$^{14}C$]methylthioadenosine at different concentrations of decarboxylated *S*-adenosylmethionine is shown. Assays were carried out by incubation for 15 min at 37°C in the presence of 0.5 mM-putrescine. Experimental details are given in the text. The formation of product was linearly related to the time of incubation for at least 30 min.

values for putrescine or spermine varied greatly according to the concentration of decarboxylated S-adenosylmethionine used as substrate (A. E. Pegg & H. Hibasami, unpublished work). In order to obtain some estimate of the relative potency of the compounds found to be inhibitory, the effect of inhibitor concentration was tested under the standard assay conditions. All the inhibitors showed clear dependence on concentration added. Results are shown in Fig. 1 for S-adenosyl-L-methionine, in Fig. 3 for S-adenosylhomocysteine sulphone and in Fig. 4 for 5′-methylthiotubercidin. S-Adenosyl-L-methionine and 5′-methylthioturbercidin were clearly much more inhibitory towards spermine synthase (producing 50% inhibition at about 30 and 15 μM respectively) than towards spermidine synthase (210 and 45 μM). S-Adenosyl-L-homocysteine sulphone (Fig. 3) was much more inhibitory to spermidine synthase, producing 50% inhibition at 20 μM, whereas spermine synthase required 210 μM.

Table 2 summarizes the inhibitory potential of those compounds showing activity in the experiments of Table 1. At least six concentrations over the range producing from 10 to 80% inhibition were tested and these curves were used to determine the concentration required for 50% inhibition, which is given in Table 2. The most potent inhibitors of spermine synthase were 5′-methylthioadenosine, 5′-ethylthioadenosine and 5′-methylthiotubercidin, which all produced 50% inhibition at 15 μM or less. Compound A9154C (50% inhibition at 35 μM), S-adenosyl-L-methionine (30 μM) and S-adenosyl-4-methylthiobutyric acid (40 μM) were the only other

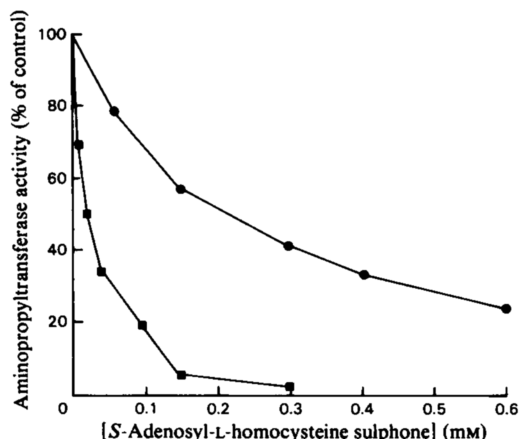


Fig. 3. *Inhibition of spermidine synthase and spermine synthase by S-adenosylhomocysteine sulphone*

The results are expressed as percentages of the control activity measured in the absence of the inhibitor. Experimental details are given in the text. Results are shown for spermidine synthase (■) and spermine synthase (●).

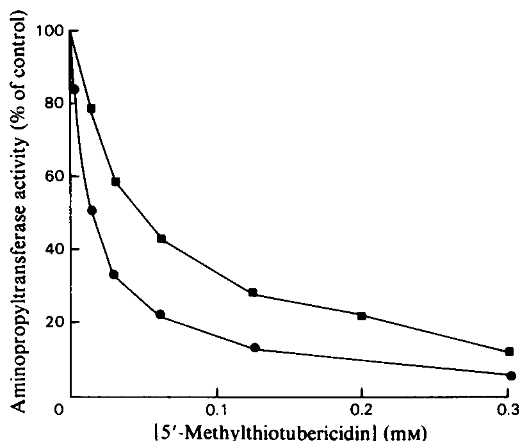


Fig. 4. *Inhibition of spermidine synthase and spermine synthase by 5′-methylthiotubercidin*

The results are expressed as percentages of the control activity measured in the absence of the inhibitor. Experimental details are given in the text. Results are shown for spermidine synthase (■) and spermine synthase (●).

Table 2. *Comparison of inhibitors of aminopropyltransferases*

Experimental details are given in the text. Only those compounds effective to inhibit by 25% at 0.1 mM were included.

| Compound | Concn. needed for 50% inhibition (μM) Spermidine synthase | Spermine synthase |
|---|---|---|
| S-Adenosyl-L-methionine | 210 | 30 |
| S-Adenosyl-L-ethionine | 185 | 95 |
| S-Tubercidinyl-L-methionine | 305 | 175 |
| S-Adenosyl-4-methylthio-butyric acid | 40 | 40 |
| 5′-Methylthioadenosine | 30 | 10 |
| 5′-Ethylthioadenosine | 30 | 15 |
| 5′-Methylthiotubercidin | 45 | 15 |
| S-Adenosyl-4-thiobutyric acid | <500 | 220 |
| S-Adenosyl-4-thiobutyrate methyl ester | 500 | 75 |
| Compound A9154C | 790 | 35 |
| S-Adenosyl-3-thiopropylamine sulphoxide | 380 | 715 |
| S-Adenosyl-L-homocysteine sulphone | 20 | 210 |
| S-Adenosyl-3-thiopropylamine sulphone | 50 | 310 |

compounds for which 50% inhibition was achieved at less than 100 $\mu$M. The only nucleosides that showed this degree of inhibition towards spermidine synthase were *S*-adenosyl-L-homocysteine sulphone (20 $\mu$M), *S*-adenosyl-3-thiopropylamine sulphone (50 $\mu$M), *S*-adenosyl-4-methylthiobutyric acid (40 $\mu$M), 5'-methylthioadenosine (30 $\mu$M), 5'-ethylthioadenosine (30 $\mu$M) and 5'-methylthiotubercidin (45 $\mu$M). The first two compounds were respectively 10 and 6 times more active against spermidine synthase than against spermine synthase. The greatest specificity towards spermine synthase was seen for compound A9154C, which was at least 20 times more active towards this enzyme than towards spermidine synthase. Other compounds that were selectively active against spermine synthesis were *S*-adenosyl-L-methionine, *S*-adenosyl-4-thiobutyrate methyl ester and 5'-methylthiotubercidin. These were 3–7 times more potent inhibitors of spermine rather than spermidine synthesis.

It was possible that some of the compounds tested in these studies were, in fact, substrates able to donate a propylamine group to the amine acceptor. This was a particularly important problem for the decarboxylated derivatives of *S*-adenosylhomocysteine, which already contain a propylamine group and therefore do not need prior decarboxylation. Such an effect would appear to be an inhibition, since the labelled substrate used in our standard assay was the usual propylamine donor, decarboxylated *S*-adenosylmethionine. All of the inhibitory compounds shown in Table 2 were therefore tested for their abilities to support the reaction with a different assay method in which conversion of labelled putrescine into spermidine or of labelled spermidine into spermine was measured. In the standard assay approx. 9000 c.p.m. was incorporated into the product, but when decarboxylated *S*-adenosylmethionine was replaced by the other compounds no significant radioactivity was found (Table 3). Neither *S*-adenosylmethionine nor *S*-adenosylethionine was active, emphasizing the absence of *S*-adenosylmethionine decarboxyalse from the preparations of enzymes used.

Inhibition of spermidine synthase by *S*-adenosyl-L-homocysteine sulphone and of spermine synthase by 5'-methylthiotubercidin was studied in more detail because of the potential usefulness of these compounds as inhibitors of polyamine production *in vitro*. Both compounds were reversible inhibitors. After dialysis overnight to remove them from the enzyme, activity was restored to that found in preparations treated similarly except for exposure to the inhibitor. Neither compound inhibited more strongly if preincubated with the enzyme in the absence of one or both substrates.

Table 3. *Abilities of various nucleosides to act as substrates for aminopropyltransferases*

Assays were carried out in the standard medium but with decarboxylated *S*-adenosylmethionine being replaced by 1 mM concentrations of the nucleoside shown and with $^{14}$C-labelled putrescine or spermidine being used to follow the reaction. Experimental details are given in the text. N.D., Not determined.

| Addition | Spermidine synthase activity (c.p.m./30 min incubation) | Spermine synthase activity (c.p.m./30 min incubation) |
|---|---|---|
| Decarboxylated *S*-adenosylmethionine | 9961 | 8555 |
| *S*-Adenosyl-L-methionine | 37 | 62 |
| *S*-Adenosyl-L-ethionine | 45 | N.D. |
| *S*-Adenosyl-L-homocysteine sulphone | 12 | 12 |
| *S*-Adenosyl-3-thiopropylamine sulphoxide | 15 | 18 |
| *S*-Adenosyl-3-thiopropylamine sulphone | 14 | 23 |

## Discussion

Mammalian aminopropyltransferases have not been studied extensively. However, the information that is available clearly indicates that, unlike *Escherichia coli*, which contains only one aminopropyltransferase preferring putrescine as acceptor but able to synthesize spermine under certain conditions (Bowman *et al.*, 1973), mammalian cells contain distinct spermidine synthase and spermine synthase (Pegg & Williams-Ashman, 1969*a*, 1970; Hannonen *et al.*, 1972; Hibasami & Pegg, 1978*a*,*b*; Pajula *et al.*, 1978). The present work confirms the existence of separate enzymes and emphasizes their different properties in that their responses to some of the inhibitors were quite different. This could have important physiological and pharmacological consequences, as discussed below.

The present results may also provide some information on the interaction between decarboxylated *S*-adenosylmethionine and the enzymes. The complete absence of inhibitory action by analogues of *S*-adenosylmethionine or *S*-adenosylhomocysteine containing bases other than adenine or its 3- and 7-deaza derivatives suggest that a purine with a 6-amino group is essential. The diminished inhibi-

tion by the $N^6$-methyl derivatives suggest that binding involves a free amino group at the 6-position. The results with the amino acid variants of *S*-adenosylmethionine suggest that the adenosyl moiety (preferably with an intact ribose ring) and a sulphonium centre are sufficient to permit inhibitory binding. The α-amino group of *S*-adenosyl-L-methionine was clearly not needed for inhibition, since *S*-adenosyl-4-methylthiobutyric acid was a potent inhibitor. However, since *S*-adenosyl-D-methionine and *S*-adenosyl-*S*-methyl-L-cysteine were less active than *S*-adenosyl-L-methionine, the presence of a side chain of appropriate length and stereospecificity is of some importance.

It should be pointed out that the synthetic sulphonium compounds used in this study (all those except *S*-adenosyl-L-methionine, *S*-adenosyl-L-ethionine and decarboxylated *S*-adenosylmethionine) are epimeric mixtures of the enantiomers at the sulphur atom. It is probable that the enzyme reactions use only one of these enantiomers of decarboxylated *S*-adenosylmethionine (Pösö *et al.*, 1976; Coward *et al.*, 1977), which is probably the *S*-configuration since Cornforth *et al.* (1977) have shown that naturally occurring *S*-adenosylmethionine exists in this form. Therefore it is quite possible that of those compounds existing as epimeric mixtures one enantiomer is much more active than the other, and care should be exercised in comparing results between two compounds one of which was a mixture and the other a single enantiomer.

Almost all of the compounds containing a sulphonium centre were more potent inhibitors of spermine synthesis than of spermidine production. This was also the case with the three 5′-alkylthio-5′-deoxynucleosides tested. Perhaps the most surprising finding in the present experiments is the inhibitory action of certain derivatives of *S*-adenosylhomocysteine, although we had previously observed some inhibition of crude preparation of spermine synthase by 1 mM concentrations of *S*-adenosylhomocysteine itself (Hibasami & Pegg, 1978*b*). The inhibitory potential of the sulphoxide derivative of decarboxylated *S*-adenosylhomocysteine might have been expected, since the polar sulphoxide could be thought of as analogous to the sulphonium centre. This compound therefore may resemble the normal decarboxylated *S*-adenosylmethionine substrate. However, the very strong inhibitory action of the sulphone of *S*-adenosylhomocysteine and its decarboxylated derivative remains to be explained. *S*-Adenosyl-L-homocysteine sulphone and its decarboxylated derivative and 5′-methylthiotubercidin may be of value in lowering cellular polyamine concentrations, particularly if given in conjunction with an ornithine decarboxylase inhibitor. The sulphone derivatives are chemically stable but inhibit some RNA methylases (Borchardt, 1977; Leboy *et al.*, 1978; Borchardt & Pugh, 1979). However, their uptake into cells and biological stability have not yet been fully evaluated. Although 5′-methylthioadenosine and 5′-ethylthioadenosine were strong inhibitors, the ubiquitous distribution and high activity of 5′-methylthioadenosine phosphorylase, which attacks both of these compounds (Pegg & Williams-Ashman, 1969*b*; Garbers, 1978; Zappia *et al.*, 1978), may limit their ability to inhibit polyamine production *in vivo*. However, 5′-methylthiotubercidin is not degraded by this enzyme but is a competitive inhibitor (Coward *et al.*, 1977; Zappia *et al.*, 1978). It could therefore inhibit polyamine synthesis both by a direct effect and indirectly by preventing the breakdown of 5′-methylthioadenosine. 5′-Methylthiotubercidin and 5′-methylthioadenosine were found to inhibit proliferation of human lymphocytes stimulated to proliferate by mitogens (Ferro, 1979), but it is not known whether the effect is mediated or accompanied by changes in polyamine concentrations. At present 5′-methylthiotubercidin appears the most likely compound to be of value in inhibiting spermine synthesis, but there are many other known analogues of 5′-methylthioadenosine that appear worthy of consideration in this respect (Montgomery *et al.*, 1974).

The results in Table 1 should also be considered when analogues of *S*-adenosylmethionine, *S*-adenosylhomocysteine and 5′-methylthioadenosine are used with the aim of inhibiting methyltransferase reactions. The present experiments suggest that a wide variety of such compounds also have the potential to interfere with polyamine synthesis. The compounds SIBA (5′-deoxy-5′-*S*-isobutylthioadenosine), Sinefungin and compound A9145C have evoked considerable interest because of their antiviral and oncostatic properties (Pugh *et al.*, 1978; Robert-Gero *et al.*, 1979; Borchardt & Pugh, 1979). These may be due to inhibition of methylation, particularly in the case of Sinefungin and compound A9145C, which are very strong inhibitors of viral mRNA (guanine-7) methyltransferase and mRNA (nucleoside-2′) methyltransferase (Pugh *et al.*, 1978; Borchardt & Pugh, 1979), but inhibition of polyamine synthesis may also occur with compound A9154C and with SIBA, which is closely related to 5′-methylthioadenosine.

Although it may appear at first sight that those compounds requiring 1 mM concentration to achieve significant inhibition are not sufficiently active to produce a substantial decrease in polyamine synthesis *in vivo*, it should be remembered that concentrations of decarboxylated *S*-adenosylmethionine *in vivo* are very low [about 1–2 nmol/g wet wt. (Pegg & Hibasami, 1979; H. Hibasami, J. L. Hoffman & A. E. Pegg, unpublished work)]. Therefore the concentration of decarboxylated *S*-

adenosylmethionine used in the enzyme assays described in the present paper may be 20–40 times higher than that present in the cell. The complex kinetics of the aminopropyltransferase reaction renders extrapolation of our results to other substrate concentrations difficult, but it is probable that inhibition by most of the compounds tested would be much greater at physiological substrate concentrations. Therefore it is possible that both *S*-adenosylmethionine and *S*-adenosylhomocysteine could influence the synthesis of spermidine and spermine under some conditions.

Finally, the very strong product inhibition by 5′-methylthioadenosine raises the question as to whether aminopropyltransferase activity might be influenced by this nucleoside. Although there are several metabolic routes to 5′-methylthioadenosine (Ferro, 1979), it is conceivable that the pathway thought of as the polyamine-biosynthetic pathway might have as its major function the production of this substance. 5′-Methylthioadenosine (Ferro, 1979) or its breakdown products have been suggested as important for cell growth (Toohey, 1977, 1978). There are at present no measurements of cellular 5′-methylthioadenosine content, but it is clearly much lower than that of polyamines (Rhodes & Williams-Ashman, 1964; Pegg & Williams-Ashman, 1969*b*). This is undoubtedly due to the activity of 5′-methylthioadenosine phosphorylase, a widespread enzyme (Pegg & Williams-Ashman, 1969*b*; Garbers, 1977; Toohey, 1978). Until the extent to which this activity lowers 5′-methylthioadenosine concentrations within the cell is known, the possible inhibition of polyamine synthesis cannot be evaluated. Also, it must be noted that, although 5′-methylthioadenosine was a potent inhibitor of rat prostate spermine synthase in our experiments, it was still less active than reported by Pajula & Raina (1979) for highly purified bovine brain spermine synthase. This could be a species difference, and it appears that another difference was that the rat prostate enzyme was much more strongly inhibited by *S*-adenosylmethionine. However, it is also possible that the greater degree of purification achieved by Pajula & Raina (1979) renders the enzyme more sensitive to 5′-methylthioadenosine.

This research was supported in part by grants GM26290, CA18138, CA18450, NS10198, MH18038 and CA10748 from the National Institutes of Health, U.S. Department of Health, Education and Welfare, and by Established Investigatorships to R. T. B. and A. E. P. from the American Heart Association.

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