Specificity of mammalian spermidine synthase and spermine synthase

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1. The specificity of rat prostatic spermidine synthase and spermine synthase with respect to the amine acceptor of the propylamine group was studied. 2. Spermidine synthase could use cadaverine (1,5-diaminopentane) instead of putrescine, but the $K_m$ for cadaverine was much greater and the rate with 1 mM-cadaverine was only 10% of that with putrescine. 1,3-Diaminopropane was even less active (2% of the rate with putrescine) and no other compound tested (including longer $\omega$-amino acids, spermidine and its homologues and monoacetyl derivatives) was active. 3. Spermine synthase was equally specific. The only compounds tested that showed any activity were 1,8-diamino-octane, sym-homospermidine, sym-norspermidine and $N$-(3-aminopropyl)-cadaverine, which at 1 mM gave rates 2, 17, 3 and 4% of the rate with spermidine respectively. 4. The formation of polyamine derivatives of cadaverine and to a very small extent of 1,3-diaminopropane was confirmed by exposing transformed mouse fibroblasts to these diamines when synthesis of putrescine was prevented by a-difluoromethylornithine. Under these conditions the cells accumulated significant amounts of $N$-(3-aminopropyl)cadaverine and $NN'$-bis(3-aminopropyl)cadaverine when exposed to cadaverine and small amounts of sym-norspermidine and sym-spermine when exposed to 1,3-diaminopropane.

Studies of the polyamine biosynthetic pathway in certain micro-organisms and in mammalian cells have established that aminopropyltransferases play essential roles in this process (Tabor & Tabor, 1976; Jänne et al., 1978; De Rosa et al., 1978; Williams-Ashman & Pegg, 1981). Mammalian cells contain two distinct aminopropyltransferases that transfer propylamine groups from decarboxylated $S$-adenosylmethionine to amine acceptors leaving 5'-methylthioadenosine. One of these uses putrescine as acceptor and forms spermidine (Hannonen et al., 1972; Hibasami et al., 1980), the other uses spermidine as acceptor producing spermine (Pajula et al., 1979; Hibasami et al., 1980). These enzymes have been much less studied than the decarboxylases forming putrescine and decarboxylated $S$-adenosylmethionine, but several recent papers have discussed their specificity with respect to the nucleoside donor and inhibition by compounds related to decarboxylated $S$-adenosylmethionine or 5'-methylthioadenosine (Coward et al., 1977; Hibasami & Pegg, 1978a; Pajula & Raina, 1979; Hibasami et al., 1980; Samejima & Nakazawa, 1980; Tang et al., 1980; Zappia et al., 1980; Pegg et al., 1981). There have been relatively few studies of their specificity with respect to the amine acceptors and some of the published literature is contradictory. Formation of sym-norspermine and sym-norspermidine by addition of aminopropyl groups to 1,3-diaminopropane occurs with enzymes isolated from thermophilic bacteria (De Rosa et al., 1978) and Euglena gracilis (Aleksijevic et al., 1979), but indirect evidence suggested that 1,3-diaminopropane was inactive with the enzyme from rat prostate (Hibasami & Pegg, 1978b). It has been claimed that 1,5-diaminopentane (cadaverine) is not a substrate for mammalian spermidine synthase (Kallio et al., 1977; Samejima & Nakazawa, 1980), whereas indirect evidence that this diamine was a substrate was published by Hibasami & Pegg (1978a,b) and it is known to be converted into $N$-(3-aminopropyl)-cadaverine by the enzyme from Escherichia coli (Dion & Cohen, 1972; Bowman et al., 1973). Spermidine apparently could not substitute for putrescine in the spermidine synthase reaction by liver or prostate extracts (Hannonen et al., 1972; Samejima & Nakazawa, 1980) but could partially replace putrescine with the bacterial enzyme (Bow-
man et al., 1973). The abilities of amines to act as acceptors of aminopropyl groups are of particular interest because several naturally occurring molecules are closely related to the physiological substrates and both natural and synthetic diamines are known to be able to support the growth of cells prevented from producing putrescine by application of inhibitors of ornithine decarboxylase (Mamont et al., 1978; Hölttä et al., 1979; Alhonen-Hongisto et al., 1979; Alhonen-Hongisto & Jänne, 1980; Mamont & Danzin, 1981; Mamont et al., 1981). Furthermore, several recent studies have shown that mammalian cells may, under certain circumstances, be capable of the production of cadaverine (Persson, 1977; Pegg & McGill, 1979; Alhonen-Hongisto & Jänne, 1980). It is known that Escherichia coli is prevented from synthesis of putrescine by substrate limitation or mutation, the production of cadaverine and its subsequent conversion into N-(3-aminopropyl)cadaverine permits growth to proceed (Dion & Cohen, 1972).

It is possible that some of the conflicting results concerning the specificity of mammalian aminopropyltransferases can be explained by difference in sensitivity of the assays employed and by the use of crude rather than highly purified enzyme fractions. In the present experiment the ability of a number of amines to serve as acceptors of the propylamine group has been studied using a sensitive assay such that rates of 1% of that with the preferred substrate could be detected. Spermidine synthase and spermine synthase purified from rat ventral prostate were used as enzyme preparations. The conversion of diamines into higher polyamines was also investigated in cultured fibroblasts, which were prevented from synthesizing putrescine by the addition of α-difluoromethylornithine, an inhibitor of ornithine decarboxylase (Metcalf et al., 1978).

Materials and methods

Materials

S-Adenosyl-l-[Me-14C]methionine (sp. radioactivity 50–60 Ci/mol) was purchased from New England Nuclear Corp., Boston, MA, U.S.A., and converted into decarboxylated S-adenosyl-[Me-14C]methionine as described previously (Pegg et al., 1981). Unlabelled decarboxylated S-adenosylmethionine was prepared in the same way. N1-acetylspermidine and N8-acetylspermidine were gifts from Dr. M. M. Abdel-Monem, Department of Medicinal Chemistry, University of Minnesota, MN, U.S.A. Monoacetylputrescine was provided by Dr. Z. Canellakis, Departments of Pharmacology and Internal Medicine, Yale University School of Medicine, Yale, CT, U.S.A. N-(3-Aminopropyl)cadaverine (1,9-diamino-4-azanonane) and sym-homospermidine (1,9-diamino-5-azanonane) were generous gifts from Dr. S. S. Cohen, Department of Pharmacology, State University of New York at Stony Brook, Long Island, NY, U.S.A. Sym-Norspermidine (3,3′-diaminodipropylamine) and sym-norspermine [NN′-bis(3-aminopropyl)propane-1,3-diamino] were purchased from Eastman Organic Chemicals, Rochester, NY, U.S.A. Other diamines were obtained from Aldrich Chemical Company, Milwaukee, WI, U.S.A., or from Sigma Chemical Company, St. Louis, MO, U.S.A. Purity of these compounds was checked by chromatography on an amino acid analyser as described below. All of the compounds used contained less than 0.1% of putrescine or spermidine. [1,4-14C]Putrescine dihydrochloride (sp. radioactivity 90–110 Ci/mol), [1,5-14C]cadaverine (sp. radioactivity 106 Ci/mol) and [5,8-14C]spermidine (1,8-diamino-4-aza[5,8-14C]octane) (sp. radioactivity 62 Ci/mol) were obtained from New England Nuclear Corp., Boston, MA, U.S.A. All other biochemical reagents were purchased from Sigma Chemical Company, St. Louis, MO, U.S.A. α-Difluoromethylornithine was kindly provided by Merrell Research Center, Cincinnati, OH, U.S.A.

Cell culture

Swiss SV40-transformed 3T3 mouse embryo fibroblasts (SV3T3 cells) were maintained as described previously (Bethell & Pegg, 1979), except that 3% horse serum/2% foetal calf serum was substituted for 10% foetal calf serum. Cells were harvested for polyamine analysis as described by Pegg et al. (1981).

Polyamine analysis

Polyamines were analysed with a Dionex D-500 Mark II amino acid analyser fitted with fluorescence detection and using an 11.5 cm (1.75 mm int. diam.) stainless-steel column packed with sulphonated polystyrene cation-exchange resin (Dionex Corporation, 1228 Titan Way, Sunnyvale, CA, U.S.A.). Separation was carried out as described by Seidenfeld & Marton (1979) and the polyamines were quantified by measurement of the fluorescence of their adducts produced by reaction with o-phthalaldehyde. This method provided excellent resolution between 1,3-diaminopropane (elution time, 11.2 min), putrescine (13.2 min), cadaverine (17.4 min), sym-norspermidine (18.3 min), spermidine (19.3 min), sym-homospermidine (21.1 min), N-(3-aminopropyl)cadaverine (22.5 min), sym-norspermine (23.2 min) and spermine (25.5 min).

Purification and assay of spermidine synthase and spermine synthase

These enzymes were purified from rat ventral prostate as previously reported (Hibasami et al., 1980). This yielded more than 400-fold purification.
The enzymes were not homogeneous but were free from each other (less than 1% contamination as shown in Table 1) and from S-adenosylmethionine decarboxylase and 5'-methylthioadenosine phosphorlyase. Aminopropyltransferase activity was routinely assayed by following the production of 5'-[Me-14C]methylthioadenosine from decarboxylated S-adenosyl[Me-14C]methionine in the presence of the appropriate amine acceptors as previously described (Hibasami & Pegg, 1978a). The assays were carried out by incubation for 30 min at 37°C in 100 mM-sodium phosphate buffer, pH 7.5, containing 5 mM-dithiothreitol. The concentrations of potential amine acceptors and the concentration and specific radioactivity of the decarboxylated S-adenosyl[Me-14C]methionine were varied according to the experimental sensitivity desired and are given in the text. The possibilities that spermidine and cadaverine were substrates for spermidine synthase were also tested using radioactivity putrescine, spermidine or cadaverine as acceptor and unlabelled decarboxylated S-adenosylmethionine. The labelled products and substrates were then separated by high-voltage paper electrophoresis (Jähne & Williams-Ashman, 1971).

### Results

Table 1 indicates the production of 5'-methylthioadenosine by prostate spermidine synthase and spermine synthase in the presence of potential amine acceptors. Only the aliphatic α,ω-diamines tested were substrates for spermidine synthase. Mono-acetylputrescine, γ-aminobutyrate, spermine and related compounds were less than 1% as effective as putrescine in stimulating the release of 5'-methylthioadenosine. Spermidine (at 1 mM) gave about 1% of the rate of putrescine but the conversion of labelled spermidine into spermine could not be demonstrated by the same enzyme preparation even at this low level. A rate of 1% of that found with putrescine could be accounted for by contamination of the spermidine with about 0.05% putrescine which is below the sensitivity with which these amines were checked for purity. Therefore, it appears that under the conditions tested (at pH 7.5) mammalian spermidine synthase cannot add propylamine groups to spermidine forming spermine. Similarly, a very slight release of 5'-methylthioadenosine was brought about by 1,6-diaminohexane, 1,8-diamino-octane and sym-homospermidine.

<table>
<thead>
<tr>
<th>Addition to assay</th>
<th>Spermidine synthase (c.p.m. in 5'-methylthioadenosine)</th>
<th>Spermine synthase (c.p.m. in 5'-methylthioadenosine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>48</td>
<td>96</td>
</tr>
<tr>
<td>Putrescine</td>
<td>3436 (100%)</td>
<td>81</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>378 (10%)</td>
<td>102</td>
</tr>
<tr>
<td>1,3-Diaminopropane</td>
<td>118 (2%)</td>
<td>105</td>
</tr>
<tr>
<td>1,6-Diaminohexane</td>
<td>70 (1%)</td>
<td>87</td>
</tr>
<tr>
<td>1,8-Diamino-octane</td>
<td>54</td>
<td>161 (2%)</td>
</tr>
<tr>
<td>1,9-Diaminononane</td>
<td>41</td>
<td>90</td>
</tr>
<tr>
<td>1,3-Diaminopropan-2-ol</td>
<td>50</td>
<td>81</td>
</tr>
<tr>
<td>Spermidine</td>
<td>69 (1%)</td>
<td>3712 (100%)</td>
</tr>
<tr>
<td>Sym-norspermidine</td>
<td>52</td>
<td>192 (3%)</td>
</tr>
<tr>
<td>Sym-homospermidine</td>
<td>68</td>
<td>715 (17%)</td>
</tr>
<tr>
<td>N-(3-Aminopropyl)cadaverine</td>
<td>42</td>
<td>232 (4%)</td>
</tr>
<tr>
<td>Sym-norspermine</td>
<td>56</td>
<td>87</td>
</tr>
<tr>
<td>Spermine</td>
<td>54</td>
<td>112</td>
</tr>
<tr>
<td>Monoacetylputrescine</td>
<td>66</td>
<td>118</td>
</tr>
<tr>
<td>N³-Acetyl spermidine</td>
<td>56</td>
<td>87</td>
</tr>
<tr>
<td>N²-Acetyl spermidine</td>
<td>44</td>
<td>81</td>
</tr>
<tr>
<td>γ-Aminobutyrate</td>
<td>58</td>
<td>84</td>
</tr>
</tbody>
</table>
but this was not greater than might have arisen from their contamination with putrescine.

The only compounds except for putrescine that enhanced the production of 5'-methylthioadenosine were 1,3-diaminopropane, which was very weakly active, and 1,5-diaminopentane (cadaverine), which was 10% as active as putrescine. The ability of prostatic spermidine synthase to produce N-(3-aminopropyl)cadaverine was confirmed by using labelled cadaverine as a substrate and separation of the product on high-voltage electrophoresis. Stoichiometric formation of 5'-methylthioadenosine and N-(3-aminopropyl)cadaverine was observed.

The apparent $K_m$ for putrescine and cadaverine was measured using a decarboxylated S-adenosylmethionine concentration of 20 $\mu$M, which gives a maximal rate of reaction [higher concentrations are inhibitory, owing to the strong substrate inhibition (Coward et al., 1977; Hibasami et al., 1980; Zappia et al., 1980)]. Under these conditions the apparent $K_m$ for putrescine was 15 $\mu$M and that for cadaverine was 0.6 $\mu$M (results not shown).

Table 1 shows similar tests for 5'-methylthioadenosine release by spermine synthase. Apart from spermidine the only compounds having significant activity were sym-homospermidine (17% of the rate with spermidine), N-(3-aminopropyl)cadaverine (4%), sym-norspermidine (3%) and 1,8-diamino-octane (2%). N-(3-aminopropyl)cadaverine and sym-homospermidine have one extra methylene group from spermidine and sym-norspermidine has one methylene group less. 1,8-Diamino-octane has the internal N atom replaced by a methylene group. The striking loss of potency as acceptors of aminopropyl groups brought about by these minor changes emphasizes the stringent specificity of spermine synthase. When assayed in the presence of decarboxylated 20 $\mu$M-S-adenosylmethionine, the apparent $K_m$ for spermidine of our spermidine synthase preparation was 20 $\mu$M, whereas the $K_m$ values for the other substrates were more than 0.5 $\mu$M (results not shown).

When mouse SV3T3 cells were cultured in the presence of 5 $\mu$M-$\alpha$-difluoromethylornithine, a potent inhibitor of ornithine decarboxylase (Metcalfe et al., 1978; Mamont et al., 1978), growth was substantially slowed (Pegg et al., 1981). This decrease in growth could be abolished completely by the addition of putrescine (Pegg et al., 1981; Table 2) and to a somewhat lesser extent by cadaverine (Table 2). 1,3-Diaminopropane was much less effective but did slightly increase growth, whereas 1,3-diaminopropanol was quite inactive (Table 2). The content of polyamines present in these fibroblasts is shown in Table 2. The cells exposed to $\alpha$-difluoromethylornithine contained no detectable putrescine, less than 1% of the spermidine and about 50% of the spermine present in control cells. In the

<table>
<thead>
<tr>
<th>Polyamines present in cells (mmol/10^6 cells)</th>
<th>N-(3-Aminopropyl)cadaverine</th>
<th>Sym-norspermidine</th>
<th>Spermidine</th>
<th>1,5-diaminopentane (cadaverine)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Putrescine</td>
<td>0.30</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>Cadaverine</td>
<td>0.08</td>
<td>N.D.</td>
<td>0.92</td>
<td>N.D.</td>
</tr>
<tr>
<td>1,3-Diaminopropane</td>
<td>0.30</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
<tr>
<td>1,8-Diamino-octane</td>
<td>0.98</td>
<td>N.D.</td>
<td>N.D.</td>
<td>N.D.</td>
</tr>
</tbody>
</table>

Table 2. Formation of polyamines in SV3T3 cells exposed to $\alpha$-difluoromethylornithine and various diamines

SV3T3 cells were grown for 4 days in dishes seeded with 4 x 10^6 cells in the presence of the additions shown. Abbreviation used: N.D., not detected.

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presence of 50 μM-putrescine, spermidine and spermine values in the cells were restored to control values. When 500 μM-cadaverine was added to the cells in the presence of α-difluoromethylornithine, two new polyamines were detected in the cell extracts. One of these co-chromatographed exactly with N-3-aminopropylcadaverine, the other was eluted after spermine in the region expected for NN'-bis(3-aminopropyl)cadaverine, although this could not be confirmed rigorously because an authentic standard was not available. These results confirm that mammalian spermidine and spermine synthases are able to convert 1,5-diaminopentane into the higher polyamine derivatives and that the replacement of putrescine and spermidine by cadaverine and N-(3-aminopropyl)cadaverine can support cell growth. When 500 μM-1,3-diaminopropanol was added to the cells, sym-norspermidine and sym-norspermine were produced, but in amounts much less than the normal polyamine concentrations (Table 1). 1,3-Diaminopropanol accumulated in the fibroblasts but did not lead to the appearance of any higher polyamines.

Discussion

The present results emphasize the stringent specificity of mammalian aminopropyltransferases with respect to amine acceptors of the aminopropyl group. Only cadaverine was a sufficiently good substrate for spermidine synthase for substantial production of the spermidine analogue in vitro and in vivo. Although the K_m for cadaverine was 40 times that for putrescine and the maximal reaction velocity almost an order of magnitude less, our results show clearly that cadaverine is a substrate. It is likely that the insensitivity of the assay prevented Samejima & Nakazawa (1980) and Kallio et al. (1977) from observing this reaction. Our results are in agreement with the reports of Mamont and his colleagues (Mamont et al., 1978, 1981; Mamont & Danzin, 1981), who detected N-(3-aminopropyl)cadaverine in HTC cells cultured in the presence of cadaverine, and Alhonen-Hongisto & Jänne (1980), who tentatively identified N-(3-aminopropyl)cadaverine in Ehrlich ascites cells. However, in our experiments we have never detected this derivative in cultured fibroblasts unless cadaverine was added to the culture medium even in the presence of α-difluoromethionine (see Table 2), whereas in the Ehrlich ascites cells, both cadaverine and the putative N-(3-aminopropyl)cadaverine were observed without addition of the diamine (Alhonen-Hongisto & Jänne, 1980). More information on the production of cadaverine in various cell types is clearly needed. The presence of cadaverine in certain mammalian, amphibian and plant cells as well as in prokaryotes is firmly documented (Heningsson et al., 1976; Hamana & Matsuzaki, 1979; Smith, 1980). It is known that this compound can be produced by the action of rat liver and mouse kidney ornithine decarboxylase acting on lysine (Persson, 1977; Pegg & McGill, 1979). The ability of mammalian HTC cells (Mamont et al., 1978, 1981) and SV3T3 cells to grow with cadaverine and N-(3-aminopropyl)cadaverine substituting for putrescine and spermidine closely resembles the situation in E. coli (Dion & Cohen, 1972) and implies that inhibitors of polyamine production would be most effective if availability of these substitutes is also prevented.

1,3-Diaminopropane was a very feeble substrate for spermidine synthase and sym-norspermidine was an equally poor substrate for spermine synthase. Even in cells depleted of putrescine and exposed to 0.5 mM-1,3-diaminopropane, there was only a very small amount of sym-norspermidine and sym-norspermine. The relative inability of 1,3-diaminopropane to serve as a precursor of higher polyamines has also been noted by Mamont et al. (1981) and may account for its relative inability to support growth in spermidine-depleted cells. Although the enzymes involved have not been fully characterized, convincing evidence has been published showing that the aminopropyl groups of sym-norspermidine and sym-norspermine are derived from decarboxylated S-adenosylmethionine in Caldariella acidophila (De Rosa et al., 1978) and Euglena gracilis (Aleksijevic et al., 1979; Villanueva et al., 1980). These organisms presumably have an aminopropyltransferase utilizing 1,3-diaminopropane as an acceptor. The present results suggest that the different specificity of the mammalian enzymes account for the lack of sym-norspermidine and other longer derivatives consisting of linear multiples of the aminopropyl groups. The strong preference of the mammalian aminopropyltransferases for the NH_2(CH_2)_nNH-R structure as acceptor of an aminopropyl group ensures that spermine is the largest polyamine that can be made in substantial amounts.

The most active acceptor for spermine synthase apart from spermidine itself was sym-homospermidine. This compound occurs in considerable amounts in certain plants, micro-organisms and amphibia (Smith, 1977; Tait, 1979; Aleksijevic et al., 1979; Hamana & Matsuzaki, 1979; Villanueva et al., 1980; Srivenugopal & Adiga, 1980). These organisms also possess spermine synthase and, if it resembles the prostatic enzyme, the presence of 1,13-diamino-4,9-diazatridecane might be expected. This compound has not been reported but is likely to be so strongly retarded on the usual ion-exchange column analysis procedure that it may have escaped detection.

Finally, rat prostate spermidine synthase apparently differs from the enzyme from E. coli in that the
latter was reported to be able to use spermidine as an acceptor at a rate 10% that of putrescine at pH 8.2 and 30% that of putrescine at pH 10, which gives an optimal rate of reaction (Bowman et al., 1973). This finding is quite puzzling since E. coli does not normally contain spermine, whereas in mammalian cells, which do contain spermine, a distinct enzyme is used to produce the higher polyamine. Our results agree with the report of Hannonen et al. (1972) that spermidine is not a substrate for prostatic spermidine synthase and the present limit of detection was 1% of the maximal rate with putrescine. In certain other respects, such as the marked substrate inhibition by excess decarboxylated S-adenosylmethionine (Coward et al., 1977; Hibasami et al., 1980; Zappia et al., 1980) and susceptibility to inhibition by S-adenosyl-3-thio-1,8-diamino-octane (Tang et al., 1980; A. E. Pegg, A. Nureddin & J. K. Coward, unpublished work) the bacterial and mammalian enzyme are very similar. However, the difference with regard to the specificity of the aminopropyl acceptor is such an important property that, if it can be confirmed that the bacterial enzyme acts on both substrates, it would be preferable to retain the term aminopropyltransferase (for which the EC number 2.5.1.16 has been assigned) for this enzyme. The mammalian enzymes are more correctly designated as spermidine synthase and spermine synthase.

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References


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