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# Polyamine synthesis in mammalian tissues

## Isolation and characterization of spermidine synthase from bovine brain

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Spermidine synthase (EC 2.5.1.16) was purified to apparent homogeneity (about 11000-fold) from bovine brain by affinity chromatography, with *S*-adenosyl-(5′)-3-thiopropylamine linked to Sepharose as the adsorbent. The enzyme preparation was free from *S*-adenosylmethionine decarboxylase (EC 4.1.1.50) and spermine synthase (EC 2.5.1.22) activities. The native enzyme had an apparent $M_r$ of 70000, was composed of two subunits of equal size, and had an isoelectric point at pH 5.22. The apparent $K_m$ values for putrescine and decarboxylated adenosylmethionine [*S*-adenosyl-(5′)-3-methylthiopropylamine] were 40 $\mu$M and 0.3 $\mu$M respectively. Cadaverine and 1,6-diaminohexane could replace putrescine as the aminopropyl acceptor, although the reaction rates were only 6% and 1% respectively of that obtained with putrescine. Ethyl, propyl and carboxymethyl analogues of decarboxy-*S*-adenosylmethionine could act as propylamine donors. Both the reaction products, spermidine and 5′-methylthioadenosine, were mixed-type inhibitors of the enzyme. On the basis of initial-velocity and product-inhibition studies, a ping-pong reaction mechanism for the spermidine synthase reaction was ruled out.

Four enzymes, including two decarboxylases [L-ornithine decarboxylase (EC 4.1.1.17) and *S*-adenosyl-L-methionine decarboxylase] and two aminopropyltransferases (spermidine synthase and spermine synthase), are known to be involved in the biosynthesis of polyamines in eukaryotic organisms (for references see Raina & Jänne, 1975). The two decarboxylases have been purified to an apparent homogeneity from rat liver (Pegg, 1974; Kameji *et al.*, 1982) and mouse kidney (Persson, 1981; Seely *et al.*, 1982). Also, we have reported the isolation of spermine synthase from bovine brain as a homogeneous protein (Pajula *et al.*, 1979).

Previous attempts to purify spermidine synthase from animal tissues by using conventional chromatographic procedures (Jänne *et al.*, 1971; Raina & Hannonen, 1971; Hibasami *et al.*, 1980*a*) have at best resulted in preparations no more than 5–10% pure. More recently Samejima and co-workers have designed a novel affinity-chromatographic adsorbent, *S*-adenosyl-(5′)-3-thiopropylamine–Sepharose, for the purification of this enzyme and isolated spermidine synthase from rat prostate as a homogeneous protein (Samejima & Yamanoha, 1982; Samejima *et al.*, 1983). Furthermore, the development of rapid and sensitive assay methods for aminopropyltransferases (Raina *et al.*, 1976; Hibasami & Pegg, 1978; Raina *et al.*, 1983*a*) has made it possible to characterize these enzymes in greater detail. In the present paper we describe purification of spermidine synthase from bovine brain to an apparent homogeneity and some properties of the purified enzyme. Some results of a kinetic analysis, including initial-velocity and product-inhibition studies, are also reported.

## Experimental

### *Chemicals*

1,4-Diaminobutane (putrescine), 1,5-diaminopentane (cadaverine), spermidine and spermine as their hydrochloride salts were obtained from Calbiochem, Los Angeles, CA, U.S.A., and 1,3-diaminopropane was from Fluka, Buchs SG, Switzerland. These amines were recrystallized twice from hot 6M-HCl/ethanol solution. In addition, chromatography on a Dowex 50 ($H^+$ form) column described previously (Pajula *et al.*, 1979) was needed to remove spermidine found as a

contaminant in many commercial spermine preparations. 1,6-Diaminohexane was a product of BDH Chemicals, Poole, Dorset, U.K. *sym*-Norspermidine (3,3'-diaminodipropylamine), diethylenetriamine and triethylenetetramine were kindly provided by CIBA–GEIGY, Basle, Switzerland. *sym*-Norspermine [*NN'*-bis-(3-aminopropyl)propane-1,3-diamine] and *NN'*-bis-(2-aminoethyl)-propane-1,3-diamine were purchased from Eastman Kodak, Rochester, NY, U.S.A. *sym*-Homospermine [*NN'*-bis-(4-aminobutyl)butane-1,4-diamine] was a gift from Dr. Masato Okada, University of Tokyo, Tokyo, Japan. *S*-Adenosyl-(5')-3-thiopropylamine hydrogen sulphate was prepared by the method of Jamieson (1963). Preparation of *S*-adenosyl-(5')-3-thiopropylamine linked to Sepharose has been previously described in detail (Samejima & Yamanoha, 1982). Dihydrogen sulphates of *S*-adenosyl-(5')-3-methylthiopropylamine (decarboxylated adenosylmethionine) and its analogues *S*-adenosyl-(5')-3-methylthioethylamine, *S*-adenosyl-(5')-3-ethylthiopropylamine, *S*-adenosyl-(5')-3-propylthiopropylamine, *S*-adenosyl-(5')-3-butylthiopropylamine and *S*-adenosyl-(5')-3-carboxymethylthiopropylamine were prepared by the published methods (Samejima *et al.*, 1978).

DL-[2-$^{14}$C]Methionine (sp. radioactivity 5.84Ci/mol) and [1,4-$^{14}$C]spermidine trihydrochloride (sp. radioactivity 10.22Ci/mol) were supplied by New England Nuclear Corp., Boston, MA, U.S.A. *S*-Adenosyl-L-[*Me*-$^{14}$C]methionine (sp. radioactivity 57Ci/mol) was purchased from The Radiochemical Centre, Amersham, Bucks., U.K. Radioactive *S*-adenosylmethionine was synthesized from DL-[2-$^{14}$C]methionine essentially as described by Pegg & Williams-Ashman (1969). Radioactive *S*-adenosyl-(5')-3-methylthiopropylamines were prepared from adenosylmethionine, labelled at the C-2 position of the methionine moiety or at the methyl group, by using adenosylmethionine decarboxylase from *Escherichia coli* as the enzyme (Pajula *et al.*, 1979). Unlabelled decarboxylated adenosylmethionine was prepared in a similar way. The products were first purified on a Dowex 50 ($H^+$ form) column, followed by preparative paper electrophoresis as previously described (Raina & Hannonen, 1971). As a final step, decarboxylated adenosylmethionine was purified on a phosphocellulose column (Cellex-P; BioRad Laboratories, Richmond, CA, U.S.A.).

5'-Methylthioadenosine was purchased from Sigma Chemical Co., St. Louis, MO, U.S.A., dithiothreitol was from Calbiochem and $(NH_4)_2SO_4$ and sucrose (both special enzyme grade) were from Schwarz/Mann, Spring Valley, NY, U.S.A. Ampholine polyacrylamide-gel plates (pH4.0–6.5) were from LKB Instruments, Stockholm, Sweden, Sephadex G-150 was from Pharmacia Fine Chemicals, Uppsala, Sweden, and Whatman phosphocellulose paper P81 was from Reeve Angel and Co., London E.C.4, U.K. ACS (Aqueous Counting Scintillant) was a product of Amersham Corp., Arlington Heights, IL, U.S.A. All other chemicals used were reagent-grade materials from various commercial sources.

*Enzyme assays*

Two methods, based on the use of $^{14}$C-labelled decarboxylated adenosylmethionine as a substrate, were used for the assay of spermidine synthase (EC 2.5.1.16) activity. The assay procedure with decarboxylated adenosylmethionine labelled at C-1 of the propylamine group has been previously described in detail (Raina *et al.*, 1976, 1983*b*; Pajula *et al.*, 1979). With methyl-$^{14}$C-labelled decarboxylated adenosylmethionine as a substrate the original method developed by Hibasami & Pegg (1978) was modified as described by Raina *et al.* (1983*b*). Dowex 50 ($H^+$ form) cation-exchanger was replaced by a weak cation-exchange material (Cellex-P). This modification made the elution of radioactive 5'-methylthioadenosine and its degradation products easier and increased the counting efficiency. Under standard incubation conditions the reaction mixture, containing 0.1M-potassium phosphate buffer, pH7.4, 5mM-dithiothreitol, 1mM-putrescine and 20$\mu$M radioactive decarboxylated adenosylmethionine, was preincubated for 10min at 37°C before the addition of the enzyme. The final incubation volume was 0.1ml. The reaction was stopped after 5 or 10min incubation by adding 0.5ml of 25mM-HCl. A 0.5ml portion of the acid solution (pH slightly below 3) was applied to a phosphocellulose column (about 0.5 ml of Cellex-P applied to a Pasteur pipette with glass-wool as a bed support; the void volume was approx. 0.8ml) previously equilibrated with 25mM-HCl. The effluent (0.5ml) was discarded and the radioactive 5'-methylthioadenosine eluted directly into a scintillation vial with 1.8ml of 25mM-HCl; 10ml of ACS scintillation solution was added and mixed thoroughly. Counting efficiency was about 82%. The column was regenerated with 2×2ml of 0.1M-NaOH and/or 2×2ml of 0.5M-HCl, which completely removed the unchanged decarboxylated adenosylmethionine. Other details of the method have been previously described (Raina *et al.*, 1983*a*,*b*).

In studies on the kinetics of the spermidine synthase reaction, the amount of the enzyme was adjusted so that no more than 5% (occasionally up to 14%) of the substrate (decarboxylated adenosylmethionine) was consumed during the reaction. To prevent adsorption of decarboxylated adenosyl-

methionine on glass at low substrate concentrations, the appropriate dilutions of the substrate were prepared in 0.15M-NaCl. Albumin (crystalline bovine serum albumin from Sigma, catalogue no. A4378, lot no. 41F-9300) was added to the diluted enzyme solutions (final concentration of albumin in the incubation mixture was 0.2mg/ml) to prevent denaturation. Some commercial samples of bovine serum albumin are known to contain amine oxidase as an impurity (see Al-Naji & Clarke, 1983), which might interfere with the enzyme assays. This possibility was ruled out in the present work by the following method. [1,4-$^{14}$C]-Putrescine and [1,4-$^{14}$C]spermidine (both at 10$\mu$M concentration) were incubated in the standard reaction mixture (minus decarboxylated adenosylmethionine) for 20min in the presence of albumin (0.2mg/ml). After incubation the recovery of the radioactive polyamines was complete, as demonstrated by paper electrophoresis (Raina *et al.*, 1976). It should also be pointed out that in most experiments of the present work the formation of radioactive 5'-methylthioadenosine was measured, which is not affected by amine oxidase unless a considerable portion of the amine substrate (putrescine) is being oxidized. This was not the case, as described above.

The enzyme assays were made in triplicates or quadruplicates that in general agreed within $\pm 5\%$. The reaction rate was linear with enzyme concentration and with incubation time up to at least 5min used in kinetic assays.

One unit of enzyme activity represents the formation of 1nmol of spermidine or 5'-methylthioadenosine in 1min under standard assay conditions.

*Protein determination*

Protein was measured by the dye-binding method of Schaffner & Weissman (1973), with crystalline bovine serum albumin as a standard, or by u.v. absorption (Kalckar, 1947).

*Paper electrophoresis*

Paper electrophoresis, used to study the efficiency of the analogues of decarboxylated adenosylmethionine to serve as substrates for spermidine synthase, was performed with 0.1M-citric acid buffer, pH3.6 (Raina, 1963). This system allowed a clear separation of radioactive putrescine from higher polyamines.

*Polyacrylamide-gel electrophoresis*

Polyacrylamide-gel electrophoresis was performed at 4°C, essentially as described by Davis (1964) with 16cm-long separating gels in 40mM-Tris/HCl buffer, pH8.9, and omitting spacer gels. Samples containing 5–50$\mu$g of protein and 20% (w/v) sucrose were applied to the column in 20–50$\mu$l of 6mM-Tris/HCl buffer, pH6.7, and electrophoresis was performed in 0.5mM-Tris/glycine buffer, pH8.3. The concentration of buffers refers to that of Tris and the pH to that found at 20°C. All buffers contained 1mM-2-mercaptoethanol and 1mM-dithiothreitol. After electrophoresis (2mA/tube until the tracking dye had migrated close to the anode end of the gel) the gels were fixed in 12.5% (w/v) trichloroacetic acid for 1h and stained at room temperature overnight with 0.05% Coomassie Brilliant Blue in 50% (v/v) methanol/5% (v/v) acetic acid solution. Destaining was performed at 37°C in acetic acid/methanol/water (3:2:35, by vol.) solution (Weber & Osborn, 1969) until the background was clear. Localization of the enzyme activity in the gel was performed as previously described (Pajula *et al.*, 1979).

Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis was performed at room temperature with 10%-acrylamide separating gels (length 7cm) and omitting spacer gels. The electrophoresis buffer and the gels contained 100mM-sodium phosphate buffer, pH7.2, 0.1% sodium dodecyl sulphate, 1mM-2-mercaptoethanol and 1mM-dithiothreitol. Protein samples were incubated for 3min at 90°C in electrophoresis buffer containing 1% (w/v) sodium dodecyl sulphate, 20% (w/v) sucrose and 0.2M-2-mercaptoethanol. After electrophoresis (8mA/gel for 4h) the gels were treated as described above. The protein standards for $M_r$ determinations were $\alpha$-lactalbumin, soya-bean trypsin inhibitor, carbonic anhydrase, ovalbumin, bovine serum albumin and phosphorylase *b* (electrophoresis calibration kit; Pharmacia).

*Isoelectric focusing*

Isoelectric focusing was performed in an LKB 2117 Multiphor apparatus with Ampholine polyacrylamide-gel plates with pH range 4.0–6.5 according to the instructions of the manufacturer. The pH gradient was determined by cutting a part of the gel at 5mm intervals and eluting with water. Staining of the gel plates was carried out with Coomassie Brilliant Blue as described above.

*Estimation of $M_r$*

The $M_r$ of spermidine synthase was estimated by the method of Hedrick & Smith (1968) with 6%, 7%, 8%, 9% and 10% polyacrylamide gels. The protein standards were soya-bean trypsin inhibitor, ovalbumin, pepsin (dimer), bovine serum albumin, conalbumin, lactate dehydrogenase (all from Sigma), fructose bisphosphate aldolase and catalase (the latter two from Pharmacia).

## Results

*Purification of spermidine synthase from bovine brain*

Fresh bovine brains (the age of the animals varied from 1 to 2 years) were obtained from a local slaughterhouse and immediately cooled on ice. All subsequent operations were performed at 0–4°C. The brains (1600g) were freed of membranes, washed in 0.25M-sucrose and homogenized in 3vol. of 0.25M-sucrose containing 1mM-EDTA, 5mM-2-mercaptoethanol and 0.1mM-dithiothreitol, with an Ultra-Turrax (Janke und Kunkel, Staufen/Br., Germany) homogenizer. The homogenate was centrifuged at 14000*g* for 30min. The supernatant fraction was filtered through glass wool.

The crude supernatant (3390ml) was fractionated with solid $(NH_4)_2SO_4$ (Mann, special enzyme grade). The proteins precipitated between 0.40 and 0.60 saturation with $(NH_4)_2SO_4$ were collected by centrifugation at 14000*g* for 30min, dissolved in 240ml of 10mM-Tris/HCl buffer, pH7.5 (at 20°C), containing 0.08M-NaCl, 1mM-2-mercaptoethanol and 0.1mM-dithiothreitol (buffer A) and dialysed for 17h against 50vol. of the same buffer, the buffer being changed twice during dialysis. The dialysed fraction was freed of insoluble material by centrifugation at 14000*g* for 15min.

The dialysed $(NH_4)_2SO_4$ fraction (325ml) was applied to a DEAE-cellulose (Whatman DE-52) column (8.5cm × 34cm) equilibrated with buffer A. The column was washed with 1100ml of buffer A containing 1mM-dithiothreitol and connected to a linear gradient of 0.08–0.4M-NaCl in buffer A (total gradient volume was 4000ml). Fractions of volume 15ml were collected. Spermidine synthase activity was eluted between 0.12M- and 0.16M-NaCl. The active fractions (497ml) were pooled, 2-mercaptoethanol and EDTA were added up to 5mM and 1mM respectively, and the fractions were adjusted to 0.70 saturation with $(NH_4)_2SO_4$ to concentrate the enzyme. The precipitate was dissolved in 17ml of 20mM-potassium phosphate buffer, pH7.2, containing 0.3M-NaCl, 0.1mM-EDTA and 0.1mM-dithiothreitol (buffer B), dialysed for 17h against 200vol. of the same buffer and centrifuged at 14000*g* for 15min.

The dialysed DEAE-cellulose fraction (29ml) was applied to an *S*-adenosyl-(5′)-3-thiopropylamine–Sepharose column (1cm × 3.5cm), equilibrated with buffer B, at a flow rate of 0.2ml/min. The column was washed with 30ml of buffer B, followed by buffer B containing 0.6M-NaCl (100ml), until no protein was being eluted (monitored by u.v. absorption). Spermidine synthase was then eluted with buffer B containing 0.5mM chemically synthesized decarboxylated adenosylmethionine (Samejima *et al.*, 1978). The active fractions (10ml) were pooled and concentrated by ultrafiltration in a Centriflo CF 25 cone (Amicon Corp., Danvers, MA, U.S.A.) to 0.6ml.

As a final step spermidine synthase was purified by gel filtration. A 0.5ml portion of the concentrated enzyme solution of the previous step was applied to a Sephadex G-150 column (1.6cm × 80cm) equilibrated with buffer B containing 1mM-2-mercaptoethanol and 1mM-dithiothreitol. The elution was carried out with the same buffer at a flow rate of 0.05ml/min. Fractions of volume 1ml were collected. The active fractions (7ml) were pooled and concentrated to a final volume of 0.35ml by ultrafiltration in a Centriflo cone. The enzyme preparation was stored at 0–4°C. No significant decrease (less than 10%) in the enzyme activity was observed during storage of several months.

Table 1 summarizes the purification. The final preparation had a specific catalytic activity about 11000-fold that of the crude supernatant enzyme. Fig. 1 indicates that after the *S*-adenosyl-(5′)-3-thiopropylamine–Sepharose step the enzyme preparation still contained traces of contaminating proteins. However, gel filtration resulted in an apparently homogeneous protein (Fig. 1), although no significant change in the specific catalytic activity of the enzyme was observed between the last two steps.

The final preparation was free of any measurable *S*-adenosylmethionine decarboxylase or spermine synthase activity. Originally (Samejima *et al.*,

Table 1. *Purification of spermidine synthase from bovine brain*

The crude extract was prepared from 1600g of bovine brain and processed as described in the text. Enzyme activity was determined under standard assay conditions.

| Purification step | Total protein (mg) | Specific catalytic activity (units/mg) | Total activity (μmol/min) | Yield (%) | Purification factor (-fold) |
|---|---|---|---|---|---|
| Crude extract | 26700 | 0.103 | 2.749 | 100 | 1 |
| $(NH_4)_2SO_4$ fractionation | 5557 | 0.266 | 1.476 | 54 | 3 |
| Chromatography on DEAE-cellulose | 498 | 2.316 | 1.154 | 42 | 22 |
| Chromatography on adenosylthiopropylamine–Sepharose | 0.504 | 1288 | 0.649 | 24 | 12510 |
| Gel filtration on Sephadex G-150 | 0.276 | 1121 | 0.309 | 11 | 10880 |

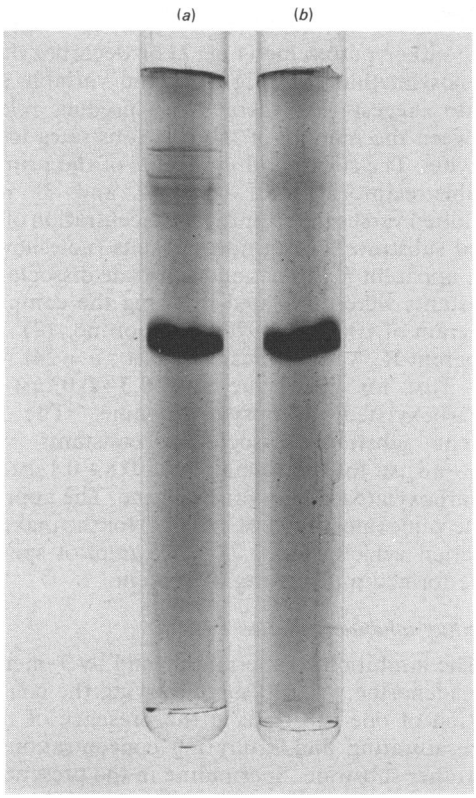


Fig. 1. *Sodium dodecyl sulphate/polyacrylamide-gel electrophoresis of spermidine synthase at final steps of purification*

(*a*) Spermidine synthase preparation (5.2 $\mu$g) after *S*-adenosyl-(5′)-3-thiopropylamine–Sepharose chromatography and (*b*) after chromatography on Sephadex G-150 (5.2 $\mu$g). For details of running conditions see the Experimental section.

1983), chromatography on hydroxyapatite was applied before the *S*-adenosyl-(5′)-3-thiopropylamine–Sepharose step to remove *S*-adenosylmethionine decarboxylase and spermine synthase activities. In the present method, hydroxyapatite chromatography was omitted, as it was observed that *S*-adenosylmethionine decarboxylase was not adsorbed on *S*-adenosyl-(5′)-3-thiopropylamine–Sepharose under the conditions described. Unexpectedly, the residual spermine synthase activity present in the enzyme preparation after the DEAE-cellulose step completely disappeared during chromatography on *S*-adenosyl-(5′)-3-thiopropylamine–Sepharose.

*Polyacrylamide-gel electrophoresis*

The purified spermidine synthase was examined by analytical gel electrophoresis in 7% and 9% polyacrylamide gels. The final preparation was apparently a homogeneous protein, which co-migrated with the enzyme activity (results not shown).

Isoelectric focusing on Ampholine polyacrylamide-gel plates with pH range 4.0–6.5 revealed one band with an isoelectric point of pH 5.22 (not shown).

*$M_r$ and subunit composition*

The determination of the molecular size of the purified spermidine synthase was carried out as described by Hedrick & Smith (1968). Comparison of the relative mobility of spermidine synthase with those of standard proteins at five different gel concentrations (see the Experimental section) gave an apparent $M_r$ of 70000 for the native enzyme.

Polyacrylamide-gel electrophoresis in the presence of sodium dodecyl sulphate revealed a single protein band (Fig. 1) and an $M_r$ of 35800. Thus it appears that the native enzyme is composed of two subunits having the same or nearly identical molecular masses.

*pH optimum*

The pH-dependence of the spermidine synthase reaction was studied in the range pH 6.5–9.8 with 0.1 M-potassium phosphate (6.5–8.0), 0.1 M-Tris/HCl (7.6–9.4) and 0.1 M-glycine/NaOH (9.0–9.8) buffers. The enzyme activity rose from 60% at pH 6.5 (the activity found at pH 7.4 was taken as 100%) and showed a plateau at pH 7.4–8.0. From pH 8.0 the activity increased up to pH 9.6 (190%) and then abruptly fell to 84% at pH 9.8, owing to denaturation of the enzyme.

*Initial-velocity studies*

Kinetic analysis of the spermidine synthase reaction is complicated by a substrate inhibition found at high concentrations of decarboxylated adenosylmethionine (Coward *et al.*, 1977; Hibasami *et al.*, 1980*a*; Samejima & Yamanoha, 1982; Raina *et al.*, 1983*a*). In the present investigation no substrate inhibition was observed at concentrations of up to 25 $\mu$M decarboxylated adenosylmethionine in the presence of 0.2 mM- or 1 mM-putrescine. It has been advised (Plowman, 1972; Allison & Purich, 1979) that the substrate concentrations should be chosen in the range 0.2–5 times the $K_m$ to avoid substrate inhibition. In the initial-velocity studies described below the concentration of decarboxylated adenosylmethionine was varied in the range 0.09–2.5 $\mu$M. Within this range the reaction followed Michaelis–Menten kinetics. The terminology recommended by the Nomenclature Committee of the International Union of Biochemistry (1982) is used.

Initial-reaction-velocity studies, based on the theory of Dalziel (1957) as further extended by Cleland (1963*a*,*b*,*c*), were carried out by varying

the concentration of one of the substrates in the presence of five different fixed concentrations of the other substrate. All data were plotted by the untransformed unweighted least-squares method.

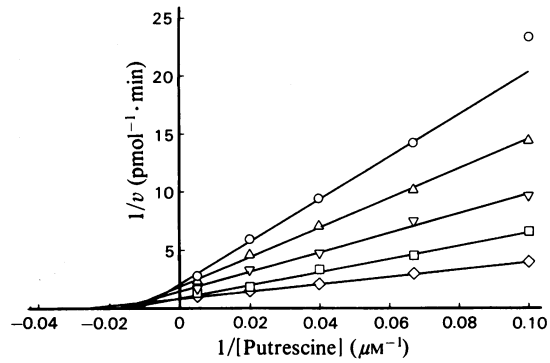


Fig. 2. *Bisubstrate kinetics of the spermidine synthase reaction at different fixed concentrations of decarboxylated adenosylmethionine*

Putrescine was the variable substrate and decarboxylated adenosylmethionine the fixed substrate at concentrations of 0.13 μM (○), 0.21 μM (△), 0.35 μM (▽), 0.71 μM (□) and 2.90 μM (◇). Incubation time was 5 min. The reaction rate is expressed as pmol/min. The lines were drawn by the untransformed unweighted least-squares method. One point (0.13 μM decarboxylated adenosylmethionine, 10 μM putrescine) was omitted in the calculation. For other details see the text.

The converging lines in the Lineweaver–Burk plot with either putrescine (Fig. 2) or decarboxylated adenosylmethionine (Fig. 3) as the variable substrate suggest that there is no product release between the binding of the two substrates to the enzyme. The slopes and intercepts of the primary double-reciprocal lines (Figs. 2 and 3) were replotted versus the reciprocal concentration of the fixed substrate, giving linear results (not shown). The apparent $K_m$ values and substrate dissociation constants were calculated by using the computer program of Cleland (1979; equation no. 17). The apparent $K_m$ values (means ± S.E.M.; $n = 24$) were 40 ± 3 μM for putrescine and 0.3 ± 0.03 μM for decarboxylated adenosylmethionine. The apparent substrate dissociation constants were 125 ± 18 μM for putrescine and 0.8 ± 0.1 μM for decarboxylated adenosylmethionine. The approximate value (mean ± S.E.M.; $n = 24$) for the maximal reaction velocity was 1.78 ± 0.05 μmol of spermidine formed/min per mg of protein.

*Product-inhibition studies*

The inhibition by spermidine and by 5′-methylthioadenosine was measured varying the concentration of one substrate in the presence of fixed non-saturating and saturating concentrations of the other substrate. Spermidine in the presence of non-saturating concentration of the fixed substrate gave double-reciprocal lines intersecting close to the $1/v$ axis both with putrescine (Fig. 4) and with decarboxylated adenosylmethionine (Fig. 5) as the

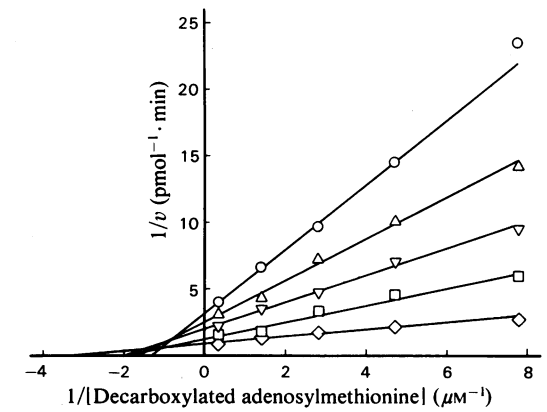


Fig. 3. *Bisubstrate kinetics of the spermidine synthase reaction at different fixed concentrations of putrescine*

Decarboxylated adenosylmethionine was the variable substrate and putrescine the fixed substrate. The concentrations of putrescine were 10 μM (○), 15 μM (△), 25 μM (▽), 50 μM (□) and 200 μM (◇). One point (0.13 μM decarboxylated adenosylmethionine, 10 μM-putrescine) was omitted in the calculation. Reaction conditions were as indicated in Fig. 2 legend.

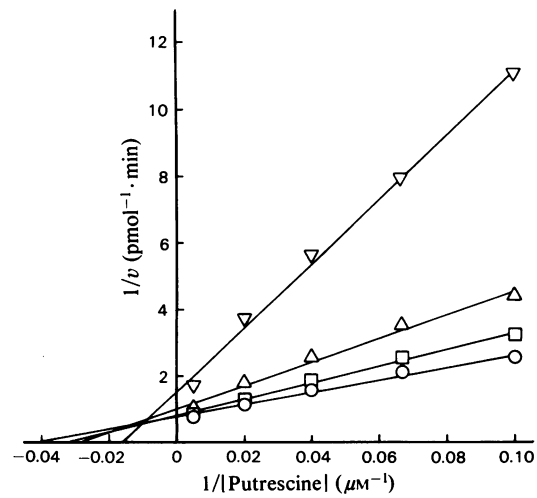


Fig. 4. *Inhibition of spermidine synthase by spermidine with putrescine as the varied substrate*

Decarboxylated adenosylmethionine was the fixed substrate (0.53 μM) and spermidine concentrations were none (○), 25 μM (□), 100 μM (△) and 500 μM (▽). For other details see the text.

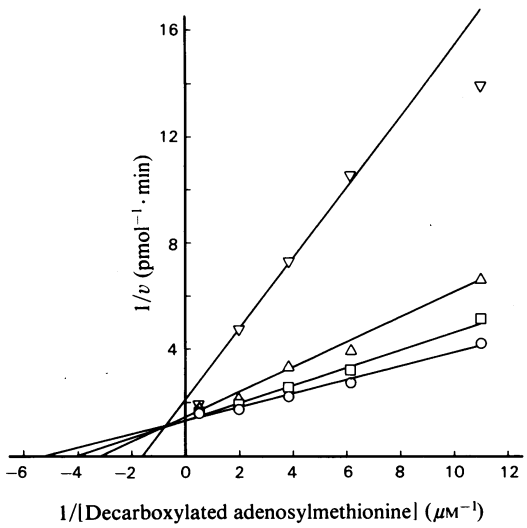


Fig. 5. *Inhibition of spermidine synthase by spermidine with decarboxylated adenosylmethionine as the varied substrate*

Putrescine was the fixed substrate (100 μM) and spermidine concentrations were none (○), 25 μM (□), 100 μM (△) and 500 μM (▽). One point (500 μM-spermidine, 0.09 μM decarboxylated adenosylmethionine) was omitted in the calculation. For other details see the text.

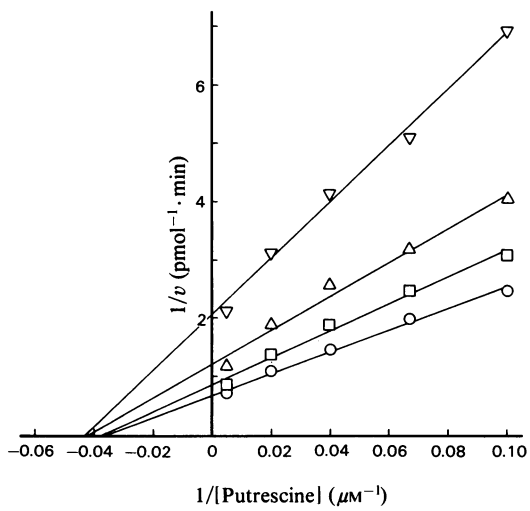


Fig. 6. *Inhibition of spermidine synthase by 5'-methylthioadenosine with putrescine as the varied substrate*

Decarboxylated adenosylmethionine was the fixed substrate (0.71 μM) and the concentrations of 5'-methylthioadenosine were none (○), 4 μM (□), 20 μM (△) and 100 μM (▽). For other details see the text.

variable substrate. However, a detailed analysis of the plots by the method of Baici (1981) revealed that the type of inhibition was not competitive but mixed in both cases. Similar patterns of inhibition were also obtained with saturating concentrations of decarboxylated adenosylmethionine (21 μM) or putrescine (2.5 mM) as the fixed substrate (results not shown). This further confirms the mixed-type inhibition of spermidine relative to both substrates. If spermidine were a competitive inhibitor, no inhibition should be seen at saturating concentrations of the fixed substrate provided that both the inhibitor and the substrate bind to the same enzyme form (Cleland, 1963*a*,*b*,*c*).

The inhibition by 5'-methylthioadenosine was clearly a mixed type with respect to both putrescine (Fig. 6) and decarboxylated adenosylmethionine (Fig. 7) at non-saturating concentrations of the fixed substrate. The result was the same with saturating concentrations of the fixed substrate (results not shown). In the latter experiments, 21 μM decarboxylated adenosylmethionine and 1 mM-putrescine were used as fixed substrate respectively.

The slopes and intercepts of the primary double-reciprocal lines (Figs. 4–7) replotted versus the concentration of the inhibitor gave hyperbolic lines that approached a finite velocity (replots not shown). Thus the apparent inhibition constants, $K_{ic}$ and $K_{iu}$ (Table 2), were calculated by the method of Baici (1981) specially designed for the analysis of hyperbolic mixed-type inhibitors. Since decarboxylated adenosylmethionine has been reported

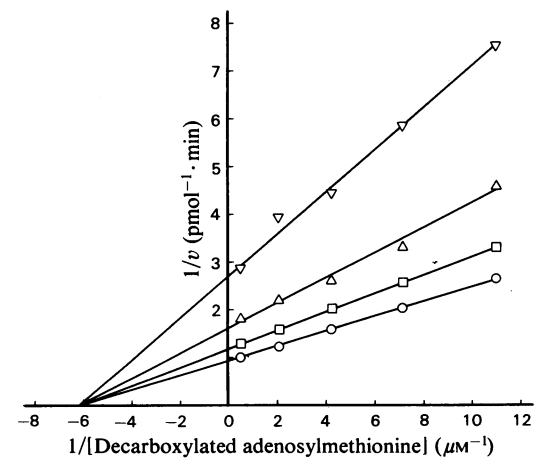


Fig. 7. *Inhibition of spermidine synthase by 5'-methylthioadenosine with decarboxylated adenosylmethionine as the varied substrate*

Putrescine was the fixed substrate (100 μM) and the concentrations of 5'-methylthioadenosine were none (○), 4 μM (□), 20 μM (△) and 100 μM (▽). For other details see the text.

Table 2. *Apparent inhibition constants for product inhibition of the spermidine synthase reaction*
The apparent inhibition constants were calculated as described by Baici (1981), and the terminology of the Nomenclature Committee of the International Union of Biochemistry (1982) is used. $K_{ic}^{app.}$ is the apparent competitive inhibition constant for inhibitor i (at non-saturating concentrations of the fixed substrates); $K_{iu}^{app.}$ is the uncompetitive inhibition constant for inhibitor i (at non-saturating concentrations of the fixed substrates).

| Inhibitor | Substrate | $K_{ic}$ (μM) | $K_{iu}$ (μM) | Type of inhibition |
|---|---|---|---|---|
| 5'-Methylthioadenosine | Putrescine | 10–20 | 10–20 | Mixed |
| | Decarboxylated adenosylmethionine | 20 | 20 | Mixed |
| Spermidine | Putrescine | 70–110 | >500 | Mixed |
| | Decarboxylated adenosylmethionine | 80–160 | >500 | Mixed |

to act as a substrate inhibitor of mammalian spermidine synthase (see above), the data obtained from experiments with saturating concentrations of fixed substrates were not used for the calculations of any of the inhibition constants shown in Table 2.

*Specificity for substrates*

A number of diamines and polyamines listed in the Experimental section were tested as substrates in the propylamine transfer reaction catalysed by the purified spermidine synthase. The reaction mixture contained 1mM amine and 15μM decarboxylated adenosyl [*Me*-$^{14}$C]methionine. The formation of labelled 5'-methylthioadenosine was measured. Besides putrescine, the only amines that showed acceptor activity were cadaverine and 1,6-diaminohexane (6% and 1% respectively of that observed with putrescine). These results are in general agreement with those obtained with partially purified (Pegg *et al.*, 1981) and purified (Samejima & Yamanoha, 1982) spermidine synthase from rat ventral prostate. No activity (less than 0.4%) was, however, found with 1,3-diaminopropane or spermidine, which agrees with Samejima & Yamanoha (1982), but is at variance with the results obtained by Pegg *et al.* (1981), who found some acceptor activity with 1,3-diaminopropane (2%) and spermidine (1%).

A number of analogues of *S*-adenosylmethionine and decarboxylated adenosylmethionine have previously been tested as aminoalkyl donors in the spermidine synthase reaction (Coward *et al.*, 1977; Samejima & Nakazawa, 1980; Pankaskie *et al.*, 1981; Samejima & Yamanoha, 1982). Spermidine synthase from rat prostate has been shown to use analogues of decarboxylated adenosylmethionine carrying an ethyl, propyl or butyl group in place of the methyl group as the propylamine donor (Samejima & Nakazawa, 1980), whereas aminoethyl and aminobutyl analogues of decarboxylated adenosylmethionine were inactive as substrates. *S*-Adenosyl-(5')-1-methyl-3-methylthiopropylamine has been shown to be a substrate for spermidine synthase from rat ventral prostate and bovine brain (Pankaskie *et al.*, 1981). Only sulphonium compounds seemed to be active (Samejima & Nakazawa, 1980; Pankaskie *et al.*, 1981). In the present work, chemically synthesized analogues of decarboxylated adenosylmethionine carrying an ethyl, propyl, butyl or carboxymethyl group (see the Experimental section) were tested as substrates for purified spermidine synthase from bovine brain. The incubation mixture (0.2ml) contained 70μM-[1,4-$^{14}$C]putrescine and 50μM biosynthetic decarboxylated adenosylmethionine or 100μM chemically synthesized analogue, corresponding to about 50μM 'active' analogue (see Samejima & Nakazawa, 1980). After 15min incubation the reaction products were separated by paper electrophoresis (see the Experimental section). Taking the amount of spermidine synthesized from biosynthetic decarboxylated adenosylmethionine as 100%, the relative activities for the ethyl, propyl, butyl and carboxymethyl analogues were 50%, 20%, 0% and 9% respectively. The chemically synthesized decarboxylated adenosylmethionine was nearly (82%) as active as was the biosynthetic one. From the above results, it appears that a bulky alkyl group causes a steric hindrance, inhibiting the propylamine transfer reaction.

*Inhibitors*

The effect of various diamines and polyamines (see the Experimental section) on spermidine synthase activity was studied in the presence of 0.1mM-putrescine and 25μM decarboxylated adenosylmethionine labelled in the propylamine moiety. The concentrations of the amine to be studied were 0.1 and 1.0mM. With the exception of cadaverine and 1,6-diaminohexane, which were shown to be substrates of spermidine synthase (see above), all the other amines only weakly (by less than 20%) inhibited the propylamine transfer at 0.1mM concentration. At 1mM concentration, the most potent inhibitors were *NN'*-bis-(2-aminoethyl)propane-1,3-diamine (52%), triethylenetetramine (39%) and 1,3-diaminopropane (34%). Cadaverine inhibited the propylamine transfer by 26% and 39%, and 1,6-diaminohexane by 23% and

30%, at 0.1mM and 1.0mM concentrations respectively.

The effect on spermidine synthesis of the analogues of decarboxylated adenosylmethionine carrying an ethyl, propyl, butyl or carboxymethyl group in place of the methyl group was studied in a system containing 1mM-putrescine and 20$\mu$M decarboxylated adenosylmethionine labelled in the propylamine moiety. None of the analogues inhibited spermidine synthesis by more than 10% at concentrations up to 20$\mu$M (the concentration refers to the 'active' form), whereas the ethylamine analogue of decarboxylated adenosylmethionine [*S*-adenosyl-(5')-2-methylthioethylamine] potently inhibited spermidine synthesis, causing 15%, 42%, 74% and 90% inhibition at concentrations of 10, 50, 200 and 500$\mu$M respectively. These results are in agreement with those reported for the prostatic spermidine synthase (Samejima & Nakazawa, 1980; Samejima & Yamanoha, 1982).

## Discussion

The procedure used in the present study for the purification of spermidine synthase from bovine brain differs somewhat from that used for the isolation of rat prostatic enzyme (Samejima & Yamanoha, 1982). Because the affinity-chromatographic adsorbent is strongly cationic, a high salt concentration (0.6M-NaCl) was used at the affinity-chromatography step for washing of the column, to diminish non-specific binding. Also, the concentration of decarboxylated adenosylmethionine used for specific elution of the enzyme was considerably lower (0.25mM) than that previously employed (Samejima & Yamanoha, 1982). It was somewhat surprising to notice that *S*-adenosylmethionine decarboxylase was not adsorbed on *S*-adenosyl-(5')-3-thiopropylamine–Sepharose, as it has been reported (see Raina & Jänne, 1975) that decarboxylated adenosylmethionine is a powerful inhibitor of this enzyme.

The apparent $M_r$, subunit composition, isoelectric point, pH optimum and substrate specificity of spermidine synthase purified from bovine brain are very similar to those of the enzyme isolated from rat prostate (Samejima & Yamanoha, 1982). The apparent $K_m$ value for decarboxylated adenosylmethionine (0.3$\mu$M) was somewhat lower than that (1.1$\mu$M) determined for the prostatic enzyme (Samejima & Yamanoha, 1982). The same was true for the $K_m$ for putrescine, i.e. 40$\mu$M and 100$\mu$M for the brain enzyme and prostatic enzyme respectively. These differences may partly be explained by high concentrations of the substrates (relative to the $K_m$ values) previously used in determining these kinetic constants. It should also be pointed out that adsorption of decarboxylated adenosylmethionine on glass at very low substrate concentrations should be prevented (see the Experimental section).

The $K_m$ values for decarboxylated adenosylmethionine and putrescine are close to the reported tissue concentrations of these compounds in the rat (Seiler & Lamberty, 1975; Hibasami *et al.*, 1980*b*). Although the concentrations of the substrates have not been determined for bovine brain, it appears plausible that in the brain spermidine synthesis is limited by the supply of both of its precursors. The activities as well as the affinities for decarboxylated adenosylmethionine of spermidine synthase and spermine synthase (Pajula *et al.*, 1979) in bovine brain are very similar. Therefore these enzymes effectively compete for a common substrate, i.e. decarboxylated adenosylmethionine.

Fairly little has been published on the enzymic mechanism of the propylamine transferase reaction. Lack of sensitive assay methods, the very high affinity of propylamine transferases for decarboxylated adenosylmethionine and difficulties arising from the substrate inhibition by decarboxylated adenosylmethionine have hindered a detailed kinetic analysis. However, the recent development of the necessary methodology has made a detailed enzymic analysis feasible.

The converging initial-velocity plot is consistent either with a random-order or an ordered mechanism in which both substrates bind to the enzyme before the release of the products (Cleland, 1963*a*,*c*), and is inconsistent with a ping-pong mechanism that has been proposed for the *Escherichia coli* enzyme by Zappia *et al.* (1980). Evidence supporting a sequential Bi Bi mechanism has also been provided by stereochemical investigations on spermidine synthesis in *E. coli* (Golding & Nassereddin, 1982) and by inhibition studies with transition-state analogues, with spermidine synthase from rat prostate as the enzyme (Tang *et al.*, 1980).

Product-inhibition studies with the purified brain enzyme demonstrated that 5'-methylthioadenosine and spermidine were both mixed inhibitors with respect to both substrates. The apparent $K_{ic}$ and $K_{iu}$ values determined for 5'-methylthioadenosine were quite similar (Table 2), suggesting that this product can effectively combine both with the free enzyme and with the enzyme–substrate complex (Dixon & Webb, 1979). Since all the kinetic studies were carried out at constant pH, $H^+$ ion was not considered as a product and no information is available of the step at which the proton release occurs.

The present kinetic results exclude a ping-pong-type reaction mechanism and suggest a complicated one that probably involves the formation of

abortive ternary complexes as well as some additional complexes (Rudolph, 1979). To define the exact mechanism followed by the spermidine synthase-catalysed reaction, more advanced methods are necessary.

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