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Rapid and Convenient Method for the Assay of Aminopropyltransferases

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A new method for the assay of aminopropyltransferase activity is described. The method measures the formation of [*methyl*-^{14}C]methylthioadenosine from decarboxylated *S*-adenosyl[*methyl*-^{14}C]methionine in the presence of an amine acceptor. When used with extracts from rat ventral prostate, kidney, liver or brain, and with putrescine or spermidine as amines, the method gave results in excellent agreement with those obtained by the much more time-consuming conventional method. It was found that 1,3-diaminopropane and 1,8-diamino-octane were not acceptors for the prostatic enzyme fraction, but 1,5-diaminopentane (cadaverine) was active and 1,9-diaminononane and 1,12-diaminododecane also lead to the production of [*methyl*-^{14}C]methylthioadenosine.

The polyamines spermidine and spermine are synthesized in eukaryotes by the addition of a propylamine group from decarboxylated *S*-adenosylmethionine to an amine acceptor (Tabor & Tabor, 1972, 1976; Raina & Jänne, 1975). Two such aminopropyltransferases have been detected in mammalian cells (Pegg & Williams-Ashman, 1969*a*, 1970; Jänne *et al.*, 1971; Raina & Hannonen, 1971; Hannonen *et al.*, 1972). One of these enzymes (EC 2.5.1.16) is similar to that characterized from extracts of *Escherichia coli* (Bowman *et al.*, 1973) and forms spermidine and methylthioadenosine from putrescine and decarboxylated *S*-adenosylmethionine. The other utilizes spermidine as the preferred acceptor of the propylamine group and forms spermine. Although polyamine biosynthesis has been the subject of many investigations in recent years, relatively little work has been carried out on these aminopropyltransferases compared with the numerous studies of the biosynthetic decarboxylases responsible for the formation of putrescine and decarboxylated *S*-adenosylmethionine (Tabor & Tabor, 1972, 1976; Raina & Jänne, 1975). In part, this is due to the lack of a rapid and sensitive assay for the aminopropyltransferases. The bacterial enzyme was sufficiently active for a coupled spectrophotometric assay for spermidine to be used in some experiments (Bowman *et al.*, 1973), but the mammalian enzymes have been assayed only by following the appearance of radioactive polyamine after incubation with either labelled amine acceptor or decarboxylated *S*-adenosylmethionine labelled in the carbon atoms of the propylamine group (Pegg & Williams-Ashman, 1969*a*, 1970; Jänne *et al.*, 1971; Raina & Hannonen, 1971; Bowman *et al.*, 1973). This assay requires laborious separation of the product from the substrates, involving column chromatography, high-voltage electrophoresis or paper chromatography. It is therefore poorly suited for assay of large numbers of tissue samples and requires radioactive substrates that are not always commercially available. In the present paper we describe a method for the assay of propylamine transferases based on the conversion of decarboxylated *S*-adenosyl[*methyl*-^{14}C]methionine into [*methyl*-^{14}C]methylthioadenosine in the presence of unlabelled amine receptors. Since methylthioadenosine is much less positively charged than decarboxylated *S*-adenosylmethionine in neutral or acidic solution it is readily separated from the substrate. Using this method, we have noted that 1,5-diaminopentane (cadaverine) may also be a substrate for mammalian spermidine synthetase.

Materials and Methods

Animals

Male Sprague–Dawley rats weighing 450–500g were purchased from Charles River Breeding Laboratories, Wilmington, MA, U.S.A., and fed on Purina Rat Chow and water *ad libitum*.

Chemicals

S-Adenosyl-L-[*methyl*-^{14}C]methionine (60.5mCi/mmol), [^{14}C]spermidine trihydrochloride {*N*-(3-aminopropyl)[1,4-^{14}C]tetramethylene-1,4-diamine; 10.03mCi/mmol}, [^{14}C]spermine tetrahydrochloride {*NN'*-bis-(3-aminopropyl)[1,4-^{14}C]tetramethylene-1,4-diamine; 61.9mCi/mmol} and [1,4-^{14}C]putrescine dihydrochloride (72.06mCi/mmol) were purchased

from New England Nuclear Corp., Boston, MA, U.S.A. Putrescine dihydrochloride, spermidine trihydrochloride, spermine tetrahydrochloride and Dowex 50W (X2) were products of Sigma Chemical Co., St. Louis, MO, U.S.A. *Escherichia coli* (strain B, A.T.C.C. 11303) was purchased from Miles Laboratories, Elkhart, IN, U.S.A. All the other chemicals used were purchased from Fisher Scientific Co., Fair Lawn, NJ, U.S.A., or Aldrich Chemical Co., Milwaukee, WI, U.S.A. Decarboxylated *S*-adenosylmethionine was prepared by the action of *S*-adenosylmethionine decarboxylase isolated from *E. coli* and purified on a Dowex 50 (H^+ form) column followed by preparative paper electrophoresis in 0.05M-citric acid buffer (Pösö *et al.*, 1976). The final preparation was free of *S*-adenosylmethionine. Decarboxylated *S*-adenosyl[*methyl*-^{14}C]methionine was prepared from *S*-adenosyl[*methyl*-^{14}C]methionine in the same way starting with 50μCi of the substrate.

Preparation and assay of extracts containing aminopropyltransferase activity

Extracts were prepared from rat ventral prostate, liver, kidney and brain. The tissue was homogenized in 2vol. of ice-cold 25mM-sodium phosphate/5mM-dithiothreitol/0.1mM-disodium EDTA, pH7.2, and centrifuged at 100000*g* for 60min. The supernatant was then fractionated by the addition of $(NH_4)_2SO_4$ (Jänne & Williams-Ashman, 1971). The protein precipitated between 40% and 65% saturation was collected and dialysed overnight against 100vol. of the homogenization buffer. This extract was used as a source of enzyme. The protein content was determined by the method of Lowry *et al.* (1951), with crystalline bovine serum albumin as standard.

Aminopropyltransferase activity in these extracts was measured by the conventional method as described by Jänne & Williams-Ashman (1971), with [^{14}C]putrescine (8.78mCi/mmol) or [^{14}C]-spermidine (3.34mCi/mmol) as substrates. The concentrations of the reagents were as given below, with the omission of the labelled decarboxylated *S*-adenosylmethionine. The results were expressed as nmol of polyamine formed/mg of protein in a 30min incubation and were corrected for recovery of the polyamines through the Dowex 50 column-chromatography and paper-electrophoresis steps by measuring the recovery of authentic labelled polyamines. This recovery was 73±6% for spermidine and 56±5% for spermine.

For the new assay of aminopropyltransferase activity the assay system had a volume of 0.2ml and contained 20μmol of sodium phosphate, pH7.5, 1μmol of putrescine or spermidine, 0.01μmol of decarboxylated *S*-adenosylmethionine, 0.4nmol of decarboxylated *S*-adenosyl[*methyl*-^{14}C]methionine (0.024μCi) and the enzyme (0.2–7.3mg of protein). After incubation for 30min at 37°C, the reaction was stopped rapidly by the addition of 0.2ml of 4M-HCl. A further addition of 0.6ml of 2M-HCl was then made and the mixture centrifuged for 10min at 1600*g* in a bench-top centrifuge. The supernatant was removed and the pellet washed with 2×1ml of 2M-HCl. The combined supernatant and washings were applied to a small column (approx. 5mm× 50mm) of Dowex 50 (H^+ form). The column was then washed with 2M-HCl. The eluate from the column was collected and the first 5ml was assayed for radioactivity after addition of 10ml of Formula-947 LSC scintillation 'cocktail' (New England Nuclear Corp.). The samples were counted for radioactivity in a Beckman LS-350 liquid-scintillation counter, and the efficiency for ^{14}C, which was determined by the use of an internal standard, was 41%. The dimensions of the Dowex 50 column are not critical, and we make up batches of the column in Pasteur pipettes plugged with glass-wool. Decarboxylated *S*-adenosyl[*methyl*-^{14}C]methionine was completely retained by the column under the conditions used, and more than 90% of [*methyl*-^{14}C]methylthioadenosine was present in the first 5ml through the column. (Recently, we have found that lowering the HCl concentration to 1M does not alter the recovery of methylthioadenosine, and this improves the radioactivity-counting efficiency. It is also possible to evaporate the samples to dryness before addition of a suitable scintillation fluid.) The results were corrected for blanks containing no acceptor amine or enzyme.

Results and Discussion

Using the new method for the assay of aminopropyltransferase activity and crude enzyme prepared from a number of tissues, we found that the reaction was proportional both to the time of incubation for at least 60min and to the protein added over the range 0.2–8mg. The production of [*methyl*-^{14}C]methylthioadenosine was entirely dependent on the addition of an acceptor amine, and no increase over the small amount of radioactivity present in samples incubated without enzyme was produced by the addition of enzyme in the absence of an amine acceptor.

Table 1 shows a comparison of the activities of spermine synthetase and spermidine synthetase in crude enzyme fractions prepared from a number of tissues and assayed by the conventional and the new method. There was excellent agreement between the two methods. The values obtained by the old method, in which the conversions of [^{14}C]putrescine into [^{14}C]spermidine or of [^{14}C]spermidine into [^{14}C]-spermine in the presence of unlabelled decarboxylated *S*-adenosylmethionine were measured, were

Table 1. *Comparison of aminopropyltransferase activity in extracts from various tissues assayed by the conventional (A) and the new method (B)*

Full details of the composition of the assay media are given in the Materials and Methods section. The assay media for methods A and B were exactly the same, except for the change in substrate, which was radioactively labelled. Sufficient protein was added to ensure that at least 500c.p.m. above the blank value in method B was obtained at the lowest protein concentration used. At least three measurements at different protein concentrations were made and the means of the results, which agreed ±5%, are shown. The results were calculated as nmol of radioactive product formed/mg of protein per 30min incubation at 37°C. For method A, the product was spermidine when putrescine was the acceptor and spermine when spermidine was the acceptor. For method B the product was methylthioadenosine.

Source of extract	Amine acceptor	Activity (nmol/mg of protein per 30 min) Method A	Method B
Ventral prostate	Putrescine	15.52	13.03
	Spermidine	1.62	1.55
Liver	Putrescine	2.00	1.93
	Spermidine	0.69	0.63
Kidney	Putrescine	0.72	0.69
	Spermidine	0.20	0.19
Brain	Putrescine	2.88	2.71
	Spermidine	1.42	1.32

slightly higher than those obtained by the new method, in which the formation of [*methyl*-^{14}C]methylthioadenosine was determined. However, this slight difference is readily explained by the fact that the values for the conventional method were corrected for recovery of the labelled products during the column chromatography and electrophoresis, but the results from the new material were not corrected for the small amount (about 5%) of [*methyl*-^{14}C]-methylthioadenosine not present in the first 5ml of the column eluate.

The activities of aminopropyltransferases as determined in Table 1 agree with previous studies showing that the rat ventral prostate is the richest source of these enzymes and that the brain has a relatively high spermine synthetase activity (Pegg & Williams-Ashman, 1969*a*; Raina & Hannonen, 1971), but are not in close agreement with other details of the relative activities of these enzymes in mammalian cell extracts (Raina & Hannonen, 1971). However, it should be noted that the extracts that we employed were used merely as convenient polyamine-free sources of these enzymes, and we did not determine if part of the original activity was lost during the preparation of the extracts. It has been reported that a partial separation of spermidine synthetase and spermine synthetase can be produced by fractionation with $(NH_4)_2SO_4$ (Hannonen *et al.*, 1972), and therefore some of the latter enzyme may have been lost in our extracts.

It is quite possible that methylthioadenosine formed in the aminopropyltransferase reaction may be further degraded by other enzymes present in crude cell extracts. Indeed, an enzyme converting this nucleoside into adenine and methylthioribose has been described (Pegg & Williams-Ashman, 1969*b*). However, this would not affect the assay unless the labelled products of further metabolism bind more strongly to Dowex 50 (H^+ form) than methylthioadenosine itself, which is not the case for methylthioribose and is unlikely for other further metabolites. The method is therefore likely to be of general value for assays of aminopropyltransferase activity in crude tissue extracts. Since the K_m for decarboxylated *S*-adenosylmethionine in the synthesis of polyamines is very low (Pegg & Williams-Ashman, 1969*a*, 1970; Hannonen *et al.*, 1972; Raina & Jänne, 1975) and methyl-labelled *S*-adenosylmethionine of very high specific radioactivity is commercially available, it should be possible to use this method to measure aminopropyltransferase activity in extracts from cultured cells where only a limited amount of protein is available. The method does require the preparation of labelled decarboxylated *S*-adenosylmethionine, but this is not more difficult than the preparation of the unlabelled compound needed for the conventional assay.

Finally, the method can be used as a rapid preliminary test to determine whether other amines can be used as acceptors for mammalian aminopropyltransferases without needing to have the amine in a radioactive form or to have a marker for the amine product of the reaction. This possibility may be of some importance, since non-physiological diamines, which repress ornithine decarboxylase, are currently being used as inhibitors of polyamine production (Pösö & Jänne, 1976; Guha & Jänne, 1977; Kallio *et al.*, 1977*a*,*b*; Pegg *et al.*, 1978), and it is critical for these experiments that these diamines do not replace putrescine in the aminopropyltransferase reaction.

Table 2 shows such an experiment, which suggests that 1,3-diaminopropane cannot be used as a substrate by the enzyme fraction from prostate, but that 1,5-diaminopentane can be used. In these respects, the prostatic extract is similar to the enzyme from *Escherichia coli* (Bowman *et al.*, 1973), but this finding differs from the report of Kallio *et al.* (1977*b*), who claim that 1,5-diaminopentane is not a substrate for aminopropyltransferases from Ehrlich ascites cells. A significant production of methylthioadenosine was also seen in response to 1,9-diaminononane and 1,12-diaminododecane, but 1,8-diamino-octane was inactive and 1,7-diaminoheptane was only very

Table 2. *Ability of various diamines to stimulate methylthioadenosine production by prostate aminopropyltransferases*

The assay medium contained 0.24mg of protein from the prostate extract and 0.5mM of the amine shown. Other components were as described in the Materials and Methods section. After incubation at 37°C for 30min the amount of [*methyl*-^{14}C]methylthioadenosine liberated was determined.

Amine added	Methylthioadenosine formed (nmol/30min)
1,3-Diaminopropane	<0.001
1,4-Diaminobutane (putrescine)	2.54
1,5-Diaminopentane (cadaverine)	0.18
1,7-Diaminoheptane	0.03
1,8-Diamino-octane	0.002
1,9-Diaminononane	0.10
1,12-Diaminododecane	0.10
Spermidine	0.23

weakly active. It is unlikely that these results are due to contamination of the amines with putrescine or spermidine, as we were unable to detect such contamination (sensitivity was better than 1%) after separation by high-voltage electrophoresis.

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