

IN VITRO SYNTHESIS OF SPERMIDINE IN THE HIGHER PLANT,VINCA ROSEA

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Cell-free extracts of Vinca rosea seedlings exhibited enzyme activities for the following reactions: S-adenosyl-L-methionine (SAM) decarboxylation, spermidine synthesis from decarboxylated SAM and putrescine, and 5'-methylthioadenosine hydrolysis to 5-S-methyl-5-thio-D-ribose and adenine. SAM decarboxylation was stimulated by putrescine and inhibited by semicarbazide. The 15-fold purified ribohydrolase possessed a  $K_m$  of  $1.03 \times 10^{-5}$  M and a high specificity for 5'-methylthioadenosine.

During the course of an investigation of a transmethylese in the higher plant, Vinca rosea (1), an enzyme-dependent cleavage of S-adenosyl-L-methionine (SAM) to 5-S-methyl-5-thio-D-ribose (MTR) via 5'-methylthioadenosine (MTA) was observed (2). Potentially this enzyme activity could be implicated in a number of pathways. In microorganisms and mammals, enzymes of polyamine biosynthesis (3) carry out a sequence of reactions from SAM to MTA which is converted to MTR by a ribohydrolase. Alternatively phage-induced SAM cleavage enzymes have been found in E. coli (4) whereas in uninfected wild type an enzyme exists which hydrolyzes S-adenosyl-L-homocysteine to adenine and S-ribosyl-L-homocysteine (5). We sought to characterize the ribohydrolase and to determine with which pathway it was associated by examining cell-free extracts of V. rosea for related enzyme activities. In this communication we wish to report on the in vitro synthesis of spermidine in higher plants.

## Materials and Methods

Chemicals - Putrescine, spermidine and spermine hydrochlorides were

obtained from Sigma Chemical Co., SAM from Boehringer-Mannheim, putrescine dihydrochloride-1, 4- $^{14}\text{C}$  (20.94 mCi/mmole), New England Nuclear, SAM (methyl- $^{14}\text{C}$ ) (0.51 mCi/mmole) and SAM (carboxyl- $^{14}\text{C}$ ) (61 mCi/mmole) from Amersham Searle.

MTA and MTR were prepared by hydrolysis of SAM (6, 7) and purified by preparative tlc on silica gel GF-254 using  $\text{CHCl}_3:\text{MeOH}:\text{H}_2\text{O}$  (65:25:4 v/v/v). Mass spectra of MTA and the trimethylsilyl derivative of MTR were in good agreement with assigned structures (8).

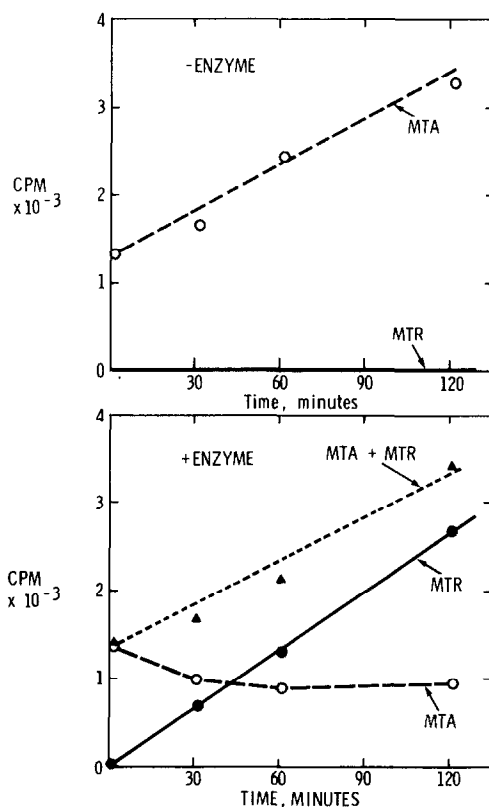
#### Preparation of crude cell-free extracts

(i) For determination of MTA ribohydrolase and spermidine synthetase activities, extracts were prepared from 5 day-old etiolated V. rosea seedlings as previously described (1) except that polyclar AT was omitted and the phosphate buffer was pH 7.5. For spermidine synthetase preparations, extracts were not centrifuged at 10,000 x g. (ii) For SAM decarboxylase, seedlings were extracted by the method of Cappoc et al. (9).

#### Enzyme assays

$^{14}\text{C}$ -MTR formation from (methyl- $^{14}\text{C}$ )-SAM was measured by passing incubation mixtures through Dowex 50W ( $\text{H}^+$ ) (Sigma) and Ag 1X8 (formate) (Bio-rad) columns (0.5 x 5 cm) consecutively, eluting with water in each case. The final eluate contained  $^{14}\text{C}$ -MTR as the only radioactive compound. It was evaporated to dryness, dissolved in methanol and an aliquot counted in a Packard liquid scintillation counter (Model 3380).

$^{14}\text{C}$ -MTR formation from  $^{14}\text{C}$ -MTA was measured by ion exchange chromatography on a Dowex 50W ( $\text{H}^+$ ) column (0.5 x 5 cm) followed by paper chromatography. (Solvent B, reference 10). The band corresponding to MTR was cut out and counted.



Time dependency of SAM (methyl-<sup>14</sup>C) conversion to MTA and MTR.

#### SAM Decarboxylase activity

CO<sub>2</sub> produced from SAM-carboxyl-<sup>14</sup>C was measured by the method of Leinweber and Walker (11).

#### Spermidine synthetase activity

Incubations were carried out anaerobically according to Miller and Gaylor (12) with catalase (1500 units) added. <sup>14</sup>C-Spermidine was isolated from incubation mixtures by Dowex 50W (H<sup>+</sup>) column chromatography followed by paper electrophoresis at pH 3.4 (8).

#### Results and Discussion

Initial experiments revealed SAM cleavage to MTR occurred to the extent of 1.3%. To probe for the intermediacy of MTA in the hydrolysis, we assayed

Table I  
Conversion of MTA (methyl- $^{14}\text{C}$ ) to MTR

Experiment		MTR dpm
1	Complete system	423
	Boiled enzyme	78
2	Complete system	1430
	Complete system + 4 mM MTA	48

Incubation mixtures contained enzyme (72  $\mu\text{g}$  protein; MTA (methyl- $^{14}\text{C}$ ) 13,615 dpm, 26 nmoles) (experiment 1) or SAM (methyl- $^{14}\text{C}$ ), 105,000 dpm, 100 nmole (experiment 2); DTT, 6 mM; in 0.16 ml potassium phosphate buffer, pH 7.2. Incubated at 32° for 2 hr.

for its presence in incubation mixtures with and without enzyme. As shown in Fig. 1, a linear non-enzymatic hydrolysis of SAM to MTA under the conditions of the incubation was observed. MTR formation was enzyme dependent, however, and nonenzymatic synthesis provided sufficient amounts of MTA to account for the sum of MTR and MTA produced in enzyme assays (Fig. 1). Additional evidence for the existence of a MTA ribohydrolase was gained by demonstrating the conversion of  $^{14}\text{C}$ -MTA to MTR with cell-free extracts, and the dilution of radioactivity of MTR by addition of cold MTA to assays in which  $^{14}\text{C}$ -SAM was used as substrate (Table 1).

A 15-fold purification of MTA ribohydrolase was attained by ammonium sulfate fractionation followed by DEAE cellulose and Sephadex G-200 chromatography. The purified enzyme exhibited an apparent  $K_m$  of  $1.03 \times 10^{-5}$  M for MTA and was highly specific. No hydrolase activity was detected with the following substrates at concentrations in the range of 10-50  $\mu\text{M}$ : adenosine,

Table II  
SAM Decarboxylase Activity

Additive	Concentration mM	Relative Activity
None		100
Putrescine	2.5	110-800
Mg <sup>2+</sup>	25	100
Isonicotinic acid hydrazide	5	100
Semicarbazide	5	40
Pyridoxal phosphate	0.2	100

Incubation mixtures contained enzyme (0.1-1 mg protein); S-adenosyl-L-(carboxyl-<sup>14</sup>C)-methionine, 1  $\mu$ Ci, 50-100 nmoles; DTT, 2.5  $\mu$ moles; 0.1 M phosphate buffer, pH 7.4 in 0.6 ml. Incubated for 1 hr at 32°.

adenine nucleotides, inosine, guanosine, SAM, S-adenosylhomocysteine, 6- $\gamma\gamma'$ -dimethylallyladenosine, 5'-tosyladenosine or 5'-trityladenosine. The high specificity for MTA appears exceptional and together with a relatively high  $V_{\max}$  (80 nmoles/hr/mg protein) suggests that this is a physiologically important hydrolysis. Possibly MTA may exert some inhibitory effect which dictates its rapid removal. Alternatively there may be an important role for its hydrolysis products in anabolic pathways.

Assays in triethanolamine buffer exhibited comparable activity to those in phosphate suggesting that the nucleosidase was not phosphorolytic as are previously investigated enzymes from yeast and animals (14,15).

Existence of other enzymes in the polyamine pathway was next investigated. Using carboxyl-<sup>14</sup>C SAM, a SAM decarboxylase activity could be

Table III  
Spermidine Synthetase Activity

Substrate	$^{14}\text{C}$ -Spermidine formed dpm
60% decarboxylated SAM	9140
SAM	1480
None	772
60% decarboxylated SAM (boiled enzyme)	1140

Incubation mixtures contained in 1 ml: 0.1 M potassium phosphate buffer pH 7.5; 1, 4- $^{14}\text{C}$ -putrescine, 3  $\mu\text{Ci}$ , 0.7 mM; SAM or decarboxylated SAM, 0.4 mM; DTT, 4 mM;  $\text{MgCl}_2$ , 10 mM; and enzyme (160  $\mu\text{g}$  protein). Incubated for 1 hr, at 32°. Solutions were  $\text{O}_2$ -depleted with glucose + glucose oxidase + catalase.

detected which was stimulated by putrescine to a variable extent from one preparation to another (Table II). SAM decarboxylases isolated from other higher plants by Williams-Ashman et al. (9) apparently were unaffected by putrescine. Although inhibited by semicarbazide, enzyme activity was neither stimulated by pyridoxal phosphate nor inhibited by isonicotinyl hydrazide leaving the question of prosthetic group for the plant enzyme unresolved. The E. coli enzyme has pyruvate as a prosthetic group (15) whereas the mammalian enzyme has been shown to utilize pyridoxal phosphate (16).

Spermidine synthetase activity from crude cell-free preparations of Vinca rosea were observed under anaerobic conditions with decarboxylated SAM as substrate (Table III). The latter was prepared chemically from SAM, pyridoxal phosphate and  $\text{Mn}^{++}$  (13). Decarboxylation was followed by adding carboxyl- $^{14}\text{C}$  SAM and measuring radioactive  $\text{CO}_2$  release. Since initial

attempts to separate SAM from the decarboxylated product were not successful, the mixture was used directly.

Spermine, spermidine and putrescine are ubiquitous constituents of higher plants (18). Nevertheless, in vitro synthesis of spermidine has not been previously reported. Our first attempts appeared to be thwarted due to interference by active degradative diamine oxidases which are prevalent in higher plants (3). Anaerobic incubation prevented this. Initially, the use of seedlings may have been fortuitous as by analogy with other systems one would expect relatively high rates of synthesis of polyamines in rapidly growing tissue (3). Efforts to further characterize the plant enzymes are in progress.

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