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S-Adenosylmethionine Decarboxylase and Spermidine Synthase from Chinese Cabbage[1]

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ABSTRACT

The enzyme, *S*-adenosylmethionine (SAM) decarboxylase (EC 4.1.1.50), has been demonstrated in leaves of Chinese cabbage, (*Brassica pekinensis* var Pak Choy). All of the enzyme can be found in extracts of the protoplasts obtained from the leaves of growing healthy or virus-infected cabbage. The protein has been purified approximately 1500-fold in several steps involving ammonium sulfate precipitation, affinity chromatography, and Sephacryl S-300 filtration. The reaction catalyzed by the purified enzyme has been shown to lead to the equimolar production of CO_2 and of decarboxylated *S*-adenosylmethionine (dSAM). The K_m for SAM is 38 micromolar. The reaction is not stimulated by Mg^{++} or putrescine, and is inhibited by dSAM competitively with SAM. It is also inhibited strongly by methylglyoxal bis(guanylhydrazone). The enzyme, spermidine synthase (EC 2.5.1.16), present in leaf or protoplast extracts in many fold excess over SAM decarboxylase, has been purified approximately 1900-fold in steps involving ammonium sulfate precipitation, affinity chromatography, and gel filtration on Sephacryl S-300. Standardization of the Sephacryl column by proteins of known molecular weight yielded values of 35,000 and 81,000 for the decarboxylase and synthase, respectively.

The reactions leading from methionine to spermidine have been studied since 1956 and have been demonstrated to proceed through the formation of SAM,[2] its decarboxylation to dSAM, and aminopropyl transfer to putrescine (22). However, several types of difficulty have tended to obscure the nature of the enzyme-catalyzed decarboxylation. Bacteria, yeast, and animal cells do contain a well characterized SAM decarboxylase (23). However, in crude extracts, the usual test substrate, [1-^{14}C]*S*-adenosylmethionine may yield $^{14}CO_2$ by enzymatic reactions other than via a direct decarboxylation of SAM (19, 24, 25). Furthermore an extensive nonenzymatic decarboxylation of SAM can be obtained in the presence of pyridoxal phosphate and Cu^{++} or Mn^{++} ions (27). Since the liberation of $^{14}CO_2$ from [1-^{14}C]SAM, particularly with crude extracts of plant materials, is not convincing evidence of the presence of SAM decarboxylase, the demonstration of a purified protein with an activity catalyzing the conversion of SAM to dSAM is required. In the presumed demonstration of this enzyme from etiolated seedlings of *Lathyrus sativus* (19), a small amount of activity was concentrated and purified by affinity chromatography. The stoichiometry of the reaction was not determined nor was the amount of activity related to the amount of spermidine synthesized in the plant. This activity, as well as that found in mung bean sprouts (6), was shown to be Mg^{++} dependent, as are some bacterial SAM decarboxylases. The levels of biosynthetic activity detected in extracts of these plant materials were extremely low and may have arisen from bacterial contamination. On the other hand, the activity of SAM decarboxylase isolated from corn seedlings (20) has been reported to be independent of Mg^{++} or putrescine, as is the enzyme we have now isolated from Chinese cabbage. Nevertheless, the low level of activity of the corn enzyme, by comparison to the amount of spermidine found in plants, has similarly raised the problem of the significance of this activity.

One product of the SAM decarboxylase reaction, dSAM, is difficultly available. Despite its recent synthesis, an extinction coefficient has not been published (15) and a direct measurement of the stoichiometry of the decrease of SAM and appearance of dSAM has not previously been attempted. For this reason the decarboxylation reaction catalyzed by several presumed SAM decarboxylases cannot be considered unequivocal. The compound, dSAM, was generated from SAM by the *Escherichia coli* enzyme in 87% yield, calculated on the assumption that dSAM had an extinction coefficient identical to that of SAM (21, 28). Low yields of dSAM via enzymatic decarboxylation of SAM, arise from the relatively high K_m of SAM in the reaction, as well as feedback inhibition by dSAM. Thus, stoichiometry in the decarboxylation has been demonstrated only by coupling spermidine synthase and putrescine to the system to trap the aminopropyl of dSAM in spermidine and to compare the yield of this triamine to that of CO_2 (12, 14).

A newly developed analytical system has been applied to the determination of picomole amounts of SAM and dSAM (9). We have determined the course of the SAM decarboxylase reaction and have established the equimolar production of dSAM and CO_2 from SAM by the cabbage enzyme in the absence of other detectable reactions.

We have been studying the control of the synthesis of spermidine in normal and virus-infected protoplasts of Chinese cabbage (4). The enzyme, spermidine synthase, is inhibited by dicyclohexylamine (17) and this inhibitor acts relatively specifically on this enzyme in the protoplasts (9). Nevertheless dSAM continued to be generated from SAM and was diverted to spermine synthesis (9). Furthermore, during the multiplication of turnip yellow mosaic virus at least, newly synthesized spermine partially replaced spermidine in the virus without affecting the course of synthesis of viral RNA, viral protein, and virions (2, 3). The inhibitor of SAM decarboxylase, MGBG, did not inhibit spermidine synthesis in the protoplasts. It was therefore necessary to determine if a SAM decarboxylase did exist in the protoplasts,

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[2] Abbreviations: SAM, *S*-adenosylmethionine; dSAM, decarboxylated *S*-adenosylmethionine or *S*-adenosyl(5′)-3-methylthiopropylamine; ATPA, *S*-adenosyl(5′)-3-thiopropylamine; MGBG, methylglyoxal bis(guanyl hydrazone); PCA, perchloric acid.

and if such an enzyme was inhibitable by MGBG. We have found a SAM decarboxylase in Chinese cabbage in amounts sufficient to account for the observed rate of spermidine accumulation. The enzyme has been purified, and its activity is independent of Mg^{++} and of putrescine. The enzyme is inhibited by MGBG in a manner comparable to the putrescine-dependent enzyme of fungi and animal cells.

MATERIALS AND METHODS

Chemicals. Putrescine dihydrochloride and spermidine trihydrochloride were purchased from Calbiochem and were recrystallized before use. Ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride, DTT, BSA, *S*-adenosylhomocysteine and 5′-thiomethyladenosine were obtained from Sigma Chemical Co. Methylglyoxal bis(guanylhydrazone) dihydrochloride monohydrate (MGBG) was purchased from Aldrich Chemical Co. and AH-, CH-Sepharose, Sephadex G-25, and Sephacryl S-300 from Pharmacia Fine Chemicals. EDTA dihydrate was obtained from Fischer Scientific Co. and $MgCl_2 \cdot 6H_2O$ from J. T. Baker Chemical Co.

[1-^{14}C]SAM (55.8 mCi/mmol), [2-^{14}C]SAM (57.2 mCi/mmol), and [1,4-^{14}C]putrescine dihydrochloride (107.1 mCi/mmol) were purchased from New England Nuclear Co. and unlabeled SAM was obtained from Sigma Chemical Co. These compounds were purified by ion exchange chromatography on SP-Sephadex (Pharmacia) (8). Decarboxylated SAM (dSAM) and *S*-adenosyl (5′)-3-thiopropylamine hydrogen sulfate (ATPA) were kindly given to us by Dr. K. Samejima of the Tokyo Biochemical Research Institute (15). All other chemicals were of the highest purity available commercially.

In addition to the proteins obtained in the gel filtration kit (Pharmacia), alcohol dehydrogenase and catalase were purchased from Sigma and creatine phosphokinase was bought from Calbiochem.

Growth of Plants and Preparation of Protoplasts. Normal Chinese cabbage (*Brassica pekinensis*, var Pak Choy) plants were grown from seeds as described previously (9). At an appropriate 4-leaf stage (approximately 23 d), the rosettes were removed and in some plants the two outer leaves were infected with turnip yellow mosaic virus. New leaves were regrown in both the healthy and the infected plants. In the latter case, at 12 to 13 d postinfection, essentially all the new leaf cells were demonstrably infected. Protoplasts were isolated from healthy and infected leaves as described (2, 4).

Preparation of Extracts. Extracts were prepared from the newly emerging leaves of both control and infected plants at 12 d postinfection and from their derived protoplasts. In several experiments half-leaves were used for homogenization to prepare extracts and the remaining half-leaves were used to isolate protoplasts.

Healthy and infected leaves were rinsed with cold distilled water and dried with absorbent paper. Chilled (4°C) 25 mM Tris-HCl (pH 7.2), containing 10 mM DTT and 1 mM EDTA (buffer A) was added in the amount of 3 ml/gm of leaf and homogenized by hand in a chilled glass homogenizer. Samples containing about 10 to 11 gm of leaf were homogenized and centrifuged at 23,000 *g* for 60 min at 4°C. Of the supernatant fraction (34–43 ml), 1.5 ml were applied to columns of Sephadex G-25 (0.8 × 18 cm), equilibrated, and subsequently washed with the Tris buffer to yield filtrates of 3.0 ml.

Protoplast suspensions derived from comparable leaf weights, *e.g.* 6.1 to 7.4 × 10^7 cells in 40 ml were disrupted completely by pumping the suspensions three times in a syringe through a 3-cm, 21-gauge needle. The suspension was then centrifuged at 28,000 *g* for 30 min at 4°C. Again 1.5 ml of each supernatant fluid was passed through Sephadex G-25 and filtrates of 3.0 ml were obtained. The yields of protoplasts from the leaves were determined from the yields of Chl (1) in the protoplasts and leaves and were in the range of 28 to 30%.

Enzyme Assays. The plants were grown at 28°C and all assays were done at this temperature. SAM decarboxylase was assayed by measuring the evolution of $^{14}CO_2$ from [1-^{14}C]SAM (5). The assay mixture contained 100 mM Tris-HCl buffer (pH 7.2), 3 mM DTT, 0.1 mM EDTA, 30 μg BSA, 75.5 μM [1-^{14}C]SAM, and the enzyme in a total volume of 0.4 ml.

The reaction was carried out in the 12 × 75 mm screw-capped tubes. A hypodermic needle (21 gauge, 1¼ inches) was inserted through the cap of the tube. To the needle was attached a 38.5-mm^2 disc of Whatman No. 3 MM filter paper, moistened with 15 μl of 1 M KOH. The reaction was started by adding the substrate. After incubation at 28°C for 60 min, the reaction was stopped by injecting 0.15 ml of 1.5 N PCA with a 1-ml syringe. The tubes were then shaken at 37°C for 1 h to release $^{14}CO_2$ from the medium. The filter paper disc carrying $^{14}CO_2$ was then transferred to a scintillation vial and counted with 1 ml of H_2O and 10 ml of Hydrofluor (National Diagnostics) in a Liquid Scintillation Spectrometer (Packard). One unit of the activity is the amount of enzyme required to form 1 nmol of $^{14}CO_2$/min under the assay conditions.

Spermidine synthase was assayed by a method reported previously (26). The reaction mixture contained 0.1 M sodium phosphate buffer (pH 7.2), 0.1 μCi [1,4-^{14}C]putrescine in 1 mM putrescine, 0.05 mM dSAM, 5 mM DTT, 0.3 mM EDTA, BSA 20 μg, and enzyme solution in a total volume of 0.5 ml. After incubation for 30 min at 28°C, 100 μl of 1.5 N PCA was added to the incubation mixture. After centrifugation for 10 min at 1000 *g*, 400 μl of the supernatant was diluted with 4 ml of 0.05 M pyridine. The solution was then applied to a small carboxymethyl cellulose column (0.75 ml, CM 22, Whatman in a column 0.6 × 2.7 cm) equilibrated with pyridinium acetate buffer (pH 5.0). After successive elution with 0.1 and 0.4 M buffer, labeled spermidine was eluted with 3 ml of 0.5 M buffer. A 2-ml aliquot of eluate was counted with 10 ml of Hydrofluor. One unit of the enzyme activity represents the formation of 1 nmol spermidine/min under the assay conditions.

Protein was measured by the method of Lowry *et al.* (11) after precipitation with 80% acetone or 1.5 N PCA. Crystalline BSA was used as a standard.

HPLC. This equipment and its capabilities in the separation, characterization, and quantification of SAM and dSAM have been described in detail (9).

RESULTS

Enzyme Activities in Crude Extracts. The activities of the SAM decarboxylase of leaves and protoplasts derived from healthy and infected plants, as described above, were compared in the centrifuged supernatants and the G-25 filtrates of the extracts. It was found that the activities were increased 5- to 6-fold in the presence of 10 mM DTT. A further small increase was obtained in 30 mM DTT, but 10 mM DTT was used in all subsequent assays.

The step of G-25 filtration was introduced when it was observed that the linearity of the rates of decarboxylation fell off significantly as the amount of extract was increased in the enzyme assay. On the other hand, the activity of the assay was linear over a 15-fold range of concentration of the G-25 filtrate. In Table I are presented data on the activities of the supernatant fluids of crude extracts compared to those of G-25 filtrates. It can be seen that the removal of low molecular weight inhibitors was obtained by gel filtration, permitting a 40 to 90% increase in the activities of the enzyme in extracts of both leaves and protoplasts.

The activity slowly decayed at 0°C in the presence of DTT, but did so more rapidly in the supernatant fluid than in the

Table I. *SAM Decarboxylase Activities of Relatively Crude Extracts*

Estimated from yields of Chl the recoveries of the protoplasts were in the range of 28 to 30%. In these experiments, approximately 2×10^7 protoplasts were present per g leaf.

Material	Healthy		Infected	
	Supernatants	G-25 filtrates	Supernatants	G-25 filtrates
Leaves[a]	465	901	624	912
Protoplasts[b]	21.9	34.9	43.4	61

[a] Values for leaves are presented in pmol per g leaf. [b] Values for protoplasts are presented in pmol per 10^6 cells.

G-25 filtrate. The $t_{1/2}$ were approximately 3 d in the former and 8 d in the latter. On heating at 100°C in a boiling water bath for 5 min, the residual activity ($^{14}CO_2$ production from 40 μM [1-^{14}C]SAM) was 20% for the supernatant fluid but fell to 5% in the G-25 filtrate of the extracts. In the latter system then, the decarboxylation was almost entirely dependent on material with the apparent heat sensitivities of most proteins.

In studies comparing extracts of leaves and protoplasts, young leaves of 5 to 7 cm in length had been used to obtain the maximal yields of protoplasts from the leaves. The larger leaves containing approximately twice the decarboxylase activity per gram of leaf were used in the subsequent isolation and purification of the enzyme.

The spermidine synthase content of the same G-25 filtrates presented in Table I revealed activities of this enzyme 22 to 27 times that of the decarboxylase. Thus spermidine synthase is in great excess in the protoplasts and plants, assuring the low concentration of dSAM previously reported (9).

The activities of the SAM decarboxylase present in the G-25 filtrates of 10^6 protoplasts of healthy or infected growing leaves were used to calculate the time necessary to provide the dSAM to duplicate the spermidine plus spermine contents of comparable protoplasts. Typical values are 4.6 and 9.1 nmols of the two amines in 10^6 healthy and infected protoplasts respectively (4). It would take approximately 5½ and 6 d, respectively for these cells to synthesize the necessary dSAM via SAM decarboxylase under the approximately optimal conditions presented.

Both the mass and polyamine content of the infected leaves above the cotyledons increased 4-fold in the interval of 7 to 25 d after infection. The increment of spermidine per plant was found to be linear with respect to time in an experiment in which six time points were taken using the leaves of three plants each (unpublished data of K. McCarthy in this laboratory). The doubling time of spermidine accumulation was found to be 7 d. Thus the SAM decarboxylase of the cells of the infected leaf appears to be sufficient to account for all of the spermidine found in the growing infected plant.

Purification of SAM Decarboxylase. In a typical experiment, a crude extract was made from 7- to 12-cm leaves (322 gm) of 35-d-old plants. The cold extract was centrifuged at 23,000 *g* for 60 min. The supernatant fraction (1,200 ml) was adjusted to 35% saturation with solid ammonium sulfate at pH 7.0 at 4°C. After stirring gently for 1 h, the inactive precipitate was removed by centrifugation at 23,000 *g* for 30 min. The supernatant fluid was similarly adjusted to 60% saturated ammonium sulfate and the precipitate, containing the enzyme, was collected by centrifugation. The protein was dissolved in 80 ml Tris Buffer A containing 0.3 M NaCl and the solution was filtered through similarly equilibrated Sephadex G-25 (5 × 40 cm) to remove ammonium sulfate. The G-25 eluate (172 ml) was then passed through a column of MGBG-Sepharose (4 × 3.5 cm) (13). The adsorbed decarboxylase was eluted with 70 ml of Buffer A containing 0.3 M NaCl and 1 mM MGBG. Aliquots of the various fractions were assayed with highly radioactive [1-^{14}C]SAM to locate the enzyme. The active fractions (50 ml) were pooled and precipitated with 80% saturated ammonium sulfate. The precipitates were dissolved in Buffer A containing 0.3 M NaCl and passed through G-25 equilibrated with Buffer A containing 0.3 M NaCl. The filtrate was concentrated by centrifugation in Centriflo cones to 1.5 ml and applied to a column of Sephacryl S-300 (1.4 × 87 cm) equilibrated with 0.1 M phosphate buffer (pH 7.2), containing 0.3 M NaCl. Elution of the enzymes was detected by assaying numerous 1-ml fractions of the eluate. As will be seen in a later figure, three protein components, in addition to the enzyme, were detected, of which two were of higher mol wt and a third of quite low mol wt. The activities of the fractions are presented in Table II. The procedure resulted in a purification of 1500-fold with a recovery of 25%.

Effects of Ions, Amines, Reaction Products, and other Compounds. The data in Table III help to develop optimal conditions for the assay and provide clues to the regulation of activity in the organism. Unlike the SAM decarboxylase of many procaryotic and eukaryotic organisms, neither Mg^{++} nor putrescine activate the enzyme purified to the third step of purification, as presented in Table II. Indeed putrescine is slightly inhibitory at concentrations above 5 mM, and this may be one of the inhibitory substances eliminated by G-25 filtration.

The reaction product, dSAM, is significantly inhibitory at 3.6 μM, which will be seen below to represent a concentration attained when the reaction has proceeded about 5% under optimal

Table II. *Purification of SAM Decarboxylase from Chinese Cabbage*

Step	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*units*	*units/mg protein*	*× fold*	*%*
Crude extract[a]	786	29.0	0.037	1	100
35–60% saturated[a] $(NH_4)_2SO_4$ ppt.	412	28.1	0.068	1.8	97
MGBG-Sepharose eluate[a]	0.35	10.7	30.4	822	37
Sephacryl S-300	0.13	7.3	56.2	1520	25

[a] Activity is tested after Sephadex G-25 filtration.

Table III. *Effects of Magnesium, Polyamines, and some Substances on SAM Decarboxylase Activity*

Compounds	Concentration	Relative Enzyme Activity
	mM	*%*
None		100
$MgCl_2$	0.1	100
	1.0	100
	5.0	101
	25.0	105
Putrescine	1.0	101
	5.0	93
	25.0	77
Spermidine	1.0	96
	5.0	87
dSAM	0.0036	89
	0.018	61
	0.036	43
S-adenosyl homocysteine	0.5	108
	1.0	109
5′-Methyl thioadenosine	0.5	105
	1.0	105
ATPA	0.5	105
	1.0	105
MGBG	0.006	76
	0.024	43

conditions. Thus maximal rates must be estimated very early in the reaction. Spermidine is far less inhibitory and is unlikely to be a major regulatory substance active on this enzyme. Adenosylhomocysteine and methylthioadenosine, related intimately to other facets of the metabolism of SAM, do not appear to affect the decarboxylase, nor does ATPA, which has been used to adsorb the plant spermidine synthase in affinity chromatography (16). Finally, MGBG is found to be a very active inhibitor of the decarboxylase, a fact which related both to the use of the substance in the purification of the enzyme in affinity chromatography and the need to remove the 1 mM MBGB from the eluate by G-25 filtration in the subsequent assay of the recovered enzyme.

The pH Dependence of the Decarboxylase. Although the activity of the enzyme changed little between pH 7.0 and 7.5 (Fig. 1), for the purpose of the assay the purified enzyme was taken as maximally active in phosphate buffer at pH 7.3 to 7.4. The activity in phosphate was almost 30% higher than in Tris buffer at the same pH. The activity was slightly higher in a glycine buffer at pH 8.3 than in the Tris buffer at pH 7.4.

SAM Requirements and Inhibitors. The early maximal rate of decarboxylation of [1-^{14}C]SAM has been determined with the 800-fold purified enzyme over a range of concentrations of SAM in the absence and presence of the two inhibitors, the reaction product, dSAM, and MGBG. The data plotted in the Lineweaver-Burk mode are presented in Figures 2 and 3, respectively.

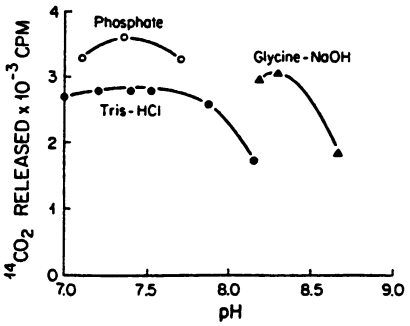

FIG. 1. Activity of SAM decarboxylase as a function of pH in various buffers. Assay conditions are described in the text; however the buffer was changed as required.

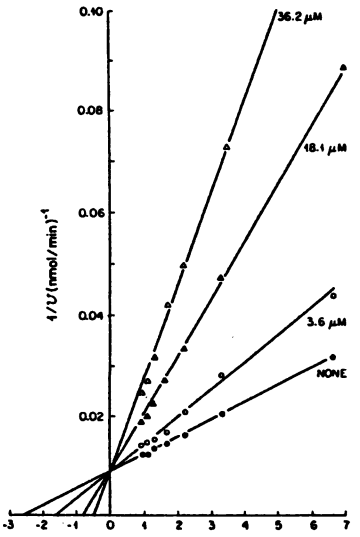

FIG. 2. SAM requirement of purified SAM decarboxylase and the inhibition of the enzyme by the reaction product, dSAM. Without dSAM (●). The concentrations of dSAM were 3.6 μM (○), 18.1 μM (▲), and 36.2 μM (△). For other details see the text.

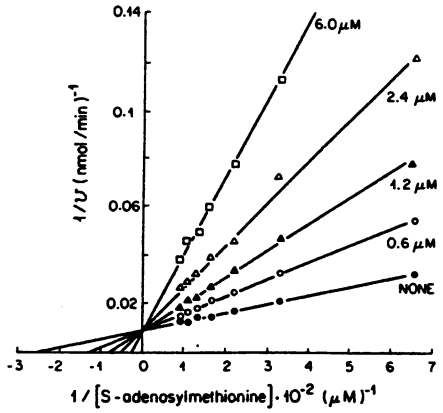

FIG. 3. Inhibition of SAM decarboxylase by MGBG as a function of SAM concentration. Without MGBG (●). The concentrations of MGBG were 0.6 μM (○), 1.2 μM (▲), 2.4 μM (△), and 6.0 μM (□). For other details see the text.

Table IV. *Decarboxylation of [2-^{14}C]SAM to [1-^{14}C]dSAM*[a]

Time	SAM		dSAM	
	Peak area (PA)[b] × 10^3	PA/cpm[c]	Peak area[b] × 10^3	PA/cpm[c]
min				
0	8034	262.5	0	0
30	7907	256.1	186.7	266.8
60	7841	235.5	298.3	284.4
90	7667	255.6	383.1	280.0
120	7686	263.3	445.6	283.8
0 enzyme	7923	259.5		0

[a] The reaction proceeded to almost 5% of completion. [b] Mean of duplicate estimations. [c] Minus background. The mean of PA per cpm of dSAM was 278.8, compared to a mean of 259.4 for five values for SAM. The 60-min value for SAM was excluded from the average.

By extrapolation of the rates of the uninhibited enzymes to the abscissa, the K_m for SAM was found to be 38 μM. Both inhibitors may be described as competitive, with the inhibitors considered to compete with the substrate, SAM, for the enzyme. The inhibition constants, K_i, for dSAM and MGBG are 6 μM and 0.6 μM, respectively.

Stoichiometry of the SAM Decarboxylase Reaction. By means of HPLC, it has been possible to estimate very low levels of dSAM and SAM, as well as to estimate the amounts of radioactivity in these separated compounds from suitably labeled precursors (9). Synthetic dSAM has been defined as $C_{14}H_{24}N_6O_3S \cdot 2H_2SO_4 \cdot 1/2$ ethanol (15). Using this material, and assuming its purity, a standard curve of nmoles dSAM *versus* peak area was constructed in the HPLC. The standard curve was linear over a range of dSAM analyzed in subsequent reaction mixtures. The yields of dSAM were then compared in the enzymatic decarboxylation of [1-^{14}C]SAM and [2-^{14}C]SAM using these substrates purified on SP-Sephadex (8). The amount of enzyme was limited to permit 5% of the reaction to proceed over 2 h.

The purified enzyme behaved essentially identically in the standard assay with both substrates; 0.4 nmol amounts of dSAM were readily estimated. To minimize any assumptions of the purity of dSAM or of the extinction coefficient of these metabolites, the conversion of [2-^{14}C]SAM to [1-^{14}C]dSAM was studied, in order to compare the peak area per units of radioactive carbon of both substances. Equivalent radioactivities of SAM and the derived dSAM are assumed to reflect identical numbers of molecules. As can be seen in Table IV, the mean peak area

per cpm of the dSAM, *i.e.* 289.8, was found in duplicate estimations at four time points to be 7.5 ± 2.1% greater than that of the mean peak area per cpm of SAM, *i.e.* 259.4. In subsequent estimations of the molar content of dSAM, the peak areas of this metabolite were divided by 1.075.

The stoichiometry of production of $^{14}CO_2$ and dSAM from [1-^{14}C]SAM was then estimated. The radioactivity of SAM was estimated under conditions in which $^{14}CO_2$ would be measured, *i.e.* on a KOH-containing paper disc, and compared with its radioactivity when SAM was dissolved in the scintillation fluid. A 3% quenching of cpm on the disc was detected. The specific activity of the substrate, *i.e.* cpm per nmol, was determined from the radioactivity and extinction coefficient of the SP Sephadex-purified [1-^{14}C]SAM, as well as the peak area per cpm of this material after HPLC separation. Duplicate measurements of decarboxylation over four time points were analyzed for yield of $^{14}CO_2$ and dSAM. The results presented in Table V indicate an average peak area per cpm of the original SAM of 150 ± 7 and the ratio of peak area of dSAM to cpm of $^{14}CO_2$ was 160 ± 7. These results indicate that the molecular ratio of the yields of dSAM and CO_2 from SAM closely approaches unity, in a reaction in which the purified enzyme catalyzes the earliest phase of the decarboxylation.

Molecular Weight of SAM Decarboxylase. The estimation of mol wt of SAM decarboxylase was made with the calibrated column of Sephacryl S-300 used for the final step of purification of this enzyme. As seen in Figure 4A the enzyme activity was obtained in a smooth curve of eight fractions; these did not coincide with protein concentrations detectable at 280 nm.

Table V. *Stoichiometry of SAM Decarboxylase Reaction with [1-^{14}C] SAM*

Time	[1-^{14}C]SAM			dSAM,	$^{14}CO_2$,	
	Peak area (PA)[a] × 10^3	cpm	PA/cpm	Peak area[a] × 10^3	cpm[b]	PA/cpm
min						
30	8,150	57,000	143	890.7	5,124	174
60	8,480	56,000	152	1234	7,718	160
90	8,020	56,000	143	1536	9,721	158
120	7,790	48,000	162	1640	11,028	149

[a] Mean of duplicate estimations. Divided by factor 1.075, as shown in Table IV. [b] Corrected for reduction of counting efficiency by disc.

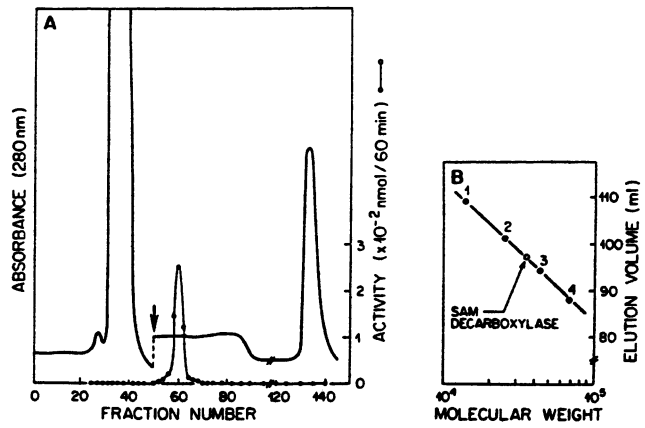

FIG. 4. A, Elution pattern of SAM decarboxylase activity from Sephacryl S-300. Arrow indicates a change to increased sensitivity (2.5-fold) of the absorbance monitor. B, Estimation of the mol wt of the decarboxylase by gel filtration compared to standard proteins. 1, Ribonuclease A (mol wt 13,700); 2, chymotrypsinogen A (mol wt 25,000); 3, ovalbumin (mol wt 43,000); 4, BSA (mol wt 67,000).

After removal of the enzyme and other proteins of step 4, standard proteins were passed through the column and the positions of elution were noted, thereby calibrating the column. The positions of the various proteins are given in Figure 4, as is the position of the SAM decarboxylase in the previous elution. It was found that the maximum of enzyme elution occurred at a position defining a protein of 35,000 D.

Purification of Spermidine Synthase. This enzyme had been detected in Chinese cabbage and purified approximately 150-fold (4, 17). The sample of ATPA in this laboratory's possession during those studies did not permit the preparation of a suitable affinity column. In earlier studies such a column had previously increased the purification of animal enzymes (16). The column constructed in this laboratory earlier did not release the enzyme at all. This experiment has not been repeated with newly synthesized ATPA; this substance has served quite well in preparing ATPA-Sepharose for affinity chromatography, the enzyme being eluted with dSAM as described previously (16).

Steps of purification of this enzyme were carried out through step 2 (Table II), as in the isolation of SAM decarboxylase. The ammonium sulfate precipitate was dissolved in 0.1 M phosphate (pH 7.2), and applied to a G-25 column (5 × 40 cm) equilibrated with phosphate buffer. The eluate was passed through an ATPA-Sepharose column (2.5 cm wide × 1 cm deep) equilibrated with 0.1 M phosphate (pH 7.2) containing 0.3 M NaCl. Spermidine synthase was eluted with 10 ml of equilibration buffer containing 5 mM dSAM. The effectiveness of this step with active ATPA has been demonstrated in three separate experiments with different extracts.

The eluted enzyme was concentrated by centrifugation in Centriflo cones to 1.5 ml and applied to a column of Sephacryl S-300 (1.4 × 87 cm) equilibrated with the phosphate buffer containing 0.3 M NaCl. The enzyme was assayed in 1.7-ml fractions (80 fractions were collected). Spermidine synthase appears to have separated in two active components of five closely associated fractions (Fig. 5A). These have been pooled and Table VI presents the complete course of the purification.

Affinity chromatography had effected almost a 500-fold increase in specific activity, with an additional 4-fold purification by Sephacryl. The last fractions in the Sephacryl separation contained dSAM. G-25 filtrates are unstable when stored at 4°C and indeed the Sephacryl-purified enzyme stored similarly is even more unstable.

When, after elution of the enzyme, the Sephacryl column was calibrated with various proteins, as described in an earlier section, the more slowly eluting fraction of synthase was found to have

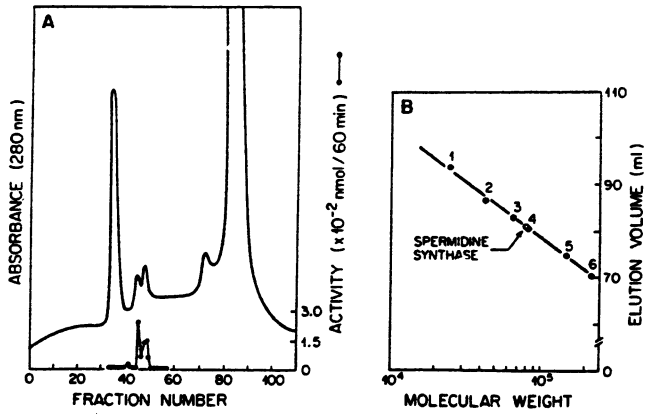

FIG. 5. A, Elution pattern of spermidine synthase activity from Sephacryl S-300. B, Estimation by gel filtration of the mol wt of spermidine synthase. 1, Chymotrypsinogen A; 2, ovalbumin; 3, BSA; 4, creatine phosphokinase (mol wt 81,000); 5, alcohol dehydrogenase (mol wt 150,000); 6, catalase (mol wt 232,000).

Table VI. *Purification of Spermidine Synthase*

Step	Total Protein	Total Activity	Specific Activity	Purification	Yield
	mg	*units*	*units/mg protein*	*× fold*	%
Crude extracts[a]	209	117	0.56	1	100
35–60% $(NH_4)_2SO_4$ ppt[a]	125	70	0.56	1	60
ATPA-Sepharose eluate[a]	0.143	39	272.7	487	33
Sephacryl S-300	0.014	15	1071.4	1913	13

[a] Activity is tested after Sephadex G-25 filtration.

appeared in a position of a protein of 81,000 D. These data are seen in Figure 5B. The pooled activities were run in gel electrophoresis (7) and the gel was sliced, extracted, and assayed. Activity was obtained in two widely separate regions of the gel. However this electrophoretic separation has only been observed once and will require further study.

DISCUSSION

A SAM decarboxylase is present in Chinese cabbage; the enzyme is present in amounts sufficient to serve as a major source of the dSAM used by the plant for the synthesis of spermidine and spermine. The liberation of $^{14}CO_2$ from [1-^{14}C] SAM by the purified enzyme has been shown to be accompanied by the formation of a stoichiometric amount of dSAM. The molar equivalence of the two products has been demonstrated by a new method, taking advantage of the sensitivity and discriminating power of computerized HPLC for SAM and dSAM, and the availability of [2-^{14}C]SAM. This substrate permitted the numbers of molecules of SAM and dSAM to be equated by means of the radioactivity contained in the substrate and product. The reaction catalyzed by our isolated plant enzyme can indeed be designated as that of a decarboxylation of SAM, and the enzyme as SAM decarboxylase.

It was found that the SAM decarboxylase is contained essentially entirely within cells, unlike the amine oxidase, reported to be present on cell walls (10). The decarboxylase is not present in excess, unlike the spermidine synthase. Thus in Chinese cabbage, which generates large amounts of putrescine under many conditions, the SAM decarboxylase appears to be the rate-limiting enzyme for the production of spermidine and spermine. It is not clear why a comparable activity in terms of both the amount of SAM decarboxylation and the properties of the enzyme has not been found in other plants.

The 20-fold excess of spermidine synthase over SAM decarboxylase is one of many possible solutions to the problem of maximizing the utilization of dSAM by this short enzyme sequence producing spermidine. The enzymes are apparently not combined in a complex which might reduce the K_m for dSAM of the second enzyme. The K_m for dSAM is 6.7 μM in the spermidine synthase reaction, a good deal lower than the K_m for SAM, *i.e.* 38 μM, in the enzymatic decarboxylation. Furthermore both enzymes appear freely soluble and essentially maximally active in a homogenate of protoplasts, suggesting that both may be cytosolic, as has been demonstrated for spermidine synthase (18). The latter, at least, does not appear to be compartmented into a tiny specialized organelle which generates spermidine from SAM. Furthermore neither enzyme appears to have a specific activator, the presence or absence of which can expand or contract the activity of the sequence. In this respect the plant SAM decarboxylase differs sharply from the animal or yeast enzyme whose activity turns on the availability of putrescine, which is potentially far more abundant in a plant. It differs also from bacterial systems activated by Mg^{++}, whose availability in plants may be controlled in growth by the deposition of Mg^{++} in Chl. In this connection it would be important to know the concentration of free Mg^{++} in early stages of leaf growth.

The result showing MGBG to be a good inhibitor of the decarboxylase, competitive with SAM, with a K_i resembling that with the animal enzyme, was surprising. In unpublished work by M. Greenberg and R. Sindhu, of this laboratory, it had been shown that millimolar MGBG did not markedly inhibit synthesis of spermidine in protoplasts; we have concluded that MGBG did not penetrate readily in these cells to the sites of decarboxylase action.

The mol wt of the cabbage SAM decarboxylase, 35,000, is clearly different from those of the *E. coli*, yeast, and rat liver enzymes, *i.e.* 108,000, 88,000, and 68,000, respectively (23). The corn seedling enzyme has been described as having a mol wt of approximately 25,000, estimated by gel filtration on Sephadex G-200 (20). The estimation reported in the present paper was conducted on Sephacryl S-300, which yielded a value for spermidine synthase, *i.e.* 81,000, essentially identical to that previously observed on Sephadex G-100 (17). It must be noted that Sephacryl may have resolved a minor component of spermidine synthase of slightly greater mol wt (Fig. 5); this had not been seen earlier (17). It may be asked if this new result relates to the observed relative instability of this enzyme.

The isolation and increased purification of the plant spermidine synthase with the aid of affinity chromatography on ATPA-Sepharose has now placed this enzyme among a similar group of analogous spermidine synthases. Both decarboxylase and synthase have, despite considerable purification (1500- and 1900-fold, respectively) not yet been purified to homogeneity. This task, as well as that of the isolation and purification of spermine synthase, remain as necessary steps in the clarification of the properties of these enzymes in polyamine biosynthesis. The inhibition of the cabbage spermidine synthase *in vivo* by dicyclohexylamine was shown to permit the continuing production of dSAM by the decarboxylase and to stimulate production of spermine (9). In cells infected by turnip yellow mosaic virus similarly inhibited in spermidine synthesis, spermine replaced spermidine in newly synthesized virus, without inhibiting the production of virus protein, virus nucleic acid, or virus particles (2). Thus the extent of a polyamine requirement for production of cells infected by turnip yellow mosaic virus has not been determined, despite the availability of elegant cellular, biochemical, and analytical systems, because suitable inhibitors of intracellular SAM decarboxylase and spermine synthase have not yet been found.

LITERATURE CITED

1. ARNON DI 1949 Copper enzymes in isolated chloroplasts, polyphenol oxidase in *Beta vulgaris*. Plant Physiol 24: 1–15
2. BALINT R, SS COHEN 1985 The effects of dicyclohexylamine on polyamine biosynthesis and incorporation into turnip yellow mosaic virus in Chinese cabbage protoplasts infected *in vitro*. Virology. In press
3. BALINT R, SS COHEN 1985 The incorporation of radiolabeled polyamines and methionine into turnip yellow mosaic virus in protoplasts from infected plants. Virology. In press
4. COHEN SS, R BALINT, RK SINDHU 1981 The synthesis of polyamines from methionine in intact and disrupted leaf protoplasts of virus-infected Chinese cabbage. Plant Physiol 68: 1150–1155
5. COHN MS, CW TABOR, W TABOR 1977 Identification of a pyruvoyl residue in S-adenosylmethionine decarboxylase from *Saccharomyces cerevisiae*. J Biol Chem 252: 8212–8216
6. COPPOC GL, P KALLIO, HG WILLIAMS-ASHMAN 1971 Characteristics of S-adenosyl-L-methionine decarboxylase from various organisms. Int J Biochem 2: 673–681
7. DAVIS BF 1967 Electrophoresis II: Method and application to human serum proteins. Ann NY Acad Sci 121: 404–427
8. GLAZER RI, AL PEARL 1978 Measurement of S-adenosyl-L-methionine levels by SP-Sephadex chromatography. Anal Biochem 91: 516–520
9. GREENBERG ML, SS COHEN 1985 Dicyclohexylamine-induced shift of biosynthesis from spermidine to spermine in plant protoplasts. Plant Physiol. In press
10. KAUR-SAWHNEY R, HE FLORES, AW GALSTON 1981 Polyamine oxidase in oat leaves: A cell wall-localized enzyme. Plant Physiol 68: 494–498
11. LOWRY OH, NH ROSEBROUGH, AL FARR, RJ RANDALL 1951 Protein meas-

urement with the Folin phenol reagent. J Biol Chem 193: 265–275
12. MANEN CM, DH RUSSELL 1974 Comparative properties of rat liver and sea urchin eggs S-adenosyl-L-methionine decarboxylase. Biochemistry 13: 4729–4735
13. PEGG AE 1974 Purification of rat liver S-adenosylmethionine decarboxylase. Biochem J 141: 581–583
14. PEGG AE, HG WILLIAMS-ASHMAN 1968 Stimulation of the decarboxylation of S-adenosylmethionine by putrescine in mammalian tissues. Biochem Biophys Res Commun 30: 76–82
15. SAMEJIMA K, Y NAKAZAWA, I MATSUNAGA 1978 An improved synthesis of decarboxylated S-adenosylmethionine and related sulfonium compounds. Chem Pharm Bull 26: 1480–1485
16. SAMEJIMA K, B YAMANOHA 1982 Purification of spermidine synthase from rat ventral prostate by affinity chromatography on immobilized S-adenosyl (5′)-3-thiopropylamine. Arch Biochem Biophys 216: 213–222
17. SINDHU RK, SS COHEN 1984 Propylamine transferases in Chinese cabbage leaves. Plant Physiol 74: 645–649
18. SINDHU RK, SS COHEN 1984 Subcellular localization of spermidine synthase in protoplasts of Chinese cabbage leaves. Plant Physiol 76: 219–223
19. SURESH MR, PR ADIGA 1977 Putrescine-sensitive (artifactual) and -insensitive (biosynthetic) S-adenosylmethionine decarboxylase activities of *Lathyrus sativus* seedlings. Eur J Biochem 79: 511–518
20. SUZUKI Y, E HIRASAWA 1980 S-Adenosylmethionine decarboxylase of corn seedlings. Plant Physiol 66: 1091–1094
21. TABOR CW 1962 Adenosylmethionine decarboxylase. Methods Enzymol 5: 756–760
22. TABOR H, SM ROSENTHAL, CW TABOR 1958 The biosynthesis of spermidine and spermine from putrescine and methionine. J Biol Chem 233: 907–914
23. TABOR CW, H TABOR 1984 Methionine adenosyltransferase (S-adenosylmethionine synthase) and S-adenosylmethionine decarboxylase. Adv Enzymol 56: 251–284
24. WILLIAMS-ASHMAN HG, A CORTI, GL COPPOC 1977 Mechanisms and regulation of adenoslymethionine decarboxylase in eukaryotes. *In* F Salvatore, E Borek, V Zappia, HG Williams-Ashman, F Schlenk, eds, The Biochemistry of Adenosylmethionine. Columbia University Press, New York, pp 493–509
25. WILSON J, A CORTI, M HAWKINS, HG WILLIAMS-ASHMAN 1979 The decarboxylation of S-adenosylmethionine by detergent-treated extracts of rat liver. Biochem J 180: 515–522
26. YAMANOHA B, K SAMEJIMA, T NAKAJIMA, T YASUHARA 1984 Differences between homogeneous spermidine synthase isolated from rat and pig liver. J Biochem 96: 1273–1282
27. ZAPPIA V, M CARTENI-FARINA, P GALLETTI 1977 Adenosylmethionine and polyamine biosynthesis in human prostate. *In* F Salvatore, E Borek, V Zappia, HG Williams-Ashman, F Schlenk, eds, The Biochemistry of Adenosylmethionine. Columbia University Press, New York, pp 473–492
28. ZAPPIA V, CR ZYDECK-CWICK, F SCHLENK 1969 The specificity of S-adenosylmethionine derivatives in methyl transfer reactions. J Biol Chem 244: 4499–4509