Dicyclohexylamine-Induced Shift of Biosynthesis from Spermidine to Spermine in Plant Protoplasts

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ABSTRACT

An improved analytical method, based on high pressure liquid chromatography, has been developed for the simultaneous determination of the polyamines and S-adenosyl-containing compounds in extracts of plant protoplasts. The method involves simple procedures for sample preparation and permits quantification of 1 picomole or less for all the compounds. This method has been used to study the effects of dicyclohexylamine, an inhibitor of plant spermidine synthase (Sindhu, R. K., S. S. Cohen 1984 Plant Physiol 74: 645–649), on biosynthesis of polyamines and 1-aminocyclopropane-1-carboxylate in protoplasts derived from Chinese cabbage leaves. Dicyclohexylamine effectively inhibits spermidine synthase in viva. Inhibition of the synthesis of spermidine by dicyclohexylamine resulted in a stimulation of spermine synthesis, without significant effect on the synthesis of 1-aminocyclopropane-1-carboxylate. Decarboxylated S-adenosylmethionine is present in control Chinese cabbage protoplasts at ~10-14 moles per cell, and dicyclohexylamine caused an increase of this metabolite of up to 10-fold in a 4-hour period. The increase in decarboxylated S-adenosylmethionine permitted an increased synthesis of spermine. These findings suggest that the availability of decarboxylated S-adenosylmethionine may be rate-limiting for the synthesis of spermine in plant protoplasts.

Polymamines have been implicated in the growth and development of bacterial and mammalian cells (6, 12, 15, 26), nucleic acid synthesis (20), and the structure and activity of viral RNA (8) and tRNA (5, 7, 19). Effects of polyamines on the activities of many enzymes in vivo (21) and in vitro (22) are well documented. Recent work in plant systems also suggests a role for the polyamines in plant growth and development (4, 25), and in the structure of an RNA plant virus, turnip yellow mosaic virus (9).

Studies from this laboratory have shown the presence and accumulation of spermidine in TYMV1 and in virus-infected Chinese cabbage plants (27). We demonstrated that protoplasts prepared from healthy and TYMV-infected leaves convert S-methionine to spermidine and spermine, and protoplasts were more active than leaf discs in the incorporation of exogenous methionine into protein and polyamines (10). Essentially all of the protoplasts derived from virus-infected leaves contained polyamines as evidence of infection (10).

The enzymes of synthesis of the polyamines, spermidine and spermine, in Chinese cabbage protoplasts, have been characterized in this laboratory. Reactions of SAM in these cells are presented in Figure 1. Decarboxylation of SAM yields dSAM, the aminopropyl donor in the reactions catalyzed by spermidine synthase and spermine synthase (10). Recent experiments in this laboratory have led to the demonstration and partial purification of a SAM decarboxylase in Chinese cabbage plants and protoplasts (B. Yamanoh, S. S. Cohen, unpublished data). Spermidine and spermine are synthesized from putrescine by the addition of successive aminopropyl moieties derived from dSAM. Adams and Yang (1) initially reported that the plant growth regulator, ethylene, was probably also derived from SAM. In 1979, Adams and Yang (2), and Lurssen et al. (17), showed that the ethylene precursor is ACC, which is derived from SAM directly. Thus SAM, in addition to participating in numerous transmethylation reactions in plants, is a key intermediate in the biosynthesis of two important plant regulatory substances, the polyamines, and ethylene (Fig. 1). SAM that is decarboxylated may be committed to polyamine biosynthesis, with ω-ω-elimination within SAM results in the formation of 5'-methylthioadenosine and ACC (1), the precursor of ethylene. Both of these pathways generate 5'-methylthioadenosine (not shown in Fig. 1) which is utilized to regenerate methionine.

An earlier sensitive method of polyamine analysis involved the TLC of their dansyl derivatives, and the estimation of the fluorescence of the separated dansyl derivatives. However, the dansyl amines are separated with difficulty, and SAM is destroyed during the derivatization reaction. A method recently reported for the analysis of polyamines in plants by HPLC (13) did not permit the detection of S-adenosyl derivatives, and did not separate the diaminos, putrescine, and diaminopropane. Newer methods based on HPLC offered improved sensitivities, more rapid and complete separations, a variety of detection modes, and the possibility of automation. These advances in HPLC analysis seemed well suited for metabolic studies involving radioactive precursors since specific activities, as well as pool sizes of precursors, intermediates, and products could be obtained. This approach was necessary to dissect the pathways involved in the utilization of S-adenosylmethionine in plants.

We have modified the method of Wagner et al. (28) so that it is suitable for the analysis of polyamines and S-adenosyl compounds present in plant extracts. This method employs reverse-phase HPLC and an ion-pairing agent to effect separation. Use

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3 Abbreviations: TYMV, turnip yellow mosaic virus; SAM, S-adenosylmethionine; dSAM, decarboxylated S-adenosylmethionine; SAH, S-adenosylhomocysteine; ACC, 1-aminocyclopropane-1-carboxylate; dansyl, 5-dimethylamino-1-naphthalene sulfonil; OPA, o-phthalaldehyde; DCHA, dicyclohexylammonium sulfonate; SP-Sephadex, sulfopropyl-Sephadex; MGGB, methylglyoxal bisguanidylhydrazone; AVG, l-2-amino-4-(2-animoethoxy)-trans-3-butenic acid.
of a UV detector and on-line derivatization of amines with a reagent containing OPA, with subsequent detection of fluorescence, allowed simultaneous determination of S-adenosyl compounds by absorbimetry and polyamines by fluorimetry. The method described in this paper permitted the detection of very small amounts of dSAM in these plant cells.

We have used this method to study the biosynthesis of polyamines and ACC from L-[3,4-14C]methionine in Chinese cabbage leaf protoplasts derived from healthy and virus-infected plants. We have used dicyclohexylamine, an inhibitor of spermidine synthase (24), to detect a shift from biosynthesis of spermidine to that of spermine.

**MATERIALS AND METHODS**

**Chemicals.** L-[3,4-14C]Methionine was from Research Products International Corp. Cellulase was from Yakult Biochemicals Co., Ltd., Nishinomiya, Japan. Pectolyase Y-23 was from Seishin Pharmaceutical Co., Ltd., Tokyo. SP-SEPHADEX was from Pharmacia Fine Chemicals. MES, dicyclohexylammonium sulfate, S-adenosylmethionine, S-adenosylhomocysteine, methionine, 5'-methylthioadenosine, and OPA were from Sigma Chemical Co. S-Adenosylmethionine was purified from commercial samples (14). MGBG was from Aldrich Chemical Co. Heptane sulfonate and octane sulfonate were from Eastman Organic Chemicals. Acetonitrile and methanol were from Waters Associates, Milford, MA. Brij-35, 1-aminocyclopropane-1-carboxylic acid, putrescine-2HCl, cadaverine-2HCl, spermidine-3HCl, spermine-4HCl were purchased from Calbiochem. The polyamines were recrystallized before use. All other reagents were from Fisher Scientific. Synthetic decarboxylated S-adenosylmethionine was a generous gift from Dr. K. Samejima, Tokyo Biochemical Research Institute, Tokyo. Synthetic dSAM had >99% of the O.D. at 254 nm associated with a single peak during HPLC analysis. The synthetic material contained a mixture of R and S isomers; only 50% of this material is a substrate in the assay for spermidine synthase. L-2-Amino-4-(2-aminoethoxy)-trans-3-butenoic acid was kindly provided by Dr. A. Stemple, Hoffman LaRoche.

**Growth of Plants.** Chinese cabbage seeds (Brassica pekinensis, var. Pak Choy) were obtained from Nichols Garden Nursery (Albany, OR). Plants were grown in a controlled environment chamber (Scientific Systems, Baton Rouge, LA) set for 18-h d at 28°C and 20,000 lux, using incandescent and fluorescent lighting, and 6-h dark periods at 22°C. When plants were ~3 weeks old, the rosette of each plant was removed, leaving two leaves. For experiments involving TYMV-infected plants, the remaining two leaves were mechanically inoculated with carbendazim and a solution of TYMV (0.1 mg/ml in 20 mM phosphate buffer, pH 7.0) The TYMV and its purification have been described (9). Leaves were harvested for experiments 10 to 14 d post pruning.

**Preparation of Protoplasts.** Leaves were deribbed, weighed, and gently abraded with carborundum on the lower epidermis, rinsed with distilled H2O and floated on a macerating solution in sterile 150-mm Petri dishes and incubated for 16 h in the dark at 22°C. The macerating solution, which liberates protoplasts, consisted of sterile 0.6 M mannitol, 5 mM MES, 1 mM CaCl2 (pH 5.6) containing 0.5% cellulose and 0.05% pectolysis. The solution was then aspirated and replaced with sterile 0.6 M mannitol, 5 mM MES, 1 mM CaCl2 (pH 5.6) (mannitol medium). Petri dishes were gently agitated to liberate protoplasts. The resulting suspension was filtered through eight layers of cheesecloth, diluted ~4-fold with sterile mannitol medium, and centrifuged at 30g for 10 min. The supernatant was removed and the pellet resuspended in sterile mannitol medium and recentrifuged as above. Protoplasts were washed until they were relatively free of chloroplasts and other debris (~6 times). Protoplasts were resuspended in mannitol medium, counted in a hemocytometer, and adjusted to approximately 10^6/ml. Essentially all protoplasts obtained from infected leaves contained chloroplast aggregates, a cytological characteristic of TYMV infection (10). The presence of virus in essentially all of the cells 2 weeks after infection, has been demonstrated with fluorescent antibody prepared against

![Diagram of polyamine biosynthesis in plant protoplasts](https://www.plantphysiol.org/content/methionine-14C-synthesized-spermidine-in-plant-protoplasts)
viral antigen. Chl was determined by the method of Arnon (3).

Incubation of Protoplasts and Sample Preparation. Six ml aliquots of the protoplast suspension were placed in sterile 60-mm Petri dishes with 1-[3,4,14C]methionine (57 μCi/μmol), final concentration 10 μM, with or without added drugs as indicated, and incubated at 23°C under 11,000 lux for varying lengths of time. A 0.3-ml aliquot of each suspension was placed into 5.0 ml of ice-cold 10% (w/v) TCA, precipitates were collected on Whatmann 3MM filters, washed three times with 5 ml ice-cold 10% TCA, and decolorized by washing three times with 5 ml ice-cold acetone/methanol (7:2, v/v). Filters were dried, and radioactivity was determined by liquid scintillation counting; 5.0-ml aliquots of the protoplast suspension (1–1.5 × 10^9/ml) were withdrawn, added to 20 ml of sterile, ice-cold mannitol medium, and centrifuged at 200g for 10 min. Protoplasts were resuspended in 10 ml of sterile mannitol medium and washed once more as above. Pellets were extracted twice with 5% (w/v) HClO_4 and the supernatants were combined. Radioactivity in aliquots of HClO_4 extracts and washes were determined by liquid scintillation counting. Extracts were stored frozen at −20°C.

Fractionation of Protoplast Extracts. Columns of SP-Sephadex (1 × 5 cm) were prepared in 10 mM HCl (14). HClO_4 extracts were thawed, the pH was adjusted to 3 to 4 with KOH, chilled on ice for 30 min, and centrifuged to remove KClO_4. Precipitates were washed with ice-cold 10 mM HCl, centrifuged, and supernatants were combined and applied to the columns. Methionine and ACC were eluted with 35 ml of 50 mM HCl. Putrescine and S-adenosylhomocysteine were eluted with 35 ml of 150 mM HCl, whereas S-adenosylmethionine, decarboxylated S-adenosylmethionine, and the polyamines were eluted with 40 ml of 0.5 M HCl. Recovery of these compounds from SP-Sephadex was greater than 95%. 5'-Methylthioadenosine and SAH, contaminants in commercial preparations of SAM, are well separated from SAM on SP-Sephadex (14). Samples were dried in vacuo at 32°C and the residues were dissolved in 10 mM HCl. Aliquots of all fractions were taken for determination of radioactivity by liquid scintillation counting.

HPLC Analysis of S-Adenosylmethionine and Polyamines. The HPLC consisted of the following equipment, all obtained from Waters Associates: two model 6000A pumps, 441 UV detector, 420-AC filter fluorimeter (334–346 nm excitation and 425 nm long pass emission), a WISP 710B autoinjector, a model 720 system controller, and an Eldex A30-S pump. The collection of HPLC data, their reduction and analysis was performed with a Nelson Analytical Model 4416 data station consisting of a Hewlett-Packard model 9816 micro-computer, equipped with a model 82901M mass storage unit and a model 2671G thermal printer, and a Nelson Analytical series 760 analog to digital converter (all from Nelson Analytical, Cupertino, CA). An LKB Super-Rac model 2211 fraction collector equipped with a sole-noid-activated three-way valve (LKB Instruments, Inc., Gaithersburg, MD) was used for the fractionation of column eluates and collection of radioactive samples. The columns used for analyses were a μBondapak C_{18} (10 μm particle size, 30 cm X 3.9 mm i.d.) and a Nova Pak C_{18} (4 μm particle size, 15 cm X 3.9 mm i.d., both from Waters Associates). Gradient elution employing two mobile phases was used for HPLC. Mobile phase A: 0.1 M NaH_2PO_4, 8 mM heptanesulfonic acid, 0.1 mM Na_2EDTA (pH 2.50) and acetonitrile (132:1, v/v). Mobile phase B: 0.2 M NaH_2PO_4, 8 mM heptanesulfonic acid (pH 3.10) and acetonitrile (73:3, v/v). All solutions were filtered through 0.5 μM filters and degassed. A linear gradient was used with initial conditions of 80% mobile phase A and 20% mobile phase B leading to final conditions of 40% A and 60% B in 21 min. At this time a reverse gradient was employed to reach initial conditions in 3 min, followed by an equilibration delay of 5 min prior to the next injection. Slight variations in the gradient were sometimes employed to improve separations, or to shorten analysis times depending upon the analytical needs, or to adjust for deterioration in column performance after extensive use. All chromatographic parameters were programmed, and the analyses and collection of fractions were fully automated. The flow rate through the column was 1 ml/min for the Nova Pak column and 1.5 ml/min for the μBondapak column. Chromatography was performed at room temperature. S-Adenosyl-containing compounds were monitored at 254 nm, and a reagent containing OPA (28) was used to derivatize amines on-line after the eluent had passed through the UV detector. The flow rate of the OPA reagent was equal to the column flow rate. Polyamines were also analyzed by the dansyl method (9).

HPLC Analysis of Methionine. The determination of the specific activity of methionine was performed on the 50-mM HCl eluate from the SP-Sephadex fractionation of the PCA extracts. The chromatographic conditions began with 100% A. Isocratic elution was used for 3 min, followed by a linear gradient leading to 75% A and 25% B at 15 min, followed by another linear gradient leading to 25% A and 75% B at 25 min, when a reverse gradient was employed to reach initial conditions in 5 min. A 7-min equilibration delay was used prior to the next injection. The mobile phase compositions, flow rates, and the OPA reagent were as described above.

ACC Analysis. ACC was analyzed by GC after its conversion to ethylene according to the method of Lizada and Yang (16). For ACC analysis, 100 μl of 1 mM HgCl_2, 0 to 200 μl of ACC solution (1 μM or 10 μM in 10 mM HCl), and water to make the volume to 0.9 ml were placed into glass vials. The vials were sealed with rubber septa and chilled on ice for 15 min. Ten drops of NaOCl/NaOH solution (2:1, v/v of 5.25% NaOCl and saturated NaOH) were introduced into the reaction vials by means of a syringe fitted with a 21-gauge needle. The vials were agitated on a shaker at 4°C for 3 min, or for 30 s on a Vortex-Genie mixer to liberate ethylene, which was then sampled for GC. The equipment consisted of an F-42 Headspace Analyzer equipped with a flame ionization detector. The output from the detector amplifier was connected to a Sigma 10 data station (all from Perkin-Elmer, Norwalk, CT). An alumina F-1, 1-mm glass column was used. Chromatographic conditions were: isothermal operation at 110°C, carrier gas (He) flow of 40 ml/min, H_2 flow of 42 ml/min, air flow of 420 ml/min. Under these conditions ethylene elutes at 36 s. The specific activity of ACC was determined from ethylene liberated. Ethylene was absorbed by mercuric perchlorate (29). The absorbed radioactivity was determined by liquid scintillation counting and the amount of ethylene absorbed was determined by GC after liberation by 4 M LiCl or 4 M HCl (29).

RESULTS

Capabilities of the Analytical Methods. The method described by Wagner et al. (28) for the simultaneous analysis of the major SAM metabolites and the polyamines was not satisfactory for work with radioactive precursors, or for the separation of the diamines. To obtain improved separations the ion-pairing agent was changed from octanesulfonic acid to heptanesulfonic acid which also significantly improved peak shapes, and allowed chromatography at room temperature. A decreased concentration of acetonitrile in mobile phase A permitted the retention of the neutral amino acids and the diamines on the column, allowing effective separation of these compounds. These changes, along with modifications of gradients employed and the use of a 4-μm column, have led to significant improvements in the chromatography.

Preliminary analyses of unfractionated PCA extracts of Chinese cabbage leaves and protoplasts revealed the presence of
numerous compounds containing primary amine groups. These compounds eluted early in the chromatogram interfering with the analysis of ACC and methionine under the conditions described above for the polyamines and SAM. For routine determinations of putrescine, spermidine, spermine, and SAM, unfraccionated PCA extracts can be applied directly to the HPLC system. The use of unfraccionated plant extracts requires more frequent cleaning of the column and changing of the guard column due to the accumulation of lipophilic substances.

For studies employing radioactive precursors, fractionation of acid extracts on an SP-Sephadex column (14) was introduced prior to HPLC analysis. A preliminary separation of radioactive methionine from the fraction containing spermidine, spermine, and SAM is useful in eliminating the trailing of radioactivity from this substrate to other fractions.

Determination of the specific radioactivity of dSAM required its complete separation from spermidine in the HPLC eluate, to obtain counts specific for dSAM. As seen in Figure 2, spermidine and dSAM can be separated by more than 1 min in a analysis in which all compounds of interest are eluted in 18 min. The poor reactivity of dSAM with the OPA reagent is evident (Fig. 2). A base line separation of more than 1 min between spermidine and dSAM allowed several fractions to be collected between these two peaks. The method also separates ACC, methionine, and SAH (not shown). ACC and methionine elute before putrescine, SAH elutes between SAM and dSAM and is well separated from spermidine. The chromatogram in Figure 2 was obtained using the longer 10-μm μBondapak column. Similar separations are possible with the shorter 4-μm Novapak column which provides improved resolutions, and has the additional advantage of reducing solvent consumption by 33%. Figure 3 demonstrates the facile separation of the diamines and polyamines possible with this method. Although cadaverine is seen frequently, we have not detected the presence of diaminopropane in acid extracts of protoplasts.

The system used for data acquisition offers several advantages. The analog to digital interface has two channels and 20-bit resolution. When the interface is connected to the 1-v output of the UV detector, a resolution of 1 μV or 1 × 10^-4 absorbance units can be obtained. During normal operation (detector operating at 254 nm) useful analyses can be conducted in the μV range for UV-absorbing compounds, such as the S-adenosyl derivatives. This has significantly improved sensitivities for compounds such as dSAM, and this capability has been essential for the quantification of the amounts of dSAM present in protoplasts of Chinese cabbage. The limit of quantification with a signal-to-noise ratio better than 10 is about 1 pmol for the S-adenosyl-containing and the amine-containing compounds. The sensitivities of the fluorescence and absorbance detectors can be varied independently in the analog-to-digital interface.

**Methionine Uptake, Specific Activity, and Conversion to SAM.** In experiments designed to measure biosynthesis of spermidine, spermine, and ACC in protoplasts from a labeled precursor, we determined the kinetics of uptake of methionine and the specific activities of the endogenous precursor pools. Figure 4 presents data from two experiments, one with healthy protoplasts and one with TYMV-infected protoplasts on the uptake of methionine, as well as the specific activity of the endogenous pools of methionine and S-adenosylmethionine. Methionine was taken up by these protoplasts, and increased in intracellular concentration, whereas the specific activity of the endogenous methionine pool reached an approximately maximal value within 1 h. The specific activity of methionine remained nearly constant for several hours. This indicated that the exogenous methionine was not exhausted during the incubation, a conclusion supported by monitoring the disappearance of radioactivity from the external medium during the experiments.

Methionine was converted rapidly to SAM in protoplasts. The specific activity of the endogenous SAM pool followed the same time-course as the specific activity of the methionine pool, reaching about 80 to 85% of the specific activity of endogenous methionine. This result has been confirmed in all subsequent
Fig. 4. Protoplasts derived from healthy and virus-infected Chinese cabbage leaves were incubated in the presence of labeled methionine as described in "Materials and Methods". A, Intracellular methionine content of healthy (●) and infected (○) protoplasts during incubation. B and C, Specific activities of endogenous methionine and SAM in healthy (●) and infected (○) protoplasts, respectively. The amount of SAM, expressed as nmol/10^6 protoplasts, was 2.6 and 2.3 for healthy and infected samples, respectively.

Fig. 5. Healthy (●) and virus-infected protoplasts (○) were incubated as described in "Materials and Methods". The specific activities of ACC, spermidine, and spermine are displayed in the left, center, and right panels, respectively. The amounts of ACC, spermidine, and spermine, expressed as nmol/10^6 protoplasts were: 0.53, 8.7, and 5.5, respectively, for healthy samples, and 0.65, 13.8, and 5.7, respectively, for infected samples. The diameter of Chinese cabbage protoplasts prepared as described is about 40 μm. Using this value to calculate the approximate volume of 10^6 protoplasts, apparent concentrations of spermidine and spermine are 260 μM and 170 μM, respectively, for healthy samples, and 410 μM and 170 μM, respectively, for infected protoplasts. Approximately 30% of the PCA extractable radioactivity elutes in the 0.5 M HCl fraction from SP-Sepadex representing SAM, SPD, SPM, and dSAM. Five to 15% of this radioactivity is in SPD; the majority of the counts represent radioactivity incorporated into SAM.

Table 1. Effect of DCHA on the Specific Activity of Methionine, SAM, and Spermidine

Protoplasts were incubated for 4 h under light as described in "Materials and Methods", in the presence of the indicated concentration of DCHA. Specific activities of metabolites were determined on acid extracts of washed protoplasts as described in "Materials and Methods".

<table>
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<th>DCHA (mM)</th>
<th>Methionine (cpm x 10^-3/nmol)</th>
<th>% of control</th>
<th>SAM (cpm x 10^-3/nmol)</th>
<th>% of control</th>
<th>Spermidine (cpm/nmol)</th>
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Inhibition of the Synthesis of Spermidine by DCHA. Dicyclohexylamine has recently been shown to be a potent inhibitor of Chinese cabbage spermidine synthase \textit{in vitro} (24). As seen in Table I, DCHA added exogenously to protoplasts in the presence of labeled methionine inhibited the synthesis of spermidine \textit{in vivo} in a dose-dependent manner. This inhibition of spermidine formation by DCHA occurred without significant effect on the uptake of methionine into protoplasts or its conversion to SAM.

Effect of DCHA on the Syntheses of ACC and Spermine. A concentration of 2 mM DCHA sharply curtailed the increases of specific activity of spermidine in both healthy and infected protoplasts without an effect on protein synthesis. Figure 6 shows that inhibition of the formation of spermidine in infected protoplasts is readily apparent whether one considers the specific radioactivity of the cellular spermidine pool, or the accumulation of total radioactivity in spermidine per protoplast. As shown in Figure 6 as well, this level of inhibition of the synthesis of spermidine is without significant effect on ACC synthesis for infected protoplasts. Similar results were obtained with healthy protoplasts.

The inhibition of spermidine biosynthesis by DCHA produced a significant stimulation of spermine synthesis in both healthy and infected protoplasts. As seen in Figure 7, 2 mM DCHA, a concentration which inhibited spermidine biosynthesis by more than 50% in both healthy and infected protoplasts, stimulated the synthesis of spermine in healthy and infected protoplasts.

**FIG. 6.** Virus-infected protoplasts were incubated as described in “Materials and Methods” in the absence or presence of 2 mM DCHA. The top two panels, from left to right, show cpm accumulated in ACC and spermidine, respectively. The bottom two panels, from left to right, show the specific activities of ACC and spermidine, respectively.

*Effects of DCHA on ACC and Spermidine Biosynthesis.*

*Fig. 7.* Healthy and virus-infected protoplasts were incubated as described in “Materials and Methods” in the absence or presence of 2 mM DCHA. The top two panels show the specific activities of SAM for healthy (left panel) and infected (right panel) protoplasts. The bottom two panels show the specific activities of spermidine and spermine for healthy (left panel) and infected (right panel) protoplasts. The amounts of SAM, SPD, and SPM expressed as nmol/10^6 protoplasts were: 1.2, 4.2, 1.0, respectively, for healthy samples, and 1.1, 10.8, 1.6, respectively, for infected samples.

Fig. 6. Virus-infected protoplasts were incubated as described in "Materials and Methods" in the absence or presence of 2 mM DCHA. The top two panels, from left to right, show cpm accumulated in ACC and spermidine, respectively. The bottom two panels, from left to right, show the specific activities of ACC and spermidine, respectively.

**Biosynthesis of Spermidine, Spermine, and ACC in Healthy and Infected Protoplasts.** Figure 5 shows the synthesis of spermidine, spermine, and ACC in healthy and infected protoplasts, expressed as specific activity of the acid-soluble cellular pools. The synthesis of each of the polyamines, estimated by total radioactivity incorporated, is similar in healthy and TYMV-infected protoplasts. However, the specific activity of spermidine in healthy protoplasts appears considerably higher than in infected protoplasts, which contain a larger total amount of spermidine (10). The situation for ACC synthesis in the two cell types is different. Most of the experiments conducted have demonstrated a greater increase of radioactivity in ACC for infected cells compared to healthy ones. The specific activity of ACC was higher in the infected protoplasts than in healthy protoplasts despite the larger ACC pool of the former. Numerous virus-infected plant tissues have been found to produce more ACC and ethylene (11, 23).
almost 2-fold. The stimulation of the synthesis of spermine has been found consistently in the experiments. This DCHA-induced shift from spermidine biosynthesis to spermine biosynthesis occurred with only a slight effect on the specific activity of the cellular SAM pools in both types of protoplasts.

Accumulation of Decarboxylated S-Adenosylmethionine during Inhibition of Spermidine Biosynthesis and Its Effect on Spermine Synthesis. Dicyclohexylamine inhibition of synthesis of spermidine did not result in the accumulation of additional radioactivity in SAM (Table II). Inhibition of spermidine formation by 2 mM DCHA had little effect on the specific activity of the SAM pool and did not result in an expansion of the cellular SAM pool. Instead, inhibition of spermidine synthesis by DCHA led to an increase of dSAM.

Decarboxylated S-adenosylmethionine has been characterized by five criteria. The first three are the appearance of a strongly cationic UV-absorbing peak during ion-exchange chromatography and HPLC analysis with the proper retention time, and eluting at the same position as an authentic sample of synthetic dSAM. The fourth criterion was the accumulation of radioactivity in a putative precursor under conditions of the inhibition of biosynthesis of spermidine. In vitro studies with S-adenosylmethionine decarboxylase of Chinese cabbage leaf extracts demonstrate that the reaction product also elutes at this same position in the HPLC separation (B. Yamanoha, S. S. Cohen, unpublished observations). Finally, the specific activity of 'dSAM' was very similar to that of its immediate precursor, SAM (Table II). This is consistent with a very small pool which is actively turned over. Calculations of turnover rate for the dSAM pool, based on the synthetic rates of spermidine and spermine, indicate that in the control cells the pool of dSAM turns over within 15 s.

Inhibition of spermidine synthesis at the level of spermidine synthase results in the accumulation of dSAM. In subsequent analyses the level of dSAM in the control protoplasts was determined. Chinese cabbage protoplasts contain approximately 2 pmol dSAM per 10⁶ cells. We have observed an accumulation of dSAM of 10-fold during a 4-h period of DCHA exposure. We have occasionally seen the intracellular content of dSAM increased to 70 pmol per 10⁶ protoplasts in 4 h (Table II).

Characteristically, dSAM was diverted to an additional synthesis of spermine during DCHA treatment. The DCHA-induced shift from biosynthesis of spermidine to spermine appears related to the increase of the substrate for both the spermidine and spermine synthase reactions, dSAM, during inhibition of spermidine synthase. This apparently results in an increased production of spermine. Additional synthesis of spermine during the inhibition of spermidine synthase has also been observed in mammalian cells (18), using another inhibitor of spermidine synthase, S-adenosyl-1,8-diamino-3-thiooctane.

**DISCUSSION**

Suspensions of protoplasts prepared from healthy and infected leaves of Chinese cabbage can provide a useful model for the study of the cellular physiology of healthy and virus-infected plants. We demonstrate the utility of this cellular system, which offers several distinct advantages to intact plant tissue. The use of suspensions of protoplasts minimizes problems of absorption of substrates and inhibitors through leaves or leaf discs. The derived protoplast preparations are more active than leaf discs in the incorporation of label from exogenously added methionine into protein, spermidine, and spermine (10). The populations of protoplasts obtained from infected leaves afford another advantage in that essentially all the cells are infected and can produce virus (10). Protoplasts from healthy plants can also be infected but infection rarely proceeds in this system in a majority of the cells.

Despite an increase in putrescine, that we have detected in the protoplasts as compared to nonprotoplasted tissue, DCHA effectively inhibits the synthesis of spermidine in protoplasts. The distortion of polyamine content that occurs during the process of protoplasting can be eliminated by the use of difluoromethylarginine without any apparent change in the efficacy of dicyclohexylamine in inhibiting the synthesis of spermidine and increasing the synthesis of spermine (R. Balint, S. S. Cohen, unpublished data). Thus the main observations on the effect of DCHA in this system do not arise from artifacts due to protoplasting.

We have described an improved analytical method for the separation and estimation of the polyamines and S-adenosylmethionine derivatives. This method provides significant advantages to recently reported procedures (13, 28). The method should prove useful to many laboratories studying polyamine metabolism, and has been essential for our work. The problems of detection and quantification of small amounts of metabolic intermediates have been addressed and solved using modern methods of liquid chromatography in conjunction with microprocessor-based data acquisition. The method described has also been used for the assay and characterization of the reaction of a SAM decarboxylase present in Chinese cabbage leaf extracts and could be used for the assay of spermidine synthase.

The availability of specific inhibitors of the polyamine biosynthetic enzymes has been an important development. Most studies employing inhibitors of the polyamine biosynthetic enzymes have been restricted to the three decarboxylases, ornithine decarboxylase, arginine decarboxylase, and SAM decarboxylase. Re-

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Table II. Incorporation of L-[3,4-14C]Methionine into SAM and dSAM

<table>
<thead>
<tr>
<th>Sample</th>
<th>SAM</th>
<th>dSAM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>cpm x 10⁻³</td>
<td>nmol</td>
</tr>
<tr>
<td>2 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>38.5</td>
<td>2.22</td>
</tr>
<tr>
<td>DCHA</td>
<td>38.4</td>
<td>2.28</td>
</tr>
<tr>
<td>DCHA + AVG</td>
<td>28.4</td>
<td>2.00</td>
</tr>
<tr>
<td>4 h</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>28.7</td>
<td>1.88</td>
</tr>
<tr>
<td>DCHA</td>
<td>31.7</td>
<td>1.74</td>
</tr>
<tr>
<td>DCHA + AVG</td>
<td>48.9</td>
<td>2.52</td>
</tr>
</tbody>
</table>

* dSAM was beneath the level of detection in these samples, i.e. <5 pmol per 4.5 x 10⁶ protoplasts in controls.
cently, specific inhibitors for the propylamine transferases have been described. Dicyclohexylamine, an easily obtainable compound, effectively inhibits the biosynthesis of spermidine in plant protoplasts. This indicates that DCHA crossed the cell membrane and reached its appropriate site of action. The results reported in this paper, on the effects of DCHA in vivo, extend our studies on the inhibition of the enzyme spermidine synthase by this compound (24).

During DCHA inhibition of spermidine synthase in vivo, SAM did not accumulate. However, decarboxylated S-adenosylmethionine, the propylamine donor for the synthesis of spermidine and spermine, increased up to 10-fold. The increase of available substrate, dSAM, appeared to increase the synthesis of spermine. The physiological consequences of such a shift from synthesis of spermidine to that of spermine, can possibly be studied by the use of an inhibitor such as DCHA. For example, the process of aging is associated with decreasing amounts of spermidine and increasing amounts of spermine. Dicyclohexylamine inhibition might facilitate studies of this aspect of senescence, though a system more complete than protoplasts would also be desired.

Low cellular concentrations of dSAM (in the range of 10^-18 mol per cell) had not previously permitted facile determination of this metabolite. Now this concentration of dSAM in plant or animal cells can be detected, and the effects of inhibitors of polyamine biosynthesis on dSAM metabolism can be studied much more easily. The finding of dSAM accumulation, during inhibition of biosynthesis of spermidine in Chinese cabbage, is similar to reports in mammalian systems employing other inhibitors of polyamine biosynthetic enzymes (18). The increase in dSAM amounts occurs rapidly, in the absence of a marked change in spermidine content, and results in an increased synthesis of spermine. This suggests that the availability of dSAM may be rate-limiting for spermine biosynthesis in plant cells.

We are interested in the interrelation of polyamine and ethylene metabolism in plant cells and have viewed SAM as the branchpoint of these two pathways (Fig. 1). We wished to know if inhibition of polyamine biosynthesis would affect SAM metabolism to ACC, the ethylene precursor. Short-term inhibition of the synthesis of spermidine in vivo did not appreciably affect ACC synthesis.

A diversion from polyamine biosynthesis to ethylene biosynthesis was not observed in these studies. In testing for a shift towards synthesis of ACC it may be necessary to use an inhibitor other than DCHA to permit SAM to accumulate. Decarboxylation of SAM continued during DCHA treatment. A role for decarboxylated S-adenosylmethionine, other than as a substrate for the aminopropyl transferases is not known, and thus dSAM formed may be committed entirely for polyamine biosynthesis. The simultaneous use of an inhibitor of spermine synthase may permit either a total accumulation of dSAM or the detection of another pathway in these cells. However, no such suitable inhibitor is yet known.

We have tried to inhibit the decarboxylation of SAM with MGBG, which is an effective inhibitor in Chinese cabbage leaf extracts (B. Yamamoyo, S. S. Cohen, unpublished data). However, a significant inhibition of the synthesis of spermidine or of the synthesis of spermine was not obtained in protoplasts at millimolar levels of MGBG. The inhibitor MGBG does not appear to penetrate easily into the protoplasts. It is possible that an as yet unavailable inhibitor of the decarboxylation of SAM would be capable of reaching its pharmacological site of action, and permit SAM to be diverted to the synthesis of ACC.

Nevertheless, DCHA has proved to be an effective inhibitor of polyamine biosynthesis in Chinese cabbage protoplasts, and provides a new tool for the study of the roles of the polyamines in plants. The metabolic systems of Chinese cabbage protoplasts, as outlined in Figure 1, have proven to be manipulatable with inhibitors and these results may permit the use of cellular models in the analysis of the physiology of plant growth.

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