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# The Synthesis of Polyamines from Methionine in Intact and Disrupted Leaf Protoplasts of Virus-Infected Chinese Cabbage[1]

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## ABSTRACT

**In exploring the role of the chloroplast in the multiplication of turnip yellow mosaic virus, the biosyntheses of the major viral polyamine, spermidine, as well as that of the tetramine, spermine were studied. The synthesis of these polyamines from [2-$^{14}$C]methionine in protoplasts of Chinese cabbage leaf cells derived from healthy plants or those infected by turnip yellow mosaic virus were examined. Populations of protoplasts of infected leaves are homogeneous with respect to containing chloroplast aggregates in contrast to those of healthy leaves. Protoplast preparations have been shown to incorporate methionine into protein, spermidine, and spermine more rapidly than do fresh leaf discs, which also show a very slow utilization of labeled arginine and ornithine into polyamine.**

**Protein synthesis is similar for 4 hours in both healthy and infected protoplasts. Accumulation of labeled spermidine stops after 2 hours in healthy protoplasts but continues in the infected protoplasts. Much of the newly synthesized protein and spermidine is present in the easily sedimentable fraction of the readily disrupted protoplasts.**

**Disrupted and diluted protoplasts have a decreased ability to metabolize methionine to protein and spermidine. The residual synthetic activity is essentially entirely in the easily sedimentable fraction. However, this fraction is unable to synthesize spermine, an activity found in protoplasts and disrupted protoplasts. Disrupted protoplasts contain spermidine synthase (EC 2.5.1.16) and about a quarter of this activity is present in a low-speed sedimentable fraction containing the chloroplasts. The protoplast system is suitable for an analysis of polyamine synthesis in turnip yellow mosaic virus infection and appears particularly suitable for study of the distribution of the enzymes involved.**

We are interested in an infection of Chinese cabbage by TYMV,[3] which is believed to multiply in chloroplast aggregates. These polyplasts are formed in the cells of the infected plant (12, 16–18) and degenerate when virus multiplication is complete. Indeed, a severe yellowing of the plant and arrest of growth are also late events in the system we are using, apparently occurring after completion of virus multiplication.

The virus is icosahedral and contains single-stranded RNA condensed within an impermeable protein shell (15). Much of the phosphorus (20–30%) of the virus RNA is neutralized by polyamines, of which the major component, spermidine (5, 14, 23), is entirely nonexchangeable and evidently becomes associated with the RNA prior to packaging in the protein outer shell (Cohen and Greenberg, unpublished observations). The aminopropyl moiety of spermidine, *i.e.* *N*-3′-aminopropyl-1,4-diaminobutane, is derived in most cells from carbons 2,3 and 4 of methionine.

In a growing plant, virus infection may cause a 3- to 4-fold increase in the spermidine content of the plant, and at late stages of infection approximately a quarter of the spermidine in plant juice is precipitable by antiviral antiserum (23; Cohen and Greenberg, unpublished observations). We have asked if the chloroplasts of the infected plant are synthesizing this polyamine and, in approaching this problem, we began by examining polyamine biosynthesis from methionine in the discs cut from normal and virus-infected cabbage leaves (23). Having established the analytical requirements for such experiments, we have now compared these results with the biosynthesis of spermidine in both intact and disrupted protoplasts isolated from normal and virus-infected leaves.

When plant leaves are incubated in the dark, or are subjected to a variety of stresses, the tissues increase in RNase and proteolytic enzymes, and lose soluble protein and Chl. This phenomenon has been termed "senescence" and is frequently accompanied or preceded by the production of the regulator, ethylene, a substance also derived from methionine (1, 2). The senescence of leaf tissue is retarded by many substances, of which spermidine and spermine are among the most potent (3). Nevertheless, it is now known that spermidine does not retard the loss of soluble protein during barley leaf senescence (6). Also, because it is possible that incubation in spermidine and incorporation of the triamine might produce a feedback inhibition of the synthesis of spermidine, we have isolated protoplasts without the use of such substances which impede senescence. Nevertheless, preparations of protoplasts obtained from young leaves are quite stable and can be shown to incorporate isotope from [2-$^{14}$C]methionine into protein, spermidine, and spermine. The presence of a spermidine synthase (EC 2.5.1.16) has also been detected in disrupted protoplasts. Furthermore, although most of this activity was soluble, an easily sedimentable fraction of lysed protoplasts containing the chloroplasts of the preparation was found to possess a significant ability to synthesize spermidine from putrescine and decarboxylated *S*-adenosylmethionine. Indeed, this fraction could also convert methionine to spermidine at a low but significant rate.

## MATERIALS AND METHODS

**Growth of Plants Inoculation with TYMV.** Chinese Cabbage seeds (*Brassica pekinensis*, var. Pak Choy) were obtained from Nichols Garden Nursery, Albany, Oregon. The TYMV and its purification have been described (15). Plants were grown in a controlled-environment chamber (Scientific Systems, Baton Rouge, Louisiana) set for 18 h days at 28 C and 20,000 lux, using incandescent fluorescent lighting, and 6-h dark periods at 22 C. When the plants were about 3 weeks old, the rosette of each plant was removed, leaving two leaves which were inoculated by abrad-

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[3] Abbreviations: TYMV, turnip yellow mosaic virus; SAM, *S*-adenosylmethionine.

ing the upper surface with carborundum and painting with a solution of TYMV (0.1 mg virus/ml in 50 mM acetate [pH 4.8]). Control plants were similarly pruned and newly emerging leaves of both control and infected plants were used 10 to 14 days later.

**Incubation of Leaf Discs with [2-$^{14}$C]DL-Methionine.** Discs of 0.9 cm diameter were punched out from the leaves with a cork borer, weighed, rinsed with cold distilled $H_2O$, and dried on paper towels. One hundred discs were floated top-side up on 13 ml of a sterile solution of 0.1 M phosphate (pH 7.0), containing 8 $\mu$Ci[2-$^{14}$C]DL-methionine (45.9 $\mu$Ci/$\mu$mol, New England Nuclear) in a sterile plastic 100-mm Petri dish. The discs were then incubated at 25 C under 10,000 lux for varying lengths of time. At each time point, 25 discs were removed, rinsed 4 times in distilled $H_2O$, homogenized in 1 ml of 3% $HClO_4$ in a cold TenBroeck homogenizer and placed on ice for 30 min. The homogenates were then centrifuged at 3,000$g$ for 15 min at 4C. The acid-soluble fraction was then analyzed for polyamine and the precipitate was resuspended in 5 ml of cold 5% trichloroacetic acid, filtered onto a Whatman 3 MM disc, washed with cold 5% trichloroacetic acid followed by 95% ethanol, dried and counted in 10 ml Aquasol (New England Nuclear) in a Packard Model 3003 scintillation spectrometer.

**Protoplast Isolation and Incubation with [2-$^{14}$C]DL-Methionine.** Young rapidly expanding leaves from 28- to 35-day-old plants grown from seed were found to give the highest yields of intact protoplasts (3–5 $\times$ $10^6$/g leaf) and were used in all experiments. For infected plants and controls, as noted above, newly emerging leaves from 31- to 35-day-old plants (10–14 days postinfection) were used. Leaves were washed with distilled $H_2O$, dried, deribbed, and the lower epidermis was breached by cross-hatching with a razor blade into approximately 1-mm squares. The leaves were then floated on a sterile solution of 0.5 M sorbitol, 1 mM phosphate (pH 5.8), containing 0.5% Cellulase (Kinki Yakult, Nishinomiya, Japan), and 0.5% Macerozyme (Kinki Yakult, Nishinomiya, Japan) in sterile 150-mm Petri dishes and incubated for 18 h in the dark at 19 C. The dishes were then rotated gently by hand to free the protoplasts and the suspension was diluted 3-fold with sterile 0.4 M mannitol, 1 mM phosphate (pH 5.8), filtered through 4 to 8 layers of cheesecloth, and centrifuged at 20$g$ for 15 min. The pellets were resuspended in the mannitol solution and layered onto cushions of 0.6 M sucrose, 1 mM phosphate (pH 5.8) and centrifuged at 100$g$ for 15 min. Nuclei and cell debris were sedimented to the bottom of the tube. The protoplasts, relatively free of chloroplasts, were collected from the interface and washed once more in the mannitol solution at 20$g$ for 15 min. The protoplasts were then resuspended in the mannitol solution, counted in a hemocytometer, and adjusted to approximately $10^6$/ml. [2-$^{14}$C]DL-methionine (45.9 $\mu$Ci/$\mu$mol) was then added to a final concentration of 1.34 $\times$ $10^{-5}$ M and 13-ml portions were placed in sterile 100-mm Petri dishes and incubated at 25 C under 20,000 lux for varying lengths of time. At each point, 3-ml aliquots were taken directly into 0.15 ml 60% $HClO_4$, vortexed and placed on ice for 30 min. Samples were processed as described above for the discs.

Disrupted protoplasts were prepared by cycling the final suspension once through a syringe equipped with a 3-cm 21-gauge needle before adding [2-$^{14}$C]methionine. Fewer than 5% of the protoplasts remained intact.

**Isolation and Quantitation of Polyamines.** Spermidine, spermine, and putrescine were quantitated after TLC of their dansyl derivatives. However, in order to remove interfering carbohydrates, the polyamines were first separated from the acid extracts by adsorption on silica gel followed by elution in dilute acid (11). Briefly, the acid extracts were adjusted to pH 9 with 1 N NaOH and loaded onto a small column containing 0.5 g silica gel (60–200 mesh, J. T. Baker) at neutral pH. The columns were then washed with 100 ml distilled $H_2O$ adjusted to pH 8 with dilute NaOH and the polyamines were eluted with 30 ml 0.03 N HCl. The column eluates were then taken to dryness under reduced pressure at 45 C and the residues were redissolved in 1.0 ml 0.03 N HCl. Recoveries were estimated to be nearly 100% by adding a known amount of [$^{14}$C]spermidine (New England Nuclear) to an unlabeled acid extract and reisolating the spermidine by silica gel chromatography.

Dansyl derivatives of the extracted polyamines were prepared and quantitated by TLC essentially as described (7). Briefly, 0.2 ml of each sample was placed in a 12-ml glass stoppered centrifuge tube to which were added 0.2 ml saturated $Na_2CO_3$ and 0.4 ml of an acetone solution of 1-dimethylaminonaphthalene-5-sulfonyl chloride (Aldrich) (30 mg dansyl chloride/ml). The reaction was allowed to proceed overnight at room temperature, stopped with 0.1 ml L-proline (Sigma) (100 mg/ml) and incubated for 30 min in the dark. The dansyl amines were then extracted into 0.5 ml benzene (J. T. Baker). Fifty $\mu$l of the benzene extract were then applied to each of six lanes of a silica gel plate (LK6D, 250 $\mu$m, Whatman, Inc.), which had been activated by heating at 110 C for 1 h just before application of samples. The plates were developed twice in ethyl acetate:cyclohexane (1:2). Dansyl fluorescence was measured by scanning the plate with a VIS-UV2 chromatogram Analyzer (Farrand Optical Co.) at 515 nm (excitation at 325 nm) and quantitated by comparing the areas under the peaks with those of standards run on the same plate. Standards consisted of 0.1 mM solutions in 0.03 N HCl of spermine tetrahydrochloride (Calbiochem), spermidine trihydrochloride (recrystallized from various commercial samples), and putrescine dihydrochloride (Calbiochem). These standard solutions were stored in 0.3-ml aliquots at −20 C and used for 2 months. Radioactivity was quantitated by scraping 0.4-cm fractions continuously from the origin up through the putrescine band into scintillation vials containing 10 ml of Aquasol and counting as described above. Recoveries were estimated to be 60% by dansylating, chromatographing, scraping, and counting a known amount of [$^{14}$C]spermidine (New England Nuclear).

Chl was extracted by homogenizing discs or protoplasts in 80% acetone. The particulates were sedimented at 460$g$ for 5 min and the Chl content of the supernatant was determined by the method of Arnon (4).

**Preparation of Decarboxylated SAM.** Decarboxylated SAM was prepared from [1-$^{14}$C]SAM with the aid of partially purified *Escherichia coli* SAM decarboxylase (21), and was isolated from the incubation mixture on a Dowex 50-$H^+$ (1 $\times$ 8 cm) by eluting with 5 N HCl after washing with 1 N HCl. The residual SAM was separated using preparative paper electrophoresis as follows: The material eluted from Dowex column with 5 N HCl was evaporated to dryness at 55 C under reduced pressure and dissolved in a small volume of 0.01 N HCl. A Whatman paper No. 1 (20 $\times$ 32 cm) was loaded with about 2 $\mu$mol (in a volume of 0.5 ml) of chromatographically purified decarboxylated SAM and subjected to paper electrophoresis at 380 v for 2.5 h using 0.1 M citrate buffer (pH 3.0). After electrophoresis, the paper strip was dried and the UV-adsorbing fractions were eluted with 20 ml of 1 N HCl for 20 min. Decarboxylated SAM migrated more rapidly than SAM. After centrifugation, the eluate was evaporated to dryness under reduced pressure at 55 C. The decarboxylated SAM thus obtained contained (per mole of adenine) 1.5% of the radioactivity of the initial substrate and was stored at −20 C at pH 4.0.

**Assay of Spermidine Synthase Activity.** To maximize enzyme activity, protoplasts were isolated after incubating the cross-hatched leaves in 0.5 M sorbitol in 1 mM $NaKPO_4$ (pH 5.8) containing 3% Cellulase and 1% Macerozyme at 30 C for only 3 h. Suspensions of 2 $\times$ $10^6$ protoplasts/ml in 1 M triethanolamine sulfate (pH 8.2) were disrupted by passing the suspension through a needle, as described under "Materials and Methods." The activity of spermidine synthase was determined by following the

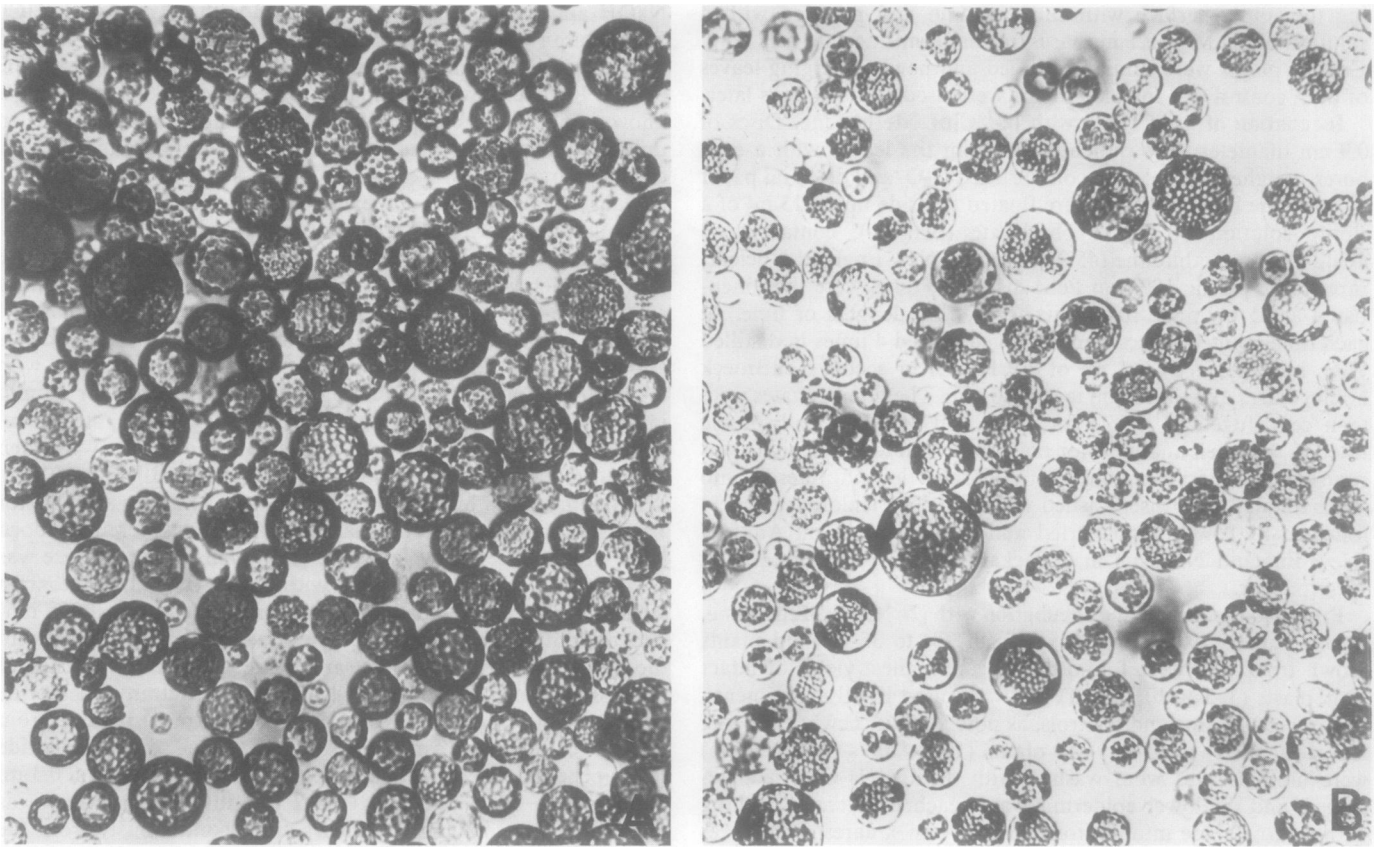


FIG. 1. Light micrographs of protoplasts from healthy and TYMV-infected Chinese cabbage leaves. A (left), protoplast suspension from leaves of healthy 35-day-old plants. B (right), protoplast suspension from leaves of comparable plants of same age 10 days after inoculation with TYMV. The cells of both preparations range from 20 to 50 μm in diameter. Brightfield illumination; magnification: 100×.

formation of [$^{14}$C]spermidine. The assay system contained 0.52 M triethanolamine sulfate (pH 8.2), [1,4-$^{14}$C]putrescine dihydrochloride, 37 μM (1.2 μCi), decarboxylated SAM (25 μM), and enzyme extract (0.17 ml) in a total volume of 0.325 ml. After incubation at 37 C for 1 h, the reaction was stopped by adding 1 ml 5% $HClO_4$. The tubes were centrifuged and the pellet was washed twice with 0.5 ml 3% $HClO_4$. After adding 0.03 μmol spermidine as the carrier, the extracts were loaded onto columns (1 × 4 cm) of Dowex −50 w ($H^+$ form 200–400 mesh, 8% cross-linked) (13). [1,4-$^{14}$C]Putrescine was eluted at 2.3 N HCl (10) and spermidine was eluted with 6 N HCl. The fraction containing spermidine was taken to dryness under reduced pressure at 55 C, dissolved in 0.03 N HCl, and dansylated. The plate containing dansylated amines was developed 6 times in benzene: triethylamine (80:16; v/v) (8) and spots corresponding in $R_F$ to standard spermidine were removed and counted.

## RESULTS

**Methionine Utilization in Discs and Protoplasts.** We have reported a quantitative similarity in the rate of conversion of [2-$^{14}$C]methionine to spermidine in discs derived from healthy or TYMV-infected leaves (23). Several circumstances might invalidate the conclusion that there is little change in the enzymic conversions in infected tissue. The penetrability of methionine into the tissue may have been a rate-limiting step. The sizes of the unlabeled metabolic pools of spermidine precursors, *e.g.* methionine and SAM, would markedly affect the apparent level of biosynthesis in each system. Also virus-infected cells in infected tissue may have been a small percentage of the total number of cells. The study of protoplasts derived from the healthy and virus-infected plants has helped to clarify the latter possibility.

Although the mechanism of infecting the plants presumably maximizes the penetration of virus into newly forming tissue, little is known at the microscopic level of the rate of spread of infection. However, examination of protoplasts derived from healthy and infected leaves revealed, as presented in Figure 1, a and b, that essentially all the cells of infected leaf tissues, isolable as protoplasts in a yield of leaf Chl in excess of 20%, show the polyplasty characteristic of the infection. By contrast, preparations of protoplasts derived from healthy tissue do not have significant numbers of such pathological cells.

Methionine was incubated with discs or with the protoplasts of healthy leaves and the conversion to spermidine and spermine were compared in the two systems. [2-$^{14}$C]Methionine was incorporated rapidly into an acid-insoluble fraction, presumably protein, in both discs and protoplasts (Table I). Similar molar rates of incorporation were obtained with [$^{35}$S]methionine, implying that the radioactivity of [2-$^{14}$C]methionine had entered protein as an intact amino acid.

The rates of incorporation into the protein fraction and into spermidine was 3.5- and 10-fold greater, respectively, in protoplasts than into these fractions of the discs. Furthermore, incorporation into spermine in the protoplasts has always proven to be active, of the order of a third as much radioactivity in the tetramine, representing about a sixth to a third as much synthesis of this polyamine as of spermidine.

In Table II are presented data on the incorporation of [2-$^{14}$C]methionine into comparable fractions of healthy and virus-infected protoplasts. The latter frequently contained slightly less Chl than did healthy protoplasts. It can be seen that they synthesize protein, spermidine, and spermine at a rate similar to that of healthy protoplasts for 2 h. However, in numerous experiments

Table I. *Time-Dependent Incorporation of Label from [2-$^{14}$C]DL-methionine into Leaf Discs and Leaf Protoplasts*

| | Time | Protein | Spermidine | Spermine |
|---|---|---|---|---|
| | *h* | *cpm/mg Chl* × $10^{-4}$ | | |
| Discs | 0 | 0.08 | 0.00 | 0.00 |
| | 1 | 5.89 | 0.11 | 0.03 |
| | 2 | 13.69 | 0.14 | 0.06 |
| | 4 | 21.06 | 0.14 | 0.06 |
| Protoplasts[a] | 0 | 0.02 | 0.00 | 0.00 |
| | 1 | 25.93 | 1.04 | 0.21 |
| | 2 | 45.33 | 1.48 | 0.48 |
| | 4 | 70.88 | 1.38 | 0.44 |

[a] In this isolation, $10^6$ protoplasts contained 0.045 mg Chl.

Table II. *Time-Dependent Incorporation of Label from [2-$^{14}$C]DL-methionine into Protoplasts of Healthy and Virus-Infected Chinese Cabbage*

| | Time | Protein | Spermidine | Spermine |
|---|---|---|---|---|
| | *h* | *cpm/$10^6$ protoplasts* × $10^{-3}$ | | |
| Healthy | 0 | 0.09 | 0.01 | 0.00 |
| | 1 | 8.38 | 0.23 | 0.06 |
| | 2 | 25.91 | 0.56 | 0.13 |
| | 4 | 61.77 | 0.59 | 0.16 |
| Virus-infected | 0 | 0.04 | 0.00 | 0.00 |
| | 1 | 6.91 | 0.16 | 0.05 |
| | 2 | 22.28 | 0.44 | 0.14 |
| | 4 | 46.51 | 0.73 | 0.29 |

(see Tables I and II), the net synthesis of the polyamines in the healthy protoplasts slows markedly or stops between 2 and 4 h, whereas this synthesis is maintained in this interval in the infected protoplasts. Indeed, in several experiments to be presented below (Tables III and V), a marked decrease in the amount of label into spermidine has been found in the healthy protoplasts between 2 and 4 h, suggesting a further metabolism of spermidine.

**Localization of Spermidine Newly Synthesized by Protoplasts.** Aliquots of a suspension of protoplasts ($10^6$/ml) from uninfected leaves incubated with labeled methionine for 2 and 4 h were disrupted by passage through a hypodermic needle. The homogenates were centrifuged at 33*g* for 15 min and the supernatant fluids were precipitated with 3% $HClO_4$ at 4 C. The initial pellets were also extracted with 3% $HClO_4$ in the cold. The acid precipitates and spermidine fractions of the supernatant fluids were analyzed for radioactivity and these were compared with similar fractions from the same suspensions of intact protoplasts. The low speed sediment derived from the disrupted protoplasts, *i.e.* organelles and membranes, contained 50 to 60% of the total spermidine of the protoplasts and 75% of the newly formed protein.

Electron microscopy of healthy protoplasts (Dewey and Kohlflesch, unpublished observations) has revealed the presence of a huge vacuole surrounded by a thin layer of cytoplasm containing an elongated nucleus and many chloroplasts. The cytoplasmic layer is only slightly wider than a single chloroplast. The chloroplasts themselves appear to comprise more than half the solid substance of the protoplasts.

**Biosynthesis in Disrupted Protoplasts.** When a portion of a suspension of healthy or infected protoplasts was disrupted, as described above, and incubated with methionine in the high osmolarity incubation medium at pH 5.8, incorporation into protein and spermidine fell to between 2 to 7% of that found in the intact protoplasts. This experiment eliminates the possibility that the low level of biosynthesis of spermidine in the protoplast can be due to microbial contamination.

To test the effect of pH on the biosynthesis by the disrupted protoplasts, the protoplasts were prepared in the usual way at pH 5.8, sedimented at and resuspended in the more alkaline medium at pH 8.0, which is frequently used for the study of protein synthesis by chloroplasts (9). Protoplasts disrupted in the pH 5.8 medium and in the pH 8.0 medium showed very little difference in incorporation into the acid-insoluble fraction. However, incorporation into spermidine was in fact doubled at the higher pH and pH 8.0 has been used in the studies of disrupted protoplasts presented in this paper.

**Biosynthesis in a Sedimentable Fraction Containing Chloroplasts.** In Table III, a comparison is given of incorporation of isotope from [2-$^{14}$C]methionine into protein, spermidine, and spermine in healthy protoplasts at pH 5.8, disrupted protoplasts at pH 8.0, and the low-speed sedimentable fraction in the pH 8.0 medium. It can be seen that the sedimented and resuspended fraction containing the chloroplasts incorporated methionine into protein at a rate similar to that found in the disrupted protoplasts. The rate of spermidine synthesis from exogenous methionine in the disrupted protoplasts was of the order of 5% of that in the intact protoplasts and did not fall between 2 and 4 h. We have observed also in several experiments, including that in Table III, that the low speed pellet has a distinct apparent increase in the rate of spermidine synthesis. In addition, whereas disrupted protoplasts are capable of a low rate of spermine synthesis, the low speed sediment, which contains the chloroplasts, has been devoid of this activity.

**Biosynthesis by the Low-Speed Sediment of Disrupted Virus-Infected Protoplasts.** A comparison was made of methionine utilization in the low speed sedimentable fractions of disrupted healthy and virus-infected protoplasts. The polyamine contents of the protoplasts and the low speed fractions used in this experiment are presented in Table IV. Although the infected protoplasts had significantly higher polyamine contents than the normal protoplasts, only minor differences were found in the polyamine con-

Table III. *Incorporation of Label from [2-$^{14}$C]DL-Methionine*

| | Time | Protein | Spermidine | Spermine |
|---|---|---|---|---|
| | *h* | *cpm/$10^6$ protoplasts* × $10^{-3}$ | | |
| Protoplasts | 0 | 0.13 | 0.00 | 0.00 |
| | 2 | 26.45 | 2.19 | 0.45 |
| | 4 | 58.10 | 0.45 | 0.09 |
| Disrupted protoplasts | 0 | 0.07 | 0.01 | 0.00 |
| | 2 | 2.32 | 0.04 | 0.01 |
| | 4 | 5.03 | 0.11 | 0.04 |
| Chloroplast-containing pellet | 0 | 0.05 | 0.01 | 0.00 |
| | 2 | 2.21 | 0.15 | 0.00 |
| | 4 | 5.75 | 0.34 | 0.00 |

Table IV. *Polyamine Content of Healthy and Infected Protoplasts and Derived Chloroplast Fractions*

| | Putrescine | Spermidine | Spermine |
|---|---|---|---|
| | *nmol/$10^6$ protoplasts* | | |
| Healthy | | | |
| Protoplasts | 5.35 | 4.18 | 0.40 |
| Chloroplast-containing pellet | 1.79 | 1.10 | 0.00 |
| Infected | | | |
| Protoplasts | 7.64 | 7.73 | 1.41 |
| Chloroplast-containing pellet | 2.16 | 1.11 | 0.14 |

tents of the low speed pellet containing the chloroplasts. In most comparisons of the polyamine contents of healthy and infected protoplasts, the infected cells contained 2.5 to 3 times as much spermidine as the healthy ones.

In Table V it can be seen that in this experiment incorporation of methionine in protein over a 4-h period were similar in the healthy and infected protoplasts and in the homologous low speed fractions derived from the disrupted protoplasts. However, in the healthy protoplasts labeled spermidine and spermine accumulated for 2 h and then fell. In the infected protoplasts, both spermidine and spermine were accumulated at greater rates and these accumulations continued for the length of the experiment.

The low speed sedimentable fractions derived from disrupted healthy and virus-infected protoplasts synthesized spermidine at 12 to 14% of the rates found in the intact protoplasts. These rates were readily determinable. As noted earlier, this synthesis occurred without any detectable spermine synthesis.

**Spermidine Synthase.** We have been assuming in this study that the synthesis of spermidine from methionine in the various biological systems described above required three reactions, *i.e.* the conversion of methionine to SAM, a decarboxylation of SAM, and a propylamine transfer from decarboxylated SAM to putrescine. The latter reaction is catalyzed by spermidine synthase and we have sought this enzyme in the soluble and low-speed sedimentable fractions of disrupted healthy and virus-infected protoplasts.

The reaction was carried out with essentially unlabeled enzymically decarboxylated SAM, purified by ion exchange chromatography and paper electrophoresis. The radioactive precursor used to estimate spermidine synthesis was [1,4-$^{14}$C]putrescine at high specific radioactivity and in 1.5-fold excess over the amount of decarboxylated SAM. To obtain reliable analyses for spermidine produced enzymically, it was found necessary to eliminate unreactive radioactive putrescine from the reaction mixture prior to dansylation of the polyamines. One % of the radioactivity of dansyl putrescine was found to contaminate the solvent track in the dansyl spermidine position. Over 95% of the residual putrescine from acid extracts could be eliminated by adsorption of the polyamines on Dowex-50-H$^+$ and elution of the putrescine selectively with 2.3 N HCl. The spermidine of the sample was then eluted with 6 N HCl and identified and counted as the dansyl derivative.

Protoplasts isolated from leaves after only a 3-h incubation with macerating enzymes to minimize enzyme loss were disrupted in the triethanolamine buffer in which the reaction was carried out. As seen in Table VI, disrupted protoplasts contained significant levels of spermidine synthase activity. Furthermore, the apparent activities of these extracts from equal numbers of healthy and infected protoplasts were similar. Since the addition of the disrupted protoplasts to incubation mixtures containing the partially purified *E. coli* spermidine synthase did not decrease this activity, it has been tentatively concluded that protoplast disruption does not release very active inhibitors of this activity.

Spermidine synthesis was not found in the absence of enzyme extract or decarboxylated SAM. The activity in the cell extract was destroyed by heating in a boiling water bath for 5 min. Unlike the stable spermidine synthase of *E. coli*, more than 90% of the activity was lost on storage at −15 C for 7 days. The amount of the enzyme, *i.e.* in terms of the spermidine synthesized 1 h/mg Chl, was more than 10 times that required to synthesize the spermidine contained in the plant during its most active growth.

It was found in several experiments that, following low-speed sedimentation of the chloroplast-containing fraction from disrupted healthy or virus-infected protoplasts, about 60% of the activity was present in the supernatant fluid. A washed, resuspended, low-speed sedimentable fraction containing the chloroplasts had about 25 to 30% of the original activity, which was sufficient to account for spermidine accumulation in the healthy plant.

## DISCUSSION

The metabolic conversions of amino acid precursors to polyamines studied in the biological materials described in this paper appear to occur at low rates and require sensitive and specific analytical systems for their demonstration. In experiments with labeled methionine, seeking incorporation into spermidine, the silica gel fractionation, which minimizes carryover of carbohydrate and other plant components into the dansylation step for the subsequent TLC of the derivatized amines, has been particularly useful. However, in experiments with labeled putrescine, it was also important to minimize labeled dansyl putrescine on the plate, because in various chromatographic solvents, traces of this component were deposited at the dansyl spermidine position. This could be effected with an ion exchange separation of putrescine and spermidine, eliminating the need for the silica gel separation. The separation of the dansylated compounds has also given some assurance of the nature of the radioactive products.

Recent experiments on the isolation and specific radioactivity of SAM, in methionine-fed normal and infected protoplasts, have

Table V. *Incorporation of Label from [2-$^{14}$C]DL-Methionine*

| | Time | Protein | Spermidine | Spermine |
|---|---|---|---|---|
| | *h* | *cpm/10$^6$ protoplasts × 10$^{-3}$* | | |
| Healthy | | | | |
| Protoplasts | 0 | 0.05 | 0.05 | 0.00 |
| | 2 | 38.69 | 0.78 | 0.07 |
| | 4 | 76.51 | 0.35 | 0.04 |
| Chloroplast-containing pellet | 0 | 0.26 | 0.02 | 0.00 |
| | 2 | 1.93 | 0.10 | 0.00 |
| | 4 | 5.71 | 0.54 | 0.00 |
| Infected | | | | |
| Protoplasts | 0 | 0.03 | 0.00 | 0.00 |
| | 2 | 38.92 | 2.00 | 0.32 |
| | 4 | 76.03 | 4.58 | 0.86 |
| Chloroplast-containing pellet | 0 | 0.04 | 0.00 | 0.00 |
| | 2 | 1.98 | 0.28 | 0.00 |
| | 4 | 5.53 | 0.75 | 0.00 |

Table VI. *Formation of Spermidine by Spermidine Synthase in Extract of Cabbage Protoplast*

| | Total Radioactivity[a] | Rate of Spermidine |
|---|---|---|
| | *cpm × 10$^{-3}$* | *nmol/h·10$^6$ protoplasts* |
| Complete system | 31.8 | 0.37 |
| Minus enzyme extract[b] | 0.7[c] | |
| Minus decarboxylated SAM | 0.8[c] | |
| Heated extract | 0.9[c] | |
| Supernatant fluid | 18.8 | 0.23 |
| Chloroplast-containing low-speed sediment | 6.3 | 0.086 |

[a] Corrected for 76% recovery. The fraction of dansyl amine plated was 0.046 of the total.

[b] The extract added to the reaction mixture was derived from 3.5 × 10$^5$ protoplasts.

[c] This is the radioactivity in the dansyl spermidine position apparently derived from the dansyl putrescine remaining after ion exchange separation.

demonstrated a 5- to 10-fold decrease in the radioactivity of the methionine moiety, as compared to the exogenous labeled amino acid (Balint and Cohen, unpublished observations). Thus, if it is assumed that the specific radioactivity of this total SAM is that of the immediate spermidine precursor, the level of synthesis of spermidine is many fold greater than that suggested by the apparently low conversion of methionine to the triamine. Further, it is probable that our method of isolation of protoplasts, involving an overnight incubation with cellulases in the dark, serves to deplete an even greater pool of methionine and SAM in leaves and discs. This result may also account for the very low level of apparent synthesis of spermidine in fresh discs (Table I). The pools of amino acids in these discs may also account in some degree for an even lower level of utilization of exogenous arginine and ornithine in the synthesis of putrescine (20; Cohen, unpublished experiments). Further, the problem of the distribution of these pools in vacuoles and other organelles will raise questions on the interpretation of experiments using exogenous precursors and these will bear on the problem of the sites of polyamine synthesis.

Nevertheless, despite the complexity of the plant protoplast and its compartments, it has been possible to use these separated leaf cells, in a healthy or infected state, to demonstrate the utilization of exogenous methionine to synthesize labeled spermidine and spermine. The fact that each population of protoplasts is homogeneous with respect to the lack of aggregation or pronounced aggregation of the chloroplasts was an unexpected result which heightened the significance of chemical difference and similarities in the various experiments comparing the two biological systems. Furthermore, the two types of cell have been found to behave differently in that the protoplasts derived from healthy plants do not continue to accumulate spermidine; after 2 h this product was degraded or deposited in an acid-insoluble form on the low-speed sedimentable fraction (Balint and Cohen, unpublished observations). On the other hand, spermidine did continue to accumulate in an acid-soluble form after 2 h in the infected protoplast. We suspect, but have not demonstrated, that this newly formed spermidine is trapped in another metabolically inert compartment, *i.e.* newly synthesized virus.

The ease of disruption of the protoplasts, enabling our ready detection of soluble and particulate spermidine synthase, has emphasized the utility of this material in the further analysis of the subcellular compartments and components of the normal and virus-infected plant cell (22). The simultaneous infection *in vitro* of cabbage protoplasts by TYMV and some biological properties of this system have been described (19). The experiments described in this paper indicate that the system is clearly suitable for a further study of metabolic events relating chloroplast functions to the viral polyamines. The present study was in fact undertaken to determine the biological suitability of protoplasts for further biochemical study in virus infection.

The initiating hypothesis of this investigation postulated a synthesis of spermidine, but not spermine, in the chloroplast, as in other procaryotic cells, including Cyanobacteria. Although about two thirds of the spermidine synthase is found in a soluble supernatant fraction, the easily sedimentable fraction, which contains the chloroplasts, contains an amount of enzyme which can account for the synthesis of cell and viral spermidine. It may be noted that this particulate fraction has lost the ability to convert methionine to spermine even though it is still capable of converting the amino acid to spermidine. On microscopic examination, the low-speed pellet has little contaminating debris other than chloroplasts, but a demonstration that this enzyme is in fact associated with the organelle will require more rigorous analysis.

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