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Biochemistry

Spermidine, an intrinsic component of turnip yellow mosaic virus

(polyamines/RNA/norspermidine/spermine)

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ABSTRACT **The major polyamine of turnip yellow mosaic virus has been identified as spermidine by gas chromatography and mass spectrometry of the trifluoroacetamido derivative. Very small amounts of putrescine and cadaverine, but not norspermidine, have been detected in the virus. The spermidine contents of numerous virus preparations were in the range 200–700 molecules per virion and were considerably in excess of those of spermine. The RNA and spermidine contents of small amounts of the virus were determined after serological precipitation in purified preparations and in the juice of infected plants. Under conditions of the precipitation or purification from juice by differential centrifugation, only small amounts of exogenous radioactive spermidine and spermine became bound to virus. Although adsorbed radioactive spermidine could be removed almost quantitatively by washing the virus in dilute buffers, only a small part of adsorbed spermine was removed by such treatment. However, >95% of the newly attached spermine was separated from the virus without loss of the original spermidine by sedimentation in buffers containing 0.5 M NaCl or 0.06 M $MgCl_2$. Crystallization of virus in 40% saturated $MgSO_4$ or 7.5% heparin or dialysis did not decrease viral spermidine. Although the virus coat may adsorb small amounts of the polyamines reversibly, the virus is impermeable to exogenous spermidine and spermine and does not exchange or leak internal spermidine. The spermidine present in purified virus is associated with viral RNA at the time of packaging and formation of intact virions.**

Turnip yellow mosaic virus (TYMV), a RNA virus known to contain polyamine (1, 2), is believed to multiply in chloroplast aggregates in Chinese cabbage (3). The virus was reported to contain far more triamine than the tetramine, spermine (1, 2), although the latter is present in the plant and binds to RNA far more tightly than does spermidine (4). The accumulation of putrescine, spermidine, and spermine is exaggerated in the infected plant (5), and we have been investigating the ability of chloroplasts to synthesize the viral triamine. We have reinvestigated the identity of the viral triamine because several recently discovered natural triamines (6), including norspermidine, originally thought to be the viral triamine (1), are not easily separated from spermidine.

To determine if the spermidine in the virus was an artifact of preparation, as a result of the sopping up of spermidine into RNA contained within a possibly sponge-like virion, we have studied the adsorption of polyamines to virus as well as the stability of polyamine content. The virus does contain spermidine as the sole triamine, which is essentially nonexchangeable, indicating that the triamine is associated with the viral RNA before packaging into virions.

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MATERIALS AND METHODS

Growth and Purification of TYMV. Chinese cabbage seeds (*Brassica pekinensis*, var. Pak Choy) were obtained from Nichols Garden Nursery (Albany, OR). Plants were grown in a growth chamber (Scientific Systems, Baton Rouge, LA) set for 18-hr days at 28°C with 20,000 lux from incandescent and fluorescent lighting and 6-hr dark periods at 22°C. At about 5 weeks, the rosettes of each plant were removed, leaving two large leaves to be mechanically inoculated with carborundum and a solution of TYMV (0.1 mg of virus per ml in 0.02 M phosphate buffer, pH 7.0). Three weeks after infection, newly emerged deribbed leaves were frozen and stored at −20°C.

Frozen leaves were homogenized in a Waring Blendor with 0.05 M pH 4.8 potassium acetate buffer at 1 ml/g. The juice was expressed through eight layers of cheese cloth and centrifuged at 3000 × *g* for 30 min. The supernatant fluid was centrifuged at 78,500 × *g* for 150 min and the drained pellets were soaked in cold acetate buffer overnight. The pellets were triturated with buffer and after another cycle of low- and high-speed centrifugation, the pellets were redissolved in the acetate buffer and centrifuged at 3000 × *g*. Such solutions contain both empty capsids and intact virions (7). Empty capsids, separated by sucrose density gradient centrifugation, are essentially devoid of both RNA and polyamine (unpublished data). These particles are significantly less stable than intact virions, and denatured virus protein precipitates on storage at 4°C. After 2–3 months the empty capsids had essentially disappeared from the preparation. Virus concentrations were estimated from the extinction coefficient $E^{1\%}_{260} = 8.4\ cm^2/mg$ (8). Virus preparations at A_{260} of 100–300 were stored at 4°C in acetate buffer pH 4.8 containing 1 mM sodium azide.

Crystallization of TYMV. Crystallization of TYMV with salts, ethanol, or heparin most commonly yields octahedra (9). In the current study, we obtained tetragonal bipyramids with salts and fragile rectangular plates with heparin. Virus preparations containing 10 and 26 mg/ml could be crystallized at 4°C from 33% saturated ammonium sulfate by mixing or by free interphase diffusion. Crystals obtained by the latter method were larger and more regular in shape than those obtained by mixing or by dehydration. TYMV crystals obtained from ammonium sulfate are not easily analyzed for amines by the dansyl method. Crystals were obtained from 40–46% saturated magnesium sulfate or from 5–10% (wt/vol) heparin. After removal of mother liquors by fine capillary pipettes, the crystals were dissolved in acetate buffer, and these solutions were extracted in 3% perchloric acid for polyamine analysis.

Abbreviations: TYMV, turnip yellow mosaic virus; norspermidine, 1,7-diamino-4 azaheptane; GC/MS, gas chromatography/mass spectrometry; dansyl, dimethylaminonaphthylsulfonyl; F_3CCONH, trifluoroacetamide.

Polyamine Analyses. Hydrochlorides of spermidine and norspermidine were recrystallized from mixtures of ethanol, water, and isopropanol. The hydrochloride of *N*-3'-aminopropyl-1,5-diaminopentane was recrystallized from absolute ethanol. These substances as well as hydrochlorides of spermine, putrescine, and cadaverine were >99% pure according to chromatography as dansyl derivatives.

Extracts of virus preparations or plant juice were made in 3% perchloric acid, chilled, and centrifuged. The cold acid extracts were neutralized with KOH, chilled, and centrifuged to remove $KClO_4$. A portion of the supernatant fluid was dansylated. Dansyl polyamines were extracted into benzene and separated by thin-layer chromatography on silica gel G plates with development with cyclohexane/ethyl acetate, 2:1 (vol/vol), two to four times. The fluorescent dansyl amines were quantified with a Farrand VIS-UV chromatogram analyzer.

Serological Method. Rabbit antisera to TYMV were obtained from the American Type Culture Collection and were used to precipitate virus specifically under conditions of equivalence or of most rapid flocculation (5, 10). In estimates of the polyamine or RNA content of virus in purified preparations or in plant juice, samples (0.2 ml) containing virus in phosphate buffer (0.05 M, pH 7.1) were incubated with diluted antiserum or buffer (0.2 ml) at 37°C for 2 hr and stored overnight at 4°C. The tubes were centrifuged at 4°C for 1 hr at 850 × *g*, and the precipitates were drained and washed similarly with phosphate buffer. The polyamines were extracted with 0.4 ml of 3% perchloric acid at 4°C. Perchloric acid (1.2 ml of 3%) was added to the drained precipitate and heated to 90°C for 15 min. After cooling and centrifugation, an ultraviolet absorption spectrum was determined and compared with that of a mixture of nucleotides prepared with the ratio of bases estimated to be that of group I type TYMV (11). The recoveries of polyamine and RNA from purified virus preparations precipitated by antisera in the presence or absence of plant juice was usually >95%. Precipitates from infected juice contained spermidine and RNA in amounts indicating the presence of virus with 200–400 molecules of the triamine per virion. Antiserum precipitated 20–26% of the triamine of the juice from an extract prepared at the height of infection (1–1.5 mg of virus per ml).

Preparation for Gas Chromatography/Mass Spectrometry (GC/MS). The polyamines were extracted from TYMV with 3% perchloric acid and were extracted at pH 13 into *n*-butanol at 30°C in the presence of saturating amounts of Na_2SO_4. The butanol extract was acidified with HCl and taken to dryness *in vacuo*. The residue was dissolved in 1.0 ml of acetonitrile, an excess (1.0 ml) of trifluoroacetic anhydride (Aldrich) was added, and the mixture was heated in a sealed tube in an oil bath for 5 min at 100°C. After cooling, solvents were removed with N_2. The trifluoroacetamido (F_3CCONH) derivatives dissolved in acetontrile were analyzed by GC/MS.

Hewlett-Packard equipment was modified and operated by Charles Iden and Philip Chang of this Department. The 5710A gas chromatograph used a 6-foot column of 3% OV-17 programmed at 16°C/min in the range 130–250°C. The mass spectrometer was model 5980A equipped with the model 5933A data system.

Eluted peaks of separated F_3CCONH diamines and triamines were analyzed in the mass spectrometer for comparison with spectra of standard derivatized diamines and triamines. In addition, the analyses were carried out by utilizing the single-ion monitor set for M-CF_3 ions—i.e., *m/e* 364 for spermidine and *m/e* 350 for norspermidine. Quantitation was achieved by constructing a standard curve with F_3CCONH-spermidine and norspermidine under isothermal conditions.

Sucrose Density Gradients. Gradients in the range 5–20% (wt/vol) sucrose were prepared in cellulose nitrate tubes with an Isco gradient mixer. A virus sample, 1 ml containing 10–11 mg of TYMV in 0.05 M KOAc at pH 4.8, was layered on 37 ml of a 5–20% gradient of sucrose in this buffer. The tube was centrifuged at 26,000 rpm for 150 min at 4°C in an SW 27 rotor in a Spinco model L5-50 ultracentrifuge. A 30% (wt/vol) sucrose solution displaced the fractions upward in an Isco density gradient fractionator; 0.9-ml fractions were collected and absorbances were determined. Aliquots (0.1 ml) of the fractions were added to 10 ml of Aquasol, and radioactivity was estimated in a Packard scintillation counter.

Radioactive Substances. Radioactive polyamines were obtained from New England Nuclear. [1,4-^{14}C]Putrescine dihydrochloride, [*tetramethylene*-1,4^{14}C]spermidine trihydrochloride, and [*tetramethylene*-1,4^{14}C]spermine tetrahydrochloride were available at 6–9 × 10^7 cpm/μmol. Spermine tetrahydrochloride was also obtained as [3-*aminopropyl*-3-^{3}H(N)]$_2$spermine·4HCl at 15–30 Ci/mmol.

RESULTS

Diamines and Triamines in TYMV. Fig. 1 compares the gas chromatographic patterns of elutions of F_3CCONH derivatives of spermidine, norspermidine, and amines extracted from samples of TYMV. These patterns were monitored in the mass spectrometer at *m/e* 126, defining the ion $CF_3CONHCH_2$-found in the analysis of all the derivatized diamines and triamines studied. Peaks from F_3CCONH norspermidine of *m/e* 126 and 350 appeared simultaneously at 6.3–6.5 min, whereas peaks from F_3CCONH spermidine of *m/e* 126 and 364 appeared at 7.2 min. The peak of the major F_3CCONH amine extracted from TYMV monitored at *m/e* 126 and 364 eluted at 7.2 min and had the additional methylene of spermidine, distinguishing it from norspermidine. The mass spectra of the F_3CCONH derivatives of both compounds are characteristically different and the F_3CCONH derivative of the viral component eluting at 7.2 min has a mass spectrum essentially identical to that of F_3CCOHN spermidine (Fig. 2). The largest mass ion obtainable from these compounds are 419 and 433 for F_3CCONH norspermidine and spermidine, respectively; these are present in small amounts in the spectra. The components monitored (*m/e* 350 and 364, respectively) have each lost CF_3. No evidence of F_3CCONH norspermidine (retention time, 6–6.5 min; *m/e* 350) was obtained in the various analyses.

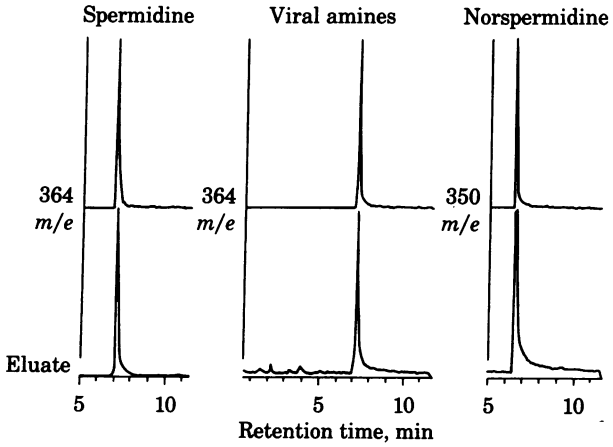

FIG. 1. Gas chromatography and mass monitoring of F_3CCONH derivatives of triamines and viral di- and triamines. The elution of the polyamines generally was monitored in the mass spectrometer at *m/e* 126 and that of the derivatives of spermidine and norspermidine, at *m/e* 364 and 350, respectively. The viral triamines were monitored at *m/e* 364 and 350; a signal was obtained only at *m/e* 364.

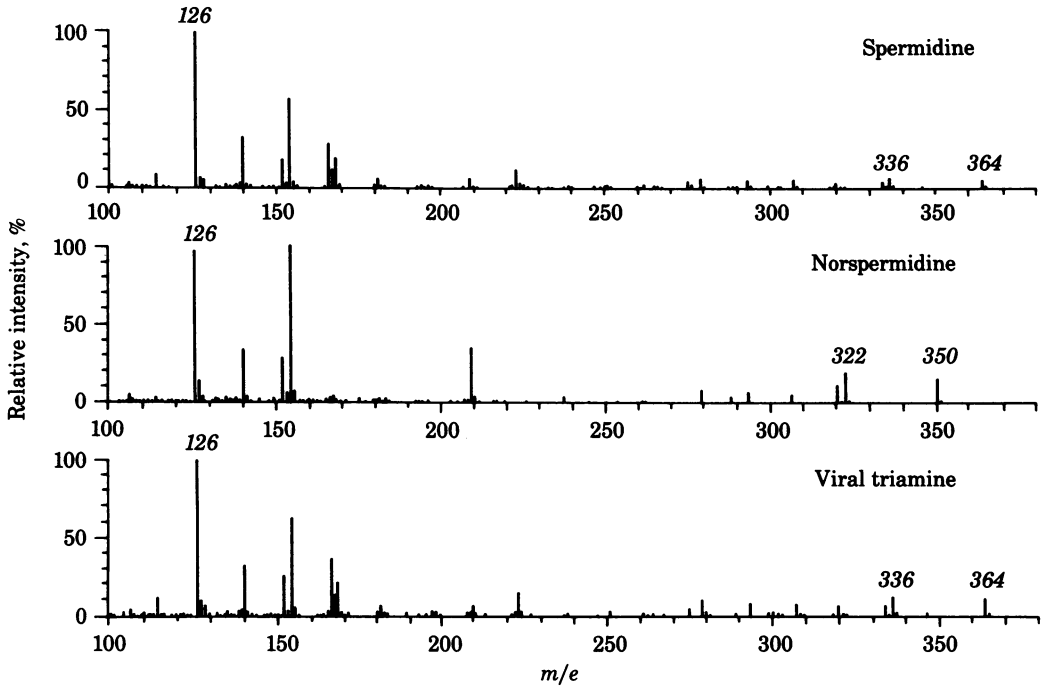

FIG. 2. Mass spectrometry of F_3CCONH derivatives of spermidine, norspermidine, and viral triamine.

Mass spectra were taken of the viral components with retention times of 2.2, 3.4, and 4.0 min. The spectra have a component of *m/e* 126. That of retention time 3.4 min is similar in major respects to that of putrescine, having an ion at *m/e* 211. The component eluting at 4.0 min gives rise to an ion at *m/e* 225, suggesting the presence of cadaverine among the amines. The GC/MS system used did not permit elution of F_3CCONH spermine.

Adsorption During Serological Precipitation. The adsorption or exchange of exogenous radioactive amines was studied under various conditions of isolation of the virus. To 0.2-ml aliquots of phosphate buffer (pH 7.1) or of virus (0.2 A_{260} unit containing 3.7 nmol of spermidine) in this buffer were added 11 μl of polyamines containing 11 nmol of radioactive polyamine (approximately 2×10^5 cpm) and 0.2-ml aliquots of diluted specific rabbit antiserum or normal serum. After the tubes were incubated, centrifuged, and washed, they were extracted with 0.4 ml of 3% perchloric acid and the extracts were assayed for radioactivity and for polyamine.

Even under conditions of a large excess of exogenous polyamines relative to viral spermidine, only small amounts of radioactivity were found in the tubes after centrifugation and washing (Table 1). Little radioactive putrescine and spermidine—i.e., 2.1% and 2.3%, respectively, of the original spermidine content—was bound to the specific precipitate (i.e., the excess in the tube containing antiserum over normal serum). However, the adsorbed [^{14}C]spermine amounted to almost 4% of the spermidine content.

The adsorption of spermidine but not of putrescine was increased by preincubation of isotopic compound at 4°C for 30 min in the tube containing virus or buffer prior to addition of the sera. The isotopic spermidine increased to approximately 10% of the spermidine initially present. On the other hand, the spermine present in the specific precipitate apparently increased to a total of 20% of the spermidine present. When exogenous spermine was reduced to a fourth, the association of the tetramine decreased considerably. Also, the nonspecific binding of spermine to the tube was found to be much higher than that of spermidine.

Association During Sucrose Density Centrifugation. TYMV (10.8 mg in 1 ml of acetate buffer, pH 4.8) was layered on a 5–20% sucrose density gradient. To the virus solution which contained 77 nmol of spermidine and 8.2 nmol of spermine in virions had been added 2 μmol (10^6 cpm) of [^{14}C]spermidine or [^{14}C]spermine—i.e., a 26- or 244-fold excess, respectively, over the respective internal amines. The solutions were centrifuged and fractionated; the results for the mixture containing spermine are presented in Fig. 3. At the peak, the virus preparation which contained 420 and 82 molecules of spermidine and spermine, respectively, per virion was found to be associated with 25.7 molecules of radioactive spermine per virion, or about a third of the total spermine. In the similar experiment in which [^{14}C]spermidine had been mixed with the virus, the peak tube contained 42 molecules of radioactive spermidine—i.e., an increment of 11% over the internal spermidine.

Association to Virus During Isolation. When 2.4 μmol of ^{14}C-labeled polyamine was added to the juice expressed from

Table 1. Association of exogenous polyamines with TYMV during serological precipitation

		Recovery of added polyamine, %	
Polyamine*	Test system†	No preincubation	Preincubated
Putrescine	V + AS	1.03	1.12
	V + NS	0.32	0.70
	B + AS	0.20	1.18
Spermidine	V + AS	1.30	6.30
	V + NS	0.54	2.71
	B + AS	0.46	0.63
Spermine	V + AS	2.00	10.74
	V + NS	0.74	3.65
	B + AS	0.90	0.05

* mol exogenous [^{14}C]polyamine/mol of viral spermidine = 3.
† V, TYMV; AS, antiserum to TYMV; NS, normal serum; B, 0.05 M phosphate, pH 7.1.

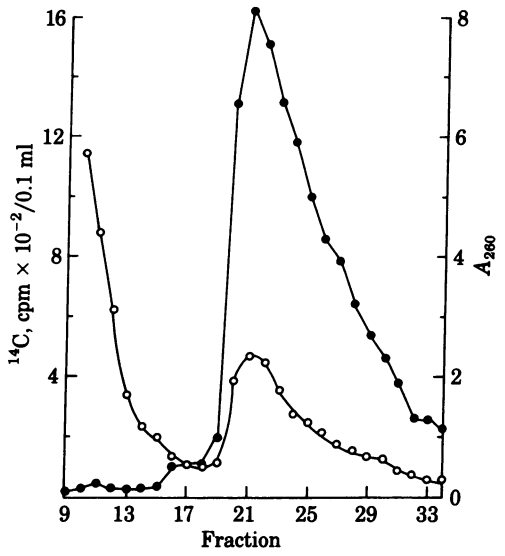

FIG. 3. Association of exogenous [^{14}C]spermine and purified TYMV in sucrose density centrifugation at pH 4.8. ●, A_{260}; ○, [^{14}C]spermine.

60-g batches of infected leaves containing 24 μmol of spermidine and then virus was isolated, the virus was found to contain only small amounts of the exogenous polyamine—i.e., 2.2 and 5.2 molecules of [^{14}C]spermidine and [^{14}C]spermine, respectively, per virion. The virus contained 216 total molecules of spermidine per virion, of which only 1% was derived from added exogenous radioactive spermidine, whereas virus containing 23 total molecules of spermine had been isolated of which 23% was derived from added radioactive spermine.

Preparations of purified TYMV derived from similar 60-g batches of infected leaves (2 ml containing 190 A_{260} units in acetate buffer at pH 4.8) were mixed with 2.4 μmol (10^6 cpm) of [^{14}C]spermidine or [^{14}C]spermine. These virus mixtures were subjected to two cycles of differential centrifugation, and the final preparations were found to contain 35 molecules of [^{14}C]spermidine and 34 molecules of [^{14}C]spermine per virion. Thus, little adsorbed spermidine was found on TYMV under conditions of virus isolation from plant juice, but a significant contamination of triamine was found when virus was reisolated from a mixture of concentrated virus and 2 mM triamine.

Washing TYMV in Sucrose Density Gradients. TYMV containing 2.2 molecules of [^{14}C]spermidine per virion or 5.2 molecules of [^{14}C]spermine per virion were subjected to 5–20% sucrose density gradients. After fractionation of the TYMV labeled with [^{14}C]spermidine, bound [^{14}C]spermidine was at most 0.3 molecule per virion, a reduction of at least 85%. On the other hand, radioactive spermine remained associated with TYMV. At the peak, the virus contained 4.0 molecules of [^{14}C]spermine per virion in contrast to the original 5.2 molecules. Spermidine had a lower affinity for the viral protein coat than did spermine.

Table 2. Removal of adsorbed spermine from TYMV by sedimentation in buffered salt

Salt solution	% radioactivity in sedimented virus	cpm/A_{260}
0.05 M acetate (pH 4.8)	68	2648
Acetate + 0.5 M NaCl	2.5	90.1
Acetate + 0.06 M $MgSO_4$	3	78.7
0.05 M phosphate (pH 7.4)	32	1033
Phosphate + 0.5 M NaCl	4.5	118
Phosphate + 0.06 M $MgSO_4$	3	72.6

Table 3. Effect of crystallization of polyamines of TYMV

Treatment	Polyamine, no./virion: Spermidine	Spermine
Preparation 1:		
Differential pelleting	187	29
Crystallization		
40% saturated $MgSO_4$	154	5
7.5% heparin	186	16
Preparation 2:		
Differential pelleting	449	99
Crystallization		
45% saturated $MgSO_4$	411	91
7.5% heparin	352	76

Washing TYMV in Salts. Virus preparations containing associated exogenous [^{3}H]spermine after several cycles of differential centrifugation were sedimented in 0.05 M acetate buffer at pH 4.8 or 0.05 M phosphate buffer at pH 7.4 containing 0.5 M NaCl or 0.06 M $MgCl_2$ or no added salt. The radioactivities and polyamine contents of the redissolved virus were determined (Table 2). Two-thirds of the radioactivity was resedimented in the acetate buffer alone, but the presence of 0.5 M NaCl or 0.06 M $MgCl_2$ decreased adsorbed spermine to 2.5–3% of the total. Phosphate alone decreased the adsorbed spermine to 32% of the total, and added salts removed radioactive spermine to very low levels. In these experiments the total spermidine contents of the virus preparations were not significantly changed on resedimentation. Spermine values (63 molecules per virion originally) tended to decrease slightly but not markedly (i.e., not greater than a third).

Effect of Crystallization or Dialysis on Polyamine Content of TYMV. In Table 3 are presented data comparing the polyamine contents of crystals of TYMV and of the original virus. In the first preparation, there was some decrease in the spermine content but no significant decrease in the spermidine content. In the second preparation, crystallization from $MgSO_4$ did not significantly affect either spermidine or spermine content per virion. Crystallization from heparin produced a 22–23% decrease in both polyamines per virion; this may have resulted from some selective degradation of the protein outer coat in some virus particles. In both instances, prolonged soaking (several weeks) in high concentrations of Mg^{2+} or of the polymeric highly charged anion heparin had not led to loss of the polyamines from the virus particles.

Aliquots of a TYMV preparation (377 molecules of spermidine per virion) were dialyzed at 4°C against water, acetate buffer, or 50% saturated $MgSO_4$ to equilibrium. The mean spermidine contents of the dialyzed preparations were unchanged—352 ± 29 molecules per virion.

DISCUSSION

The GC/MS analyses identify spermidine unequivocally as the major polyamine found associated with TYMV. The amounts of this substance vary from preparation to preparation over a 3.5-fold range—i.e., from about 200 molecules per virion to almost 700 molecules per virion—indicating neutralization of about 10–35% of the RNA phosphorus by this polycation. The spermine molecules per virion has been observed to vary from 3–5% of the spermidine content as a lower value (2) to as high as 22% of the spermidine content (Table 3).

Spermidine was not lost from TYMV under conditions of

dialysis or of prolonged soaking during crystallization in concentrated Mg salt solution or heparin. Small amounts of exogenous spermidine, always less than 10% of that contained in the particle, would associate with TYMV in solutions up to 2 mM with respect to the triamine but could be displaced from the particle during centrifugation in 0.05 M salt. On the other hand, exogenous spermine was bound tightly to TYMV in 0.05 M acetate buffer at pH 4.8. However, spermine was almost totally displaced in 0.05 M phosphate buffer at pH 7.4 containing 0.5 M NaCl or 0.06 M $MgCl_2$. It might be useful to incorporate one or another of these salts in a stage of virus purification.

The association of exogenous spermine with TYMV is an additional instance of the significant affinity of spermine for all kinds of anionic surfaces: glass, nucleic acids, cells and organelle membranes, and a number of proteins. Among such spermine-binding proteins, which bind spermine noncovalently much more tightly than spermidine, have recently been described an androgen-sensitive protein in the rat ventral prostate (12) and IgGs of human and rat sera (13–15). The increased affinity of spermine for certain eukaryotic proteins may relate to the evolutionary origin and survival of spermine biosynthesis in eukaryotic cells and suggests that spermine may regulate the functions of such proteins.

Because we have not found significant amounts of spermidine in RNA-free protein shells isolated from virus preparations, we have concluded that the spermidine is associated with the internal RNA of TYMV. The fact that soaking or washing in concentrated salt solution does not reduce the spermidine content of the virus preparations reinforces the conclusion that the protein shell of TYMV is impentrable to the organ cations. The data on nonexchangeability indicate that this polyamine is associated with the RNA at the time at which the RNA is packaged into impenetrable particles. This demonstrates a nonexchangeability in a RNA virus that is already known in certain DNA viruses—e.g., the wild-type T-even phages (16).

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