# Ultrastructure of Extracted Carnation Etched Ring Virus Inclusion Bodies Treated with Proteolytic Enzymes and DNase

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ABSTRACT

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Carnation etched ring virus (CERV) inclusion bodies were extracted from infected leaves of *Saponaria vaccaria* 'Pink Beauty' and treated with proteolytic enzymes. The effect of the enzymes on inclusions in ultrathin sections was compared to the alteration produced in the ultrastructure of inclusions that were exposed to enzymes and subsequently fixed and embedded. Ultrathin sections of extracted inclusions embedded in glycol methacrylate (GMA) were more susceptible to Protease VIII and trypsin when fixed in formalin than in glutaraldehyde or acrolein. Enzymatic treatment removed the matrix before digesting the virions. The time required for digestion decreased as the concentrations of the enzymes increased. Formalin-fixed inclusions embedded in GMA were unaltered after treatment with deoxyribonuclease. Pelleted inclusions and inclusions in suspension that were treated with protease and then fixed in glutaraldehyde and embedded in Epon showed digestion of the matrix before the virions were morphologically altered. Protease digestion occurred more uniformly and rapidly with suspended inclusions than with those that had been pelleted prior to treatment. The greater susceptibility of the inclusion matrix to proteolytic enzyme digestion compared to the virions may indicate a difference in the proteins of the matrix and virions.

*Additional key words*: electron microscopy, enzyme digestion, cytoplasmic inclusions.

Previous ultrastructural investigations have established that carnation etched ring virus (CERV) induces cytoplasmic inclusion bodies (5, 14, 19) that are characteristic of the caulimoviruses. Members of this group include cauliflower mosaic virus (CauMV) (4), mirabilis mosaic virus (2), strawberry vein-banding virus (11), dahlia mosaic virus (DaMV) (13), and cassava vein mosaic virus (12).

The cytoplasmic inclusions induced by CERV and other members of the caulimovirus group are composed of virions embedded in an electron-dense matrix (20) containing electron-transparent vacuole-like openings throughout the matrix. These inclusion bodies can be stained with protein stains (6, 9, 13, 17, 18) and observed with the light microscope. The proteinaceous nature of the CauMV inclusion matrix has been confirmed by treating ultrathin sections with proteolytic enzymes (3, 17). A light-microscope observation which suggested that DaMV inclusions were surrounded by a pellicle (18) has not been confirmed by ultrastructural studies of this virus or of any other member of the group.

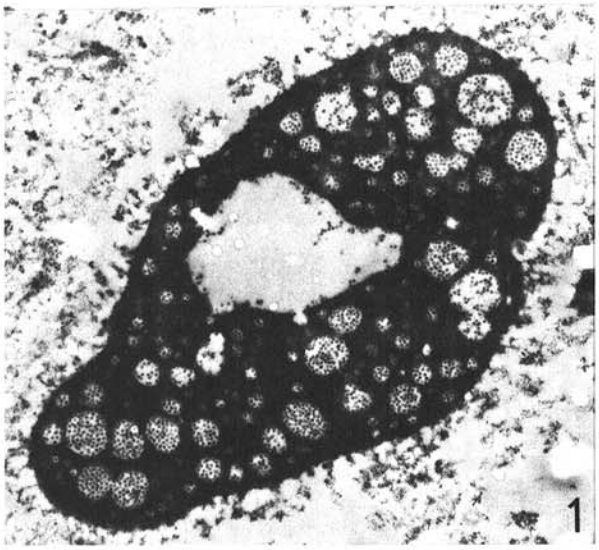
Reports on the presence of DNA and RNA in the inclusion matrix are conflicting. Cauliflower mosaic virus (17) and DaMV (18) inclusions stained positively with methyl-green pyronin for RNA. However, no reaction with methyl-green pyronin was observed after ribonuclease treatment (18). In another report, a faint reaction with methyl-green pyronin for RNA was observed in DaMV inclusions after ribonuclease (RNase) digestion (13). Cauliflower mosaic virus inclusions also stained faintly for DNA with methyl-green pyronin but results with the Feulgen DNA stain were inconclusive (13). Treatment of ultrathin sections of CauMV inclusions in leaf tissue with RNase had no effect on the virions or matrix; deoxyribonuclease (DNase) had no effect on the matrix but the virions were more lightly stained (3).

Labeling studies have shown that thymidine-$^3$H is incorporated by CauMV inclusion bodies (4, 10). Whether the matrix as well as the virions were labeled could not be determined; however, CauMV inclusions may be sites of virus assembly and DNA replication (20).

Limited information is known about the strucutral composition or function of CERV-induced inclusions, but incorporation of thymidine-$^3$H into CERV virions has established the presence of DNA in the virus particles (5).

Cytochemical studies of inclusions induced by caulimoviruses have been reported only in situ but the isolation and partial purification of CauMV induced inclusions (21) has provided a means to study the inclusions in vitro. Cytochemical investigations of CERV inclusions in vitro allowed uniform exposure of inclusions to fixatives and enzymes and avoided the problem of nonuniform penetration in situ. In addition, these techniques permitted observation of numerous inclusions; consequently, the extent of variation within a population could be more reliably assessed.

The purpose of our study was three fold: (i) To examine the effects of proteolytic enzymes on extracted CERV inclusions before and after fixation and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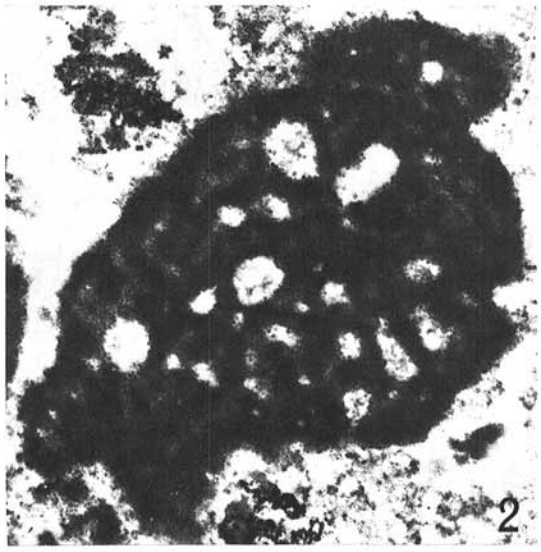
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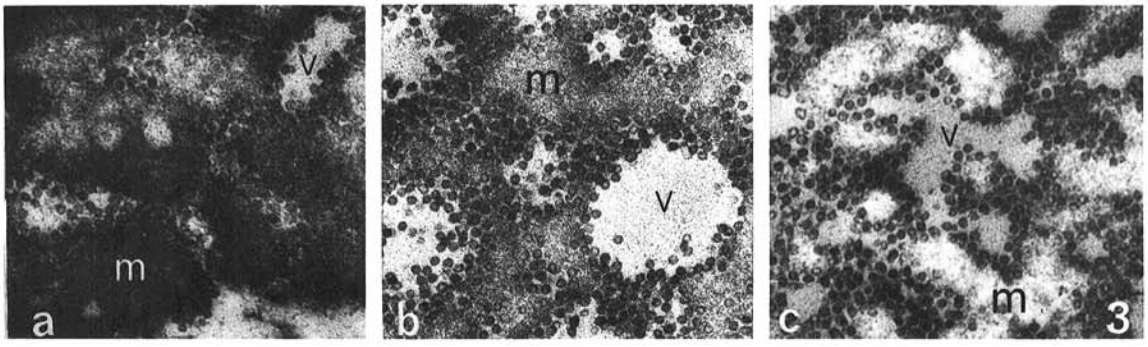
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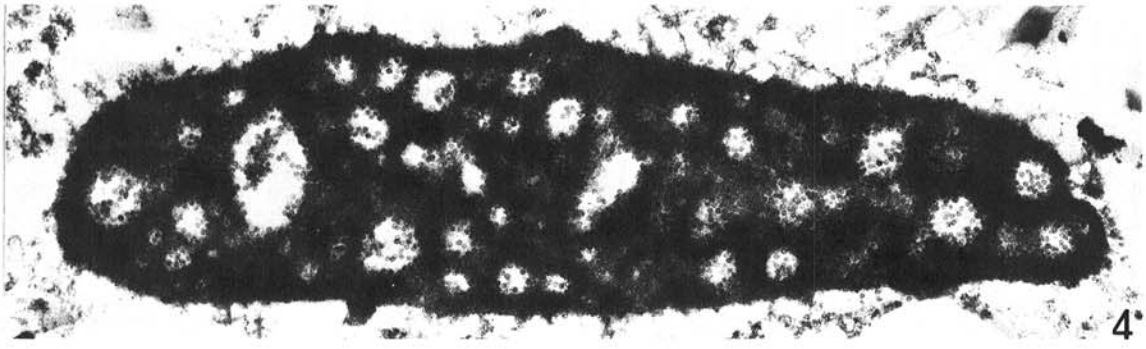
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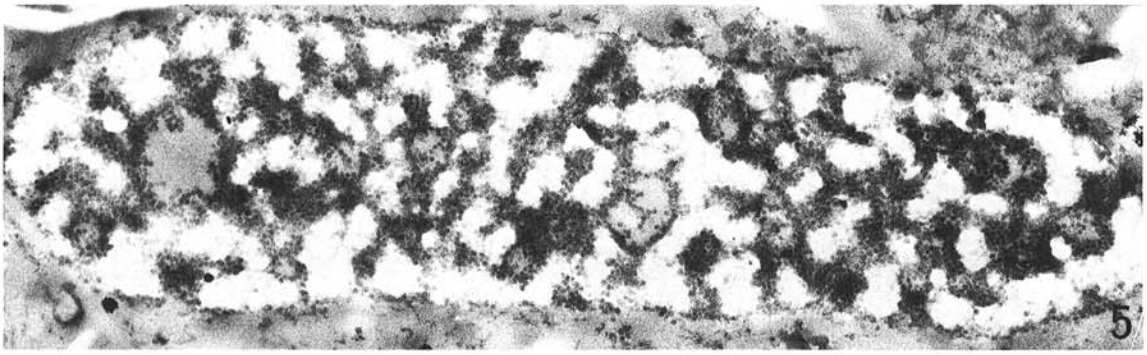
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embedding. Enzyme concentrations, treatment times and pH's were varied and compared to the effects of glutaraldehyde, formalin, and acrolein on inclusion fixation and on enzyme digestion; (ii) To determine the effects of proteolytic enzymes on the degradation of inclusions by attempting to remove the matrix and disperse the virions in vitro, thereby improving the yield of purified virus; (iii) To evaluate the effect of DNase on the ultrastructure of inclusions embedded in glycol methacrylate.

## MATERIALS AND METHODS

**Inclusion extraction.**—Inclusions were extracted from *Saponaria vaccaria* 'Pink Beauty' plants mechanically inoculated with carnation etched ring virus. Twenty to 30 g of mechanically inoculated leaves showing red-ring and red-line symptoms, and the first set of systemically invaded leaves showing leaf curl, were harvested 18-20 days after inoculation. The leaves were homogenized in five volumes of 0.05 M Tris buffer, pH 7.3, containing 0.25 M sucrose, 1 mM $MgCl_2$, and 25 mM KCl (complete Tris) (21). Triton X-100 at a final concentration of 5% was added to the homogenate and the mixture was stirred for 2 hr at 4 C. The extract was filtered through a 701-$\mu$m wire mesh screen and subsequently passed through 149-$\mu$m, 105-$\mu$m, and 74-$\mu$m mesh screens. The filtrate was centrifuged at 3,000 *g* for 10 min. The pelleted inclusions were twice resuspended in 0.2 the original volume of complete Tris. The final pellet was resuspended in 0.1 the original volume of complete Tris. In later experiments, extractions were made in Tris containing cations, but without sucrose.

**Glycol methacrylate embedding.**—Inclusions extracted in Tris-HCl buffer with ions were fixed in various fixatives and embedded in glycol methacrylate (GMA) (15). Inclusion pellets 4-6 mm in diameter were resuspended in cold 3-5% glutaraldehyde, 10% formalin (1/10 dilution of a commercial 40% formaldehyde solution), or 5% acrolein. Fixatives were made in Tris-HCl buffer or Tris-HCl buffer with ions, and adjusted to pH 7.0-7.3. The samples were centrifuged at 9,000 *g* for 15 min and fixed for 30-80 min. The pellets were rinsed overnight with the same suspension medium that was used to dilute the fixative.

The pellets were dehydrated with GMA and infiltrated overnight in partially polymerized resin. The resin consisted of seven parts of 97% 2-hydroxyethyl methacrylate (Polysciences, Inc., Warrington, PA 18976) inhibited with hydroxyquinone and three parts of *n*-butyl methacrylate (Polysciences) with 2% benzoyl peroxide (granular form from Electron Microscopy Sciences, Fort Washington, PA 19034). The mixture was prepolymerized by heating to 115-118 C and then rapidly cooled in an ice bath. The pellets were cut into several pieces and placed in fresh prepolymer in gelatin capsules. All steps were done in the cold. Polymerization was completed by placing the capsules over a 15-W General Electric blacklight (F15T8·BLB) having a wavelength emission peak at 365.4 nm at 4 C. After polymerizing the block for one to two days, pale-gold sections were cut and mounted on Formvar-coated, rhodium-plated copper or gold grids.

**Enzyme treatments of ultrathin sections.**—Grids with freshly cut GMA sections were floated on 0.05-0.1 ml of prewarmed enzyme or control solutions in the wells of a spot plate, which was covered with glass and placed in a petri dish on moist filter paper. Sections were treated at 36-39 C for 5 min to 4 hr. The sections then were washed thoroughly in double-distilled water and stained with aqueous 4% uranyl acetate and Reynold's lead citrate.

The enzyme solutions were prepared as follows: (i) Protease VIII (Sigma Chemical Co., St. Louis, MO 63178; activity of 11 units/mg when one unit will hydrolyze casein to produce color equivalent to 1.0 $\mu$mole tyrosine per minute at pH 7.5 and 37 C). A 1% stock solution was prepared in double-distilled water and found to be stable for at least 1-2 mo at 4 C. Dilutions of enzyme were made in .05 M Tris-HCl buffer. Enzyme solutions were adjusted to pH 5.2, 7.2, or 9.2 with HCl or NaOH as necessary. (ii) Trypsin (Sigma; pancreatic type II, crude; activity 720 BAEE units/mg and 690 ATEE units/mg). A 0.5% stock solution was made in 0.05 M Tris-HCl, pH 8.1, with .0115 M $CaCl_2$ and sonicated briefly before five- or tenfold dilutions were made in buffer with $CaCl_2$. Stock solutions were used for only a few days because the enzyme precipitated. (iii) Deoxyribonuclease type I (Sigma and Calbiochem, Los Angeles, CA 90063) was made in double-distilled water and tested at concentrations of 0.1-0.5% and from pH 5.3-6.8. The activity of the enzyme was verified by testing its ability to reduce the viscosity of salmon sperm DNA. Controls consisted of solutions of buffers or aliquots of the appropriate enzyme solution that had been boiled for 10-15 min.

The effect of different concentrations of protease and DNase on inclusions fixed in glutaraldehyde, acrolein, or formalin with and without ions was compared. The effect of protease at pH 5.2, 7.2, and 9.2 on formalin-fixed inclusions also was observed. The susceptibility of glutaraldehyde-fixed and formalin-fixed inclusions to trypsin was compared.

Inclusions either suspended in Tris buffer with cations or centrifuged into pellets were treated with protease

**Fig. 1-5.** Ultrathin sections of carnation etched ring virus (CERV) inclusion bodies fixed in glutaraldehyde, embedded in glycol methacrylate, and nontreated or treated with protease for different times and at different pH. **1)** Nontreated glutaraldehyde-fixed CERV inclusion body with a uniformly dense matrix (× 9,000). **2)** Nontreated acrolein-fixed CERV inclusion body with a mottled matrix and light and dark areas (× 12,000). **3)** Formalin-fixed CERV inclusion bodies treated with 0.0001% Protease VIII for (a) 15 min, (b) 22 min, and (c) 30 min. Note the progressive loss of matrix (m) with the increased treatment time. The vacuoles (v) are distinguished from the matrix. Samples (a) and (c) were treated with cations (× 30,000); sample (b) was without cations (× 34,400). **4)** Formalin-fixed CERV inclusion body treated with 0.0005% Protease VIII, pH 5.2, for 20 min. The matrix is dense without evidence of digestion (× 17,200). **5)** Serial section of the inclusion in Fig. 4 treated with 0.0005% Protease VIII, pH 9.2, for 20 min. The matrix is extensively digested (× 17,200).

before Epon embedding. Two ml of the inclusion suspension was mixed with an equal volume of protease in Tris buffer with cations, pH 7.3, to give final enzyme concentrations of 0.5 and 1.0%. The inclusions were pelleted after 1- or 2-hr incubation periods at 21-22 C by centrifuging at 9,000 *g* for 15 min. The supernatant liquid was replaced with fixative. Simultaneously, undisturbed pelleted inclusions were treated with 2 ml of 0.5 and 1% enzyme in Tris buffer, pH 7.3. After a 1- or 2-hr incubation the enzyme was removed and replaced with fixative. The treated inclusions and controls were fixed in cold 3% glutaraldehyde for 75 min in Tris buffer containing cations. The pellets were rinsed twice in buffer, postfixed for 1 hr in Tris buffered 1.0% $OsO_4$, dehydrated in a graded series of ethanol-propylene oxide, and embedded in Epon 812 (16). The pellets were cut into several pieces whose location in the original pellet was recorded. Thick-sections were stained with lactofuchsin for observation with the light microscope and thin sections were stained with uranyl acetate and lead citrate.

## RESULTS

**Shape of inclusions.**—Partial purification of CERV inclusions permitted observation of the shape of the inclusions as they tumble in liquid. The inclusions vary in size, shape, and density of the matrix material within the inclusion. Previously, inclusions have been observed only in cells. The DaMV inclusions were considered circular if the inclusions were large, or irregularly shaped if they were smaller (13). The CauMV inclusions have been described as differing in shape from broadly elongated to globosely rounded (17). From observations of CERV inclusions in vitro we can conclude that although some inclusions appear nearly circular in the horizontal plane, they appear thin and elongated when rotated 90 degrees. Thus, many inclusions are disk or cushion shaped. Smaller inclusions appeared to be more nearly spherical. These observations are consistent with those from thin sections with elongated and flattened profiles (Fig. 4-7) of large inclusions and circular profiles (Fig. 8, 9, and 14) of smaller inclusions.

**Proteolytic enzyme digestion of ultrathin sections containing inclusions embedded in glycol methacrylate.**—Results reported here are based on observation of many inclusion bodies and differences within the native population of untreated inclusions have been considered throughout this work. Wherever possible, enzyme treatments were applied to serial ultrathin sections of the same inclusion body and compared to control sections of the same inclusion.

Extracted inclusions were adequately preserved in GMA by the fixatives used. They appeared similar to those fixed with glutaraldehyde-osmium and embedded in Epon 812. The amount of matrix material in the extracted inclusions varied from inclusion to inclusion with all fixatives. Glutaraldehyde-fixed inclusions (Fig. 1) appeared slightly swollen or enlarged and had a uniformly dense matrix as compared to those fixed in formalin or acrolein. The formalin- and acrolein-fixed inclusions were more mottled with light and dark areas in the matrix (Fig. 2). Dense matrix areas existed where no virions could be resolved; other light matrix areas within the inclusion contained virions. Virions without any apparent matrix between the particles were clustered in some of the vacuole-like regions which were present in most inclusions.

The effects of protease and trypsin at different enzyme concentrations, treatment times, and pH's on thin sections of inclusions embedded in GMA are summarized in Table 1. Formalin-fixed inclusions were susceptible to protease and trypsin digestion. The time required for digestion was inversely proportional to the concentration of the enzyme. At concentrations of 0.1% protease and higher, the matrix was removed within 5 min. Low concentrations of protease (0.0001%-0.0005%), pH 7.2, removed the inclusion matrix after about 30 min. Intermediate stages in the digestion could be observed with shorter incubation periods. The densely stained matrix areas containing no recognizable virus particles gradually lost electron density beginning in the center (Fig. 3-a), and moving outward (Fig. 3-b). As a result,

TABLE 1. Effects of protease and trypsin at different concentrations, pH's, and treatment times on thin sections of carnation etched ring virus inclusion bodies fixed in formalin, glutaraldehyde, or acrolein and embedded in glycol methacrylate

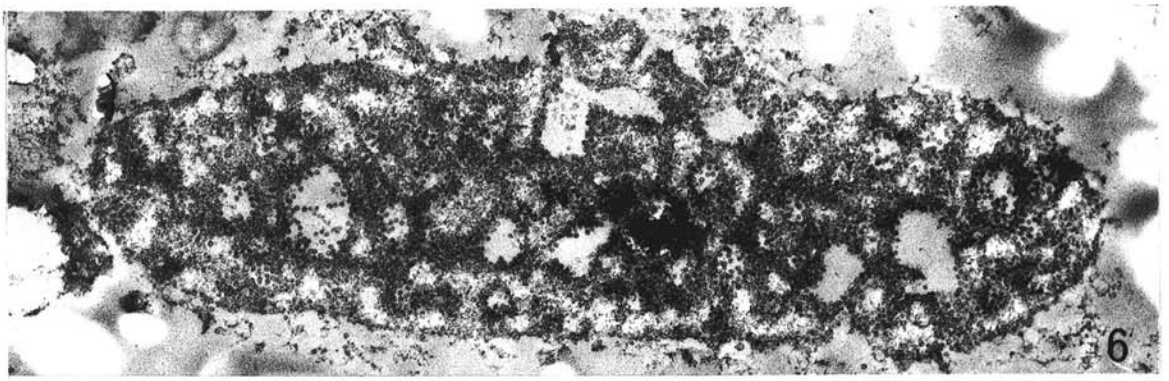
| Fixation[a] | Enzyme | Concentration (%) | pH | Treatment time (min) | Matrix digestion in ultrathin sections[b] |
|---|---|---|---|---|---|
| Formalin | Protease | 0.0005 | 5.2 | 20 | 0 |
| Formalin | Protease | 0.0005 | 7.2 | 20 | ++ |
| Formalin | Protease | 0.0005 | 7.2 | 5 | 0 |
| Formalin | Protease | 0.0005 | 9.2 | 5 | +++ |
| Formalin | Trypsin | 0.0002 | 8.1 | 20 | 0 |
| Formalin | Trypsin | 0.002 | 8.1 | 20 | ++ |
| Glutaraldehyde | Protease | 0.5 | 7.2 | 30 | +++ |
| Glutaraldehyde | Protease | 0.1 | 7.2 | 30 | ++ |
| Glutaraldehyde | Trypsin | 0.2 | 8.1 | 180 | + |
| Acrolein | Protease | 0.1 | 7.2 | 30 | ++++ |

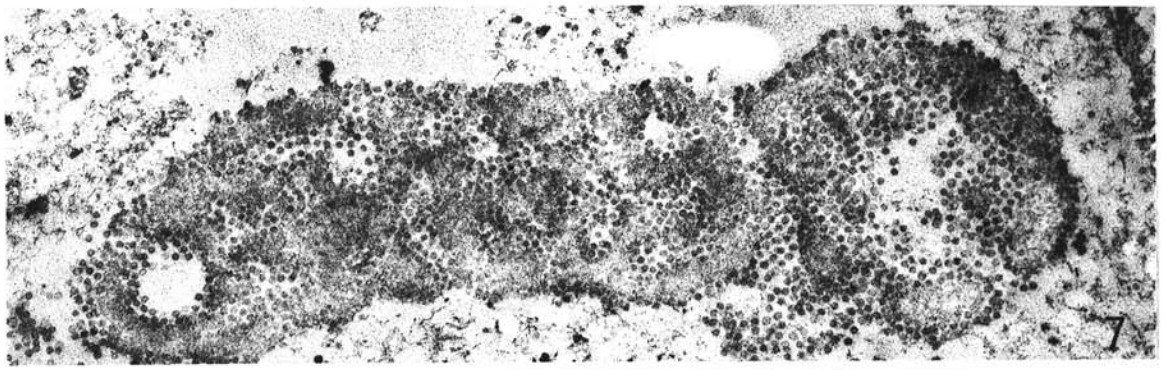
[a]Inclusions were fixed in 10% formalin, 3-5% glutaraldehyde, or 5% acrolein.

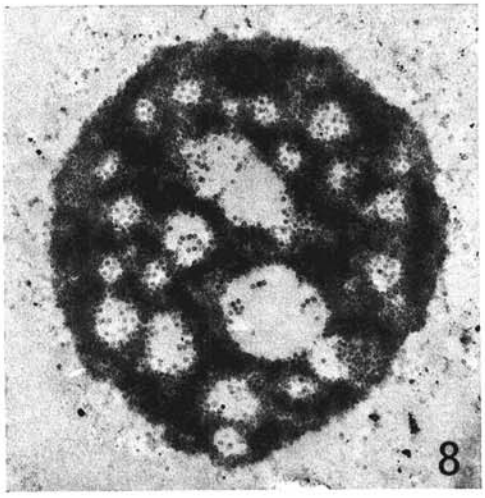
[b]Matrix digestion rated from 0, with no apparent digestion, to ++++, with complete removal of the matrix: + = matrix lightening, just detectable; ++ = moderately lightened; +++ = matrix digested; ++++ = matrix digested and holes in the GMA.

holes developed in the GMA (Fig. 3-c). The virions were digested only with the high concentrations of protease or after incubation for 1-2 hr at low concentrations of enzyme. Clusters of virions within the pellet were recognizable after most of the cellular debris had been removed. Inclusions fixed in formalin with and without ions and treated with 0.0001% protease showed a similar rate of inclusion digestion.

Comparisons of the rates of digestion with 0.0001-0.0005% protease at pH 5.2-9.2 showed that the rate of digestion was greater at the alkaline pH. After 20 min of incubation, the inclusions on protease at pH 5.2 were not digested (Fig. 4) but the matrix was removed from those incubated at pH 9.2 (Fig. 5). At the same concentration of



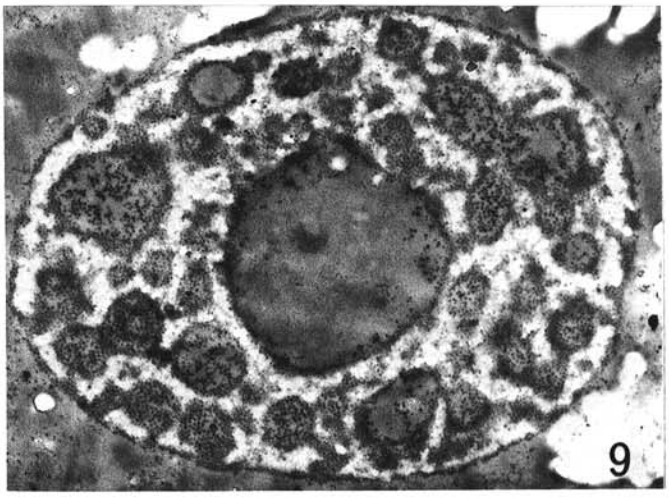




**Fig. 6-9.** Ultrathin sections of carnation etched ring virus (CERV) inclusion bodies fixed in formalin, glutaraldehyde, and acrolein, embedded in glycol methacrylate (GMA), and treated with protease and trypsin. **6)** Serial section of the inclusion in Fig. 4 treated with 0.002% trypsin for 18 min. The matrix of the inclusion is partially digested (× 17,200). **7)** Glutaraldehyde-fixed inclusion treated with 0.1% protease for 30 min. The matrix is only partially digested (× 23,400). **8)** Glutaraldehyde-fixed inclusion treated with 0.2% trypsin for 3 hr. There is only a slight decrease in density of the matrix but the background debris is removed (× 10,000). **9)** Acrolein-fixed inclusion embedded in GMA and treated with 0.1% protease for 30 min. Note the complete digestion of the matrix and holes in the GMA compared to Fig. 7 (× 11,000).

enzyme the matrix was removed after a 5-min treatment at pH 9.2, but not at a pH of 7.2.

Trypsin digestion of formalin-fixed inclusions gave results similar to those obtained with protease. A trypsin concentration of 0.002% digested the inclusion matrix in 15-20 min (Fig. 6), but no detectable digestion occurred with 0.0002% in that time. Trypsin also digested the virions, but only after long incubation periods or higher enzyme concentrations.

No matrix digestion was detectable in formalin-fixed controls incubated on buffer or boiled protease or trypsin solutions for 15-30 min. An overall loss of contrast in the sections occurred with 1-2 hr of incubation but this was easily distinguished from the effects of the active enzymes.

Glutaraldehyde- and acrolein-fixed inclusions were less sensitive to attack by protease than formalin-fixed inclusions. Protease concentrations required to achieve digestion were $10^3$ times greater than those needed for formalin-fixed inclusions and longer incubations were required. Treatment with 0.5% protease for 30 min removed the matrix from most glutaraldehyde-fixed inclusions, leaving holes in the GMA. Treatment of glutaraldehyde-fixed inclusions with 0.1% protease for 30 min produced partial digestion of the matrix (Fig. 7). The same treatment completely removed the matrix and left holes in acrolein-fixed inclusions (Fig. 9). An exposure of 1-3 hr was required to remove the matrix from glutaraldehyde-fixed inclusions at this enzyme concentration and the degree of removal was variable among inclusions within a section. Many inclusions gradually were lightened without leaving holes in the GMA, making it difficult to locate inclusions and assess the degree of digestion at a given time.

When glutaraldehyde-fixed inclusions were treated with 0.2% trypsin for 3 hr, the sections contained intact, though somewhat less electron-dense, inclusions (Fig. 8). However, most of the cellular debris in the pellet was removed. Longer incubations at higher concentrations of trypsin were not practical because the enzyme precipitated from the solutions. No changes in the ultrastructure of the GMA-embedded inclusions could be detected after DNase treatment of ultrathin sections regardless of the fixative that was used.

**Protease treatment of inclusions prior to fixation and Epon embedding.**—The effects of protease on light microscope staining and matrix digestion of inclusions treated after pelleting and in suspension before pelleting are summarized in Table 2. Epon-embedded inclusions were examined with the light microscope by staining 4- to 5-$\mu$m-thick sections with lactofuchsin. In thick-sections of pellets treated with 0.5% protease for 1 hr and then fixed in 3% glutaraldehyde and embedded in Epon, the inclusions near the bottom and mid portions of the pellet stained densely with lactofuchsin (Fig. 10). The staining intensity of inclusions near the surface of the pellet varied, but most were lightly stained (Fig. 11). Inclusions that were treated with protease for 1 hr in suspension and then pelleted were only very lightly stained, regardless of the position of the inclusions in the pellet. Observations of the ultrastructure of the inclusions embedded in Epon showed that the intensity of lactofuchsin staining was correlated with the amount of matrix protein in the inclusions.

No apparent alterations were observed in ultrathin sections of inclusions in the middle or at the base of pellets of samples that were pelleted and then treated with 0.5% protease for 1 hr as compared to control pellets (Fig. 12). Many of the inclusions close to the upper surface of the pellet showed variation in the amount of matrix remaining (Fig. 13 and 14), which may reflect degrees of enzyme digestion. The dense matrix areas appear more susceptible to protease than the virions and matrix. Vacuole-like areas were present in some inclusions, but were less distinct in others with an apparent

TABLE 2. Effects of protease at pH 7.3 on carnation etched ring virus inclusion bodies treated in suspension or in pellets before fixation in glutaraldehyde and Epon embedding

| Treatments and enzyme concentration (%) | Treatment time (hr) | Location in pellet | Light microscope staining of inclusions in thick section[b] | Matrix digestion in ultrathin sections[c] |
|---|---|---|---|---|
| Inclusions pelleted and then treated with enzyme | | | | |
| 0.5% | 1 | Top[a] | + | + to +++ |
| 0.5% | 1 | Middle and bottom | +++ | 0 |
| 1.0% | 1 | Top, middle, and bottom | − | ++++ |
| 0.5% | 2 | Edge of pellet | − | ++++ |
| 0.5% | 2 | Center of pellet | − to + | +++ |
| 1.0% | 2 | Top, middle, and bottom | − | ++++ |
| Inclusions incubated in suspension before embedding | | | | |
| 0.5% | 1 | Top, middle, and bottom | + | ++++ |
| 0.5% | 2 | Top, middle, and bottom | − | No recognizable inclusions |
| 1.0% | 1 | Top, middle, and bottom | − | ++++ |

[a]The position of the inclusions in the pellet was designated top, middle, or bottom with inclusions at the top of the pellet surface nearest the enzyme suspension, those midway through the pellet, and at the bottom next to the centrifuge tube.

[b]Staining intensity of the inclusion bodies was rated as no recognizable inclusions (−), light (+), medium (++), and densely (+++) stained with lactofuchsin.

[c]Matrix digestion rated from 0, with no apparent digestion, to ++++, with complete removal of the matrix: + = matrix lightening, just detectable; ++ = moderately lightened; +++ = very light, extensive digestion; ++++ = matrix completely digested.

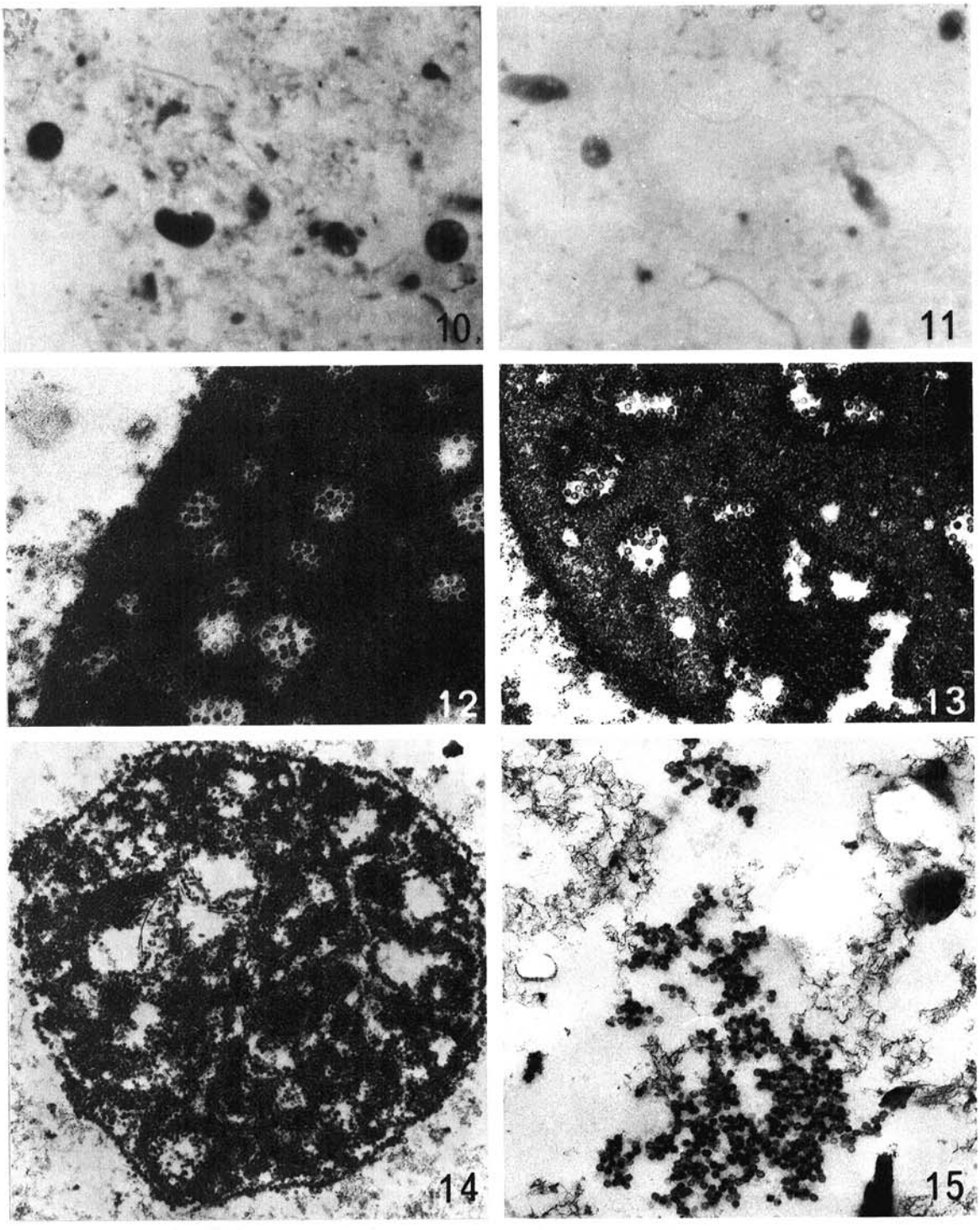



**Fig. 10-15.** Thick and ultrathin sections of carnation etched ring virus (CERV) inclusion bodies treated with protease after pelleting and before embedding in Epon. **10)** Thick-section from the bottom of a pellet treated with 0.5% protease, pH 7.2, fixed in glutaraldehyde, embedded in Epon, and stained with lactofuchsin. Note the densely stained inclusions with vacuoles (× 865). **11)** Thick-section near the surface of the pellet treated as in Fig. 10. Note the very lightly stained inclusions that are partially digested with protease (× 865). **12)** Ultrathin section of an untreated inclusion fixed in glutaraldlehyde and embedded in Epon. The matrix is dense and the virions are well defined (× 30,500). **13)** Portion of an inclusion near the upper surface of a pellet treated with 0.5% protease for 1 hr. Note the partial removal of matrix and less-dense areas (× 30,000). **14)** Ultrathin section of an inclusion without dense matrix near the upper surface of the same pellet as in Fig. 13. Note the border of virions at the margin of the inclusion. Many virions have a distinct margin (× 15,200). **15)** Dispersed cluster of CERV particles in a pellet treated with 1% protease for 2 hr. No intact inclusions remain (× 33,400).

rearrangement of the virions as the matrix was digested (Fig. 14). A few of the inclusions showed a complete loss of matrix but the virions retained a distinct outline and dense staining property. Virions were aggregated along the margin of some inclusions. In some inclusions, the virions in one portion remained closely packed in either a random or regular orientation and the vacuoles were still present. In another portion of the same inclusion, the particles were more loosely aggregated and no vacuole-like regions remained.

After treatment of pellets with 0.5% protease for 2 hr, small clusters of virus particles occurred at the edge of the pellet. Toward the center of the pellet virions were more densely stained and the particle clusters were larger. These inclusions were similar in appearance to those found in the suspensions treated with 0.5% protease for 1 hr.

No intact inclusions and only a few clusters of virions remained in pellets treated with 1% protease for 2 hr (Fig. 15). The sample was composed of diffuse masses of strands with dense particles, presumably virions, of heterogenous size among the strands. These inclusions were similar to those treated with 1% protease for 1 hr.

Inclusion suspensions that were treated with 0.5% protease for 1 hr and then concentrated into a pellet contained many clusters of virus particles with little or no surrounding matrix (Fig. 16). The size and shape of some of the virion clusters were similar to those of control inclusions but many were smaller. Single virions dispersed throughout the pellet and small particle clusters adjacent to the larger virus aggregates showed a distinct outline and density similar to virions in control inclusions. No differences in diameter of virions in treated and control samples were found. No vacuoles occurred in the treated inclusions.

Inclusions were not present in the pellets from the suspension treated with 0.5% protease for 2 hr. The pellets consisted of masses of strands and a few dense virions of heterogenous size.

A few medium-sized clusters of virions occurred following treatment of inclusions in suspension with 1% protease for 1 hr. Virions in the clusters were more widely spaced. The virions were distorted and had a diffuse outline, which may indicate partial degradation (Fig. 17).

## DISCUSSION

Few investigations have been made comparing the suitability of different fixatives and water-soluble embedding media for enzyme treatments of normal

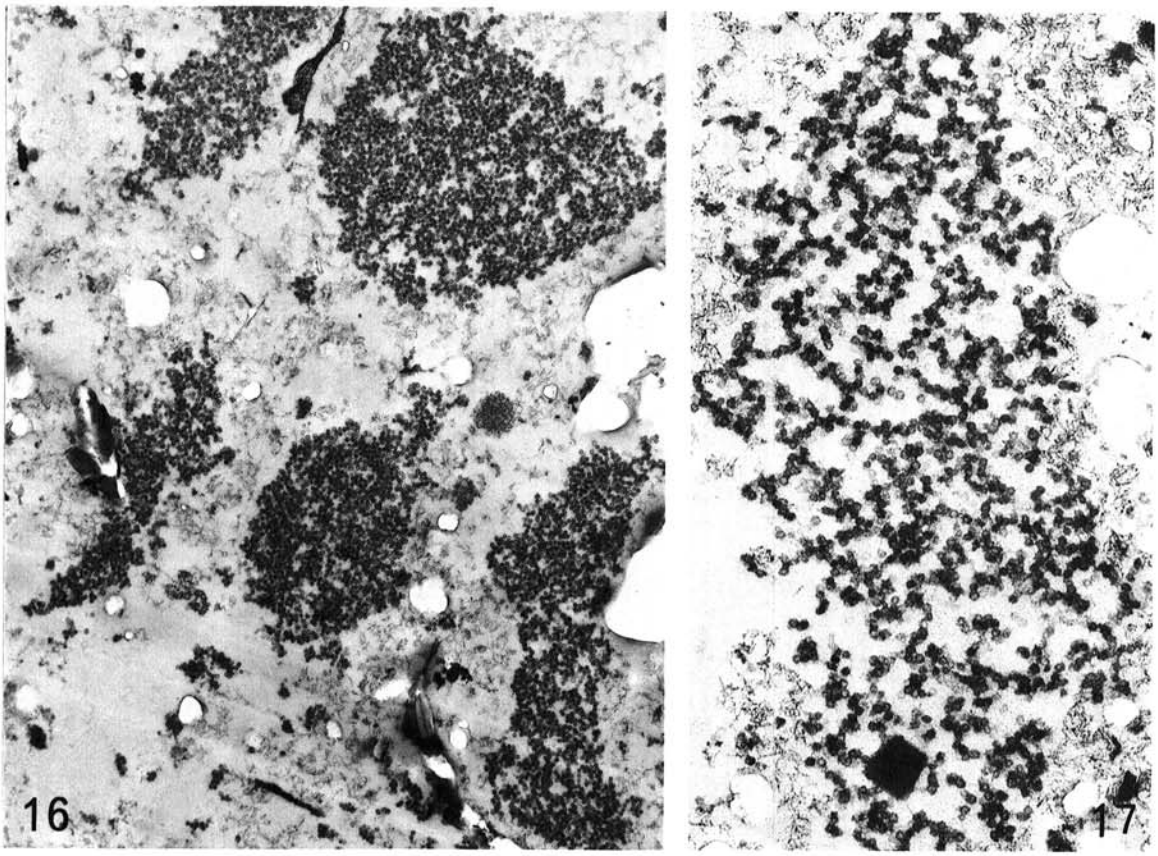



**Fig. 16 and 17.** Ultrathin sections of carnation etched ring virus inclusions treated with protease in suspension before pelleting and embedding in Epon. **16)** Inclusions treated with 0.5% protease for 1 hr. Note the clusters of well defined virions without surrounding matrix (× 13,800). **17)** Clusters of virions remaining after treatment with 1% protease for 1 hr. The matrix is digested and the virions are not well defined (× 32,700).

components or virus-induced abnormalities in plant tissues. The combination of fixative, enzyme treatment, and embedding media can influence structural preservation and susceptibility to enzyme digestion (22).

Formalin-fixed CERV inclusions gave more reproducible results than those fixed in glutaraldehyde. Increased sensitivity of formalin-fixed inclusions to proteolytic enzymes may result from less-stable bonding of the protein than in glutaraldehyde fixation. Formaldehyde is a single carbon monoaldehyde. Although formalin penetrates rapidly, cross-linking of proteins is not as extensive with formaldehyde fixation due to the small size of the molecule (8) and the one reactive site. Glutaraldehyde is a five-carbon dialdehyde and both aldehyde groups may react to form stable cross-links.

The CauMV protein contains a high percentage of basic amino acids consisting of an estimated 18% lysine and a total of 23% lysine and arginine (1). If CERV protein has a composition similar to CauMV, we would expect that the virions would be readily attacked by trypsin with preferential hydrolysis of the peptide bond linking the lysine or arginine to the adjacent amino acid. The rapid digestion of the matrix and virions may indicate that both are rich in basic amino acids. However, because the virions are more resistant to degradation by trypsin than the matrix, they may contain more lysine and arginine or more available sites for enzyme attack. This conclusion would be consistent with the known property of formaldehyde to bind to proteins containing many free amino groups.

The CauMV inclusions fixed in glutaraldehyde and acrolein/formalin and treated with 0.1% pronase, pH 7.0, for 8 hr, showed a loss of matrix and reduced size of the virions, but not a complete loss of matrix (3).

Enzyme concentration, treatment time, and pH are variables which should be studied in experiments in enzyme histochemistry. Protease VIII is referred to as alkaline protease and has a maximum degrading effect at pH 10 to 11 (7). A striking pH effect was observed in our tests with the rapid removal of inclusion matrix at pH 9.2 compared to pH 7.2 after 5 min incubation, indicating the reaction was a protease-specific degradation.

Repeated attempts to demonstrate DNA association with the inclusion matrix in formalin-fixed inclusions embedded in GMA were unsuccessful. This result does not indicate that DNA is absent from the inclusion matrix because no obvious effect of the treatment on the virions could be observed. However, unlike some DNA animal viruses, the plant viruses containing DNA have no obvious central core of nucleic acid and the centers of the virions vary greatly in density from particle to particle.

Suspensions of inclusions treated with protease before embedding in Epon were degraded much like inclusions treated in ultrathin sections following glutaraldehyde, acrolein, or formalin fixation and embedding. Treatment of inclusions in suspension with 0.5% protease for 1 hr digested the matrix without apparent degradation of the virions. There was no evidence of initial digestion of the matrix at the border of the inclusion with progressive removal toward the center of the inclusion. Perhaps the vacuolate nature of the inclusion allows rapid diffusion of the enzyme throughout the structure. Virions in the inclusions treated in suspension are rearranged and randomly distributed with the disappearance of the vacuole-like openings as the matrix is degraded. The virions apparently remain in clusters that are similar to the original inclusion body after the suspension treatment and reconcentration by centrifugation.

A border effect was observed in some inclusions that were treated with protease after pelleting, but prior to fixation, as well as in some that were fixed and embedded in GMA and treated with enzyme after sectioning. This effect was observed at a stage of digestion when dense-staining virions were still present in the inclusion, but were confined to the margin of the inclusion or bordering the vacuoles. Large areas with lightly stained matrix or holey areas where matrix was digested were present in these inclusions. No virions were present in these matrix areas. The apparent stability of the virions in the border areas probably cannot be explained by more limited accessibility of these particles to the enzyme since inclusions treated with protease before fixation and embedding were affected similarly. Virions in CauMV inclusions in situ not exposed to proteolytic enzyme tend to crowd together at the border of the vacuole-like spaces of the inclusions and within the vacuoles (6). Perhaps the close proximity of the virions to one another along the borders of CERV inclusions acts to retard digestion in these areas. In addition, the inclusion matrix may be composed of more than one kind of protein in which the matrix with closely associated virions differs structurally from the protein in the matrix areas without virions.

The initial stages of degradation of CauMV inclusions treated with Protease V in situ were similar to the effects on CERV inclusions with initial degradation of the matrix (17). The CauMV inclusions stained weakly for protein. However, the staining reaction may result from either residual undigested matrix or CauMV capsid protein.

Our results show that protease digestion does not release virions from the inclusion by selectively digesting only the matrix. In addition, they indicate that the matrix differs from the protein of the virions. Whether this difference is related to the types of protein in the light and dense areas of the matrix and in the virions or a conformational change in proteins is not known. Although our results indicate that the inclusion matrix is composed of protein, other constituents that might be present in the matrix protein could be lost by leaching as the matrix is degraded.

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