Carnation Etched Ring Virus Inclusions: Serology and Ultrastructure of Alkaline-Treated Inclusions

Roger H. Lawson and Suzanne S. Hearon

Research plant pathologists, Agricultural Research, Science and Education Administration, U. S. Department of Agriculture, Beltsville, MD 20705.

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ABSTRACT

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Carnation etched ring virus (CERV) cytoplasmic inclusion bodies induced the formation of antibodies in immunized rabbits to intact virions (S-Ag) and to two rapid-diffusing antigens. One of the rapid-diffusing antigens (R-AG) may be matrix protein. The second rapid-diffusing antigen was absorbed by sap from healthy *Saponaria vaccaria*. The effects of extraction medium, cations, and pH on the stability of CERV inclusions were determined by serological tests and electron microscopy. Agar gel double diffusion tests and electron microscopy showed that R-Ag was dispersed more readily from inclusions extracted in distilled water than from those extracted in Tris buffer. Increased amounts of R-Ag were released from inclusions treated in distilled water at increasingly alkaline pH. Inclusions in distilled water showed a progressive increase in the loss of dense-staining matrix protein in ultrathin section with increasing pH. In Tris buffer at high pH the inclusion matrix remained more electron-dense than in distilled water. Weak precipitin lines were formed to R-Ag in Tris. S-Ag was detected with prolonged incubation in both distilled water and Tris at neutral and alkaline pH. The addition of cations stabilized the matrix protein. R-Ag was detected when protoinclusions were first observed in infected *S. vaccaria* cells.

Carnation etched ring virus (CERV), a member of the caulimovirus group (11), has been partially characterized (6,7). The virus forms cytoplasmic inclusions composed of virions embedded in an electron-dense matrix (9). The proteinaceous nature of the inclusion matrix of members of this group is indicated by their susceptibility to protease digestion (4,10,12) and other protein-degrading conditions (12). Protease treatment of partially purified CERV inclusions showed that the matrix protein was more susceptible than the virions to enzymolysis (10).

The antigenicity of CERV particles associated with inclusion bodies and of the inclusion matrix protein is the subject of this report. The extraction medium, presence of cations, and the pH of the medium all affected the serological detection of the virus and inclusion matrix. The effects of these factors on the ultrastructure of extracted inclusions also was examined. A preliminary report has been published (8).

MATERIALS AND METHODS

The antigenicity of CERV inclusions was tested by injecting rabbits with extracts of concentrated inclusions. Antiserum was then reacted with crude sap from CERV-infected plants and with partially purified and concentrated inclusions treated in conditions

that either stabilized or degraded them.

Inclusion extraction and fractionation. Mechanically inoculated *Saponaria vaccaria* 'Pink Beauty' was grown in a growth chamber at 27 C with 21,520 lux (2,000 ft-c) fluorescent illumination on a 16-hr light period for 13–15 days, then maintained 20 hr in the dark before harvesting. About 30 g of tissue, including inoculated leaves showing red concentric ring lesions and the first pair of systemically infected leaves, were homogenized in four or five volumes of distilled water or 0.05 M Tris (Tris) buffer, pH 7.2. Initially, Ca^{++}, Mg^{++}, and K^{+} salts were added to the extraction medium (10). However, cations stabilized cell membrance fragments and induced clumping of cellular contamination that trapped inclusions and increased contamination in the final preparation. In the experiments described herein, cations were not added to the extraction medium.

Leaf homogenates were stirred for 2 hr at 4 C after the addition of Triton X-100 to a final concentration of 5% (v/v). The extract was sieved successively through 417-, 149-, 105-, 74-, and 45-μm (mesh) screens and the inclusions were concentrated by centrifugation at 3,000 *g* for 10 min. The pellets were resuspended in a total of one-half the volume of the medium used to extract the tissue. The pellets were resuspended by repeated aspiration with a Pasteur pipette and washed twice following low-speed centrifugation. The final pellet was resuspended in 8.0 ml of the original extraction medium.

Further purification of inclusions by density gradient centrifugation was attemped in some experiments. Inclusions in distilled water or Tris buffer were layered on linear 50–80% (w/v) sucrose density gradients and on 50-80% discontinuous sucrose density gradients (5, 5, 8, and 10 ml, respectively). Volumes of 1.5 to 2.0 ml containing 9 to 10×10^6 inclusions per milliliter, counted in a hemocytometer (3), were layered on the freshly prepared gradients and centrifuged for 5 min at about 3,000 rpm in a Spinco SW 25.1 rotor. The original volumes of each concentration of sucrose were removed by puncturing the side of the tube with a needle except that the 70% sucrose zone was removed as two separate 4.0-ml fractions. The inclusions were reconcentrated from each fraction by centrifuging at 5,000 *g* for 15 min. Resuspended inclusions were stained with phloxine and differences in size and staining properties of the inclusions were evaluated. Counts from the reconcentrated 60, 70, and 80% fractions were made.

Antiserum production. Rabbits were injected with either unfractionated inclusion bodies (8 to 10×10^6/ml) extracted in distilled water on inclusions pelleted from the 60% and top of the 70% sucrose zone from the discontinuous 50–80% sucrose gradients (6.5 to 7.2×10^6/ml) resuspended in distilled water. Two intravenous injections (IV) of 0.6 ml each were administered 9 days apart. This was followed 2 wks later by an intramuscular injection (IM) of 0.5 ml of inclusions emulsified with an equal volume of Freund's Complete Adjuvant. A second IM was given 25 days after the first. Rabbits were bled 14, 24, and 34 days after the last IM injection. The antisera used here were from the second and third bleedings and had a similar titer. Antiserum also was prepared to partially purified CERV (6). Antiserum was titered in a microprecipitin test with purified CERV and all subsequent tests were double diffusion reactions in agarose gel.

Effects of suspension medium, pH, and cations on serological detection of inclusion antigens. Gel double diffusion tests were performed using 12 ml of 1% agarose (Bio-Rad) in distilled water and 0.1% NaN_3 with or without 0.85% NaCl dispensed into a 9-cm-diameter plastic petri plate. Wells were cut with a No. 2 cork borer around a central well at a distance of about 7 mm edge-to-edge. Inclusion extracts in distilled water or Tris buffer were prepared as described above omitting the sucrose gradient fractionation, and tested against the antiserum to CERV inclusions (As-I) and antiserum previously prepared to purified CERV (As-V) (6). In addition, extracted inclusions in distilled water or Tris buffer were treated with a range of several alkaline pHs for 18 or 72 hr in the presence or absence of cations to observe the effect of the treatment on serological reactions with As-I and As-V.

Eight 0.3-ml aliquots of the inclusion extracts in distilled water were centrifuged at 3,000 *g* for 10 min. Distilled water (0.4 ml) was added to each of four pellets and distilled water containing 1 mM $MgCl_2$, 3 mM $CaCl_2$, and 25 mM KCl was added to the other four pellets. The pellets were resuspended with a glass rod and two samples, one with and one without cations, were adjustsed to pH 9.0, 10.0, and 11.0 with NaOH. The same cation and pH treatments were given to the inclusions extracted in Tris buffer. The pHs of unadjusted control samples were pH 6.0 in the distilled water extract and pH 7.2 in the Tris buffer extract with and without cations. Inclusion extracts were incubated in a desiccator that was flushed with N_2 and contained a NaOH sink to absorb CO_2. After 18 or 72 hr a portion of the treated extracts was removed and tested directly in the agarose plates. In some experiments the samples were then centrifuged at 3,000 *g* for 10 min and the supernatant also was tested in the agarose plates. This procedure was used to detect virions and soluble antigen released from the inclusions into the supernatant in contrast to the antigens that were still bound to the inclusions following the treatments but which later were released from the inclusions after placing the complete extract in wells of the agarose plates.

Effects of suspension medium, pH, and cations on inclusion ultrastructure. Inclusions suspended in distilled water and Tris buffer were pelleted following 18 hr of treatment with and without cations at different pHs as previously described and were processed for electron microscopy. Pelleted inclusions were fixed in 2% glutaradehyde and 1.5% acrolein for 1.5 hr. After dehydration in ethanol-propylene oxide, the inclusions were embedded in Epon 812. Pellets were usually cut into four pieces and each piece was embedded separately. Thick sections were stained with lactofuchsin to locate the areas in the pellet with greatest inclusion concentration. Ultrathin sections were stained with uranyl acetate and lead citrate.

RESULTS

Inclusion extraction and fractionation. Extraction of inclusions in distilled water or Tris buffer gave good yields of inclusions that varied in size and staining density in the light microscope. Attempts to further purify the inclusions and separate them according to size or density resulted in a heterogenous distribution throughout linear sucrose gradients and the method was abandoned. Although inclusion distribution also was heterogenous in discontinuous sucrose gradients, the method resulted in partial separation of smaller inclusions and larger, lightly stained inclusions from large inclusions that were darkly stained. Many inclusions in the 50% fraction were too small and lightly stained to count accurately. About 60% of the inclusions were retained in the 60% fraction; the remainder of the inclusions were distributed in the 50, 70, and 80% zones and in the pellet (Table 1). The 50% zone contained many small and lightly stained inclusions 1.0 μm in diameter with some measuring 2.0–8.0 μm (Fig. 1). The 60% fraction contained many densely stained inclusions that measured 2–10 μm (Fig. 2) and a few smaller inclusions. Fewer inclusions were in the top of the 70% zone and the lower portion of the 70 and 80% zones contained only a low concentration of inclusions and cellular debris.

Antiserum to inclusions. Injections of rabbits with inclusion extracts resulted in a multivalent antiserum with three distinct

TABLE 1. Counts of carnation etched ring virus inclusion bodies in density gradient fractions[a]

Sucrose (%)	Inclusions in three replicates[b] ($\times 10^6$/ml)		
	1	2	3
60	5.62	4.88	5.48
70 (top 4.0 ml)	2.20	1.92	1.89
70 (bottom 4.0 ml)	0.52	0.44	0.60
80 (+ pellet)	1.32	1.24	1.14

[a] Extract was centrifuged 3,000 rpm, 5 min in a Spinco SW 25.1 rotor.
[b] Inclusions were pelleted and resuspended to achieve comparable dilution of each gradient fraction.

antibody-antigen reactions in agarose double diffusion tests. In microprecipitin tests As-I reacted with partially purified virus (40 μg/ml) and had a dilution endpoint of 1/64. At this concentration of virus the As-V had a dilution endpoint of 1/128. In gel double diffusion tests As-I gave detectable reactions with concentrations of purified virus as low as 0.5 μg/well.

As-V antiserum produced a single recurved precipitin band with purified virus and the band was confluent with a band produced by a 30-day-old inclusion body extract in distilled water (Fig 3). This reaction was identified as the intact virus reaction and was designated S-Ag because of the slow migration rate. A second band formed in reactions of As-I and aged inclusion extracts were detected and the antigen was designated R-Ag because of the rapid migration in agarose gel (Fig. 4). This reaction was not observed with purified virus and As-I.

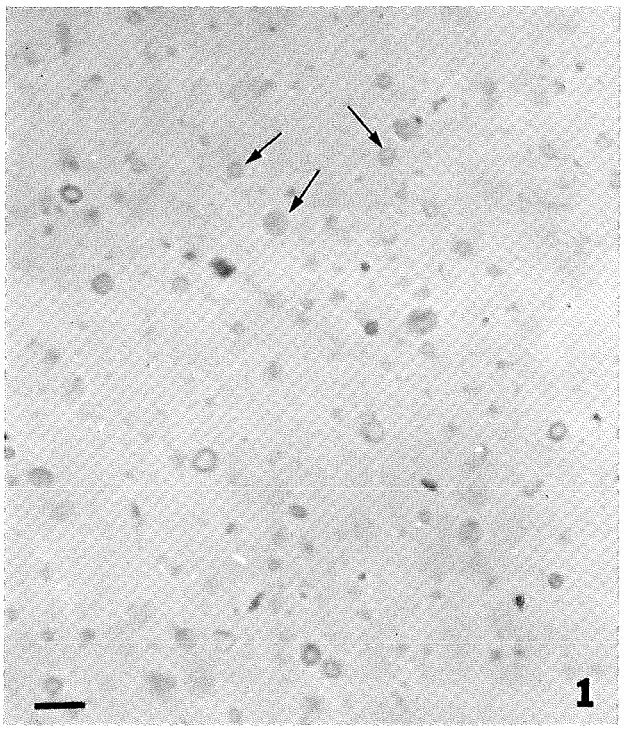
A third precipitin line was detected when As-I was reacted with crude sap extracts of either healthy or CERV-infected *S. vaccaria* in agarose gel containing 0.85% NaCl. This healthy reactant occurred close to the R-Ag precipitin band but unlike R-Ag it was not detectable or only weakly formed when NaCl was omitted from the agarose (Figs. 5 and 6). R-Ag reacted equally well in both media. Both the rapidly migrating component in healthy *S. vaccaria* sap and the R-Ag from inclusions treated at pH 11.0 formed precipitin zones that overlapped with crossing spurs (Fig. 6). Two precipitin lines were formed opposite wells containing crude sap from CERV-infected *S. vaccaria* (Fig. 6). One of the precipitin lines was confluent with the line from healthy *S. vaccaria* sap. The addition of NaCl resulted in slower migration of the virions from the antigen well and increased time for the reaction to appear. The use of NaCl was discontinued and only water agarose with NaN_3 was used in all subsequent tests.

Effects of pH on serological reactions of inclusions in distilled water with and without cations. R-Ag is released from uncentrifuged inclusions after 18 hr of incubation at neutral and alkaline pH both in the presence and absence of cations (Table 2).

TABLE 2. Formation of precipitin lines in agarose gel by uncentrifuged, distilled water-extracted CERV inclusions and supernatants from these extracts in tests with antiserum to partially purified inclusion bodies (As-I) after treating inclusions at neutral and alkaline pH

Inclusions suspended in distilled water	Control extract[a]	Precipitin line formation following incubation of inclusions at			
		pH 7	pH 9	pH 10	pH 11
	U[b] S[c]	U S	U S	U S	U S
Incubation 18 hr					
Without cations					
S-Ag[d]	0[e] 0	0 0	0 0	+ 0	+ +
R-Ag[f]	+[g] +	+ 0	+ +	+ +	+ +
With cations					
S-Ag		0 0	0 0	0 0	0 0
R-Ag		+ 0	+ 0	+−[h]	+ +
Incubation 72 hr					
Without cations					
S-Ag	+ 0	+ 0	+ +	+ +	+ +
R-Ag	+ +	+ −	+ +	+ +	+ +
With cations					
S-Ag		0 0	0 0	0 0	0 0
R-Ag		+ 0	+ 0	+ +	+ +

[a] Control extract is the original inclusion extract in distilled water at pH 6.0.
[b] Uncentrifuged inclusion preparation.
[c] Supernatant from extract tested in (b) following centrifugation at 3,000 *g* for 10 min.
[d] S-Ag = slow diffusing antigen
[e] 0 = no precipitin line.
[f] R-Ag = rapid diffusing antigen.
[g] + = precipitin line.
[h] − = very weak precipitin line.



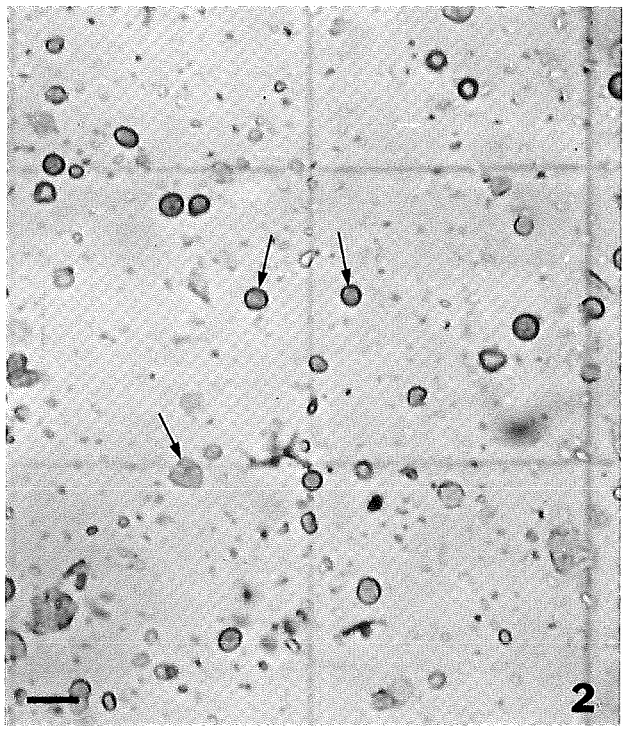


Fig. 1 and 2. Inclusion bodies (arrows) of carnation etched ring virus. **1,** From the 50% fraction of a discontinuous sucrose density gradient. Some inclusions measured from 2 to 5 μm, but many were less than 2 μm and lightly stained with 1% phloxine. Starch was also present in this fraction. **2,** From the 60% sucrose gradient fraction. Note the larger more densely Phloxine-stained inclusion bodies (arrows). Photo is of inclusions in a Levy counting chamber (note grid lines) Bars = 10 μm.

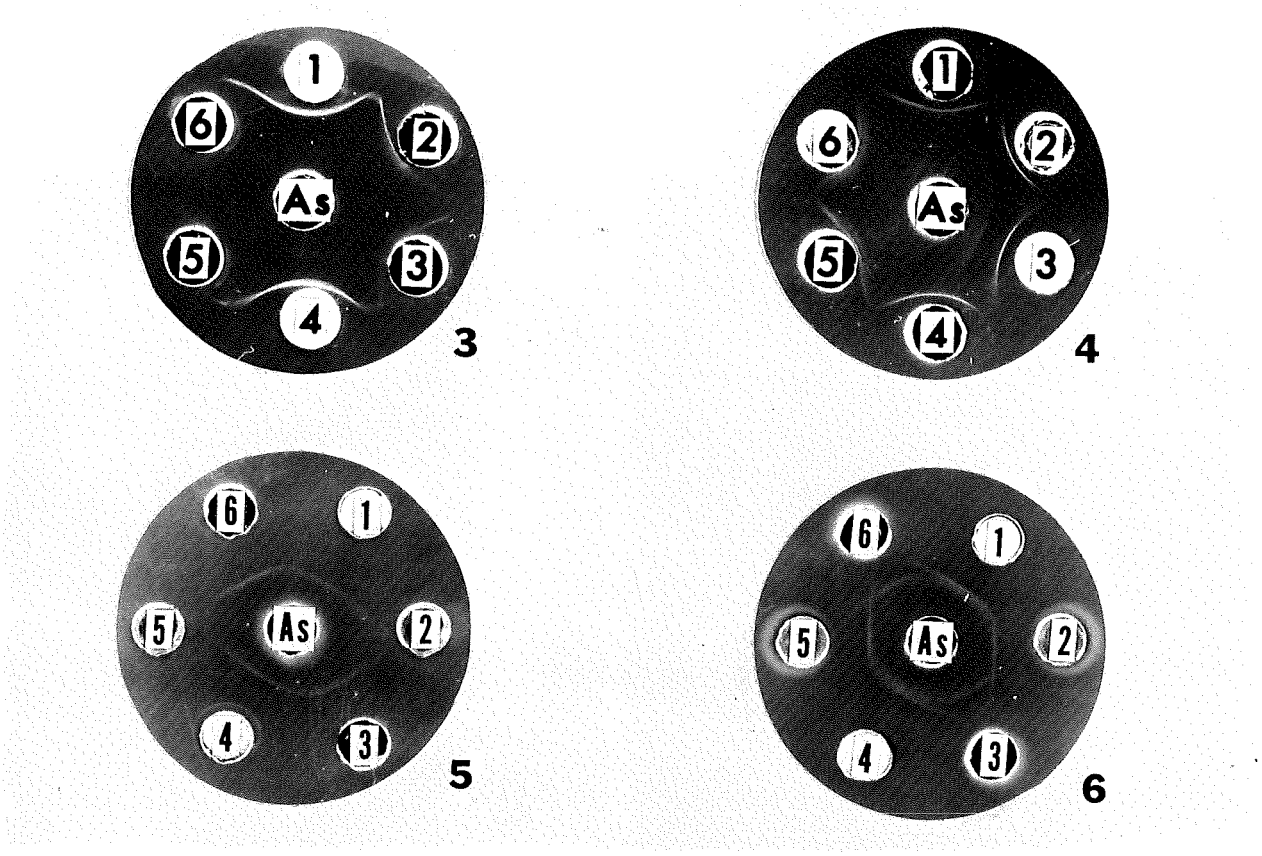
In contrast, S-Ag was detected in uncentrifuged inclusions and supernatants only at pH 10.0 and 11.0, respectively, and this antigen was not detected following the addition of cations at any pH (Table 2). R-Ag and S-Ag were detected after 72 hr of incubation at all alkaline pHs tested in uncentrifuged inclusions and supernatants, but S-Ag was not detected in the presence of cations and R-Ag only at pH 10.0 and 11.0 in supernatants (Table 2).

Effect of pH on serological reactions of inclusions in Tris buffer with and without cations. R-Ag was detected in uncentrifuged inclusions and supernatants both with and without cations after 18 hr of incubation only at high pH. S-Ag was detected after 18 hr of incubation in uncentrifuged inclusions at neutral and alkaline pH, but in the supernatant only at pH 11.0 in the absence of cations (Table 3). After 72 hr of incubation R-Ag was detected in uncentrifuged inclusion extracts at neutral and alkaline pH, but in supernatants only at pH 11.0 with or without cations (Table 3). S-Ag was detected in both uncentrifuged inclusions and supernatants at all pHs tested without cations, but only in uncentrifuged inclusions and not in supernatants in the presence of cations (Table 3).

Effects of suspension medium, pH, and cations on inclusion ultrastructure. Inclusions extracted at pH 6.0 in distilled water (Fig. 7) resembled inclusions treated at pH 9.0. Similar dense-staining inclusions were observed in situ in infected *S. vaccaria* in thin-section electron microscopy after staining with uranyl acetate and lead citrate. The amount of matrix in inclusions treated at pH 10.0 was reduced and the inclusions often appeared mottled with light and dark areas of electron density within a single inclusion (Fig. 8). At pH 11.0, the inclusions were very lightly stained and most of the matrix was removed (Fig. 9). Many inclusions retained their original outline and shape although the matrix was dispersed. Some virion aggregates were in the preparation but some of the virions were very lightly stained and apparently degrading. The appearance of the R-Ag precipitin band is correlated with the decreased matrix density at increasing pH. This electon microscope observation is consistent with the appearance of the S-Ag precipitin band only in the supernatant of the inclusions treated at pH 11.0 and is correlated with the rapid removal of matrix with the release of virions and virion clusters from the inclusions.

Inclusions in distilled water with cations at pH 10.0 contained a more uniformly dense matrix than did those without cations. At pH 11.0 the effect of cations was more obvious than at pH 10.0. Some loss of matrix occurred at this pH, but many inclusions remained more electron dense (Fig. 10) than inclusions at pH 11.0 without cations. Cations prevent the release of R-Ag in the supernatant of inclusions treated at pH 7.0 and 9.0. R-Ag reactions at pH 10.0 and 11.0 show that inclusions are not completely stabilized and some R-Ag is removed. However, in the presence of cations the inclusions are stabilized sufficiently to prevent S-Ag reactions in both uncentrifuged inclusions and supernatants.

Tris buffer-extracted inclusions retained matrix and appeared electron-dense staining in treatments at pH 7.0 and 11.0 in the absence of cations. This stability is correlated with the presence of R-Ag in the supernatant only at pH 11.0 in both the 18-and 72-hr treatments, and S-Ag in the supernatant at pH 11.0, but not lower pHs, after 18 hr. More extensive fragmentation of inclusions was observed at the high pH. S-Ag reactions at neutral and alkaline pH often appeared after 72 hr in Tris buffer and are associated with the appearance of many inclusions with an irregular profile.



Figs. 3-6. Reactions of carnation etched ring virus (CERV) antisera to purified virus (As-V) and inclusion bodies (As-I). **3,** AS-V reacted with: well 1, CERV inclusion body extract (30 days old); well 2, purified CERV (1 μg), pH 7.0; well 3, purified CERV (1 μg), pH 11.0; well 4, same as well 1; well 5, purified CERV (0.2 μg), pH 7.0; well 6, same as well 5. **4,** As-I reacted with: well 1, purified CERV (1.5 μg), pH 7.0; well 2, CERV inclusion body extract (14 days old); well 3, same as well 2 but 21 days old; well 4, same as well 1; well 5, purified CERV (1.0 μg), pH 7.0; well 6, purified CERV (0.5 μg), pH 7.0. **5,** As-I reacted with: well 1, crude sap from 10-day-old CERV-infected *Saponaria vaccaria;* well 2, healthy crude sap of *S. vaccaria;* well 3, CERV inclusions incubated 18 hr at pH 11.0 before testing; well 4, same as well 1; well 5, same as well 2; well 6, same as well 3. **6,** Same as Fig. 5 except the agarose contains 0.85% NaCl. Note the single precipitin line opposite well 2 that forms an overlapping line with the CERV inclusion extract in well 3. A double line is opposite the well with crude sap from CERV-infected *S. vaccaria.*

DISCUSSION

Formation of two distinct and specific antibody populations in rabbits immunized with CERV inclusion bodies indicates that a sufficient concentration of separate virions is available in the animal to form antibody to capsid protein as well as to the associated inclusion matrix. Inclusions in the concentrated, unfractionated, and partially purified extracts are heterogenous in size and shape and may contain variable amounts of matrix protein and virions. Antibodies may be formed primarily to those inclusions that are only partially formed and are composed of aggregates of protoinclusions with associated virions as well as to virions on the surface of mature inclusions.

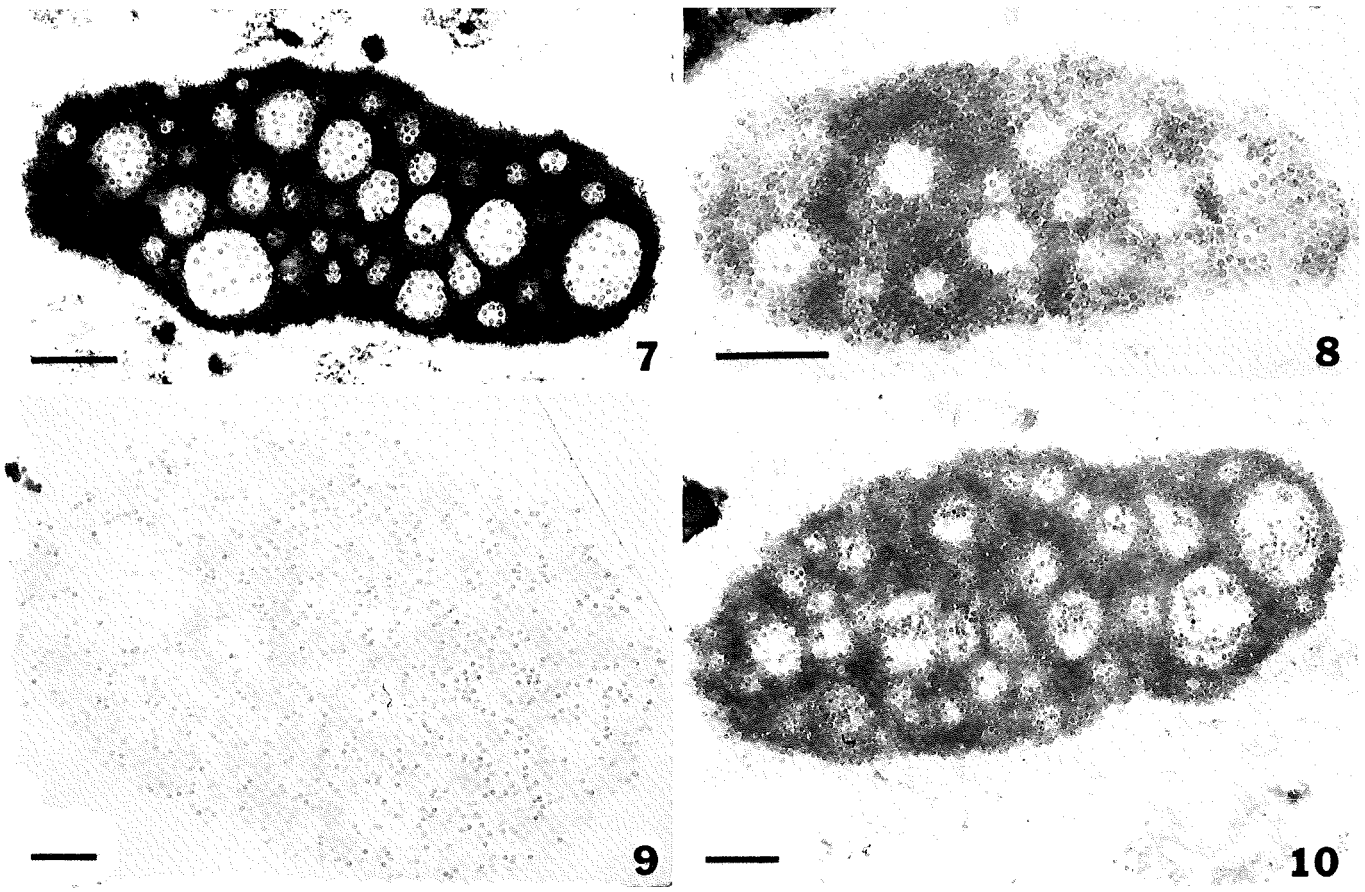
Alkaline degradation of CERV inclusions releases matrix protein. However, alkaline pH does not completely disrupt the inclusions to free populations of individual virions as with high pH treatment of baculovirus inclusion bodies (5). Although small clusters of CERV virions may separate as fragmentation occurs from the parent inclusion, many inclusions retain the original size and shape following exposures to high pH. However, exposure to high pH results in reduced staining density of virions with associated virion degradation as observed in ultrathin sections of treated inclusions. The stabilizing effect of added cations with subsequent treatment at high pH is consistent with the reduced serological reactivity of cation-treated inclusions and the retention of dense-staining matrix and virions in ultrathin section.

Inclusions extracted in Tris buffer and in distilled water at neutral pH appear similar in ultrathin section. However, Tris buffer apparently provides and added stability to the R-Ag that retards its solubilization at neutral or slight alkaline pH. The appearance of the S-Ag reaction and the associated weak R-Ag reaction from the same extract may result from better preservation of protoinclusions and protoinclusion aggregates. These structures show many virions at the margin of the inclusion at an early stage of infection (9). In ultrathin section the Tris-extracted inclusions often appear more irregular in outline, but it is not possible to determine if this represents increased stability of partially formed inclusions or fragmentation of mature inclusions.

CERV inclusion bodies are first observed in situ as small, electron-dense protoinclusions (9). Protoinclusions are first observed when the R-Ag reaction from crude sap extracts from CERV-infected *S. vaccaria* is first detected. This observation, along with the appearance of the R-Ag in alkaline treated inclusion extracts, is consistent with the hypothesis that the rapidly migrating antigen is inclusion matrix protein.

Ultrastructural examination of inclusions in situ shows ribosomes incorporated in and surrounding the protoinclusions. The electron-dense matrix apparently is formed by ribosomal activity. If the ribosomes are incorporated as part of the matrix protein, the in situ observation may explain the formation of a population of antibodies to normal host protein. A much stronger serological reaction is produced from healthy crude sap of *S. vaccaria* than from inclusion extracts or healthy extracts prepared according to the protocol described for partial purification of inclusion bodies. This result may indicate that the strong healthy component reaction in crude extracts results from cytoplasmic ribosomes that are removed from inclusion extracts by repeated washing.

Purified virus, treated at high pH or aged for several weeks at 4 C, failed to react to the antibody in the inclusion antiserum that



Figs. 7–10. Ultrastructure of carnation etched ring virus inclusion bodies extracted in distilled water. **7,** From original extract at pH 6.0. Note the dense-staining inclusion matrix. **8,** Treated at pH 10.0 for 18 hr before fixation and embedding. Note the light-stained area adjacent to the more-densely stained matrix. **9,** Treated at pH 11.0 for 18 hr before fixation and embedding. Note the absence of dense-staining matrix. **10,** Treated with cations before incubation at pH 11.0. Although the matrix shows less dense staining than the pH 7.0 treated inclusion, it has been partially stabilized. All bars equal 500 nm.

reacted to the R-Ag in inclusion extracts. It should be noted, however, that CERV virions are very stable. A mild disruptive condition that would produce serologically reactive R-Ag can't be ruled out, but this condition was not found.

Although differing greatly in gross morphology and structure, it is of interest to compare insect polyhedra with those of caulimoviruses. Polyhedra produced by nuclear polyhedrosis viruses of insects contain about 95% polyhedron protein by weight and the virus particles are about 5% (2). Alkaline digestion of polyhedra of nuclear polyhderosis virus release virions and polyhedron protein (1). Antiserum was prepared to highly purified intact polyhedra, polyhedron protein, or virions (1). Polyhedron protein cross reacted with virions when partially purified antigens were used in agar gel double diffusion tests, but highly purified polyhedron protein and virions reacted only with their homologous antisera and no cross reactions were observed.

Further work must be done to purify CERV inclusion body matrix protein and determine the relationship between this protein and viral capsid protein.

TABLE 3. Formation of precipitin lines in agarose gel by uncentrifuged, Tris buffer-extracted CERV inclusions and supernatants from these extracts in tests with antiserum to partially purified inclusion bodies (As-I) after treating inclusions at neutral and alkaline pH

Inclusions suspended in 0.05 M Tris buffer	Control extract[a]	Precipitin line formation following incubation of inclusions at			
		pH 7	pH 9	pH 10	pH 11
	U[b] S[c]	U S	U S	U S	U S
Incubation 18 hr					
Without cations					
S-Ag[d]	+[e] +	+ 0[f]	+ 0	+ 0	+ −[g]
R-Ag[h]	+ +	0 0	0 0	− 0	− +
With cations					
S-Ag		+ 0	+ 0	+ 0	+ 0
R-Ag		0 0	0 0	− 0	− −
Incubation 72 hr					
Without cations					
S-Ag	+ +	+ +	+ +	+ +	+ +
R-Ag	+ +	− 0	− 0	− 0	− −
With cations					
S-Ag		+ 0	+ 0	+ 0	+ 0
R-Ag		− 0	0 0	− 0	− −

[a]Control extract is the original Tris buffer inclusion extract at pH 7.2.
[b]U = Uncentrifuged inclusion preparation.
[c]S = Supernatant from extract tested in (b) following centrifugation at 3,000 *g* for 10 min.
[d]S-Ag = slow diffusing antigen.
[e]+ = precipitin line.
[f]0 = no precipitin line.
[g]− = very weak precipitin line.
[h]R-Ag=rapid diffusing antigen.

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