Virions and Ultrastructural Changes Associated with Blueberry Red Ringspot Disease

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ABSTRACT

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An ultrastructural study of red ringspot-diseased specimens of highbush blueberry (*Vaccinium corymbosum* L.) revealed viruslike particles (VLPs) in both cytoplasm and nuclei of leaf cells. In the cytoplasm, the particles were embedded in circular inclusions composed of an electron-dense matrix with electron-lucent areas and surrounded by rough endoplasmic reticulum. In the nucleus, particles were associated with the nucleolus or lipid globules. Purified preparations from diseased blueberry leaves revealed large spherical VLPs 42–46 nm in diameter. Density gradient centrifugation of purified preparations in either sucrose or cesium chloride gradients always resulted in two absorbance peaks. Infectivity of purified preparations could not be demonstrated. Although the ultrastructural studies showed similarities to caulimoviruses, no serological reaction could be demonstrated with cauliflower mosaic virus antiserum.

Additional key words: virus-host interaction.

Red ringspot disease of highbush blueberry occurs in many areas in the United States (19). Its widespread distribution is apparently due to the movement of diseased planting materials. The causal agent of red ringspot disease was transmitted by grafting from diseased to healthy plants (7) and was therefore assumed to be a virus. Varney (19) cited an unpublished report by Chen of viruslike particles (VLPs) in ultrathin sections of infected leaves.

The studies reported here confirm that a virus is associated with the red ringspot disease. The ultrastructure, virus purification, and serology indicate certain similarities as well as differences to caulimoviruses.

MATERIALS AND METHODS

Electron microscopy. Leaf samples of highbush blueberry (*Vaccinium corymbosum* L.) cultivars Blueray, Burlington, Darrow, Coville, and Bluetta grown in northwestern Arkansas and Blueray from a greenhouse-grown plant collected originally near Nunica, MI, were prepared for examination by electron microscopy. Tissue specimens, 1–2 mm^2, were taken from the green-to-chlorotic centers and the border of reddish rings from leaves showing typical red ringspot symptoms (Fig. 1). Specimens from Arkansas were sampled three times—in September, October, and November—while the Michigan specimens were collected once in October.

Specimens were fixed for 2 hr at room temperature under a low vacuum in modified Karnovsky's paraformaldehyde-glutaraldehyde fixative (8), which consisted of 2% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.0. After three washings with buffer, the tissues were postfixed in 1% osmium tetroxide for 2 hr, then stained in bulk overnight at 0–4 C in 0.5% aqueous uranyl acetate. The tissues were dehydrated in an ethanol series and propylene oxide, embedded in Spurr's medium, and sectioned. Sections were double stained in 2% aqueous uranyl acetate for 10 min and lead citrate for 2 min before examination under the electron microscope.

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Purification of VLPs. Mature, symptom-bearing leaves were collected from mature Blueray bushes near Nunica, MI, and held at −20 C until processed. A modification of an improved method for purification of cauliflower mosaic virus (CaMV) was used (6). All procedures were carried out at 4 C. Five hundred grams of leaves was ground in a Waring Blendor with 2 ml/g 0.5 M potassium phosphate buffer, pH 7.2, containing 0.75% (w/v) sodium sulfite. The juice was expressed through cheesecloth and made 1 M with respect to urea and 2.5% (v/v) with respect to Triton X-100. After stirring overnight in the cold, the preparation was given a low-speed centrifugation (5,000 rpm for 10 min) in an IEC No. 872 rotor (IEC Co., Needham Heights, MS 02194), followed by a high-speed cycle in a Beckman No. 30 rotor at 28,000 rpm for 75 min. The pellet was resuspended overnight in a small volume of 0.01 M potassium phosphate buffer, pH 7.2, and the preparation was given a second series of differential centrifugation. The VLPs were passed through linear-log (1) or 5–30% linear sucrose gradients made in resuspension buffer in a Beckman SW 41 rotor at 38,000 rpm for 1 hr. Gradients were fractionated at 254 nm by using ISCO equipment (Instrumentation Specialties, Inc., Lincoln, NE 68504). Fractions containing red ringspot VLPs were diluted 3:1 (v/v) with resuspension buffer and the VLPs were pelleted from sucrose in a Beckman No. 40 rotor at 38,000 rpm for 3 hr.

In some cases VLPs purified by sucrose gradient centrifugation were further purified in a step gradient of CsCl made in resuspension buffer. The step gradient consisted of 1 ml each of CsCl solutions $\rho = 1.55, 1.45, 1.40$, and 1.35 gm/cm^3. The gradients were run in a Beckman SW 50.1 rotor at 35,000 rpm for 15–20 hr and fractionated as before, into 0.25-ml fractions, using Fluorinert® (Instrumentation Specialities, Inc.) as a chase solution. Refractive indices of alternate fractions of CsCl were determined in an Abbé 3L refractometer. Density of CsCl was determined from a formula (2).

Purified VLPs were stained with 2% ammonium molybdate, pH 7.2, on formvar-coated carbon grids. Size was determined by use of a carbon replica grating grid.

Attempts to associate infectivity with purified VLPs. Suspensions of purified VLPs adjusted to a concentration of 0.3 mg/ml (using $E_{260\ nm}^{0.1\%} = 7$[17]) were rub-inoculated to the following herbaceous host range on two occasions: *Brassica nigra*

'Tendergreen,' *Chenopodium amaranticolor*, *C. foetidum*, *C. quinoa*, *Cucumis melo*, *C. sativus* 'National Pickling,' *Cucurbita pepo*, *Datura stramonium*, *Dianthus barbatus*, *Dolichos biflorus*, *Gomphrena globosa*, *Lycopersicon esculentum* 'Marglobe,' *Nicotiana clevelandii*, *N. clevelandii* ×*glutinosa* (Christie's Hybrid), *N. sylvestris*, *N. tabacum* 'Kentucky 16 Burley,' *N. tabacum* 'Havana 425,' *N. tabacum* 'Turkish,' *Phaseolus vulgaris* 'Tendergreen,' *P. vulgaris* 'Bountiful,' *Petunia hybrida*, *Sesbania exaltata*, *Torenia fournieri*, *Vigna sinensis*, *V. unguiculata* 'Early Ramshorn,' *Vinca rosea*, and *Zinnia elegans*. All plants were lightly dusted with 48-μm (300-mesh) carborundum prior to inoculation.

Ten Blueray blueberry seedlings, 5–10 cm tall, were similarly inoculated by rubbing leaves and roots with the above concentration of virus. Five control plants were rub-inoculated with buffer only. All test plants were held in a greenhouse at 20–25 C with supplemental cool-white plus cool-white Gro-Lux wide spectrum fluorescent lighting programmed for a 16-hr day length.

Serology. A series of six injections of purified virus emulsified in Freund's Incomplete Adjuvant (Difco Laboratories, Detroit, MI 48202) was given intramuscularly to a New Zealand white rabbit at 7–10 day intervals. Antigen concentration ranged from 0.3 to 0.4 mg per injection. Test bleedings were begun after the fourth injection. Titer tests were done by using gel double diffusion in agarose gel consisting of 0.8% (w/v) agarose containing 0.1% NaN_3 (w/v) and 0.85% NaCl (w/v). Purified red ringspot VLPs were used as test antigen and a purified extract from healthy Blueray blueberry leaves as a healthy control antigen.

The B strain of cauliflower mosaic virus and its homologous antiserum (titer, 1:512) were obtained from R. J. Shepherd. The gamma globulin (IgG) was purified and an alkaline phosphatase conjugate was made following the published standard protocol (3). Virus was multiplied in Tendergreen mustard and purified according to the published method (6).

Gel double diffusion tests were performed by using CaMV antiserum at twofold dilutions before its homologous end point. The test antigens used were purified CaMV, purified red ringspot VLPs (both at a concentration of 0.2 mg/ml) and a purified preparation from healthy Blueray blueberry leaves. A six-hole circular well pattern was used (Grafar Co., Detroit, MI 48238). Well diameters were 6 mm and were 100 mm apart center to center.

One-way ELISA tests were performed by using the CaMV gamma globulin and enzyme conjugate according to the standard procedure (3). Flat-bottomed M 220-29A type plastic plates were employed (Dynatech Co., Alexandria, VA 22314). The IgG concentration was 10 μg/ml and the enzyme conjugate dilution was 1:200 (v/v). Test antigens and concentrations were as follows: purified CaMV, red ringspot VLPs and a preparation from healthy blueberry leaves purified by the same procedures as those used for the blueberry VLPs used were each at 10, 100, 250, 500 and 1,000 μg/well (assuming $E_{260\ nm}^{0.1\%} = 7$ for both antigens); purified tomato bushy stunt (TBSV) or tobacco ringspot virus (TRSV) at the above concentrations were also employed as controls. Spectrophotometric readings were taken at 405 nm, following the addition of enzyme substrate and incubation for 20–30 min at room temperature.

Fig. 1. A blueberry leaf showing typical red ringspot symptoms.

RESULTS

Electron microscopy. In all leaf samples showing the red ringspot symptoms (Fig. 1), either from Arkansas or Michigan, cells containing electron-dense inclusions (Fig. 2) were consistently found. The inclusions, which occurred only in the cytoplasm, were well-defined circular bodies ranging in size from 0.5 to 2.5 μm in diameter. Many cells contained a single large inclusion (Fig. 3), but two closely associated inclusions were not uncommon (Fig. 2). Although the inclusions were found in most types of parenchyma cells, they were most often seen in palisade and spongy mesophyll cells.

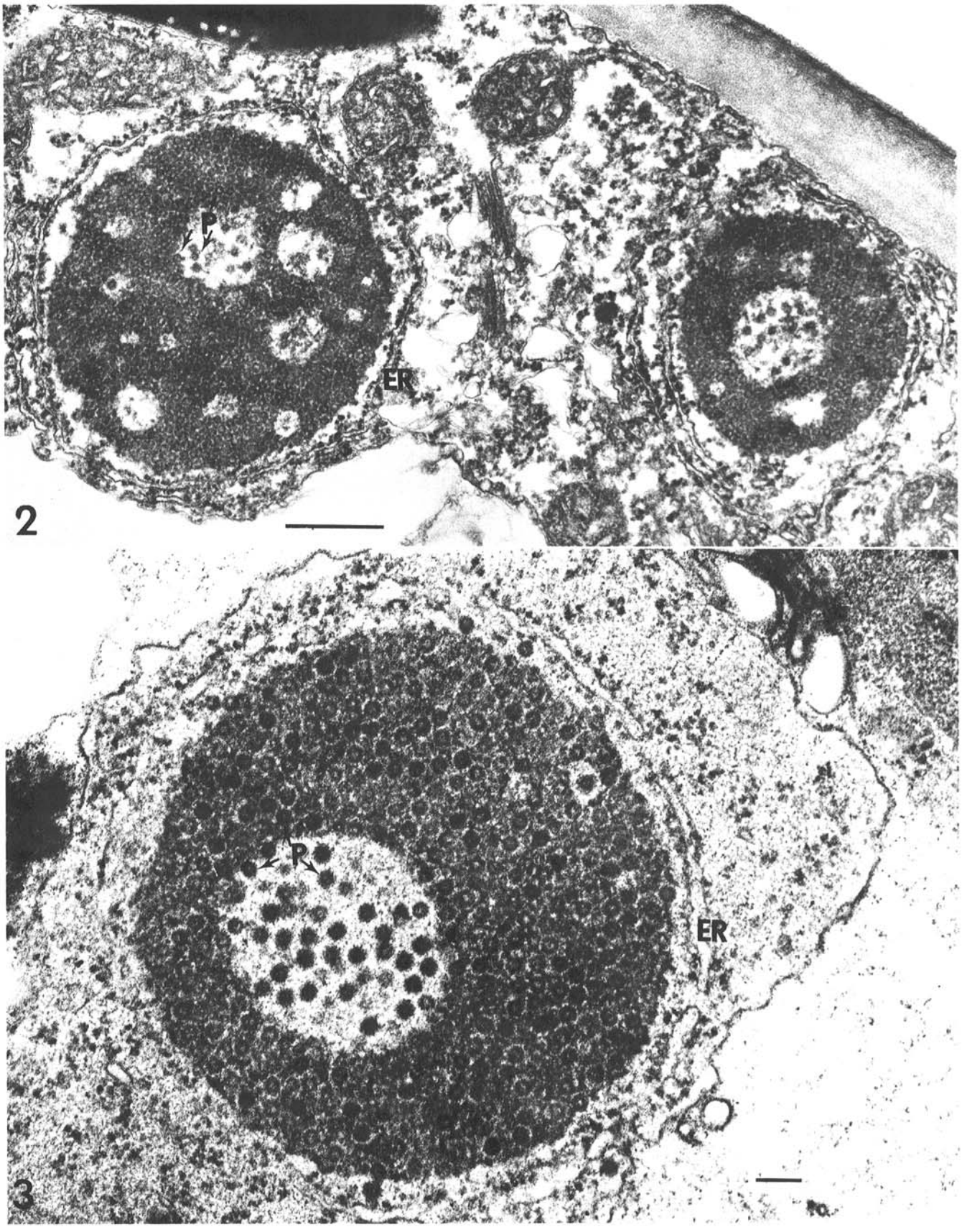
The inclusions consisted of a finely granular, electron-dense matrix in which homogeneous spherical particles were embedded. They contained one to several electron-lucent areas of variable size in which the particles were well defined (Figs. 2 and 3). The inclusions were surrounded by a cisterna of rough endoplasmic reticulum (Figs. 2 and 3). The particles, 45–50 nm in diameter, usually had an electron-lucent central core surrounded by a roughly surfaced circular coat. Cisternae of rough endoplasmic reticulum arranged somewhat circularly and containing free ribosomes and patches of electron-dense substance, but no particles, also were often observed, especially in green tissue from the central areas of ringspots (Fig. 4). No inclusions or particles were noted in specimens from symptomless plants.

Inclusions of different sizes were found in the various lesions sampled. These differences seemed to be associated with the age of the lesions. Smaller inclusions were frequently encountered in specimens from green tissue in the central area of ringspots. (Fig. 5). Larger inclusions were evident in specimens from chlorotic centers or from the reddish borders of lesions. Smaller inclusions usually contained fewer particles scattered loosely in an electron-dense matrix (Fig. 6) and occasionally no particles at all (Fig. 5). Regardless of size, the inclusions were always surrounded by rough endoplasmic reticulum (Figs. 2, 3, 5, and 6). Cell wall thickenings and protrusions were observed in cells with inclusions, especially those containing smaller inclusions (Fig. 5). Large wall protrusions penetrating deeply into the cytoplasm often contained many plasmodesmalike structures (Fig. 5). Although some of these structures were filled with electron-dense material, none of them contained VLPs.

Although the particles were usually associated with inclusions in the cytoplasm, some nuclei contained particles. In such nuclei, the particles were either scattered randomly, grouped together near the nucleoli (Fig. 7A and B), or closely associated with the lipidic globules (Fig. 8A and B). Inclusions were never observed in the nuclei.

Purification of VLPs. Typically, 500 gm of purified leaves yielded 0.3–0.4 mg of purified VLPs. Two diffuse peaks always resulted from sucrose gradient centrifugation of purified preparations (Fig. 9). Electron microscopic examination of samples from these peaks revealed large spherical virions (Fig. 10) measuring 42–46 nm in diameter. There also were some virions present in fractions above and below the two peaks in the gradients. Extracts from healthy leaves purified in an identical manner resulted in a broad diffuse peak in the same region. Electron microscopic examination of such preparations did not reveal any spherical VLPs. Further purification of the VLP preparation through step gradients of CsCl revealed two sharp peaks of densities, 1.30 and 1.40 gm/cm^3. Both of these peaks contained a high concentration of VLPs 42–46 nm in diameter. Electron microscopy revealed no discernible morphological difference between the two species of particles. Both peaks contained broken particles, however. Fractions either side of or between those peaks did not contain VLPs. A sibling tube containing CaMV resulted in a single sharp peak with a density of 1.35 g/cc. Preparations from healthy plants further purified through CsCl did not reveal any VLPs. Only one peak with $\rho = 1.20$ gm/cc was observed. The net yield of virus after SDG and CsCl gradient purification was about 0.15 mg total from 500 gm of blueberry leaves.

It is apparent that red ringspot VLPs differ from CaMV in that the former sediment as two buoyant density species while the latter



Figs. 2–3. Ultrastructure of inclusions in red ringspot-affected blueberry leaves. **2,** Two typical inclusions in the cytoplasm of a palisade cell. The inclusions are composed mainly of viruslike particles (P), embedded in an electron-dense matrix with several electron-lucent areas where the particles are well defined. Each inclusion is surrounded by a cisterna of rough endoplasmic reticulum (ER). Scale bar represents 500 nm. **3,** A higher magnification of an inclusion containing numerous viruslike particles (P) in a cell from the chlorotic center of a ringspot lesion. ER = endoplasmic reticulum. Scale bar represents 100 nm.

sediments as only one (17).

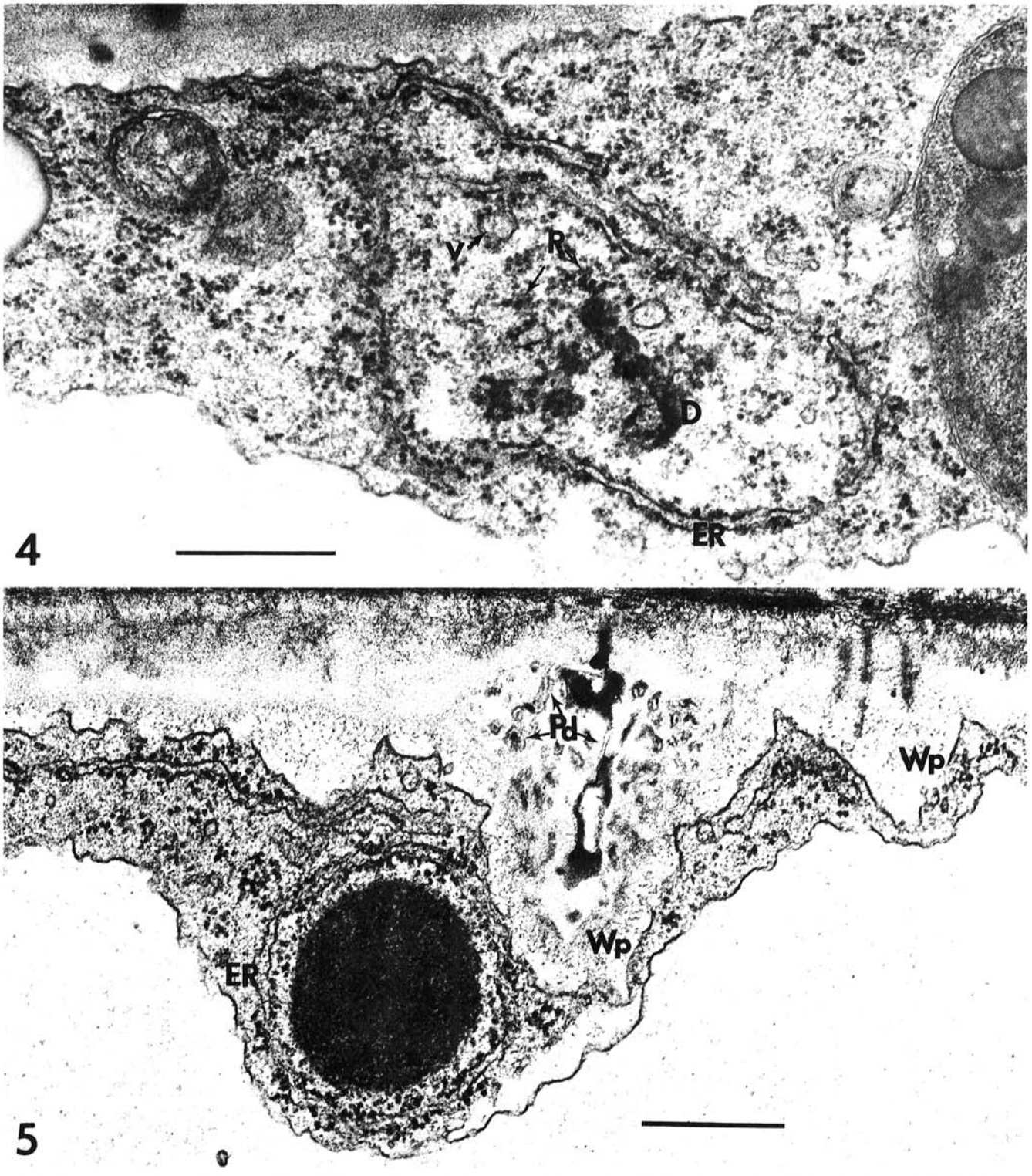
Transmission to herbaceous hosts or blueberry. No herbaceous plants exhibited symptoms as a result of two separate inoculation tests.

After 5 mo incubation in the greenhouse, no symptoms developed on any of 10 blueberry seedlings. In previous work (13), inoculation of blueberry seedlings with purified blueberry shoestring virus resulted in infection and symptoms within 4–5 mo. Until successful inoculations occur with blueberry, only an association of the VLPs with the disease can be claimed.

Serology. The attempt at making an antiserum to red ringspot VLPs failed. The virions may have degraded in the rabbits. An attempt will be made again with CsCl-purified VLPs fixed in 0.5% formaldehyde.

In gel double diffusion tests against CaMV antiserum, purified red ringspot VLPs did not react. Purified VLPs and a preparation from healthy blueberry leaves reacted identically to an antiserum dilution of 1:2. Purified CaMV reacted to an antiserum dilution of 1:512 in the same test.

In ELISA tests against CaMV IgG and enzyme conjugate, CaMV



Figs. 4–5. Ultrastructure in a red rinspot-affected blueberry leaves. **4,** Cisternae of the rough endoplasmic reticulum (ER) encircling vesicles (V), ribosomes (R), and electron-dense materials (D). Scale bar represents 500 nm. **5,** A small inclusion consisting mainly of the matrix substance surrounded by rough endoplasmic reticulum (ER); one large and two small wall protrusions contain many plasmodesmalike structures (Pd). Scale bar represents 500 nm.

at 10 ng reacted fairly strongly ($A_{405\ nm} = 1.08$); concentrations of 100 ng and above gave readings greater than 3.0. Red ringspot VLPs at 10, 100, 500, and 1,000 ng/ml gave $A_{405\ nm}$ readings of 0.36, 0.50, 0.45, and 0.50, respectively; similar results were obtained with preparations from healthy blueberry, TBSV, and TRSV.

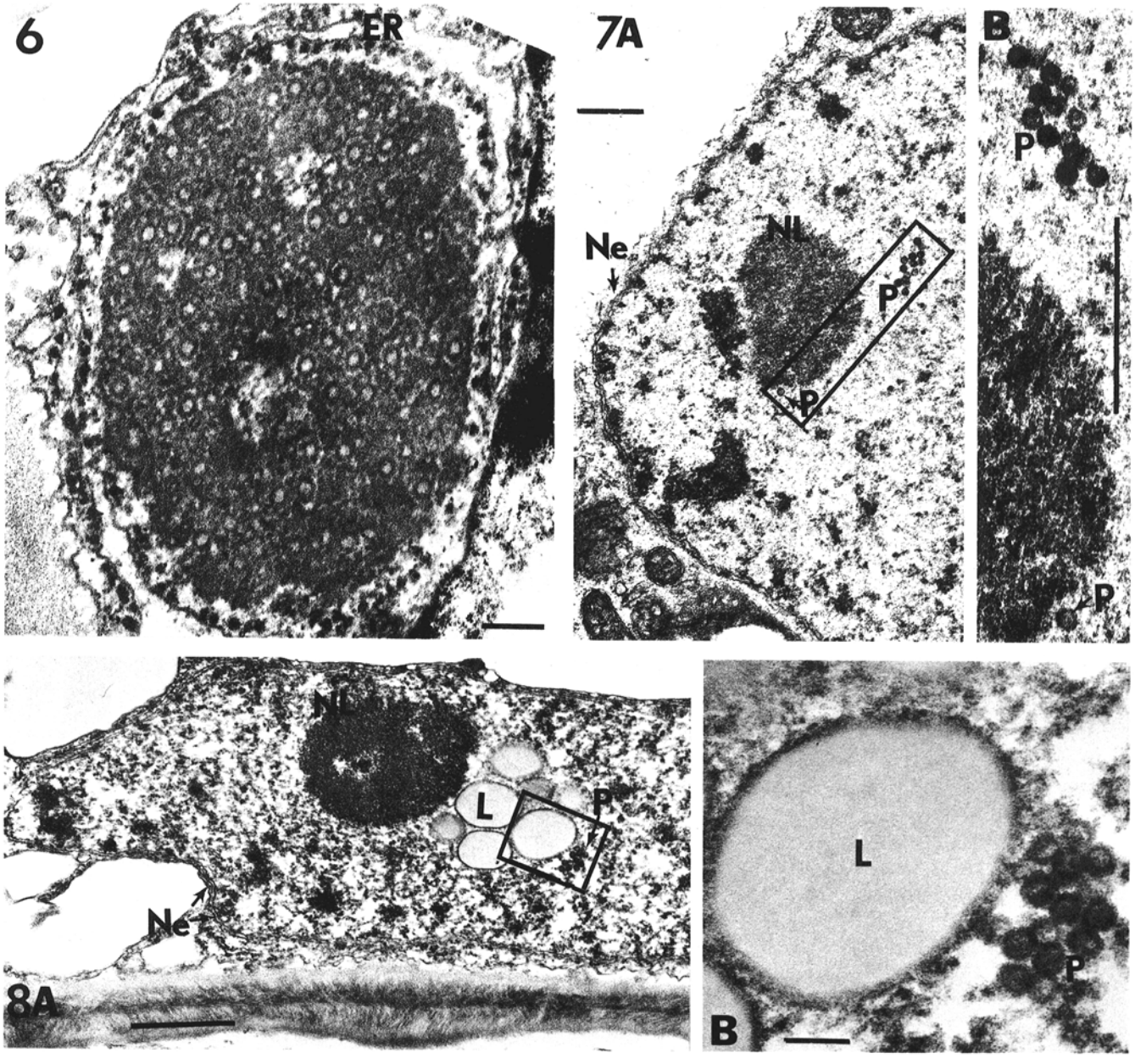
Based upon one-way serology tests, red ringspot virus is not related to CaMV.

DISCUSSION

The inclusions and the particles in blueberry leaves infected with red ringspot disease are similar in morphology to those found in cells infected with caulimoviruses (15,18) such as dahlia mosaic virus (10), cauliflower mosaic virus (4,5,14), strawberry vein-banding virus (9,16), and carnation etched ring virus (11). The inclusions in blueberry leaves affected with red ringspot are predominantly spherical. The irregularly shaped small inclusions found in cells infected with dahlia mosaic virus (10) as well as with other caulimoviruses (9,11, 16) were not found. However, free ribosomes associated with vesicles and patches of electron-dense substances surrounded by rough endoplasmic reticulum (Fig. 4) are suggestive of such small or elementary (11) inclusions.

The rough endoplasmic reticulum tightly encircling each inclusion, regardless of size, was so consistent that it appeared as a boundary membrane. Caulimovirus inclusions, however, lack a boundary membrane although an abnormal increase in endoplasmic reticulum and/or dictyosomes (9–11, 16) has been noted. With petunia vein-clearing virus (12), however, the general appearance of inclusions and associated proliferation of rough endoplasmic reticulum was similar to those observed in this study although some differences were evident.

Virions have not been reported to occur in the nucleus with most caulimoviruses. Lawson and Hearon (11), however, observed virions but no inclusions in the nuclei of *Saponaria vaccaria*



Figs. 6–8. An inclusion and viruslike particles in red ringspot-affected blueberry leaves. **6,** An inclusion in the green center of a ringspot lesion in which the particles are sparsely embedded in an electron-dense matrix. ER = endoplasmic reticulum. Scale bar represents 100 nm; **7A,** A low magnification of a portion of a nucleus containing viruslike particles (P) near the nucleolus (NL). Ne = nuclear envelope. Scale bar represents 500 nm. **7B,** A higher magnification of a portion from Fig. 7A showing the viruslike particles (P). Scale bar represents 500 nm. **8A,** A low magnification of a nucleus containing lipidic globules (L) near the nucleolus (NL) and viruslike particles (P) closely associated with the globules. Ne = nuclear envelope. Scale bar represents 1,000 nm. **8B,** A higher magnification of a portion of Fig. 8A showing the details of the globules (L) and the viruslike particles (P). Scale bar represents 100 nm.

infected with carnation etched ring virus. The particles observed in nuclei of blueberry red ringspot-diseased cells were also not associated with inclusions. Particles of petunia vein-clearing virus also occurred in nuclei of infected cells (12). Lipidic globules, which occurred only in nuclei of infected cells, were, however, often associated with virus particles. The significance of the presence of these globules in not known.

Although electron micrographs of the inclusion bodies and VLPs associated with red ringspot disease are suggestive of a caulimovirus, there are several factors that dictate against it being a member of that grouping. First, the presence of a rough endoplasmic reticulum around the red ringspot inclusion bodies contrasts with its absence around those induced by members of the caulimovirus group. Petunia vein-clearing virus (12) induces a similar involvement between the rough endoplasmic reticulum and the inclusion bodies, but this virus has not been shown to be a member of the caulimovirus group. Second, the presence of two types of VLPs with distinctly different buoyant densities in preparations from red ringspot virus-infected leaves compared to the single species in CaMV-infected leaves indicates a marked difference in physical properties of the two agents. And last, the lack of a serological reaction between CaMV antiserum and the VLPs from blueberry rules out any affinity between red ringspot VLPs and those members of the caulimovirus group that are serolically related to CAMV; ie, dahlia mosaic virus, carnation etched-ring virus, and strawberry vein-banding virus.

The vector of the etiologic agent of red ringspot disease is still unknown. If the agent were classifiable in the caulimovirus group the vector would likely be an aphid, since all members of that group have an aphid vector. It is interesting to note that in New Jersey, red ringspot spreads actively (A. W. Stretch, *personal communication*), whereas in Michigan spread is virtually nonexistent in the field. In 1974, one of us (D.C.R.) counted 47 bushes infected in the Nunica, MI, field and in 1979, no spread was observed from the same 47 infected bushes in the same field. None had been rogued out during that time.

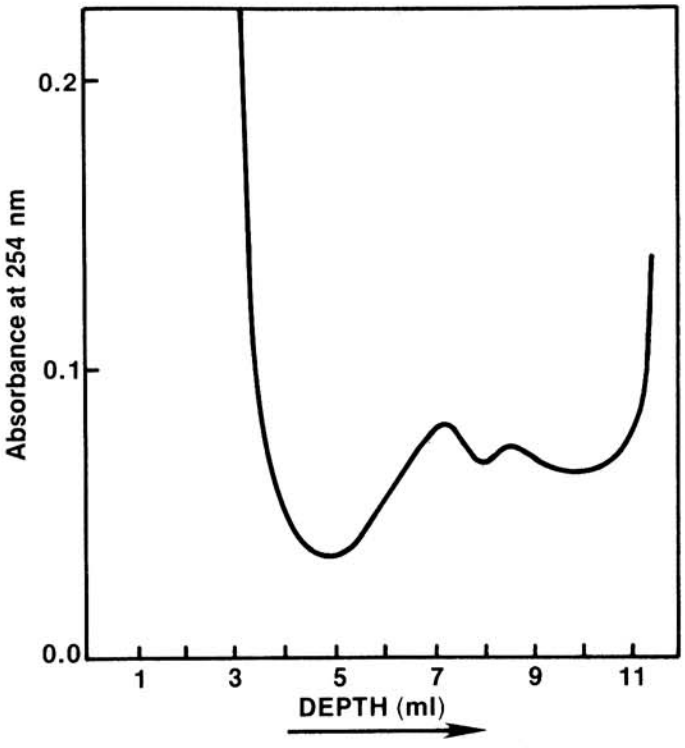


Fig. 9. Photometric scan at 254 nm of an ultracentrifuged linear-log sucrose gradient containing purified blueberry red ringspot viruslike particles from disease cultivar Blueray blueberry leaves. Gradients were run in a Beckman SW 41 rotor at 38,000 rpm for 1 hr at 4 C.

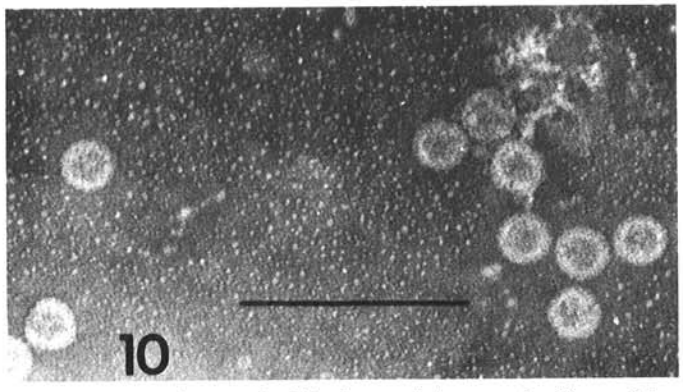


Fig. 10. Electron micrograph of blueberry red ringspot viruslike particles purified from diseased cultivar Blueray blueberry leaves. The viruslike particles were stained in 2% ammonium molybdate, pH 7.2. Bar represents 185 nm.

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