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Cereal Chlorotic Mottle Virus—Purification, Serology and Electron Microscopy in Plant and Insect Tissues

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Abstract

Cereal chlorotic mottle virus (CCMV) was shown to accumulate in the perinuclear space and cytoplasmic vesicles of infected plant cells. The virus was present in both vascular and mesophyll tissues. In infected leafhoppers *Nesoclutha pallida* (Evans) the virus particles had a similar appearance and intracellular location but sometimes appeared to be in a granular matrix and were dispersed throughout some degenerated nucleii.

The virions (63 by 230 nm), when viewed in cross-section, showed 17 densely staining points around the circumference and had three concentric ring structures but no stained axial core. Tangential sections confirm the hexamer structure shown in negative stain.

CCMV was purified by extraction in tris–HCl buffer and concentrated on a discontinuous sucrose gradient before filtering through a celite pad. The filtrate was pelleted and then given two sucrose gradient separations before the final pelleting. Purified virus was used to prepare an antiserum which had a titre of 128 and no detectable host reaction. In gel-diffusion tests with the homologous antiserum, CCMV produced two major reaction lines, with an additional line present in some tests.
[Other keywords : rhabdovirus.]

Introduction

Cereal chlorotic mottle virus (CCMV) is a newly described rhabdovirus which infects several species of Gramineae in Australia and is transmitted by the leafhopper *Nesoclutha pallida* (Evans). The distinctive structural appearance of CCMV particles in potassium phosphotungstate (PTA) negative stain has already been reported (Greber 1979*b*) and this is complemented by the additional information from thin-section electron microscopy put forward in the present paper.

In those rhabdoviruses which mature on or near the inner nuclear membrane, particles normally accumulate in both the perinuclear space and cytoplasmic vesicles. Within these spaces the particles are often surrounded by a relatively clear area, but some associated structures or 'viroplasms' have been reported in the cytoplasm for barley yellow striate mosaic virus (BYSMV) (Conti and Appiano 1973) and in the nucleus for sowthistle yellow vein virus (SYVV) (Lee and Peters 1972).

Several leafhopper-transmitted rhabdoviruses have been demonstrated in both plant and vector tissue (Chiu *et al.* 1970; Herold 1972; Shikata 1972; Conti and Plumb 1977; Bell *et al.* 1978; Greber 1979*a*). These studies show that if a virus has a mainly perinuclear location in the plant, e.g. wheat striate mosaic virus (WSMV) and maize mosaic virus (MMV), it also has a major nuclear association in the insect. BYSMV and Digitaria striate virus (DSV), which are cytoplasmic in the plant, are also located away from the nucleus in the insect. The tissue locations of virus in the insect are

reviewed by Francki (1973). Some rhabdoviruses of Gramineae and particularly those associated with cytoplasmic membranes occur mainly in phloem-associated cells such as companion cells and phloem parenchyma (Conti and Appiano 1973; Greber 1979*a*). Others such as MMV and WSMV are present in most plant tissues (Herold 1972; Sinha and Behki 1972).

Several different antigens are usually detected in serological reactions of plant rhabdoviruses as would be expected of particles constituted from several different proteins (Knudson 1973). Soluble antigens from untreated virus extracts have been reported for LNYV (McLean *et al.* 1971), PYDV (Knudson and MacLeod 1972), BNYV (Lin and Campbell 1972) and WSMV (Thottappilly and Sinha 1973). Viral degrading agents such as sodium dodecyl sulfate (SDS), Tween-ether, Triton X-100, lipase and sodium deoxycholate increase the number of diffusable antigens but can also produce some spurious reactions if proper precautions are not observed (Purcifull and Batchelor 1977).

This paper reports on the reactions of an antiserum to CCMV with purified virus preparations and extracts from infected plants. It also presents details of the structure and localization of the virus in both infected plant and insect tissue.

Materials and Methods

Sources of Isolates, Infected Plant Material and Vector Insects

The isolate of CCMV used for purification and preparation of antisera was transmitted from XL389 maize collected at Ayr in northern Queensland. This same isolate was used for host range tests (Greber 1979*b*) and electron microscopy but other isolates from both New South Wales and Queensland were also used for comparative serological tests and electron microscopy. Colonies of infective *N. pallida* leafhoppers were maintained for production of infected plants for virus purification and electron microscopy.

Healthy colonies of *N. pallida* were also maintained and some were separated off and given access to CCMV-infected *Dinebra retroflexa* (Fahl.) Panz. for 10 days before being individually caged on healthy *D. retroflexa* seedlings for several consecutive 3-day transmission feeds. Proven vector individuals and insects from the healthy colonies were then used for electron microscopy experiments.

Electron Microscopy

Neutral 2% PTA was found to be the most useful of the various staining compounds and pH ranges tried and was used routinely for negative stain preparations. Plant material for thin sectioning was fixed in 3% buffered glutaraldehyde at pH 7·2 for 3–6 h. After washing in buffer, specimens were post-fixed in 1% osmium tetroxide, then dehydrated in a graded series of acetone and embedded in Spurr's embedding medium. Sections were cut on an LKB Ultrotome III and stained with uranyl acetate and lead citrate before viewing with a Siemens 101 electron micrsocope. Insects for thin sectioning were partially dissected in 3% buffered glutaraldehyde by removal of legs and wings and then processed by the same techniques as for plant tissue. The whole insects were flat embedded to facilitate orientation.

Purification

The method used was essentially that of Jackson and Christie (1977). 20–40 g of infected *D. retroflexa* or maize leaf tissue was crushed with mortar and pestle, squeezed through cheesecloth and given a low speed centrifugation before being layered onto discontinuous (600 and 300 mg/ml) sucrose gradients in Beckman 25·1 rotor tubes. The extracting buffer used was tris–HCl (0·1 M) at pH 8·4 with 0·04 M sodium sulfite added and the pH was adjusted to 7·4 after extraction. This follows Jackson and Christie (1977) except that no magnesium acetate or manganese chloride was added at any stage of the procedure. The final pellets were resuspended in water or tris buffer (0·02 M, pH 7·4) and some virus was stored frozen for use in antiserum production.

Serology

Rabbits were given four intramuscular injections of virus suspension plus Freund's incomplete adjuvant at weekly intervals. Most serological reactions were in 0·75% (w/v) Ionagar with 0·85% (w/v) NaCl, 0·1% (w/v) sodium azide and either 0·005 M phosphate buffer, pH 7·0, or 0·01 M borate buffer, pH 8·0. SDS agar (Purcifull and Batchelor 1977) was used in some tests. Wells were 4 mm in diameter and mostly in circular patterns with six peripheral wells. Reactions were noted after 1 day and 3 days. A Beckman type 65 rotor run at 40000 rev/min was used for sedimentation tests on viral antigens.

Antisera to WSMV were provided by Professor P. E. Lee and Dr R. C. Sinha, Ottawa, Canada, and an antiserum to MMV was supplied by Dr R. Lastra, Caracas, Venezuela.

Results

CCMV in Plant Cells

The virus particles were found in mesophyll, parenchyma and vascular tissues. The structure and organelles of most infected plant cells were apparently intact except for the distension of the perinuclear space by particle accumulations. Nuclear disruption was only evident in near-necrotic tissues. Most cells with included virions showed

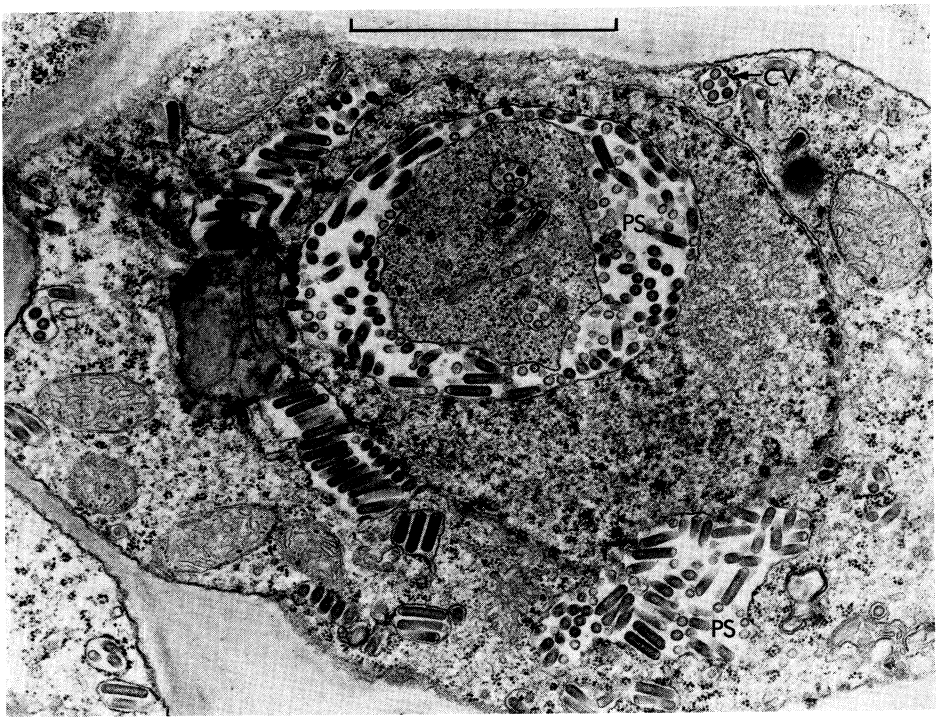

Fig. 1. Cell of *Dinebra retroflexa* infected by CCMV. Virions are present both in the perinuclear space (*PS*) and cytoplasmic vesicles (*CV*). The cytoplasm shown in the centre of the section is apparently the result of indentation of the nuclear membrane in this area. Bar represents 1000 nm.

distention of the outer nuclear membrane into the cytoplasm with cytoplasmic vesicles beyond this (Fig. 1). Invagination *into* the nucleus in plant cells was uncommon in contrast to insect cells. Palisade orientation of bullet-shaped particles on the inner nuclear membrane was frequently observed and particles detached from the membrane

sometimes showed one end with a less dense area under the hemispherical cap (Fig. 2*d*). The space between particles within the vesicles or enclosed within the nuclear membrane lamellae in plant cells appeared to be empty of any other densely staining material or organized structures.

Particles in cross-section showed 17 densely staining protrusions around the outer circumference (Fig. 2*a*). There were either two or three distinctly stained concentric rings. One contained the projections, another was just inside or contiguous with this and there was a distinct inner ring of about half the total particle diameter. There was never any central dark-staining core such as is present with DSV and most other plant rhabdoviruses. Tangential longitunal sections (Figs 2*b* and 2*c*) showed a

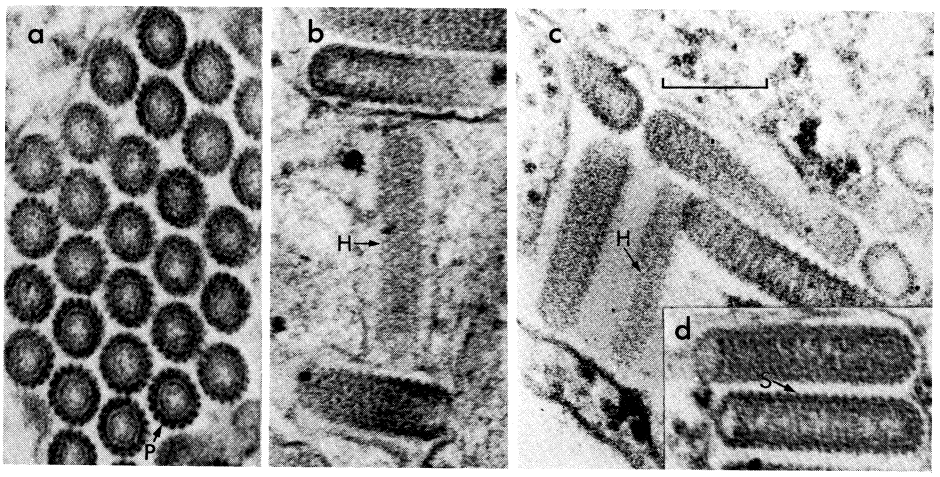

Fig. 2. (*a*) Transverse sections of CCMV particles in a plant cell showing densely stained protrusions (*P*). (*b*) and (*c*) Tangential longitudinal sections showing the hexamer pattern (*H*). (*d*) Medial longitudinal sections showing one lightly stained end and spikes (*S*) around the perimeter. Bar represents 100 nm.

hexamer pattern which can be correlated with similar patterns shown by the virions in PTA (Greber 1979*b*). Medial longitudinal sections showed external spikes (Fig. 2*d*) which correlate well with those shown in PTA preparations. Particle dimensions in thin sections measured 63 nm diameter by 230 nm.

There were few, if any, 'dimeric' particles and no obvious uncoated nucleocapsids (Kitajima *et al.* 1969) were observed.

CCMV in Leafhopper Cells

Individual virions in cells of the vector were similar to those in plant cells. The same concentric rings and peripheral pattern of cross-sections, spikes and hexamer patterns in longitudinal sections were all evident. The palisade arrangement of bullet-shaped particles on the inner nuclear lamella was also present (Fig. 3) but there were more instances of particles laterally appressed to the inner nuclear lamella than in perpendicular palisades.

In insect cells there was a marked tendency for the perinuclear space to invaginate *into* the nucleus. Also, many insect cells showed varying degrees of nuclear disruption

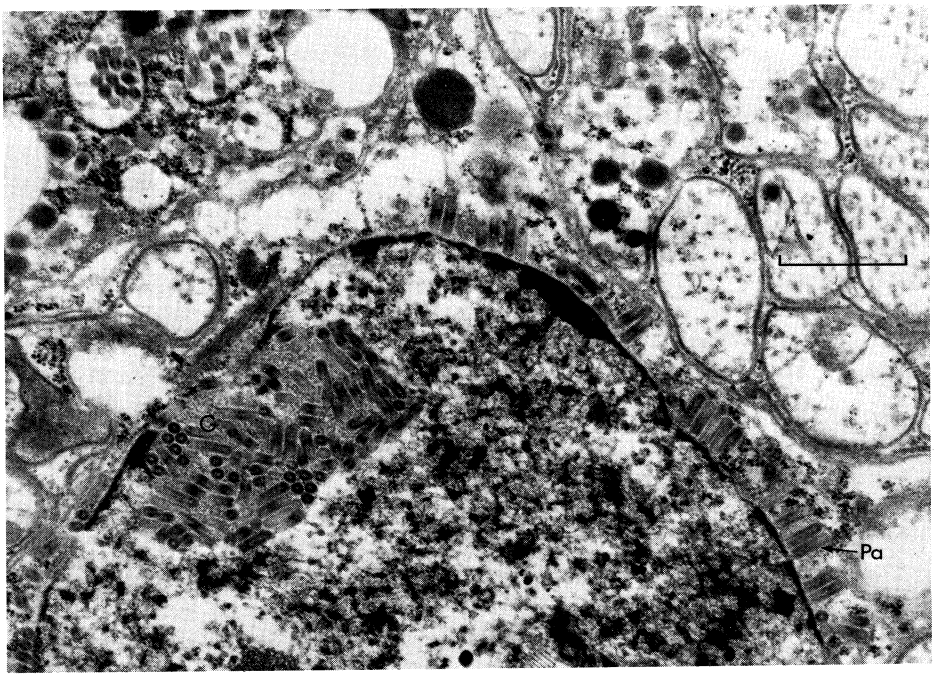

Fig. 3. Cell of *Nesoclutha pallida* infected by CCMV, showing particles in a palisade (*Pa*) between the lamellae of the nuclear membrane and some which appear to be in granular matrix (*G*). Bar represents 500 nm.

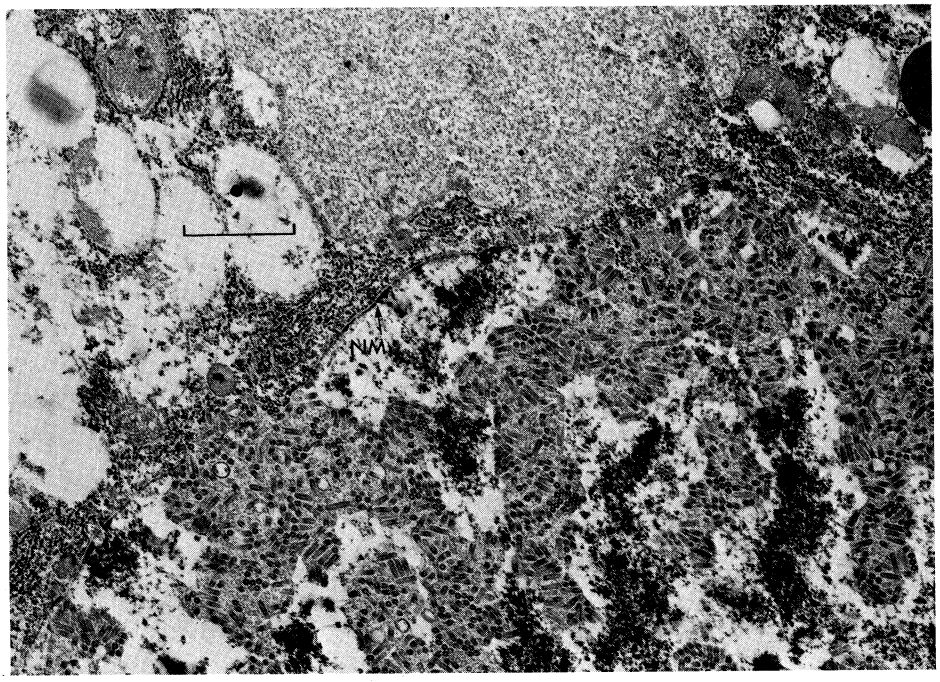

Fig. 4. Salivary gland cell of *N. pallida* with extensive nuclear disorganization after CCMV infection. *NM* is the nuclear membrane. Bar represents 1000 nm.

(Fig. 4). In some almost fully disrupted nucleii the particles and residual nucleoplasm appeared to be intimately mixed. In leafhopper cells the particles were embedded in a granular ground mass (Fig. 3) whereas the perinuclear space in plants (Fig. 1) had a clear appearance. The ground mass surrounding the particles in insect cells had a texture similar to parts of the nucleus itself. Even when groups of particles within the nucleus had no obvious connection with the nuclear membrane, they were mostly retained as discrete groups and not dispersed into the nucleoplasm (Fig. 3).

The virions were found only in the salivary glands and brain tissue of 10 infective insects examined. No insects have been examined after recent aquisition feeds which could be expected to enhance the chances of particles being present in the gut wall. All actively transmitting insects examined had virus particles in the salivary glands and brain tissue. Two insects from a previously reported experiment (Greber 1979*b*) which had ceased to transmit 3 weeks earlier, were found to have virus only in brain tissue. No virus particles were found in healthy insects with no access to CCMV-infected plants.

Purification

The method used gave an adequate yield of purified virus from about 30 g of maize or *D. retroflexa* leaf tissue. Grinding finely in a mortar and re-extracting fibrous tissue helped improve yield. This weight of tissue gave about the maximum quantity of extract that could be handled in three Beckman 25·1 rotor tubes on the first (discontinuous) gradient. The celite pad removed all obvious green pigment and the first pellets were glassy and about 4–5 mm in diameter. These pellets contained virus particles in good condition along with some host membrane material which was removed by the first (low density) sucrose gradient. The sucrose gradients showed one dense band (Fig. 5*a*) and sometimes a much weaker band about 5 mm below this. When both bands were pelleted separately they were both found to contain CCMV

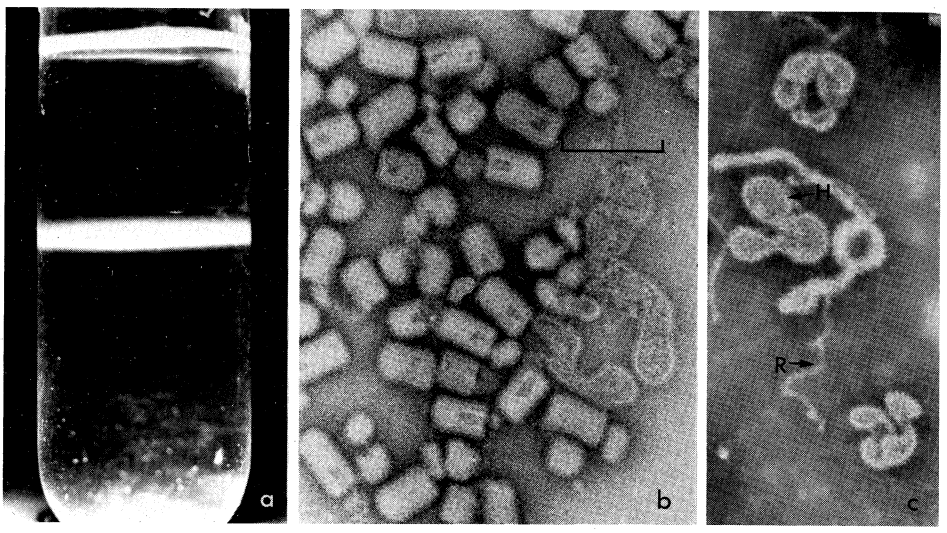

Fig. 5. (*a*) Sucrose gradient tube with dense band of CCMV particles. (*b*) Purified CCMV preparation with PTA negative stain. (*c*) Some disrupted particles from a purified CCMV preparation, showing hexamer debris (*H*) and uncoiled ribonucleoprotein helix (*R*). Bar represents 200 nm.

virions and appeared similar when examined with an electron microscope. Concentrated suspensions of the virus were obviously milky. No host debris was detectable by electron microscopic examination of the final pellet but there were more distorted virus particles in this pellet than in the first (Figs 5*b* and 5*c*). Most particles were still intact after storage for 10 days at 4°C.

Serology

When purified virus was reacted in gel-diffusion tests against its homologous antiserum a precipitation line was produced very close to the antigen well. However, when Triton X-100 (*c.* 1%) was added to the virus, two strong lines were formed (Fig. 6*b*). Because of the sedimentation characteristics indicated below, these antigens were designated *p* (pellet) and *s* (supernatant). The titre of the antiserum for the more diffusable antigen (*s*) (Fig. 6*a*, arrow) was 128. There was no host reaction with healthy sap.

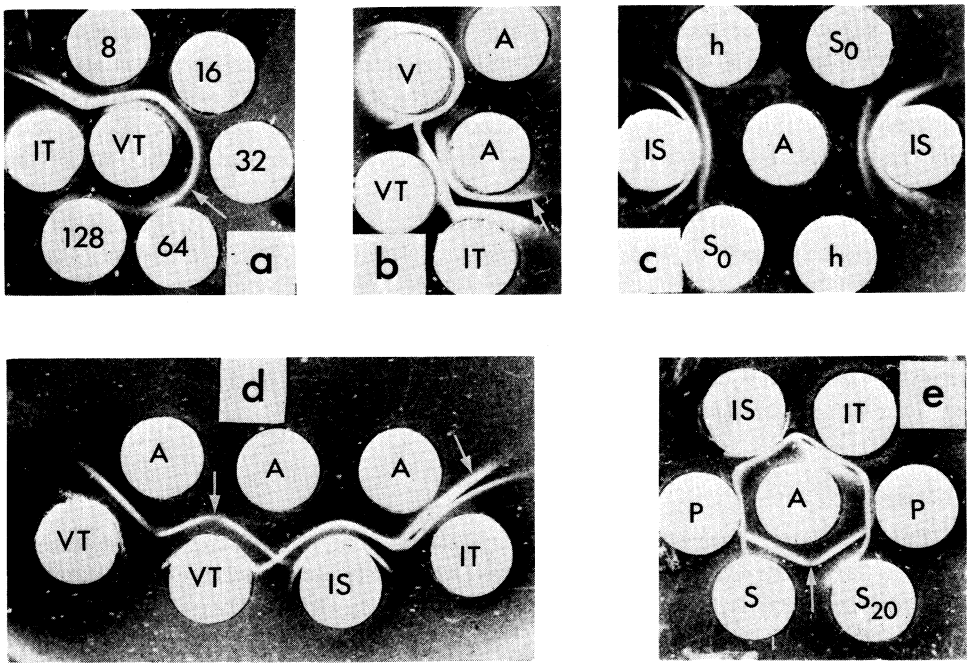

Fig. 6. Agar gel-diffusion serology of CCMV. (*a*) Antiserum dilution series (1 : 8 to 1 : 128). Arrows indicate *s* antigen lines. (*b*) Effect of adding Triton X-100 to purified virus. (*c*) No soluble antigen was detected in the supernatant from fresh infective sap without addition of Triton X-100. (*d*) Note crossing of lines. The *p* line from freshly purified virus is very close to the well. A third antigen appears to be present in infective sap without Triton X-100. (*e*) Almost complete separation of *p* and *s* (arrowed) antigens is obtained after 110 min at 100 000 *g* while both are present in the supernatant after only 20 min.

Key to legends: *IT*, infective sap with 1% Triton X-100; *VT*, purified virus with 1% Triton X-100; *A*, CCMV antiserum, dilutions varying from 1 : 1 to 1 : 8; *V*, purified virus; *IS*, infective sap without Triton X-100; *h*, healthy sap; S_0, supernatant 100 000 *g* 110 min, no Triton X-100; S_{20}, supernatant 100 000 *g* 20 min, Triton X-100 added; *S*, supernatant 100 000 *g* 110 min, Triton X-100 added; *P*, pellet 100 000 *g* 110 min, Triton X-100 added.

The same two reactions were also produced by infective sap treated with Triton X-100 (Figs 6*b* and 6*d*), but there was some indication of an additional reaction from untreated infective sap. The relative position of the *p* and *s* lines was reversed between detergent-treated and untreated infective sap and it was found that the position of these lines could also be changed or made to coincide by manipulating the molarity of buffer used for sap extraction. The third reaction was found in tests with sap extracted in water or very dilute buffer and in tests of concentrated purified virus with undiluted antiserum, but was difficult to separate entirely from the *p* line.

Use of SDS at 1% with either infected or healthy sap or purified virus produced a spurious line with both CCMV antiserum and normal serum and interpretation of the other reactions was difficult. No significant reaction lines resulted when SDS-treated antigens were reacted in SDS-agar (Purcifull and Batchelor 1977). Use of sodium deoxycholate at 1–2% with Triton-treated infective sap removed the *p* line.

When freshly extracted sap was centrifuged at low speed (10 000 *g*) to remove cell debris and then at 100 000 *g* for 110 min, both the *p* and *s* antigens were sedimented (Fig. 6*c*). However, when sap was allowed to stand for 24 h or more at room temperature or Triton X-100 (1%) was added before centrifuging, there was substantial though slightly incomplete separation (Fig. 6*e*) of the antigens between the pellet and the supernatant when examined by gel-diffusion serology. The sap was centrifuged through a band of 15% (w/v) sucrose to minimize contamination of the pellet by the supernatant. Additional sedimentation experiments (100 000 *g* for 20 min) determined that neither of the antigens released by Triton X-100 sedimented at the rate of the intact virion.

There was no definite indication of serological relationship to WSMV or MMV when antisera to these viruses were reacted with purified CCMV and its homologous antiserum under the conditions recommended for each. However, when the WSMV antiserum from Dr R. Sinha was reacted undiluted in a well adjacent to CCMV antiserum, there was a tendency for the precipitation lines from the latter to curve in front of the WSMV well. This effect may have been caused by a very weak reaction with the latter antiserum.

Discussion

The combined information from PTA and thin-section micrographs enable a model to be envisaged for CCMV. The surface layer is readily penetrated by PTA in unfixed preparations (Greber 1979*b*) and reveals a hexamer layer resembling that of BNYV (Hills and Campbell 1968). The latter is probably in the matrix region of the particle and the surface projection correlate with the hexamers which are each based on two helix striations. The hexamer layer has some intrinsic structural strength as shown by the maintenance of this configuration in debris free of the ribonucleoprotein (RNP) helix (Fig. 5*c*). In sap preparations this layer is seldom penetrated by PTA stain and particles showing cross-striations are rarely seen. The uncoiled helix seen in some deteriorated purified preparations (Fig. 5*c*) resembled that described for other plant rhabdoviruses (Wolanski *et al.* 1967; Peters and Kitajima 1970), while the sac-like structures with surface spikes still in position (Fig. 5*b*) probably represent other damaged virions. Lack of any densely staining core in cross-sections of CCMV particles contrasts with many other rhabdoviruses such as DSV. Assuming this core is a real structure, this difference could possibly explain why CCMV appears to

flatten more than DSV in PTA preparations. In thin section, the particles widths of these two viruses (DSV 55 nm, CCMV 63 nm) are much more comparable than in PTA (DSV 55 nm, CCMV 75 nm) (Greber 1976, 1977). Another obvious difference in the cross-sectional appearance of these two viruses is the ring of densely stained structures around the outer circumference shown only by CCMV. These would be best expressed when the section is cut so that the structures are aligned to reinforce the images in the tubular portions of particles (*c.* 80 nm long) which are included in each thin section. The number of these apparent protrusions may be characteristic of the virus, e.g. CCMV shows 17, while MMV shows 13 (Martelli and Russo 1977).

Virions are readily located in both plant and insect cells and have a similar cellular location in each. However, the particles in the insect are usually embedded in a stained ground mass which mostly protrudes into the nucleus while in the plant the perinuclear space containing the particles is clear of other staining material and usually protrudes into the cytoplasm. These differences are possibly due to inherent properties of the plant and insect cells. No uncoated nucleocapsids or very long particles were seen. The long particles found for DSV (Greber 1979*a*) were accumulated in cytoplasmic vesicles.

CCMV particles appear to be more durable *in vitro* than many plant rhabdoviruses. It was not necessary to provide protection during purification by adding the metal salts used for some other viruses of this type (Ahmed *et al.* 1970; Jackson and Christie 1977). The antisera prepared had no apparent host reaction and were found to be very satisfactory for sap diagnosis of CCMV. Although soluble antigens have been associated with many plant rhabdoviruses, including WSMV (Sinha and Thottappilly 1974), none were detected in fresh plant extracts containing CCMV particles. The major serological activity of some rhabdoviruses has been shown to be associated with the G and N proteins (Knudson and MacLeod 1972; Knudson 1973). The sedimentation characteristics of the *p* antigen of CCMV indicate it is probably associated with the nucleocapsid, where the N protein is located. The *s* antigen of CCMV is readily detached from the particle by Triton X-100. It is much less readily sedimented than the *p* antigen and is probably associated with the spike or G protein. Now that purified preparations of CCMV are available, electrophoretic separation of the proteins should enable these hypotheses to be investigated more accurately.

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