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Light and electron microscopy of red clover vein mosaic virus in pea (Pisum sativum)

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Abstract

Epidermal strips of pea infected with the RK31 isolate of the red clover vein mosaic virus were found to contain many diagnostic crystalline inclusions, recognizable two, but more clearly three weeks after inoculation. They could be easily detected by light microscopy, even at low magnification, when stained with phloxine and methylene blue, but more rapidly with a phase-contrast microscope in unstained preparations in water. They were always found after the onset of external symptoms. With the pea streak strain (P42) they were usually absent.

In ultrathin sections made 22 days after inoculation or later, virus distribution and accumulation were comparable to those of other members of the carlavirus group. Sometimes extensive irregular bundles were observed. These were distinct from the crystals seen by light microscopy. Sections of the three-dimensional crystals studied in the electron microscope showed a very regular internal striation with a periodicity of about 11 nm, or were sometimes composed of spherical particles in looser array. When crystals were isolated intact and stained negatively, the majority of the material appeared to consist of spherical or polyhedral particles 11–12 nm in diameter.

Introduction

Red clover vein mosaic virus (RCVMV) (R/1:*/5:E/E:S/Ap) is widely distributed and causes severe stunting in pea and vein mosaic in various clovers (Varma, 1970). The virus has rather stiff elongated particles, 650–670 nm in length. It is known to induce the formation of crystalline inclusion bodies, but these have not been systematically investigated and reports concerning them are fragmentary.

Porter and McWhorter (1952) first found the hyaline crystals in epidermal cells of infected broad bean (*Vicia faba*) and pea (*Pisum sativum*) and considered their presence highly diagnostic for the virus. Sander (1959) found amorphous and crystalline inclusions in leaf hairs of diseased red clover plants. McWhorter (1965) observed the crystals in various clovers, pea, *Lathyrus odoratus* and broad bean infected with different strains.

Bos (1963) published a photograph by Porter and McWhorter of a crystal in an infected broad bean cell. More illustrations of such crystals were given by Rubio-Huertos (1964), although at the time he did not identify his virus with certainty as RCVMV. He isolated intact crystals and published electron micrographs of carbon replicas. From examination of ultrathin sections of infected pea plants he thought that the crystals consisted of elongated particles arranged in three dimensional order. McWhorter (1965) supposed the crystals to be composed of pure virus.

In a recent study on an aberrant strain of RCVMV and on the differentiation

between RCVMV and pea streak virus Bos et al. (1972) made several light microscopy observations. Further results on light and electron microscopy of pea streak virus have just been published (Bos and Rubio-Huertos (1972)). The present paper provides more detailed information on the intracellular changes induced by RCVMV.

Materials and methods

The *virus isolates* used were the Dutch red clover isolate RK31 and the American pea streak strain (P42) provided by Dr R. W. Goth, Beltsville, USA. For information on their origin, properties and cultivation see Bos et al. (1972).

For *light microscopy* epidermal strips from young stems, petioles and main leaf veins of systemically infected pea plants, mainly of the cultivar 'Koroza', were stained with 1 % phloxine and 1 % methylene blue in Christie's solution (Bos, 1969). Sometimes phase-contrast microscopy was used.

For *ultrathin sectioning* leaf material was treated with 4% glutaraldehyde in 0.1 M Sörensen buffer, fixed in 2% osmium tetroxide, stained in 0.5% uranyl acetate, dehydrated in a graded series of ethanol and propylene oxide and embedded in Epon Araldite. For details see Bos and Rubio-Huertos (1972).

Intact crystals for negative staining in 2% PTA were isolated as described earlier by Rubio-Huertos (1950, 1964).

Results

Light microscopy. In the course of the investigations reported by Bos et al. (1972), several groups of pea plants (22 in total), were tested for inclusion bodies for various purposes.

In 'Koroza' and most other pea cultivars, the first symptoms of systemic infection with either isolate were usually systemic vein clearing and leaf curling, especially of stipules, observed about 11 days after inoculation. With RK31 necrotic stem streaking sometimes occurred. With P42, stem necrosis, followed by irregular yellowing and premature plant death, predominated.

With RK31 in pea, inclusions appeared after the 14th or more certainly after the 21st day following inoculation. They persisted until the end of the period of observation. They were also observed in broad bean plants.

With P42, inclusions were found on only three out of 14 occasions. Contamination of the three plants with RK31 cannot be excluded, but could not be proved as there are no differential indicator hosts for RK31. The crystals were characteristic of the RK31 isolate.

The inclusions found were always more or less hyaline and clearly crystalline, never granular or amorphous. With phloxine and methylene blue they usually stained slightly red. Sometimes some blue stain accumulated. They were easily detected even at low magnification (Fig. 1A). However, they varied greatly in shape, from very regular triangular or hexagonal (Fig. 1B and E) to composite forms or more irregular and rounded structures (Fig. 1C, D, E and F). Sometimes eight or more of the irregularly shaped crystals were seen in a cell (Fig. 1D). Using phase-contrast microscopy the crystals could be found almost immediately in epidermal strips mounted in water without staining.

Fig. 1. Light micrographs of epidermal strips from systemically infected pea petioles 22 (B, C, D) and 39 days (A, E, F) after inoculation with the RK31 isolate of RCVMV; stained with phloxine and methylene blue. Bars represent 10 μm. (Photographs Dr R. E. Labruyère).

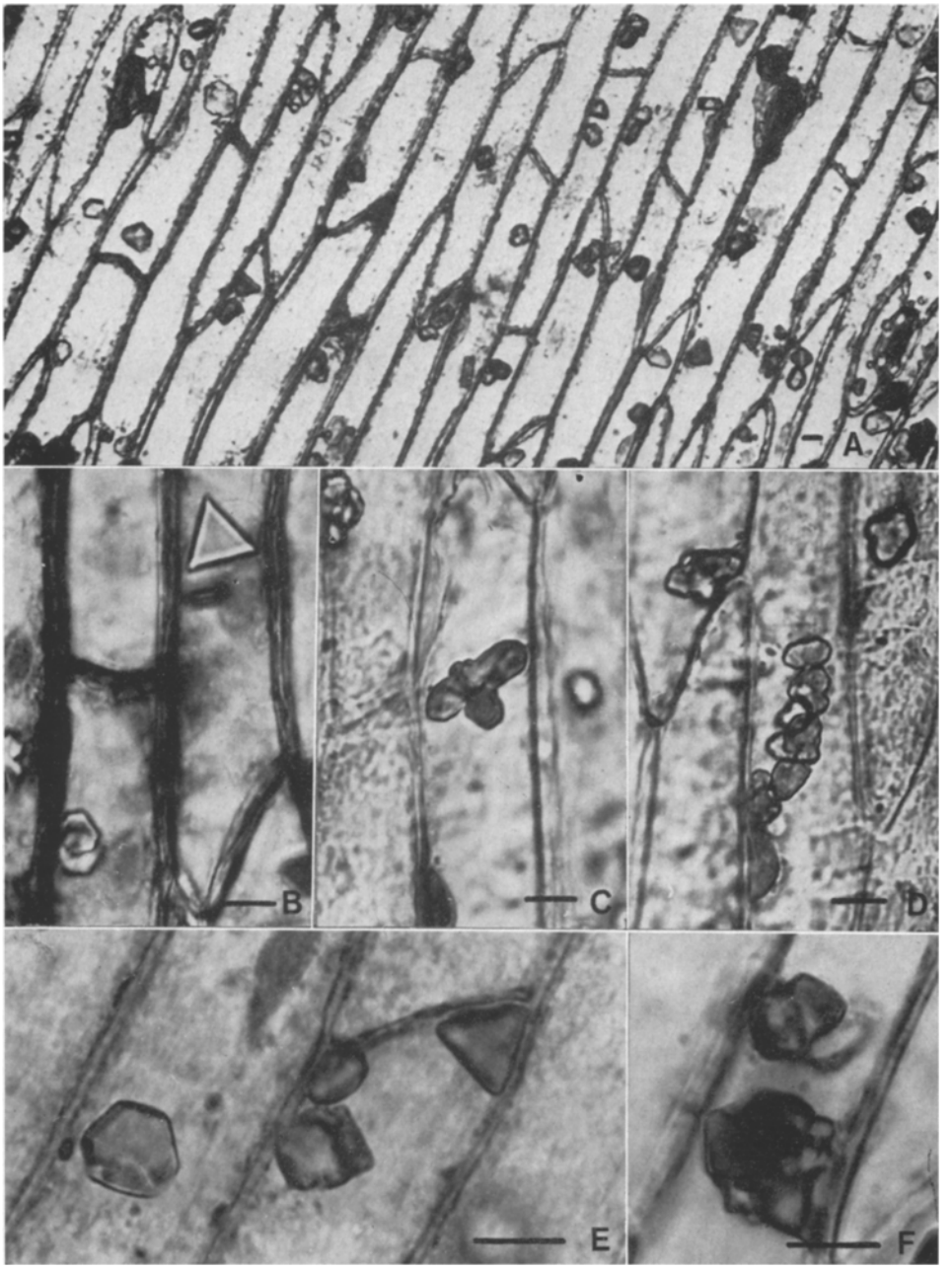

Fig. 1. Lichtmicroscopische foto's van epidermisreepjes van systemisch geïnfecteerde bladstelen van erwt 22 (B, C, D) en 39 dagen (A, E, F) na inoculatie met het RK31-isolaat van het nerfmozaïekvirus van rode klaver; gekleurd met floxine en methyleenblauw. Staven geven 10 μm aan. (Foto's Dr. R. E. Labruyère).

Fig. 2. Electron micrographs of virus accumulations (*vi*) in pea leaf cell; *A* P42 20 days and *B* RK31 22 days after inoculation; *m* mitrochondrion, *c* chloroplast, *v* vacuole, *w* cell wall; bars represent 500 nm.

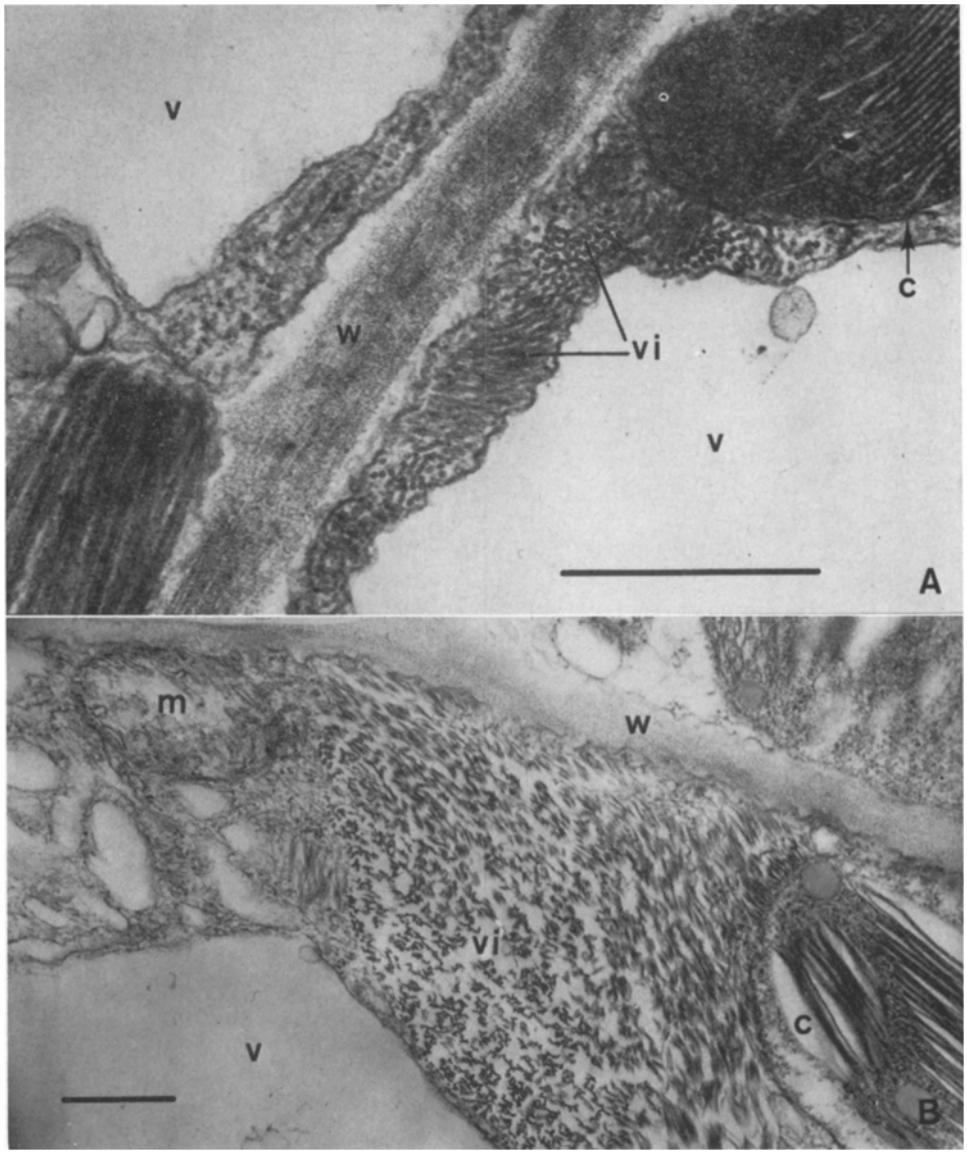

Fig. 2. Elektronenfoto's van vrirusophopingen (vi) in erwtebladcel; A *RK31 22 dagen en* B *P42 20 dagen na inoculatie;* m *mitochondrium;* c *chloroplast;* v *vacuole;* w *celwand; staven geven 500 nm aan.*

Electron microscopy. With both the RK31 and P42 isolates, ultrathin sectioning of pea material embedded 3 weeks after inoculation usually revealed little virus. When present, the virus particles could be detected occurring in small groups in protoplasmic strands or in thin protoplasmic layers against cell walls (Fig. 2A) with their extremities attached to tonoplast, as commonly occurs with members of the carlavirus group. With RK31, extensive accumulations of elongated particles were occasionally observed near cell walls and chloroplasts (Fig. 2B), and big, more or less irregularly shaped crystals were frequently found. These crystals were regularly striated, the periodicity of striation being about 11 nm (Fig. 3 and 4). Often parts of such crystals showed a looser array, clearly suggesting that they are built up of spherical particles (Fig. 5).

When crystals were separated from infected cells using a light microscope and then negatively stained with PTA and viewed in the electron microscope, few elongated particles and abundant spheres or polyhedra of about 11–12 nm in diameter were observed (Fig. 6).

In tissues infected with RK31 sometimes membrane-bound structures, similar to the anomalous crystals recently reported by de Zoeten et al. (1972), were found.

Fig. 3. Ultrathin section showing a big crystal in a RK31-infected vacuolated pea cell; bar represents 500 nm.

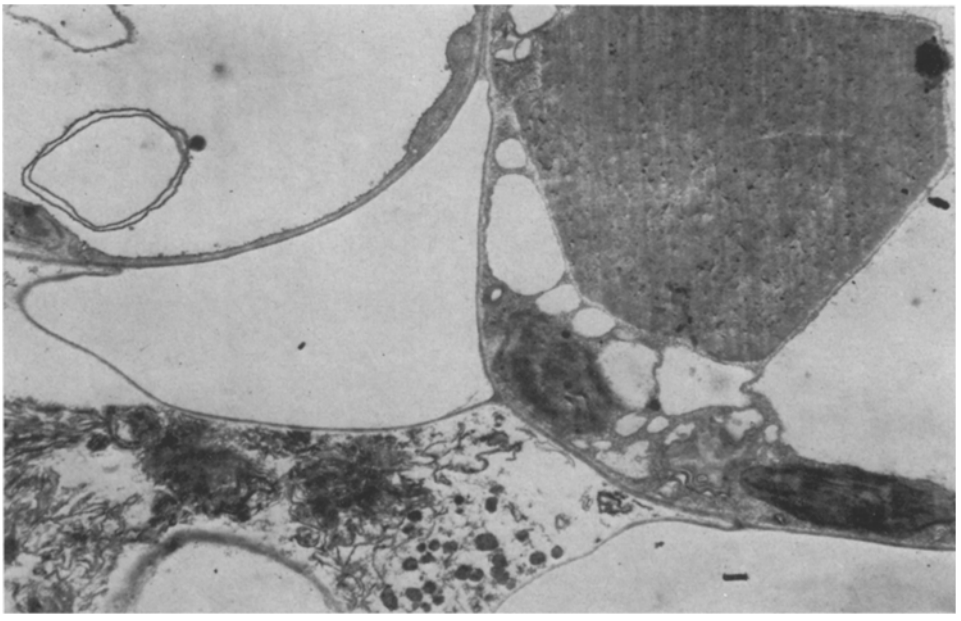

Fig. 3. Ultradunne coupe met een groot kristal in een met RK31 geïnfecteerde, van vacuoles voorziene erwtecel; staaf geeft 500 nm aan.

Fig. 4. Cross sections of RK31 crystals showing striations; bars represent 500 nm.

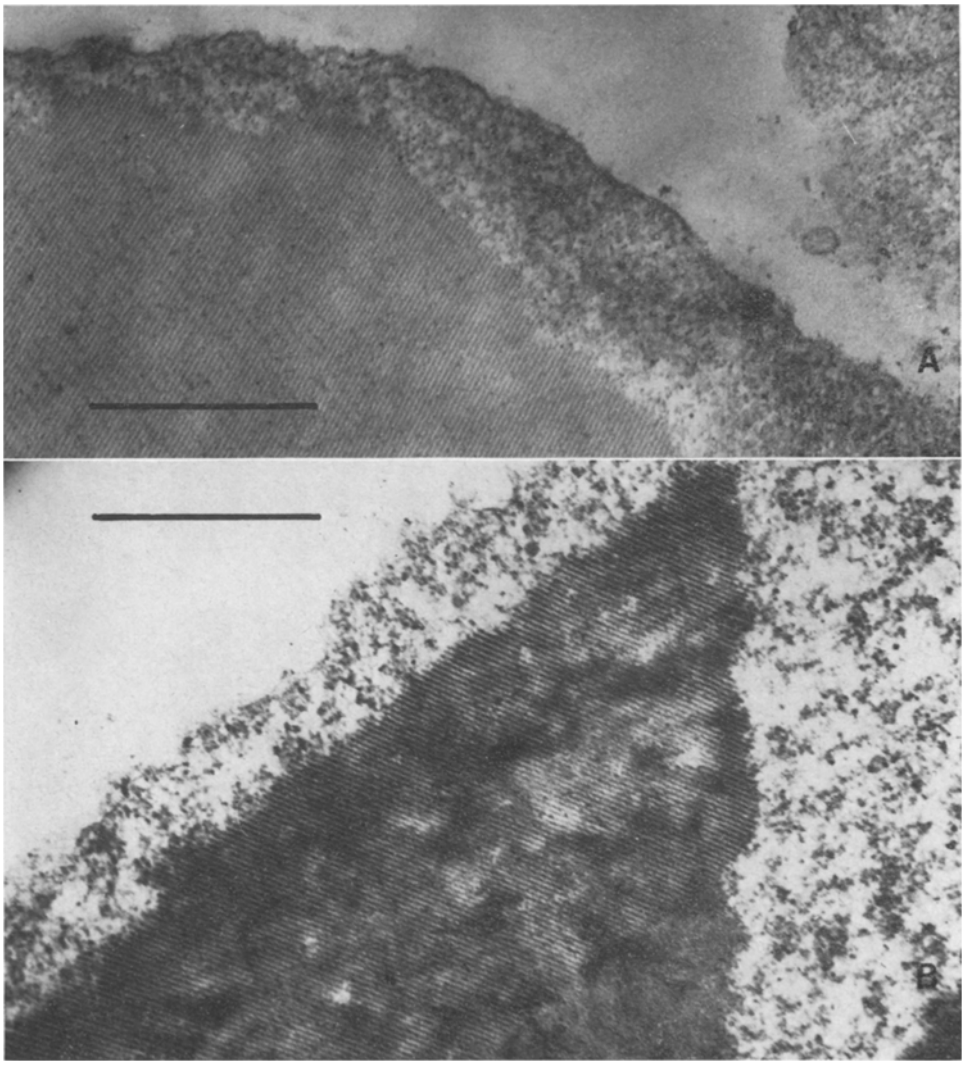

Fig. 4. Dwarsdoorsnede van een RK31-kristal met dwarsstreping; staven geven 500 nm aan.

Discussion

Our results show that RCVMV has an intracellular distribution comparable to that of other members of the carlavirus group studied so far: potato virus M (Tu and Hiruki, 1970), potato virus S (de Bokx and Waterreus, 1971), *Passiflora* latent virus (Bos and Rubio-Huertos, 1971), carnation latent virus (Castro et al., 1971), pea streak virus (Bos and Rubio-Huertos, 1972), and the recently reported cole latent virus (Kitajima et al., 1970) apparently also belonging to the group.

Fig. 5. Ultrathin section of RK31 crystal showing spherical particles partly in loose array; bar represents 500 nm.

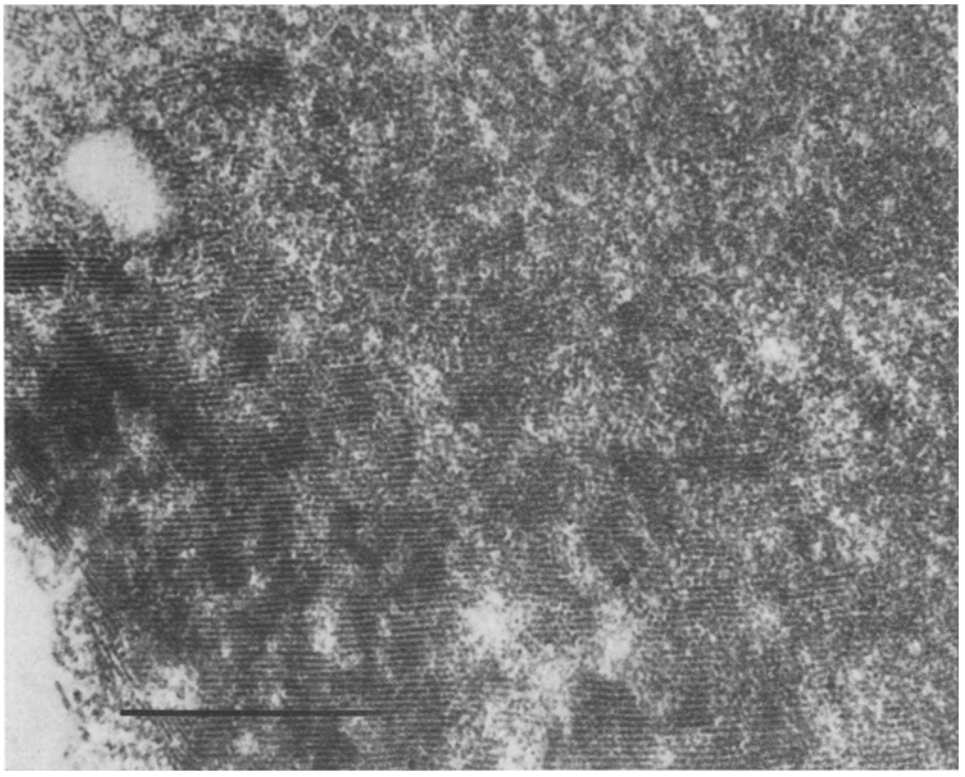

Fig. 5. Ultradunne coupe van RK31-kristal met bolvormige deeltjes gedeeltelijk in losse rangschikking; staaf geeft 500 nm aan.

With none of these viruses has true crystallization been observed, perhaps because their particles are not sufficiently straight although rather rigid. Therefore accumulations tend to be more or less extensive bundles or plates, sometimes occurring in groups. Only with pea streak virus can the virus accumulations in the cytoplasm be stained and observed with the light microscope.

We did not find amorphous inclusions accompanying the crystals in red clover leaf hairs, as reported by Sander (1959). Sander's inclusions may resemble the inclusions we observed with pea streak virus (Bos and Rubio-Huertos, 1972). The amorphous inclusions found by Rubio-Huertos (1964) together with crystals in peas contained pinwheel structures and presumably were caused by a contaminating virus, possibly bean yellow mosaic virus.

So far, RCVMV is the only member of the group known to induce the formation of extensive true crystals in infected plants. We have never observed them in healthy pea or broad bean plants or in legumes infected with any other virus. They can be rapidly detected and are of diagnostic value (see also McWhorter, 1965), although they may

Fig. 6. Negatively stained dip preparation of RK31 crystal, isolated intact from epidermal cell but disintegrated during staining; bar represents 500 nm.

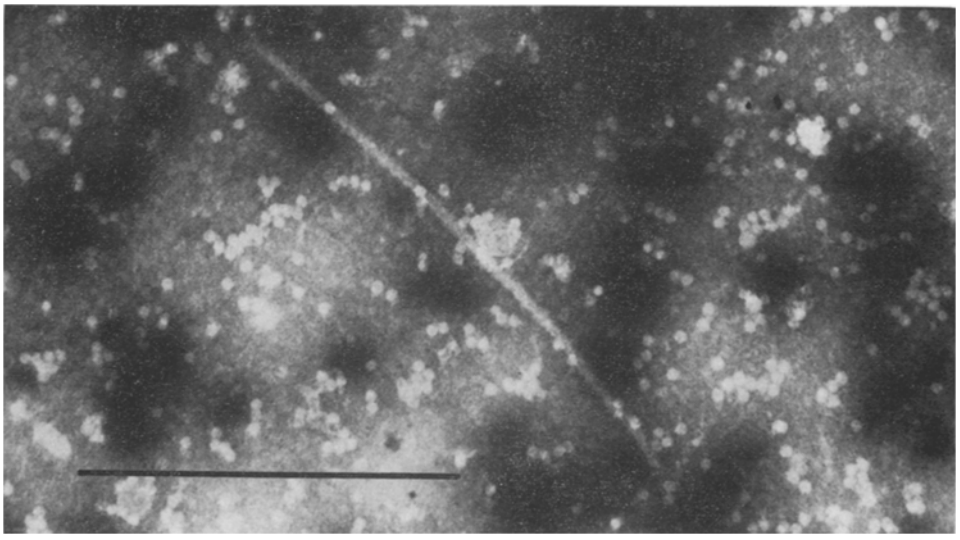

Fig. 6. Negatief gekleurd indooppreparaat van RK31-kristal, in intacte toestand uit epidermiscel afgezonderd maar bij kleuren uiteengevallen; staaf geeft 500 nm aan.

not be induced by all strains. The few cases where they occur with P42 are suspected to have been caused by contamination with RK31. The aberrant strain E207, latent in pea but occurring in high concentration, was never found to produce crystals (Bos et al., 1972; Bos and Rubio-Huertos, in preparation).

The crystals are evidently not composed of virus particles, as suggested by McWhorter (1965), but consist of uniform spherical or polyhedral particles about 11–12 nm in diameter. The main arguments are (1) the gradual transition between crystalline array and erratic distribution of clearly separate particles as in Fig. 5, (2) a similar picture at any angle of sectioning and a too great uniformity of these particles to represent cross sections of rods, and (3) the results of negative staining of isolated crystals as shown in Fig. 6. Such particles could also be observed directly in negatively stained extracts of diseased leaves. It is not known whether the arrangement in Fig. 5 is due to dispersion of particles caused by the preparation process, or is a step in the process of aggregation.

The three-dimensional crystals are more or less comparable to those found with a number of viruses of the potyvirus group and composed of non-infectious material, perhaps protein. With bean yellow mosaic virus their internal structure shows a periodicity of 7 nm, first thought to be indicative of a parallel array of elongate particles (Weintraub and Ragetli, 1966). Later by means of enzyme digestion these crystals were found to consist entirely or primarily of protein. There was no evidence that they contain nucleic acid (Weintraub and Ragetli, 1968). Other examples are the crystals with a striation periodicity of 11–13 nm caused by *Atropa* mild mosaic

virus (Harrison and Roberts, 1971) and by Sharka virus (van Bakel and van Oosten, 1972). However, especially those caused by the *Atropa* virus seem to be of different structure. They have been interpreted as composed of hexagonally arrayed tubules of unknown composition.

Further research is needed to elucidate the true nature of the RCVMV-induced crystals and their role in the infection process. Even if they are by-products of a virus-deranged host metabolism, their accumulation means a considerable drain of metabolites or building materials.

The anomalous membrane-bound structures sometimes found with RK31 have been found by de Zoeten et al. (1972) in pea with pea enation mosaic virus and once in non-infected material. In their discussion these authors also mention their observation in peas with RCVMV. They are either due to virus infection in general, or more probably, normal structures.

Samenvatting

Licht- en elektronenmicroscopie van nerfmozaïekvirus van rode klaver in erwt (Pisum sativum)

Epidermisreepjes van erwtebladstelen en -hoofdnerven en van jonge erwtestengels, geïnfecteerd met het RK31-isolaat van het nerfmozaïekvirus van rode klaver, bleken vele voor de diagnostiek waardevolle kristallijne insluitsels te bevatten (Fig. 1). Ze waren twee weken, maar met meer zekerheid drie weken na inoculatie waar te nemen. Ze konden gemakkelijk, zelfs bij zwakke vergroting, met de lichtmicroscoop worden aangetoond na kleuring met floxine en methyleenblauw, maar nog sneller met een fasecontrastmicroscoop in ongekleurde preparaten in water. Ze werden slechts gevonden nadat uitwendige ziekteverschijnselen begonnen te ontstaan. Met de P42-stam werden ze slechts een paar maal waargenomen en verontreiniging met het RK31-isolaat kan in die gevallen niet worden uitgesloten.

In ultradunne coupes, gemaakt 22 dagen na inoculatie of later, bleken de verdeling en de plaatselijke ophoping van het virus (Fig. 2) vergelijkbaar met die van andere virussen uit de carlavirusgroep (aardappelvirus-S-groep). Soms werden omvangrijke bundels virusdeeltjes gezien (Fig. 2B). Deze verschilden van de lichtmicroscopisch waar te nemen drie-dimensionele kristallen. Ultradunne coupes van deze laatste (Fig. 3) vertoonden bij sterke vergroting een regelmatige inwendige streping met een periodiciteit van ongeveer 11 nm (Fig. 4). Soms bestonden ze uit min of meer los gerangschikte bolletjes (Fig. 5). Na isolering van intacte kristallen en negatieve kleuring van een indooppreparaat daarvan werden behalve enkele virusdraden overwegend bolvormige of polyedrische deeltjes met een diameter van 11–12 nm gevonden (Fig. 6). De aard en betekenis van deze, als gevolg van infectie optredende kristallen zijn nog niet bekend.

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