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# CELL INCLUSIONS OF THE CUCUMBER GREEN MOTTLE MOSAIC VIRUS AND THE ODONTOGLOSSUM RINGSPOT VIRUS

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## Introduction

Both cucumber green mottle mosaic virus (CGMMV) and odontoglossum ringspot virus (ORSV) belong to the tobacco mosaic virus group which has been recently termed Tomoviruses (*to*bacco *mo*saic) by Harrison *et al.* (1969). This group is characterized by 300 mμ long rod-shaped virus particles, a high virus concentration in plant sap, a high thermal inactivation point, great infectivity, and serological relationship between members. Harrison *et al.* (1969) distinguish seven Tomoviruses.

Wittmann (1965) considers that Tomoviruses probably originated monophyletically and then gradually developed by a large number of small mutations. Today it is possible to establish by serological methods (Wetter, 1968) or amino acid exchanges in coat protein (Knight, 1963; van Regenmortel, 1967) that they are remotely related to each other.

We investigated the main types of crystalline inclusions induced by the Tomoviruses. This approach was justified by the following facts. As it is known, the common strain of the tobacco mosaic virus (TMV) builds cell inclusions in the form of hexagonal prisms, the structure of which was studied in detail by Iwanowski (1903), Steere and Williams (1953) and Wehrmeyer (1959). However, it was recently found that some Tomoviruses such as sunn hemp mosaic virus (SHMV) (Resconich, 1961) and Holmes' ribgrass virus (HRV) (Miličić, 1968) build rounded plates instead of prisms. The name of rounded plates is derived from their round, oval or irregular shape when viewed in flat position.

The hexagonal prisms are true virus crystals in which virus particles are densely arranged in layers and are always oriented perpendicularly to the hexagonal surface of crystals. In the rounded plates the virus particles are also arranged in layers and are oriented in the same direction (Resconich, 1961; Miličić, 1968).

In order to gather more information about the spread of these bodies in plants infected by various Tomoviruses we have studied the cell inclusions of CGMMV and ORSV. Vovk (1948) established that in cucumber cells infected with CGMMV many spindlelike crystals formed under the influence of 0,1 N HCl. However, the inclusions of CGMMV described in this paper were first noticed by Goldin (1954). This author defined them correctly as layered crystals which resemble thin plates. In this paper we could also indirectly confirm the observations of Brčák and Hršel (1961) that the CGMMV particles aggregate together laterally with ends aligned forming plate like inclusions.

## Material and Methods

The strain CV 4 of CGMMV (cp. Martyn, 1968) used in this work was obtained by courtesy of Dr. C. Wetter (Saarbrücken). This virus is remotely related to the common TMV and differs from it in 19 amino acid exchanges (van Regenmortel, 1967).

ORSV was sent to us by courtesy of Dr. L. Paul (Braunschweig). This virus was shown to be remotely related to the common TMV (Paul *et al.* 1968).

The investigations carried out by a light microscope on living material enabled us quickly to examine many plants grown under various conditions of light intensity and temperature and in various seasons. The drawings presented in Fig. 1 and 2 were made with a camera lucida.

## Results

### *Inclusions of the Cucumber Green Mottle Mosaic Virus*

We studied the inclusions of CGMMV in many cultivars of the cucumber. Most sections were paradermal along the main veins on the underside of infected leaves. The inclusion have the form of thin plates immersed in the parietal cytoplams and are oriented with their flat and rounded surface against the cell wall. When the rounded plates are placed next to the outer or inner cell wall they are viewed flat. In this position their outlines are sometimes irregular (Fig. 1). By moving in cytoplasm they change their position and can come next to the lateral cell walls. When they are viewed edgeways they are often very thin. On the basis of the change of their aspect it can be established that they are built either of one or more disks of various size. When they consist of only one disk they are translucent and can hardly be observed. This is probably the reason why the rounded plates of CGMMV were discovered so late. If they consist of more disks they are often striated in side view and show a layered structure with concentric or eccentric lines in flat view. Their form corresponds well with that of rounded plates of SHMV (Resconich, 1961) and HRV (Miličić *et al.*, 1968).

As the rounded plates were never found in healthy control plants they are undoubtedly virus inclusions. Their chemical character was examined with several reagents. They turned red with Millon's reagent and yellow with concentrated nitric acid (xanthoprotein reaction). These reactions demonstrate that the plates contain protein.

After treatment with diluted HCl a great number of spindlelike crystals arise in the cells of cucumber (Fig. 4 A). This reaction found by V o v k (1948) is very specific and it can be used for a rapid detection of CGMMV. By means of polarized light it was established that the $n\gamma$ of spindles lies in direction of the length of these inclusions.

The inclusions can easily be found in infected plants during all seasons except in hot summer months when they are rather scarce. Only in one case spindle-shaped inclusions were observed in the cells instead of rounded plates.

### *Inclusions of the Odontoglossum Ringspot Virus*

According to P a u l *et al.* (1965) ORSV can be transmitted systemically in *Nicotiana clevelandii*. This plant is very appropriate for studying the inclusions of this virus. We investigated them in elongated epidermal cells along veins (Fig. 2), in tabular epidermal cells with undulate outlines which are placed between veins (Fig. 3 E) and in hair cells. In all these cells we often found rounded plates which were slightly thicker and consisted of more disks than the plates of CGMMV. They were sometimes elongated in the direction of cell length (Fig. 2 C, 3 D) and sometimes their shape was very complicated (Fig. 2 E, 3 E). Fig. 3 C shows us a plate in side view which has more disks in its upper part than in the lower one. Like the plates of CGMMV the plates of ORSV often have a round form with concentric (Fig. 2 D) or eccentric lines (Fig. 2 B). The whole plate or just some of its disks sometimes show the tendency to attain a hexagonal form instead of the round one (Fig. 2 D; upper plate).

The rounded plates of ORSV turn red with Millon's reagent and yellow with concentrated $HNO_3$. They become brown with aqueous solution of iodine and potassium iodine. Thus, this type of inclusions contains protein.

The rounded plates of ORSV are also very stable. Although we observed them in various seasons and under various external conditions we could never notice any important change in the structure of plates or the appearance of other forms of inclusions.

### *Orientation of Virus Particles within Rounded Plates*

The rounded plates of CGMMV and ORSV shine between crossed polarization prisms in side view (Fig. 4 D) but do not shine in face view. This birefringence points out that the virus particles in crystals are arranged regularly. Also we noted that the $n\gamma$, i. e. the big refractive index (see S i t t e, 1965, p. 36), is oriented perpendicularly to the rounded surface of the plates. These optical properties of the rounded plates of CGMMV and ORSV correspond exactly to these of the rounded plates of HRV M i l i č i č *et al.* 1968) or the hexagonal prisms of TMV (Wehr-

Fig. 1. Epidermal cells of *Cucumis sativus*. Rounded plates of CGMMV consisting of one to three disks in face view.

Sl. 1. Epidermske stanice krastavca. Okruglaste pločice virusa zelenog pjegavog mozaika krastavca, koje se sastoje od jednog do tri pločasta dijela, vide se u plošnom položaju.

Fig. 2. Epidermal cells of *Nicotiana clevelandii* affected by ORSV contain polylayered rounded plates. One disk of the upper plate in D is nearly hexagonal.

Sl. 2. Epidermske stanice vrste *N. clevelandii* inficirane virusom prstenaste pjegavosti odontoglosuma. U stanicama se nalaze višeslojne okruglaste pločice. Jedan pločasti dio gornje okruglaste pločice na D gotovo je heksagonalan.

Fig. 3. Epidermal cells of cucumber infected with CGMMV (A, B); epidermal (D, E) and hair cells (C) of *N. clevelandii* infected with ORSV. Rounded plates, in face view except in C where they are viewed edgeways.

Sl. 3. Epidermske stanice krastavca inficirane virusom zelenog pjegavog mozaika krastavca (A, B); epidermske (D, E) i dlačne stanice (C) vrste *N. clevelandii* inficirane virusom prstenaste pjegavosti odontoglosuma. Okruglaste pločice u plošnom položaju osim na C gdje se vide sa strane.

Fig. 4. (A) Epidermal cells of cucumber infected with CGMMV. A great number of spindle shaped paracrystals appear after treatment with 0.1 N HCl. (B—D) Hair cells of *N. clevelandii* affected by ORSV. Next to the left cell walls rounded plates in side view. The plate in (B) shows striations derived from the desintegration of the plate in a great number of needle crystals after treatment with 0.1 N HCl. The needles have remained in the boundaries of the plate. (C, D) The same plate in ordinary (C) and in polarized light (D); the latter shines between crossed prisms.

Sl. 4. (A) Epidermske stanice krastavca inficirane virusom zelenog pjegavog mozaika krastavca. Velik broj vretenastih parakristala nastaje poslije obrađivanja sa 0.1 N HCl. (B—D) Dlačne stanice vrste *N. clevelandii* inficirane s virusom prstenaste pjegavosti odontoglosuma. Blizu lijeve stanične stijenke okruglaste pločice u bočnom položaju .Pločica na B pokazuje poprečno pruganje koje je nastalo zbog raspadanja pločice u velik broj igličastih kristala poslije obrađivanja sa 0.1 N HCl .Igličasti kristali zadržali su se na mjestu gdje se prije toga nalazila pločica. (C. D) Ista pločica u običnom (C) i u polariziranom svjetlu (D); posljednja svijetli među unakrštenim prizmama.

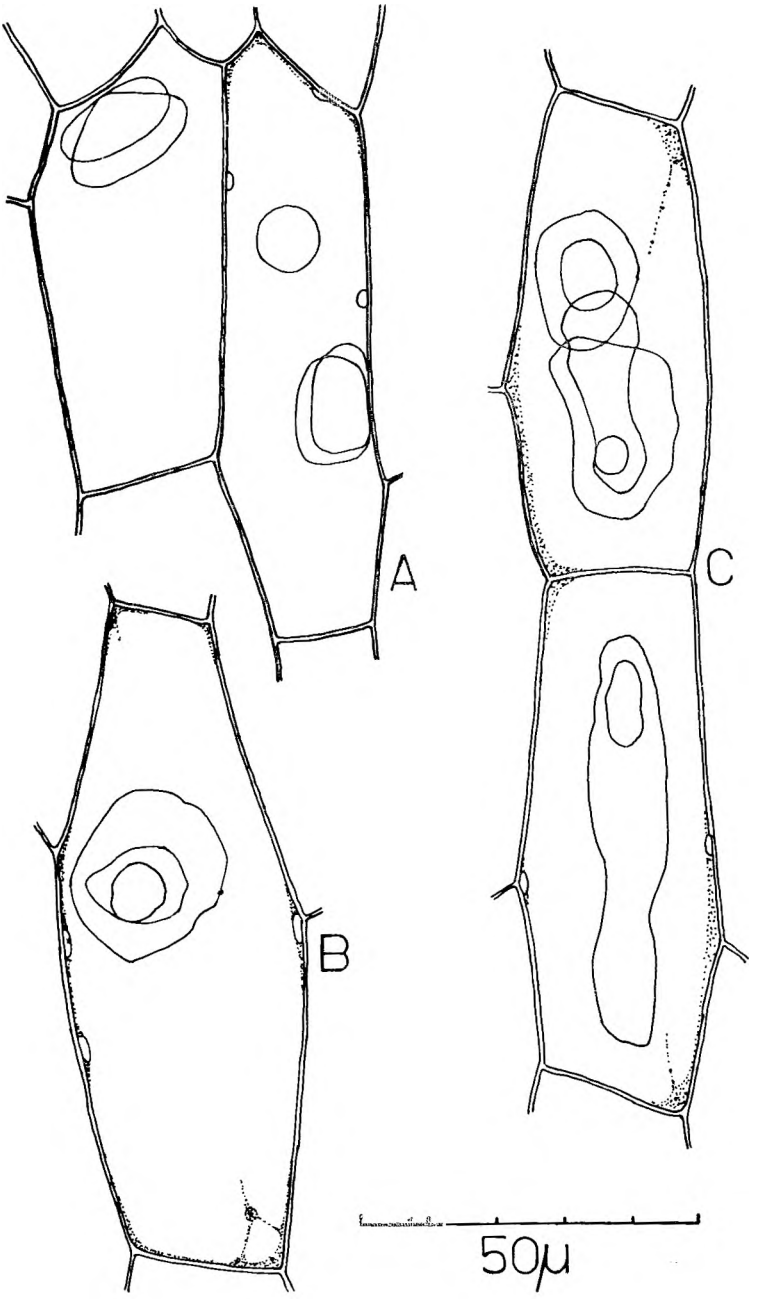


Fig. 1. — Sl. 1.

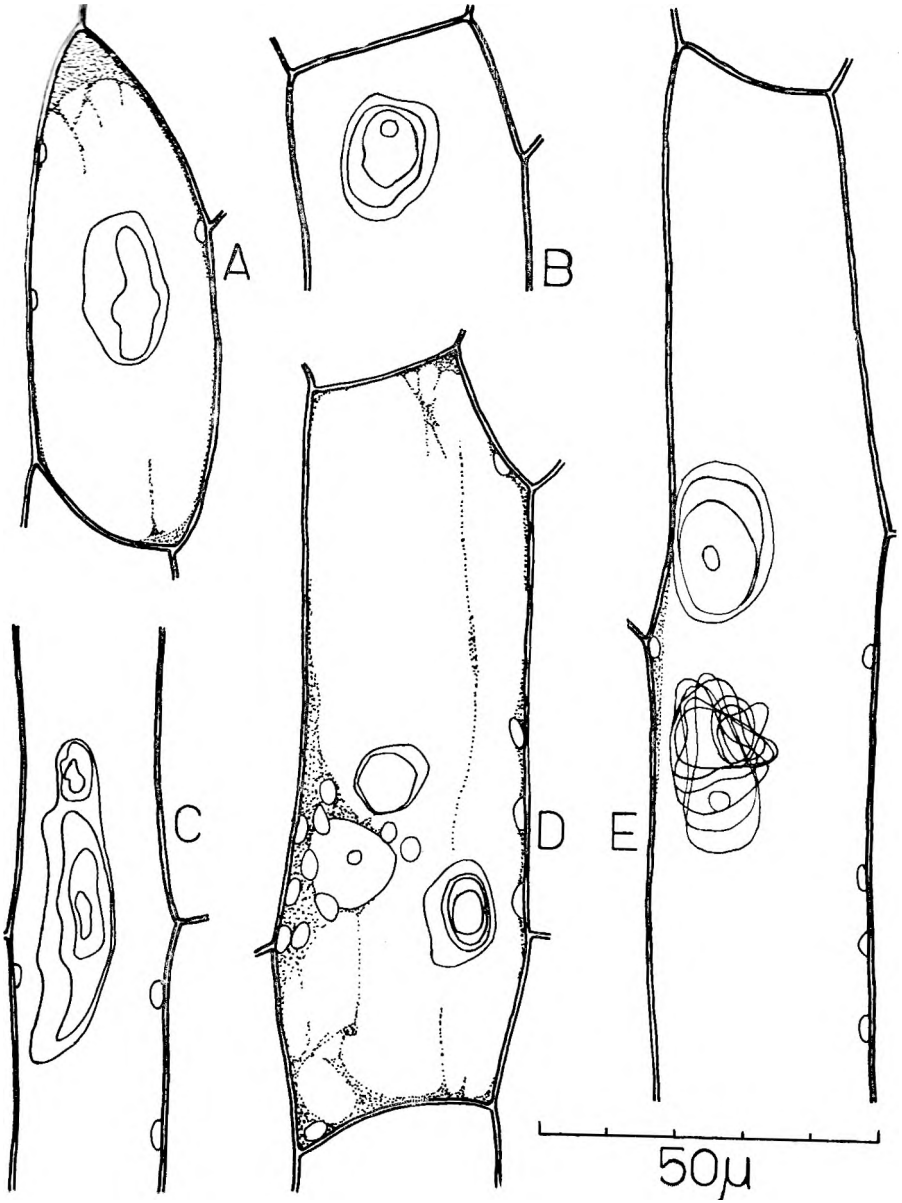


Fig. 2. — Sl. 2.

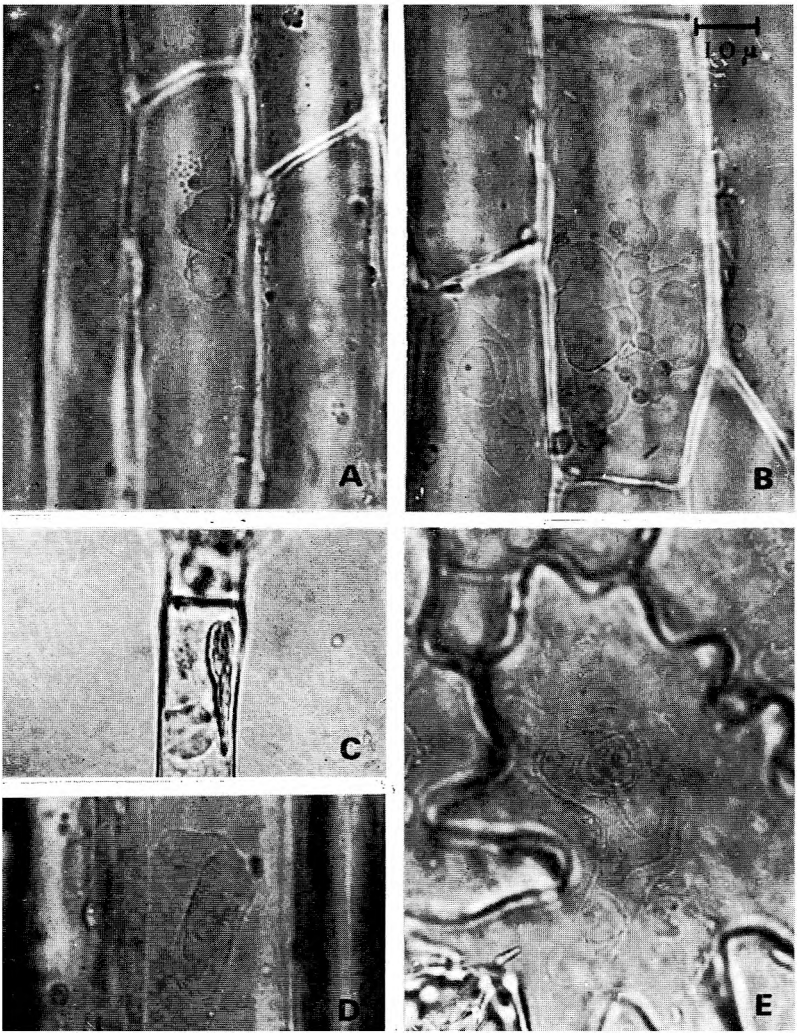


Fig. 3. — Sl. 3.

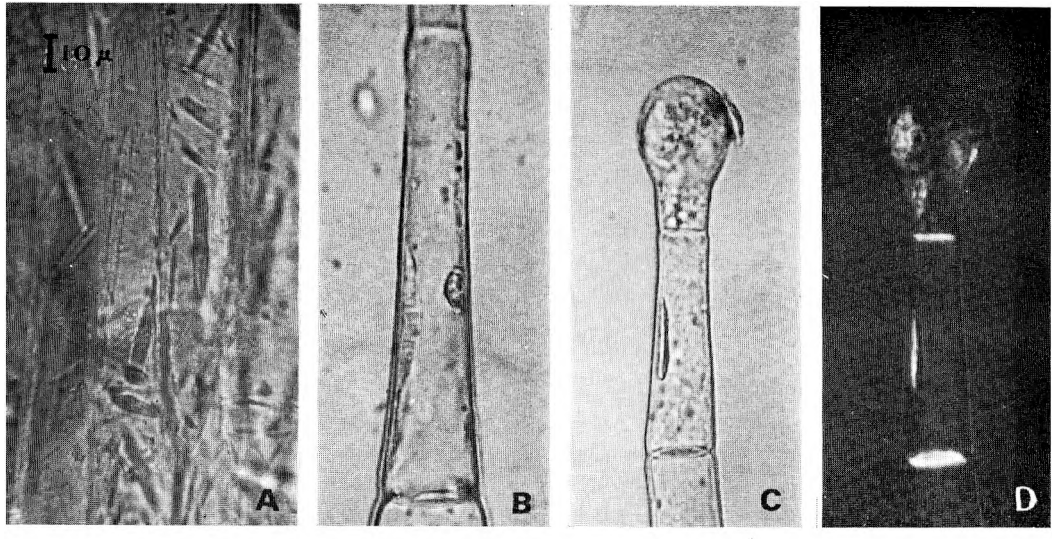


Fig. 4. — Sl. 4.

meyer, 1959). Conformity in optical properties also implies similarity in fine structure. As the nγ of inclusions of HRV and TMV lies in the same direction as their virus particles, the virus particles in the inclusions of CGMMV and ORSV are also likely to be oriented in the direction of nγ.

To establish whether the virus particles are really oriented perpendicularly to the flat surface they were treated with diluted HCl or with saturated aqueous solution of picric acid (cp. Purdy Beale, 1937; Resconich, 1961). After treatment the striations of rounded plates disappeared and the typical virus needle crystals took rise. These needles were first produced within the boundaries of original rounded plates (Fig. 4 B; cp. Warmke and Edwardson, 1966, p. 51). They were packed at right angles to the positions of the original striations and to the rounded surface of inclusions. It seems therefore that the virus particles are oriented in these inclusions in the same direction as in the rounded plates of SHMV and HRV, and in hexagonal prisms of TMV.

This conclusion is further supported by the investigations of Brčák and Hršel (1961) who have studied the inclusions of CGMMV in cucumber. They have noted that the virus particles in monolayered plates are oriented perpendicularly to the surface of inclusions.

## Discussion

CGMMV, ORSV and some other Tomoviruses form very similar inclusions called rounded plates. The plates have to a certain extent the same fine structure as the hexagonal prisms which are characteristic inclusions for the TMV (Iwanowski, 1903; Wehrmeyer, 1957; Juretić, 1969) and for the tomato mosaic virus (ToMV) (Brčák, written communication; Juretić, 1971). These two types of crystalline bodies are the main inclusions of Tomoviruses. Their distribution in the group is presented in Table 1.

Table 1. — Distribution of inclusions among Tomoviruses

| Virus | Inclusion |
|---|---|
| Tobacco mosaic | Hexagonal prisms |
| Tomato mosaic | Hexagonal prisms |
| Holmes' ribgrass | Rounded plates |
| Sunn hemp mosaic | Rounded plates |
| Cucumber green mottle mosaic | Rounded plates and intermediate forms between plates and prisms |
| Odontoglossum ringspot | Rounded plates and intermediate forms between plates and prisms |

The number of strains of these six Tomoviruses, inclusions of which have been till now investigated, is relatively small. Therefore it is necessary to continue these examinations because it is not excluded that other strains react differently from the type strain and produce other forms of inclusions. By these investigations it is also necessary to take care that the examined strains are in pure culture. So for instance in tobacco plants which were infected with a strain of the common TMV

and slightly contaminated with another Tomovirus, rounded plates were found (Juretić, written comunication). On basis of this finding we can not conclude that this strain produces rounded plates because the contaminating virus could also influence the form of inclusions.

The peculiar structure of rounded plates, the monolayers of which have a rounded form instead of a hexagonal form, is probably due to the difficulties in lateral aggregation of virus particles. On account of that their layers do not reach a hexagonal form but a round or irregular form.

However, it is interesting to know which types of inclusions are frequent in cells infected by some other Tomoviruses. Chessin *et al.* (1967) have recently examined the inclusions of *Lychnis* virus, which is related to the HRV. This virus does not cause the appearance of hexagonal prisms but builds so called "gray plates" which are a young form of inclusions. According to Bald and Solberg (1961) the gray plates are mostly monolayered virus bodies from which crystalline inclusions develop. The rounded plates differ from gray plates because they are completely developed inclusions, they are mostly multilayered and have a greater diameter. It would be interesting to know which type of inclusions, if any, is present in fullgrown leaves infected with *Lychnis* virus for a long time.

According to Chessin *et al.* (1967) neither the strain U 2 nor the strain U 5 is able to produce hexagonal prisms (Solberg and Bald, 1962, p. 155). The U 2 is closely related to G-TAMV and the para-tobacco mosaic virus (Wittmann, 1965) so that it differs from G-TAMV only in one amino acid pair (van Regenmortel, 1967). Bald and Solberg (1961) explain the inability of U 2 and U 5 to aggregate into hexagonal crystals by the weakness of the end-to-end binding of virus particles; the binding is too weak to hold a number of monolayers together. Instead of hexagonal crystals U 2 and U 5 later build acicular aggregates (Chessin *et al.*, 1967).

## Summary

Rounded plates are a widely spread type of crystalline inclusions of the cucumber green mottle mosaic virus (CGMMV) and the ondotoglossum ringspot virus (ORSV). They appear a few days after infection and persist for quite a long time. Inclusions are built of one or more rounded disks of different size which are stacked one above the other. This structure can be established by examining the inclusions in side and in face view in a light microscope. Observations in polarized light and treatments with acids exhibit that the virus particles are oriented perpendicularly to the flat surface of the plates.

Inclusions similar in appearance to the hexagonal prisms of the tobacco mosaic virus (TMV) or the tomato mosaic virus (ToMV) also develop in cells infected with CGMMV and ORSV but they are rather thin and usually do not have a typical hexagonal shape. They are connected with rounded plates by a number of intermediate forms.

The rounded plates are very frequent inclusions in the group of Tomoviruses (= the group of the tobacco mosaic virus) because they also occur in the cells infected by the sunn hemp mosaic virus (SHMV; syn. bean form of TMV) and Holmes' ribgrass virus (HRV).

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## SADRŽAJ

### STANIČNE INKLUZIJE VIRUSA ZELENOG PJEGAVOG MOZAIKA KRASTAVCA I VIRUSA PRSTENASTE PJEGAVOSTI ODONTOGLOSUMA

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Okruglaste pločice vrlo su raširen tip kristaličnih inkluzija u stanicama inficiranim virusom zelenog pjegavog mozaika krastavca (VZPMK) i virusom prstenaste pjegavosti odontoglosuma (VPPO). One se pojavljuju nekoliko dana poslije infekcije i zadrže se dosta dugo u stanicama. Inkluzije su izgrađene od jednog ili više pločastih dijelova različite veličine koji su postavljeni jedan iznad drugoga. Da okruglaste pločice imaju ovu strukturu, moglo se ustanoviti nakon istraživanja inkluzija u bočnom i plošnom položaju s pomoću svjetlosnog mikroskopa. Promatranja u polariziranom svjetlu i obrađivanja kiselinama, pokazala su da su virusne čestice u inkluzijama orijentirane okomito na plosnatu površinu pločica.

Inkluzije, koje su po izgledu slične heksagonalnim prizmama virusa mozaika duhana ili virusa mozaika rajčice, također se razvijaju u stanicama inficiranim sa VZPMK i VPPO, ali su prilično tanke i obično nemaju tipični heksagonalni oblik. One su povezane s okruglastim pločicama nizom prijelaznih oblika.

Okruglaste pločice vrlo su česte inkluzije u skupini tomovirusa (tobacco mosaic group) budući da nastaju i u stanicama inficiranim virusom mozaika krotalarije (sunn hemp mosaic virus) i virusom mozaika trpuca (Holmes' ribgrass virus).

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