Light and Electron Microscopy of the Intracellular Inclusions of Cauliflower Mosaic Virus

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SUMMARY

The development and constitution of the inclusion bodies induced by Italian and Californian isolates of cauliflower mosaic virus was studied by light and electron microscopy. Cytochemical and enzyme-digestion tests revealed that the inclusion bodies are essentially proteinaceous, and contain RNA and some DNA. These inclusions have a matrix composed of densely packed, finely granular or fibrillar material and vacuole-like areas not bounded by a membrane. Virus particles are interspersed at random with the matrix and are not aggregated in regular crystalline arrays. The developmental sequence of the inclusions suggests that they are not merely structures for virus accumulation but, rather, that they are sites of virus synthesis and/or assembly.

INTRODUCTION

Cauliflower mosaic virus (CIMV) and the serologically related dahlia mosaic (Brunt, 1966) and carnation etched ring 50 (Hollings & Stone, 1969) viruses, all have isometric particles of the unusual diameter of about 50 nm. In cells these particles appear to be intimately associated with conspicuous cytoplasmic inclusion bodies whose ultrastructural organization has been recently studied in some detail (Kitajima, Oliviera & Costa, 1965; Kitajima, Lauritis & Swift, 1969; Fujisawa et al. 1967; Petzold, 1968; Rubio-Huertos et al. 1968; Moreno, Castro & Rubio-Huertos, 1969). The interpretations of the various authors relative to the formation, significance and nature of these bodies are, however, not always in complete agreement. Investigations with the light and electron microscopes were therefore undertaken to study primarily the constitution and the development of the inclusions.

METHODS

Viruses and hosts. Three isolates of CIMV originating respectively from California, Central and Southern Italy were used. The viruses were cultured in Brassica rapa L. and B. perviridis Bailey plants grown in a glasshouse at 20 to 24° and were periodically transferred from plant to plant by mechanical inoculation of sap.

Light microscopy. Epidermal strips were removed with forceps from the underside of midribs and petioles of leaves with severe symptoms of infection. The samples were dipped for 5 to 10 min. in cold acetone or were stained immediately without fixation, mounted in water and viewed under a Zeiss Photomicroscope II. Some strips were observed without previous staining. Photographs were taken using interference contrast. Cytochemical staining was done with (i) 1 % (w/v) phloxine dissolved in water (Rubio-Huertos, 1950) or
alcohol-cellosolve solution (Bos, 1969) for showing the presence of the inclusion bodies; (ii) mercuric bromophenol blue (Mazia, Brewer & Alfert, 1953) for total protein; (iii) methyl green-pyronin (Jordan & Baker, 1955; Robb, 1964) for RNA and DNA; (iv) Feulgen for DNA.

**Enzyme digestion tests.** Epidermal strips as above were fixed for 1 to 4 hr in glutaraldehyde-paraformaldehyde (Karnovsky, 1965) in the cold (4°), washed free of fixative and incubated for 1, 2, 3, 4 and 6 hr at 38° in a protease (type V, Sigma Chemical Co) solution containing 1 mg./ml. enzyme in tris+HCl buffer 0.05 M, pH 7.5. Controls were incubated in the same buffer without enzyme. After treatment the samples were thoroughly washed with buffer and stained with mercuric bromophenol blue or methyl green-pyronin for light microscopy or they were post-fixed with osmium tetroxide and processed for thin sectioning as specified below.

**Electron microscopy.** Small fragments of *B. rapa* leaf tissue were excised from the yellowish local lesions induced by CIMV as soon as these became evident (9 to 13 days after inoculation) and from leaves of *B. rapa* and *B. perviridis* with severe systemic symptoms (3 to 6 weeks after inoculation).

The samples were fixed in paraformaldehyde-glutaraldehyde in cacodylate or phosphate buffer 0.1 M, pH 7.2 (Karnovsky, 1965) at room temperature. They were postfixed in 1 to 2 % (w/v) osmium tetroxide in s-collidine buffer pH 7.4 and dehydrated through graded ethanol dilutions with two final passages in propylene oxide. A mixture of Epon-Araldite was used for embedding (Mollenhauer, 1964).

Thin sections were cut with glass knives and stained with uranyl acetate and lead citrate.

**RESULTS**

**Light microscopy of inclusion bodies**

Inclusion bodies were present in epidermal strips of infected but not of healthy plants. Under the light microscope, using interference contrast optics and without staining, the inclusions were visible as variously shaped refringent masses (Fig. 1 b). Under the same conditions, the nuclei were barely discernible, and the cells from healthy leaves appeared 'empty' (Fig. 1 a).

The inclusion bodies were more easily seen when treated with phloxine (Fig. 1 c). They appeared as structures of varying dimensions (up to 15 to 18 µm.), sometimes exceeding the size of the nucleus, differing in shape from broadly elongated to globose rounded. Usually, one body was present per cell, and often it was near or apposed to the nucleus.

The staining reactions were as follows: (i) with mercuric bromophenol blue the inclusion bodies stained deep blue (Fig. 1 e, f) indicating the presence of substantial amounts of protein. The nuclei, and particularly their nucleoplasm, became pale blue (Fig. 1 d to f); (ii) treatment with methyl green-pyronin produced a definite red coloration of the bodies with occasional faint shades of blue, suggesting presence of RNA and, possibly, of some DNA. As expected, the nucleoplasm became blue and the nucleolii bright red—the reaction of DNA and RNA, respectively; (iii) Feulgen staining was evident in the nuclei but uncertain in the inclusion bodies. In some preparations the bodies assumed a pale shade of purple, again indicative of the possible occurrence of DNA; (iv) after exposure to protease for 1 hr, the inclusion bodies were barely visible when stained with mercuric bromophenol blue, but became evident, although showing unmistakable signs of digestion, when treated with methyl green-pyronin (Fig. 1 h). The nuclei were also much affected by the enzyme and only the nucleoli withstood the treatment well (Fig. 1 h). The bodies were completely
Inclusion bodies of CIMV

Fig. 1. Light microscope pictures of healthy and infected epidermal cells of *Brassica rapa*. All photographs are taken using interference contrast. (a) Healthy unstained cells; (b) infected unstained cells with two inclusion bodies; (c) a large inclusion body adjacent to a nucleus, stained with phloxine; (d) healthy cells stained with mercuric bromophenol blue; (e) and (f) inclusion bodies in infected cells heavily stained by mercuric bromophenol blue; (g) infected cells kept for 4 hr in tris + HCl buffer and stained with methyl green-pyronin. A nucleus and the adjacent inclusion body are clearly discernible; (h) after 1 hr incubation with protease the nucleus is almost totally digested and the inclusion body is also strongly attacked; (j) complete digestion of cell contents after 4 hr incubation with enzyme; (h) and (j) stained with methyl green-pyronin. *lb* = inclusion bodies; *N* = nucleus.
digested within 2 hr. After 4 hr, the treated cells appeared empty (Fig. 1j) and were no longer stained, while the controls retained their normal appearance (Fig. 1g).

**Electron microscopy**

**Local lesions.** In plant cells within local lesions, the inclusions existed primarily as small, irregularly shaped bodies, some scattered in the cytoplasm but most near the nucleus. These structures consisted of an electron-dense finely granular or fibrillar matrix surrounded by rows and/or clusters of rounded particles 15 to 18 nm. in diameter, indistinguishable from normal cytoplasmic ribosomes (Fig. 2). Larger inclusions containing groups of virus particles, as described below, were also occasionally present. A few individual virus particles, scattered at random and apparently unconnected with the inclusions, could also be seen. These solitary particles occurred also in cells of systemically invaded tissues.

**Systemic infections.** In systemically infected cells, the inclusions appeared as large electron-opaque cytoplasmic structures not bounded by a membrane, often located near the nucleus. They were irregularly shaped or elongated in *B. rapa*, and more or less rounded or with circular profile in *B. perviridis* (Fig. 4). Their basic matrix was irregularly punctuated by vacuole-like areas not bounded by a membrane. CIMV-like particles, appearing as heavily stained rounded or doughnut-shaped bodies about 45 nm. in diameter, were interspersed with the ground substance or were located in the less electron-dense areas (Fig. 3, 4).
The virus particles seemed to vary greatly in number from one inclusion to another. They never formed regular crystalline arrays even when many particles occurred in contact (Fig. 3).

Fig. 4. Cells of infected Brassica perviridis with two large rounded inclusion bodies containing virus particles. $W =$ cell wall; $I_b =$ inclusion bodies.
The large inclusions were surrounded by ribosome-like particles which, however, were less evident than in the smaller ones. Some of them contained or were surrounded by fine fibrils similar to those visible in the nucleoplasm (Fig. 3). These fibrils resembled nucleic acid strands as seen in thin sections.

Fig. 5. (a) Nucleus of infected cell kept for 3 hr in tris + HCl buffer. Although some extraction of material might have occurred, the organelle appears essentially normal; (b) an inclusion body after 1 hr exposure to protease; (c) and (d) remnants of inclusion bodies after 3 hr incubation with enzyme. Only a few particles, some of which are partially digested, are visible. The chloroplasts and nucleus seem empty. Arrows point to nuclear envelope. \( \text{Ib} = \) inclusion bodies; \( \text{ch} = \) chloroplast; \( N = \) nucleus.
No difference was found in the structure, organization and general appearance of the inclusion bodies produced by the three CIMV isolates studied.

After 1 hr exposure to protease, the inclusion bodies were severely damaged and much of the ground substance had disappeared. Many clustered virus particles were still visible (Fig. 5b). The effects of the enzyme were more evident after 3 hr (Fig. 5c, d) when the inclusions were totally digested. At this stage only a few, more-or-less intact virus particles could be identified and most of the organelles were digested (Fig. 5c, d) and could scarcely be recognized.

**DISCUSSION**

Some of our findings on the intracellular appearance and composition of the inclusion bodies confirm the conclusions of other workers. In particular, the evidence provided by cytochemical and enzyme digestion tests that these inclusions are essentially proteinaceous and contain RNA and some DNA, conforms with reports on dahlia mosaic virus (DMV) and CIMV (Robb, 1964; Mamula & Milicic, 1968; Kamei, Rubio-Huertos & Matsui, 1969; Kitajima *et al.* 1969).

Kitajima *et al.* (1969) listed a few ‘subtle differences’ between the inclusions of CIMV and DMV: the circular profile of DMV inclusions contrasted with the elongated or irregular contours of those of CIMV, and there was no association of ribosomes and Golgi structures with CIMV inclusions. Our findings and those by Mamula & Milicic (1968) indicate that the first difference is difficult to maintain because CIMV induces inclusions of different shapes, some spherical, in different plants. Thus, the host, rather than the virus, may have a bearing in determining the shape of the inclusion body. Conceivably, the inclusion bodies tend to grow preferentially in one direction (i.e. parallel to the cell wall), thus becoming elongated, in mesophyll cells of plants like *B. rapa* where a large central vacuole confines the cytoplasm to a narrow parietal strip.

We also found ribosomes around CIMV-induced inclusions, so the only remaining difference from DMV lies in the lack of association of dictyosomes with inclusion bodies. Indeed, this difference is consistent and unlikely to depend on the host species. This association was recorded in all the plants in which DMV was studied (Petzold, 1968; Kitajima *et al.* 1969), but in none of those used by us and others (Fujisawa *et al.* 1967; Rubio-Huertos *et al.* 1968; Kamei, Goto & Matsui, 1969) to study CIMV.

Golgi structures are thought to be involved in the formation of DMV inclusions (Petzold, 1968; Kitajima *et al.* 1969). The development of these inclusions was described in detail by Petzold (1968) who postulated that they begin as accumulations of ribosomes which form small electron-opaque units, fuse together with strands of endoplasmic reticulum, and form larger bodies where virus particles are produced. Kitajima *et al.* (1969) reported similar findings and emphasized the importance of ribosomes and dictyosomes in the possible genesis of the inclusions.

We have observed a similar developmental sequence with CIMV-induced inclusions, although without the apparent involvement of Golgi structures. This is in contrast with the interpretation of Rubio-Huertos *et al.* (1968) who suggest an opposite sequence: i.e. virus particles are synthesized first, independently of the matrix, which later accumulates around them to form the inclusion body. If this were true, many more free virus particles than were found should have occurred in the cytoplasm, both in early stages of infection (e.g. local lesion) and later, during active growth of the inclusions. A more plausible explanation is that CIMV inclusion bodies are not merely structures formed by virus accumulation, but sites of virus synthesis and/or assembly. This hypothesis, already put
forward for DMV (Petzold, 1968; Kitajima et al. 1969), seems compatible with the chemical constitution of the bodies, which possess the material (RNA) needed for accomplishing an important step of virus synthesis (coat-protein production).

If the inclusions are virus factories, the commonly found doughnut-shaped particles may be either incomplete virus particles (Fujisawa et al. 1967) or fully formed particles cut to show a hollow centre (Kitajima et al. 1969).

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REFERENCES


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