Ultrastructure of Tobacco Mesophyll Protoplasts Inoculated with Cucumber Mosaic Virus

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ABSTRACT

Mesophyll protoplasts isolated from tobacco plants were inoculated in vitro with cucumber mosaic virus and were examined by thin sectioning at intervals after inoculation. Inoculum virus particles entered the protoplasts by a pinocytic process. Progeny virus particles were readily detectable 24 hr after inoculation as aggregates and were associated with the plasmalemma, tonoplast, and nucleolus, or were present in the cytoplasm. No virus particles were found in chloroplasts, mitochondria, or central vacuole. In the nuclei containing CMV particles, heterochromatin was not seen and the nucleolus was sometimes vacuolated. No other degenerative changes were found in the protoplasts until 48 hr after inoculation.

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Plant virus study using tissue systems suffers from many technical difficulties which arise from low frequency of primary infection and asynchronous virus multiplication. These difficulties can be largely eliminated by using mesophyll protoplasts, a newly developed experimental system of free naked leaf cells (12). It has been shown that the mesophyll protoplasts of tobacco can be inoculated in suspension with tobacco mosaic virus (TMV) (11, 13), and synchronous virus multiplication in the majority of protoplasts was confirmed by electron microscopy (10).

Recently, the tobacco mesophyll protoplast system was successfully inoculated with cucumber mosaic virus (CMV) (9), a spherical plant virus of which little is known about the ultrastructure of infection (4, 5, 7). In the present work, we exploited the advantages of protoplast system for studying the fine structure of CMV infection and multiplication in tobacco leaf cells.

MATERIALS AND METHODS.—Isolation of protoplasts from *Nicotiana tabacum* L. 'Xanthi', their inoculation with CMV-Y, a yellows strain, and the incubation of inoculated protoplasts were carried out as described in a previous paper (9). Staining with fluorescent CMV antibody (9) showed that 70-93% of protoplasts were infected in the experiments of this study. Protoplasts sampled at intervals after inoculation were processed for electron microscopy. Protoplasts suspended in 2 ml of 0.7 M mannitol solution were fixed for 1 hr by adding 2 ml of 12% glutaraldehyde solution in 0.01 M phosphate buffer (10). They were postfixed for 2 hr with 2% osmium tetroxide (10). Noninoculated protoplasts incubated under the same conditions were also examined for comparison.

RESULTS.—*Inoculum virus.*—For inoculation with CMV, protoplasts were mixed into the virus suspension for 15 min (9). Various stages of virus penetration into protoplasts could be observed in sections of the protoplasts fixed immediately after 15 min contact with the inoculum virus. The inoculum particles were adsorbed onto the plasmalemma singly (Fig. 1) or in aggregates (Fig. 2). Invaginated plasmalemmae with adsorbed virus particles (Fig. 2, 3) and intracytoplasmic vesicles containing virus (Fig. 4) were also seen. Serial sections showed that the structures like that shown in Fig. 4 are closed vesicles and are not an extension of the plasmalemma invagination. The structures illustrated in Fig. 1-4 were not seen in noninoculated protoplasts; intracytoplasmic vesicles were present also in healthy protoplasts, but they did not contain virus-like particles.

Protoplasts fixed 40 min after inoculation showed profiles in section which were similar to those of noninoculated protoplasts; no virus particles were thus seen at the plasmalemma surface or within protoplasts. By that time the virus particles which had entered the protoplasts were apparently transformed into vegetative forms which were not detectable by electron microscopy.

Progeny virus.—Protoplasts incubated for 24 and 48 hr after inoculation with CMV contained a number of progeny virus aggregates. At lower magnifications, these aggregates appeared as dark areas of various size and were present in association with the plasmalemma (Fig. 5), the tonoplast (Fig. 5, 6), and (less frequently) free in the cytoplasm (Fig. 6). They were also very frequently found in the nucleolar region of nuclei (Fig. 7). At higher magnifications, the aggregates consisted of uniformly electron-dense particles approximately 25 nm in diameter (Fig. 8). The virus aggregates in sections corresponded in number, size, and distribution to the masses of virus antigen which were visualized by staining with fluorescent CMV antibody. In reasonable agreement with the results of fluorescent antibody staining, the virus aggregates were present in 60-80% of the sectioned protoplasts. There is no doubt that the particles in these aggregates are newly formed CMV, since they far exceed in number the inoculum particles which entered the protoplasts. A measurable amount of increased infectivity developed in the protoplasts with the formation of these aggregates (9).

Progeny CMV particles were arranged in the aggregates more or less randomly (Fig. 5-8). Sections with a tangentially cut profile of the plasmalemma showed. however, a regular array of particles, suggesting that the particles directly attached to the plasmalemma are aligned in an orderly fashion. Virus particles in a chain were frequently encountered near the central vacuole (Fig. 5, 9). In addition, doughnut-like particles arranged in a hexagonal array were often found in the same region (Fig. 10). These structures presumably represent perpendicularly (Fig. 5, 9) and tangentially (Fig. 10) cut

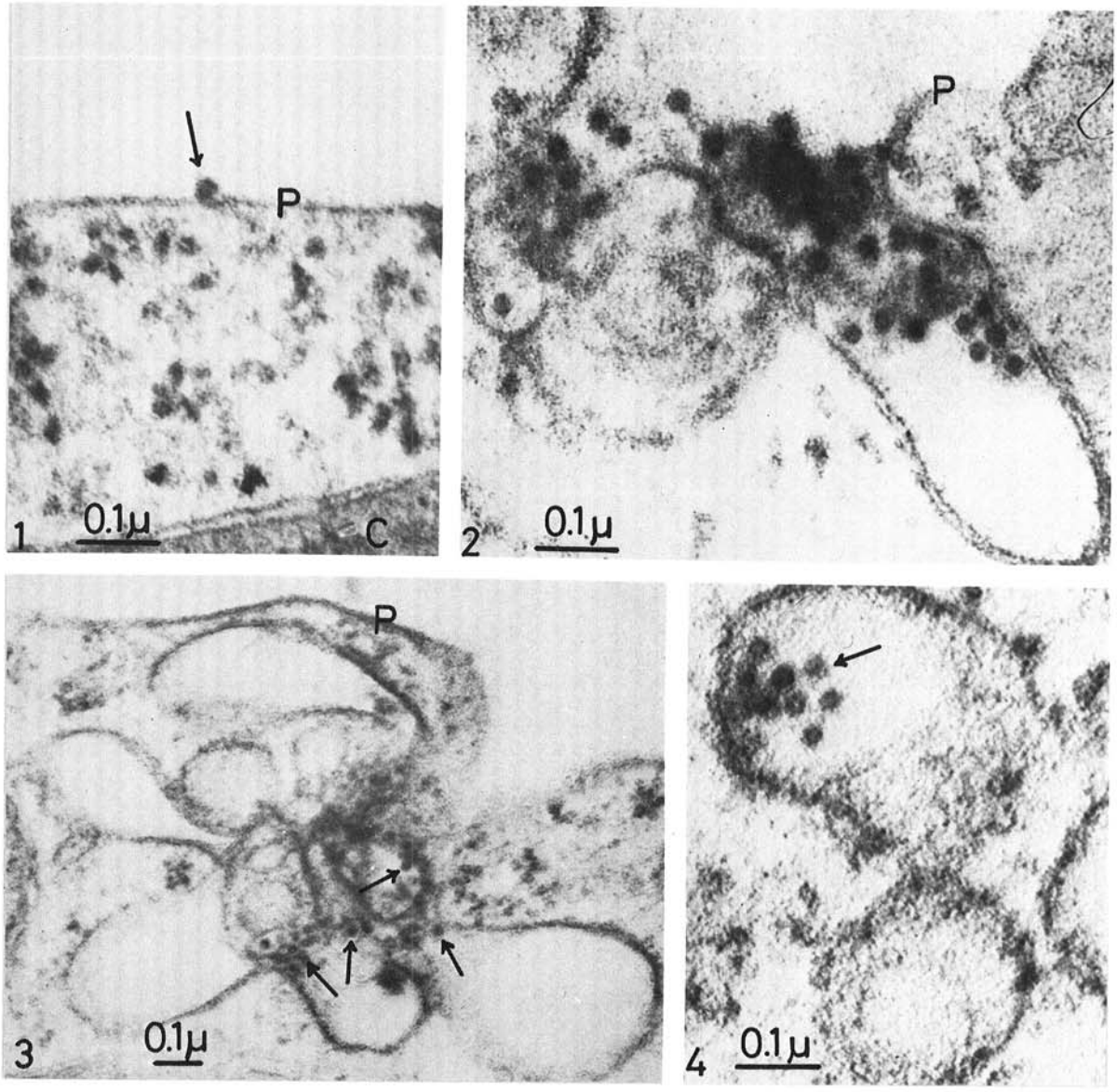

Fig. 1-4. Inoculum CMV particles associated with protoplasts. **1)** A CMV particle (arrow) adsorbed to the plasmalemma (× 120,000). **2)** Pinocytic invagination of the plasmalemma (× 120,000). **3)** Plasmalemma at a later stage of pinocytic invagination. CMV particles are indicated by arrows (× 77,000). **4)** CMV particles (arrow) in a pinocytic vesicle (× 120,000).

Abbreviations used in Figs. 1-15. C, chloroplast; CV, central vacuole; M, mitochondrion; NE, nuclear envelope; NP, nucleoplasm; NS, nucleolus; NV, nucleolar vacuole; OG, osmiophilic globule; P, plasmalemma; T, tonoplast; V, intracytoplasmic vacuole.

views of monolayer virus aggregates which are sandwiched between the tonoplast and the membrane of intracytoplasmic vacuoles.

Some aggregates of progeny CMV were present in the cytoplasm either free (Fig. 6) or attached to the outer surface of intracytoplasmic vacuoles. The presence of virus particles scattered in the cytoplasm cannot be excluded, but was difficult to demonstrate conclusively, because of their dimensional resemblance to ribosomes. Virus particles were not found in association with the membranes of chloroplasts and mitochondria, nor were they present within those organelles. No CMV particles were found in the central vacuole.

Protoplasts infected by CMV consistently contained aggregates of progeny virus in the nucleus. The intranuclear virus aggregates were usually located at the nucleolus (Fig. 7, 11, 12) but were occasionally present in the nucleoplasm. The nucleoli with virus particles were sometimes vacuolated (Fig. 7, 11). The nuclei containing virus lacked electron-dense heterochromatin (Fig. 7, 13)

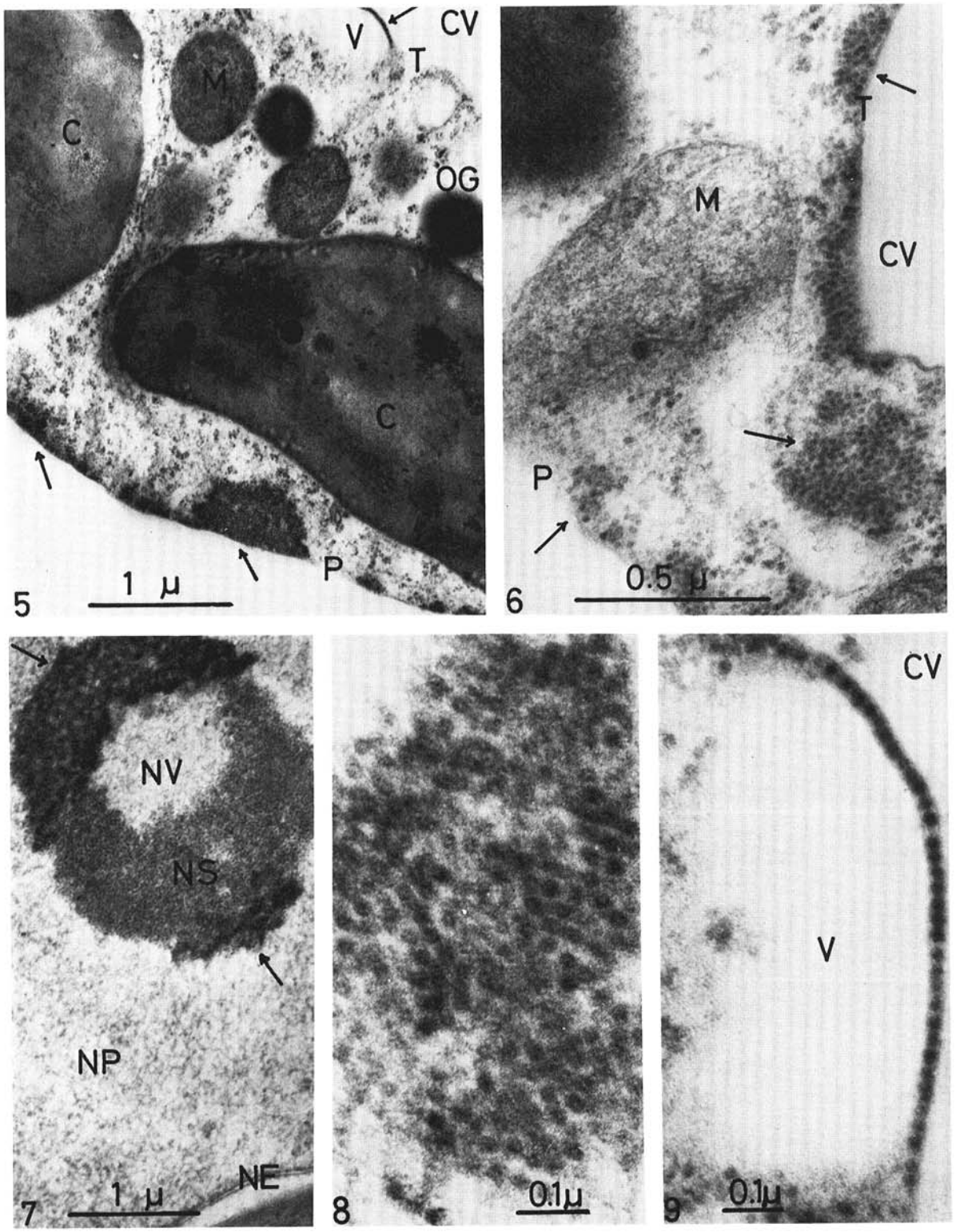

Fig. 5-9. Progeny CMV particles in protoplasts. Fig. 5, 6, 8, and 9 were sampled at 24 hr after inoculation. **5)** Aggregates of CMV particles (arrows) associated with plasmalemma and tonoplast (× 24,000). **6)** Aggregates of CMV particles (arrows) seen in association with plasmalemma and tonoplast or free in the cytoplasm (× 65,000). **7)** Aggregates of CMV particles (arrows) associated with the nucleolus. Note the absence of heterochromatin. From a protoplast cultured for 48 hr (× 22,000). **8)** Details of an aggregate of CMV particles associated with the plasmalemma (× 120,000). **9)** CMV particles in narrow cytoplasm between the central and an intracytoplasmic vacuole (× 100,000).

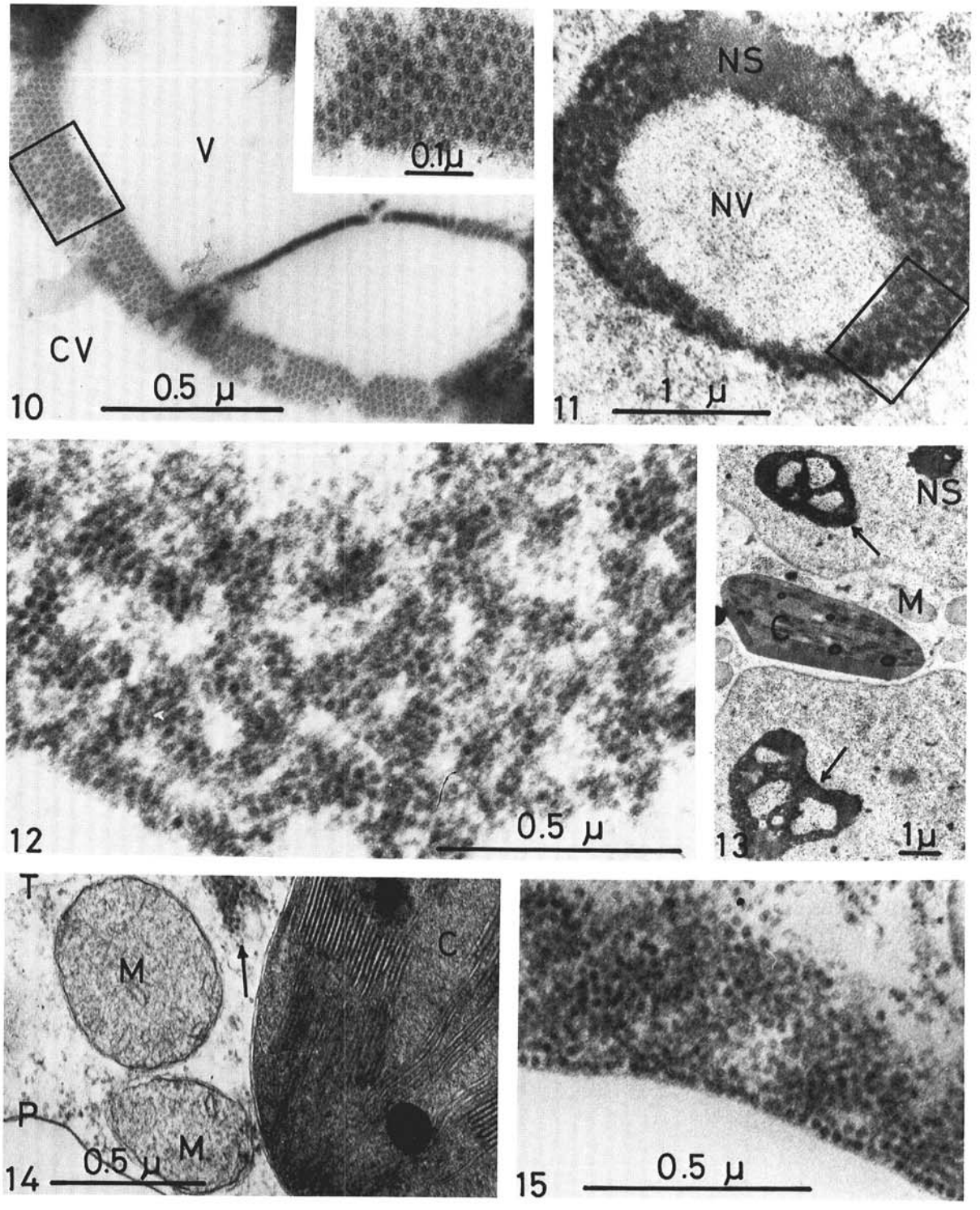

Fig. 10-15. Progeny CMV particles in protoplasts. **10)** Doughnut-like particles aligned in a regular array on the tonoplast. From a protoplast cultured for 24 hr (× 60,000). Inset shows a part of the aggregate marked by the square in Fig. 10 (× 110,000). **11)** Large aggregates of CMV particles associated with the nucleolus. From a protoplast cultured for 48 hr (× 26,000). **12)** Detailed picture of CMV aggregate marked by the square in Fig. 11 (× 78,000). **13)** A multinucleate protoplast infected by CMV. Virus aggregates in the nuclei are indicated by arrows. From a protoplast cultured for 48 hr (× 7,000). **14)** A chloroplast and two mitochondria in a protoplast cultured for 48 hr after inoculation with CMV. A small aggregate of CMV (arrow) is associated with the tonoplast (× 51,000). **15)** Aggregate of CMV particles associated with the plasmalemma. Protoplasts were isolated from tobacco leaves infected for 4 days (× 75,000).

which is prominent in the nucleus of healthy protoplasts (14). Some of the protoplasts were multinucleate as the result of spontaneous fusion during isolation (14). Each nucleus in these multinucleate protoplasts contained virus aggregates (Fig. 13).

The number and the size of CMV aggregates increased during 24 and 48 hr of incubation. However, they did not become the giant aggregates which are common in TMV-infected protoplasts (10). This observation again agrees with the results of staining with fluorescent antibody (9). Although an unusual morphology was induced in the nucleus by CMV-infection (see above), no degenerative change was evident in the cytoplasm, chloroplasts, and mitochondria (Fig. 14) at least until 48 hr after inoculation. Recently, Motoyoshi et al. (8) reported that tobacco mesophyll protoplasts infected with cowpea chlorotic mottle virus show abnormal endoplasmic reticulum. Such a change was not found in CMV-infected protoplasts.

Virus in leaf tissues.—Tobacco leaves were fixed and sectioned 4 days after inoculation with CMV. Virus aggregates could not be demonstrated electron microscopically in the mesophyll cells, suggesting that progeny CMV particles are scattered in the cytoplasm. However, when protoplasts were prepared from these inoculated leaves, virus aggregates were seen in association with the plasmalemma (Fig. 15) or with the tonoplast.

DISCUSSION.—Ultrastructural observation of the penetration of CMV into tobacco mesophyll protoplasts (Fig. 1-4) showed that this process is pinocytic in nature, as was reported previously for TMV (2, 6, 10). CMV particles taken up by this mechanism appear to be uncoated rapidly, since they are no longer detectable in the pinocytic vesicles or elsewhere 40 min later. In a recent paper on the uptake of cowpea chlorotic mottle virus by tobacco mesophyll protoplasts Burgess et al. (1) reported that localized lesions of the plasmalemma are favored sites for virus adsorption. These workers proposed that the virus is directly incorporated into the cytoplasm through the plasmalemma lesions, although they found virus particles also in vesicular structures (1, 8). In contrast to their observations, we were unable to find any evidence which argues against the involvement of pinocytosis in virus uptake, such as preferential binding of the input virus to points of damage in the plasmalemma.

In CMV-infected protoplasts, most of the progeny virus aggregates in the cytoplasm were attached to the plasmalemma or to the tonoplast (Fig. 5, 6). Leaf cells infected in tissue apparently contain scattered virus particles but showed similar localization of virus aggregates, if they were transformed into protoplasts (Fig. 15). It may, therefore, be assumed that the hypertonic medium for suspending protoplasts alters the intracellular environment in such a way as to facilitate aggregation of CMV particles and their attachment to the membranes.

Protoplasts infected in vitro by CMV invariably contained progeny virus in the nucleus (Fig. 7, 11, 12, 13). The amount of intranuclear virus particles (Fig. 11, 12), their association with the nucleolus (Fig. 7, 11, 13), as well as the unusual nuclear morphology (Fig. 7) suggest that these particles were produced in the nucleus. CMV has been reported to occur occasionally in the nucleus in infected tobacco leaf tissues (3).

Degeneration of chloroplasts is one of the most conspicuous ultrastructural symptoms caused by CMV-infection (5, 7). The plastids remained, however, quite normal in CMV-infected protoplasts at least for 48 hr after inoculation, during which time the virus multiplied substantially. It is likely that the chloroplast abnormalities reported in infected leaves represent a cytopathic effect which appears only in later stages of infection.

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