

# ASSEMBLY AND AGGREGATION OF TOBACCO MOSAIC VIRUS IN TOMATO LEAFLETS

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## ABSTRACT

Cells of tomato leaflets (*Lycopersicon esculentum* Mill.) were studied by phase and electron microscopy at various intervals after inoculation with a common strain of tobacco mosaic virus (TMV). Forty-eight hours after inoculation, prior to the development of assayable virus, individual TMV particles, and also particle aggregates, were observed in the ground cytoplasm of mesophyll cells. The most rapid synthesis of virus occurred between 80 and 300 hours after inoculation. Cytological changes during this time were characterized by an increased number of individual particles in the cytoplasm, growth of some aggregates, distortion and vacuolation of chloroplasts, and formation of filaments in the cytoplasm which were approximately four times the size of TMV. These filaments were interpreted as possible developmental forms of the TMV particle. Vacuoles in chloroplasts commonly contained virus particles. Evidence indicated that TMV was assembled in the ground cytoplasm and, in some cases, subsequently was enveloped by distorted chloroplasts.

## INTRODUCTION

Events relating to the intracellular development of tobacco mosaic virus (TMV) are poorly understood. There is indirect evidence that after cells become infected the ribonucleic acid (RNA) of TMV is synthesized in the nuclei and is subsequently liberated into the ground cytoplasm where it assembles with TMV-protein to form characteristic nucleoprotein tubes (1, 14, 26, 31). Other evidence indicates that viral RNA and protein are synthesized concurrently and that their formation is coordinated with a linear extension of the virus's RNA fiber and of the helical array of protein subunits (3). Chloroplasts have also been considered possible sites of TMV formation. Recently, Zaitlin and Boardman presented strong evidence that chloroplasts extracted from infected tobacco plants contain TMV (29).

Thin sectioning techniques for electron microscopy provide a direct means of studying virus infection. With the introduction of certain

methods, notably the use of epoxy resin embedding media (6) and staining after sectioning (5, 15), the ability to view TMV at relatively high levels of resolution in well preserved cells has been greatly improved. These methods were used in the present study in conjunction with phase contrast microscopy of living material to study the sites and manner of TMV development in tomato leaflets during progressive stages of infection.

## MATERIALS AND METHODS

**VIRUS AND PLANT MATERIAL:** Leaflets of young tomato plants (*Lycopersicon esculentum* Mill. var. Improved Pearson) were inoculated with a common strain of TMV similar to the U-1 strain as described by Siegel and Wildman (18). Individual leaflets were inoculated by rubbing their upper surface with a cotton swab dipped in a purified virus suspension. Inoculum contained approximately 38  $\mu\text{g/ml}$  TMV in 0.05 M phosphate buffer (pH 7.2). After inoculation, leaflets were rinsed with distilled water and plants were placed in a controlled temperature

chamber at 13°C with 12-hour alternating periods of darkness and white fluorescent lights (350 ft-c). Samples were removed for study at selected intervals after inoculation. In each case, samples were taken after the plants had been exposed to 1 hour of light following a 12-hour dark period. Only leaflets which were directly inoculated were used.

Since the experiments usually extended over a period of several weeks, plants varied in age from the beginning to the end of an experiment. As a check against age effects, in some experiments all plants were placed in the growth chamber simultaneously and inoculated at different intervals. These samples subsequently were taken at the same time and stage of plant growth. The results reported here were the same under both conditions of inoculation and sampling.

**VIRUS ASSAY:** Rates of increase of virus were measured by infectivity assay on leaves of *Nicotiana glutinosa* L. Leaflets of tomato plants were weighed and then ground in several drops of buffer. They were then diluted according to stage of infection, to give no more than 80 local lesions per half leaf on assay plants. Opposite half leaves were rubbed with a standard purified suspension of TMV which produced between 50 and 100 lesions per half leaf. Relative infectivity was expressed as the ratio of lesions produced on half leaves of *N. glutinosa* by a gram of sample (after correcting for dilution) to the number of standard lesions on opposite half leaves. The  $\log_{10}$  of each value was plotted as a function of time after inoculation to yield a virus increment curve.

**ELECTRON MICROSCOPY:** Immediately before the samples were ground for infectivity assay, small pieces of tissue were cut from the leaflets and prepared for electron microscopy. They were killed and fixed in Dalton's chrome-osmium solution (4) at 2 to 5°C for 24 hours, dehydrated in ethanol, and embedded in Araldite 6005 epoxy resin (CIBA). Castings were cured for 24 hours at 70°C in a nitrogen atmosphere. Serial sections with silver to gold interference colors were cut on a Porter-Blum microtome with glass knives, expanded with xylene vapor (13), and mounted on collodion-coated grids. Sections then were stained on one side according to the method of Gibbons and Bradfield (5) or on both sides (17) using 1.0 per cent aqueous strontium or potassium permanganate for 10 minutes, followed by a rinse with distilled water (8).

Sections were examined using an RCA-EMU3 electron microscope with a 50  $\mu$  objective aperture and operating at 50 kv.

**PHASE CONTRAST MICROSCOPY:** For studies of living cells, epidermal strippings were removed from leaf samples and mounted in distilled water.

A Zeiss microscope, Model GFL with neofluar objectives, was used.

## RESULTS

**VIRUS INCREMENT CURVE:** The development of assayable virus in tomato leaves followed an increment curve (Fig. 1) similar to that reported for TMV in tobacco and for other plant viruses (22, 28). The first detectable virus occurred about 80 hours after inoculation. This was followed by a logarithmic increase in infective material during which the concentration of virus doubled every 32 hours. After about 300 hours there was a decline in the rate of increase, followed by a stationary phase. The latent period (80 hours) and generation time (32 hours) were longer than those previously reported for TMV. This may have resulted from the low temperature of incubation (13°C), as the generation time for TMV in tobacco has been reported to vary inversely with temperature (28).

Fig. 1 represents the average virus content of all cells in the sample. Since not all cells are infected to the same degree at any given time, it is impossible to correlate cytological events in individual cells with the over-all curve. In the present study, specific changes in cell ultrastructure attending the course of infection were related to the curve either because they were the only ones observed or because they occurred consistently in the majority of cells sampled at any time. The following results were based on observations of several thousand cells.

**INTRACELLULAR IDENTIFICATION OF TMV:** In the course of infection, particles believed to be TMV were observed in cells viewed with the electron microscope. Identification of these structures as virus was based, in part, on a comparison of their morphology with that of purified TMV which had been similarly embedded, sectioned, and stained. The intracellular particles presumed to be virus and the particles in sections of purified virus had the following characteristics in common: (1) a particle width distribution which ranged from 80 to 200 A, with most particles being 160 to 200 A wide (Fig. 2); (2) a tubular shape with a central, heavily stained core, 50 to 60 A wide and parallel to the long axis (Figs. 3, 4); and (3) an apparent length which depended on its orientation in the section and on section thickness (17). Further evidence that the intracellular particles were

TMV was their absence in non-infected cells, the apparent correlation between their number and the virus increment curve, and their aggregation in the form of intracellular crystals known to contain infective material (24). For these reasons, the particles observed in infected cells may reasonably be identified as virus and will be referred to as such throughout this report.

**PATHOLOGICAL CYTOLOGY:** Most electron microscope observations were made on mesophyll cells. A non-infected mesophyll cell is shown in Fig. 5.

transparent zones. They did not appear to be associated with any structural component of the cell, such as chloroplasts, mitochondria, or nuclei.

Seventy hours after inoculation, just prior to the beginning of the logarithmic increase of virus, individual and aggregated virus particles were encountered more frequently in ground cytoplasm. Certain cells apparently contained a large number of individual aggregates since sometimes as many as 7 were observed in one thin section of a cell.

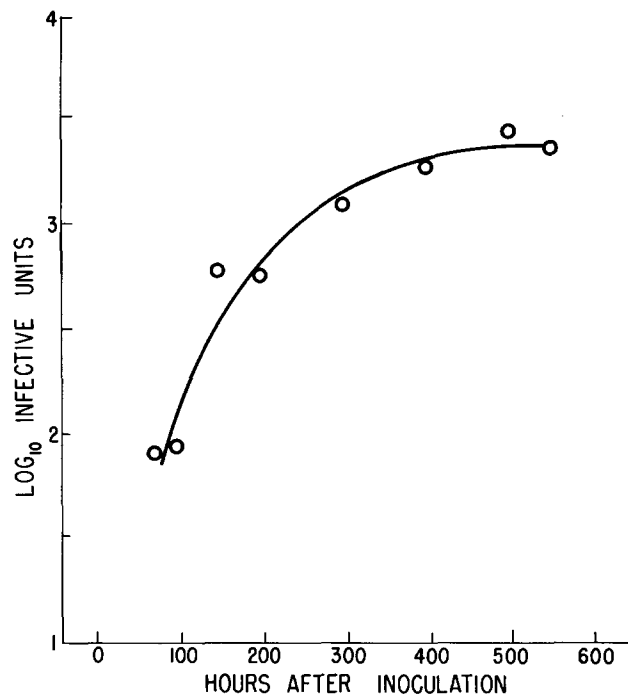


FIGURE 1 Increment curve for tobacco mosaic virus produced in leaves of tomato plants at 13°C. Infective units represent lesions per gram of leaf sample on leaves of *Nicotiana glutinosa* L. per lesions produced by a standard TMV suspension applied to opposite half leaves of the assay plant.

### 1. LATENT PERIOD

The first evidence of infection was observed with the electron microscope 48 hours after inoculation. This was during the latent period of infection. Virus particles were found, individually (Fig. 6) or in small aggregates (Figs. 7, 8), in peripheral ground cytoplasm of some cells. Transverse and longitudinal serial sections of the aggregates revealed that they were cylindrical, about 0.3  $\mu$  long and 0.1 to 0.3  $\mu$  in diameter, and composed of several to a hundred virus particles. These were interpreted as developmental stages of crystalline inclusion bodies. Aggregated and non-aggregated particles were surrounded by electron-

### 2. LOG PHASE

Subsequent events observed during the period of most active synthesis of virus were marked by increased numbers of TMV particles scattered in the cytoplasm (Fig. 9), growth of virus aggregates (Figs. 10 to 12), formation in the cytoplasm of relatively large filamentous structures (Figs. 13 to 15), and changes in chloroplasts (Figs. 16 to 19).

As the infection progressed, some of the virus aggregates increased in diameter and became multilayered (Figs. 10, 11, 12). The axes of particles in adjacent layers sometimes were oriented at different angles to one another to

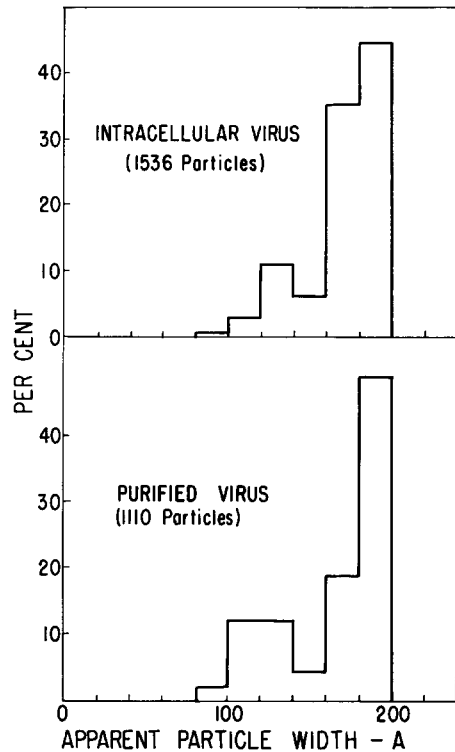


FIGURE 2 Width distribution of TMV particles in tomato mesophyll cells and of purified virus preparations which were embedded in Araldite, sectioned, and stained with strontium permanganate.

produce the "herring-bone" arrangement observed in frozen replicas of large crystalline inclusions (23). The presence of large, multi-layered aggregates was accompanied by increased numbers of virus particles scattered randomly in the vacuole as well as in the cytoplasm. From results of previous studies, virus in the vacuole is believed to originate from crystalline inclusions dispersed during fixation (2, 7, 16).

During this period many cells contained electron-opaque filaments in the ground cytoplasm which were usually distinct from the characteristic TMV particles (Figs. 13, 14). These filaments were 60 to 70  $\mu$  in diameter and were variable in length, sometimes nearly 2  $\mu$ . Usually they were arranged in orderly bundles having a pattern similar to that of virus aggregates. They were associated with endoplasmic reticulum and ribosomes (Figs. 13, 14, *er*). In addition to being present in the ground cytoplasm, they were the primary structural component of X bodies (Fig. 15). The X bodies also contained mitochondria, TMV particles, and numerous membranes.

During periods of most active synthesis of virus, many chloroplasts were distorted and appeared to possess large vacuoles, some of which contained TMV particles (Figs. 16, 17) or various cytoplasmic organelles such as mitochondria (Fig. 18, *m*). Other chloroplasts possessed thin

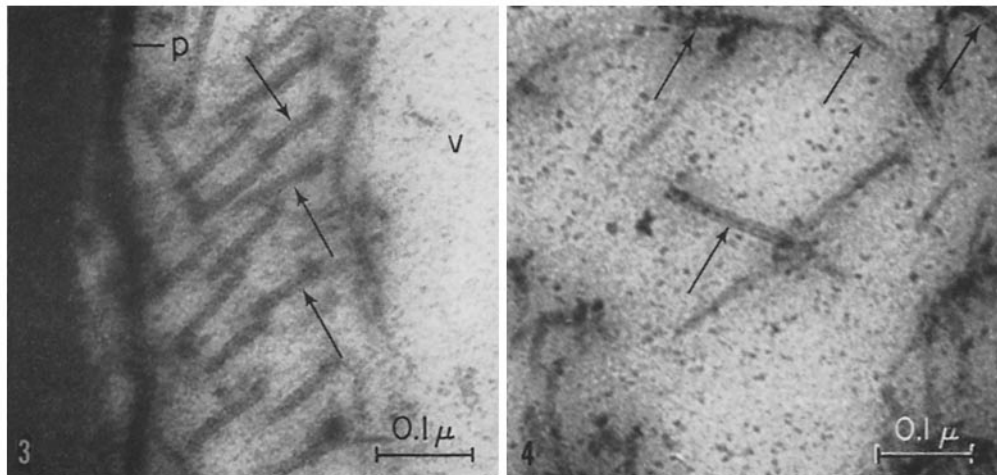


FIGURE 3 Intracellular TMV particles in peripheral cytoplasm between the plasma membrane (*p*) and the cell vacuole (*v*). Some particles have a heavily stained central core (arrows). 500 hours after inoculation.  $\times 136,000$ .

FIGURE 4 Section of a purified preparation of TMV embedded, sectioned, and stained with strontium permanganate. A heavily stained central core is apparent in many of the particles (arrows).  $\times 136,000$ .

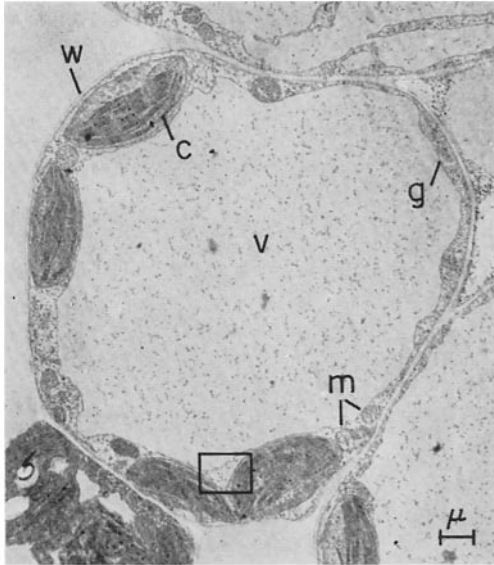


FIGURE 5 Non-infected mesophyll cell of tomato leaf showing the cell wall (*w*), chloroplasts (*c*), cell vacuole (*v*), mitochondria (*m*), and ground cytoplasm (*g*). The nucleus is not shown. Micrographs taken at higher magnifications during progressive stages of virus infection represent regions of peripheral cytoplasm similar to the one indicated (rectangle).  $\times 5,000$ .

projections which appeared to envelop virus particles and organelles of the ground cytoplasm (Fig. 19).

In living epidermal cells, chloroplasts became abnormally active about 8 days after inoculation. They developed long appendages which extended and contracted. Contraction sometimes occurred in a few seconds. When the appendages contracted, transparent swollen regions 1 to 2  $\mu$  in diameter often developed in the chloroplasts. Some of these regions contained bodies presumed to be mitochondria. The origin of the swellings was not clear. Sometimes they appeared to arise within the chloroplast stroma; at other times, they seemed to result from the envelopment of ground cytoplasm during rapid contraction of appendages. They corresponded, in size, location, and time of appearance, to the regions which appeared under the electron microscope as vacuoles in the chloroplasts.

Electron microscopy of mesophyll cells also revealed vesicles 10 to 150  $m\mu$  in diameter in the stroma of many chloroplasts (Fig. 18). These vesicles were devoid of electron-opaque material. Usually they were situated near the limiting membranes of the chloroplast, often distending

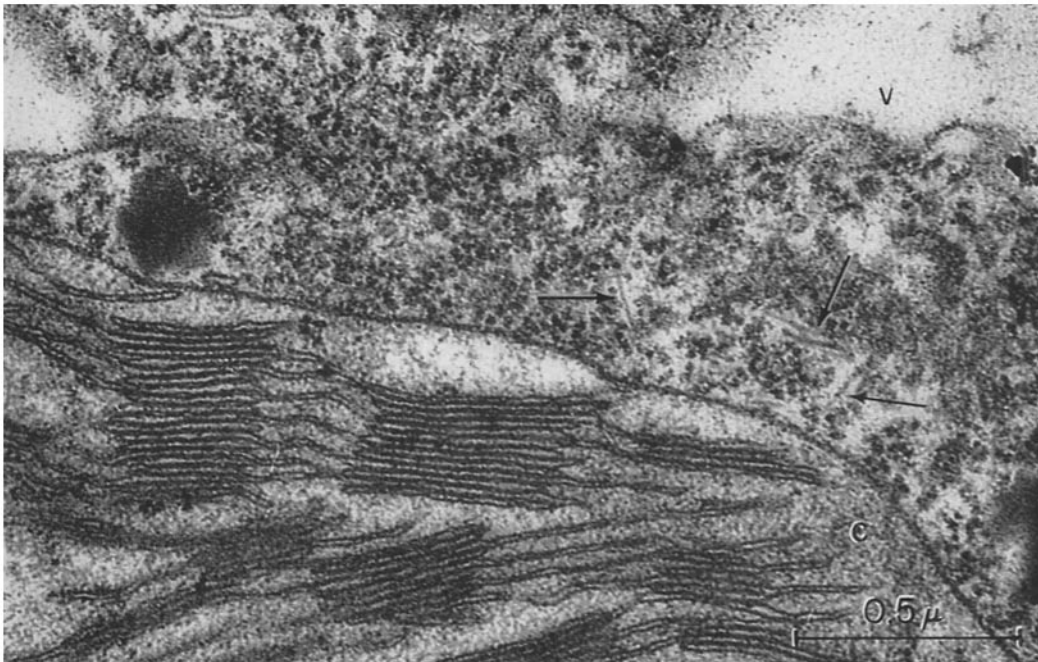


FIGURE 6 Layer of ground cytoplasm, between the cell vacuole (*v*) and a chloroplast (*c*), containing several scattered TMV particles (arrows). 48 hours after inoculation.  $\times 60,000$ .

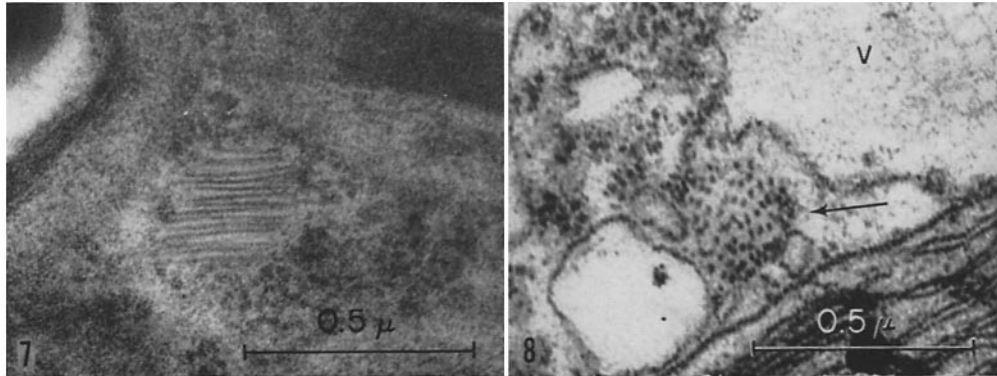


FIGURE 7 Longitudinal section through a cylindrical aggregate of TMV particles in the ground cytoplasm. 48 hours after inoculation.  $\times 60,000$ .

FIGURE 8 Transverse section through a cylindrical aggregate of TMV particles (arrow) in the ground cytoplasm near the cell vacuole (*v*). 48 hours after inoculation.  $\times 60,000$ .

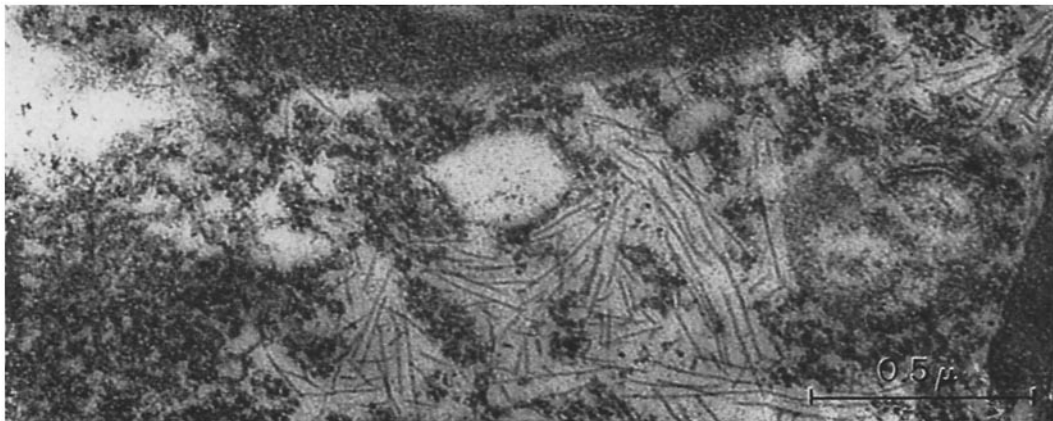


FIGURE 9 Scattered TMV particles in the ground cytoplasm of a mesophyll cell. 140 hours after inoculation.  $\times 60,000$ .

them or apparently being released into the ground cytoplasm.

### 3. STATIONARY PHASE

Twenty-three days after inoculation, during the stationary phase of virus synthesis, most cells contained large virus crystals which were mostly disrupted when cells were killed, filling the cell vacuoles with numerous virus particles (Fig. 20). The cytoplasm contained small aggregates of virus, similar to those observed in early infection, and also many individual particles. Where there were numerous virus particles in the ground cytoplasm, there were notably few ribosomes (Fig. 3).

The cytoplasm also contained inclusion bodies composed of filamentous structures (Fig. 20, *i*) similar to those observed during the logarithmic phase. These inclusions differed from the X-bodies, described earlier, in being generally smaller, containing no membranes, vacuoles, or mitochondria, and having the component filaments arranged in a random manner (Fig. 21).

Chloroplasts which contained vesicles or were distorted in the manner described above were observed less frequently than during the period of maximum increase of virus. No consistent changes were detected in the ultrastructure of nuclei, mitochondria, or Golgi bodies.

## DISCUSSION

Previous identification of intracellular TMV by electron microscopy has been limited to particles in or derived from crystalline inclusion bodies (2, 7, 9, 16, 26, 27). Consequently, no conclusions could be made concerning sites of particle assembly. In the present study, particles which were

latent period as used here was the time between inoculation and the formation of assayable virus. Some infective particles undoubtedly were formed during this period but were not detected by the assay method. Some of the cells sampled for electron microscopy during the apparent latent period were probably in advanced stages of in-

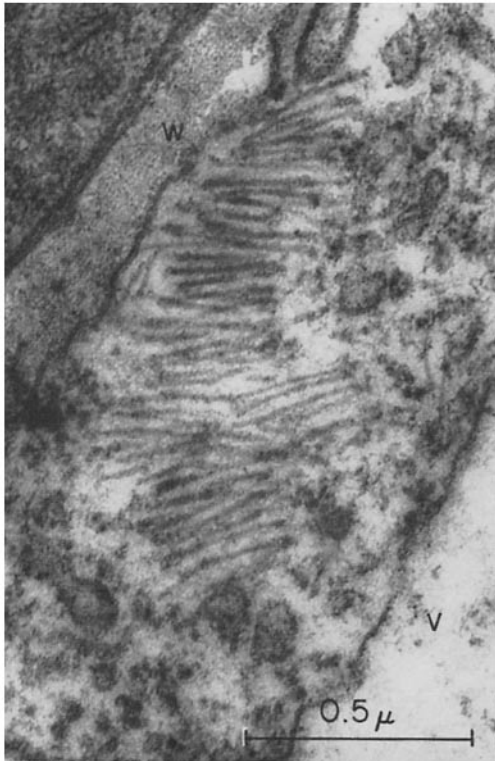


FIGURE 10 Monolayer aggregate of TMV in the ground cytoplasm. 100 hours after inoculation. Cell wall (*w*), vacuole (*v*).  $\times 60,000$ .

believed to be not derived from inclusion bodies were observed in the ground cytoplasm. They occurred early in the infection process, before large inclusion bodies developed. Although small aggregates of virus were present, unlike large crystals they were not highly dispersed by the killing solution. The apparent absence of particles in any other part of the cell in early infection leads to the conclusion that they were assembled in the ground cytoplasm.

The presence of particles during the latent period of infection may have been due to the relatively low sensitivity of infectivity assay. The

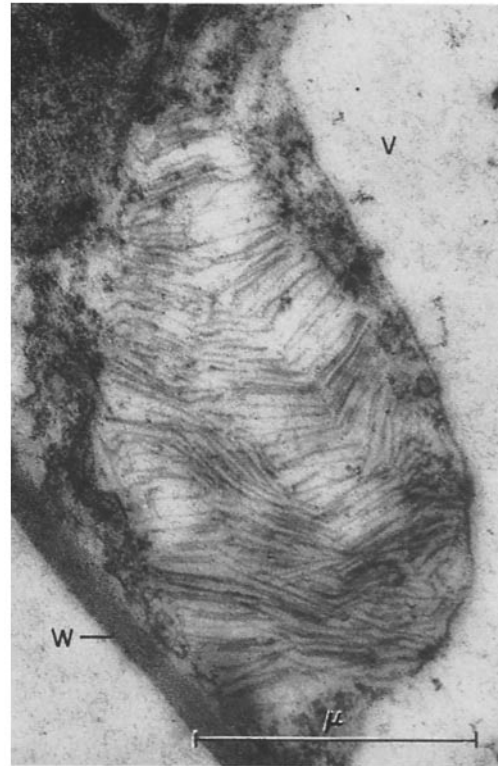


FIGURE 11 Multilayered aggregate of TMV in the ground cytoplasm. 100 hours after inoculation. Cell wall (*w*), vacuole (*v*).  $\times 37,500$ .

fection, and the cytological changes actually may have been events of logarithmic development of virus.

In later stages of infection, virus particles also were observed in what appeared to be vacuoles of the chloroplasts. Vacuolation of tomato chloroplasts in response to TMV infection was reported by Sorokin from studies with the light microscope (21). However, the origin of these vacuoles was not clearly established. In the present studies, their formation was accompanied by severe distortion of chloroplasts, including formation of projections which rapidly extended and con-

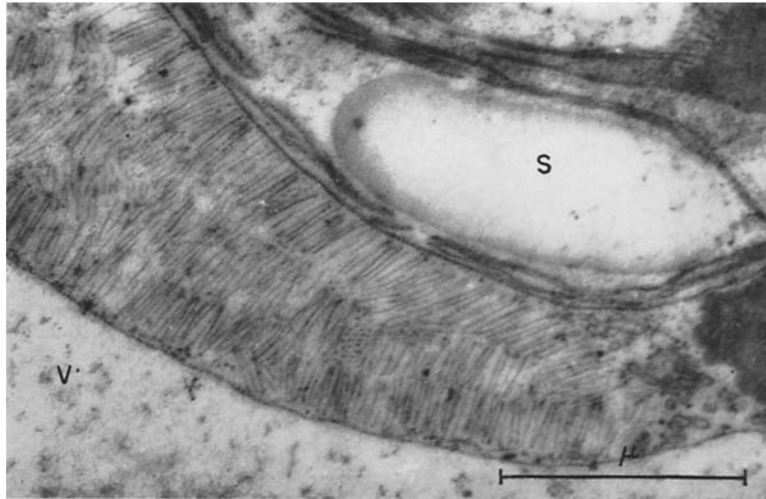


FIGURE 12 Multilayered aggregate of TMV. Chloroplast starch grain (s), vacuole (v). 200 hours after inoculation.  $\times 32,000$ .

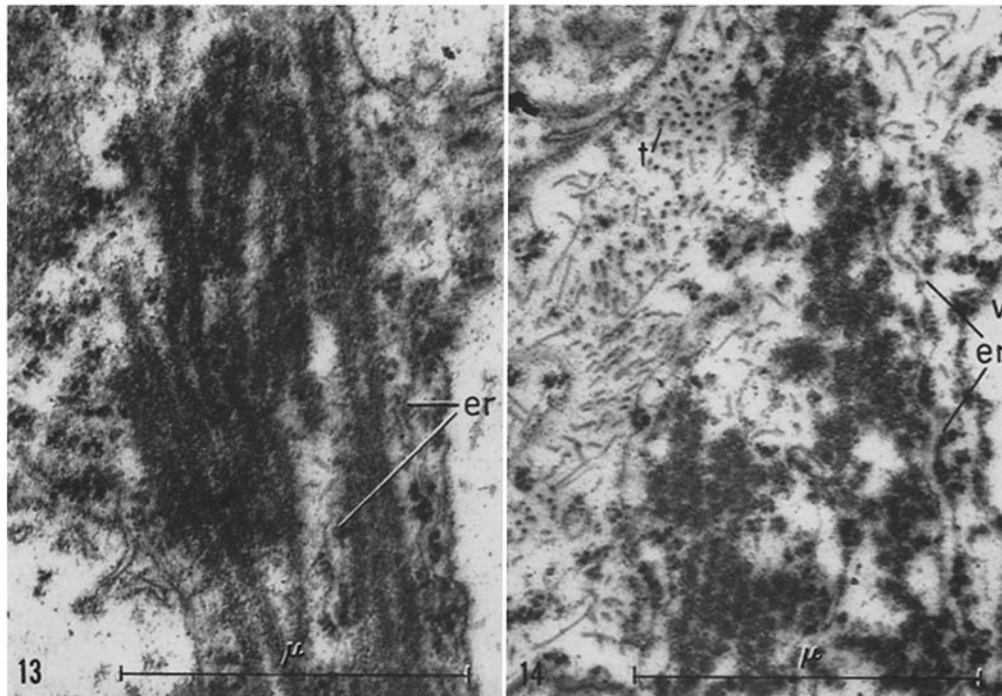


FIGURE 13 Longitudinal view of filaments in the ground cytoplasm interpreted as possible developmental forms of TMV. They are oriented parallel to one another and to elements of endoplasmic reticulum (er). 260 hours after inoculation.  $\times 46,000$ .

FIGURE 14 Transverse view of filaments in the ground cytoplasm similar to those shown in Figure 13. Also shown are profiles of endoplasmic reticulum (er) and aggregates of TMV (t). Vacuole (v). 260 hours after inoculation.  $\times 46,000$ .



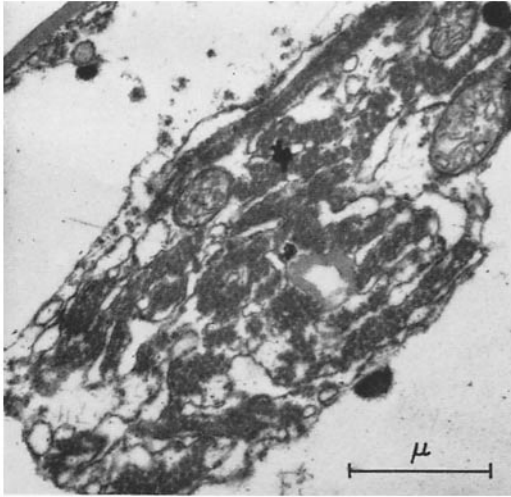


FIGURE 15 X body in an epidermal cell. The main structural feature appears to be filaments, similar to those viewed in transection in Fig. 14, arranged in an ordered manner and associated with a system of membranes. The X body also contains mitochondria and some TMV particles. 200 hours after inoculation.  $\times 19,500$ .

tracted. This behavior, together with the common inclusion of mitochondria and other cytoplasmic organelles, and such views as shown in Fig. 19 suggested that envelopment of ground cytoplasm by cup-like projections of the plastid was one means by which the vacuoles arose. Although it is not known whether any of these structures observed with the electron microscope were closed vacuoles, many of the transparent swellings observed in living material seemed quite stable once they were formed, giving the impression that they were closed.

Under such conditions of vacuolation, virus and other cytoplasmic components could become closely bound to or incorporated into chloroplasts. Such a process could account for the associating of TMV with chloroplasts of infected plants as has been reported (11, 12, 25, 29). In view of this possibility, the presence of virus in chloroplasts would not necessitate its synthesis or assembly therein, and would not conflict with the evidence for assembly in the ground cytoplasm.

The vacuolation and distortion of chloroplasts commonly observed in cells during logarithmic

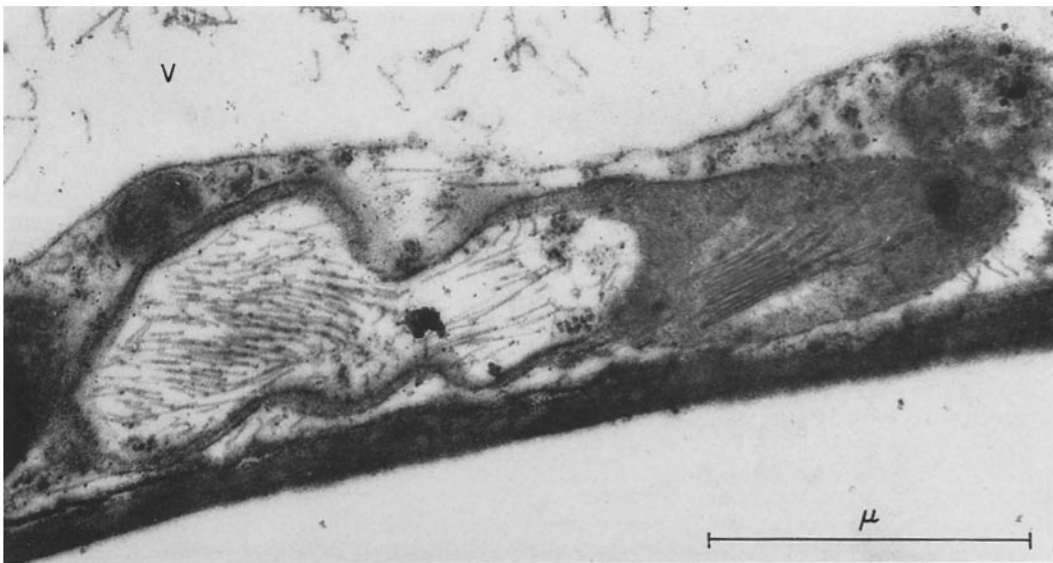


FIGURE 16 Chloroplast, during period of rapid synthesis of virus, containing a large vacuole and numerous TMV particles. Grana lamellae may be seen in the right portion of the plastid. The cell vacuole (*v*) also contains TMV particles, presumably a result of inclusion body dispersion. 260 hours after inoculation.  $\times 43,000$ .

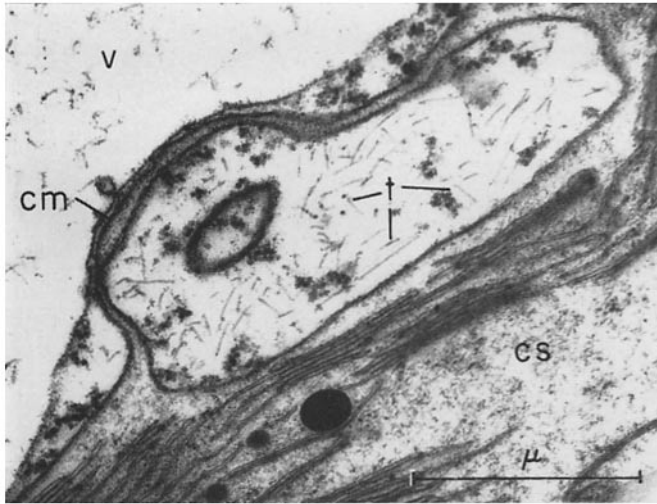


FIGURE 17 TMV particles (*t*) in chloroplast vacuole. Cell vacuole (*v*), chloroplast membrane (*cm*), chloroplast stroma (*cs*). 260 hours after inoculation.  $\times 30,000$ .

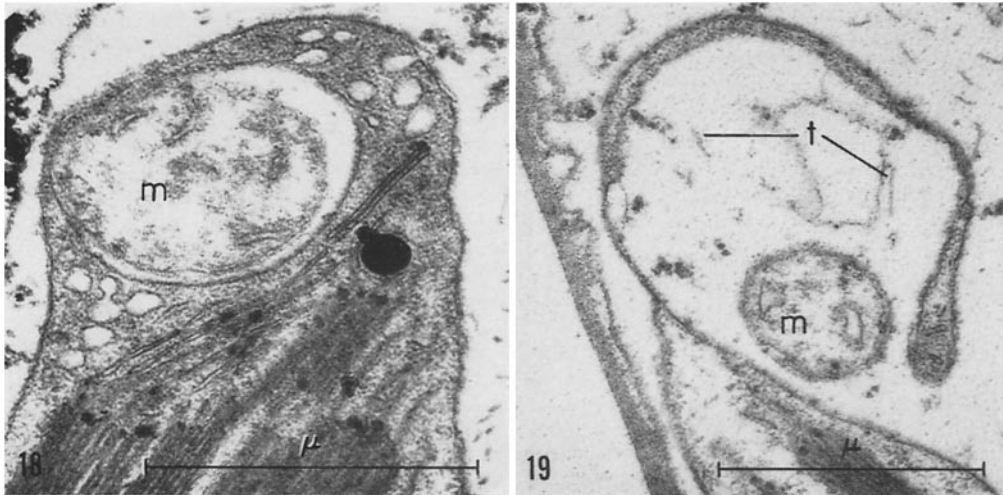


FIGURE 18 Chloroplast with a large vacuole containing a mitochondrion (*m*). Numerous small vesicles may be seen in the chloroplast stroma, one of which is in contact with the ground cytoplasm. 260 hours after inoculation.  $\times 44,500$ .

FIGURE 19 Extension of distorted chloroplast encircling a mitochondrion (*m*) and several TMV particles (*t*). 260 hours after inoculation.  $\times 35,000$ .

development of virus are not believed to be specific to virus infection, since in a few instances they were observed in non-infected cells. Similar changes also were observed by Sitte in chloroplasts of *Elodea callitrichoides* Casp. when cells were plasmolyzed with sucrose solutions (19). Such changes may be general pathological reactions.

The nature of the large filaments shown in Figs.

13 and 14 is not known. Their shape and pattern of aggregation suggest a structural relationship to TMV tubes. Their size, however, is approximately four times that of TMV. Analogous structures have been observed in cells of *Vicia faba* L. infected with petunia ring spot virus (10). Here they were aggregated in a pattern suggesting a structural relationship to rod-shaped particles

presumed to be virus, and had the staining properties of nucleoprotein. They were interpreted as immature forms of the virus.

Evidence for intermediate forms of TMV which are larger than the final particle has been presented by Smirnova (20) and Zech (30). According to Zech, flexible, large strands appeared in extracts of tobacco hair cells approximately 16 hours after infection. These strands contained threads which were sensitive to ribonuclease and which appeared to contract, with progressing infection, to form stiff TMV tubes. Zech proposed

served in early stages of infection. This argues against their being developmental forms. However, their apparent absence in early infection could be due to the low frequency with which they might be encountered and to possible difficulties in recognizing them when they are not present in large numbers or in aggregates.

Shrinkage of the strands to form the stiff TMV tubes might explain the electron-transparent zones which characteristically surround the mature particles (Fig. 6). The association of the large filaments with endoplasmic reticulum and ribo-

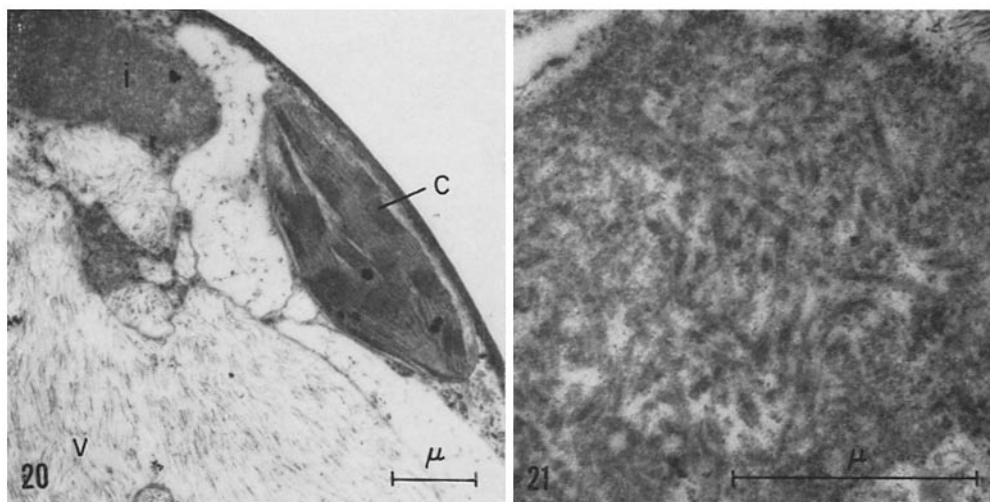


FIGURE 20 Portion of mesophyll cell sampled during the stationary phase of virus development (550 hours after inoculation). The cell contains numerous virus particles in the cell vacuole (*v*), some apparently normal chloroplasts (*c*), and heavily stained inclusion bodies (*i*).  $\times 10,500$ .

FIGURE 21 Portion of an inclusion body, similar to the one shown in Fig. 20 (*i*), containing filamentous structures arranged at random. 550 hours after inoculation.  $\times 32,000$ .

that the RNA-rich and biologically active part of the TMV was synthesized first, and then underwent a stepwise coating with TMV protein which was usually complete 30 hours after infection. He suggested that these infection products were rich in water, and that most of this was lost with time, resulting in a shrinking of intermolecular distances and formation of stiff TMV particles.

The strands in Zech's micrographs were comparable in size and morphology to the filaments observed in sections of tomato leaflets. Most of the cytological observations in the present study agree with the above model of TMV formation, with the exception that the strands were not ob-

served (Figs. 13, 14) suggests a spatial relation to possible sites of protein synthesis. An association of endoplasmic reticulum with TMV or products of infection has been previously observed (9, 26).

Further studies, possibly employing specific staining techniques, with still closer control over the sequence of events in infection, are needed before a more precise relation between such possible intermediate forms and infective particles can be determined.

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