

AN ELECTRON MICROSCOPE STUDY  
OF TOBACCO MOSAIC VIRUS  
LESIONS IN *NICOTIANA GLUTINOSA* L.

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ABSTRACT

Ultra-thin sections of *Nicotiana glutinosa* L. leaves inoculated with a concentrated solution of tobacco mosaic virus were made at short intervals from 0 to 78 hours after inoculation. Eight hours after inoculation, the size of starch grains increased. This was followed by rupture of cytoplasmic and chloroplast membranes. At about 24 hours there was a great increase in number of mitochondria, which persisted until about 60 hours, when some became electron opaque while others appeared to disintegrate. Finally, the cell contents were compressed into one area of the cell, where they became electron opaque. This was accompanied by collapse of the rest of the cell and tearing away of the cell walls from adjacent cells. The nucleus remained stable and intact for as long as observations could be made. No identifiable virus particles were seen.

INTRODUCTION

The early events and consequent changes that lead to cell death in a local lesion produced by virus infection have not yet been described in a detailed and systematic fashion. The sequence of these events is of interest in itself. Moreover, studies of such infected leaf tissues have showed (17) that they respire at a much higher rate than healthy tissues, the rise occurring some hours before the first detectable signs on the surface. Since the infection results in metabolic changes before its overt expression, it seemed possible that a study of the infected cells, using the electron microscope (EM), might yield information that could be correlated with the physiological effects. We tried to detect the earliest cytological changes following inoculation, and then to follow their course until the terminal stages. The use of the EM made it possible to observe changes in cellular ultrastructure at a much later stage of infection than is possible with the light microscope, which is limited by the extreme density of the infected cells as necrosis proceeds.

MATERIALS AND METHODS

Plants of *Nicotinana glutinosa* L. were kept for 3 weeks in a room with controlled temperature and continuous illumination. The leaves of some of the plants were lightly dusted with Carborundum, and then inoculated with a purified solution of tobacco mosaic virus (TMV) of such concentration that the leaves were covered with confluent lesions. This procedure minimized the chance of including non-infected areas when samples of the leaves were taken prior to the appearance of symptoms. Two controls were included: from leaves dusted with Carborundum and rubbed with water; and from leaves that were not treated.

Pieces 2 to 3 mm<sup>2</sup> were cut from the leaves at 0 hours, and then every 4 hours until symptoms appeared 33 hours after inoculation. At this time, samples were taken hourly until 42 hours, then at about 4-hour intervals. The last sample was taken 78 hours after inoculation, when the leaves were completely wilted and dying.

The samples were fixed for 1 to 1½ hours in 1 per cent osmium tetroxide buffered to pH 7.3 with acetate-Veronal buffer, then dehydrated through a

graded series of ethanol dilutions, and embedded in Epon 812 (10). Thin sections cut with glass knives on a Servall Porter-Blum microtome, were examined unstained or stained with uranyl acetate, and photographed at screen magnifications of 10,000 to 40,000 in a modified Philips EM 100, at 60 and 80 kv.

## RESULTS

For the first 8 hours the inoculated cells appeared normal, with the cytoplasmic contents apposed closely to the cell wall, and most of the chloroplasts containing either 1 or 2 starch grains (Fig. 1). The starch grains were inserted into the lamellar structure of the chloroplast, or surrounded by what von Wettstein (22) refers to as granular ground substance or stroma (Fig. 2).

The first consistent changes were noted between 8 and 16 hours after inoculation, in the chloroplasts, when the starch grains appeared swollen to as much as 5 times the thickness of those in healthy cells (Fig. 3). The grains were surrounded by a non-structured opalescent vacuole, and the lamellae were markedly distorted (Fig. 4). The swelling did not persist, and few enlarged starch grains were found 28 to 32 hours after inoculation. During this period there was little change in the appearance of the chloroplasts and starch grains of healthy cells.

About 24 hours after inoculation a movement of the cytoplasmic contents was seen, away from the cell wall into the vacuole, reminiscent of plasmolysis (Fig. 5). Accompanying this movement, the cytoplasmic membranes began to

rupture. By the time lesions appeared, most of the membranes were broken or no longer adhered to the cytoplasm and its inclusions, but hung freely in strands or coils (Fig. 6) in the vacuole.

Beginning at about 24 hours, and continuing for about 20 hours, there was a marked increase in the number of mitochondria. The relative number of mitochondria in infected and healthy cells was not determined quantitatively, but scanning a large number of cells gave an estimate that there were 2 or more times as many mitochondria in the infected cells by the time lesions were fully developed.

Forty-eight to 52 hours after inoculation, disintegration of the cytoplasmic membranes was virtually complete. The contents of the cell were evenly distributed throughout the cell space, and small vesicle-like inclusions were prevalent. The chloroplasts showed marked disintegrative changes as the membrane loosened from the contents (Figs. 6 and 7) and began to rupture. Nearly all the chloroplast membranes had disintegrated 52 to 60 hours after inoculation, so that only traces could be detected. Simultaneously, large vacuoles developed in the remaining portions of the chloroplasts, further distorting the lamellae (Fig. 8). Despite the loss of membrane integrity, many lamellae persisted in stacks, until the terminal stages.

In contrast with these destructive effects, the nucleus appeared to stay intact. Amidst bits of membranes and other debris, the characteristic double nuclear membrane could be traced unbroken, and the nuclear contents were not no-

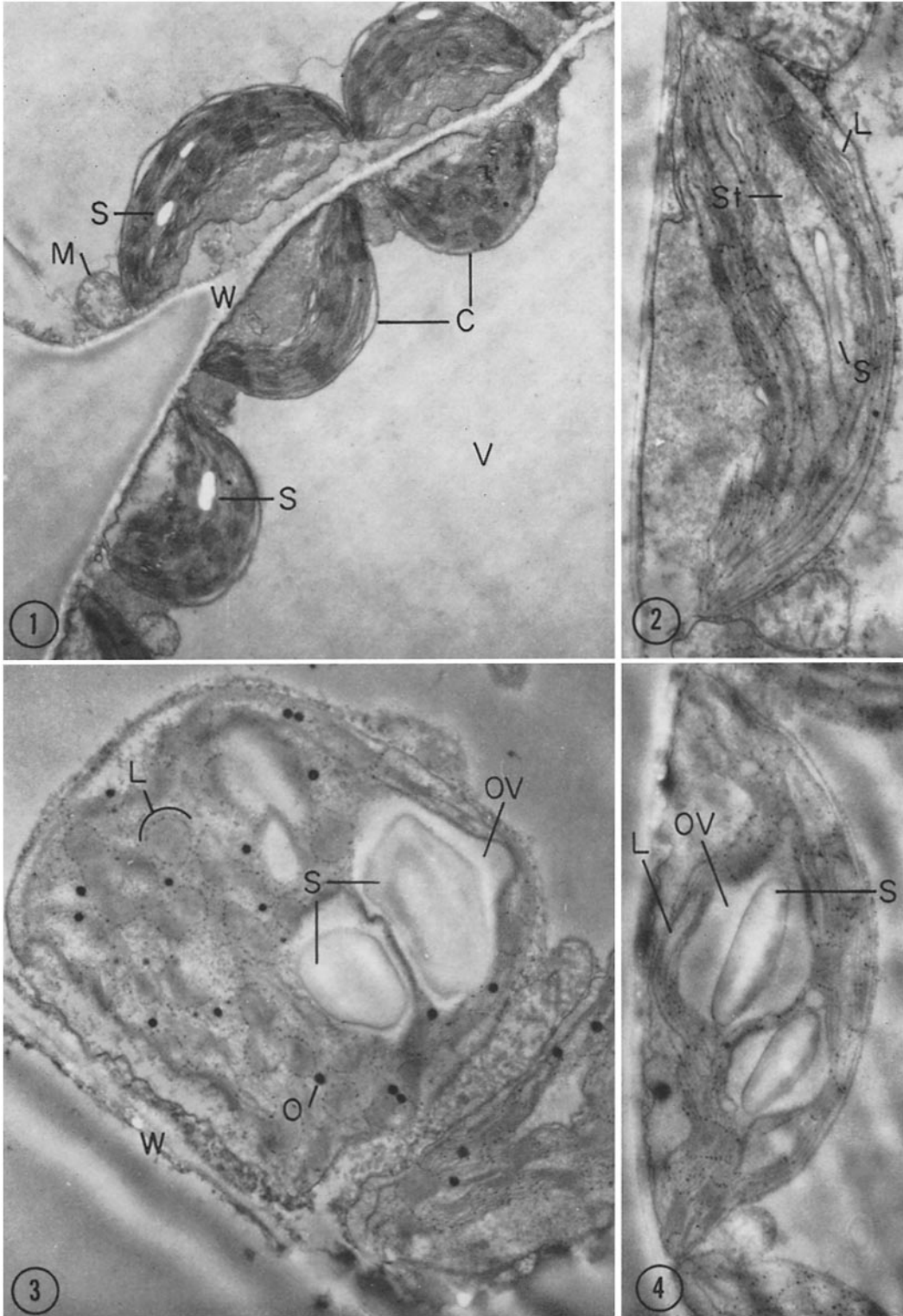
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FIGURE 1 Portion of a cell 0 hours after TMV inoculation. Chloroplasts (*C*) with occasional starch grains (*S*), and cytoplasm including mitochondria (*M*) are appressed to cell wall (*W*), while the vacuole (*V*) is empty.  $\times 6,500$ .

FIGURE 2 Chloroplast showing lamellae (*L*) in cell 0 hours after TMV inoculation. Small starch grain (*S*) is surrounded by stroma (*St*).  $\times 16,000$ .

FIGURE 3 Cross-section of a chloroplast 15 hours after TMV inoculation. Most of the lamellae are seen in cross-section as a series of points in a circle (*L*). The starch grains (*S*) are greatly swollen and each one is surrounded by an opalescent vacuole (*OV*). Osmiophilic granules (*O*) are dense. Cytoplasmic contents are, at this time, still close to the cell wall (*W*).  $\times 12,000$ .

FIGURE 4 Chloroplast in cell 8 hours after TMV inoculation. Starch grains (*S*) and the surrounding opalescent vacuoles (*OV*) are greatly swollen, resulting in marked distortion of the lamellae (*L*).  $\times 14,500$ .



ticeably different from those in healthy cells (Fig. 9).

Between 60 and 78 hours, the mitochondria showed degenerative changes (Fig. 7), many becoming electron opaque, while a few had discontinuous outer membranes. Ruptured mitochondrial membranes were found only infrequently. Cells were found, after 78 hours, with no intact mitochondria to be seen, but containing elongated lamellar structures, not previously found, that might have been cristae released from ruptured mitochondria (Fig. 10). However, since intermediate stages of ruptured mitochondria were encountered so infrequently, it is also possible that these elongated structures were portions of the endoplasmic reticulum.

Seventy-eight hours after inoculation most of the cells appeared to be in the terminal stages of infection. The contents of the cell were aggregated in one end (Fig. 11), so that the vacuole was once again nearly clear. The final stages of necrosis were marked by all the cell contents becoming closely pressed together and electron opaque. Only traces of inclusions, such as chloroplast lamellae, could be distinguished (Fig. 12). Frequently, adjoining cells were at various stages of this process (Fig. 13).

Finally, the infected cell collapsed, and the walls tore away from adjoining cells wherever there were no longer any cell contents. The contents were reduced to a small, compact, and extremely electron-opaque mass. The collapsed portion of the cell consisted of a double wall separated by a small amount of electron-opaque material, and was surrounded by space previously occupied by the cell before its collapse (Fig. 14).

#### DISCUSSION

The effects of a few systemic virus infections on the ultrastructure of cells have been described, in

detail in the case of cucumber virus 4 (7). Other electron microscope studies (*e.g.* references 4, 9, 23), although they include valuable incidental observations on structural changes, are aimed primarily at detecting and describing intracellular virus particles. In localized virus infections, Shalla (14, 15) also has the same aim, although he includes a description of the final disintegrative changes that occur in the cytoplasm and chloroplasts. None of these studies, however, follow the sequence of events that occur in the cell subsequent to inoculation.

It is apparent from the highly destructive changes during the development of a local lesion that the total organization of the cell is thrown into chaos. All of the systems that normally regulate physiological and metabolic stability are disrupted and finally destroyed, including membrane systems, mitochondria, and chloroplasts.

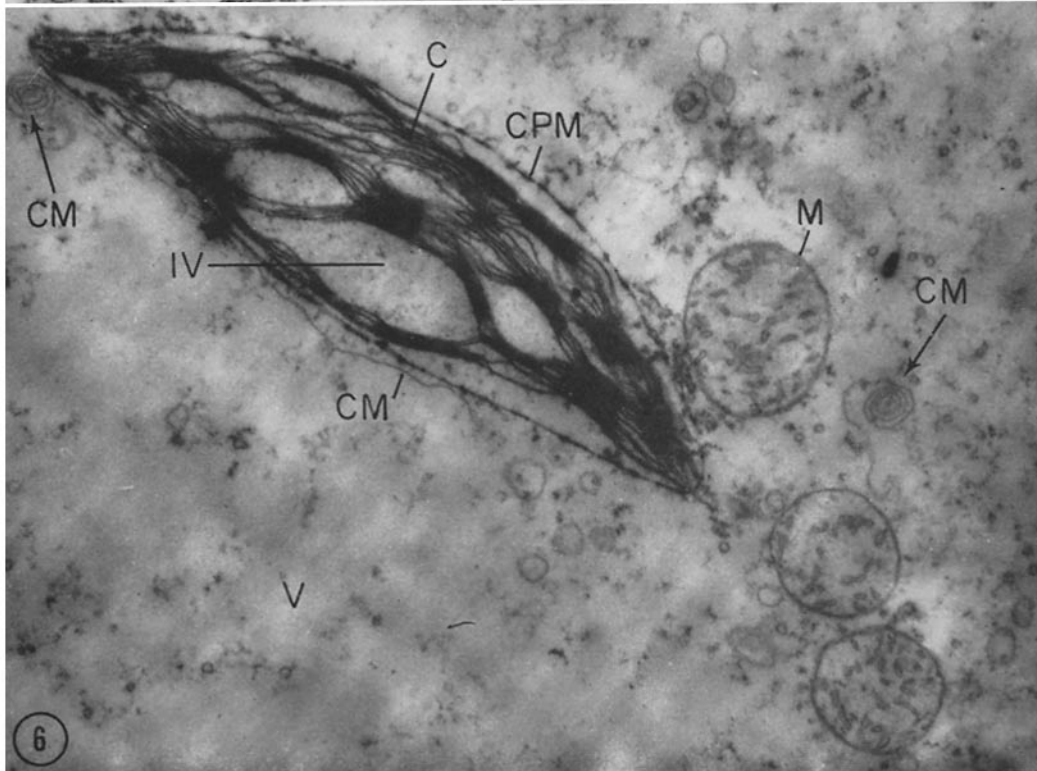
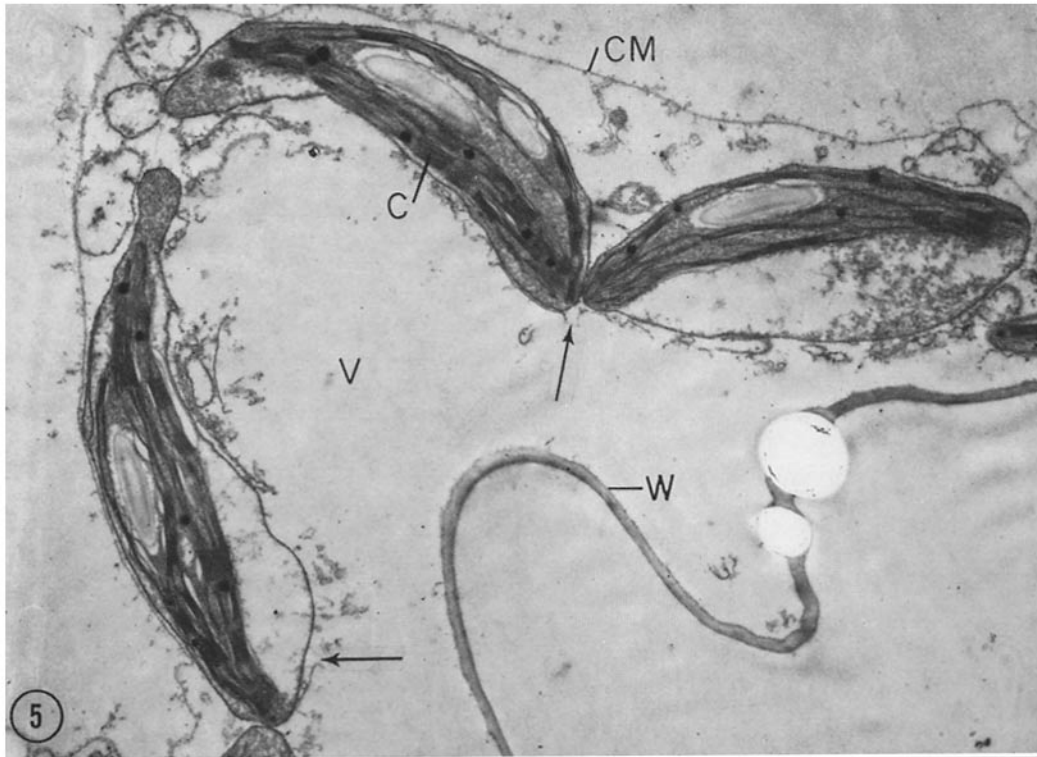
The disruption of the cytoplasmic membranes, detectable even 24 hours after inoculation, indicates a loss of organized osmotic control. This is immediately reflected in the loss of water that occurs soon after lesions develop (12), which continues with increasing severity until the leaves are dried out. The bits of cytoplasmic membranes are of random lengths and shapes (Figs. 6 and 9), but the coils seen in Fig. 6 are commonly found. These might be artifacts, but might also indicate that the cytoplasmic membranes are normally in a state of bilaterally unequal tension, and that fragmentation releases this tension and permits the broken fragments to coil.

The proliferation of mitochondria is of particular interest, in that it occurs before symptom expression on the leaf, and in that an increase in the number of mitochondria should result in an increase in the total amount of mitochondrial enzyme in the cell. We have, indeed, found (21) that mitochondrial preparations made from in-

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FIGURE 5 Portion of cell, 40 hours after TMV inoculation, in which cytoplasmic contents have moved away from cell wall (*W*) into vacuole (*V*). Chloroplasts (*C*) in this cell are still intact, but cytoplasmic membranes (*CM*) are loosened or ruptured (arrows).  $\times 7000$ .

FIGURE 6 Portion of cell containing a chloroplast (*C*) in which internal vacuolation (*IV*) can be seen, as well as the loosening of the chloroplast membrane (*CPM*) from the contents. The cytoplasmic membranes are fragmented, and are seen as strands of varying lengths (*CM*) or as coils (*CM* with arrows). The vacuole (*V*) contains a great deal of vesicular debris. Mitochondria (*M*) in this cell appear to be normal.  $\times 19,500$ .



infected tissues have at least twice the nitrogen content of those from healthy tissues. This supports our estimate, from scanning the sections, of a doubling in mitochondrial numbers. Furthermore, the enzymatic activity of such preparations is much higher than that of preparations from healthy tissues, as evidenced by succinoxidase activity (21). Thus, the effect of the virus infection on proliferation of mitochondria helps to explain the over-all rise in respiration of intact, infected tissues (17). Increase in numbers of mitochondria is not restricted to the infection studied here, but often results from other pathological conditions (2, 8).

The degeneration of the mitochondria later in the course of infection is well correlated with the observation (17) that at this time the respiration of intact leaf disks falls rapidly to a rate below that of healthy disks, and with our findings that mitochondrial preparations at this time yield very low succinoxidase activity (21). The changes that are seen to occur in the mitochondria are found in other systems, and appear to be common in animal cells infected with virus, and even in normal cells undergoing degeneration as a result of aging (13).

The appearance of electron-opaque bodies also seems to be characteristic of animal cells infected with cytoplasmic viruses. The origin of these bodies is uncertain (8, 11). However, within the cells of TMV-infected *N. glutinosa*, dense bodies that were sufficiently electron-transparent to show detail contained structures resembling cristae. Since the number of dense bodies appeared to increase as infection proceeded, this suggested that they were formed by degeneration of mitochondria.

The first changes in the chloroplasts were correlated with the starch lesions, first observed by Holmes (5), that can be demonstrated by staining

an infected leaf with iodine. The electron micrographs show clearly that these lesions result from a rapid increase in size of the starch grains already present, rather than from formation of additional ones. The non-structured, opalescent vacuoles surrounding the swollen starch grains have not been identified with published descriptions of chloroplasts, except possibly with the vacuole development that accompanies degradation of chloroplasts in yellowing tobacco (16).

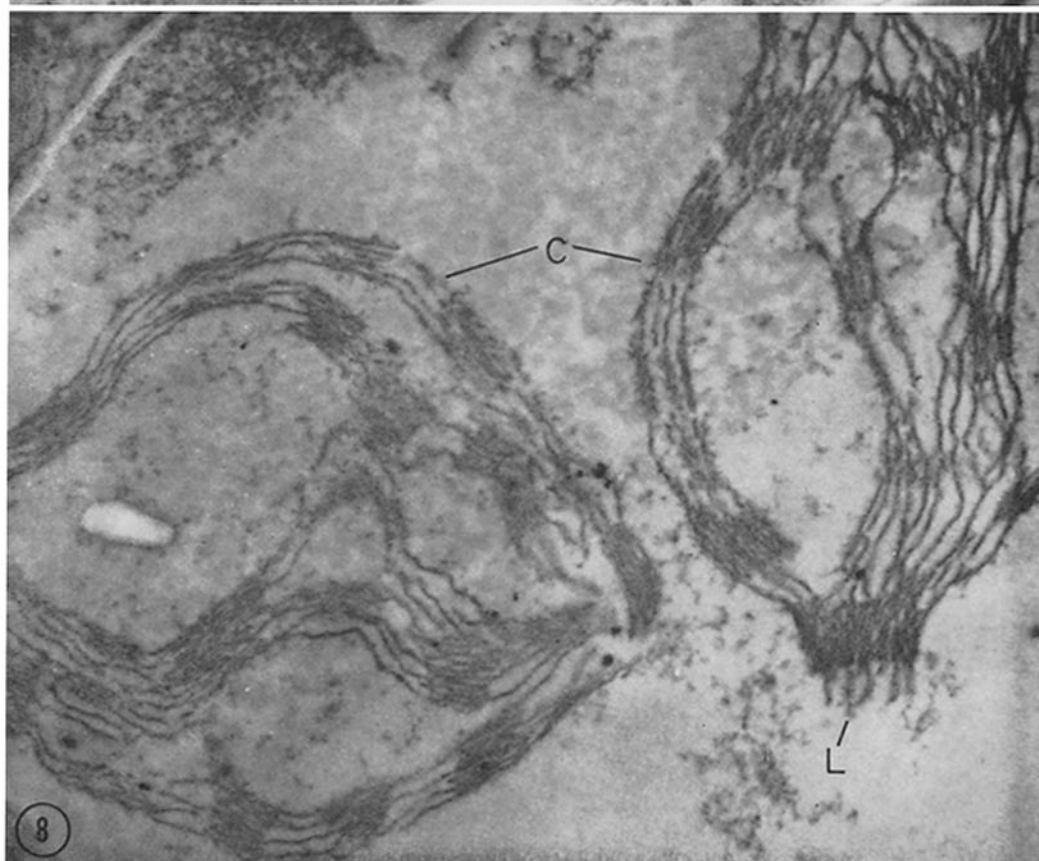
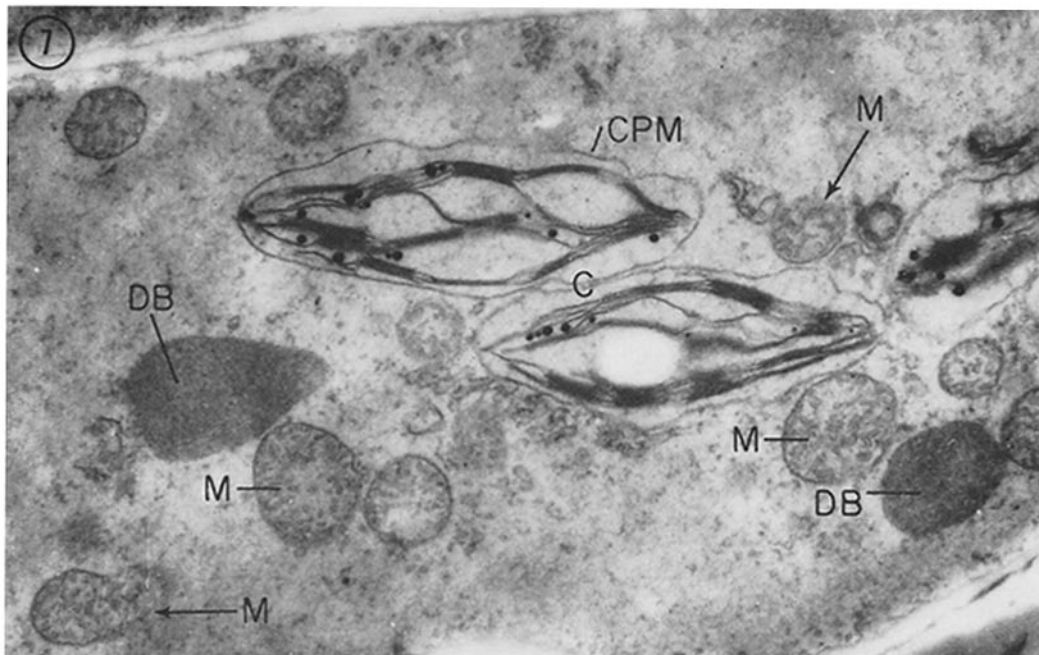
The apparent stability of the nucleus is remarkable, considering the surrounding destruction. Using a phase contrast microscope, Hooker and Bald (6) described a series of optical changes in the cytoplasm and nuclei of cells of tobacco leaves on which early lesions were developing after infection with tobacco necrosis virus. They observed the emission of a substance from the nucleolus into the nucleus, which increased in opacity. We observed no comparable changes. But it must be borne in mind that many of the changes described by Hooker and Bald are transient and so cannot be seen with the EM. Furthermore, with the latter instrument, the total number of cells examined and the volume of any one cell in a section are small compared with what can be seen with any light microscope. The chances for what Rose and Morgan call sampling error (13) are therefore great. However, cells in a lesion can be studied with the EM at a much later stage of degeneration than with the phase contrast microscope. Such cells contained intact nuclei.

The terminal stages of infection were marked by the increasing electron opacity of the remaining cell contents which were packed tightly into a corner of the cell. Presumably this darkening of the contents is the effect of polyphenol oxidation, and the deposition of polyphenols and tannins. The tearing away of cell walls from adjoining cells in the terminal stages may be brought about

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FIGURE 7 Portion of cell 78 hours after TMV inoculation. Chloroplasts (C) are vacuolated, and their membranes (CPM) loosened. The mitochondria (M) show varying degrees of electron opacity, while several appear to be disrupted (M with arrows). Dense bodies (DB) are present, the internal contents of which cannot be seen.  $\times 11,500$ .

FIGURE 8 Two chloroplasts (C) in a cell 78 hours after TMV inoculation. The chloroplast membranes have disintegrated completely, and the lamellae (L) lie freely in the cell, but are still in stacks.  $\times 20,000$ .



by changes in the composition of the middle lamella to water-soluble pectins, which are very soft materials (18).

No structures were seen at any stage that could be identified as TMV particles, which agrees with the findings of Shalla (15). This is not surprising since our calculations (19) indicate that no more than  $10^3$  virus particles per cell are synthesized in a local lesion. Recently, Hayashi and Matsui (3) claimed to have found TMV particles in local lesions on *N. glutinosa*. However, it has been suggested (20) that their particles are better interpreted as stacks of lamellae from disintegrated chloroplasts.

The absence of recognizable virus particles in

our sections, and our calculation that a very small amount of virus is synthesized, fits well with the hypothesis of Ackermann (1) that the primary cytopathogenic effect of viruses may be not in the synthesis of foreign materials but in the uncoupling of the normal metabolic processes and controls. This could well explain the devastating effects on cellular structure of a limited virus synthesis.

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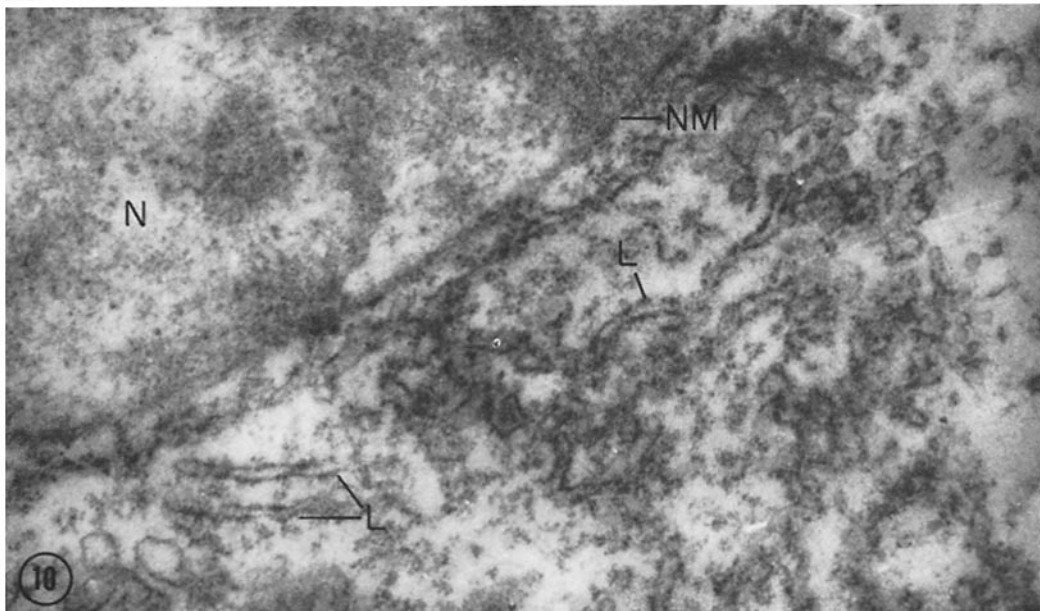
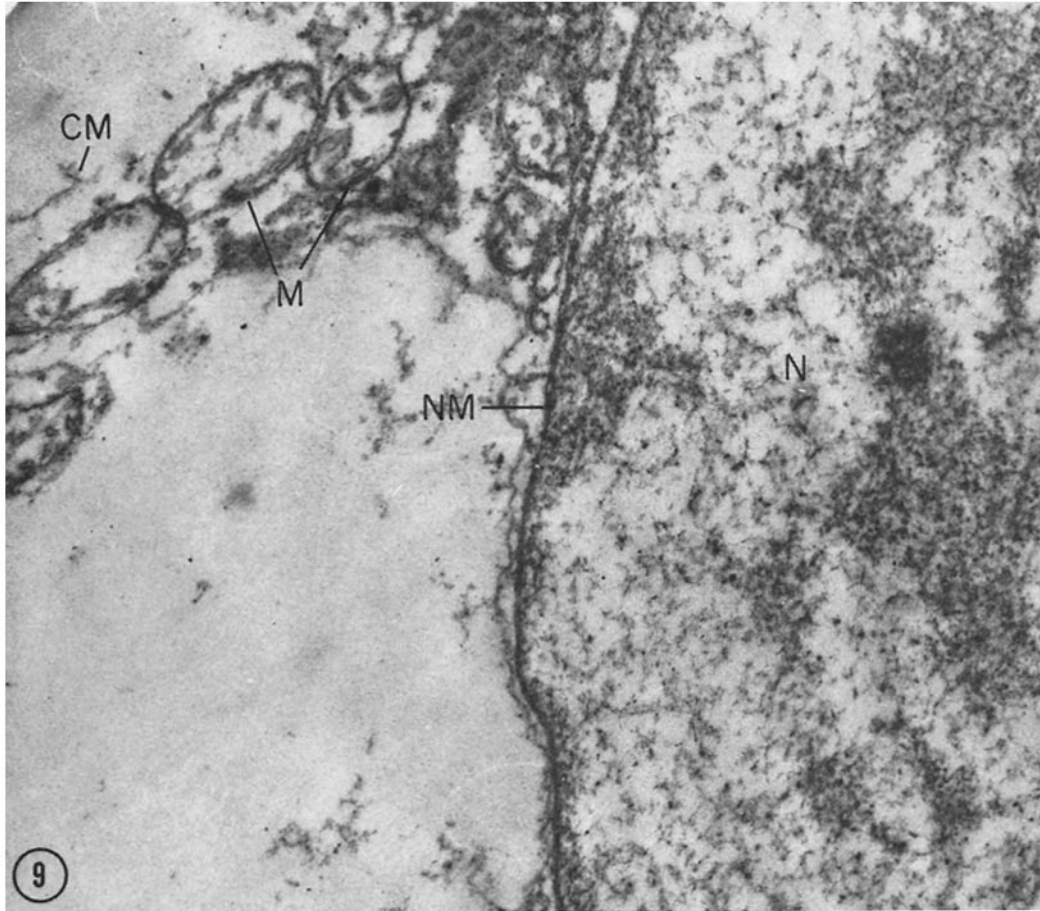
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FIGURE 9 Portion of a cell, 40 hours after TMV inoculation, containing intact nucleus (*N*) and double nuclear membrane (*NM*). Other membranes are in various stages of disintegration (*CM*), and mitochondria (*M*) have increased greatly in number.  $\times 19,000$ .

FIGURE 10 Portion of a cell, 78 hours after TMV inoculation, in which no intact mitochondria were found. Long lamellar structures (*L*) are seen in abundance. The nucleus (*N*) appears normal, and its membrane (*NM*) intact.  $\times 39,000$ .





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FIGURE 11 The chloroplasts (*C*) and other cytoplasmic contents including mitochondria (*M*) begin to move into a compact mass. At this stage all components of the organelles, such as the grana of the chloroplasts (*G*), and the cristae of mitochondria (*Cr*), are still readily distinguishable.  $\times 10,000$ .

FIGURE 12 A late stage in the death of the cell, 78 hours after TMV inoculation. The contents are compressed into an electron-opaque mass, and few details of the internal organelles can be seen except for traces of the chloroplast lamellae (*L*), and more rarely the vague outline of a complete chloroplast (*C*) including the osmiophilic granules (*O*).  $\times 13,500$ .

FIGURE 13 Portions of 2 adjoining cells, 78 hours after TMV inoculation, showing the uneven progression of cell death. In the upper cell the cell contents, including degenerated chloroplasts (*C*) and assorted debris, are in the process of moving towards the cell wall (*W*) in one corner of the cell. The lower cell has passed this stage, and the contents are compressed and electron opaque, with only traces of lamellae still visible.  $\times 7500$ .

FIGURE 14 Portions of cells in the last stages of death. The cell contents are packed into an extremely electron-opaque mass (*DM*) in one portion of the cell. Only very faint traces of structures, probably lamellae (*L*), can be detected. The rest of the cell has collapsed, the cell walls tearing away from adjoining cells, so that the walls extend across an empty space (*S*) in the form of a double process (*W*) separated by a small amount of electron-opaque material. The cells in the lower portion of the photograph are in earlier stages of disintegration, where both chloroplasts (*C*) and fragments of cytoplasmic membranes (*CM*) are visible.  $\times 4000$ .

