

Effects of Preparative Procedures on the Preservation of Tobacco Mosaic Virus Inclusions

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ABSTRACT

Tobacco mosaic virus (TMV) inclusions could be preserved consistently in the crystalline state if five variables were met in the following order: (i) continuous light-treatment of turgid plants for 36 to 144 hr; (ii) Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) citrate as the fixative buffer; (iii) chromic acid-formaldehyde as the fixative; (iv) acetone as the dehydrant, and (v) Epon 812 or Spurr low-viscosity resin for final embedding. No stabilizing influence of divalent cations or anions on the crystalline

inclusions was found. Inclusions were dispersed, either partly or completely, by dehydrating agents other than acetone and by embedding resins other than Epon 812 or Spurr low-viscosity resin.

Phosphate-buffered 3-5% glutaraldehyde, or Karnovsky's fixative, invariably dispersed TMV inclusions, irrespective of pretreatment or postfixation of the tissue. The entire preparative process could be followed in selected haircells.

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There are few objective criteria by which to judge the quality of preservation of tissue fixed and embedded for electron microscopy. One such criterion is the preservation of structures that can be seen in living cells by phase microscopy. Crystalline inclusions of tobacco mosaic virus (TMV) can be readily observed by phase microscopy, and the observation that inclusions are present in embedded tissue, as they were in the same tissue when it was living, is an excellent criterion of good preservation.

We have used this criterion to develop procedures that

consistently preserve all the TMV inclusions in tissue as a prerequisite to studies on sites of synthesis of TMV. Previous results (14) showed unsatisfactory fixation of cell contents, though the virus appeared to be immobilized. Preservation of crystalline inclusions does not guarantee that virus in other structures will be preserved without artifact, but if the inclusions are disrupted, it is certain that artifacts will be present.

Numerous reports, beginning with that of Black et al. (2), have appeared on the ultrastructure of TMV-infected tissue. The results have been reviewed recently (5, 18).

Commonly used chemical fixatives, or freeze-drying, preserve some of the crystalline inclusions, as shown by the many published micrographs of intact, or nearly intact, inclusions (5, 9, 13, 20, 22, 24, 26, 27, 28, 29, 30). However, we were not able to preserve all inclusions by the commonly used procedures, nor has any data been published showing that this can be done. Usually, most inclusions are partly or wholly disrupted, but a search will reveal some which have remained intact. We report here a procedure that consistently preserves the crystalline inclusions present in a piece of tissue.

MATERIALS AND METHODS.—*Virus.*—The common strain of TMV, American Type Culture Collection (1) no. 2, was maintained in *Nicotiana tabacum* L. cultivar (cv.) 'Xanthi' in the greenhouse. Infected plants were placed under continuous low light 4,300 lx (400 foot-candles) at 25 C for 36-144 hr, and then in the dark to reduce the starch grains in the chloroplasts before tissue was harvested for fixation. Potential source leaves were examined in the light microscope without staining, and a leaf that had virus crystals in many cells was selected for fixation.

Fixative and embedding procedure of choice.—Tissue pieces were fixed, dehydrated, and embedded in Spurr's resin (23) as described previously for tobacco tissue (15). Tissue was cut in 1 × 1 mm pieces with a razor blade. It was vacuum-infiltrated to replace air in the intercellular spaces with the following fixative: 0.008 M chromic acid, 0.13 M formaldehyde (from stock 37% formaldehyde stabilized with 10-15% methanol), 0.1 M Hepes (N-2-hydroxyethylpiperazine-N'-2-ethane sulfonic acid) and 0.05 M sodium citrate. The pH of the buffer was raised to 7.2 with NaOH. Tissue was fixed at 3 C for 8 to 24 hr. Then it was dehydrated with a cold acetone series and infiltrated with Epon 812, Spurr low-viscosity resin (23), or a mixture of the plastics. Tissue was also fixed with Karnovsky's (12) fixative, dehydrated, and embedded as described by Esau & Cronshaw (6).

Sections were stained with uranyl acetate and lead citrate according to standard procedures (21) and viewed at 100 kV in a RCA-3G electron microscope.

Embedding.—All tissues were flat-embedded and examined with the light microscope for the preservation of TMV crystals. Spurr-resin-embedded tissues were also macerated on a glass slide in resin, the drops were transferred to a carbon-coated slide, and the resin was polymerized under a carbon-coated coverslip (15). TMV crystals in cells, or free in the released resin sheet, were photographed under oil immersion with Zeiss phase optics and with Nomarski interference-contrast. Selected tissue pieces or inclusions were cut out and mounted at the desired angle for thin-sectioning with glass or diamond knives.

RESULTS.—The presence of crystalline inclusions of identical appearance in the living tissue and in the same piece of tissue after fixation and embedding was taken as

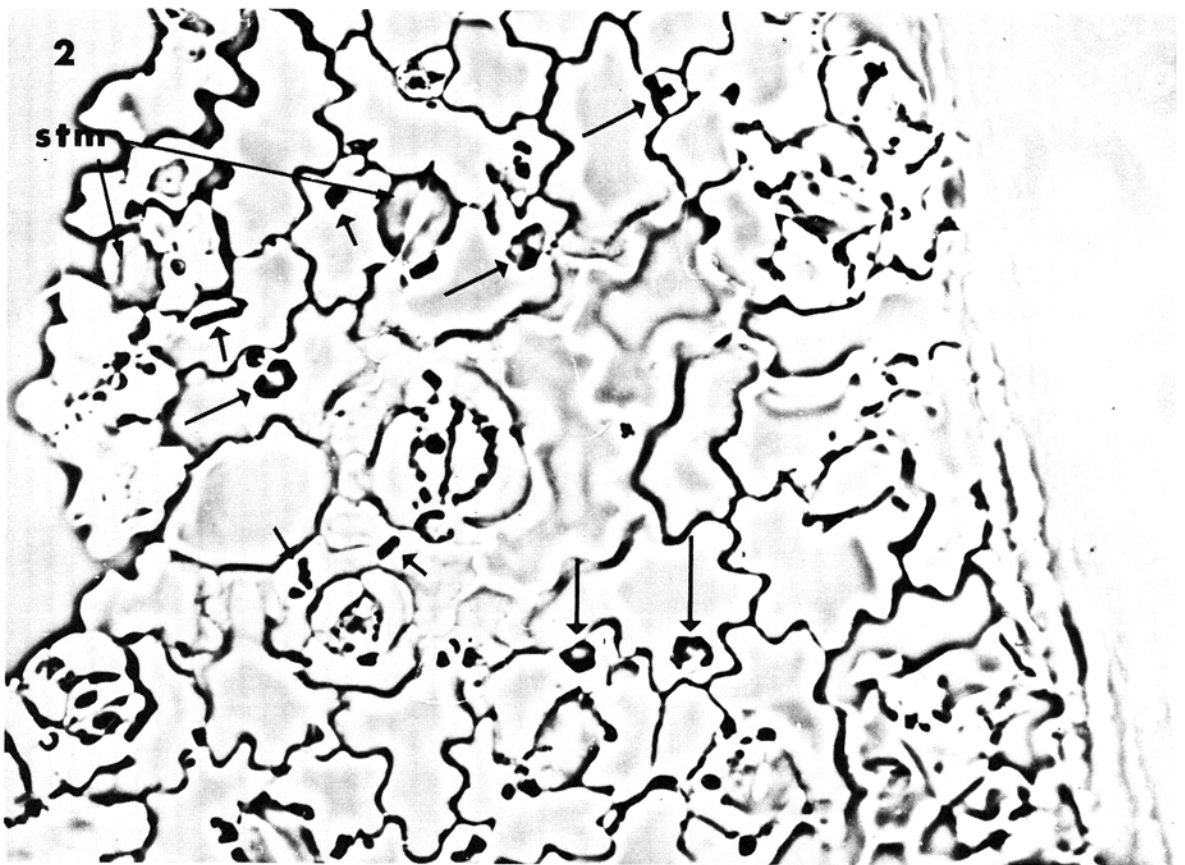
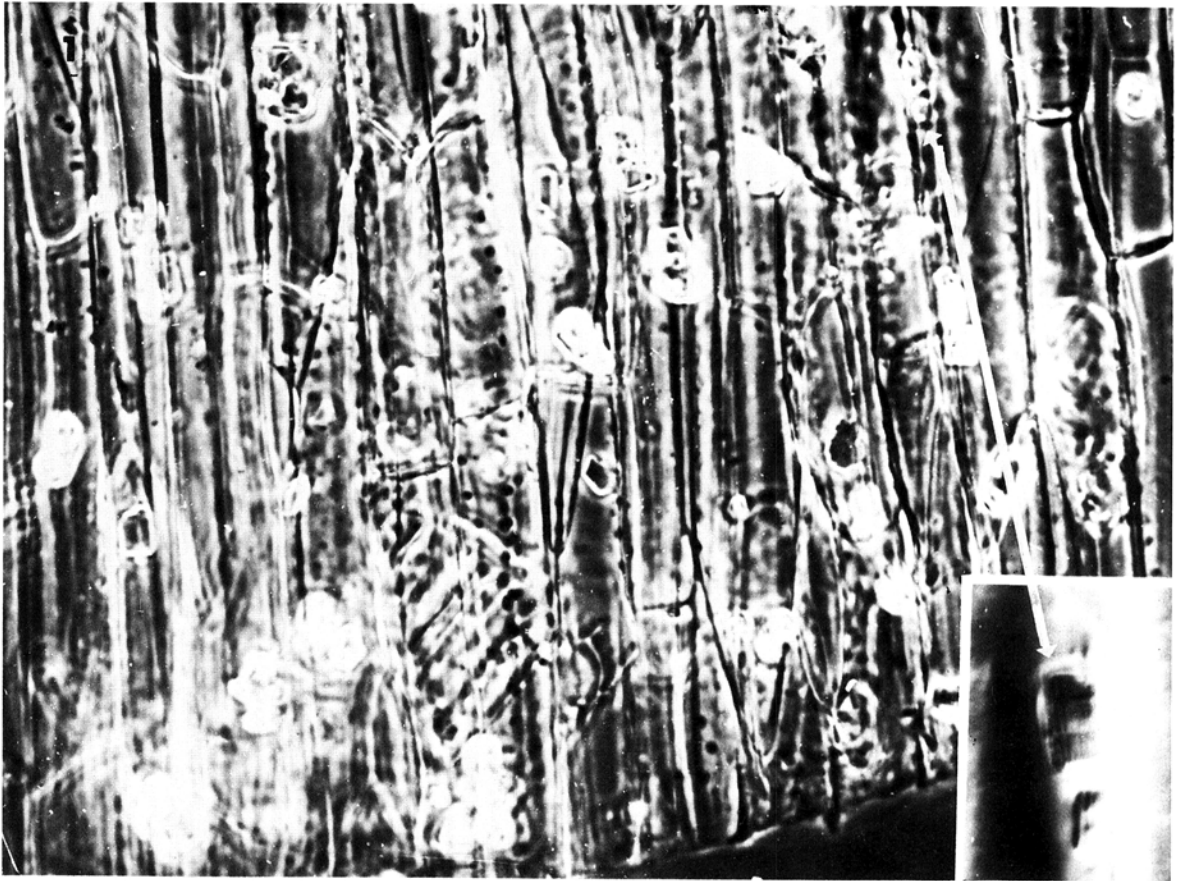
a sign of good preservation. A flat-embedding procedure allowing high resolution phase-light microscopy for the selection of tissue or subcellular organelles was developed to follow the preservation procedure (15). The TMV fixation procedure, as described, was highly reproducible, and TMV inclusions present in the living tissue were preserved. Figure 1 is a phase-contrast light micrograph of a piece of stem epidermis embedded in Spurr's low-viscosity epoxy. Crystalline inclusions are visible as bright birefringent inclusions, or in outline when exactly in focus. Two crystals (inset) were photographed under oil immersion with interference-contrast optics.

Several conditions had to be met to preserve all inclusions present in living cells: (i) continuous low-light treatment for at least 36 hr, but not more than 144 hr, followed by 8 hr dark treatment; (ii) Hepes-citrate as fixative buffer; (iii) chromic acid as the main fixative; (iv) acetone as the dehydrant; and (v) use of Epon 812 or Spurr-resin (23) as the embedding medium. Often, many inclusions were preserved when the above conditions were altered; if chromic acid remained as the fixative, phosphate-citrate buffers could be substituted for Hepes-citrate buffer, but results were not as consistent.

Inclusions in haircells were more resistant to disruption than those in epidermal cells under conditions unfavorable for crystal preservation. The sequence of disruption in intact haircells was from the base to the top. Inclusions in the basal cell could be dissolved while inclusions in the cells towards the top or in the tip itself remained well preserved. Fixative, dehydrant, and resin apparently penetrated the basal cell and from there progressed from cell to cell toward the tip with very little, if any, penetrating the exterior cell wall directly to the tip cells. If the rate of infiltration with resin was accelerated, the haircells collapsed, whereas the rest of the tissue showed no sign of plasmolysis. Under unfavorable conditions, crystals in epidermal cells dissolved before dissolution of crystals in the basal haircells.

Sections of 1 to 1.5 microns were cut and examined in the light microscope to determine when tissue suitable for ultrathin sections had been reached. Figure 2 shows a light micrograph of a paradermal section of TMV-infected tobacco tissue. Fifty TMV crystals were counted in this section, part of which constitutes Fig. 2. A few compound hexagonal crystals have been sectioned nearly across the whole crystal, making identification somewhat easier from the micrograph (arrows). It seemed improbable that a one-micrometer, or thinner, section would show many crystals present. However, TMV infection of tobacco tissue often produces more than one crystalline inclusion per cell. The likelihood of sectioning several crystals simultaneously is thus increased (Fig. 2, 3). Ten crystal-bearing cells are shown in the ultrathin section of Fig. 3. The crystals are well preserved and unbroken, and there is no virus in the central vacuole. The

Fig. 1-2. 1) Phase-light microscope micrograph of Spurr-resin-embedded, TMV-infected, tobacco stem epidermis. Crystalline TMV inclusions were birefringent and show as bright areas (× 430). Inset is a light micrograph taken with Nomarski interference-contrast optics of two TMV inclusions (arrow) (× 1,050). 2) Phase-light micrograph of a paradermal, one-micron section through the epidermis of TMV-infected tobacco leaf tissue. Fifty well-preserved TMV inclusions were counted in this tissue section. Several inclusions are identified with arrows. Stomate (stm) (× 485).



cytoplasm of the fully expanded cells is extremely thin in places, but the crystals are undisrupted. The crystals have been preserved and are not held in position by the surrounding fixed cytoplasm, as might be expected with small inclusions.

It has been reported that TMV-infected tobacco tissue contains undifferentiated cells (18, Fig. 9, 13 B). We have not found undifferentiated cells in the chlorotic areas of *N. tabacum* cv. Xanthi when the tissue was cut across from upper to lower epidermis. Figure 4 shows a 1- μ section of chlorotic tissue infected with TMV. The section contains all the leaf constituents normally found in a healthy leaf. From left to right: upper epidermis, palisade layer, spongy mesophyll with a small vascular bundle, calcium oxalate storage cells, and lower epidermis. Part of the section (arrow) is shown with Nomarski interference-contrast to show the presence of a TMV inclusion.

Tissue sectioned paradermally, yielded hexagonal inclusions most frequently (Fig. 2); whereas, tissue cut from upper to lower epidermis showed layered aggregates in cross section (Fig. 3, 4). Hexagonal inclusions, although appearing intact and in one piece by phase-light microscopy, often possessed cracks not visible in the light microscope (Fig. 12). It is suspected that the preservation was somewhat less than perfect in these cases, and that the cracks were nonexistent in inclusions in the living plant. The least indication of imperfect fixation was cracking of the crystal, followed by slow or complete dissolution.

Less-than-perfect preservation of crystals resulted in what appeared to be a gradation from slight to complete disruption. The first sign in the process of disruption of an inclusion was loss of the herringbone arrangement, with virus particles still more or less aligned. The next apparent step was dissolution from the edge of the TMV inclusion. Figure 5 shows a crystal arrested in the disruption process by the polymerizing embedding plastic.

Figure 6 shows part of a well-fixed inclusion without major cracks. The virus particles in intact, well-fixed inclusions were always visible but were never as distinct as in disrupted inclusions. It is postulated that crystals contained virus and another substance, a precipitant, which obscured the individual particles.

Other fixatives and additives tested.—No proteinaceous, large anionic-, or di-, or trivalent cationic additives were found to have a stabilizing influence on the virus inclusions during fixation. Hepes was used as a buffer in tests of the influence of divalent and trivalent ions on the preservation of TMV crystals. High-ionic-strength salts (0.1 to 0.5 M sodium citrate) did not disrupt the TMV inclusions if the five conditions for adequate preservation were met. Plasmolysis of the cell contents was the only visible result.

Osmic acid (0.01-1%) was used in postfixation experiments because of reported successful preservation

of TMV inclusions (25, 26, 29, 30). The crystals showed an increasing instability with increasing concentration of osmic acid used as postfixative. With the chromic acid-formaldehyde fixative buffered with Hepes, no search had to be made for intact crystals, since all crystals were well preserved from the edge of the tissue piece to the center. The use of osmic acid was therefore omitted as not necessary and in fact was considered to be disruptive.

Results with all the above tests were compared with those obtained with buffered 3-5% glutaraldehyde as the fixative. In no instance did glutaraldehyde preserve TMV inclusions in our experiments. It is obvious that sometimes intact inclusions, or parts thereof, may be preserved with glutaraldehyde as shown by Granett & Shalla (9). These are exceptions, however, and not the rule.

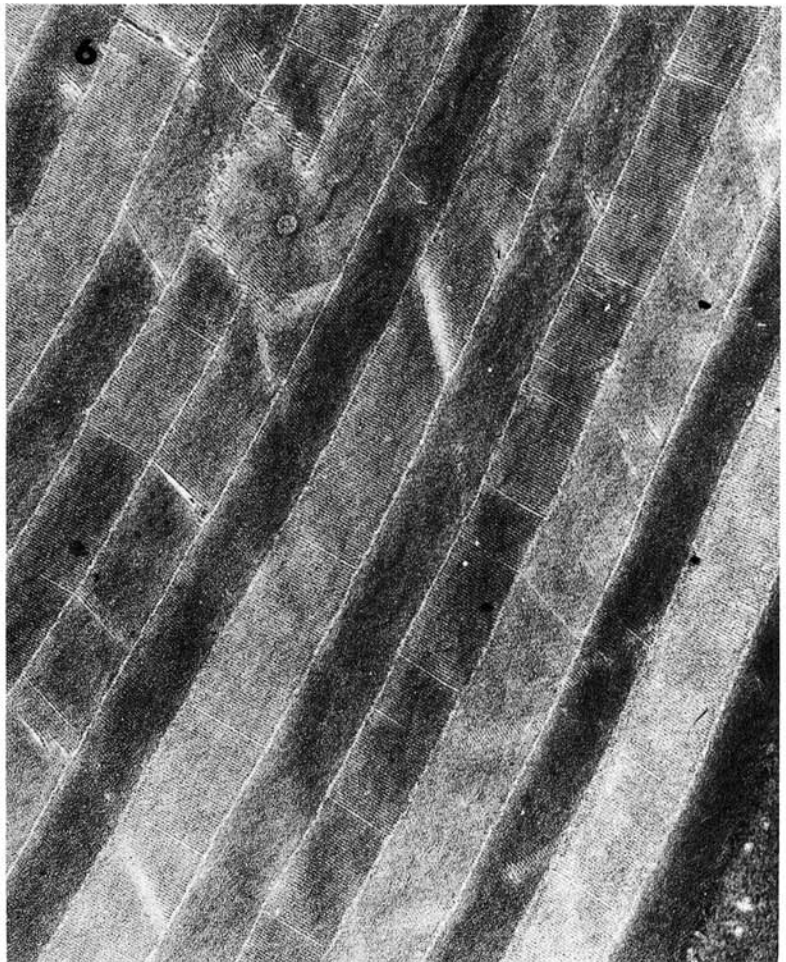
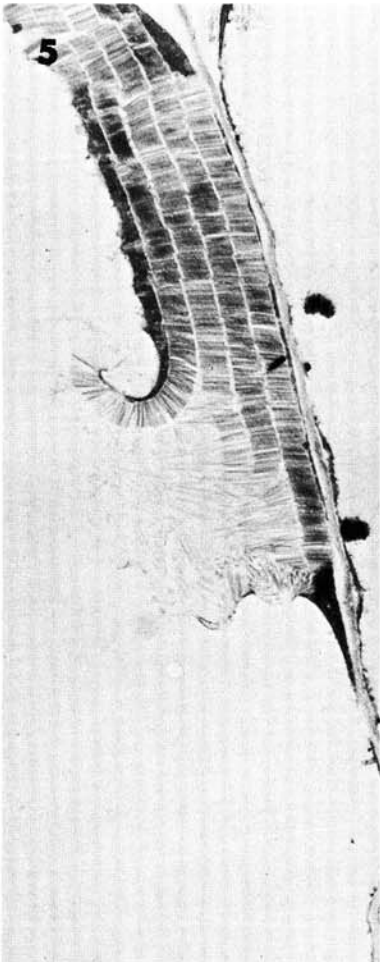
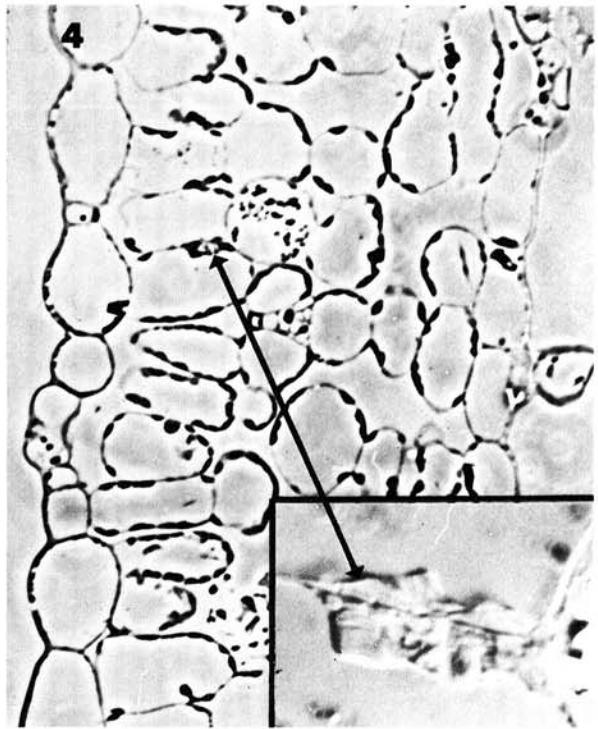
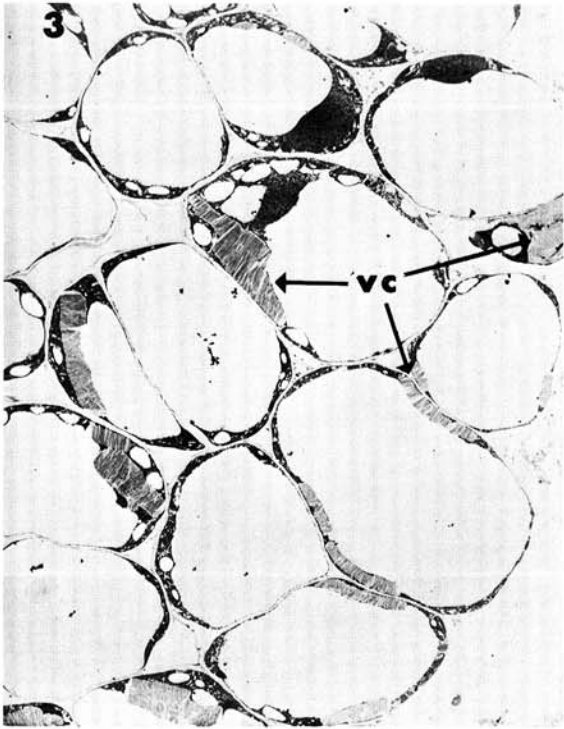
Classical fixation procedures as used in botanical histology: HgCl₂, I₂, picric acid, potassium bichromate, and formaldehyde (11) were not successful.

Light influence.—The influence of light treatment of host plants on crystal preservation was not immediately realized. A comparison of notes taken over several years showed that during the early summer months, successful preservation occurred more frequently than during the winter months. Temperature differences were tested and found to have little influence. Prolonged low-light treatments of plants, however, had a marked effect. Crystalline inclusions were best preserved by a 48- to 72-hr light treatment, followed by an 8-hr dark treatment. The dark treatment was used only to reduce the size of starch grains in chloroplasts and was not necessary for inclusion preservation. Prolonged light treatment of plants (> 168 hr) was detrimental to crystal preservation. Progressively more virus crystals disappeared from the final embedded tissue with increased light treatment of host plants.

Influence of dehydrants and embedding plastics.—Of all the dehydrants tested, cold acetone consistently gave best results. Alcohols necessitated the use of an intermediate solvent, such as propylene oxide. In parallel experiments, tissues infiltrated with Spurr plastic directly from acetone had intact TMV inclusions, whereas tissues embedded by means of propylene oxide had disrupted inclusions. Dehydrations with methanol, hexylene glycol, and methyl cellosolve preserved inclusions better than dehydration with ethanol, tetrahydrofuran, or ethylene glycol.

Resins tested.—Spurr-plastic was the best plastic for the preservation of inclusions, whereas Epon 812 (17), DER 332-732 (16), and Araldite 6005, used according to the schedule for Araldite 502 (21), were less effective in that order. Maraglass 655 (4), Vestopal W, Durcupan, methacrylate, and polyampholyte (19) were not usable. Equal mixtures of Epon and Spurr resin were also used successfully, as were mixtures of Spurr-resin and Araldite. More than 50% Araldite could not be used

Fig. 3-6. 3) Electron micrograph of TMV-infected tobacco tissue, showing preservation of virus inclusions (vc) in cells below the epidermis ($\times 1,400$). 4) Phase-light micrograph of a cross-section through chlorotic area of TMV-infected tobacco leaf tissue. Leaf structure essentially equal to that in healthy tobacco leaf, showing normal-appearing leaf palisade and spongy mesophyll cells ($\times 425$). 5) Crystalline inclusion of TMV that appears to have been arrested in slow dissolution, depositing virus particles in the central vacuole. Tissues had not received prolonged light treatment, and were Epon-embedded ($\times 10,500$). 6) Inclusion of TMV as encountered after prolonged (36-144 hr) light treatment ($\times 25,000$).



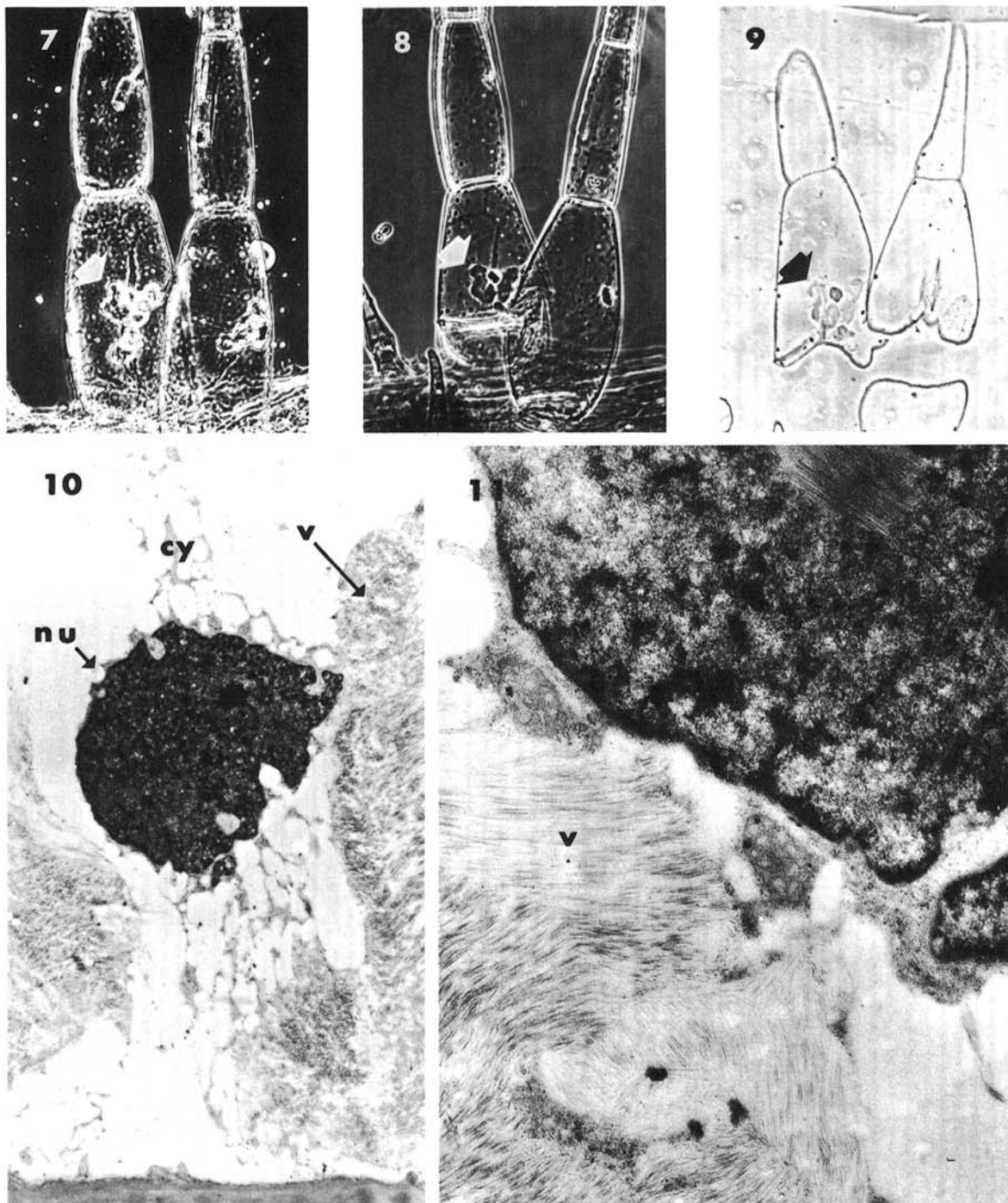


Fig. 7-11. 7) Phase-light micrograph of tobacco haircells immediately after placing in Karnovsky's (12) fixative. Birefringent intact TMV inclusions visible as bright areas ($\times 185$). 8) The same haircells after fixation, dehydration, and embedding according to Esau & Cronshaw (6). The previously birefringent inclusions completely (right haircell and cells above basal cells) or partly disrupted (left basal haircell), resulting in crystal ghosts. Phase-light micrograph ($\times 185$). 9) One-micron section of the same haircells. Phase-light micrograph ($\times 185$). 10) Electron micrograph of a thin section through the nucleus (nu) and the cytoplasmic strand (cy) visible in Fig. 7 and 8. Disoriented virus particles in the partly dissolved crystal (v). Notice the cytoplasmic invaginations in the nucleus ($\times 4,100$). 11) Enlarged portion of the lower left of the nucleus in Fig. 10. Micrograph taken from a serial section through the same area ($\times 17,500$).

without sacrificing some hexagonal inclusions, however.

Fixation with glutaraldehyde-formaldehyde mixtures.—Because of Esau's considerable efforts in the area of ultrastructural studies of TMV in the host cell, which were superbly and generously illustrated (5, 6, 7, 8), we have tested this procedure extensively.

With a recently described technique (15), it was possible to follow the influence of fixation, dehydration, etc. on a selected haircell, which contained a large birefringent TMV inclusion. The same haircell was photographed at the start of the fixation in Karnovsky's fixative (Fig. 7), after having been embedded in a thin film of polymerized plastic (Fig. 8), during 1- μ sectioning (Fig. 9), and finally at higher magnification (Fig. 10, 11). This experiment was repeated with four other haircells.

TMV inclusions were disrupted in all five instances, but some crystal ghosts were preserved (Fig. 8, left haircell, crystal surrounding the nucleus). The ghost is visible because a large concentration of disoriented virus has more or less remained in the original position. More often the crystals disappeared completely, with only the surrounding fixed cytoplasm indicating where the crystal had been (Fig. 8, right haircell). In contrast, Fig. 12 depicts a hexagonal TMV inclusion as found after

fixation with the chromic-acid procedure described herein.

Haircells had deeply invaginated nuclei containing virus and mitochondria. Nuclei of the underlying tissue were not as invaginated. The condition of nuclei in healthy haircells is unknown. Virus was found free in haircell nuclei not surrounded by membranes. The nucleus in the haircell shown in Fig. 10, 11 had virus, either as individual rods or as small aggregates.

Virus has been reported in nuclei on previous occasions (6, 9, 10).

DISCUSSION.—The crystalline inclusions of TMV in the plant cell are exceedingly fragile, a condition commented on by many authors (18). The fragility of the hexagonal prisms may best be exemplified by the fact that to our knowledge no micrograph of a paradermal section through a complete hexagonal inclusion has been previously published. Yet the majority of the inclusions seen in the living tissue consist of hexagonal virus inclusions or compound hexagonal inclusions.

The purpose of this research was to develop a procedure in which the crystalline inclusions would be preserved after fixation and embedding, a goal which has been accomplished. Previously used procedures

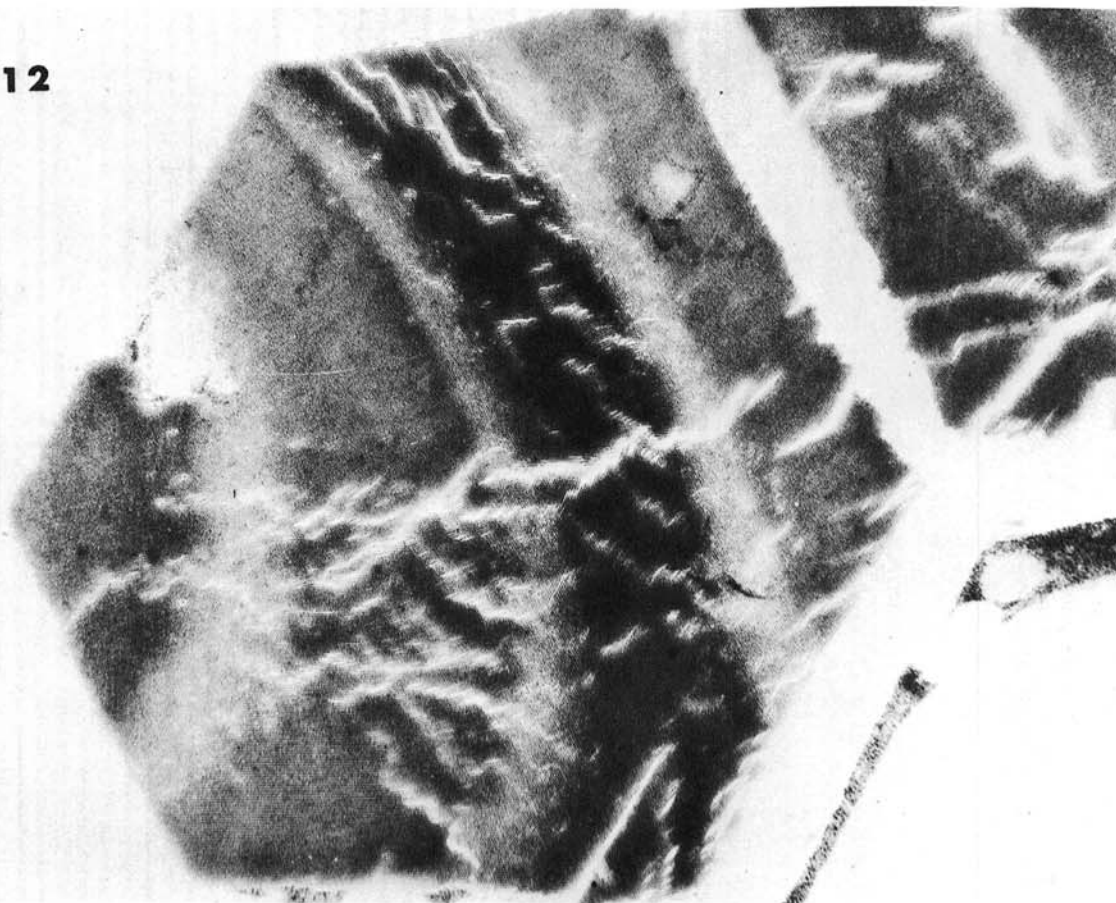
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Fig. 12. Hexagonal TMV inclusion as encountered after chromic-acid fixation and embedding procedure as described in text. Sectioned obliquely, showing at least seven virus layers in the crystal ($\times 19,000$).

preserved only part of the inclusions, others being disrupted to varying degrees. In such a situation, it is possible to mistake a sequence of artifacts for a sequence of developmental stages in TMV crystal formation (6).

Crystalline TMV inclusions could not be consistently preserved by use of the chrome-formalin fixative alone. The physiological state of the plant as influenced by light treatment, the fixative, the buffer used in the fixative, the dehydrant, and the final embedding medium all were important. It is interesting to note that Cheo (3) recently reported a higher virus-replicating capacity in tobacco cultures under continuous light. Epoxy plastics differed in their interaction with the fixed tissue. We were unable to preserve TMV inclusions in Maraglass 655, yet Warmke and Edwardson could do so following permanganate fixation (27, 28).

Each component of the complex affects the final result. Therefore, one must consider the interaction of virus, tissue components, fixatives, dehydrants, and embedding plastics in procedures designed to preserve cell contents as they exist in living tissue.

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