Electron Microscopy of Unstable Inclusions Induced in Maize by Maize Dwarf Mosaic Virus

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ABSTRACT

Maize dwarf mosaic virus (MDMV) virions and virus-induced laminated aggregates (LA) were seen in glutaraldehyde-fixed and osmic acid-postfixed tissues if sulfhydryl compounds or enzyme inhibitors were present during glutaraldehyde fixation, but were absent, or rarely present, after conventional glutaraldehyde-osmic acid fixation. Laminated aggregates and virions were also observed with fixation procedures considered detrimental to enzymic action; i.e., osmic acid-only, formaldehyde, or chromic acid-Formalin fixations. Pinwheels were observed with all fixation procedures, but were more abundant after fixation with glutaraldehyde alone. The results suggest that the partial disappearance of MDMV and LA can be attributed to cellular lytic action during conventional glutaraldehyde fixation.

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Additional key words: sulfhydryl compounds, electron microscopy.

Relatively few virions, and no laminated aggregates (LA), have been reported in ultrastructural studies of tissue infected with maize dwarf mosaic virus (MDMV) (7, 13, 28, 31). Based on results with viruses of similar morphology, both virions and laminated aggregates should be observable. We have obtained 0.5—2.8 mg of purified MDMV virions from 100 g of infected tissue (14) similar to quantities of wheat streak mosaic virus (1) virions, which are readily seen in thin sections (25, 30).

MDMV belongs to the potato virus Y group, and laminated aggregates have been reported for many members of this group (for literature, see reference 33). Furthermore, MDMV is related to sugarcane mosaic virus (SCMV) H (26), and Edwardson (4) found laminated inclusions after osmic acid fixation of tissue infected with sugarcane mosaic virus. Later, Krass & Ford (13) in a comparative study could not distinguish ultrastructural differences in tissue infected with MDMV strains A and B and SCMV strain H. They did not report the presence of laminated aggregates.

Investigation of the problem has shown that the selection of the fixative is crucial for the observation of virions and laminated inclusions in MDMV-infected tissue. We report here that these structures are readily observed in tissue fixed with a buffered chrome fixative, with osmic acid alone, after formaldehyde fixation, or with glutaraldehyde prefixation in the presence of reducing agents or enzyme inhibitors and osmic acid postfixation. A preliminary report has appeared (15).

MATERIALS AND METHODS.—Maize dwarf mosaic virus (MDMV-B strain) was obtained from naturally infected sweet corn in Nebraska in 1967, and its identity determined by its failure to infect wheat or to systemically infect Johnson grass, and by serology (antiserum supplied by R. E. Ford, Ames, Iowa). Greenhouse-grown plants were kept in the dark overnight to rid chloroplasts of starch grains. In order to standardize observations and reduce sampling errors, chlorotic areas of unfolding leaves of sweet corn (*Zea mays* L. cv. 'Goldencross Bantam'), systemically infected with MDMV, were cut into 2 × 2 mm pieces 10-14 days after manual inoculation, vacuum-infiltrated to replace air in the intercellular spaces with fixative, and fixed for a specified time. Only laterally adjoining strips of tissue

Fig. 1. Sweet corn plant systemically infected with maize dwarf mosaic virus; area from which tissue was sampled is marked (× 1/4).

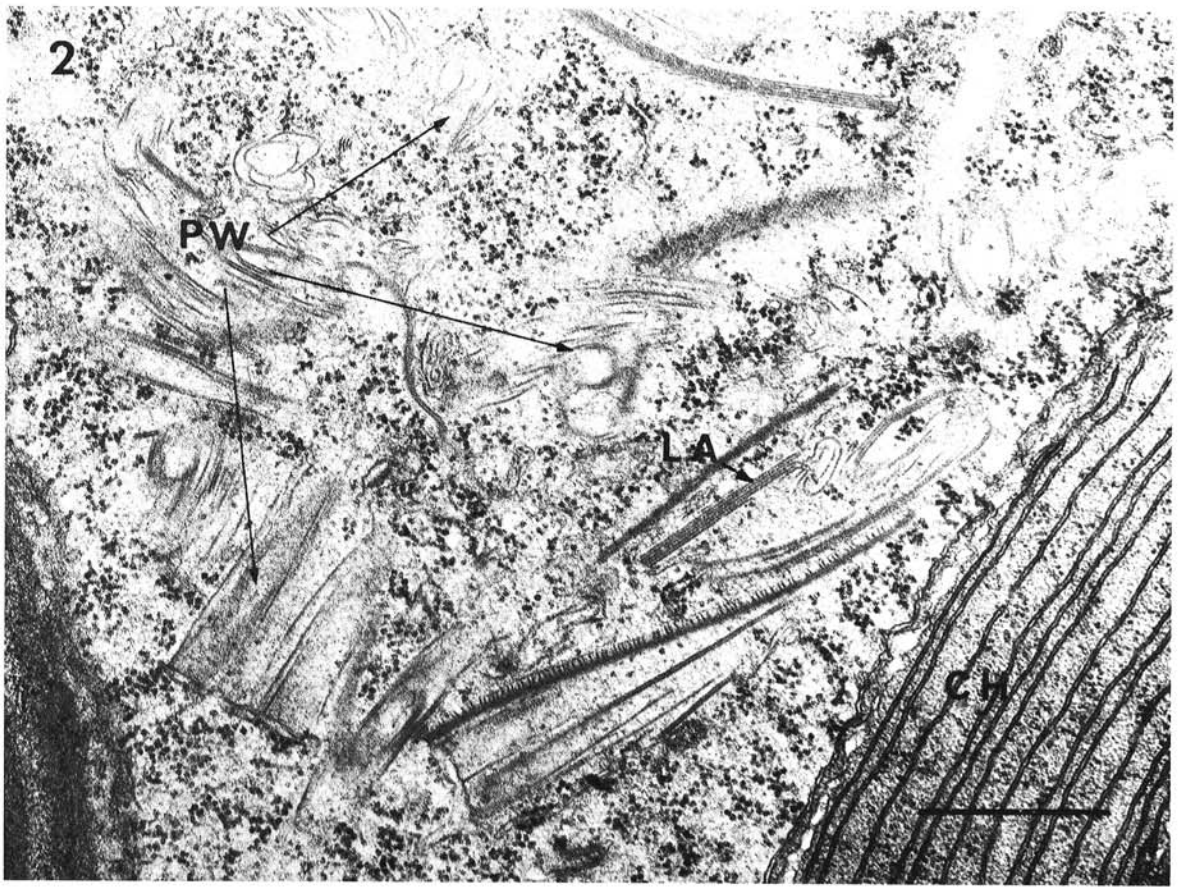


Fig. 2. Sheath cell of a vascular bundle of maize dwarf mosaic virus (MDMV)-infected corn. Laminated aggregates occurred rarely in cytoplasm of glutaraldehyde-osmic acid-fixed cells. Pinwheels (PW, arrows) were encountered in greater number than with other fixation methods and were sectioned in different planes. Chloroplast (CH) of the lamellar type were observed in sheath cells of healthy and diseased plants (9). Scale bar = 0.5 micrometer.

from one developmental stage of the leaf (Fig. 1) were chosen for comparison of fixative influences. Infected tissues were taken over a 3-year period from greenhouse-grown corn plants.

Four main fixatives were used with, or without, additives. The figure in brackets following a fixative procedure denotes the number of times tissue was sampled and fixed with the particular fixative during the course of this investigation. The following fixation procedures were followed: (i) Cold 5% glutaraldehyde (Biological grade, Fisher Scientific Co., St. Louis, Mo.) in 0.05 to 0.1 M phosphate buffer, pH 7.2, for 3 hr followed by six 15- to 30-min rinses in cold buffer and postfixation in cold 0.5-1% osmic acid in the same buffer for 1 hr [ten]. This fixation procedure was varied by adding one of the following materials to the glutaraldehyde (GA) fixative and to the buffer used for rinsing tissue between GA and osmic acid: 0.1 – 0.2% sodium thioglycolate, dithiothreitol, cysteine, or 2-mercaptoethanol (2-ME); 0.01 M butanethiol, 2-methyl-1-propanethiol, sodium iodoacetate, sodium diethyldithiocarbamate (Na-DIECA), or 0.03 M diethyl pyrocarbonate (DEP) [twenty-five, with additives]. Emulsification of the complete fixative was sometimes necessary when the additive was oily; e.g. DEP. (ii) Cold 1% osmic acid in 0.05 – 0.1 M phosphate buffer, pH 7.2, with fixation for 1 hr at 3 C [two]. (iii) A buffered chrome fixative of pH 7.2. The fixative was prepared from stock solutions just before use and consisted of 0.008 M chromic acid, 0.13 M formaldehyde (from 37% commercial formaldehyde stabilized with 10-15% methanol) in 0.1 M phosphate buffer. Tissues were vacuum-infiltrated with the fixative and then placed at 3 C for 18-22 hr. Osmic acid was then added to 0.3–0.5%, and fixation continued for 2 hr at 3 C. Sodium thioglycolate (0.2%), 0.01 M sodium iodoacetate, or 0.1 M DEP was added in some experiments. Dithiothreitol, cysteine, and Na-DIECA were incompatible with chromic acid at pH 7.2. Tissues were not rinsed with buffer after fixation in the chromic acid fixative and before osmic acid fixation [fourteen]. (iv) Formaldehyde (4%) prepared from paraformaldehyde (19) in 0.1 M phosphate buffer, pH 7.2, for 22 hr and with and without 0.01 M 2-ME. The tissues were washed with cold phosphate buffer, with and without 2-ME, and postfixed in 1% osmic acid in cold buffer for 1 hr [three].

All tissues were dehydrated after fixation in a cold

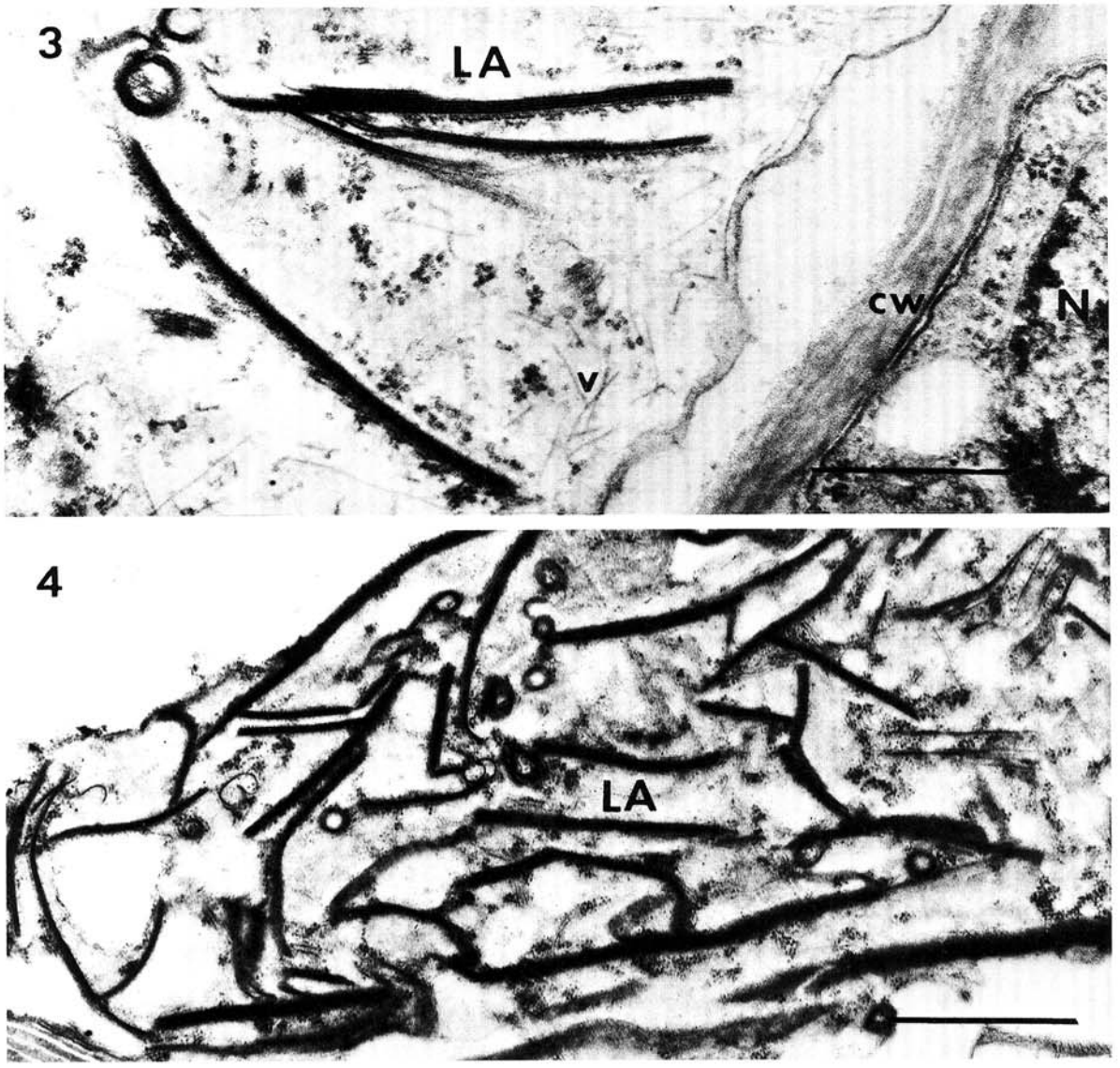
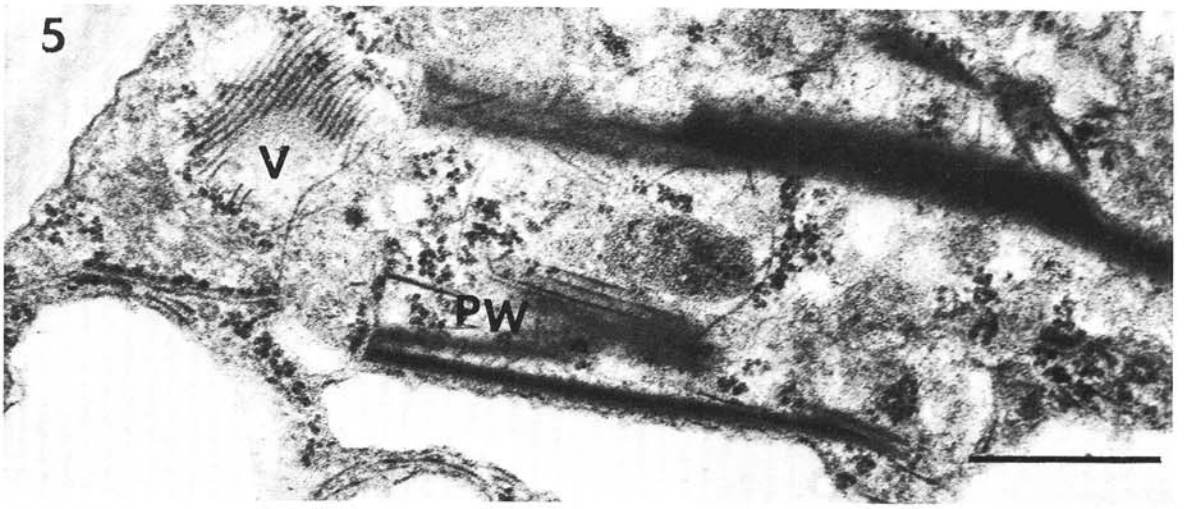


Fig. 3-4. 3) Epidermal cell of MDMV-infected sweet corn and part of stomate guard cell. Tissue was fixed in glutaraldehyde containing thioglycolate and Na-DIECA and postfixed in osmic acid. Laminated aggregate (LA), virus (v), cell wall (cw), and nucleus (N) in guard cell. Scale bar = 0.5 micrometer. **4)** Conglomerate of laminated aggregates (LA) and pinwheels in corn cell infected with MDMV. Fixation as in Fig. 2 but in the presence of 0.2% thioglycolate. Scale bar = 1 micrometer.

methanol or acetone series. Changes were made at 15-min intervals to 100%, when the fixation vessels were brought to room temperature. Tissues were changed to 100% propylene oxide via a 50:50 propylene oxide-methanol, or propylene oxide-acetone, mixture and embedded in Spurr's low-viscosity plastic (29), Araldite 6005, or a combination of these two plastics.

Fifty-four independent fixation experiments were made. From each experiment, one to four tissue blocks were sectioned and thin sections examined. Sections were

Fig. 5-7. 5) Parenchyma cell in MDMV-infected sweet corn. Virus (V) in small orderly aggregate, pinwheel (PW) cut parallel to the long axis. Prefixation in glutaraldehyde with thioglycolate and postfixation with osmic acid. Scale bar = 0.5 micrometer. **6)** Thin section of pellet of partially purified, glutaraldehyde-osmic acid-fixed MDMV. Virus (V) is visible under the conditions of fixation for tissue but is not frequently seen in tissue after this fixation. Scale bar = 0.5 micrometer. **7)** Part of parenchyma cell of sweet corn infected with MDMV. Fixation in osmic acid only. Laminated aggregates (LA) and virus (V) more visible than with glutaraldehyde-osmic acid fixations. Scale bar = 0.5 micrometer.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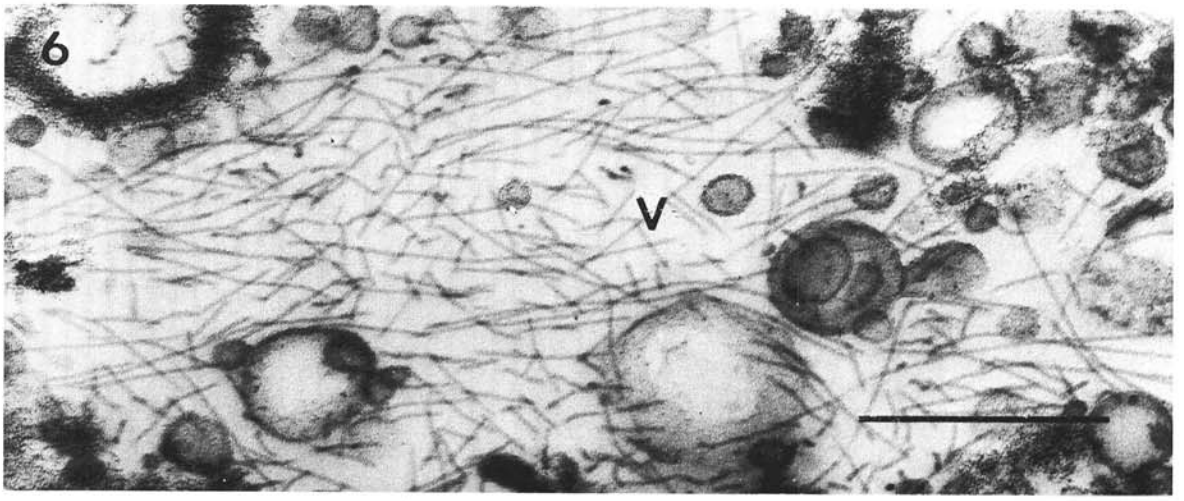
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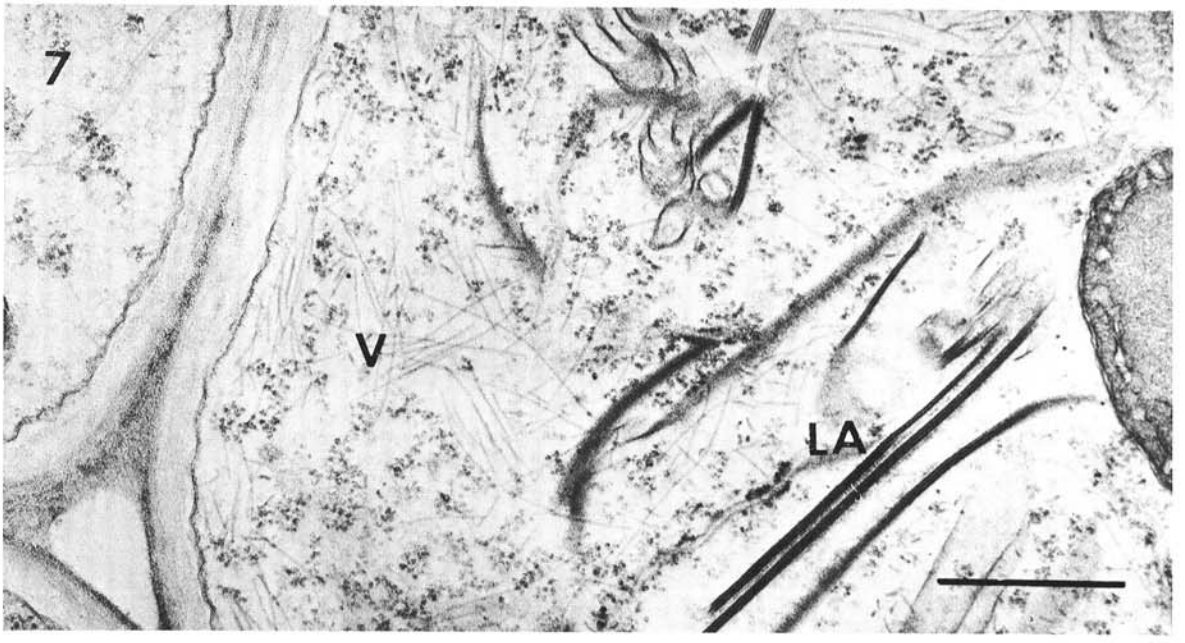
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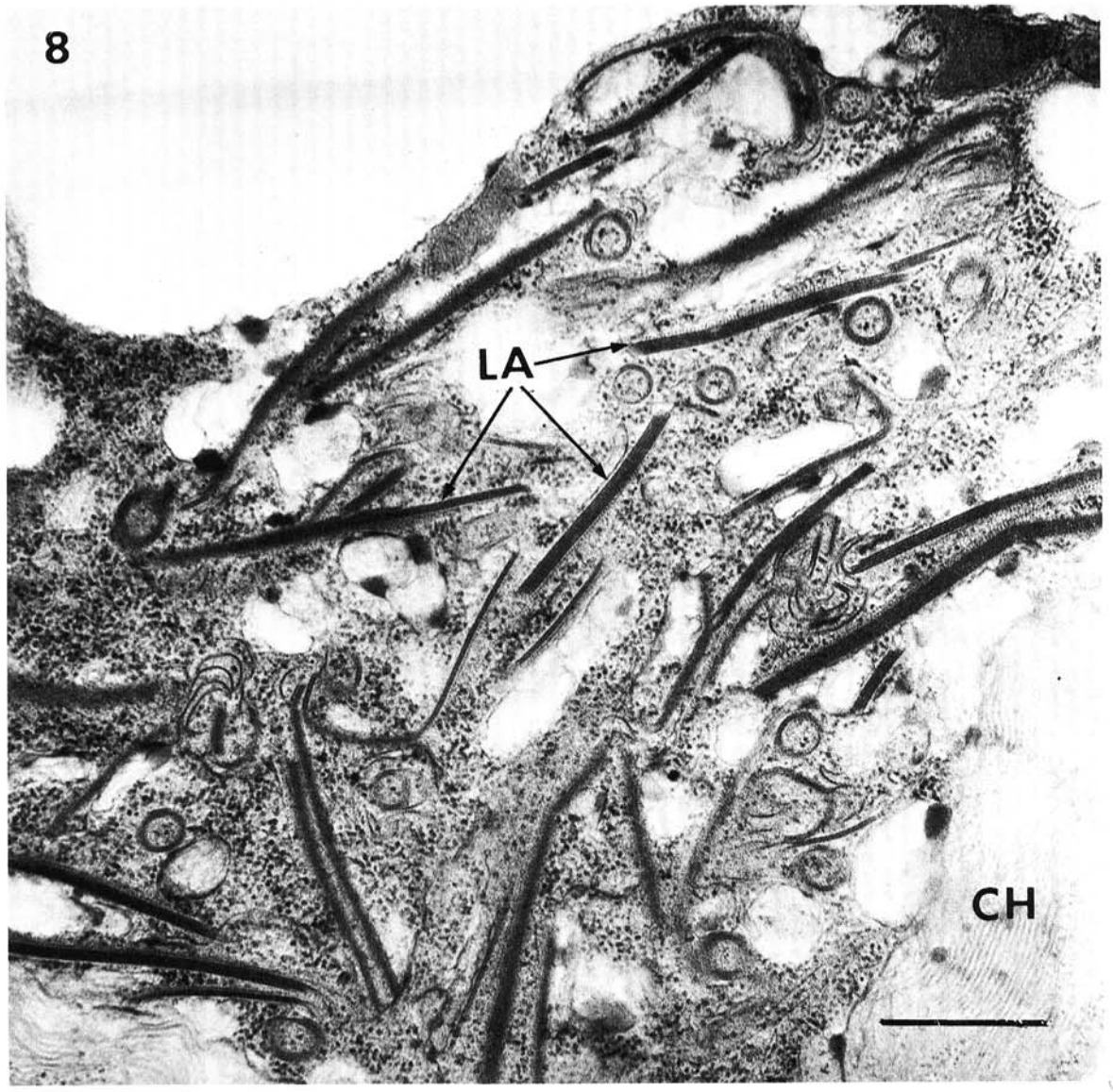
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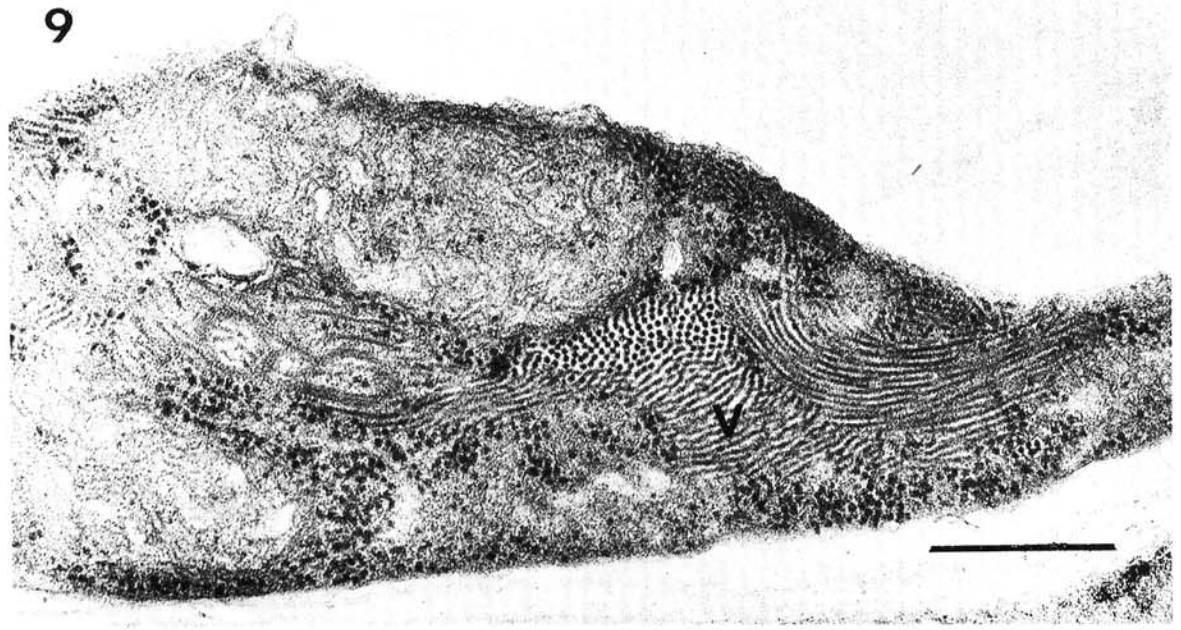
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V

stained with uranyl acetate followed by lead citrate according to standard procedures (19), and viewed at 100 KV in a RCA-3G electron microscope.

RESULTS.—*Fixation in glutaraldehyde and osmic acid.*—Virus and LA were rarely observed in sections of leaf tissue fixed in glutaraldehyde without additives and followed by osmic acid. Of the 10 experiments in which tissue was fixed in GA and postfixed in osmic acid, LA were encountered in only one tissue block (Fig. 2). Pinwheels, sectioned all angles, were abundant (Fig. 2). The results were similar to those reported earlier for similarly fixed tissue (7, 13).

Results were quite different when sulfhydryl compounds or enzyme inhibitors were added to the glutaraldehyde. Individual virus particles were scattered through the cytoplasm of cells, including guard cells of stomates and other epidermal cells (Fig. 3). Laminated aggregates and virus were preserved equally well by glutaraldehyde in the presence of any of the sulfhydryl compounds or enzyme inhibitors. Dithiothreitol was used more than the other additives because of convenience and less odor. Reducing agents other than mercaptals were not tested.

In this study, LA were encountered in cells fixed with GA if an enzyme inhibitor (e.g. DEP) or reducing agent was present. Laminates consisted of three to nine parallel plates. Dimensions of laminated aggregates differed from one to another, as did the length of plates within one aggregate (Fig. 3). Moreover, some LA consisted of two or more joined aggregates as determined by serial sections. Such complexing of LA has also been reported for parsnip mosaic virus, a virus with a particle morphology similar to MDMV (17). Right-angle bands occurred in other aggregates (not shown). Laminated aggregates were often associated in masses in the cytoplasm (Fig. 4).

Laminated aggregates and pinwheels did not occur simultaneously in appreciable numbers. When LA were present in great abundance, pinwheels were few (Fig. 3, 4, 8). Several laminated aggregates were interconnected by pinwheels (Fig. 2, 4, 8).

Virus was mostly dispersed; however, there were exceptions; e.g., the small aggregate shown in Fig. 5 and the larger one in Fig. 9. Figure 5 also shows a tangential cut through a pinwheel arm plate. Such tangential sections through a pinwheel could be misconstrued as a LA. The laminate appearance, however, is lacking.

Adjacent tissue pieces were used for fixation in GA with and without additives. Pinwheels were always present, but LA and virus were much more visible when reducing agents or enzyme inhibitors were included. Plumb & Vince (20) reported that the Rothamsted culture of henbane mosaic virus stretched when glutaraldehyde fixation was followed by osmic acid. We observed a similar result with MDMV; however, the stretching was inconsistent. Partially purified, pelleted, virus was readily visible after fixation in GA without additives, followed by osmic acid fixation, embedding, and sectioning (Fig. 6). The same fixation procedure used for tissue, resulted in poorly visible virus in tissue cells. However, partially purified virus had been removed from most cellular contents before fixation. Virus in tissue during fixation would be subject to reactions of cellular components.

Osmic acid-only.—Fixed tissue showed virus, pinwheels, and LA present (Fig. 7). They were found in sections without difficulty. Osmic acid is detrimental to enzymic action (23).

Chromic acid-based fixative.—Pinwheels and LA were found in abundance with chromic acid fixation (Fig. 8). The appearance of virus, laminated aggregates, pinwheels, etc., was essentially the same as described above for the GA fixation with inhibitors. However, tissue appeared more electron-dense after chromic acid fixation. The increased density is partially attributed to chromic acid staining and partially to better preservation of ribosomes in the cytoplasm than occurred with glutaraldehyde or osmic acid-only fixation.

Formaldehyde fixation.—Fixation in formaldehyde also revealed virus present in infected corn tissue but not always in as large an aggregate as the one shown in Fig. 9. Laminated aggregates and pinwheels were present and well preserved. Long-term formaldehyde fixation was used when experience with wheat tissue showed insufficient fixation after 3 hr. The addition of 2-ME during formaldehyde fixation did not yield different results.

DISCUSSION.—The scarcity of LA and virus in tissues after fixation in glutaraldehyde and postfixation in osmic acid is demonstrated by the fact that in four previous ultrastructural investigations of MDMV, LA apparently were not observed and that only small amounts of virus was reported present in thin sections (7, 13, 28, 31). Although SCMV and MDMV are closely related (26), other investigators have not noted or discussed the apparent absence of laminated inclusions of MDMV. As previously reported for all fixation methods used (7, 13, 28), pinwheels and bundle-type inclusions were found in plant cells infected with MDMV. The pinwheels thus represent a stable structure in MDMV infection which is similar to the findings described for infections caused by viruses in the potato virus Y group (4, 5, 6, 11, 12, 13, 16, 17, 32, 33), or by longer viruses, such as Atropa mild mosaic virus (8) and wheat spindle streak mosaic virus (34). However, particle lengths of Atropa mild mosaic virus (925 nm) and of wheat spindle streak mosaic virus [at least 1,000 nm (27)] exclude them from the potato virus Y group (2).

Laminated aggregates have been reported for some viruses with a morphology similar to MDMV; these include SCMV (4), tobacco etch virus (5), turnip mosaic virus and bean yellow mosaic virus (11), pokeweed mosaic virus (12), and parsnip mosaic virus (17). Laminated aggregates have not been reported for watermelon mosaic virus.

Fig. 8-9. 8) Area in cytoplasm of sweet corn parenchyma cell rich in laminated aggregates (LA). Membranes in chloroplasts (CH) do not stain well after fixation in the chromic acid fixative. Notice interconnections of laminated aggregates. Scale bar = 0.5 micrometer. **9)** Corn leaf cell with large amount of MDMV (V). Fixation with formaldehyde for 22 hr followed by osmic acid. Scale bar = 0.5 micrometer.

MDMV is not the only virus of the potato virus Y group for which observation of the virus in tissue after GA fixation is more difficult than would be expected based on its concentration. Weintraub & Ragetli (32) stated that bean yellow mosaic virus had a low concentration of recognizable virus particles in sections, yet the number of particles found in leaf exudates was high.

Four explanations are proposed to interpret the differences between our results and those of four previous investigations (7, 13, 28, 31): (i) Variations in concentration of GA in commercial lots could account for observed differences. (ii) Glutaraldehyde does not fix all proteins present in the cell and those that are not fixed are subsequently extracted during processing of the tissue. (iii) Glutaraldehyde alters the morphology of LA and virions with which it reacts so as to change their stainability in thin sections. (iv) Glutaraldehyde does not prevent postmortem changes in plant cells sufficiently rapidly; consequently, lytic action decomposes LA and virus.

Variable GA concentration.—Although the purity and concentration of commercial glutaraldehyde may differ from supplier to supplier, the observed differences with GA fixation in the presence or absence of inhibitors, cannot readily or reasonably be ascribed to differences in effective concentration of GA. Thirty-five samples were fixed and embedded during the 3-year period of this investigation, and several lots of GA were used. The concentration of glutaric acid in the different lots was never so high that a few drops of 1 N NaOH added to a 100-ml solution of 5% GA (=0.5 M GA) would not neutralize the solution. Sulfhydryl compounds will react with GA (23), but the resulting hemithioacetal is unstable and the reaction is reversible in aqueous solution so that the effective concentration of GA would not be appreciably lowered by the free-SH groups of added mercaptals. Results were consistent with all batches of GA; fixation in GA without additives did not preserve LA and virus as well as did fixation in GA with additives. Distillation of glutaraldehyde, to obtain a purer product, was not attempted. Robertson & Schultz (22) have shown that the basis for distillation of GA is unfounded.

Nonfixation by GA.—To our knowledge there are no reports which indicate that some proteins do not react with GA. Many enzymes are not inactivated by GA, but this does not mean that they are not fixed or insolubilized by cross-linking. However, a report of diffusion of a large protein (hemoglobin) through tissue during GA fixation was recently published (21). The possibility of rather large proteins migrating in the cell during GA fixation cannot be dismissed. However, we do not believe that the failure of GA to fix or cross-link proteins was the primary difficulty in the present case because it seems unlikely that addition of compounds such as iodoacetate would increase the cross-linking of proteins by GA.

Morphology alteration.—Resulting changes in LA and virions after glutaraldehyde fixation are not sufficient to prevent staining as was indicated in the electron micrographs of purified virus (Fig. 6) and of LA after fixation in the presence of a reducing agent.

Cellular lytic action is responsible for the disappearance of LA and virions.—We regard the presence of LA and virus in tissue fixed in GA plus enzyme inhibitors, and their absence in tissue fixed in GA without inhibitors, as evidence that GA does not prevent cellular lytic action. It is more probable that the enzyme inhibitors act by inhibiting lytic enzymes, than by directly increasing the cross-linking of proteins by GA. The inhibitor DEP was used as a general enzyme inhibitor. Iodoacetate is more specific than DEP, and inhibits those enzymes dependent upon -SH groups for their function.

The presence of an occasional LA in GA-fixed tissue is entirely expected if enough different samples are sectioned. Presence or absence of LA and virions was determined for leaf tissue taken within the same leaf area and fixed in the different fixatives.

Not only does GA preserve activity of many enzymes (23), it may actually activate others. Nuclear acid phosphatase is activated (3) in the nuclei of cultured tobacco cells after fixation with 2% GA. Furthermore, complex biochemical events can still take place after fixation in glutaraldehyde; e.g., spinach chloroplasts showed light-dependent H^+ uptake (18), and bacterial ribosomes retained substantial activity (10, 24).

Glutaraldehyde is widely used as the primary fixative in ultrastructural studies. It is not always appreciated that it is a gentle fixative, originally employed because it left most enzymes active so that they could be localized histochemically (23). It is not known whether compartmentalization of enzymes is maintained when the cell is killed by GA prior to fixation. Lysis of cellular components by released enzymes may take place either during the GA fixation, or during the customary washing of the tissue to remove unreacted aldehyde. Possible interpretive errors resulting from unknown enzymic action during conventional GA fixation could be reduced by control fixations made with other methods.

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