THE STRUCTURE OF CELLS DURING TOBACCO MOSAIC VIRUS REPRODUCTION

Mesophyll Cells Containing Virus Crystals

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ABSTRACT

The submicroscopic organization of mesophyll cells from tobacco leaves systemically infected with tobacco mosaic virus (TMV) is described. After fixation with glutaraldehyde and osmium tetroxide the arrangement of the TMV particles within the crystalline inclusions is well preserved. Only the ribonucleic acid–containing core of the virus particles is visible in the micrographs. Besides the hexagonal virus crystals, several characteristic types of "inclusion bodies" are definable in the cytoplasm: The so-called fluid crystals seem to correspond to single layers of oriented TMV particles between a network of the endoplasmic reticulum and ribosomes. Unordered groups or well oriented masses of tubes with the diameter of the TMV capsid are found in certain areas of the cytoplasm. A complicated inclusion body is characterized by an extensively branched and folded part of the endoplasmic reticulum, containing in its folds long aggregates of flexible rods. Certain parts of the cytoplasm are filled with large, strongly electron-scattering globules, probably of lipid composition. These various cytoplasmic differentiations and the different forms of presumed virus material are discussed in relation to late stages of TMV reproduction and virus crystal formation.

INTRODUCTION

In a previous paper (33) the submicroscopic organization of the nucleus and cytoplasm during successive phases of tobacco mosaic virus (TMV) reproduction was described for singly infected hairs of tobacco leaves. For further studies on the sites of virus multiplication and especially on the synthesis as well as assembly of the virus coat protein in the cell, it was thought desirable to look for improved fixation and embedding techniques. For this purpose various procedures have been tried on leaves systemically infected with a TMV flavum strain. Uninfected leaves have been studied for comparison. Fixation with glutaraldehyde and osmium tetroxide has been found especially useful in elucidating the structure of infected cells. In this paper the organization of systemically infected mesophyll cells with TMV inclusions, representing the final stage in TMV reproduction, is outlined

MATERIALS AND METHODS

Two- to four-month-old plants of *Nicotiana tabacum* (hybr. Samsun × White Burley) systemically infected with a flavum strain of TMV (kindly supplied by Dr. Wittmann) were used. For comparative studies pieces from leaves about 3 cm long with symptoms were fixed and embedded. The following method gave the best results:

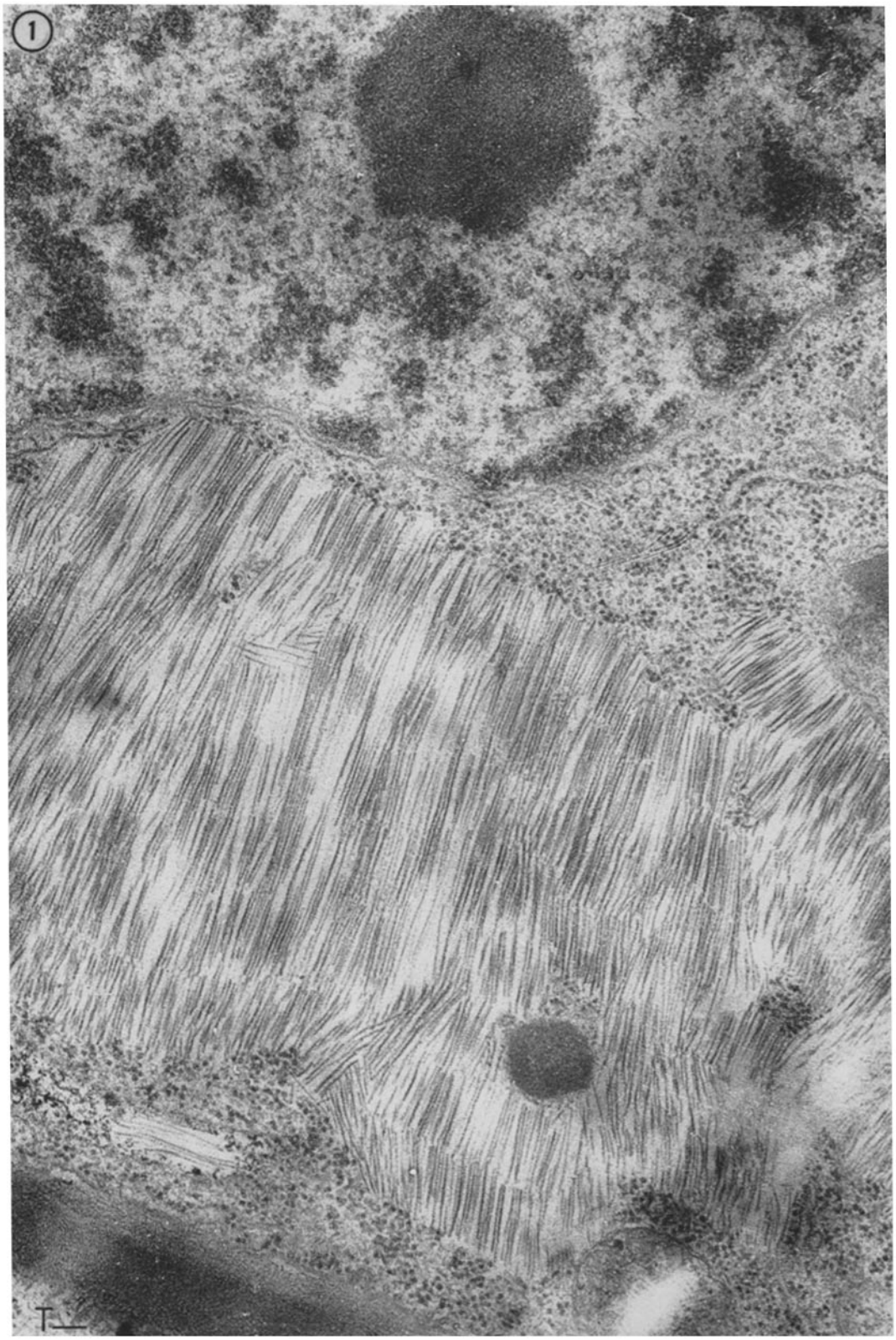
Small leaf pieces were fixed for 3 hours in 6.5 per cent glutaraldehyde (23) in 0.07 M phosphate buffer (pH 7.0) at 4°C. After 6 hours of washing in 0.07 M phosphate buffer, postfixation was carried out in 2 per cent buffered OsO_4 solution at 4°C. The material was dehydrated by passing it through increasingly concentrated acetone solutions up to pure acetone. After 10 minutes in propylene oxide, the pieces were kept for 12 hours in a 1:1 mixture of propylene oxide and Epon, using Luft's formula (16). After embedding in Epon, polymerization was carried out for one day at room temperature, followed by one day at 38°C, and finally at 60°C. Sections were cut on an LKB ultrotome and examined in a Siemens Elmiskop Ib after treatment with 1 per cent uranyl acetate and/or lead citrate (21). Electron microscopic magnifications were determined with the aid of a diffraction grating replica.

RESULTS

The general appearance of the cell structures in the leaf cells after fixation with glutaraldehyde and OsO_4 is identical with that described for root cells by Ledbetter and Porter (15). The ribosomes are clearly defined whether free in the cytoplasm or bound to the endoplasmic reticulum and nuclear membrane (Figs. 1, 8, and 9). The Golgi apparatus, the mitochondria, and the chloroplasts show the well known pattern of fine structure. The microtubules are frequently seen adjacent to the cell wall, sectioned longitudinally as in Figs. 1 and 8 or transversely as in Fig. 3 (upper left corner).

The hexagonal TMV crystals are well preserved as to outline (Figs. 1 to 3). Generally, the crystals embedded in the bulk of the cytoplasm reveal a more compact structure and a better parallel orientation of the individual TMV particles than those lying in the vacuole surrounded only by a thin film of cytoplasm. The latter seem thus to be more liable to swelling during preparation. The complete crystals consist only of virus particles, except for some cytoplasmic elements such as ribosomes in a few localized areas (Figs. 1 and 2). The crystals lie free in the ribosome-rich cytoplasm without any type of membrane or other visible boundary. Steere (29) has concluded from replica studies of frozen TMV crystals that the virus rods are oriented parallel within the individual layers of the hexagon, and that the rods of adjacent layers are oriented parallel to each other or at a characteristic angle leading to a herringbone pattern, if the crystal is cut perpendicular to its hexagonal face. The same pattern is observed in the sectioned crystals. The particles within individual layers are oriented more or less parallel. The rods of adjacent layers seem either to be arranged more or less parallel (Fig. 2) or to display the characteristic herringbone pattern (Fig. 3). The length of the particles has been measured at places where the layers of individual rods are well defined. Particles which were tilted with respect to each other were preferred for the measurements, to assure that the entire length of the particle was contained in the section. Such particles were found to be 2986 ± 19 (SE) A (n = 79) long. This is in close agreement with the commonly accepted length of 3000 A for dried TMV rods (14) or with that of 2890 A recently determined by Markham *et al.* (17). According to the x-ray diffraction data and electron microscopic measurements, the mean and maximum diameters of TMV virus particles are 150 and 180 A, respectively (5, 10, 14, 17). The diameter of the electron-scattering rods in the present micrographs has been measured to 79 ± 1 (SE) A (n = 100). As concluded earlier (33), the strongly electron-scattering strands seen in thin sections thus represent only the ribonucleic acid–containing cores of the virus particles, the capsid being invisible. The value obtained is in good agreement with the established diameter of 80 A for the nucleic acid helix in TMV (5, 10). On close inspection of the micrographs the strongly scattering strands can in many places be resolved into two irregularly curled dark lines and a lighter interspace of the order of 40 A. With regard to the electron-scattering properties, the sectioned TMV crystals behave like formalin-OsO_4–fixed cyrstals of the Coxsackie viruses, in which only the ribonucleic acid–containing core of the spherical particle is visible in thin sections (19). Besides these clearly defined crystals, the cyto-

FIGURE 1 Section through a mesophyll cell of a tobacco leaf systemically infected with TMV. A virus crystal is contained in the cytoplasm between the nucleus (above) and the cell wall (below). Microtubules at cell wall (T). × 42,000.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plasm of the mesophyll cells revealed a variety of characteristic structural differentations unique to the TMV-infected cells. They should correspond to the various types of inclusion bodies known from light microscopy to occur in crystal-containing epidermal and hair cells (*cf.* 4).

In many places loosely aggregated and poorly oriented small groups of particles identical in structure and dimensions with those in the virus crystals are found within clear areas of the cytoplasm. A typical example is given in Fig. 4.

Another type of organization of the virus material in the cytoplasm is reproduced in Fig. 5. It consists of single layers of oriented TMV particles between a network formed by the endoplasmic reticulum and cytoplasmic strands rich in ribosomes. It can be imagined that this arrangement is an intermediate stage in the condensation of the hexagonal crystals. The crystallization, known to take many hours, would be accomplished by aggregation of the virus monolayers and successive elimination of the endoplasmic reticulum and ribosomes.

Restricted areas of the cytoplasm are filled with long tubes. They may be dispersed in small, randomly oriented groups between parts of the endoplasmic reticulum (Fig. 6) or they may form large, well oriented and evenly spaced masses as in Figs. 7, 8, and 16. The diameter of the tubes measures 191 $\pm$ 2 (SE) A (n = 151). The diameter of the little electron-scattering central hole is estimated to be 60 to 90 A. The walls of the tubes occasionally give the impression of being composed of subunits. The diameter of the tubes corresponds to that of the capsid of TMV particles. If these tubes represent aggregated rods of virus material, their structural organization must be different from that of the virus particles in the crystals, as evidenced by their different affinity for OsO_4 and lead or uranyl ions. The borders of areas containing the tubes are rich in mitochondria, elements of the endoplasmic reticulum, Golgi vesicles, and double-membraned organelles with a diffusely granular inner structure (Fig. 8).

Relatively large areas of the cytoplasm are filled with strongly electron-scattering globules of highly variable size (Figs. 9 and 10), which tend to fuse into relatively large masses. The affinity for osmium suggests a lipid nature of the homogeneously structured globules. As in other regions of the cell, small "vacuoles" containing a few rods reminiscent of virus particles in the crystals are found in the cyto plasm betweeen the globules (*cf.* Fig. 10). They are 80 to 100 A in diameter and in longitudinal sections give the impression of spirals. We should like to suggest that these inclusions with the globules represent X bodies, since the latter, as indicated by cytochemical reactions, are rich in lipids (25, 27, 38).

The structurally most complicated type of inclusion bodies is that depicted in Figs. 11 to 14. These bodies are frequently associated with fully condensed crystals. The inclusion body consists of an extensively branched and folded part of the endoplasmic reticulum. The membranes of the endoplasmic reticulum are of both the smooth and the rough type, ribosomes being attached to the membranes only at certain regions. Long aggregates of diffusely structured and apparently flexible rods lie in the folds of the endoplasmic reticulum. This can be seen best at places where the rods are cut transversely (marked by arrows in Figs. 11, 12, and 13). It is then frequently observed that one or several aggregates are partly surrounded by a transversely cut membrane of the endoplasmic reticulum. The aggregates are found on the cytoplasmic side of the membranes, *i.e.* on the same side as the attached ribosomes. Usually ribosomes are lacking at the part of the membrane closest to the rods. The diameter of the aggregates in longitudinal sections ranges from 350 to 650 A. In cross-sections (Figs. 13 and 17) the aggregates appear as groups of rings with a hole of less density. The outer diameter of these rings is estimated to be 250 to 300 A, that of the hole 80 to 100 A. Most frequently three rings are associated with one another, forming a triangular structure (Figs. 11, 12, 13, and 17), but configurations of two or rarely four or five rings in contact with one another are also found. The transverse sections of these aggregates of presumed virus material suggest that they consist of two to five attached tubes. It is found, on the other hand, that only one member of a group of three shows a clearcut tube cross-section, the other two having diffuse outlines as if sectioned obliquely. The configurations could represent cross-sections through the loosely spiralized voluminous forms of TMV particles found earlier in isolated samples (36) and considered to be stages during the formation of the capsid around the nucleic acid core.

The infected mesophyll cells under study contained many small rhomboidal crystals with a lattice spacing of 100 A (Figs. 11 and 15). The

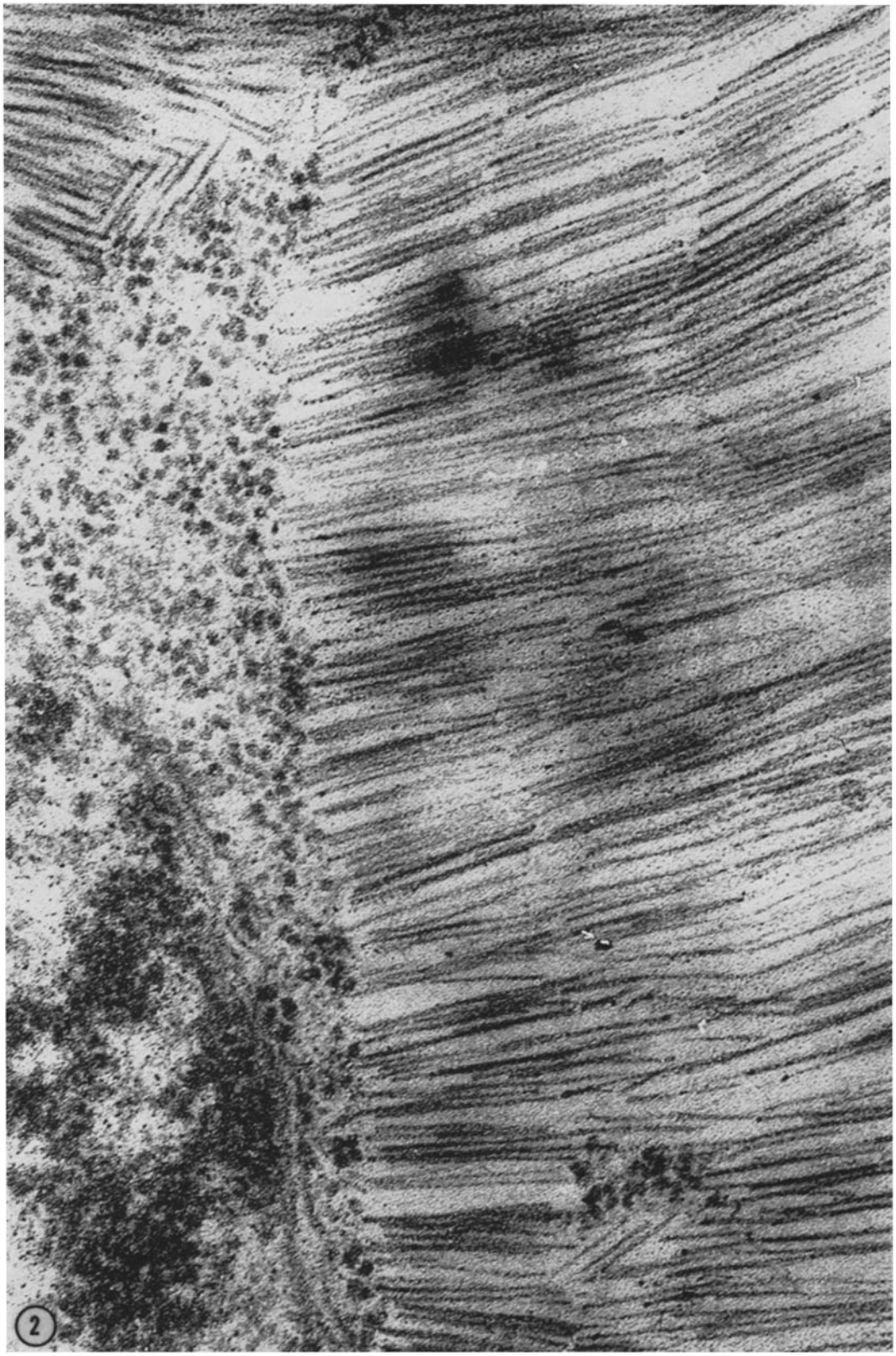


FIGURE 2 Border of virus crystal showing three layers of TMV rods. No membrane is visible between the ribosomes in the cytoplasm and the virus crystal. Nucleus at lower left corner. × 99,000.

crystals are contained in a single membrane-bounded organelle. Although they were especially frequent in the cells with the TMV crystals, they also occurred in uninfected control leaves. They seem to correspond to the crystal-containing bodies recently described by Thornton and Thimann in *Avena* coleoptiles (32).

The chloroplasts of the virus crystal–containing cells are of two types. Some of them appear entirely normal in their organization, with a well developed lamellar system, the grana structure, and some starch grains. Others are degenerated, the lamellar system being deranged and swollen or completely eliminated except for a few vesicles. The degenerated plastids are filled with strongly electron-scattering lipid globules. Both types of plastid can occur in the same cell. None of the virus particles described or the different forms of presumed virus material could be identified unequivocally within the plastids.

DISCUSSION

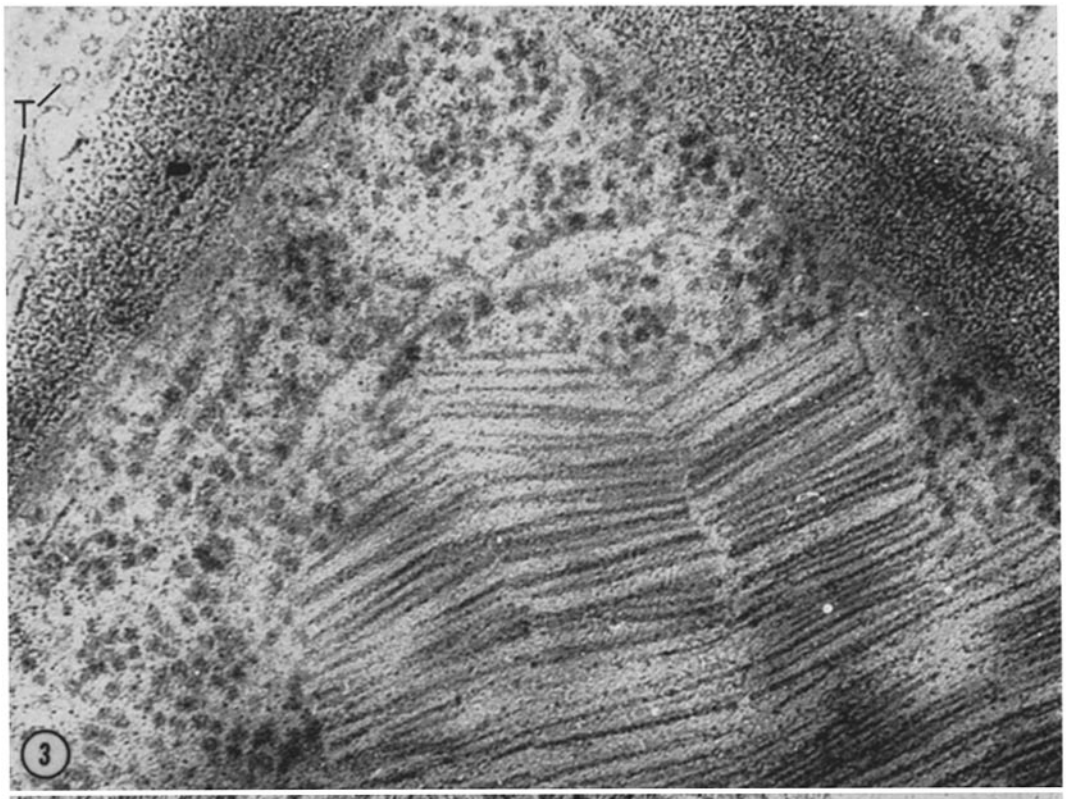
The characteristic cytoplasmic differentiations and the various types of inclusion bodies found in the TMV-containing mesophyll cells of tobacco leaves are comparable to those just reported by Shalla (24) for tomato leaves infected with a vulgare strain of TMV. It is especially interesting to note that the filaments described in the cytoplasm of the tomato leaves seem to correspond to the long, well oriented tubes of our Figs. 7, 8, and 16. The complicated inclusion bodies of Figs. 11 to 14 resemble those with the "randomly arranged filamentous structures" in Shalla's paper.

Later stages of infection in *Datura stramonium* leaves by another filamentous plant virus have recently been studied by Matsui and Yamaguchi (18). Single-layered aggregates of the virus particles were scattered in the cytoplasm, which also contained "microcrystals embedded in masses of fine granules." The microcrystals seem to be identical with the small rhomboidal crystals within the membrane-bounded organelle found in tobacco leaves. Late stages of TMV reproduction, like those investigated in the present paper, exhibit the following features, when studied in epidermal hair cells by phase contrast microscopy (1–3, 26, 28, 33–35, 39, 40): The broad, slowly streaming cytoplasmic strands contain associations of round or ellipsoidal bright masses, which can be seen to transform into fluid TMV crystals, also called gray plates (Bald, Solberg). The fluid crystals can be seen to disperse and recondense, and finally the typical hexagonal plates of TMV appear. Repeated recrystallization of the crystals in the cell with a successive decrease in their ribonucleic acid content can be observed (35, 40). Evidence that the synthesis and assembly of the capsid protein is carried out in localized areas of the cytoplasm with a folded differentiation of the endoplasmic reticulum has been presented earlier (33). The organization of single layers of complete virus rods within a network of the endoplasmic reticulum and ribosomes depicted in Fig. 5 provides a good link to this picture. This association obviously represents a section through such a bright mass or fluid crystal observable in the cytoplasm by phase contrast microscopy. The repeated dispersion and condensation leads to the elimination of the cytoplasmic constituents from the fluid crystal. Such a successive elimination of ribosomes could also explain the decrease in the ribonucleic acid content of the virus crystals. The dispersion and recondensation of the fluid crystals in the streaming cytoplasm may lead to loosely aggregated and poorly oriented groups of particles like those in Fig. 4.

Of the final nucleocapsid in the TMV crystals, only the ribonucleic acid–containing core is visible in the micrographs as a double-contoured rod. It is conceivable that the tight nucleic acid helix in the TMV particle gives such an image in thin sections owing to contrast effects. If the entire particle is contained within the section, the two highest electron opacities should be located close to the periphery of the helix. Free nucleic acid in the same tightly coiled configuration as that inside the nucleocapsid would—if it occurs in the cyto-

FIGURE 3 Section through TMV crystal cut perpendicular to hexagonal face revealing the angle between particles of adjacent layers. Microtubules in cross-section (*T*). × 95,000.

FIGURE 4 Loose aggregate of TMV particles in cytoplasmic inclusion body. × 121,000.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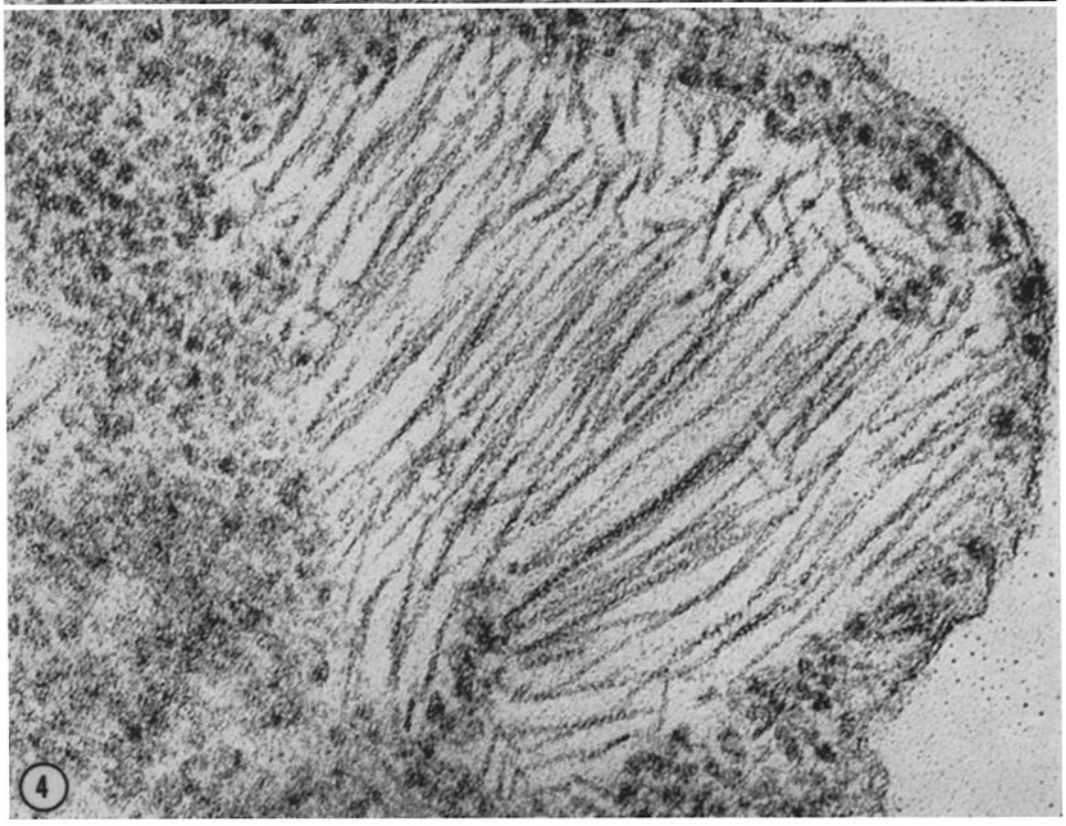
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FIGURE 5 Single layers of TMV particles separated by a network of the endoplasmic reticulum and ribosomes. × 50,000.

FIGURE 6 Cytoplasmic area containing groups of long tubes oriented at random. × 47,000.

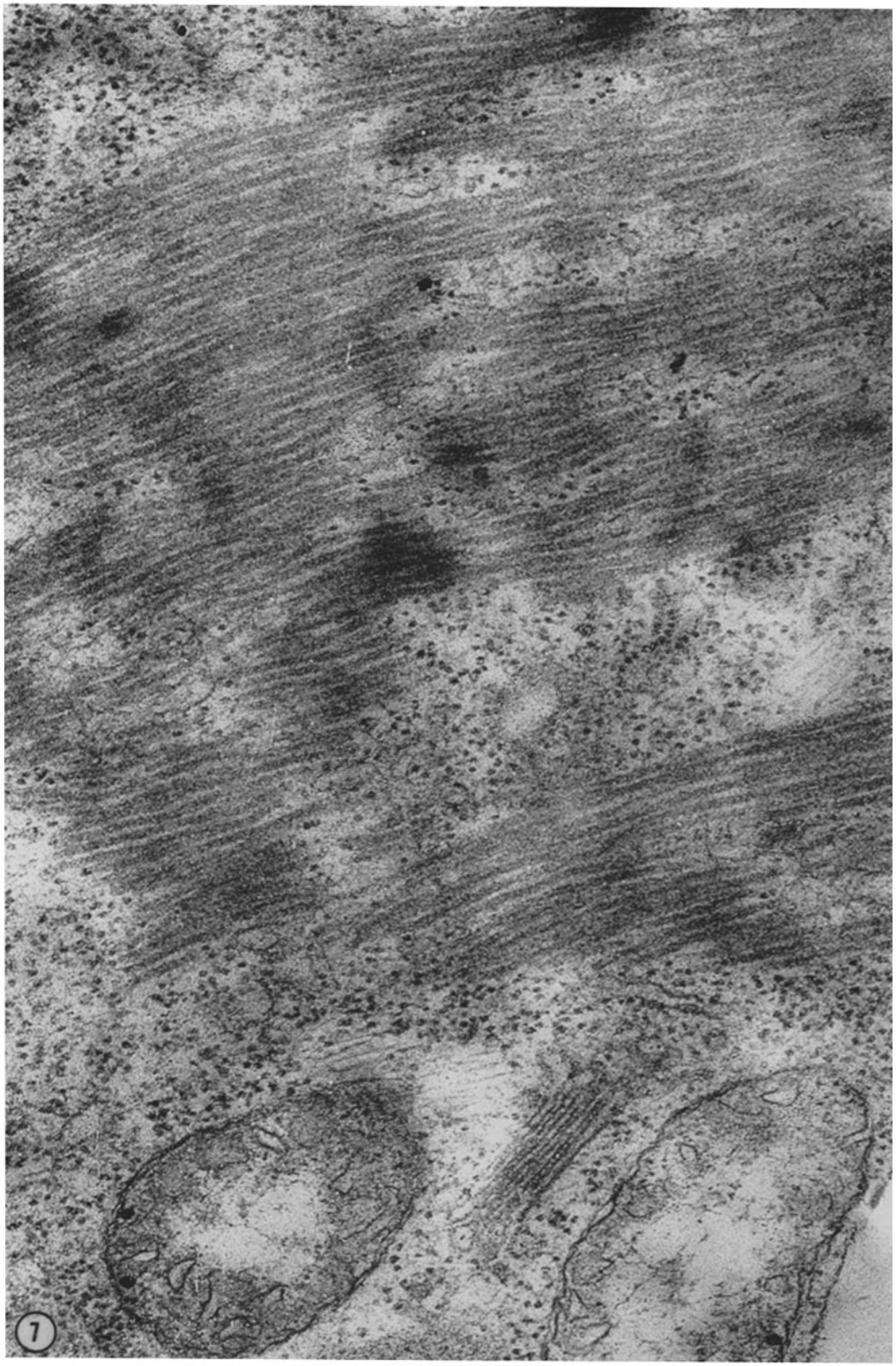

FIGURE 7 Oriented mass of tubes in cytoplasm. × 68,000.

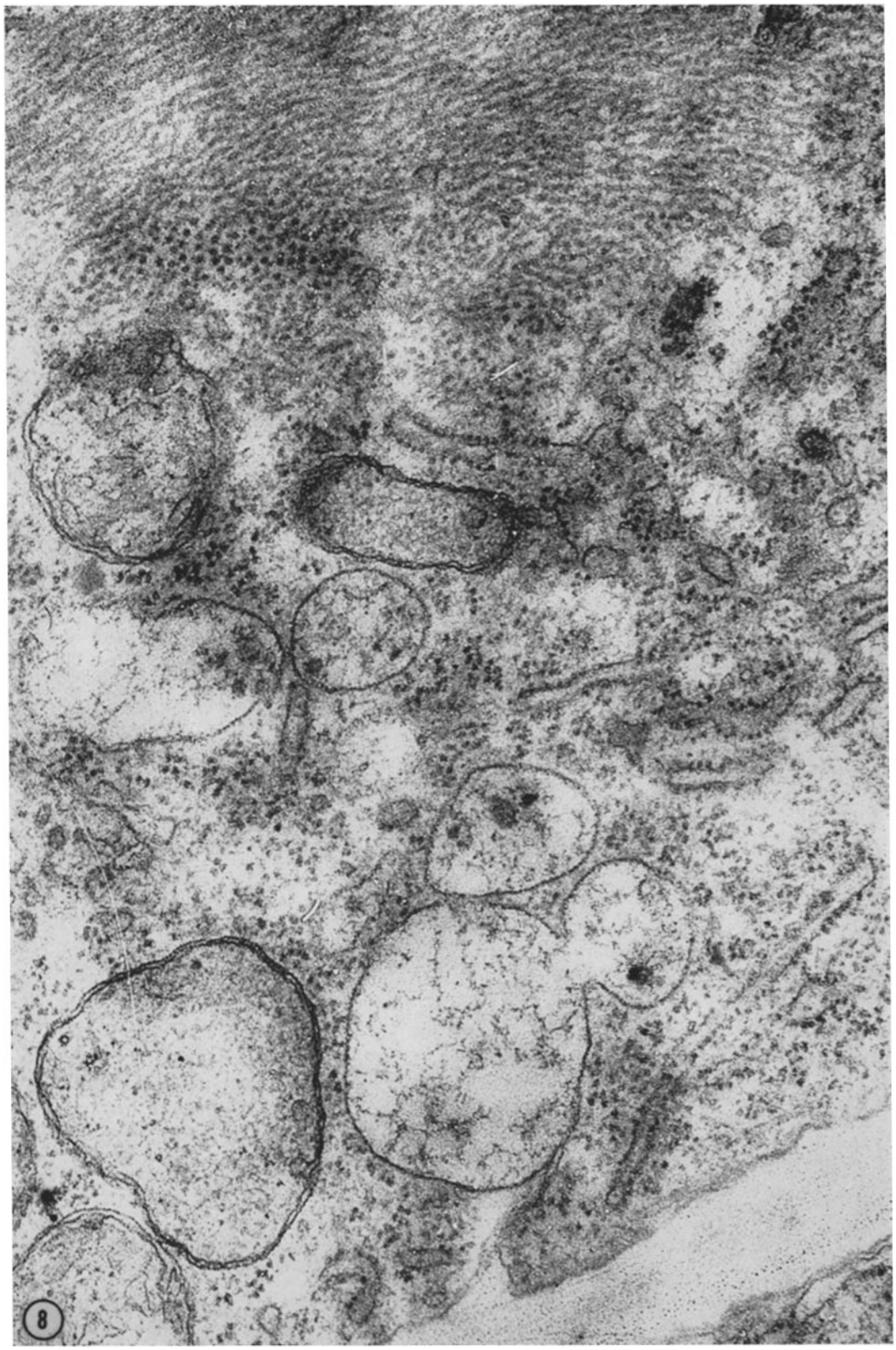

FIGURE 8 Organization of cytoplasm adjacent to oriented mass of tubes. × 68,000.

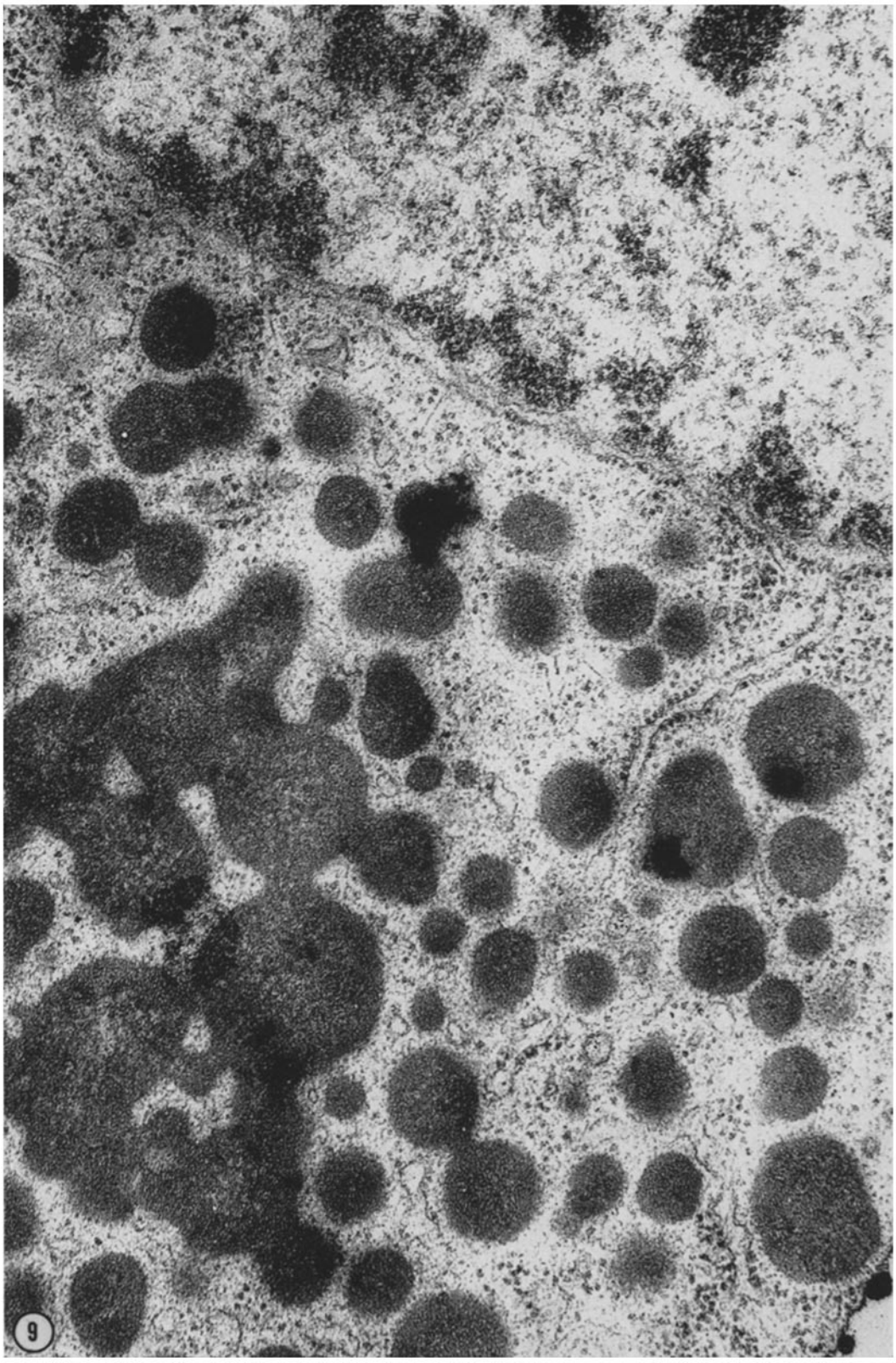

FIGURE 9 Area containing strongly electron-scattering globules close to a nucleus. This possibly corresponds to an X body. × 51,000.

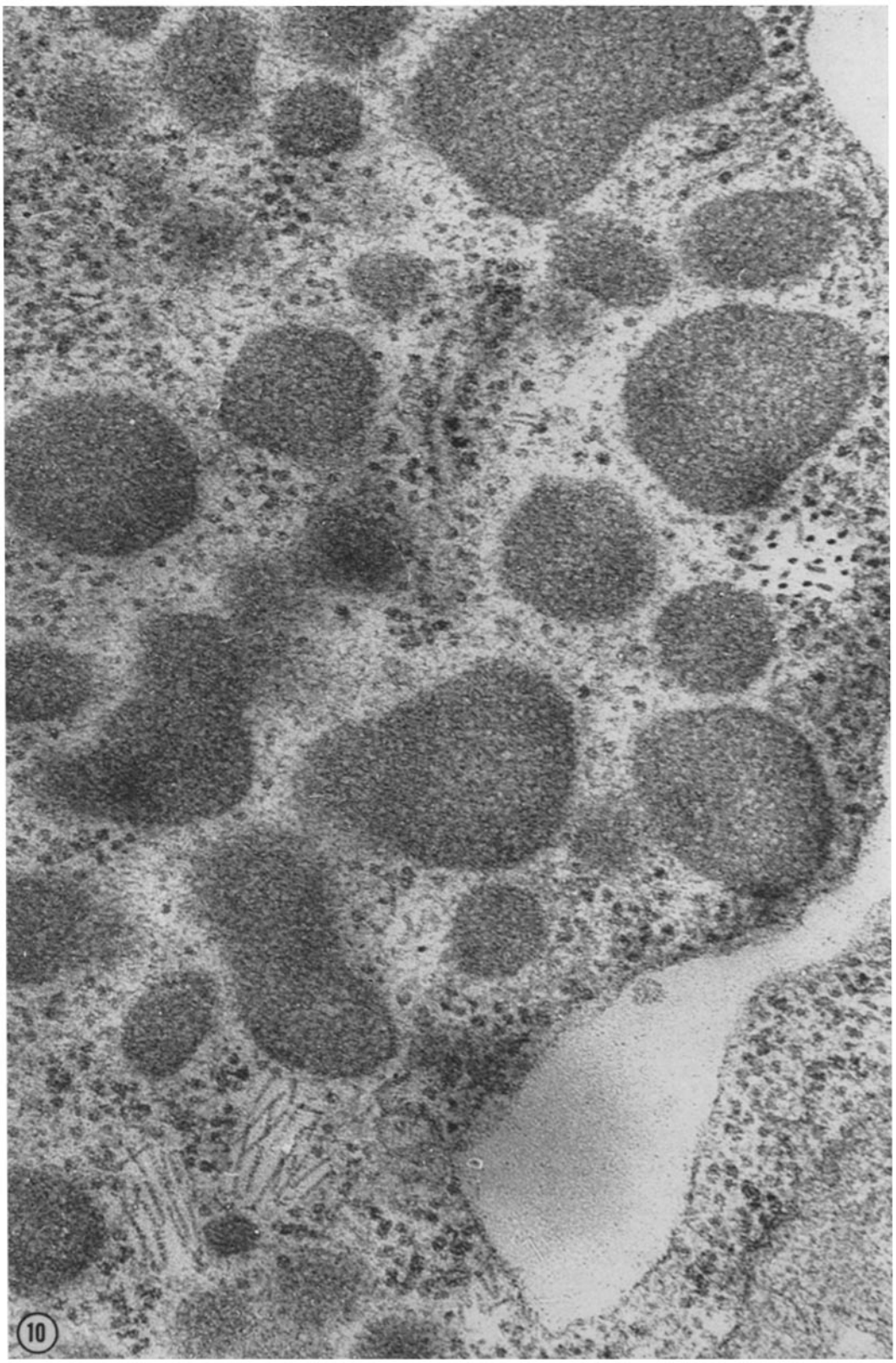

FIGURE 10 Cytoplasmic inclusion body containing electron-scattering globules and viruslike rods in cross-section (middle right) and in longitudinal section (lower left corner). × 88,000.

plasm—not be distinguishable from complete virus particles in the micrographs. The small aggregates of strands found in the cytoplasm could therefore represent either free virus nucleic acid in its coiled form or whole TMV nucleocapsids. The occurrence of free infectious nucleic acid in the cell has been inferred from various experiments (6–9, 11, 13, 20, 37). The long tubes of Figs. 6, 7, 8, and 16 have, as mentioned, the diameter and form of the TMV capsid. If they are TMV capsids, their different affinity to OsO_4 and lead or uranyl ions may be due to the absence of nucleic acid. Alternatively, a different state of aggregation of the protein subunits could cause their different electron-scattering behavior. A good candidate for the tubes is the so-called X protein found in infected cells (12, 22, 30, 31). It consists of nucleic acid–free TMV protein subunits which can be polymerized to various degrees. The X protein can be regarded as newly synthesized virus protein to be polymerized around the viral RNA (14). When formed in excess of the amount used in the formation of complete virus particles, it may aggregate in the cell into the masses of tubes, which are laid down in the cytoplasm without intermingled endoplasmic reticulum and ribosomes.

The inclusion bodies with the elaborate net of the endoplasmic reticulum and the clusters of ribosomes in polysomal configuration (Figs. 11 to 14) are interpreted as further developmental stages of the earlier described differentiations of the endoplasmic reticulum in hair cells during TMV reproduction (33) and as precursors of the fluid crystal of Fig. 5. The close association of the diffusely structured rods with the endoplasmic reticulum and the ribosomes makes it an attractive hypothesis that the synthesis of the virus protein and/or the coating of the virus nucleic acid with the protein is carried on in these areas. In this respect the structure of the subunits which compose the big rods is of special interest. The latter are likely to be aggregates of small discs, having a central hole and a diameter distinctly larger than the TMV.

It seems unlikely that the different forms of presumed virus particles discussed are degradation products of the virus or cell components, since the cells investigated represent early stages in TMV crystal formation. Further studies on the development of these various cytoplasmic differentiations and on the formation of the different forms of virus particles are in progress.

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FIGURE 11 Cytoplasmic area with extensively folded network of the endoplasmic reticulum. In the folds of the endoplasmic reticulum long aggregates of presumed virus material are found. Cross-sections of these aggregates are marked with arrows. Obliquely sectioned TMV crystal at the right. Small crystal in organelle at upper right corner. × 43,000.

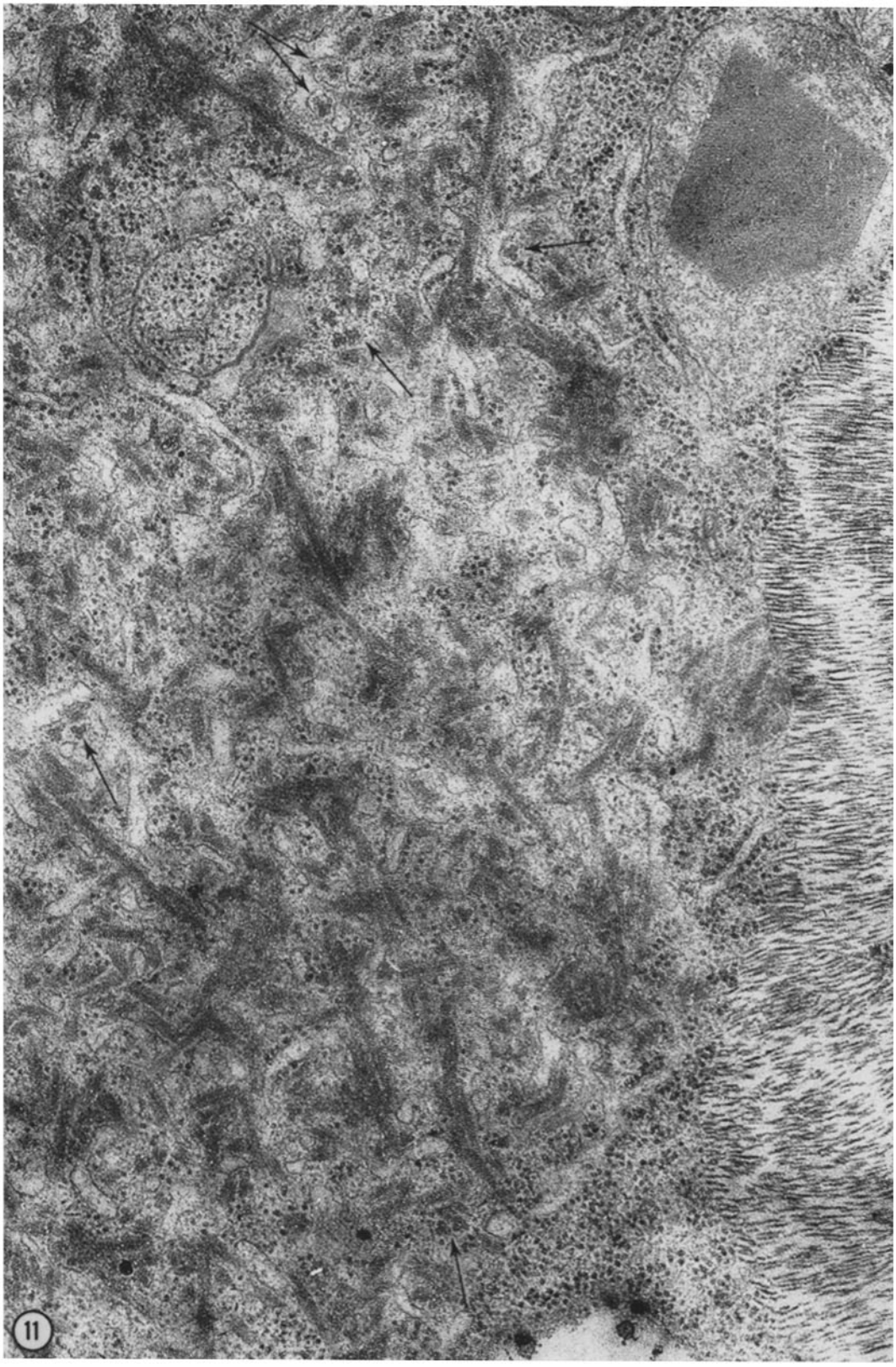
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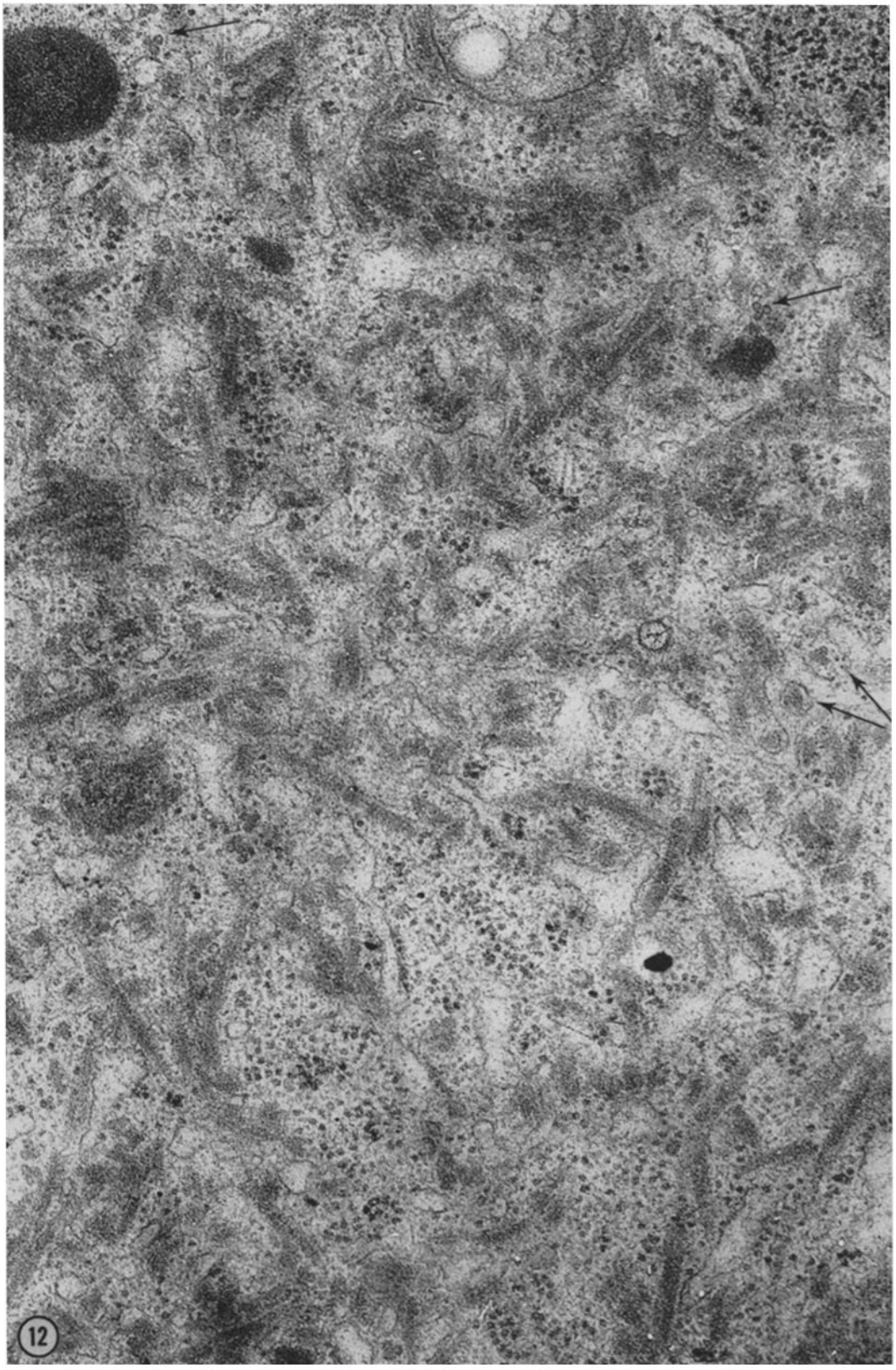
FIGURE 12 Cytoplasmic area similar to that in Fig. 11. Cross-sections of aggregates in folds of the endoplasmic reticulum are marked with arrows. × 53,000.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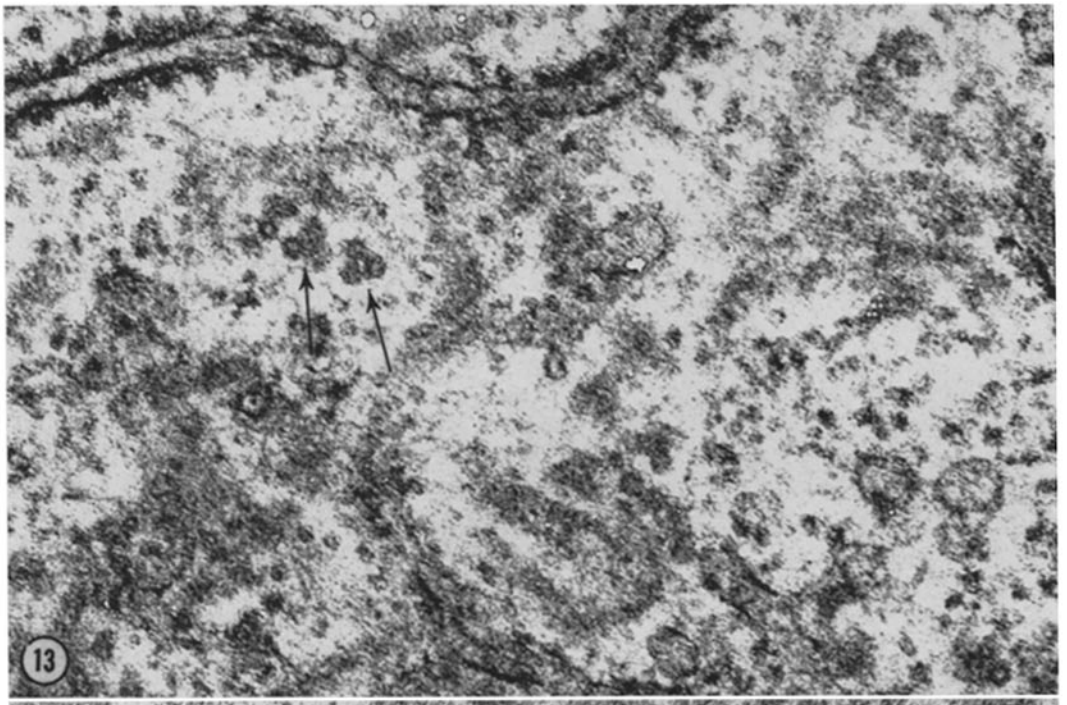
FIGURES 13 AND 14 Aggregates of presumed virus material within the network of the endoplasmic reticulum. Cross-sections, marked with arrows, give the impression that the aggregates consist of groups of rods with a central hole. Frequently three rods appear to be in contact with one another, forming a triangular structure. × 130,000.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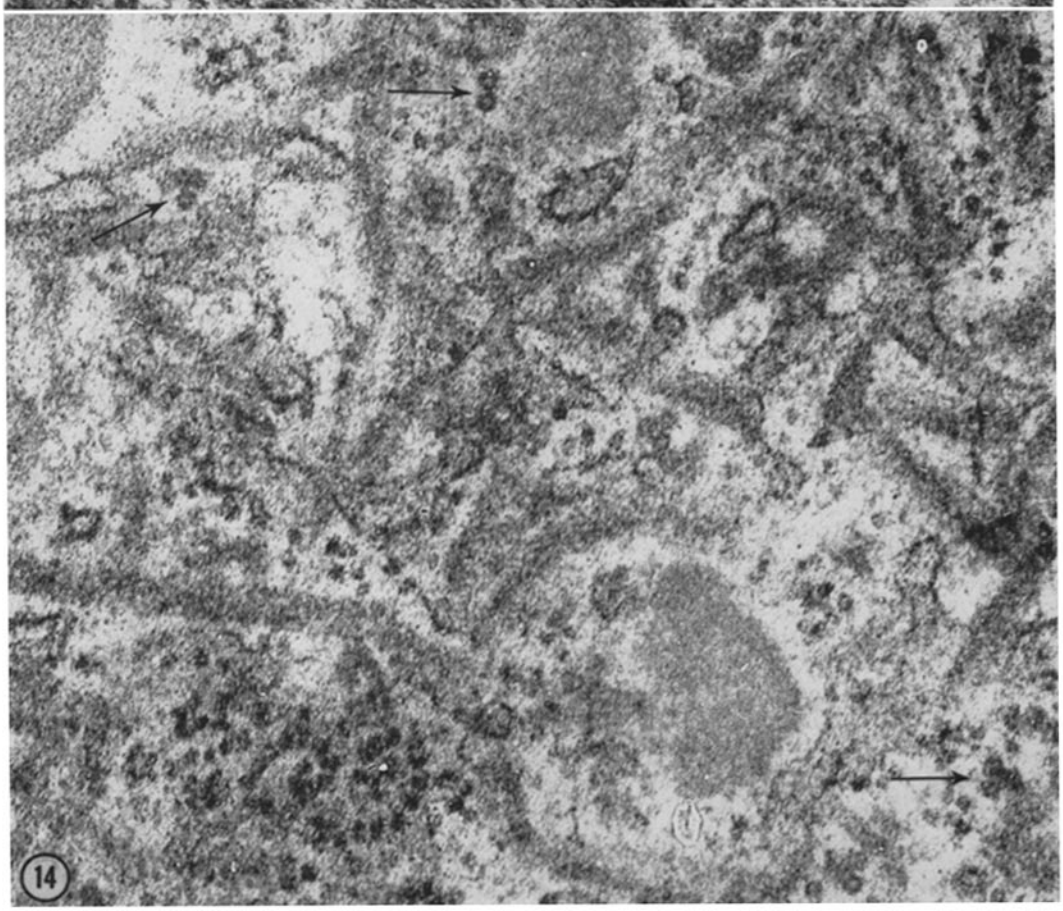
14

FIGURE 15 Small crystal-containing body. The lattice of the crystal has a spacing of 100 A. × 123,000.

FIGURE 16 Cross-section of oriented mass of tubes in cytoplasm. × 190,000.

FIGURE 17 Higher magnification of Fig. 13 with three aggregates of presumed virus material in cross-section (indicated by arrows in Fig. 13). × 350,000.