STUDIES OF A MYXOVIRUS RECOVERED FROM PATIENTS WITH INFECTIOUS HEPATITIS

II. Fine Structure and Electron microscopic Demonstration of Intracytoplasmic Internal Component and Viral Filament Formation*

By PHILIP H. PROSE, M.D., SAMUEL D. BALK, HARVEY LIEBHABER,‡ M.D., and SAUL KRUGMAN, M.D.

(From the Departments of Pathology and Pediatrics, New York University School of Medicine, New York)

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The subgroup of larger myxoviruses, *i.e.*, mumps, Newcastle disease (NDV), and parainfluenza viruses, when negatively stained, possess distinctive structural features (1, 2). Their virus particles are irregular in size, about 1000 to 3000 A in greatest diameter, and irregular in shape. These particles contain an internal component composed of ribonucleoprotein. The internal component (nucleocapsid) is an elongated structure with helical symmetry, 170 to 180 A in width, which is packed or tightly wound within an outer, lipid containing viral envelope. The latter, derived from the cytoplasmic membranes of host cells, has well defined projections on its surface, and shows hemagglutinating and neuranimidase activities.

Electron microscopic studies of the intracellular development of myxoviruses has shed light on little more than the end stage of virus release. Completely assembled particles are observed leaving the cell in bud-like or villus-like projections of the cell membrane (3, 4). Assembly of the internal component and surface structure into complete virus particles has never been observed. Moreover, internal component has never been identified satisfactorily within

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infected cells. This seems noteworthy in view of (*a*) the elegance and comparative ease with which investigators have demonstrated these structures in negatively stained virus particles or fractions thereof, and (*b*) the immunohistochemical evidence for the elaboration of internal component in the cytoplasm of infected cells (5).

Liebhaber *et al.* (6) recently isolated a virus from the serum and urine of patients with infectious hepatitis. Thus far, antigenically identical viruses have been isolated from 4 patients (7). This virus (WB virus) has clearly been identified as a member of the parainfluenza group. At the present time there does not appear to be any etiologic relationship between virus and infectious hepatitis.

This report describes the fine structure of the WB virus. In addition, it describes changes in virus-infected tissue culture cells which have not previously been reported; (*a*) the identification of nucleocapsids (internal component) within the cytoplasm, and (*b*) the morphologic delineation of several stages in the process of virus assembly and release.

Materials and Methods

Negatively Stained Viruses.—Purified WB virus was prepared by adsorbing infected WGM-1 (continuous African green monkey kidney cells) tissue culture fluid with washed guinea pig erythrocytes (2.5 per cent v/v), at 4°C for 3 hours. The erythrocytes were sedimented by low speed centrifugation in the cold and washed once with 5 volumes of cold phosphate-buffered saline (PBS). The cells were then suspended in PBS, one-tenth the volume of the original culture fluid, and placed in a water bath at 37°C for 2 hours. The red cells were sedimented and discarded. The supernatant fluid was subjected to a second cycle of adsorption to and elution from erythrocytes. The resultant virus suspension was clarified by centrifugation at 10,000 *g* for 15 minutes. The virus was then sedimented from this clarified suspension in the No. 40 rotor of a Spinco model L at 20,000 RPM for 60 minutes. The pellet was resuspended in 0.5 ml of 1 per cent ammonium acetate solution. This suspension was mixed with an equal volume of 2 per cent potassium phosphotungstate, pH 7.4, and then sprayed onto formvar covered or carbon coated, formvar covered grids.

Preparation of Thin Sections of Tissue Culture Cells.—Diploid human embryonic lung fibroblasts (WI-38) or WGM-1 tissue cultures were grown in a confluent monolayer in 60 mm plastic Petri dishes containing Eagle's minimal essential medium supplemented with 10 per cent inactivated calf serum (MEM-10CS) and maintained in MEM-2CS. These cultures were infected with 100 plaque forming units (p.f.u) of WB virus. When cytopathic effects were observed in 50 to 75 per cent of the cell sheet, the nutrient fluid was gently aspirated from the surface of the monolayer, which was then washed three times with PBS. Uninfected tissue cultures were used as controls. Following removal of the final wash fluid, the monolayers were fixed in phosphate-buffered 3 per cent gluteraldehyde for 10 minutes, postfixed in 1 per cent buffered osmic acid for 10 minutes, rapidly passed through graded alcohols, and embedded in epon. Propylene oxide–epon mixtures were not used in the embedding procedure. The plastic dish was removed from the polymerized epon by cutting through both with a shearing force. The embedded cells were cut on a Porter-Blum microtome with glass or diamond knives. Sections with gold or silver interference colors were stained with either uranyl acetate or uranyl acetate and lead citrate and examined in a Siemen's elmiskop I electron microscope. The specimens were magnified up to 31,000 diameters and photographically enlarged to the desired size.

Preparation of Negatively Stained Whole Tissue Culture Cells.—Monolayers were prepared according to the procedure detailed in the preceding paragraph through the stage of washing with PBS. Cells from several infected and control monolayers were gently dislodged from the surface of the plastic dish and floated onto carbon coated, formvar covered grids and subjected to one cycle of rapid freezing and thawing. The grids were then stained with 1 per cent potassium phosphotungstate, pH 7.4, and examined in the electron microscope.

RESULTS

Negatively Stained Virus.—Concentrated virus preparations, stained with potassium phosphotungstate, showed virus particles (virions) 140 to 330 mμ in greatest diameter. The particles were observed as light (electron translucent) structures on a dark (electron opaque) background. Although generally pleomorphic, many virions were spherical or ovoid in shape. The viral envelope consisted of a membrane, approximately 30 to 50 A in width, surrounded by cylindrical or club-shaped spikes (approximately 130 to 160 A in length) which projected outward from its periphery (Figs. 1 *a* and 2). Most particles were centrally covered by many electron translucent (light) dots on a dark background (Fig. 2), suggesting spikes viewed on end; some particles showed well defined nucleocapsids (internal component) within their envelopes (Fig. 1 *a*); and a few, rents in their envelopes through which a nucleocapsid had erupted (Fig. 1 *a*). Attesting to the ease with which the particle is ruptured, large amounts of free nucleocapsids were seen. When tightly wound, the internal component, 160 to 180 A in width, had a "fish bone" appearance with protein subunits appearing as serrations (about 70 A in length and 30 A in width) on either side of a central, "hollow" core, approximately 40 to 60 A wide (Fig. 3). The periodicity between serrations measured approximately 45 A. In some fragments of internal component, the helix was partially unwound (Fig. 4). In these, the strand formed a single rather than a double helix. In addition, the strand appeared ellipsoid in cross section since its width was least (about 30 A) when seen in side view, and greater (about 70 A) when viewed at angles more normal to the long axis of the helix. When a coil of the helix was seen on end, it appeared round with periodicity (about 30 A) on its circumference (Fig. 5), suggesting a strand formed by protein molecules packed about the nucleic acid helically.

Thin Sectioned Tissue Culture Cells.—Since the main pathologic findings in infected WI-38 and WGM-1 cells were very similar, only observations on the latter cells will be presented here. The uninfected kidney cell (Fig. 6) was usually ovoid with one or two nuclei. The latter contained a nucleolus and showed slight peripheral condensation of nuclear karyoplasm. The cytoplasm contained a moderate number of organelles; mitochondria with regularly placed cristae in a matrix of low electron density, a few scattered myelin figures probably representing degenerated mitochondria, one or two Golgi apparati, many scattered small vesicles surrounded by smooth surfaced endoplasmic reticulum, several somewhat dilated cisternae surrounded by rough surfaced

endoplasmic reticulum, and scattered, small clusters of ribonucleoprotein (RNP) particles. The outer cytoplasmic membranes showed only rare microvillous projections.

By contrast, the infected cell was very large, multinucleated (Figs. 7 and 8), with most irregular outlines due to the presence of many large, broad, polypoid cytoplasmic excrescences. The nuclei showed focal, peripheral condensation of karyoplasm, one or two nucleoli, and well defined inner and outer nuclear membranes. Mitochondria were numerous and often contained many small, irregularly arranged cristae in a moderately dense matrix. Small vesicles and several dilated cisternae lined by smooth surfaced endoplasmic reticulum were scattered throughout the cytoplasm. Some of the cisternae lined by rough surfaced endoplasmic reticulum formed large whorls; these were usually situated in the peripheral cytoplasm. RNP particles were extremely numerous.

Internal Component (Nucleocapsid) was Present in the Cytoplasm of Infected Cells.—These cells contained many angulated or serpentine, thread-like structures with uniform width, approximately 150 A, and varied length (Figs. 9 and 10). These structures were composed of linear rows of small rings or ellipsoids tangent to one another in their long axes; their appearance was consistent with that of sectioned helices. The center of the ring or ellipsoid was "clear" approximately 70 A in diameter; the circumference was moderately electron opaque. These "threads" were found in all zones of the cytoplasm, but they were not seen in the nuclei (Fig. 10). Although these structures were occasionally closely packed into large aggregates, the morphologic integrity of the individual "thread" was always maintained. The frequent absence of cytoplasmic organelles in these aggregates seemed to indicate that these "threads" and/or the surrounding cytoplasmic matrix were relatively rigid, had displaced organelles, and mechanically prevented their flow into the region. In some areas, the thread-like structures were less densely aggregated and presented a somewhat reticulated pattern.

Viral filaments and spheres, composed of internal component or nucleocapsid surrounded by an envelope, were observed most frequently at the cell surface and (Figs. 7 to 9 and 11) occasionally within cytoplasmic cisternae. When the internal component within the viral filaments was compared with the thread-like intracytoplasmic structures just described, they appeared identical. Their identity was confirmed, (*a*) when continuity between the intracytoplasmic thread-like structures and internal component within filaments was observed (Fig. 11), and (*b*) when negatively stained, freeze-thawed, infected cells showed pools of internal component (fish bones), similar in appearance to that already described in negatively stained preparations of concentrated virus (Fig. 12).

Viral Assembly and Release Occurred at the Cell Surface.—Within the cytoplasm of infected cells, internal component was haphazardly oriented in relation to the cell surface. Just beneath the cell surface, however, internal

component formed loose spirals with the long axes of the spirals parallel to the cell surface. The outer cytoplasmic membrane above the spiralled internal component was somewhat more electron dense than the adjacent membrane, suggesting transformation into envelope (Figs. 13 and 14). The cytoplasm at either side of or below the spiral contained varied sized vacuoles lined by membranes with somewhat increased electron density (Figs. 11 and 15). When the vacuoles coalesced and the intervening walls disappeared, filaments were produced. These filaments, up to several microns in length, were parallel to the cell surface. They were attached to the cytoplasm by a narrow base, 100 to 110 mμ in narrowest diameter. Occasionally, several filaments separated from each other by vacuoles, appeared to form at the same time (Figs. 11 and 15). At a later stage, or perhaps when the cell was sectioned in another plane, the filament often extended directly outward from the cell surface (Fig. 11).

In the viral filaments, the internal component was also loosely spiralled with the long axis of the spiral parallel to the long axes of the filaments. The surrounding envelope when resolved, showed a densely stained inner membrane, 30 to 60 A in width, on which rested a row of rounded or cuboidal vesicles, approximately 130 A in greatest diameter (Fig. 16). When these vesicles could not be resolved, a "clear" space, approximately 130 A in width, separated the inner dense membrane from an outer, continuous membrane of lesser density. Some filaments were ruptured and internal component was extruded from them (Fig. 1 *b*). Other filaments appeared to be attached to the cytoplasm by an "inner body" which was found at a short distance from the cell surface (Fig. 8). At the tips of some filaments, "empty", bulbous or club-like expansions of the envelopes (Figs. 7 and 8), 200 to 280 mμ in diameter, were seen. Occasionally they contained a tubular or cisternal structure, 27 to 33 mμ in diameter, lined by densely staining membrane, and/or internal component.

Spheres, approximately 100 to 110 mμ in diameter, composed of envelope and circularly arranged internal component, were seen just outside the cell, and rarely within intracytoplasmic vesicles and cisternae. The relatively uniform diameter of these particles would suggest that they were distinct structures rather than sectioned filaments. Occasionally, these spheres appeared to bud from the cell surface (Fig. 17); the possibility that these buds represented the bases of sectioned filaments could not be entirely excluded. Rarely, spherical "buds" were seen on filaments (Fig. 18).

DISCUSSION

On the basis of its biological, physical, and antigenic properties, WB virus clearly belongs in the mumps-NDV-parainfluenza group of viruses (7). The fine structure of negatively stained WB virus further justifies its inclusion in this group.

The internal component of WB virus appears to have the same general

structure as tobacco mosaic virus in that it is an elongated structure consisting of a hollow core surrounded by an RNA helix, about which protein subunits are regularly arranged (8). In negatively stained preparations, the appearance of WB virus nucleocapsid is similar to that of SV-5 virus as described by Choppin and Stoeckenius (9). The helix appears to be single stranded, and in cross-section seems to have an elliptical shape. A linear strand of nucleocapsid with a tightly wound helix measures 170 to 180 A in width, and has the characteristic "herring bone" appearance. Ellipsoidal protein subunits appear to be helically arranged about the hollow appearing core which measures 40 to 60 A in diameter. The subunits measure 70 A along their major axis and 30 A along their minor axis. The periodicity between subunits on adjacent coils of the helix is 45 A. In photographs with the highest resolution and contrast a regular periodicity of 30 A was observed along the circumference of the helical strand when it was viewed on end. This periodicity is approximately equal to the thickness of the protein subunits, which suggests that the subunits are packed side to side along the helical strand of RNA.

To our knowledge, previous electron microscopic studies have not demonstrated the presence of nucleocapsids within the cytoplasm of myxovirus infected cells. This is somewhat surprising in view of the ease with which this structure has been identified within infected cells with fluorescein-labelled antibody (5, 10, 11). In the present study the undulated, randomly oriented thread-like structures found in the cytoplasm of infected cells are clearly shown to be continuous with and identical in appearance to the loosely coiled internal component seen within virus filaments. These electron micrographs demonstrate in a direct manner, that these intracytoplasmic structures are indeed viral nucleocapsids. Less direct confirmation of their identity was obtained from freeze-thawed, negatively stained cells. These preparations contained large numbers of elongated herring bone structures, which in form and by actual measurement proved identical to nucleocapsid from disrupted virus particles. The observed localization of nucleocapsid to the cytoplasm is consistent with the data from immunofluorescence experiments which demonstrate that soluble antigen appears only in the cytoplasm of NDV-infected cells throughout most of the virus growth cycle (5).

In two previous reports, thread-like filaments (12) and filaments and granules (13), thought to represent viral nucleoprotein were observed in the cytoplasm of cells infected with parainfluenza virus. These structures were not well enough resolved to have demonstrated much structural detail, furthermore the width of the thread-like structure measured 100 A which is far smaller than the corresponding dimension in a strand of parainfluenza virus nucleocapsid.

The factors which made the demonstration of intracytoplasmic nucleocapsid possible in the present study are not at all clear. The techniques employed in the preparation of specimens have been widely used with one possible exception,

namely, the use of glutaraldehyde as a primary fixative. It is also possible that some unique property of the virus-cell systems studied enabled us to observe these structures.

Although caution must be exercised in drawing dynamic conclusions from static photographs, the evidence presented in this study indicates some of the morphologic changes that occur during viral assembly and release. In reconstructing events so as to best coincide with our observations, it seemed likely that as an aggregate of haphazardly arranged nucleocapsids neared the cell surface it became less tightly packed. Individual nucleocapsid strands formed loose spirals with long axes parallel to the outer cytoplasmic membrane. The spiral was usually more organized at one end of the coil than at the other. The outer cytoplasmic membrane above the spiral was denser than the adjoining membrane; this morphologic alteration was thought to represent transformation of cell membrane to viral envelope. Subsequently, the altered membrane became invaginated around the spiralled internal component. Separation of the virus filament from the cell occurred when the opposing margins of the invaginated membrane met and coalesced. One can account for the vacuoles found neighboring on spiralled nucleocapsids by assuming that invagination of the altered cytoplasmic membrane is dysynchronous. Fusion of these vacuoles appears to be in part responsible for viral release. We cannot exclude the possibility that in addition to altered outer cytoplasmic membrane, intracytoplasmic vacuoles, normally present near coiled nucleocapsid, take an active part in filament formation. In contrast to the present demonstration of filament formation, earlier work by others (4) suggested that viral filaments were produced by outward "spaghetti-like" extrusions of cytoplasm or that filament formation was initiated with an "inner body" followed by a tubular structure being drawn out from the cell membrane. We did not observe these modes of filament formation but cannot entirely exclude them.

In our material, nearly all the viral filaments were attached to infected cells. It seems likely that many freed filaments were removed in processing the tissues, since the surface of the cultures were washed several times prior to fixation and embedding. One can only speculate on the reason for the continued attachment of filament to the cell by a narrow base. The only explanation we can offer is based on our morphologic studies. These would lead us to assume that a well formed spiral of nucleocapsid is required for invagination of viral envelope into the cytoplasm. The nucleocapsid spiral, as previously stated, is usually better formed at one end than at the other. The well formed spiral is usually encircled by viral envelope and resides in that part of the filament that is free of the cell surface; the imperfectly formed spiral at the other end of the nucleocapsid is usually found in the filament's base of attachment.

Viral filaments and spheres were most often seen at the cell surface. They were occasionally found in intracytoplasmic vesicles or cisternae. The possibility

that these spaces were connected with the cell surface could not be excluded in the sections examined. The spheres were rather uniform in diameter and we therefore suspect that they represent distinct entities rather than sectioned filaments. It appeared to us that the spheres originated as buds at the cell surface and buds on filaments. Filaments with attached spheres have been observed by others (14).

The electron micrographs presented in this report represent the first direct observations of intracellular nucleocapsid and its incorporation into a myxovirus particle at the cell surface. The availability of an experimental system such as this should prove useful in the study of several important and, at present, poorly understood aspects of myxovirus biology.

SUMMARY

WB virus has been shown to have a fine structure which is characteristic of members of the mumps-NDV-parainfluenza group of viruses.

Viral internal component has been demonstrated within the cytoplasm of infected cells. The incorporation of internal component into virus filaments at the cell surface has also been demonstrated. The evidence presented suggests that virus filament formation occurs by a process of invagination of the cell membrane about the coiled strands of internal component lying beneath the membrane.

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EXPLANATION OF PLATES

PLATE 69

FIG. 1 *a*. Negatively stained viral particle showing envelope with cylindrical or club-shaped spikes (↔) projecting outward. Internal component with "fish bone" appearance is present inside the particles. It is partly extruded from the particle through a rent (→) in the envelope. × 108,000.

FIG. 1 *b*. Part of an infected WGM-1 (continuous African green monkey kidney) cell showing several viral filaments projecting from the cell surface. These contain loosely spiralled internal component. One of the filaments has ruptured, and extruded internal component (→) is seen in its vicinity. A viral sphere is shown near the upper right corner of the micrograph. × 96,000.

FIG. 2. Negatively stained, intact viral particle showing envelope with cylindrical or club-shaped spikes (→). The center of the particle is covered by electron translucent (light) dots on a dark background; the dots may represent spikes seen on end. × 156,000.

FIG. 3. Negatively stained, tightly wound strand of internal component showing fish bone appearance. The protein subunits are seen as serrated structures on either side of the central, "hollow" core. × 158,000.

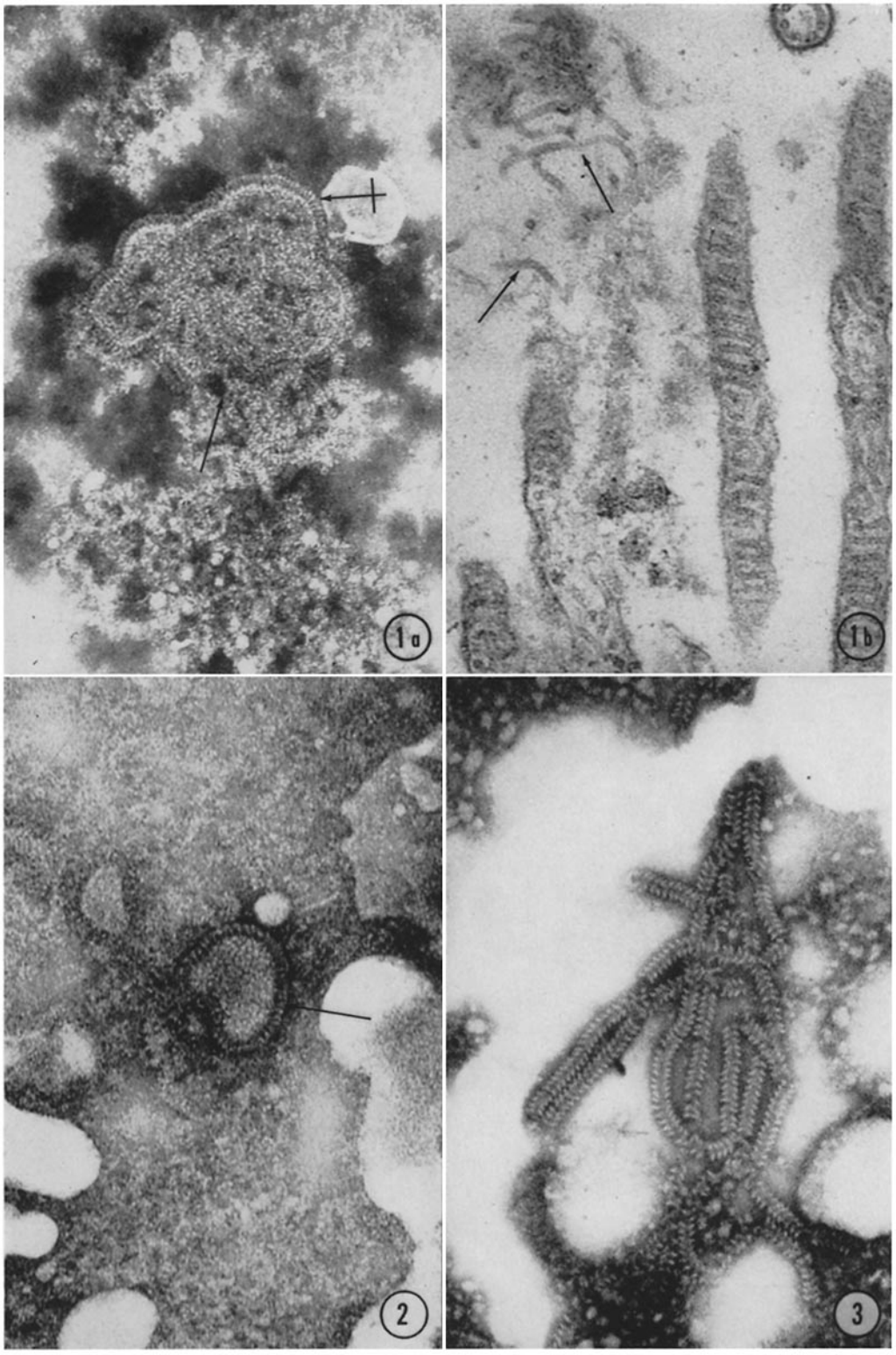

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PLATE 70

FIG. 4. Negatively stained, partly unwound strand of internal component showing a segment of the coil assuming a serpentine configuration (→) consistent with a single helix. The strand appears thinner in side view (↔) and thicker when seen at an angle more normal to the long axis of the helix (↔). × 187,000.

FIG. 5. Negatively stained internal component showing "fish bone" appearance. When the coil is viewed on end (→) it appears round. Periodicity is seen on its circumference and is shown most clearly in center of micrograph. × 120,000.

FIG. 6. Section of a normal WGM-1 cell showing nucleus (*N*) with slight peripheral condensation of karyoplasm and nucleolus (*nu*). The cytoplasm is bordered by a smooth outer membrane and contains mitochondria (*m*), myelin figures (*my*), some rough surfaced endoplasmic reticulum (*r*), and a Golgi apparatus (*g*). × 11,000.

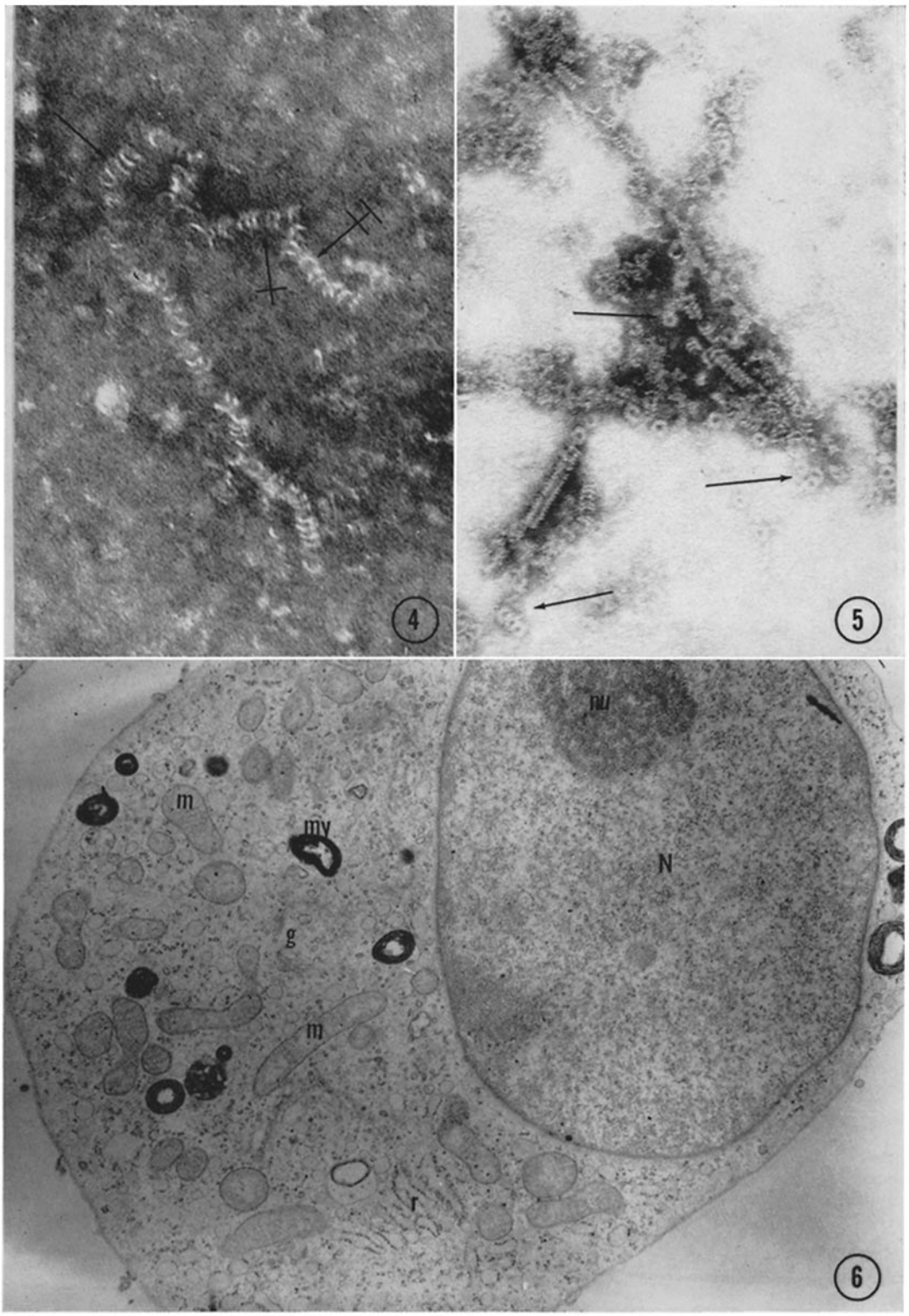

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PLATE 71

FIG. 7. Section of part of an infected WGM-1 cell showing polypoid cytoplasmic excrescences at the periphery of the cell. Some of the excrescences contain whorls of rough surfaced endoplasmic reticulum (*W*). The cytoplasm in addition to many ribonucleoprotein particles, contains mitochondria (*m*), vesicles (*v*) and a viral filament (→, right side of micrograph), with bulbous, "empty" tip, lying in a closely contoured space. Viral filaments (→) are also shown at the cell surface. A nucleus (*N*) shows focal, peripheral condensation of karyoplasm. × 17,000.

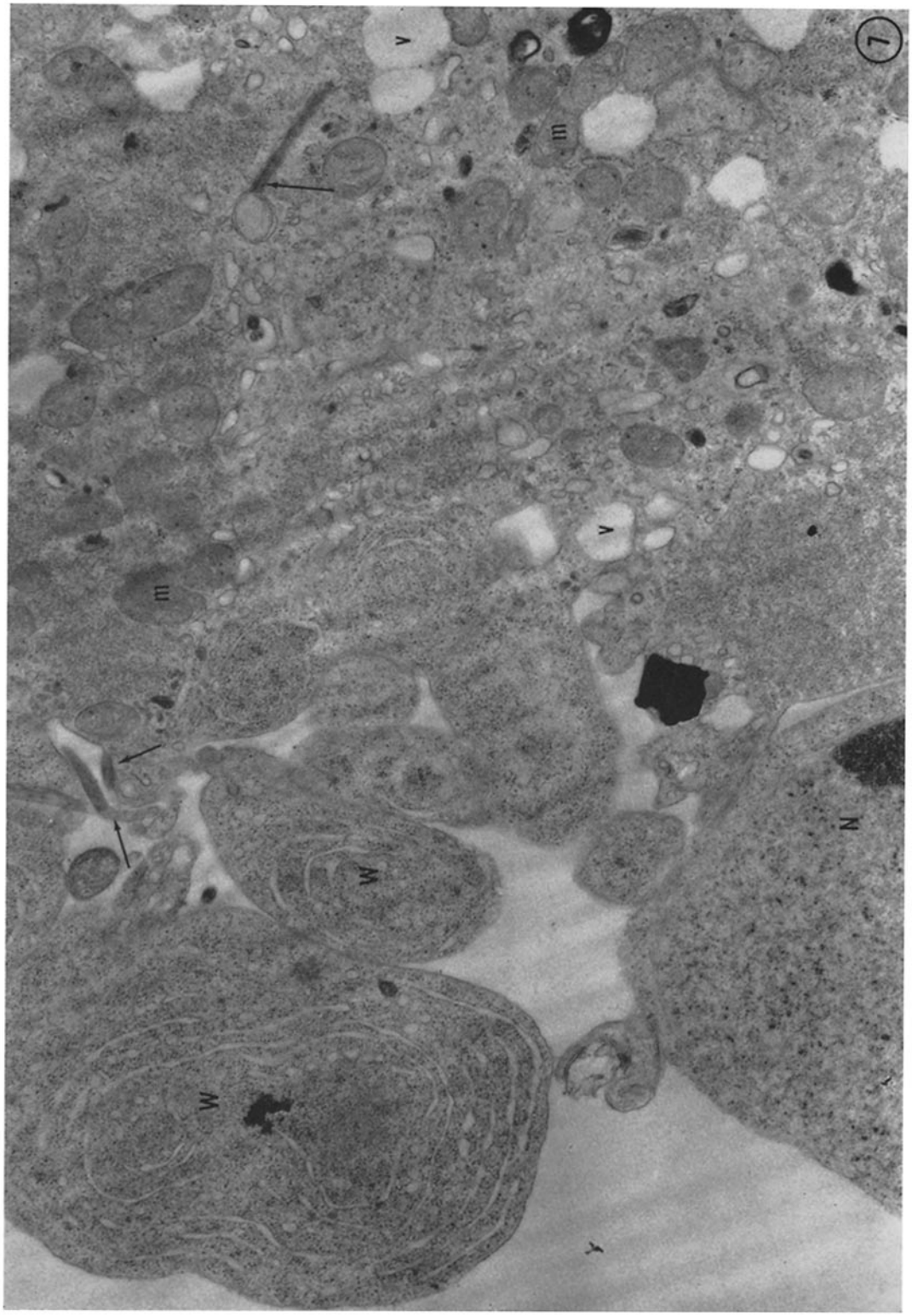

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PLATE 72

FIG. 8. Section of part of an infected WGM-1 cell showing viral filament (→) and spheres (*s*) at the cell surface. This filament has an inner body (*IB*) and contains internal component surrounded by an inner, electron dense membrane and, in part, by an outer, less dense membrane. A narrow, empty space separates the two membranes. The filament ends in a bulbous expansion. The cytoplasm contains mitochondria (*m*), many small vesicles, some of which suggest pinocytotic vesicles, and aggregates of long, narrow filaments (*f*). × 48,000.

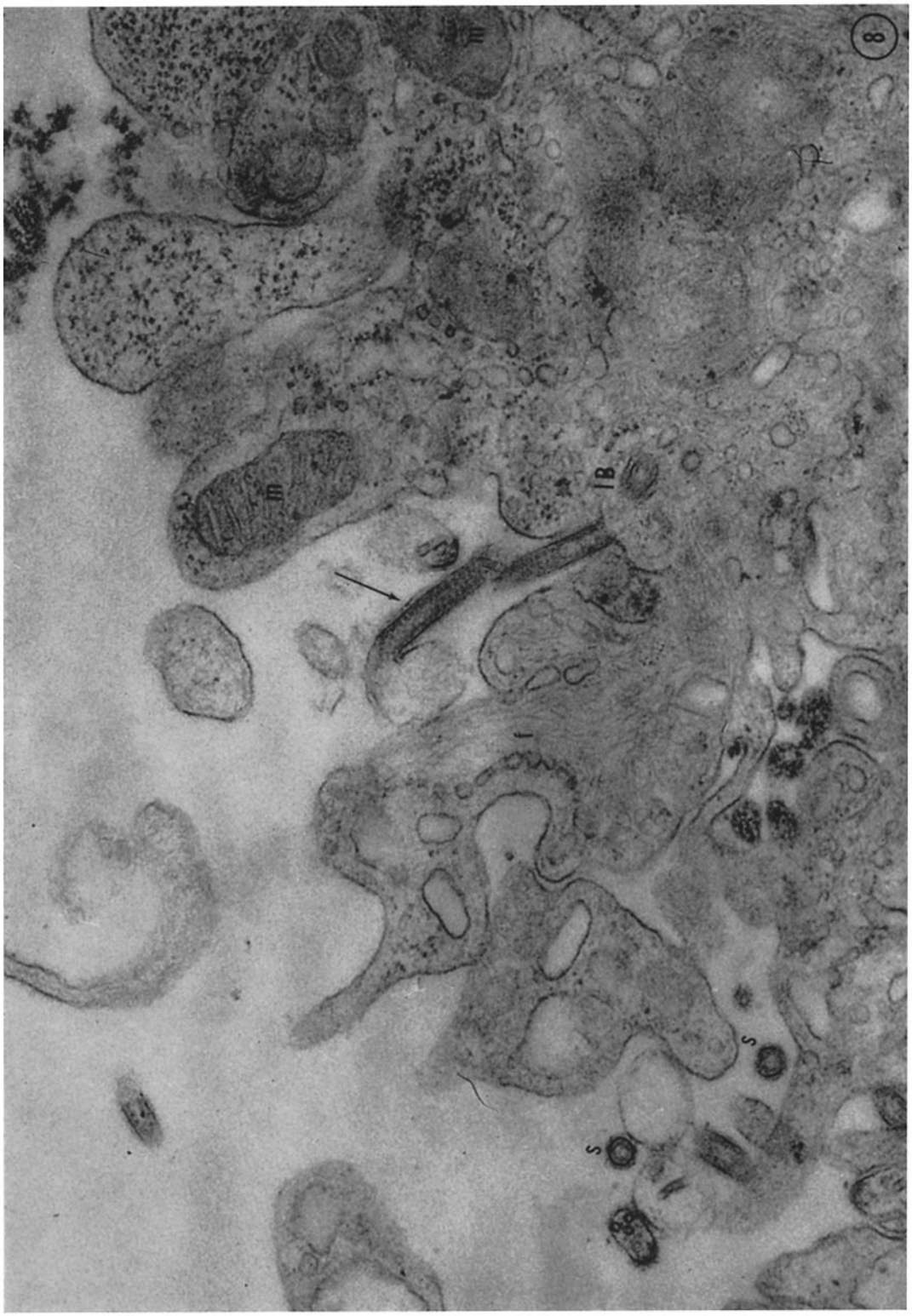

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PLATE 73

FIG. 9. Part of an infected WGM-1 cell showing filament (→) parallel to the cell surface. The filament contains loosely spiralled internal component surrounded by a dense inner membrane. It is free of the cell surface in the left half of the micrograph, but it is still, in part, connected to the cell by cytoplasmic bridges extending between vacuoles (*v*). The cytoplasm of the cell is almost completely filled with randomly arranged internal component identical in appearance to that in the filament. × 93,000.

FIG. 10. Part of nucleus (*N*) and perinuclear cytoplasm in an infected WGM-1 cell. The nucleus is separated from the cytoplasm by an outer nuclear membrane. The cytoplasm appears to be entirely filled with internal component. These are seen as thread-like structures made up of small ellipsoids or rings (→) tangent to one another in the long axes of the "threads". Internal component is not seen in the nucleus. × 104,000.

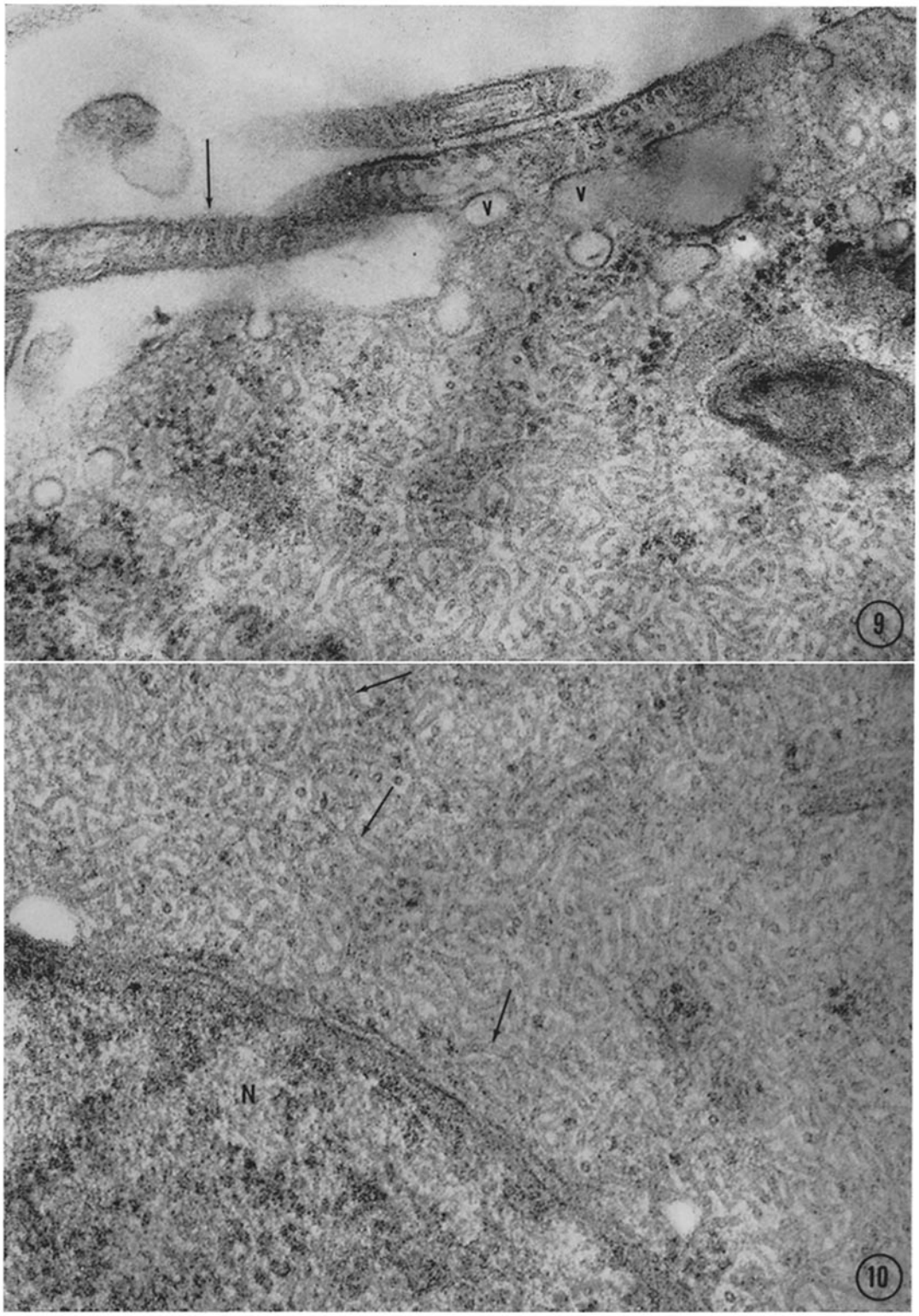

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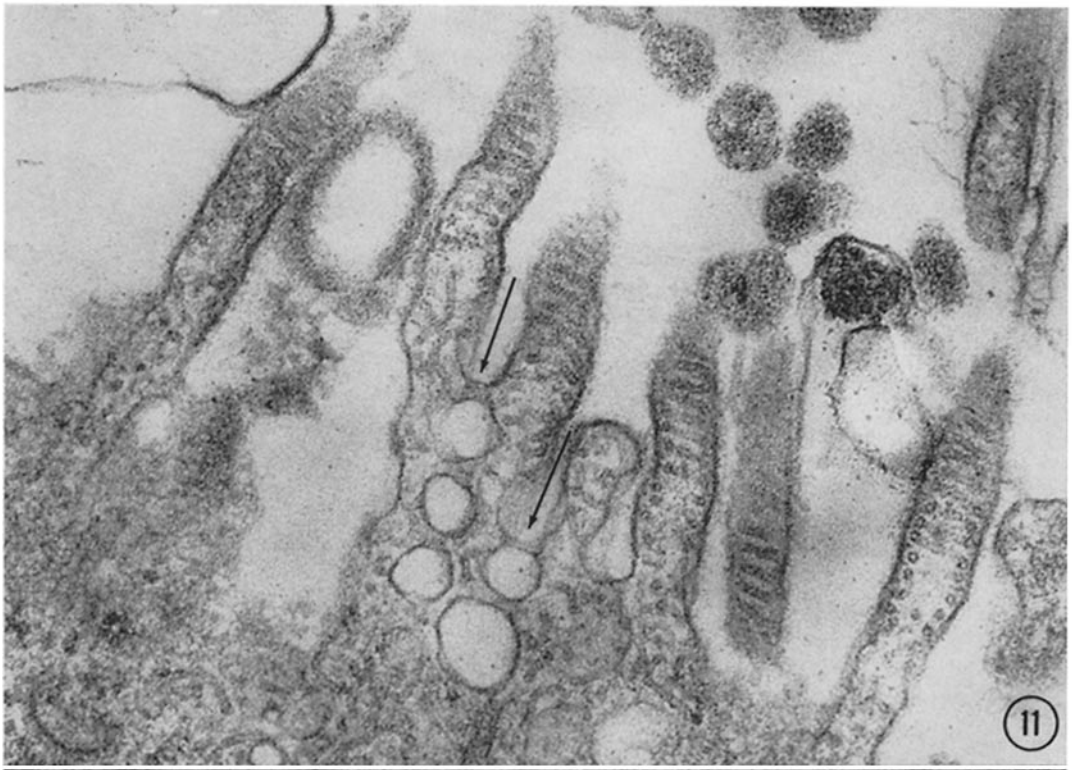
PLATE 74

FIG. 11. Part of an infected WGM-1 cell showing viral filaments apparently extending outward from the cell surface or tangentially cut. Three of the filaments are separated by vacuoles (→) and connected to each other by thin cytoplasmic or membranous bridges. It is thought that dissolution of these bridges results in growth of the filament. The internal component at the distal ends of the filaments is more regularly arranged than it is at the base of the filaments or within the cytoplasm. The cytoplasm at the base of most filaments contains internal component similar in appearance to that within the filaments. × 102,000.

FIG. 12. Negatively stained, frozen and thawed, infected WGM-1 cell showing internal component with "fish bone" appearance in the cytoplasm. In an area suggestive of the cell surface (→), the long axes of the nucleocapsids parallel the surface. A large structure with laminated membranes is suggestive of a viromicrosome (*vm*). × 64,000.



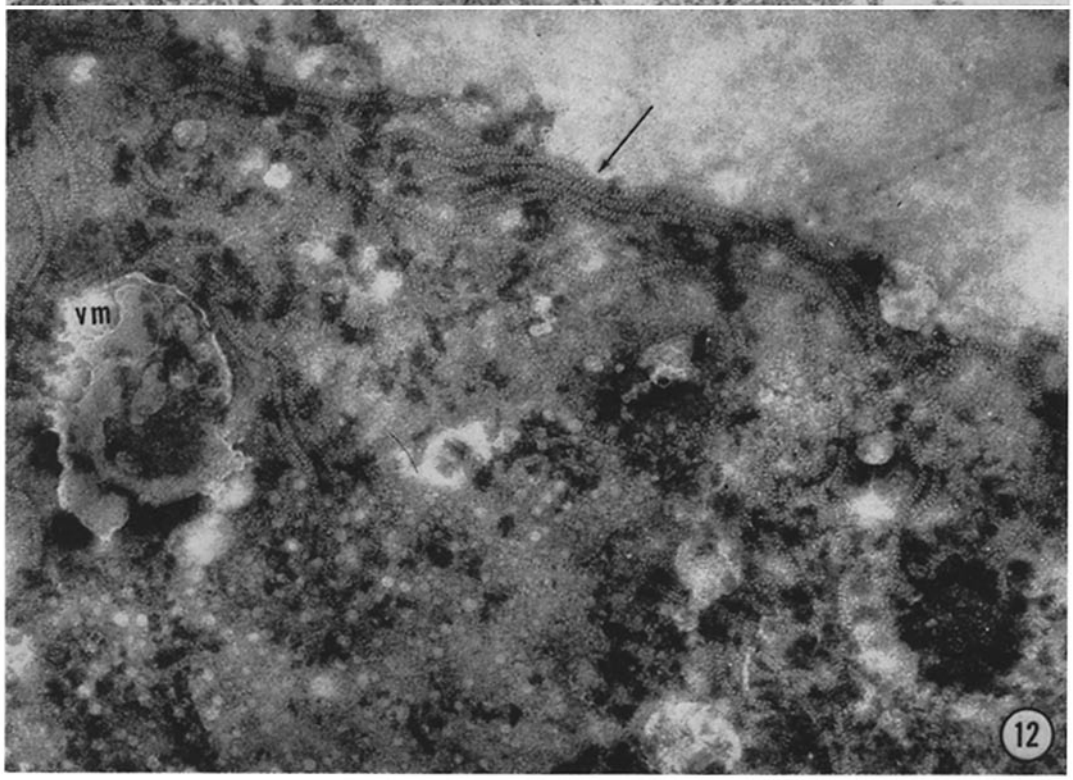


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PLATE 75

FIG. 13. Part of an infected WGM-1 cell showing cytoplasm filled with haphazardly arranged, thread-like, internal component made up of long rows of small ellipsoids or rings (→) tangent to one another in the long axes of the "threads". At the cell surface, the internal component forms a loose spiral (↔). The outer cytoplasmic membrane shows increased density suggesting alteration to viral envelope. × 94,000.

FIG. 14. Part of an infected WGM-1 cell shows viral filament free of cell surface (→). Its nucleocapsid is continuous with intracytoplasmic spiralled nucleocapsid (↔) beneath the altered cell surface. More proximally and within the subjacent cytoplasm, the nucleocapsid is more randomly arranged. A small vacuole (*v*) is present within the cytoplasm. × 113,000.

FIG. 15. Part of an infected WGM-1 cell showing 3 long viral filaments with long axes parallel to the cell surface. The uppermost filament is free of the cell in this region. The middle filament is attached to the cytoplasm by a broad pedicle on its lower surface. A small vacuole is present near the base of the filament. The lowermost filament shows attachment to the cytoplasm at its base and on its lower surface. The latter is attached to the cytoplasm by narrow cytoplasmic bridges separated by vacuoles. The appearance of these filaments would suggest that the filament is freed from the cytoplasm by fusion of vacuoles. Internal component is also present within the cytoplasm (→). × 76,000.

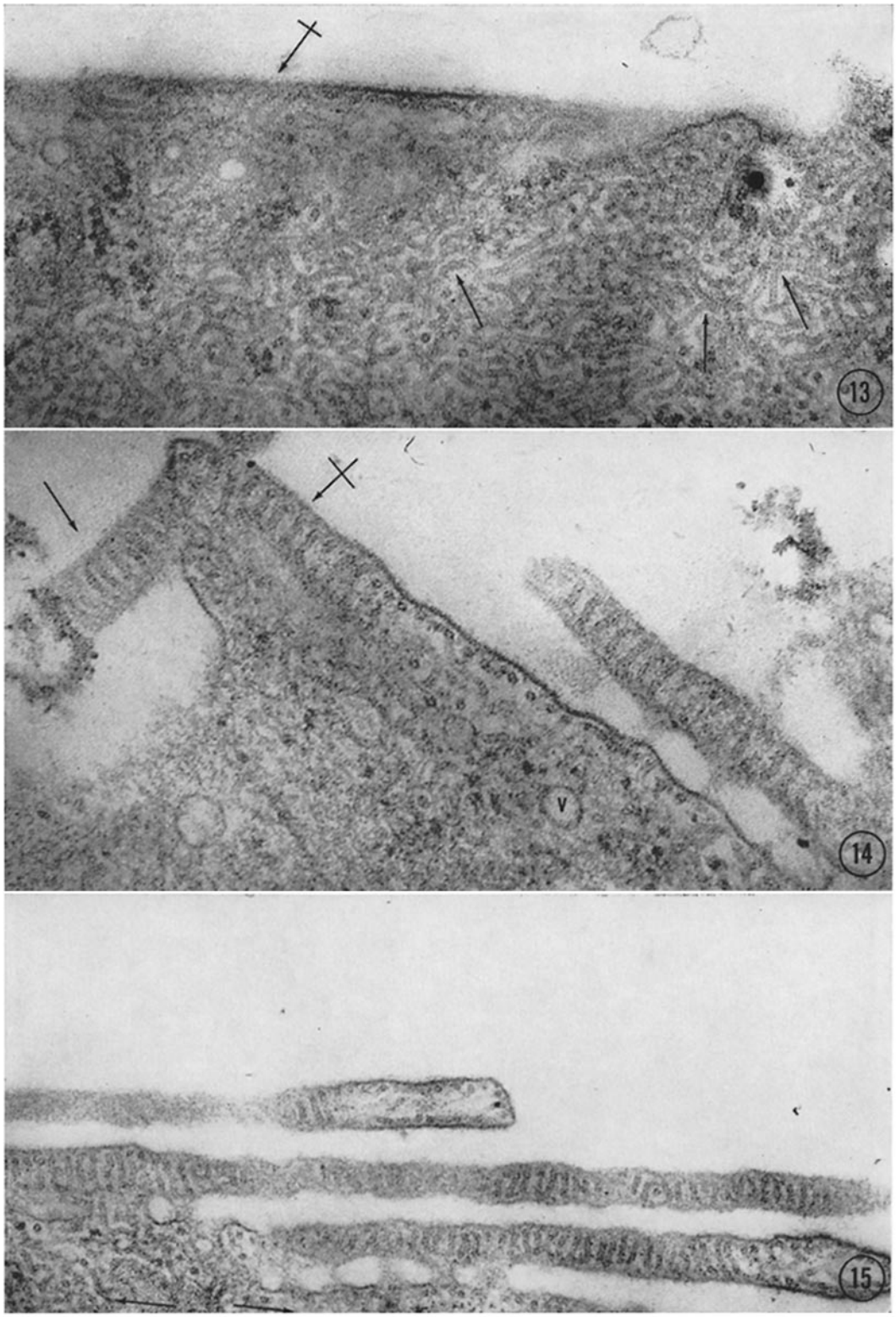

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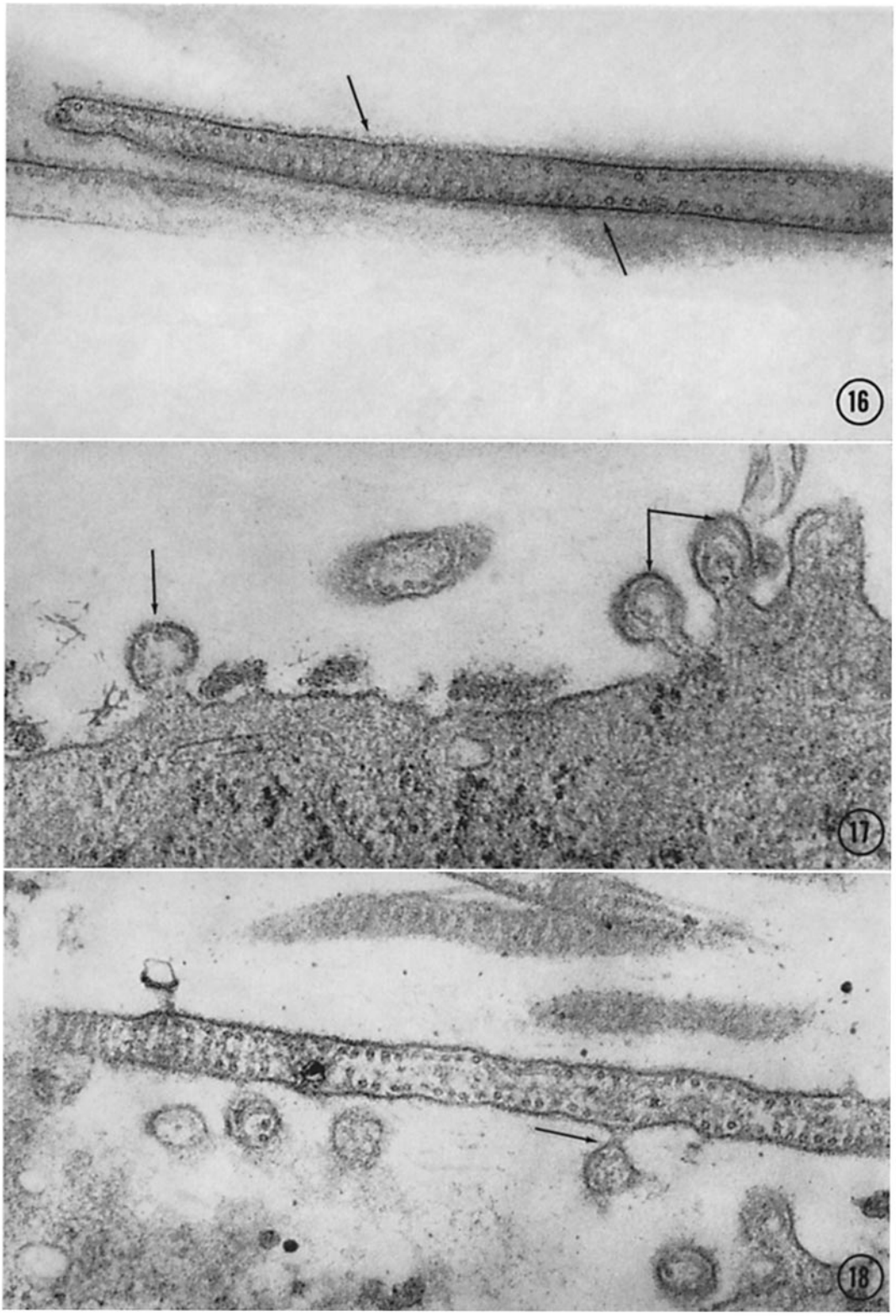
PLATE 76

FIG. 16. Section of a viral filament showing internal component surrounded by an electron dense inner-membrane separated from an outer membrane of lesser density by a narrow "clear" space. In some areas (→), the inner membrane appears to be covered by a single row of closely apposed cuboidal or round vesicles. × 95,000.

FIG. 17. Section of part of an infected WGM-1 cell showing bud-like processes (→) projecting outward from the cell surface. These contain internal component; the latter is for the most part surrounded by an envelope. These processes suggest beginning viral sphere formation. × 106,000.

FIG. 18. Filament with lateral bud-like processes (→) suggesting sphere formation from a filament. × 99,000.



(Prose *et al.*: Myxovirus and infectious hepatitis. II)