STUDIES ON PNEUMONIA VIRUS OF MICE (PVM) IN CELL CULTURE

II. Structure and Morphogenesis of the Virus Particle*

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Plates 20–25

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Pneumonia virus of mice (PVM) is a usually latent virus which may induce a fatal pneumonia when serially passed in mice. The structure of the virus has not been described, and it has not been classified within the major groups of animal viruses. The limited knowledge of the properties of PVM has been due in large part to the lack of a suitable cell culture system. The preceding paper (1) described the growth of the virus in a line of baby hamster kidney cells, and presented evidence suggesting that PVM is an RNA virus that replicates in the cytoplasm.

The propagation of PVM in cultured cells has enabled an electron microscopic study of the structure and morphogenesis of the virus. The results of this investigation, reported here, indicate that the morphology and development of PVM are typical of the myxovirus group.

Materials and Methods

Virus and Cells.—The procedures employed in the growth of BHK21 cells and the propagation of PVM were described in the preceding paper (1).

Negative Staining.—PVM grown in BHK21 cells was sedimented by centrifugation for 3 hr at 19,600 *g*. Pellets were held overnight at 4°C in approximately 0.2 ml of the residual supernatant and then resuspended in that volume. A small drop was spread over a Formvar-coated grid with a heavy carbon film, rinsed with phosphate-buffered saline (PBS) (2), and fixed with a drop of 1% glutaraldehyde (3) in PBS. The grid was then rinsed with distilled water and stained with 2% phosphotungstic acid, pH 6.2 (4).

Thin Sections.—Monolayers of cells were detached from Petri dishes by scraping or treatment with 0.25% trypsin and 0.05% EDTA (5). Cells were centrifuged for 4 min at 200 *g*, and the pellet was fixed for 4 min in 1% glutaraldehyde in PBS, and postfixed with 1%

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osmium tetroxide (6) in isotonic phosphate-buffered NaCl, pH 7.2. In some experiments specimens were stained immediately after fixation with 0.5% uranyl acetate in veronal acetate buffer, pH 5, for 2 hr (7). The cell pellets were dehydrated in a series of alcohols and embedded in epoxy resin (8). Thin sections were stained by a 1 min application of a saturated solution of uranyl acetate diluted 1:1 with ethanol, followed by a 1–2 min application of lead citrate solution (9). Specimens were examined in a Hitachi HS-7S electron microscope.

RESULTS

Structure of the PVM Virion

Virus Particles.—The morphology of the virus particle was examined in thin sections as well as by the negative staining technique. PVM particles are extremely fragile (1), and no virus particles were seen in unfixed, negatively stained preparations. However, if PVM suspensions were fixed with glutaraldehyde prior to negative staining, and grids were scanned extensively at low magnification, a number of long, filamentous particles were observed.

These filaments (Figs. 1 and 2) usually have a uniform diameter of 100–120 mμ, and a total length of 2–3 μ. Frequently one end of the filament shows an enlarged, bulbous tip (Fig. 1). The outer surface consists of an envelope covered with a layer of short projections or spikes 80–90 A in length. In most instances the particles were not penetrated by the negative stain, and it was not possible to discern the internal structure. Occasionally an irregular, strandlike internal component has been seen in some filaments. Much of the grid surface was covered with strandlike material which may represent disrupted internal component from broken virus particles.

Thin sections of BHK21 cells infected with PVM showed frequent extracellular filamentous particles which correspond in size to those seen with negative staining (Figs. 3 and 4). In addition, circular profiles 80–120 mμ in diameter were seen in thin sections (Fig. 3). The frequency with which circular profiles were observed suggests that they are sections of spherical virus particles, rather than cross-sections of filamentous particles. The failure to detect small, spherical particles in negatively stained preparations probably resulted from inability to recognize such particles when scanning grids at low magnification.

The sectioned particles are bordered by a membrane with outer projections, corresponding to the envelope seen with negative staining. The internal structure is seen more clearly in sectioned particles than within negatively stained particles. Electron-dense internal strands extend across the circular profile in Fig. 3, and a similar component occurs coiled within filamentous particles (Fig. 4).

The particles described above were found only in PVM-infected cells and were the only virus-like structures which could be associated with PVM, either in sections or with negative staining. It was therefore concluded that these represented PVM virions. Additional evidence which supports the conclusion

that these are PVM particles includes the time course of their appearance and their interaction with murine erythrocytes (see below).

Release of a Helical Component from Cells Infected with PVM.—The PVM particles described above bear an obvious morphological resemblance to the myxoviruses, having an outer, spike-covered envelope and a strandlike internal component. Since it was not possible to visualize the exact structure of internal component within virions, isolation of this component was attempted.

As described in the preceding paper (1), prominent eosinophilic inclusions appear in the cytoplasm of BHK21 cells after infection with PVM. These inclusions appear to consist of strandlike elements (cf. Fig. 9). Similar inclusions in BHK21-F cells infected with the parainfluenza virus SV5 consist of aggregates of the helical SV5 internal component (5), and large amounts of SV5 internal component have been isolated from such infected cells (10). The procedure developed for isolation of SV5 internal component from cells was therefore applied to PVM-infected cells.

Cells were inoculated at a multiplicity of 0.5 $TCID_{50}$ per cell, and after 3–4 days were suspended by treatment with 0.25% trypsin and 0.05% EDTA. The cells were pelleted at 200 *g* for 5 min, and resuspended in distilled water at a concentration of approximately 1.5×10^7 per ml. Many cells were disrupted, and a large number of nuclei free of cytoplasm were observed. These extracts were clarified by centrifugation at 6500 *g* for 15 min, and the supernatant (4.5 ml) was mixed with cesium chloride (2.0 g). The resulting solution (average density = 1.30) was centrifuged for 7–10 hr at 45,000 rpm in the SW50 rotor. After centrifugation, fractions were collected from the bottom of the tube, and densities determined by refractive index measurements.

A diffuse band with a density of 1.29 g/ml was observed near the center of tubes that contained extracts of PVM-infected cells; no bands were observed in this position in control tubes containing extracts of uninfected cells. The material in the band was examined in the electron microscope. As illustrated in Figs. 5 and 7, the observed structures have the general appearance typical of the helical nucleocapsids of myxoviruses. However the diameter of the PVM component is 120–150 A, which differs from the diameters of the nucleocapsids of the two subgroups of myxoviruses (11). For comparison, the component from PVM-infected cells (Fig. 5) and the helical nucleocapsid of the parainfluenza virus SV5, a subgroup II myxovirus (Fig. 6), were photographed at the same magnification in the microscope, and enlarged by the same factor. It is apparent that the internal component of SV5, which has a diameter of 150–180 A (12), is larger than the component from PVM-infected cells.

The helical PVM component is usually seen somewhat stretched or extended; tightly coiled helices, such as the segment of SV5 nucleocapsid shown in Fig. 6, were not observed. In selected regions, the turns of the helix of the PVM component are well separated, and their orientation suggests that the structure is single-stranded (Fig. 7).

Observations on Thin Sections of PVM-Infected Cells

Formation of Virus Particles.—Uninfected BHK21 cells are similar in morphology to the BHK21-F cells previously described (5). They are fibroblastic, contain an extensive rough-surfaced endoplasmic reticulum and few smooth-surfaced cisternae, and possess the other usual cytoplasmic organelles. The surfaces of BHK21 cells show relatively few microvilli. Bundles of fibers are frequently seen in the cytoplasm, particularly in cells examined several days after monolayers have become confluent. Uninoculated BHK21 cells also contain the virus-like particles which have been previously described (13, 14, 5).

Cells examined 24 hr after inoculation at a multiplicity of 0.5 $TCID_{50}$ per cell showed no characteristic alterations in structure, and extracellular virus particles were seen infrequently. By 48 hr, extracellular PVM particles were numerous, and examples are shown in Figs. 3, 4, 11, 13, and 17. In addition, many cells observed at this time contain dense cytoplasmic inclusions (Figs. 8–11) which appear to consist of elements with a threadlike appearance (Fig. 9). Occasionally, similar dense inclusions are seen extracellularly, apparently resulting from rupture of the cells in which they were formed. These intra- and extracellular inclusions are similar in size, shape, and distribution to the inclusions seen in the light microscope, which stain flame-red with acridine orange (1).

Virus particles acquire their envelope by budding at the outer cell membrane (Figs. 10–12, 14, 16). The interior of budding virus particles (Figs. 10–12) has a dense appearance similar to that of the material in inclusions, and in some areas the inclusion appears to extend into the budding particle (Fig. 11). In most regions no well-defined organization is visible in the dense interior of budding virus particles; occasionally, however, a strand is seen (Fig. 12) which resembles the internal component seen in mature, extracellular virions. Filamentous particles are also seen budding at the cell surface (Figs. 14 and 16). All of the budding particles are bordered by an electron-dense membrane which is continuous with the outer cell membrane. Increased electron density on the outer surface of the particles corresponds to the spikes seen with negative staining.

The leaflets of the unit membrane (15) were not readily resolved in the BHK21 cell membrane by the usual postembedding staining procedure. However, in specimens stained with uranyl acetate prior to embedding, the staining of membranes was enhanced. With this procedure it was possible to observe a typical unit membrane in the envelope of mature, extracellular virus particles (Fig. 13), and the leaflets in the BHK21 cell membrane were also resolved. The membrane in the envelope of budding virus particles is continuous with, and appears morphologically identical to, the outer cell membrane (Fig. 14).

Cells on which budding virus particles are present also exhibit rows of closely spaced vesicles projecting inward from their surfaces (Fig. 11). These vesicles

have a rather uniform diameter of 60–100 mμ, and are seen with greatest frequency on regions of the cell surface between the regions where virus is budding. Occasionally, similar vesicles are seen closely adjacent to, or just beneath, budding virus particles (Fig. 10). Although the relationship of these vesicles to virus formation is not clear, they are numerous only on surfaces of cells which exhibit budding virus, suggesting a possible connection between virus maturation and vesicle formation.

Apart from the cytoplasmic inclusions and surface phenomena involved in virus maturation, no consistent virus-specific structural changes were observed in infected cells. Dense masses were observed in a few nuclei, but these could not be positively identified as virus-related.

Interaction of PVM and Infected BHK21 Cells with Murine Erythrocytes.— The interaction of PVM particles and PVM-infected cells with murine erythrocytes, which the virus agglutinates (16, 1), was examined in thin sections of cells harvested 48 hr after infection.

Monolayers of cells were rinsed twice with PBS, and overlayed with 2 ml of a 0.8% suspension of murine erythrocytes in buffered saline. After gentle agitation at 10-min intervals for 30 min, the unadsorbed erythrocytes were removed by washing twice with PBS. The erythrocytes adsorb firmly to infected BHK21 cells, but are readily washed from uninfected cells. The cells were then suspended, fixed, and sectioned by the usual methods.

Erythrocytes were seen in direct contact with budding virus particles at the surfaces of infected cells (Fig. 15). Frequently, however, erythrocytes were observed attached to areas on the BHK21 cell surface where no virus-specific morphological changes were visible (Fig. 16). Attachment of erythrocytes to apparently unmodified cell surfaces may be due to budding virus which is not in the plane of sectioning; however, this seems unlikely because of its frequency. In Fig. 16 the shape of the erythrocyte is distorted to conform to that of the cell surface, and a space separates the adsorbed erythrocyte from the cell membrane. Such an apparent gap has been observed with measles virus–infected cells (17), and may contain material which is not stained by the procedures employed, or result from separation of the two membranes during processing.

In addition to adsorption of erythrocytes to surfaces of infected cells, adsorption of extracellular virus particles to erythrocytes was also seen (Fig. 17). The virus particles observed in these studies thus exhibit the interactions with mouse erythrocytes which would be expected of PVM virions.

DISCUSSION

The preceding paper (1) presented evidence that PVM is an RNA virus that replicates in the cytoplasm. The present results indicate that PVM virions are spheres or filaments, 80–120 mμ in diameter, which contain a strandlike internal component that is enclosed in an envelope acquired by a budding process

at the cell surface. The evidence that the observed particles are PVM virions includes their appearance in infected cells at the time PVM infectivity and hemagglutinin are detected, the absence of such particles in uninfected control cells, and their interaction with murine erythrocytes. In addition, no other virus-like particles have been observed in preparations of PVM, and the structure of these obviously fragile, membrane-bounded particles is compatible with the known ether sensitivity (18) and extreme fragility (1) of PVM. The PVM hemagglutinin with an estimated diameter of 40 mμ described by Curnen and coworkers (19) probably represented fragments of 80–120 mμ particles such as those observed in the present studies. Evidence for hemagglutination by fragments of PVM virions was presented in the preceding paper (1).

The internal component of PVM appears to be a single-stranded helix with a diameter of 120–150 A. It was thus smaller than the ~180 A helical nucleocapsid of subgroup II myxoviruses, but larger than the ~90 A internal component of influenza virus (11, 20).[1] That the dense inclusions seen in PVM-infected cells represent aggregates of internal component is suggested by their strand-like appearance at high magnification, and by continuity of the inclusion with the dense interior of some budding virus particles. In addition, the inclusions stain red with acridine orange, indicating the presence of RNA (1). It has been shown previously (5) that the cytoplasmic inclusions in BHK21-F cells infected with the parainfluenza virus SV5 consist of large aggregates of the helical internal component of this virus.

As with myxoviruses (5), the outer cell membrane is continuous with the membrane forming the envelope of the budding PVM particle. It thus appears that cellular membrane is incorporated into the virion. The membrane in the viral envelope possesses an outer covering of spikes which are individually visible with negative staining, and appear as a dense outer layer in sections. Some virus-specific proteins may be present on the cell surface before any morphological change can be detected, because virus-specific adsorption of erythrocytes can occur on regions of the cell surface where no budding virus or layer of spikes is apparent. This type of hemadsorption was first demonstrated in influenza-infected cells by Hotchin and coworkers (21). It is possible that some precursor of the viral spikes may be responsible for the hemadsorption seen in such regions.

The structure and morphogenesis of pneumonia virus of mice suggest that it is a member of the myxovirus group. Although there is no available evidence that PVM adsorbs to a neuraminic acid-containing receptor on erythrocytes,

[1] There have been conflicting reports of the diameter of the internal component of respiratory syncytial virus, one indicating a diameter slightly larger than that of the subgroup I myxoviruses (Bloth, B. N. T., and E. C. J. Norrby. 1965. *Arch Ges. Virusforsch.* **15**:249), and another, a diameter closer to that of subgroup II viruses (Eckert, E. A., H. F. Maassab, and J. J. Cavallaro. 1965. *J. Bacteriol.* **90**:1784).

other viruses, such as measles, which are now considered to be members of the myxovirus group also apparently do not require such a receptor (20).

The myxoviruses have been segregated into two well-defined subgroups (11, 20) on the basis of structural and biological properties. As summarized in Table I, PVM does not fit into either of these subgroups, but shares some properties with both. The particle size of PVM is similar to that of the influenza viruses (subgroup I). There is no indication that PVM possesses hemolytic or cell-fusing activity, and in this respect, also, it resembles influenza virus. However, the synthesis of PVM internal component appears to occur in the cytoplasm

TABLE I

*Comparison of Properties of Pneumonia Virus of Mice with the Two Subgroups of Myxoviruses**

	PVM	Subgroup I (influenza)	Subgroup II (parainfluenza, mumps, NDV)
Particle size	80–120 mμ	80–120 mμ	120–500 mμ
Diameter of internal component	120–150 A	90 A	180 A
Filamentous particles	+	+	±‡
Easily disrupted	+	−	+
Apparent site of synthesis of internal component	Cytoplasm	Nucleus	Cytoplasm§
Cytoplasmic inclusions	+	Usually absent	+
Hemolytic and cell fusing activities	−	Weak or absent	+

* Summary of properties of the two subgroups adapted from Waterson (11) and Hirst (20).

‡ It was thought (11, 20) that filaments were rare among subgroup II myxoviruses; however, many filamentous virus particles have been observed recently in fixed, sectioned preparations of parainfluenza viruses (22, 5, 23).

§ Although in most virus-cell systems nucleoprotein synthesis appears to be limited to the cytoplasm, in some instances nuclear synthesis has been reported (24).

and eosinophilic cytoplasmic inclusions are prominent; these properties are characteristic of subgroup II myxoviruses. PVM also resembles these viruses in the ease with which it is disrupted, whereas the influenza viruses show greater structural stability. The failure of PVM to fit into either subgroup of myxoviruses, and particularly the 120–150 A diameter of its internal component, suggest that a third subgroup of myxoviruses may exist.

SUMMARY

Pneumonia virus of mice (PVM) particles are spheres 80–120 mμ in diameter, or filaments of similar diameter with lengths up to 3 μ. The particles possess an outer spike-covered envelope and helical internal component 120–150 A in diameter. Virus particles acquire their envelope by a budding process at the cell membrane; mature particles are seen only extracellularly.

Dense inclusions are prominent in the cytoplasm of PVM-infected BHK21 cells by 48 hr after inoculation. The inclusions appear to consist of aggregates of the internal component of PVM, and the helical component has been isolated in a cesium chloride gradient from extracts of osmotically shocked cells.

Murine erythrocytes, which are agglutinated by PVM, adsorb to the surface of infected cells and to budding and extracellular PVM particles.

On the basis of its structure and morphogenesis, PVM appears to be a myxovirus; however, it does not fit into either of the established subgroups of myxoviruses. The 120–150 A diameter of the PVM internal component differs from the diameters of the internal components of the two established subgroups of myxoviruses, and suggests that a third subgroup of these viruses may exist.

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EXPLANATION OF PLATES

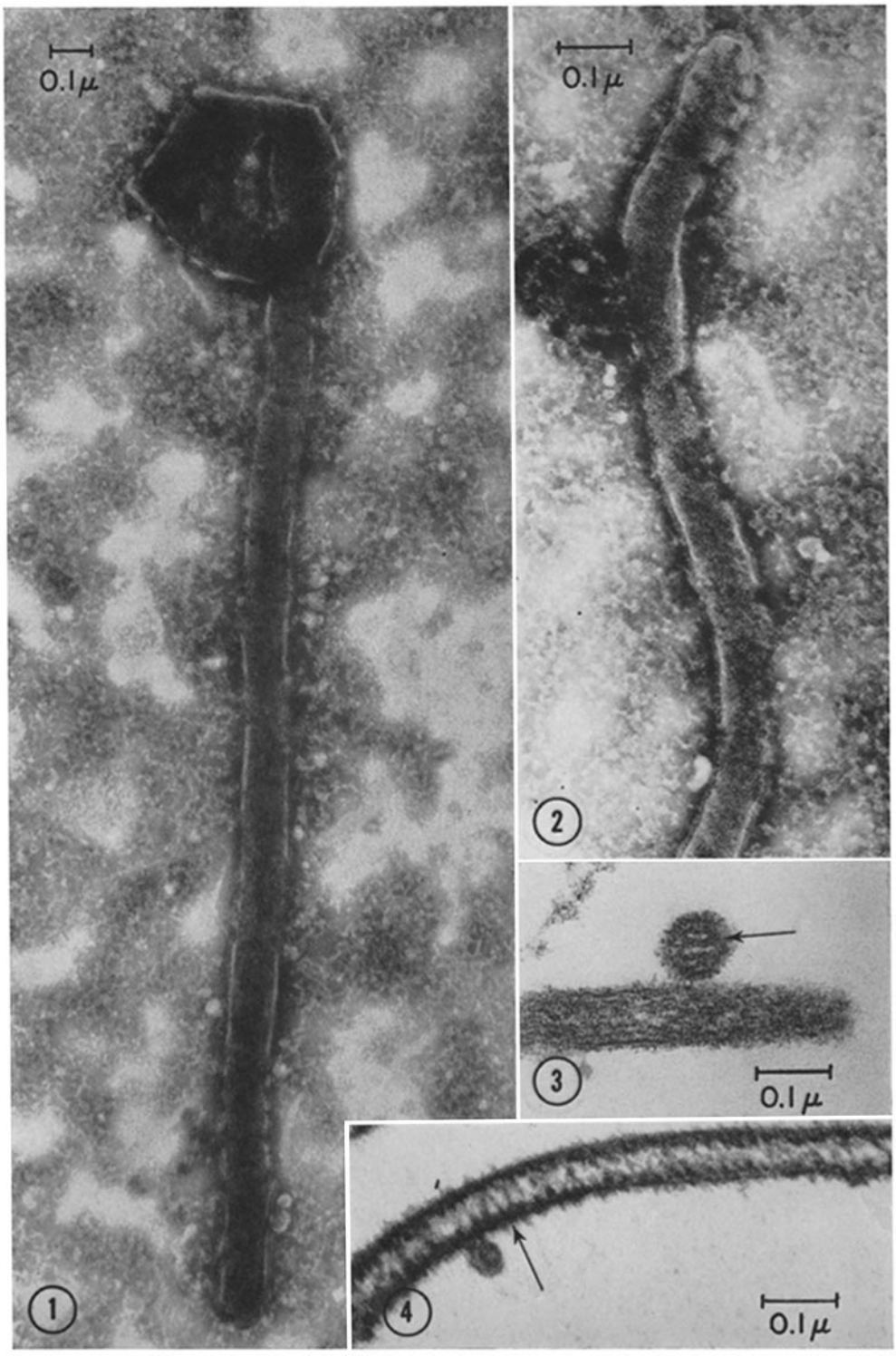
PLATE 20

FIG. 1. A filamentous PVM particle, 3 μ in length and 110 mμ in diameter, with a bulbous tip 400–500 mμ in diameter. Much of the background in negatively stained PVM preparations is covered with material which in some areas has an irregular strandlike appearance. × 60,000.

FIG. 2. A portion of a PVM filament showing the layer of projections or spikes covering its surface. × 106,000.

FIG. 3. A circular profile of a PVM virion which is traversed by well-defined strands approximately 120 A in diameter (arrow). Below is a section of a filamentous particle whose internal structure is not well defined. × 108,000.

FIG. 4. Section of a PVM filament in which electron-dense strands (arrow) traverse a portion of the interior. × 108,000.



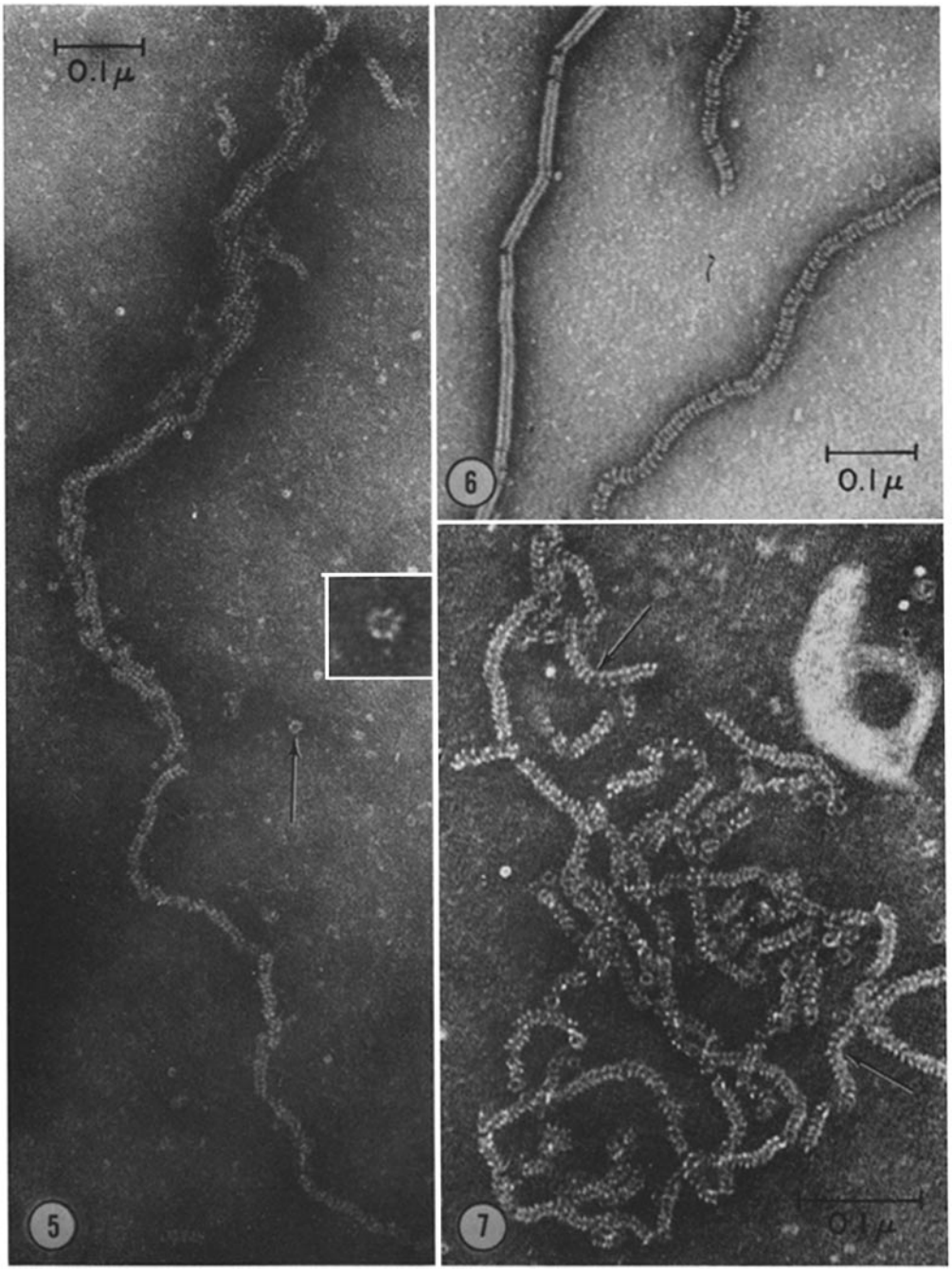
(Compans, Harter, and Choppin: Structure and morphogenesis of PVM)

PLATE 21

FIG. 5. Helical component (nucleocapsid) 120–150 A in diameter isolated from PVM-infected cells. It appears to be loosely coiled and fragmented. The arrow indicates an end view of a segment of the nucleocapsid suggesting the presence of subunits. This is shown at higher magnification in the insert. × 120,000; insert × 275,000.

FIG. 6. Segments of the helical nucleocapsid of the parainfluenza virus SV5, 160–180 A in diameter, shown for comparison with PVM. Both loosely and tightly coiled segments are seen. Figs. 5 and 6 were photographed at the same magnification and enlarged by the same factor; the smaller diameter of the PVM nucleocapsid is apparent. × 120,000.

FIG. 7. The PVM internal component at higher magnification. In several regions (arrows), the helix is stretched into a loose coil which suggests that it is a single-stranded structure. × 170,000.



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FIGS. 8–17. Sections of BHK21 cells 48 hr after infection with PVM. Key to abbreviations: *N*, nucleus; *E*, murine erythrocyte; *I*, inclusion.

PLATE 22

FIG. 8. Low magnification view showing a dense, well-circumscribed cytoplasmic inclusion near the periphery of the cell. × 17,000.

FIG. 9. Higher magnification of a cytoplasmic inclusion (outlined by arrows). It consists of tangled, electron-dense, threadlike elements. × 51,000.

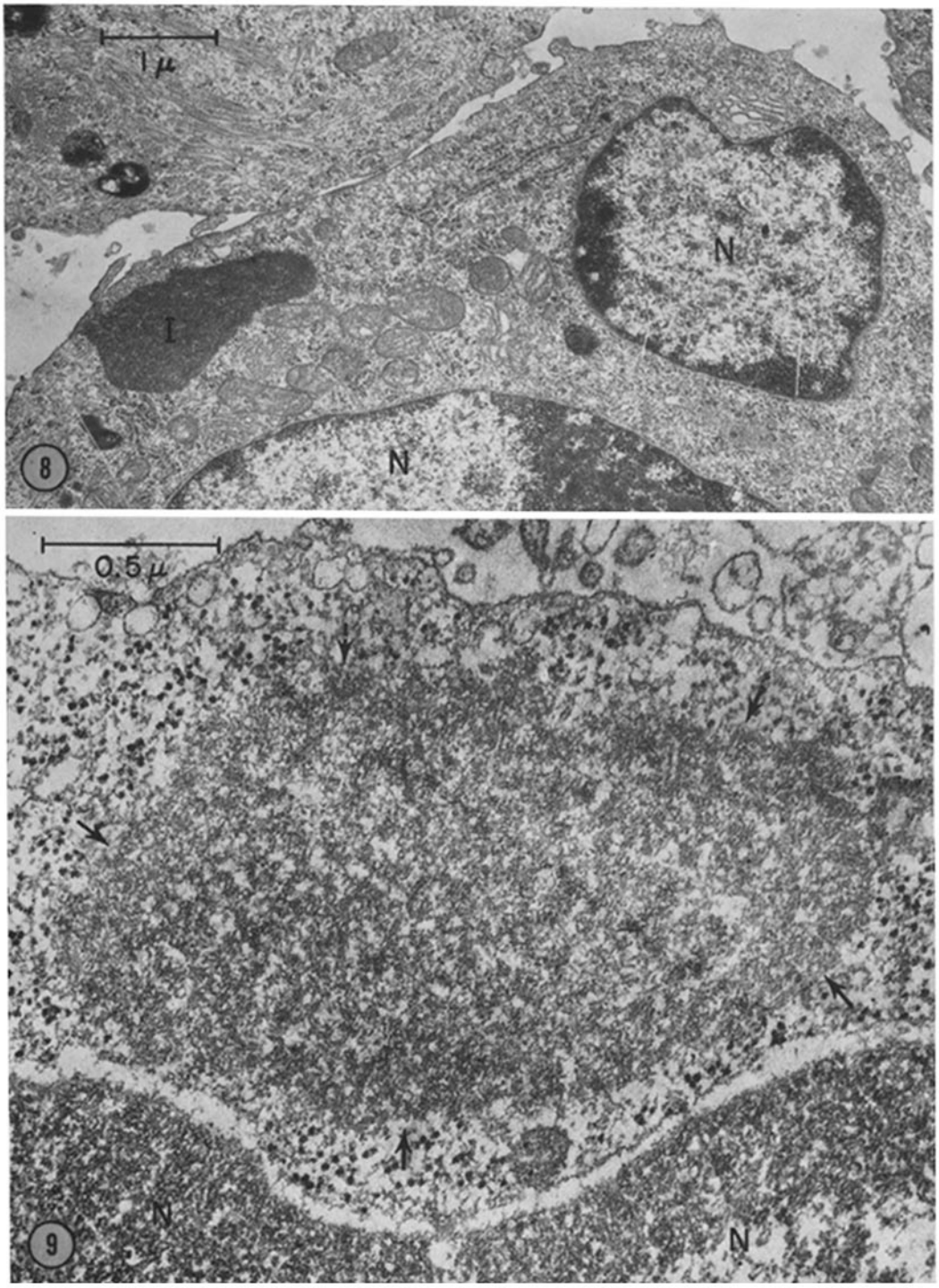

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PLATE 23

FIG. 10. A region of the cell surface on which eight budding PVM particles are arranged in a glovelike configuration. Other regions of the cell surface, indicated by arrows, show underlying dense threadlike material, but do not exhibit outfolding of the cell membrane. These may be regions from which buds will develop. × 104,000.

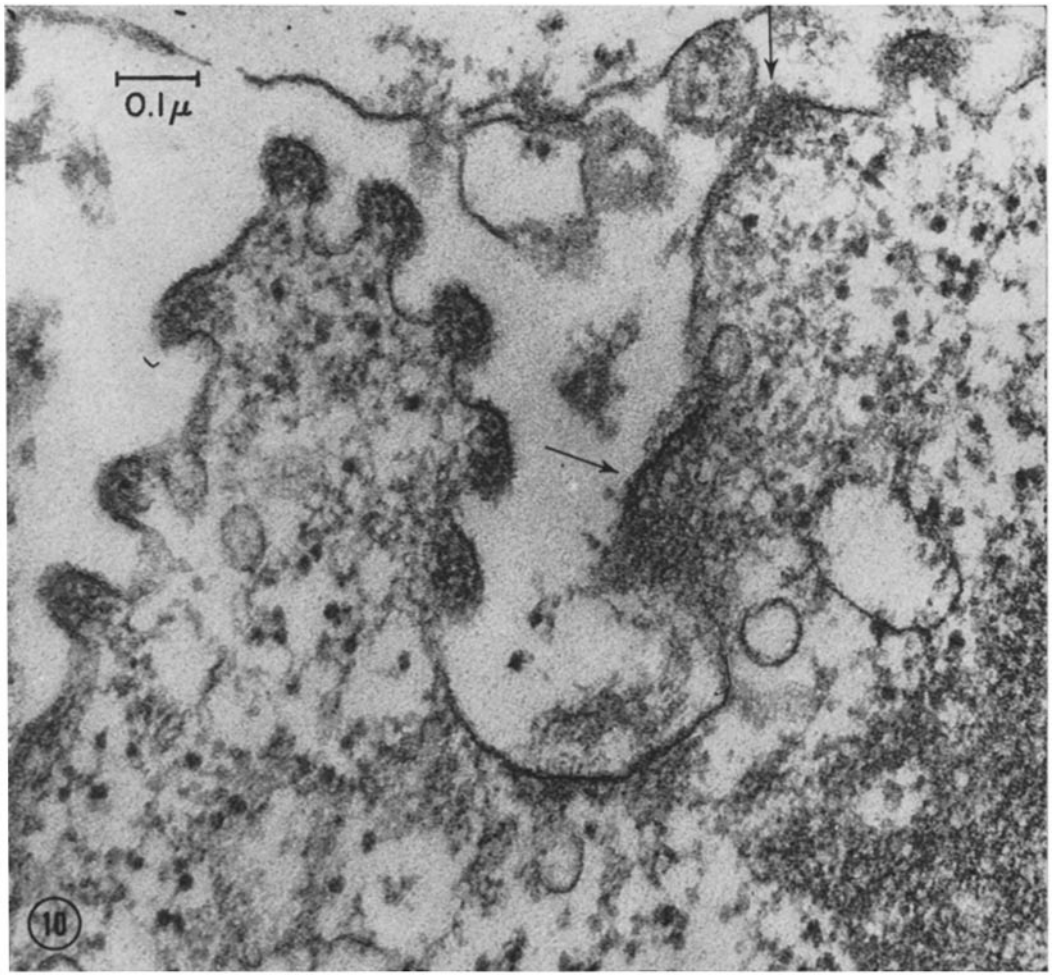

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PLATE 24

FIG. 11. Portion of the cell surface studded with budding virus particles, and a PVM filament which appears to have been released. A dense inclusion occupies much of the cell interior. The inclusion consists of dense material similar in appearance to the interior of virus particles, and this material appears to extend into some of the budding particles. Ovoid vesicles or invaginations (arrows), 60–100 mμ in diameter, are frequently observed in regions of the cell surface near budding virus particles. × 67,000.

FIG. 12. Budding virus particles at higher magnification; one particle shows an internal strand (arrow). × 94,000.

FIG. 13. Extracellular virus particles in a specimen stained with uranyl acetate prior to embedding. This procedure is effective for demonstrating the unit membrane, i.e. two electron-dense layers separated by a less dense layer. Such a unit membrane is visible in the envelope of the filament with its bulbous tip as well as in the small circular profile (arrows). The internal structure of virus particles is not well defined by this staining procedure. × 111,000.

FIG. 14. PVM filaments at the cell surface in a specimen stained with uranyl acetate. The unit membrane structure is resolved in some areas of the cell surface and in the envelope of budding virus particles. The cell membrane appears to be incorporated into the emerging virus. × 87,000.

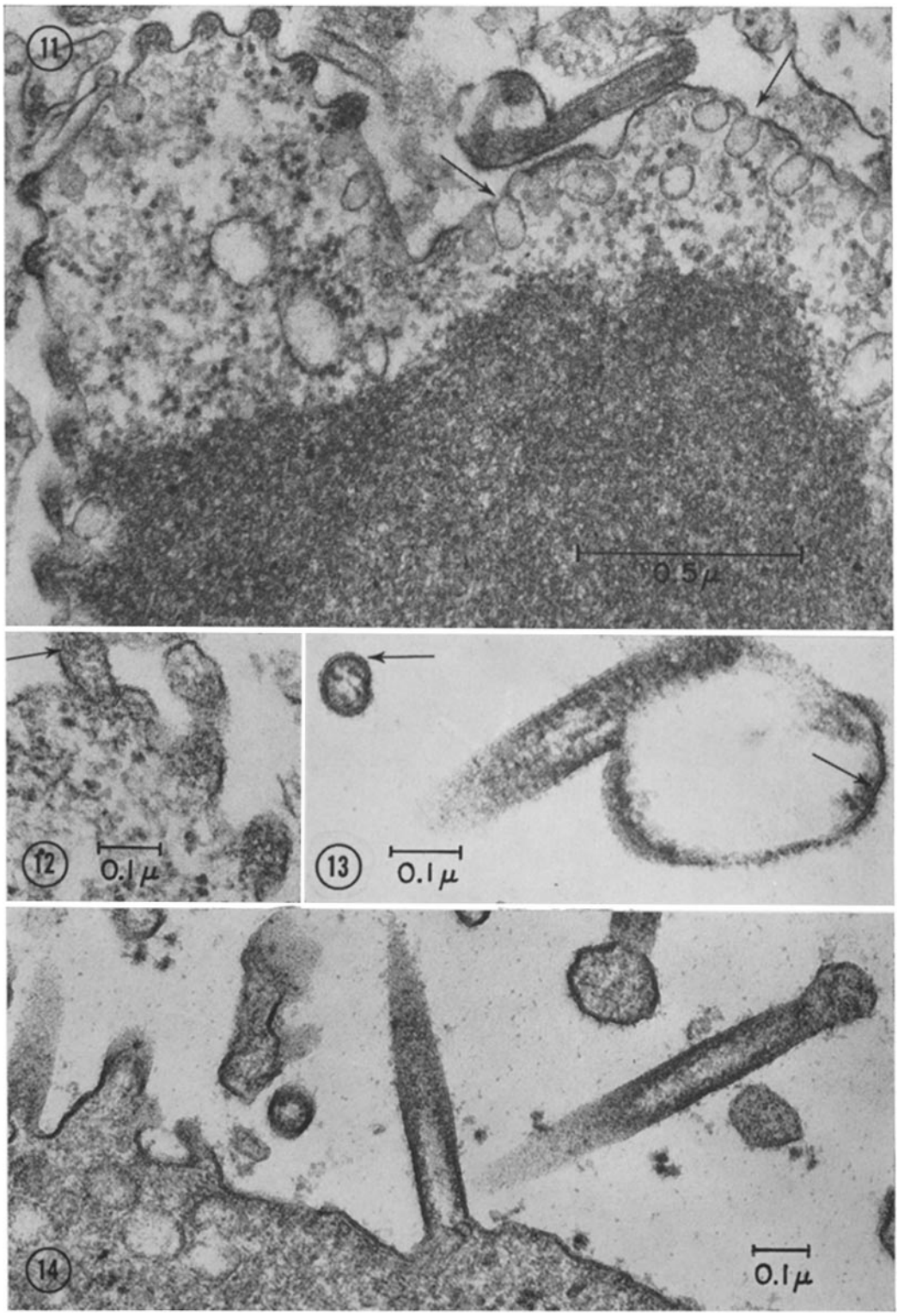

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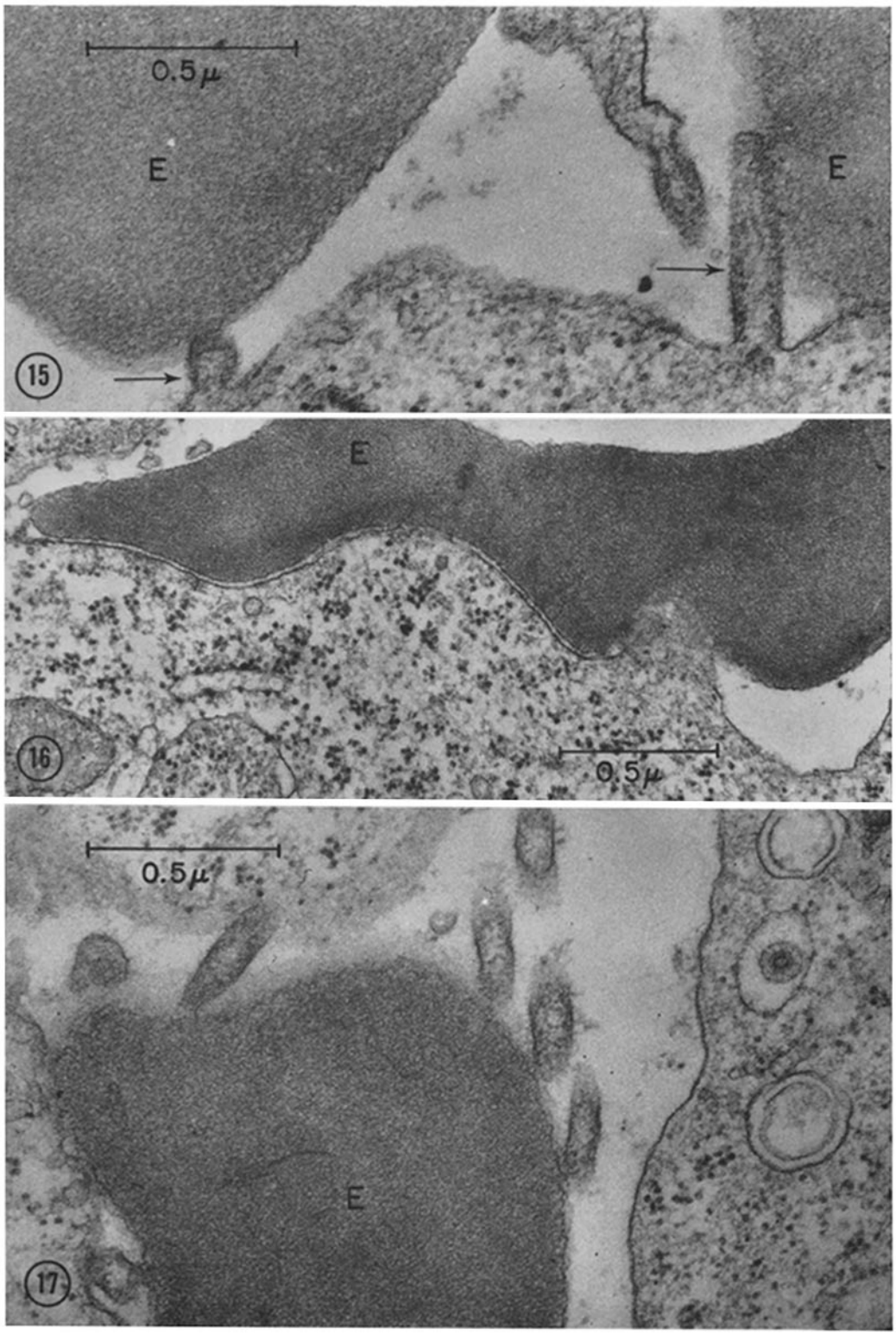
PLATE 25

FIG. 15. Erythrocytes in contact with PVM particles (arrows) which are budding from the surface of an infected cell. × 65,000.

FIG. 16. An adsorbed erythrocyte (*E*) whose contours closely follow those of the BHK21 cell surface. The erythrocyte membrane and the BHK21 cell membrane are separated by an apparent gap approximately 150 A in width. No virus-specific structures are seen on the BHK21 cell in the zone of adsorption. × 48,000.

FIG. 17. Sections of filamentous PVM particles in contact with the surface of an erythrocyte. A virus-like particle is shown within a vesicle in the cell on the right. Such particles are also found in uninoculated BHK21 cells, and do not resemble PVM virions. × 59 000.



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