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Micromorphological Aspects of the Development of Rubella Virus in BHK-21 Cells[1]

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Sequential effects of rubella virus infection in BHK-21 cells were studied by electron microscopy of thin sections of control and infected cells, 2 to 7 days after infection. Vacuolization of cytoplasm in Golgi areas apparently preceded budding of virions from vacuole membranes and involvement of the endoplasmic reticulum. Newly formed endoplasmic reticulum cisternae encircled and segregated virion-forming vacuoles together with other cellular elements. Large vacuolar complexes with numerous virus particles developed, and virus release from these areas occurred with disruption at the cell periphery. The viral particles, with a mean diameter of about 56 nm, consisted of an electron-dense core surrounded by a less dense capsid, enveloped by a typical unit membrane derived from the vacuole membrane.

The rubella virus particle has been described in recent studies of strains propagated in cell cultures of BHK-21 (3, 5, 6), RK (6; T. E. Hobbins and K. O. Smith, Bacteriol. Proc., p. 181, 1968), and SIRC (8). The findings, in general agreement, have indicated that rubella virus is not a myxovirus as suggested by an earlier report (13) but may fall into a taxonomic group yet to be defined (8, 9, 11). Limited data on the development of rubella virions in SIRC cells have been reported (8). The progressive events, however, in the development of the rubella virions in infected cells have not been clarified. The present study describes the ultrastructural changes of BHK-21 cells infected with rubella virus and the successive stages in the formation and maturation of the virions.

MATERIALS AND METHODS

Virus strain. M33 (N.Y.S. no. 65-0287), isolated by Parkman et al. (12), was obtained from The American Type Culture Collection as the 20th passage in primary cultures of African green monkey kidney cells, and was adapted in this laboratory to the BHK-21 clone 13 cell line and used in the 9th and 11th passages for electron microscopy.

Cell cultures. The BHK-21 cells were grown in monolayers with Eagle's modified medium containing 10% fetal bovine serum and 10% tryptose phosphate broth. Two to 4 days after seeding, the cultures were inoculated with virus at a multiplicity of about 0.2 to 1.0 $TCID_{50}$ per cell. Maintenance medium was Eagle's with 3% fetal bovine serum. Control and infected cells were examined from 2 to 7 days post-infection.

Preparation for electron microscopy. Cell monolayers were fixed with glutaraldehyde-osmium tetroxide (15) or chrome-osmium tetroxide (A. J. Dalton, Anat. Rec., p. 281, 1955), washed with buffer, scraped into a fresh change of buffer, and sedimented by centrifugation. After dehydration in a graded series of ethyl alcohol solutions followed by propylene oxide, the material was infiltrated and embedded in an Epon mixture (7). Thin sections were obtained with Reichert or LKB ultratomes, stained with uranyl acetate and lead citrate (17), and photographed with Siemens Elmiskop models I and IA at magnifications of 20,000 to 80,000 ×.

RESULTS

In the infected cell cultures, cytopathic effects were evident in 48 hr. Infectivity titers of supernatant fluids in repeated tests were $10^{5.8}$ to $10^{6.5}$ $TCID_{50}$ per ml at 48 hr and $10^{6.5}$ to $10^{7.5}$ at 72 hr. The presence of rubella viral antigen was confirmed by immunofluorescence with rabbit homologous antiserum or human convalescent-phase serum (4) before examination of duplicate cell cultures for electron microscopy.

Control cells exhibited normal Golgi structures (1), generally abundant in actively growing cells, with closely packed stacks of lamellae and a profusion of small vesicles usually budding off from the lamellae. Large vesicles or vacuoles were rarely found. Profiles of the endoplasmic reticulum varied widely in different cell sections. Typical mitochondria, free or attached ribosomes,

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and cytoplasmic filaments were generally abundant. Nuclei were of normal configuration.

In infected cells, vacuolization of the cytoplasm was apparent in at least 50% of the cells at 48 to 72 hr postinoculation. In many instances, the origin of vacuoles could be attributed to dilation of Golgi cisternae (Fig. 1) and may possibly be correlated with viral penetration in cycles of infection subsequent to the initial infection of the cell culture. Particles were seen extracellularly in areas of cell membranes displaying pinocytotic activity with little further change from normal cells (Fig. 1). Involvement of the endoplasmic reticulum was evident after the appearance of virions in vacuoles. Profiles of the endoplasmic reticulum were readily identified around these vacuoles containing virions (Fig. 2), sometimes encircling them completely (Fig. 7). Virions budding from the vacuole membrane were frequently found (Fig. 2, 4). The triple-layered structure of a typical unit membrane (14) could clearly be seen in the vacuole membrane and in the viral envelope (arrow Fig. 2, 8, 9). Budding from plasma membranes was not observed in our preparations.

Cells at more advanced stages of viral infection, 4 and 7 days, were in various stages of disintegration. Infectivity titers of fluids from the 4-day culture were $10^{5.5}$ $TCID_{50}$ per ml. Later effects of infection were characterized by segregation of cortical cytoplasmic areas that contained numerous membranous structures and large vacuoles with increased numbers of viral particles. The newly formed smooth endomembranes appeared to originate from the rough endoplasmic reticulum which branched in various directions. They encircled the areas containing virus particles and separated them from other portions of the cytoplasm. The segregation process, generally extensive, also enclosed normal organelles, such as mitochondria and lysosomes, with resulting structures that may correspond to the phagocytic vacuoles described by Holmes et al. (6). The vacuolar complexes apparently became permeable, dilated, and burst at the cell surface, discharging masses of virions and cell debris (Fig. 3, 10).

Mature spherical virions ranged in diameter from about 45 to 65 nm (Fig. 8 to 10). The rubella virion with a diameter of 57.5 nm was enveloped by a triple-layered single membrane approximately 8 nm in width (Fig. 8). The interior of the particle consisted of a highly electron-dense core, ca. 18.5 nm in width, surrounded by a capsid of medium to light electron density, ca. 11.5 nm wide. Variations in density and size of cores (Fig. 8, 9) may be due to different planes of sectioning or preparatory methods. In Fig. 10, however, particles are seen with light or dense cores, possibly as a result of staining variations. Coupled virions were also seen (Fig. 4 to 6). Particles resembling latent hamster virus (2, 16), but readily distinguishable from rubella virions, were found only in endoplasmic reticulum cisternae as single particles or in small groups, with equal frequency in control and infected cells.

DISCUSSION

Our observations on sequential effects of rubella virus infection in BHK-21 cells revealed preliminary signs of infection in the Golgi regions followed by budding of virions from vacuole membranes and involvement of the endoplasmic reticulum cisternae. Frequently, the endoplasmic reticulum cisternae developed around initially small vacuoles containing virions. They apparently continued to proliferate, segregating extensive affected areas and enclosing large, complex, vacuolar structures and also islets of cytoplasm with organelles such as mitochondria and lysosomes. Formation of such structures by confluence of the small vacuoles seems to be an autophagic process rather than phagocytic as suggested by Holmes et al. (6). Increased permeability and dilation of the vacuoles could at final stages cause their disruption at the cell periphery with virus release. The virus particles were clearly seen to be enclosed by a typical unit membrane derived from the vacuole membrane. Our findings are in general agreement with those of others on the size and morphology of rubella virions of different strains isolated and propagated in various cell lines.

A paper by Murphy et al. (10) on electron microscopy of the development of rubella virus in BHK-21 cells appeared just as this paper was being completed. Their observations differ from ours in several important respects: no morphological changes were seen in infected cells before budding of virus from cell membranes; budding was more prominent from the cell membrane than from endomembranes and was seen in cisternae of the endoplasmic reticulum; the envelope of the virion was not indicated as a typical unit membrane; latent hamster virus seemed to be more numerous. Certain of these differences may have resulted from the high passage level of their virus strain in BHK-21 cells (38 as compared with 9 or 11 in our study) or from the preparatory methods.

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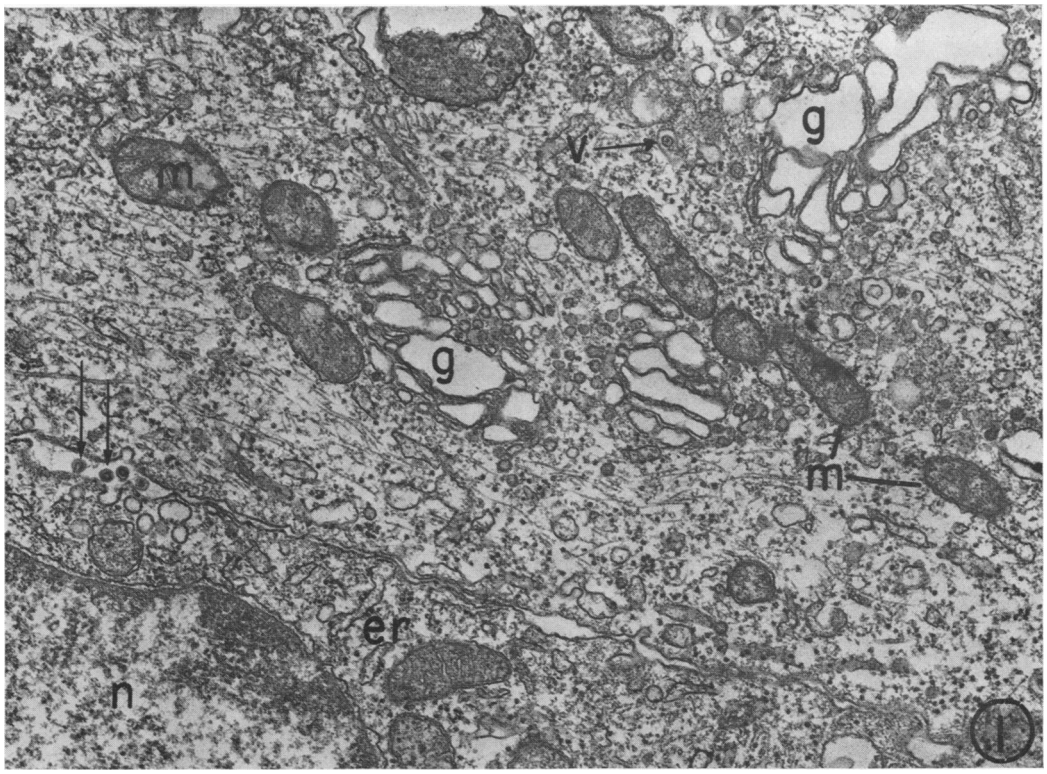


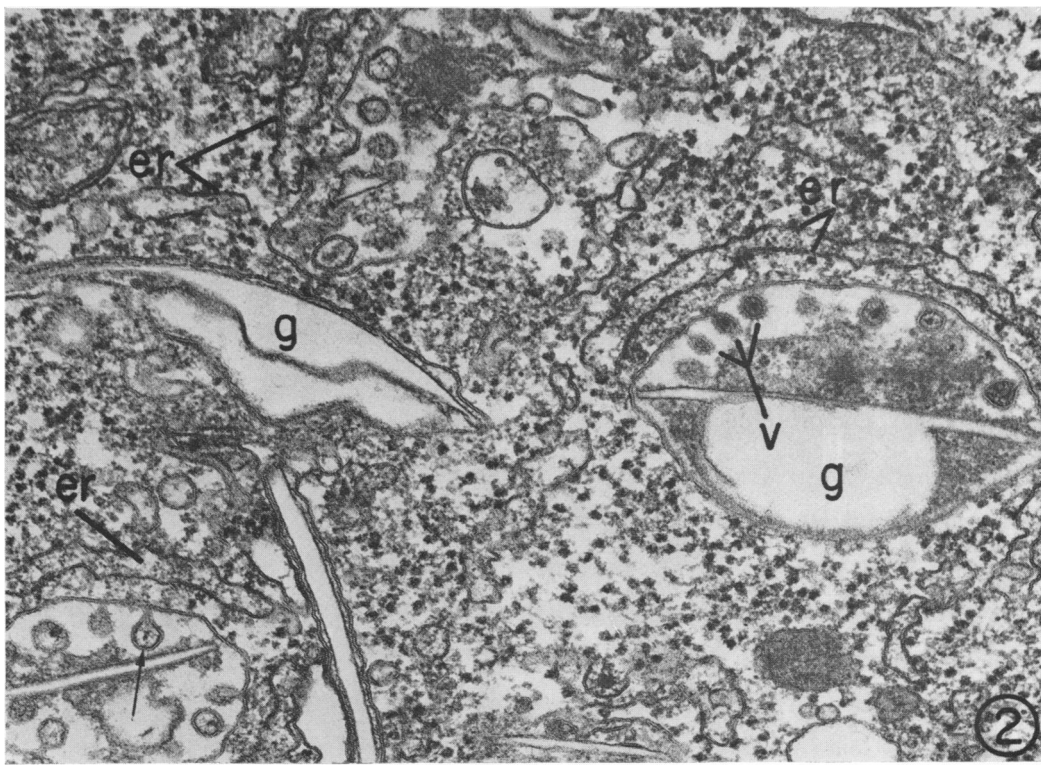
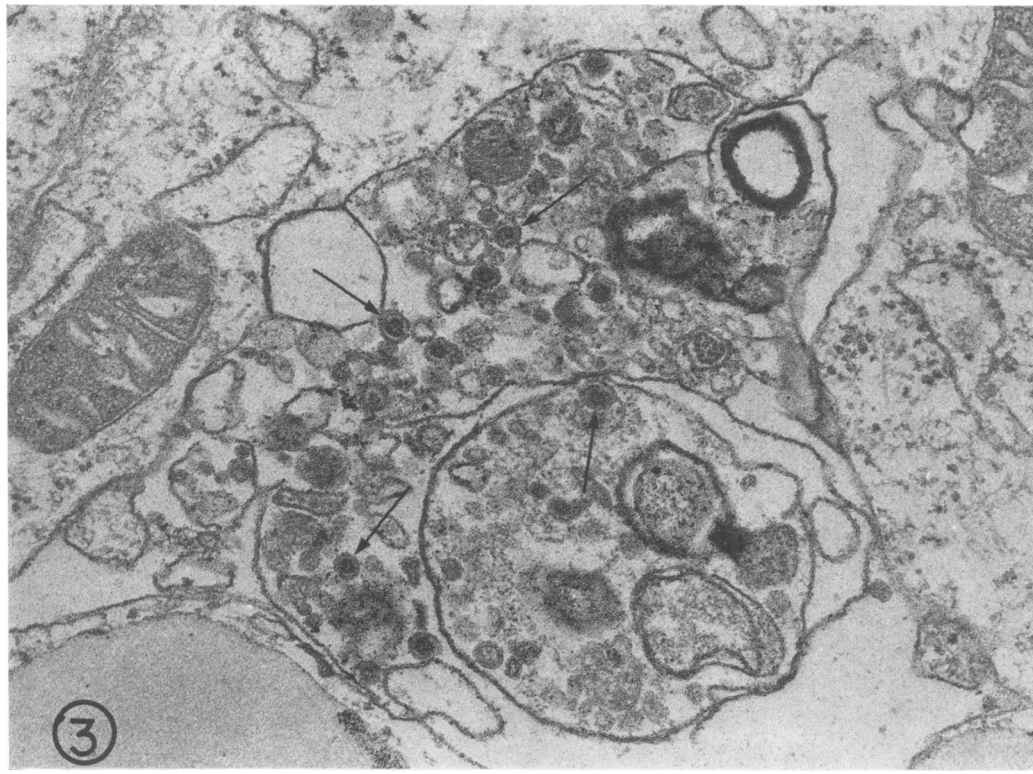
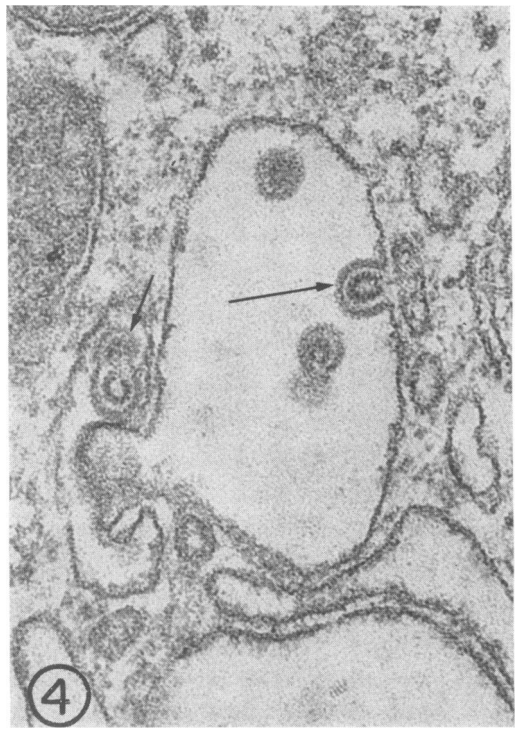
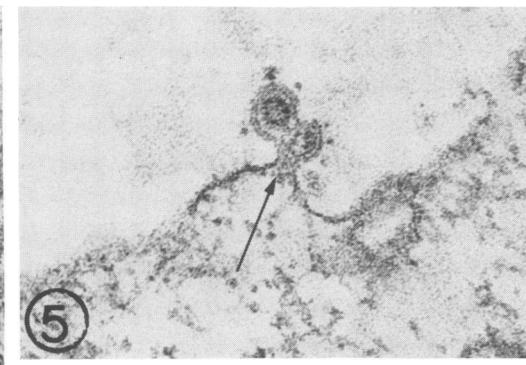


FIG. 1. *Portions of two adjacent cells showing nucleus (n), mitochondria (m), dilated Golgi cisternae (g), scarce elements of the endoplasmic reticulum (er), cytoplasmic filaments, and free ribosomes. Note, at intercellular space, virions (arrows) and pinocytotic vesicles in this region. A virion (v) is seen in a small vesicle. Glutaraldehyde-osmium fixation.* × *30,000.*

FIG. 2. *Portion of infected cell revealing vacuoles presumably derived from Golgi cisternae (g) and virions (v). At the lower left (arrow), note the continuation between vacuole membrane and envelope of virion. Profiles of the endoplasmic reticulum (er) in the vicinity of vacuoles are indicated. Glutaraldehyde-osmium fixation.* × *60,000.*







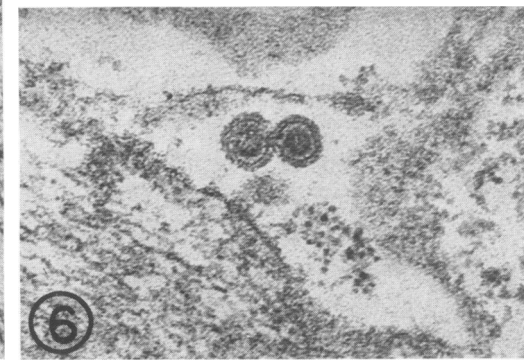


FIG. 3. *Virions (arrows) in partially disrupted vacuole or vacuoles which also contain degenerated membranous organelles and amorphous material. Glutaraldehyde-osmium fixation.* × *60,000.*

FIG. 4. *Budding particle exhibiting envelope still continuous with the vacuole membrane (long arrow). Short arrow points to a double particle. Glutaraldehyde-osmium fixation.* × *120,000.*

FIG. 5. *Double virion with common base at point of origin in vacuole membrane (arrow). Glutaraldehyde-osmium fixation.* × *120,000.*

FIG. 6. *Further example of double virions. Glutaraldehyde-osmium fixation.* × *120,000.*

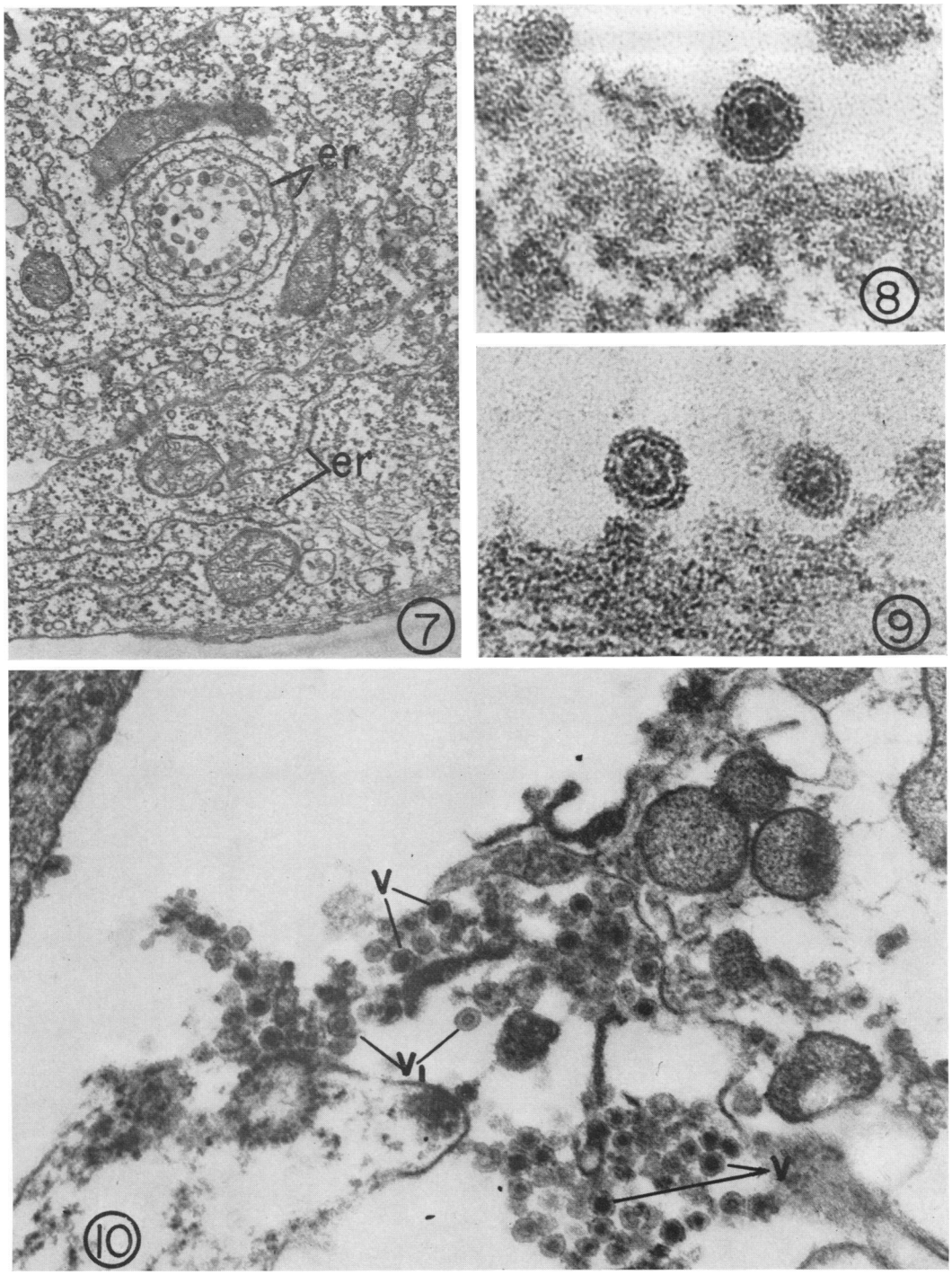


FIG. 7. *Cytoplasmic portions of adjacent cells, one of which exhibits numerous virions within a vacuole completely encircled by a cisternal element of the endoplasmic reticulum (er). Mitochondria and attached or free ribosomes are seen in both cell portions. Chrome-osmium fixation.* × *30,000.*

FIG. 8. *Mature virion exhibiting electron-dense core, capsid, and enveloping membrane (unit membrane). Chrome-osmium fixation.* × *240,000.*

FIG. 9. *Virions with slightly less dense cores than that of Fig. 8, but also enveloped by unit membranes. Glutaraldehyde-osmium fixation.* × *200,000.*

FIG. 10. *Portion of degenerating cell, at advanced stage of infection, displaying masses of virions associated with broken membranes and amorphous material. Note virions with cores of varying density (v, vi). Glutaraldehyde-osmium fixation.* × *60,000.*

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