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Morphogenesis of Sindbis Virus in Cultured *Aedes albopictus* Cells

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Cultured mosquito cells were found to produce Sindbis virus nearly as efficiently as BHK-21 cells at 28 C. In virtually all of the cells observed in the electron microscope, virus morphogenesis was found to occur within complex vesicular structures which developed after viral infection. Viral nucleocapsids were first seen in these vesicles and appeared to be enveloped within these structures. The process of envelopment within these inclusions differed in some respects from the process previously described for the envelopment of nucleocapsids at the plasma membrane of vertebrate cells. Free nucleocapsids were only rarely seen in the cytoplasm of infected mosquito cells, and budding of virus from the cell surface was detected so infrequently that this process of virus production could not account for the amount of virus produced by the infected cells. The vast majority of extracellular virus was produced by the fusion of the virus-containing vesicles with the plasma membrane releasing mature virions and membrane nucleocapsid complexes in various stages of development.

Togaviruses are perpetuated in nature by their ability to infect and replicate within the cells of both vertebrate and invertebrate hosts. The morphogenesis of these viruses in vertebrate tissue-cultured cells has been extensively investigated by electron microscopy (4, 6, 8, 12, 15). Alpha togavirus morphogenesis takes place in two distinct and separate processes in cultured vertebrate cells. (i) Virus nucleocapsids are assembled in the cell cytoplasm from virus capsid protein and RNA. (ii) The completed nucleocapsid is enveloped by budding through the plasma membrane of the infected cell after that membrane has been modified by the insertion of virus-specific glycoproteins. The development of Flavi togaviruses (group B) in vertebrate cells may differ from the above scheme in that envelopment by budding of the cytoplasmic nucleocapsid does not take place at the plasma membrane but rather occurs through the limiting membrane of internal vesicles (12).

The development of stable lines of cultured mosquito cells by Singh (21) has facilitated a limited number of investigations of the morphological processes related to togavirus production in cultured cells of the invertebrate vector. Studies of the growth of a number of togaviruses in cultured mosquito cells have revealed that although the onset of virus production, rate of virus synthesis, and yield of virus is similar to that found in cultured vertebrate cells, the virus-infected mosquito cells do not show the acute cytocidal effects observed in vertebrate host cells (5, 16, 22). Togavirus-infected mosquito cells establish a state of chronic infection in which the infected culture continuously produces virus for periods of several months (16).

Filshie and Rehacek (8) studied the morphology of *Aedes aegypti* cells infected with Murray Valley encephalitis and Japanese encephalitis viruses (both Flavi togaviruses) and concluded that the primary site of virus development was the endoplasmic reticulum of the infected cell. The cisternae of the endoplasmic reticulum were found to contain many mature virions, suggesting that envelopment of the virions took place through this intracellular membrane. Although these authors saw large numbers of mature virions associated with the outer surface of the cell, no budding of virions through the plasma membrane could be detected.

The production of alpha togaviruses in cultured *A. albopictus* was examined morphologically by Raghow et al. (18, 19). These authors described electron-dense vesicles which contained viral capsids and mature virions in infected mosquito cells. Although the authors concluded that the capsids were probably enveloped within the vesicles, they discredited these structures as a source of progeny virus as they felt that these inclusions fused with lysoso-

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mal vesicles, thus destroying the contents. Raghow and co-workers further concluded that extracellular viruses were produced by budding of nucleocapsids through the plasma membrane, a process similar to that which has been described in the vertebrate cell system.

We have investigated the development of the alpha togavirus Sindbis in cultured *A. albopictus* cells in an attempt to further understand the morphogenesis of the togavirus in invertebrate cells and the establishment of the noncytopathic persistent state of infection.

(A summary of this work was presented at the annual meeting of the American Society for Microbiology, 27 April–2 May 1975, New York, and at the 4th International Congress on Invertebrate Tissue Culture, 5–8 June 1975. Mont Gabriel, Canada.)

MATERIALS AND METHODS

Virus, cells, and media. Sindbis virus was donated by Elmer Pfefferkorn (Dartmouth Medical School) and maintained by passage at low multiplicity on chicken embryo fibroblasts or BHK-21 cells. Titrations of virus were carried out in BHK-21 by the method of Pfefferkorn and Clifford (17).

A. albopictus cells were provided by Sonya Buckley (Yale Arbovirus Research Unit) and the American Type Culture Collection. The cells were propagated as monolayers in the medium described by Mitsuhashi and Maramorosch (M + M) (14) supplemented with 20% fetal calf serum and adjusted to pH 7.2 by addition of sodium bicarbonate. The growth of arboviruses in tissue-cultured mosquito cells has been described (5, 16, 22). *A. albopictus* cells were infected with a small volume of Eagle medium (7) containing sufficient virus to produce a multiplicity of infection of 50 to 100 PFU per cell. After 1 h at room temperature, the cells were washed once with M + M media, given new media, and incubated at 28 C. For comparison, similar procedures were used to infect BHK-21 cell cultures grown in Eagle medium.

Electron microscopy. Negative staining was carried out by the procedure of Anderson (2). Phosphotungstic acid was prepared as a 2% solution and adjusted to pH 7.2 with NaOH.

For ultrathin sectioning, cells were fixed with ice-cold 2% glutaraldehyde in phosphate-buffered saline. After fixation for 1 h, the cells were washed in phosphate-buffered saline and postfixed for 1 h with 1% osmium tetroxide in the phosphate buffer of Millonig (13). The fixed cells were embedded in Epon 812 by the procedure of Luft (10), and ultrathin sections were cut with a Reichart OMU-2 microtome.

Freeze-etching of virus-infected cells was carried out by the procedure described by Brown et al. (4).

All specimens were photographed in a Siemens 101 electron microscope, the magnification of which was calibrated with a carbon grating replica having 2,160 lines per mm (Ernest Fullam Co.; no. 1002).

The micrographs of freeze-etched specimens presented here were produced from contact copies of the original photographic plates so that the metal source appears as the source of illumination; the shadows containing no metal are dark.

RESULTS

Growth of Sindbis virus in vertebrate and invertebrate cells. Figure 1 shows the growth kinetics of Sindbis virus in both BHK-21 cells and *A. albopictus* cells at 28 C. The onset of virus production occurs soon after infection in both cell systems. The production of virus in the mosquito cells lags somewhat behind that in BHK cells. At about 14 to 18 h after infection, production of virus in the BHK cells is complete, and gross cytopathic effects are observed microscopically at about 20 h postinfection. Maximum virus production in the mosquito cell system is obtained at 18 to 20 h after infection, but is not accompanied by observable cytopathic effects. In the invertebrate system a decrease in virus titer is observed at about 24 to 26 h after infection. By 40 to 42 h, virus production is reduced by approximately one log (not shown) and is maintained at this lower level for a period of weeks.

Morphology of Sindbis virus-infected mosquito cells. Figures 2 and 3 show at low magnification uninfected (Fig. 2) and Sindbis virus-infected cells at 6 h postinfection (Fig. 3). Infected cells differed from uninfected or mock-infected cells primarily by the presence of a number of vesicular structures in the infected cell cytoplasm. Many of the vacuoles seen at those early times were found to contain aggregates of membranes which seemed to traverse the vesicle from side to side in a complex "stacked" organization (Fig. 3 and 4). It has not been possible to determine the origin of these vesicular structures or to establish from what, if any, pre-existing cellular structures they were derived. In some instances layered membranes of the type seen in the vesicles could be detected in the cell cytoplasm without a surrounding limiting membrane (Fig. 4), suggesting that such a structure might be a precursor to the membrane-limited vesicles. The vacuoles contained much amorphous electron-dense material and ribosomes, suggesting that some cytoplasmic constituents became entrapped in the vacuoles during their formation.

Freeze-etching of these early membranous vesicles at 4 to 8 h after infection (Fig. 5) revealed typical cleavage planes in the stacked membranes and the presence of interior membrane beads (presumably glycoprotein [11, 23]). Freeze etching further suggested that, although the stacked membranes approach the limiting membrane of the vesicle, they are not continuous with it, and therefore do not represent an

invagination of the limiting membrane. The freeze-etched vesicles also revealed the presence of smaller closed membrane structures within the vesicle itself. Viral nucleocapsids or mature virions were not found associated with the infected cells at this time.

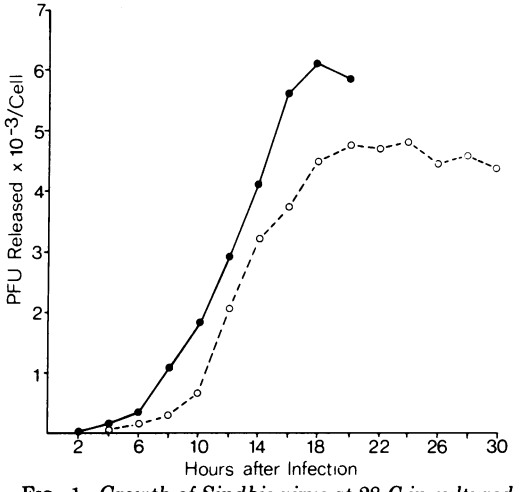

FIG. 1. *Growth of Sindbis virus at 28 C in cultured A. albopictus* (○) *and BHK-21* (●) *cells.*

At later times after infection (8 to 12 h), the cytoplasmic vesicles were found to contain many viral nucleocapsids and mature virions (Fig. 6). Occasionally, partially enveloped capsids were seen. Cells examined by thin sectioning contained few nucleocapsids in the cell cytoplasm and no mature virions associated with the cell surfaces.

At 14 to 20 h postinfection the cells contained a large number of very electron-dense vacuoles (Fig. 7a) which were found to contain amorphous electron-dense material and mature virions when examined at high magnification. In many instances the virions in the inclusions appeared to be packed in a paracrystalline array having hexagonal symetry (Fig. 7b). Cells having large numbers of these virus-rich inclusions showed no evidence of virus production at the plasma membrane by a classical budding process (Fig. 7a).

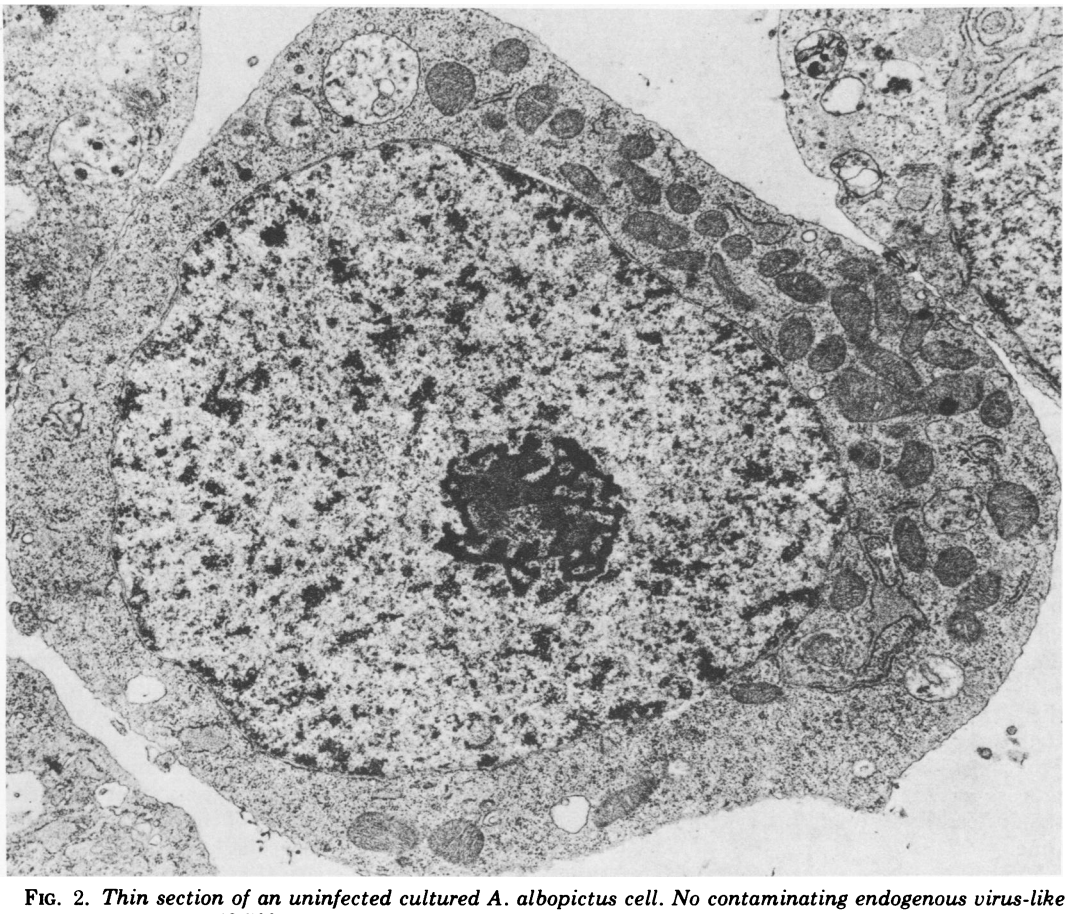

FIG. 2. *Thin section of an uninfected cultured A. albopictus cell. No contaminating endogenous virus-like structures are seen.* ×*18,500.*

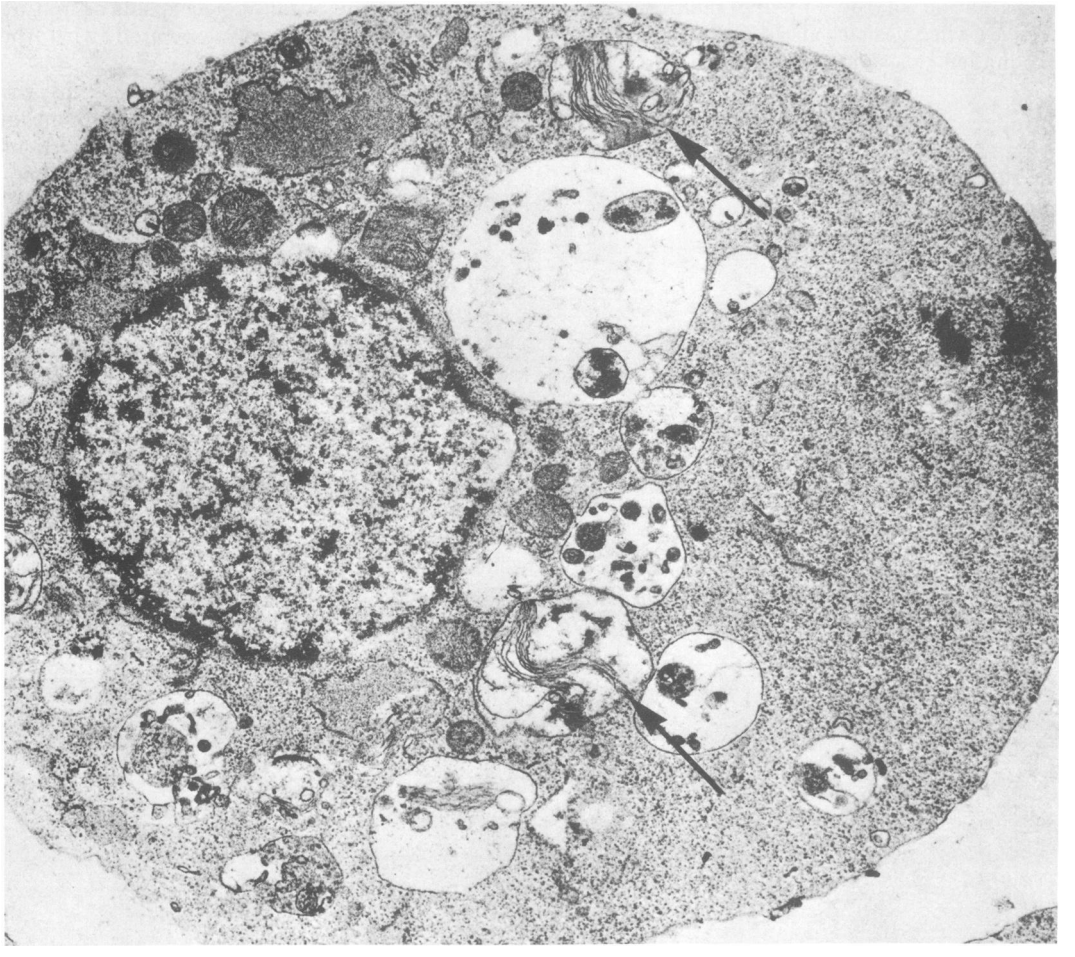

FIG. 3. *Thin section of A. albopictus cell 6 h after infection with Sindbis virus. Arrows point to membranous vesicles.* ×*18,500.*

The vesicles appeared to release their contents into the surrounding medium by fusing with the plasma membrane (Fig. 8). As release from the cell occurred the uniform electron-dense contents of the vesicle appeared to break up releasing virions with much of the dense material associated with the virus surface (Fig. 8). This material did not seem to be tightly bound to the virions as negative staining of the virions released into the media before and after density gradient purification revealed the virus to be free of bound surface contaminants. At these later times all of the described vesicular structures were present in the infected cells. This suggests that if indeed some sequential process occurs in the development of these structures, the system is not synchronous at this time.

Images of freeze-etched cells at later times after infection revealed an alteration in the morphology of some of the vesicular membranes (Fig. 9). These vesicles contained some membranes which, when cleaved, contained a higher than normal density of interior membrane beads, and others which, after cleaving, appeared to be completely free of interior membrane particles. Other membranes in the same cells contained a normal distribution of membrane particles, suggesting that the atypical situation viewed in some of the intravesicular membranes may result from topological alterations in the membranes accompanying virus development. That the production of mature virions from the vesicular membranes is accompanied by loss of the interior membrane particles is supported by the observation that the freeze-cleaved envelopes of Sindbis virions produced from mosquito cells lack particles in the intramembrane space (Fig. 10).

Although the vast majority of infected cells

produced virus exclusively in the above described vesicular structures, a few cells have been found which had nucleocapsids located in the cytoplasm and mature virions associated with the outer surface of the cell (Fig. 11). These cells occasionally possessed structures which suggested envelopment of nucleocapsids by budding through the plasma membrane. However, the small amount of virus associated with the surface of the infected cells and the infrequency of budding at the plasma membrane suggest that few if any of the extracellular virions were produced by this route in these experiments.

Electron microscopy of cell-free virus-related structures. The possibility that the total contents of the vesicles are released into the extracellular fluid prompted us to examine by negative staining the structures present in the media of infected cultures. One might expect that if the entire contents of these vesicles were discharged from the infected cells, intermediate structures in virus maturation would also be released.

Examination of the extracellular material revealed large numbers of intact mature virions of normal size and the morphological variants described by Brown and Gliedman (3). In addition to these "normal particles" a number of interesting aberrant virus structures were also observed. These structures were found in untreated growth media and could be purified away from cell debris by isopycnic centrifugation in potassium tartrate gradients. The abberrant structures were less dense than the main virus band which appeared at a density of 1.2 g/cm^3. Membranes were found in the media to which viral capsids appeared firmly attached (Fig. 12a). The capsids attached to the illustrated membrane fall into three different size classes, corresponding to the three capsid sizes predicted for the Sindbis morphological variants (3). In some of the capsids it is possible to discern the presence of capsomeres (Fig. 12a, insert). These capsomeres have a center-to-center spacing of about 7.0 nm, as described by Brown et al. (4) for the normal-size nucleocapsid produced from vertebrate cells.

The membrane to which the nucleocapsids are attached (Fig. 12a) does not show the presence of spikes on its surface opposite the nucleocapsids. This suggests that either the topological alteration in the membrane resulting in the appearance of spikes has not taken

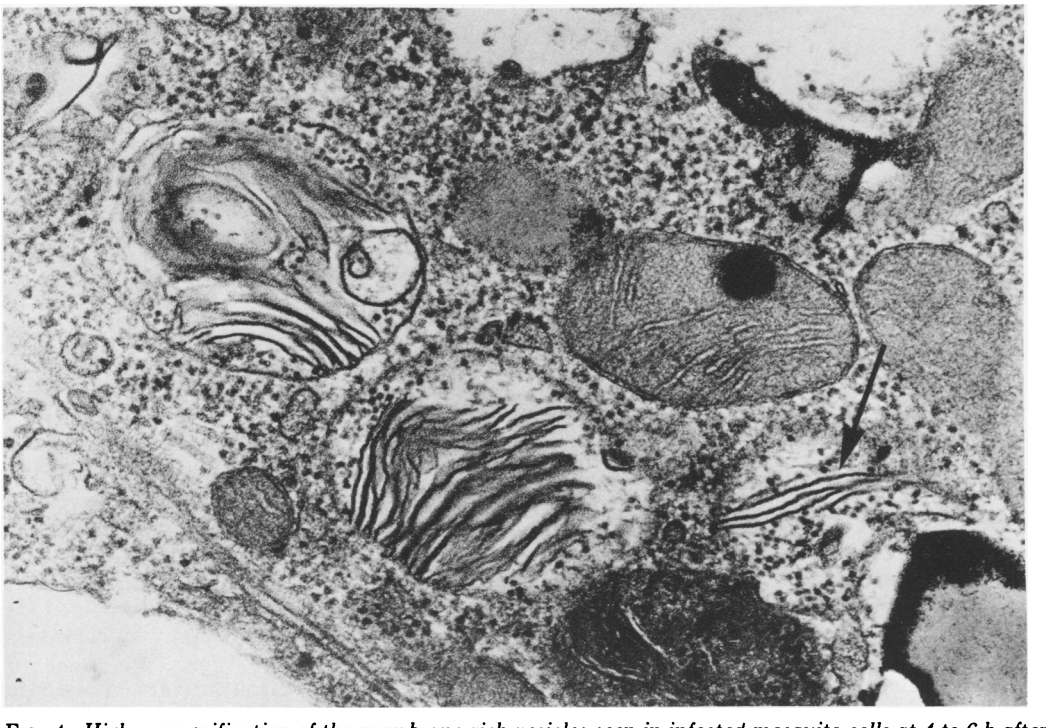

FIG. 4. *Higher magnification of the membrane-rich vesicles seen in infected mosquito cells at 4 to 6 h after infection. The vesicles contain ribosomes in addition to the stacked membranes. One set of membranes (arrow) appears not yet to be enclosed in a limiting membrane.* ×50,000.

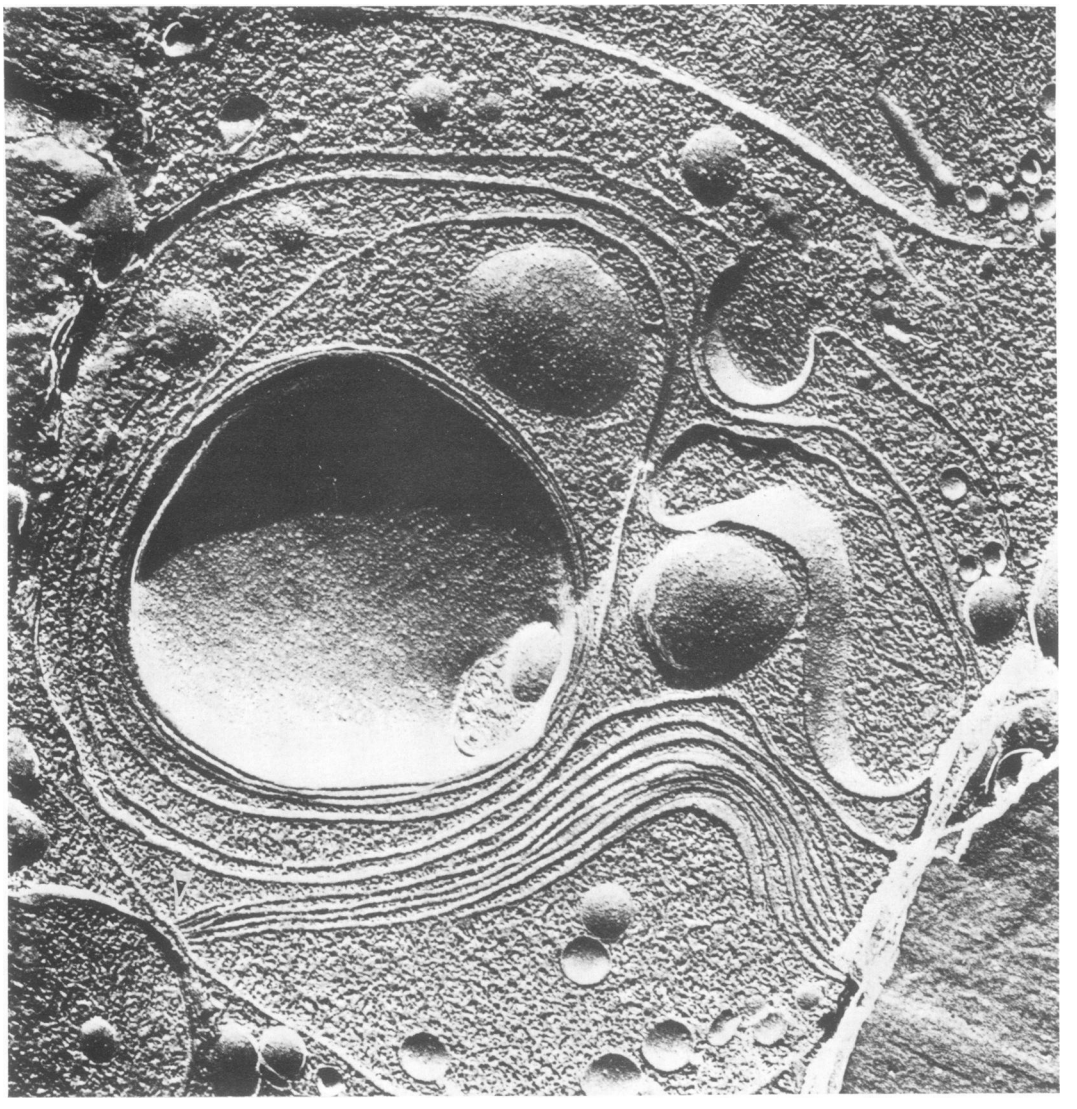

FIG. 5. *Freeze-etch replica of a membranous vesicle in an A. albopictus cell 6 h after infection with Sindbis virus. The arrow indicates the point of contact of the stacked membrane with the surrounding limiting membrane. The cleaved membrane interiors have the typical distribution of interior membrane beads.* ×95,000.

place or that these structures were lost from the membrane prior to examination. Other large membranous structures with associated nucleocapsids could also be found in the culture media (Fig. 12b and c). These structures differed from that illustrated in Fig. 12a in that the viral nucleocapsids appear to be partially enveloped in the membrane. Some areas of the membrane are contoured as though they surrounded nucleocapsids which were later lost from the structure (Fig. 12b). The entire outer surface of the membrane appears to be covered with spike-like structures similar to those seen in intact virions. The presence of the spikes on this membrane surface suggests that the topological rearrangement of the membrane accompanying its conversion to the viral envelope is complete and that this membrane represents a more advanced state of virus development than that shown in Fig. 12a. The culture media also contained large numbers of closed spiked viral membranes surrounding many nucleocapsids (Fig. 12c).

Examination of ultrathin sections of virus-infected mosquito cells suggested that viral nucleocapsids were enveloped within the vesicular structures by interaction with the intravesicular membranes (Fig. 6). Further evi-

dence for such a process of envelopment was obtained by examination of extracellular viral structures (Fig. 13). Many membrane fragments were found with attached single virions in the final stages of envelopment. The nucleocapsids of such viruses were enveloped to the extent that only a small area of attachment remained. In some instances the membrane of the viral envelope was continuous with the membrane from which it was derived. Such

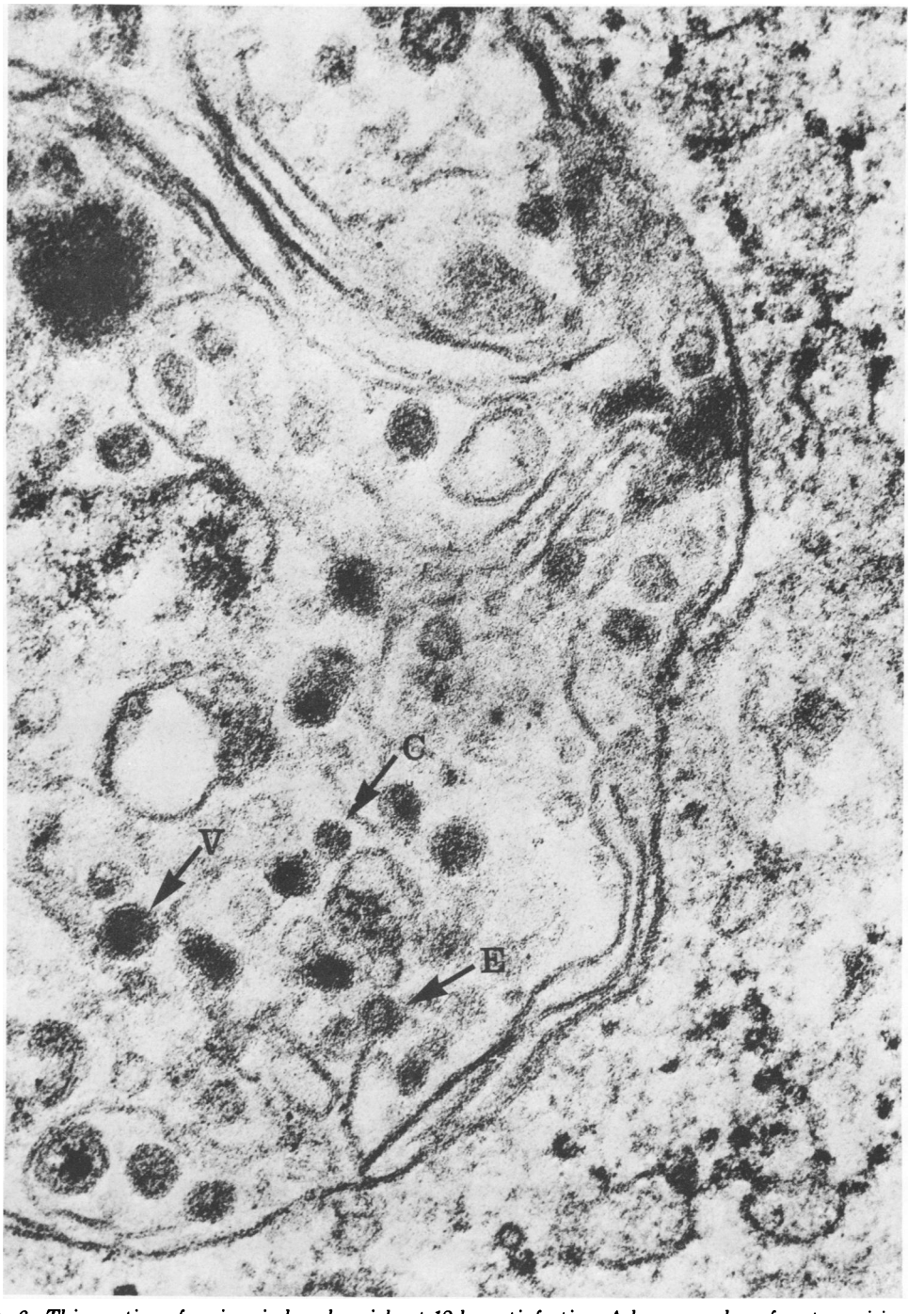

FIG. 6. *Thin section of a virus-induced vesicle at 12 h postinfection. A large number of mature virions (V) can be seen in addition to viral nucleocapsids (C); one virion is partially enveloped (E).* ×*135,000.*

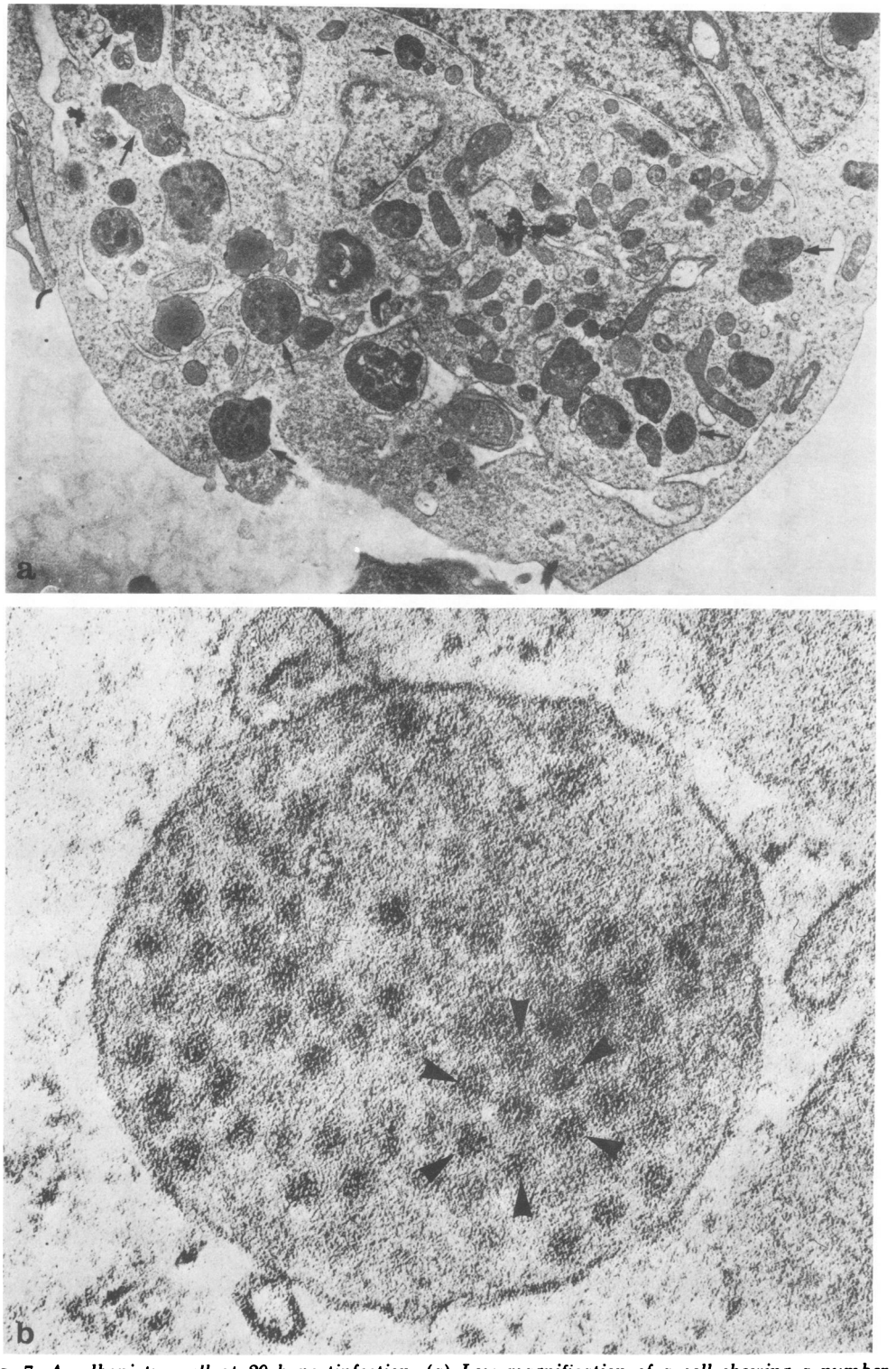

FIG. 7. *A. albopictus cell at 20 h postinfection. (a) Low magnification of a cell showing a number of electron-dense inclusions (arrows) which were found to contain large numbers of virions. Budding of virus from the plasma membrane is not evident. ×18,000. (b) A high magnification of one of the electron-dense vesicles. Virions are packed in a hexagonal lattice. ×134,000.*

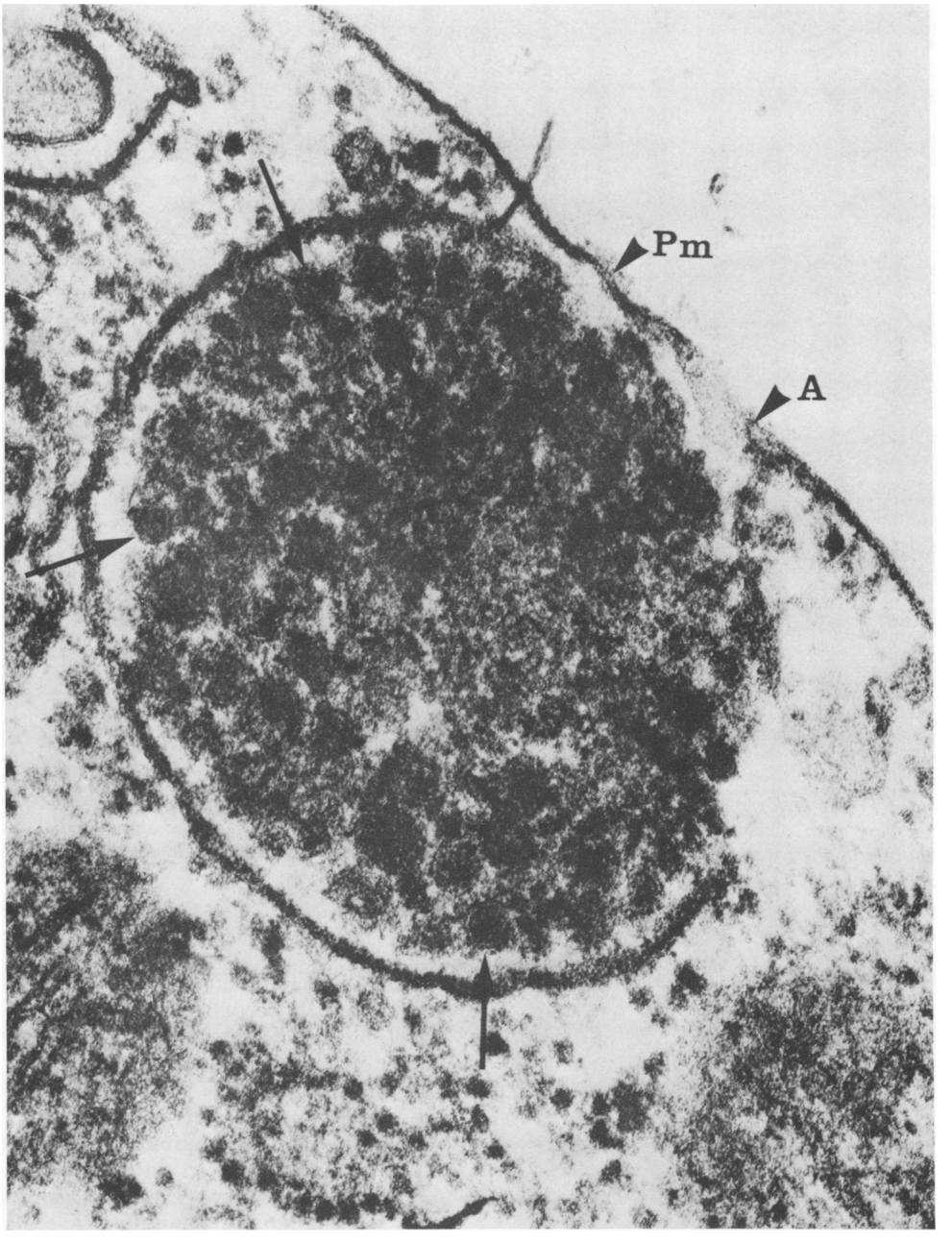

FIG. 8. *A thin section of a cytoplasmic vesicle containing many virions (unmarked arrows). The vesicle is close to the plasma membrane (Pm) and it appears that the limiting membrane of the vesicle has fused with the plasma membrane at the point indicated by the arrow (A). The uniform appearance of the vesicle (seen in Fig. 7b) has been lost. The virions are coated with the contents of the vesicle.* ×120,000.

structures are very similar to the last stage of envelopment of Sindbis virus from the plasma membranes of vertebrate cells previously reported (4). This suggests that as in vertebrate cells, the final release of the virions from the parent membrane occurs with fusion of the envelope membranes into a closed sphere at the point of attachment.

DISCUSSION

Although the growth kinetics of Sindbis virus are similar in BHK-21 and *A. albopictus* cells at

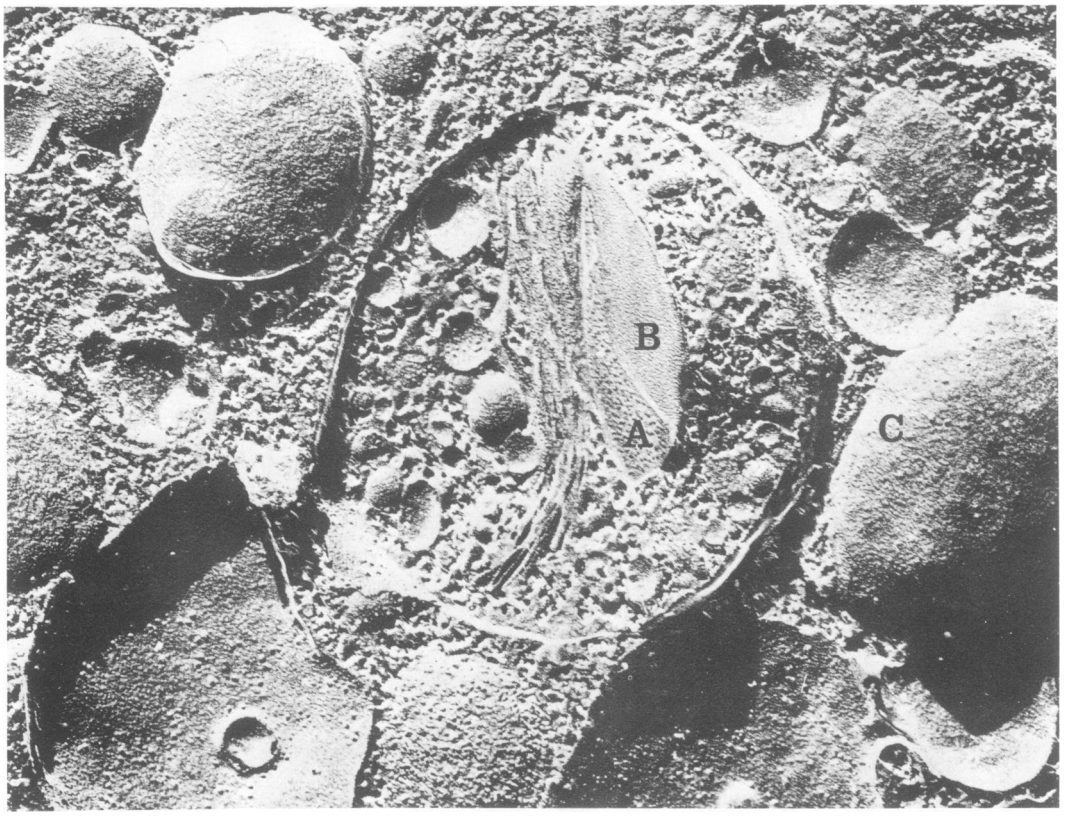

FIG. 9. *Freeze-etch replica of a virus-induced vesicle at 18 h postinfection. Some of the stacked membranes have a very high density of interior membrane particles (A), whereas others are smooth (B). Normal distribution of the interior membrane particles can be seen on other membranes outside of the vesicle (C). ×80,000.*

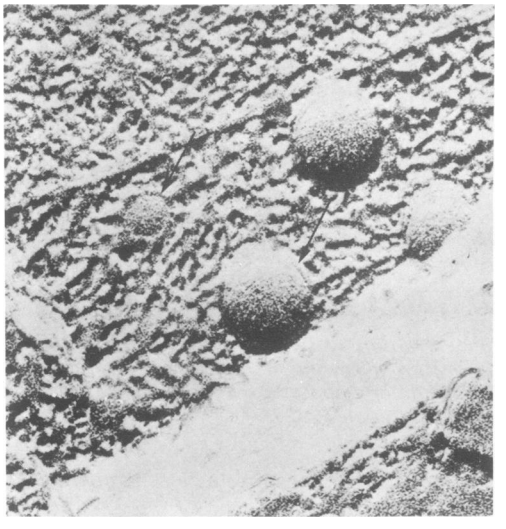

FIG. 10. *Freeze-etch replica of pelleted virions produced from A. albopictus cells. The interior of the viral envelope is free of interior membrane beads. Arrows indicate the cross-fractured outer leaflet of the viral membrane. ×180,000.*

28 C, the pattern of virus morphogenesis was found to be very different in the two cell systems. The production of mature virions from vertebrate cells occurs by the budding of free cytoplasmic nucleocapsids through the plasma membrane of infected cells (1, 4). This process is accompanied by gross cytopathic effects and cell death within 20 h postinfection. Morphogenesis of Sindbis virus in *A. albopictus* cells occurred primarily in cytoplasmic vesicles; budding of virions through plasma membranes and accumulation of cytoplasmic nucleocapsids occurred only in a very small percentage (less than 1%) of the observed cells. The infection of the invertebrate cells by Sindbis virus does not result in noticeable cytopathic effect or in a detectable reduction in the rate of cell growth.

The observation of virus development in internal vesicles presented here is similar to the report of Raghow and co-workers for two other alpha togaviruses (18, 19). Unlike these authors, however, we did not find any evidence for the destruction of the virus-rich inclusions by fusion with lysozome-like bodies. We suggest that the

vesicles release matured virions into the surrounding medium by fusion with the plasma membrane. Examination of cells persistently infected with Sindbis virus for periods of time up to 5 days after infection revealed the continued presence of virus-containing vesicles in the cytoplasm of the infected cells, although fewer in number than seen in Fig. 7a. With the exception of the reduction in number of vesicles the general morphology of the persistantly infected cells was found to be similar to that of the cells at about 20 h after infection when the logarithmic stage of virus production is completed. It is not yet clear whether the apparent sequestering of virus morphogenesis in this manner enables mosquito cells to survive infection.

The origin of the nucleocapsids which appear in the cytoplasmic vesicles described here and by Raghow et al. (18, 19) is unclear. The presence of free nucleocapsids within the vesicles implies that the nucleocapsids are either assembled in the vesicles, transported into the vesicles after assembly in the cell cytoplasm, or that the vesicles are developed around the nucleocapsids which are first assembled in restricted areas of the cytoplasm. The last two possibilities seem less likely to us considering the scarcity of free cytoplasmic nucleocapsids and the fact that we have never observed nucleocapsids attached to or budding into the cytoplasmic vesicles. However, it is possible that transport of free nucleocapsids into the vesicles or elsewhere occurs very rapidly and that the small percentage of cells having cytoplasmic nucleocapsids may result from a slowing or an interruption in this transport process. We are presently conducting a study of infected cells by pulse-chase autoradiography with protein and RNA precursors in an attempt to localize RNA and protein synthesis and the fate of these products in infected mosquito cells.

The envelopment of the nucleocapsids in the vesicles presumably takes place through interaction of the nucleocapsids with the membranes present in the vesicles and appears to possess some differences from the interaction of nucleocapsids with the plasma membrane that occurs during "budding" in the vertebrate cell system (1, 4).

The attachment of the viral nucleocapsids to the membranes found within the vesicles of the infected mosquito cells seems to be followed by a topological change in the entire membrane structure as envelopment occurs. This generalized morphological reorganization of the membranes was seen in negatively stained preparations of premature nucleocapsid-membrane

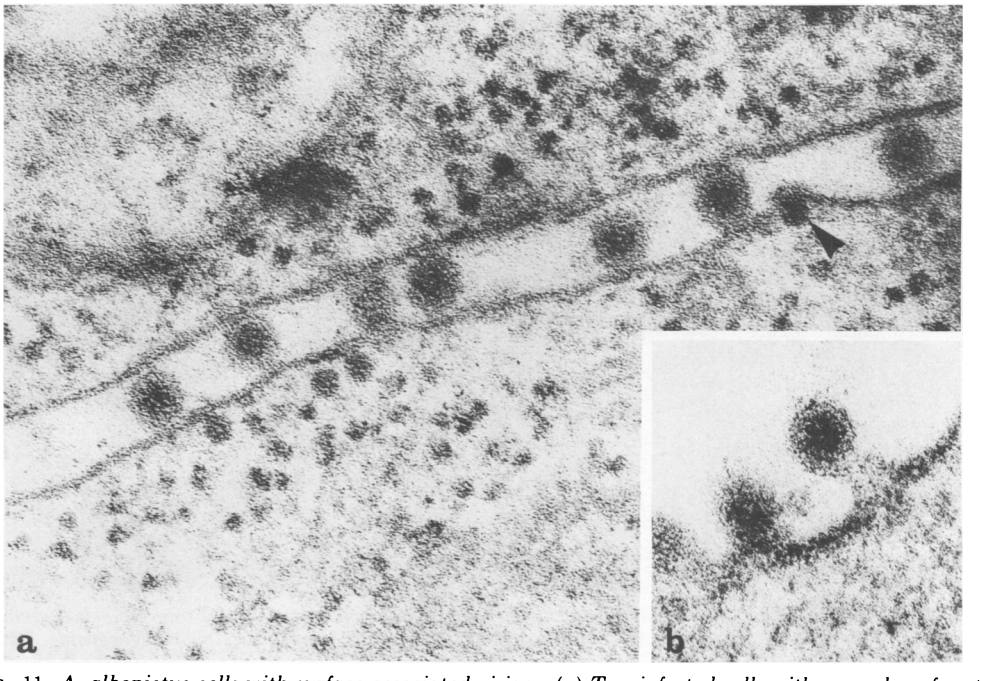

FIG. 11. *A. albopictus cells with surface-associated virions. (a) Two infected cells with a number of mature completed virions in the extracellular space. The cytoplasm of the lower cell contains nucleocapsids of which one is associated with the plasma membrane as though in an early stage of budding (arrow). ×160,000. (b) The surface of an infected cell with virions in what may be a late stage of budding. ×160,000.*

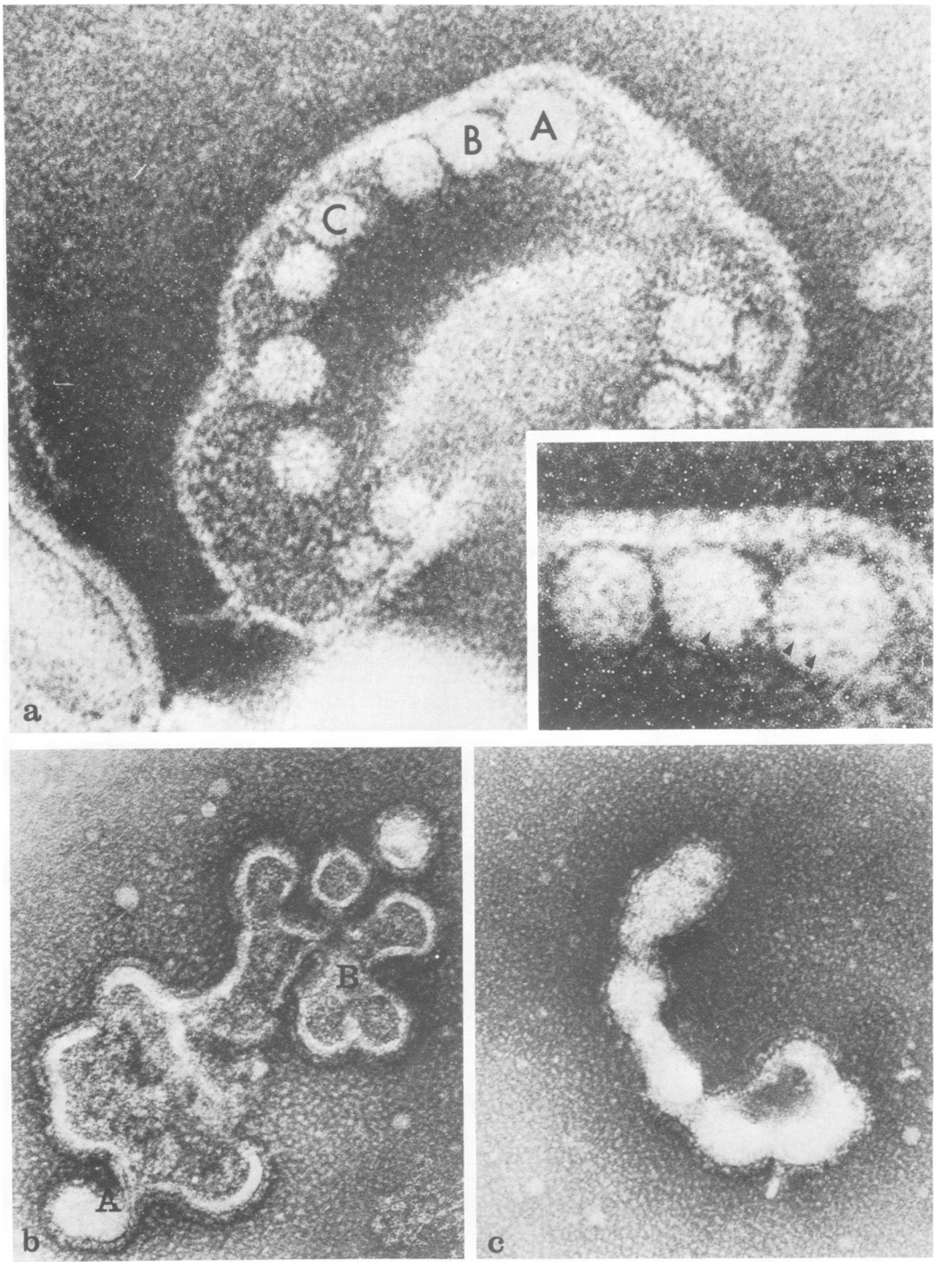

FIG. 12. *Negative stain of extracellular nucleocapsid-membrane complexes. (a) A large spikeless membrane fragment with attached normal size (A), medium size (B), and small size (C) Sindbis nucleocapsids. The inset at higher magnification shows three of the attached nucleocapsids in which capsomeres (arrows) are visible. ×320,000; insert ×597,000. (b) A large fragment of a modified membrane with externally located spikes and one or two enveloped nucleocapsids (A). Other areas of the membrane appear to have lost capsid structures (B). ×179,000. (c) Membrane fragment containing several virus nucleocapsids. This membrane is apparently closed and has spikes on its outer surface. ×179,000.*

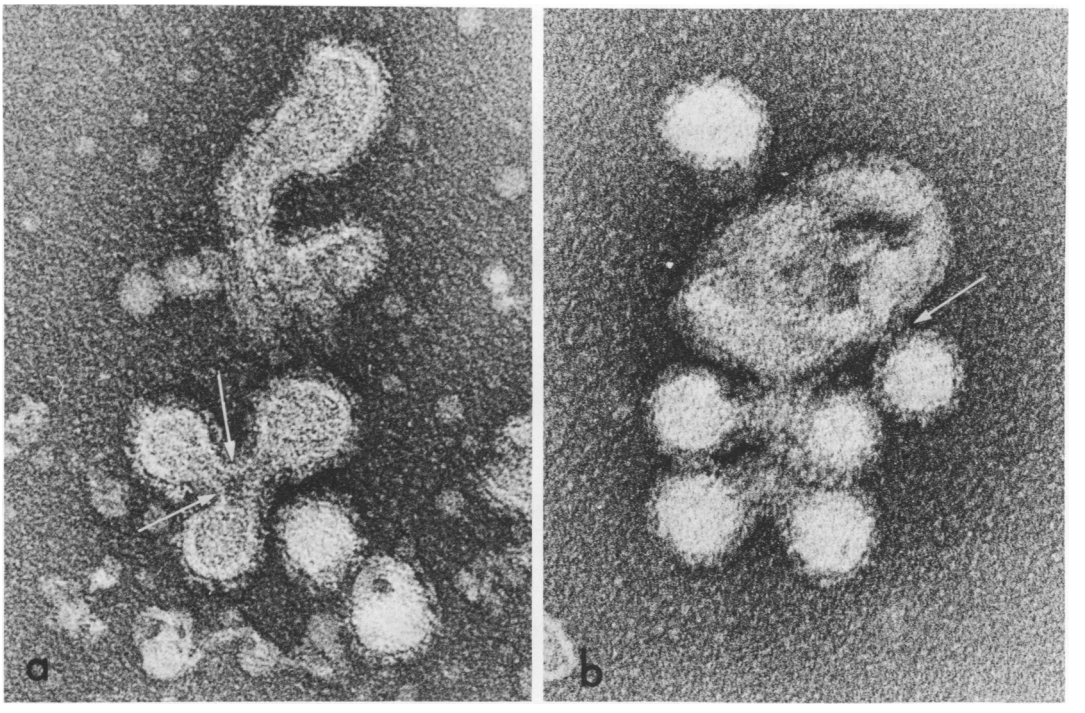

FIG. 13. *Negative stain of extracellular complexes containing modified membranes and maturing virions. In some areas the viral envelopes are still continuous with the modified membrane from which they have developed (arrows). ×180,000.*

complexes (Fig. 12 and 13) and in freeze-etch images of the interior regions of the intravesicular membranes (Fig. 9). The total alteration of the internal and external morphology of the membrane, including regions of the membrane which are not being wrapped around nucleocapsids, is very different from the localized topological change described for the vertebrate cell systems (4).

The presence of the multi-nucleocapsid-containing enveloped structures which are found in preparations from invertebrate cells (Fig. 12b, 12c, and 13) suggests that the process of envelopment within the vesicles is not as specific as the individual nucleocapsid packaging process seen at the plasma membrane in vertebrate cells. It appears that once the modification of the membranes in the invertebrate vesicles is complete, the membranes close around the attached nucleocapsids, occasionally producing the aberrant multicapsid structures. The release of partially enveloped nucleocapsids from the infected cells raises the possibility that the process of envelopment may continue even after such structures are released from the host cells.

An exhaustive search for the budding of virions from the plasma membrane (as described for virus maturation in vertebrate host cells [1, 4]) utilizing freeze etching and ultrathin sectioning of Sindbis-infected mosquito cells during the period of maximum virus production was generally negative. The presence of large numbers of intact virions on the surface of the infected cells as described by other investigators (8, 18, 19) was also not seen. The presence of surface-associated virus is of itself not an indication that budding of virus has taken place, as under appropriate conditions of glutaraldehyde fixation one would expect a certain amount of free virus to be cross-linked to the cell (9, 24). In no study of togavirus production in invertebrate cells has extensive budding at the plasma membrane been reported. The few images of what appears as budding virions produced here (Fig. 11) and in other studies are infrequent compared to the ease with which they are obtained in the vertebrate cell system under conditions producing similar amounts of virus. The few incidences of apparent budding produced in this study could actually represent stages in penetration during reinfection by extracellular virus.

It is possible, however, that the occasional budding figures and the infrequently observed free cytoplasmic nucleocapsids represent an alternative process of virus maturation. This

process occurs in a small percentage of the infected cells and reflects either a heterogeneity in the cell population or an alteration in the physiological condition of a few of the infected cells. It is also possible that both processes of virus production can occur in a particular cell and whether one pathway or the other prevails is determined by factors effecting the physiology of the cell. Support for this latter notion has been obtained by experiments conducted in this laboratory in which large numbers of cytoplasmic nucleocapsids were accumulated in Sindbis-infected mosquito cells after treatment with colcemid (to be published elsewhere). Treatment with colcemid did not increase the number of budding virions observed and reduced the amount of virus produced.

ACKNOWLEDGMENTS

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