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Semliki Forest Virus in HEp-2 Cell Cultures

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The growth and development of Semliki Forest virus (SFV), an arbovirus of serological group A, in HEp-2 cells in tissue culture was examined by various techniques at frequent intervals. Infectivity and fluorescent-antibody studies demonstrated the presence of infective virus and viral antigens within the cells at 8 hr after infection. The antigen was particulate and distributed throughout the cytoplasm. Thereafter, there was rapid progression of virus production and cell destruction. By electron microscopy, tubular structures bounded by a fine membrane were observed in cytoplasm at 12 hr. Rows of small (25 mμ) virus particles were often present on the outer surface of these membranes, and at later times they became progressively more encrusted with the small virus particles. These structures subsequently increased rapidly in number, size, and complexity, and the space between the membrane and the tubules increased, thus forming vacuoles which contained tubules and were covered with the small particles. At later times (24 hr and later) larger (42 to 50 mμ) particles were observed, usually inside of the vacuoles. These larger particles (and occasionally the smaller ones) were also seen at the cell periphery and in the extracellular space. The large SFV particles appear to form by three distinct processes: (i) from the smaller particles, (ii) by development on an intravacuolar membrane, and (iii) at the ends of the tubules. The mode of development of SFV is unique among viruses studied to date, but in some characteristics it resembles that of other group A arboviruses. Its development differs from that of most arboviruses of group B and other serological groups.

A previous study of West Nile and Guaroa viruses revealed that these two arboviruses, of different serological groups, differed markedly in the manner of their development and release in human cell cultures (26). This is a report of a similar study of Semliki Forest virus (SFV), an arbovirus of still another serological group (5), isolated by Smithburn and Haddow (25) in Uganda, East Africa.

MATERIALS AND METHODS

SFV was originally supplied to this laboratory by the Rockefeller Foundation Laboratories as the fifth mouse brain passage. The virus stock used in these experiments was the supernatant fluid from a 10% suspension of SFV-infected mouse brain, representing the twelfth mouse brain passage since isolation. It had an infectivity titer of $10^{-8.7}$ determined by intracerebral inoculation of 0.03 ml of the test material into young adult Swiss white mice. The LD_{50} titers were calculated by the method of Reed and Muench (22).

Calculations of the number of infectious doses of virus particles were based on the arbitrary assumptions that a single infectious particle was sufficient to kill a mouse on intracerebral inoculation, and that the total number of infective particles in any suspension was 0.7 times the number of LD_{50} doses. The probability of getting one or more units of virus in 50% of the mice at the end point of titrate is $1 - e^{-1/2}$.

Tissue cultures were of the human epidermoid cancer cell line HEp-2 (17) grown as monolayers on glass in milk dilution bottles in a nutrient composed of Eagle's basal medium (10) containing 20% "agamma" calf serum (Hyland Laboratories, Los Angeles, Calif.). Tissue culture media also contained penicillin (200 units/ml) and streptomycin (0.2 mg/ml). Monolayer cultures for study by the fluorescent-antibody (FA) technique, acridine orange, or light microscopy were obtained by placing a cover glass (13 × 50 mm) in the bottom of each culture bottle before the cells were planted and removing it just before the cultures were harvested.

Three studies were done. In the first, cultures were studied 1, 2, and 3 days after inoculation with the virus. In the others, cultures were examined at 4, 8, 12, 18, and 24 hr after addition of the virus. At "time-zero" of each study, 1 ml of 10% mouse brain stock of SFV was added to each of approximately 20 bottle cultures of HEp-2. This provided approximately 500 infective virus units per cell (as judged by the

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mouse intracerebral LD_{50}). The virus inoculum was left in the cultures, in conformity with our studies of other arboviruses. About half as many bottles were not inoculated, but served as the uninfected controls.

At each study interval, two to five infected cultures (bottles) were removed from the incubator and examined for cytopathology. The medium was removed and pooled. Cells were harvested for cell counts, infectivity titrations, and electron microscopy by trypsinization of the monolayers with 0.25% trypsin in isotonic (0.85%) saline after removal of the nutrient medium. These cell suspensions were pooled, washed twice with Gey's saline solution, and then resuspended in Gey's solution (13); the cells were counted in a hemocytometer. At appropriate intervals, uninfected control cultures were prepared in the same way.

The pooled culture media were titered for infectivity at each study interval. For the day 2 and day 3 studies, when the media contained much cellular debris, the media were centrifuged, and the clear supernatant fluid and the sediment were titered separately.

For routine microscopy of cells on the cover slips, the stain used was May-Grünwald Giemsa. Acridine orange studies were done by the technique of Niven et al. (20).

For FA studies, the direct technique was used on the cover-slip preparations. The anti-SFV serum was prepared in this laboratory by pooling sera collected from four rabbits 14 days after the last of four intramuscular injections of live SFV mouse brain stock containing approximately 10^8 mouse intracerebral LD_{50} per injection. This serum had a neutralizing index of more than 50,000 (i.e., neutralized 4.7 logs of virus). Globulins were prepared from this anti-SFV serum as Cohn fraction II, and were labeled with fluorescein isothiocyanate (9). They were then fractionated on diethylaminoethyl cellose to reduce nonspecific staining (14). Control studies at every time period included SFV-infected cells pretreated with an unlabeled Cohn II fraction of the anti-SFV serum or with a normal rabbit serum pool collected from the same rabbits prior to immunization. In addition, cells from the uninfected control cultures were examined simultaneously with the first and last time periods of each study.

For electron microscopy, the cell suspensions were centrifuged at 150 × *g* for 10 min and the cell pellets were fixed in phosphate-buffered 2% osmium tetroxide (*p*H 7.4) containing glucose (final osmolality 390 milliosmols/kg of water) for 1 hr (16). After dehydration in graded alcohols, the specimens were embedded in Maraglas (11). Thin sections were stained with uranyl acetate (27) followed by lead citrate (23) and were examined in a Siemens Elmiskop 1 electron microscope.

RESULTS

The three experiments, one studied at daily intervals for 3 days and two studied at shorter intervals over a total period of 24 hr, gave results which were consistent and supplementary, so the data will not be discussed separately. A graphic representation of changes observed by all of the study techniques is presented in Fig. 1.

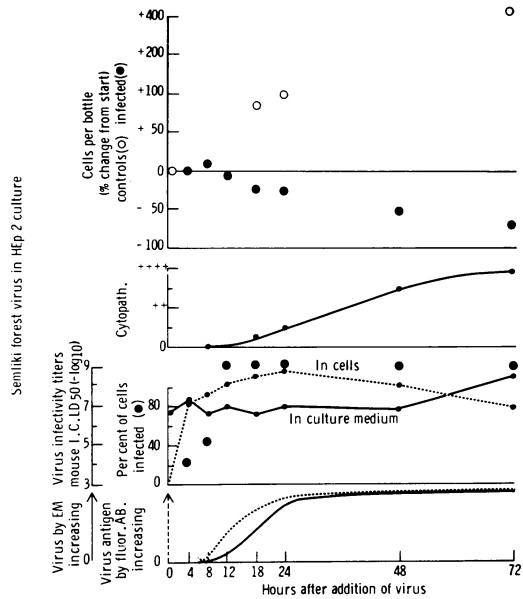


FIG. 1. *Graphic representation of changes in cells and virus at various times after addition of SFV to the HEp-2 cultures. Numbers of cells remaining in the monolayer is presented as percentage change from time zero, rather than actual count, to permit inclusion of data from all three experiments. Degree of cytopathology is based on examination of living cultures. Virus infectivity data are presented in three ways: the solid line indicates mouse intracerebral LD_{50} titers in the culture medium (undiluted medium is 10^0); the dotted line indicates LD_{50} titers in the suspension of cells harvested from the monolayer, and is calculated on the basis of 10^9 cells per gram of packed cells (i.e., 10^8 cells per ml is a 10^{-1} dilution); the large black dots indicate the approximate percentage of cells which carry infectious virus, as judged by comparison of cell counts and infectivity titers of the whole-cell suspensions. The curves denoting the frequency with which virus particles and viral antigens were seen (by electron microscopy and fluorescent-antibody technique, respectively), are unquantitated estimates of the progressive change from zero to maximum.*

Microscopic examination of the living cultures showed no apparent abnormality during the first 12 hr after virus was added. By 24 hr, the cultures were considered suspicious of cytopathology. By 48 hr, cell destruction was clearly evident, and by 72 hr most cells had detached from the glass and most of those remaining on the monolayer were damaged. Examination of fixed and stained monolayers showed no abnormality at 4 or 8 hr, but by 12 hr there was an occasional damaged cell evident as rounding-up, or by "bubbling" of the cell periphery. By 18

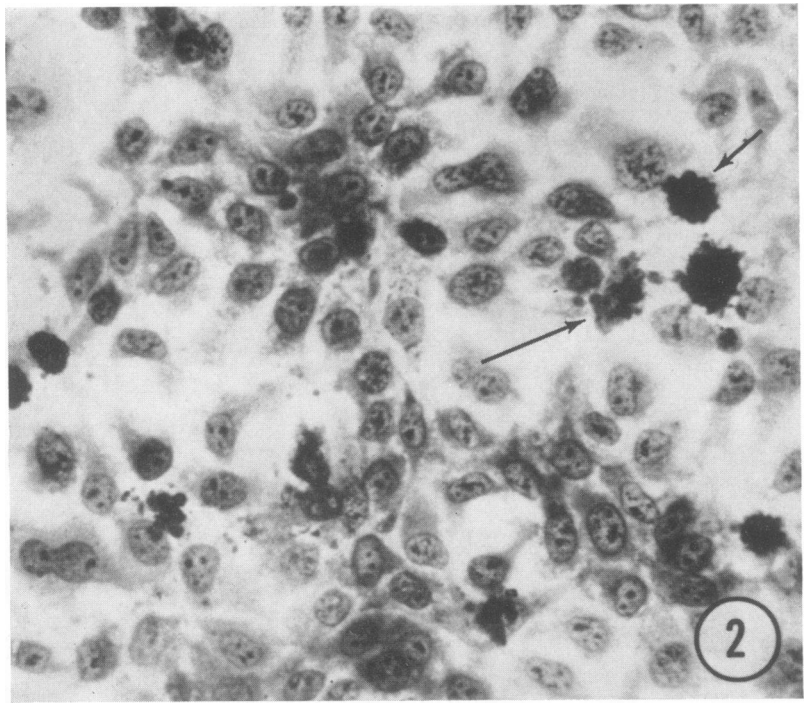


FIG. 2. *Monolayer of HEp-2 cells 24 hr after addition of SFV. May-Grünwald-Giemsa stain. Cell damage is obvious in numerous but scattered cells. Short arrow indicates cell with "bubbling" cytoplasm. Larger arrow indicates cell with karyorrhexis. Graded as one-plus cytopathology.* × *250.*

hr, these changes were obvious in at least 2% of the cells in the monolayer. By 24 hr, about 10% of the cells in the sheet showed these changes and the population density of the cells in the monolayer appeared less than that in the controls (Fig. 2). In the 2- and 3-day preparations, cell damage was obvious and the cell density was strikingly diminished from that of the parallel uninfected control cultures.

Cell counts (of cells remaining on the glass) are in general agreement with the impressions derived from the stained preparations. In the uninfected cultures, the cell count had increased twofold by 24 hr, and by 72 hr it had increased about fivefold. In contrast, the number of cells in the infected cultures had decreased slightly by 18 and 24 hr and was reduced to about one-half of the original number by 2 days, and to one-third by 3 days.

The amount of infective virus in the washed whole-cell suspensions was already high within 4 hr after the start of the experiment. The mouse intracerebral LD_{50} titer of the packed whole cells was $10^{-7.2}$. From this titer and the cell count, it was calculated that there was infective virus in or on about 20% of the cells in the monolayer. Similarly, at 8 hr about 40% of the cells carried infectious virus. At 12 hr, the virus titer had increased somewhat, the number of cells remaining in the monolayer was beginning to decrease, and 100% of the cells appeared to be infected. At later intervals too, comparison of cell counts and infectivity titers of the whole cell suspensions indicated that all cells were infected.

The amount of virus in the culture medium did not change significantly during the first 48 hr (titers ranged from $10^{-6.5}$ to $10^{-7.5}$), indicating that decay of the unadsorbed virus inoculum roughly paralleled production and release of new virus during this period. In retrospect, it was unfortunate that the inoculum was not removed after a brief exposure period because, with this relatively stable and rapidly propagating virus, the infectivity titers of the culture medium gave no information on early release of virus or amount of virus released per cell. At 72 hr, there was an increase of infective virus in the culture medium to $10^{-8.5}$.

The acridine orange studies showed red-orange fluorescence in cytoplasmic masses in many cells, particularly in the studies done at 2 and 3 days after infection (Fig. 3). These masses were several microns in diameter and were usually in contact with or partially overlying the nucleus. They were not found in the uninfected cultures. These observations suggest an increased concentration of single-stranded ribonucleic acid (RNA), resulting from virus infection and possibly

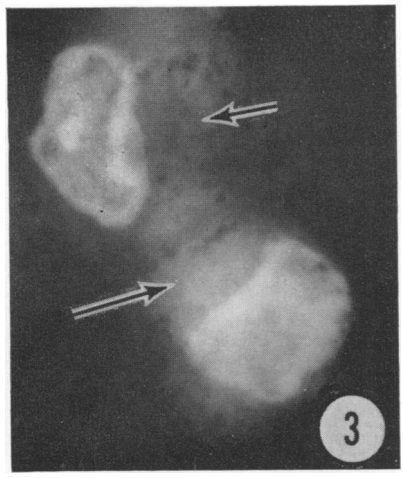


FIG. 3. *Acridine orange study at 72 hr after addition of SFV to HEp-2 culture. Reproduced from color photograph. The nucleus of the cell at lower right is indented by an adjacent cytoplasmic mass of orange-staining material (long arrow) which may be viral RNA. A larger but less intensely stained mass is also present in the upper cell (short arrow) adjacent to an indentation of the nuclear membrane.* × *1,000.*

representing the virus itself. However, studies with nucleases to determine whether this was in fact RNA were not attempted, and the great variation in diffuse cytoplasmic staining by acridine orange in cultures of different population densities precludes any definite conclusions concerning the relation of this localized RNA type of staining to viral content. No changes in the intensity of localization of deoxyribonucleic acid (DNA)-type (yellow-green) staining were apparent.

In the FA studies (Fig. 4–6), no evidence of viral antigen was detected at 4 hr, but by 8 hr (Fig. 4) scattered cells showed intense fluorescence which was shown to be specific for Semliki Forest virus antigen. This was widely distributed in the cytoplasm and consisted in large part of finely particulate material. At 12 hr (Fig. 5), the pattern of fluorescence was the same but the frequency of infected cells was increased. At 18 hr and thereafter, fluorescent cells were extremely common, probably constituting more than 50% of the cells in the monolayer. After 12 hr, however, many of the cells were distorted and showed a more diffuse pattern of fluorescence, often interrupted by small nonfluorescent areas which gave the cells a "moth-eaten" appearance (Fig. 6). Fluorescence in the nuclear area was uncommon, and presumably represented viral particles in the overlying cytoplasm rather than any localization of virus antigen within the nucleus.

No significant abnormalities were detected by electron microscopy 4 to 8 hr after infection. At 12 hr, tubular structures measuring 30 to 40 mμ in diameter (most commonly 36 mμ) and up to 1.7 μ in length were occasionally observed in the perinuclear cytoplasm. These solitary or paired tubular structures (rarely multiple) were enclosed

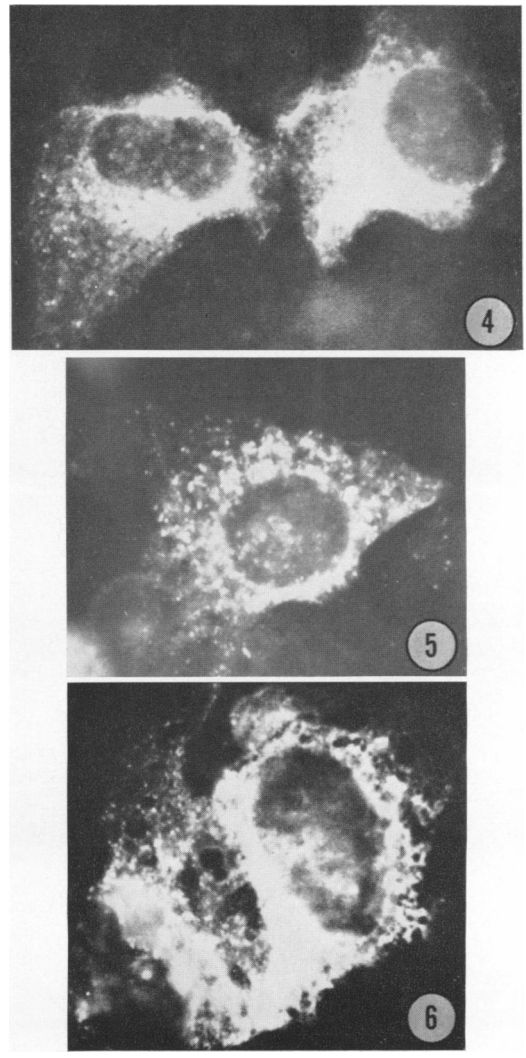


FIG. 4. *Eight hours after addition of virus. Diffuse fluorescence throughout most of the cytoplasm, with some particulate fluorescence toward the cell margins.* ×*720.*

FIG. 5. *Twelve hours after addition of virus. Particulate fluorescence throughout most of the cytoplasm.* ×*720.*

FIG. 6. *Eighteen hours after addition of virus. Intense fluorescence throughout the cytoplasm except for numerous nonfluorescent vacuoles.* ×*720.*

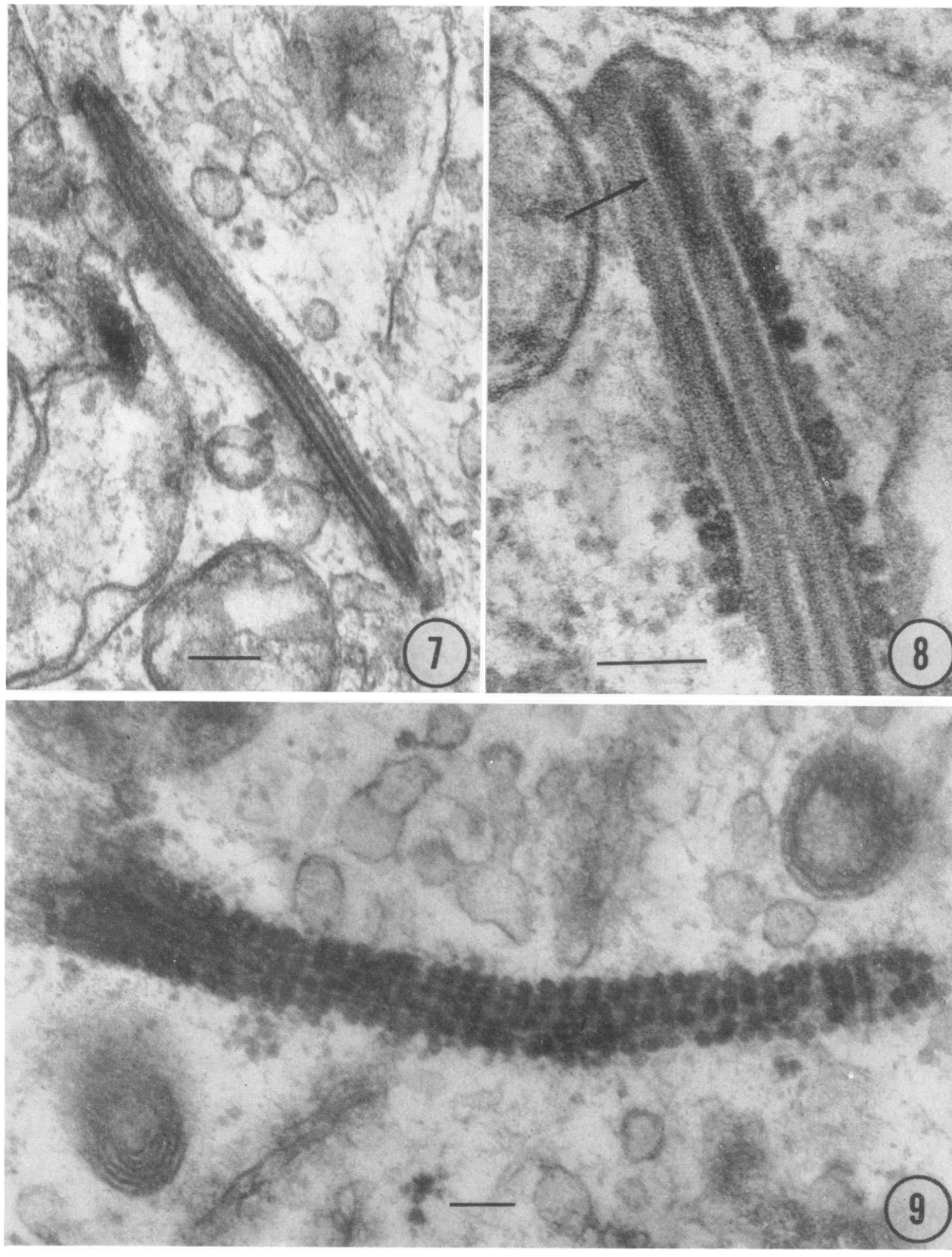


FIG. 7–24. *Electron microscopy studies of HEp-2 cells from SFV-infected cultures. The scale line represents 0.1 μ except in Fig. 14 and 21, in which it represents 1 μ. Figures 11–12 and 14–24 are from day 3 infected cultures. Figures 7–10 and 13 are from earlier times, which are recorded in the figure legends.*

FIG. 7. *Two tubules surrounded by a single membrane in the cytoplasm of a 12-hr infected cell. × 99,000.*

FIG. 8. *High-magnification micrograph of tubules similar to Fig. 7, but also showing small particles attached to the outer surface of the limiting membrane. Note the dense material within the lumen of one of the tubules (arrow). Twenty-four hours after addition of virus. × 150,000.*

FIG. 9. *A 24-hr infected cell showing a glancing section through an angulated tubular structure encrusted with small particles. × 87,000.*

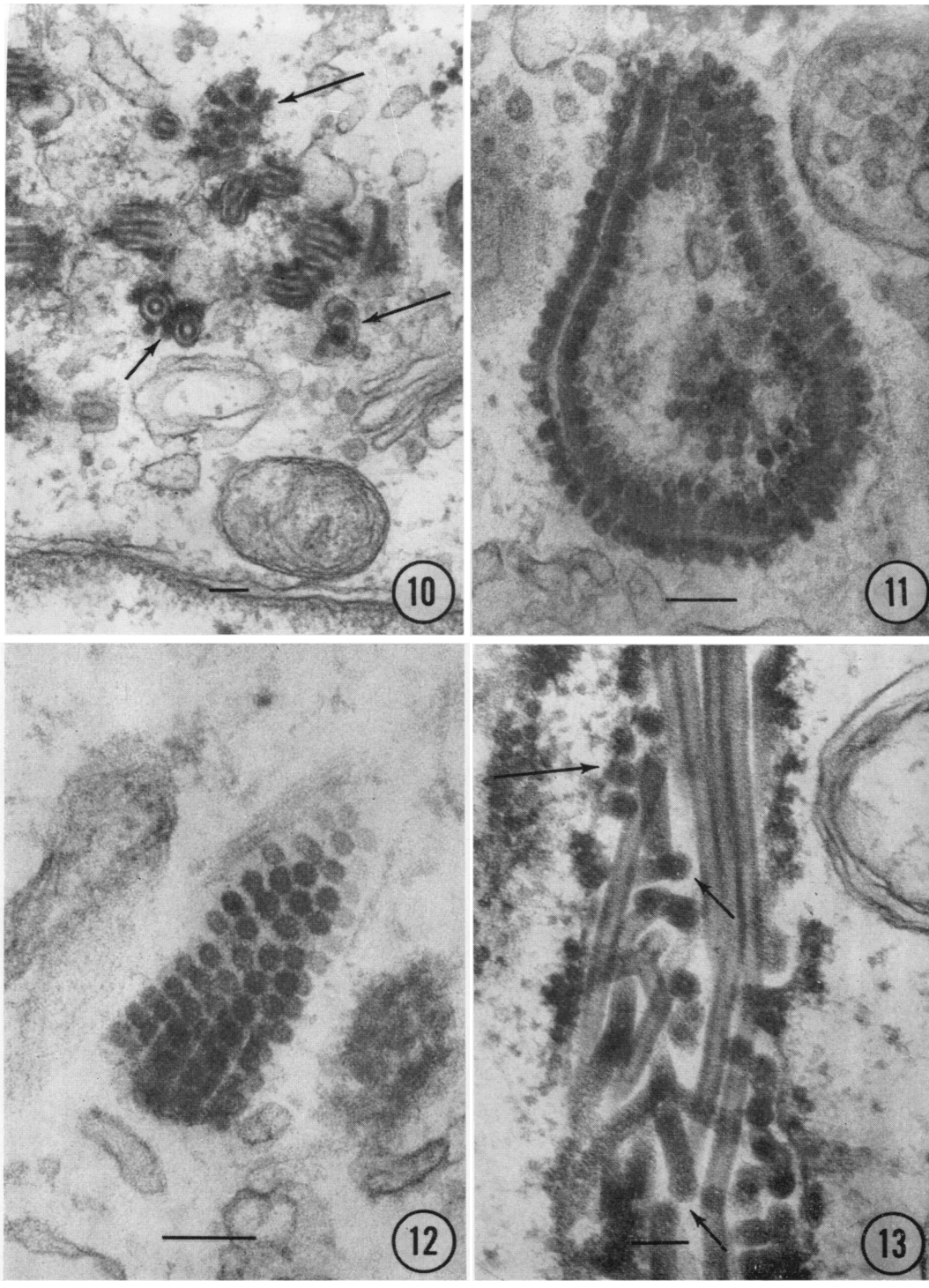


FIG. 10. *Numerous tubular structures in the cytoplasm of a 24-hr infected cell. The two small vacuoles contain large virus particles (long arrows). Note the two membrane-surrounded tubular structures cut in a cross section (short arrow).* × *62,000.*

FIG. 11. *"Pear-shaped" structure presumably formed by invagination of a simple virus-studded vacuole.* × *105,000.*

FIG. 12. *Oblique section through the small particle-encrusted membrane which is most likely surrounding two tubules at their terminus.* × *140,000.*

FIG. 13. *Area of cytoplasm showing numerous tubules within a membranous enclosure. The long arrow indicates a small particle which may be acquiring a coat while migrating through the membrane. Note also the intimate association of some of the large virus particles with the tubules (short arrows). Twenty-four hours after addition of virus.* × *87,500.*

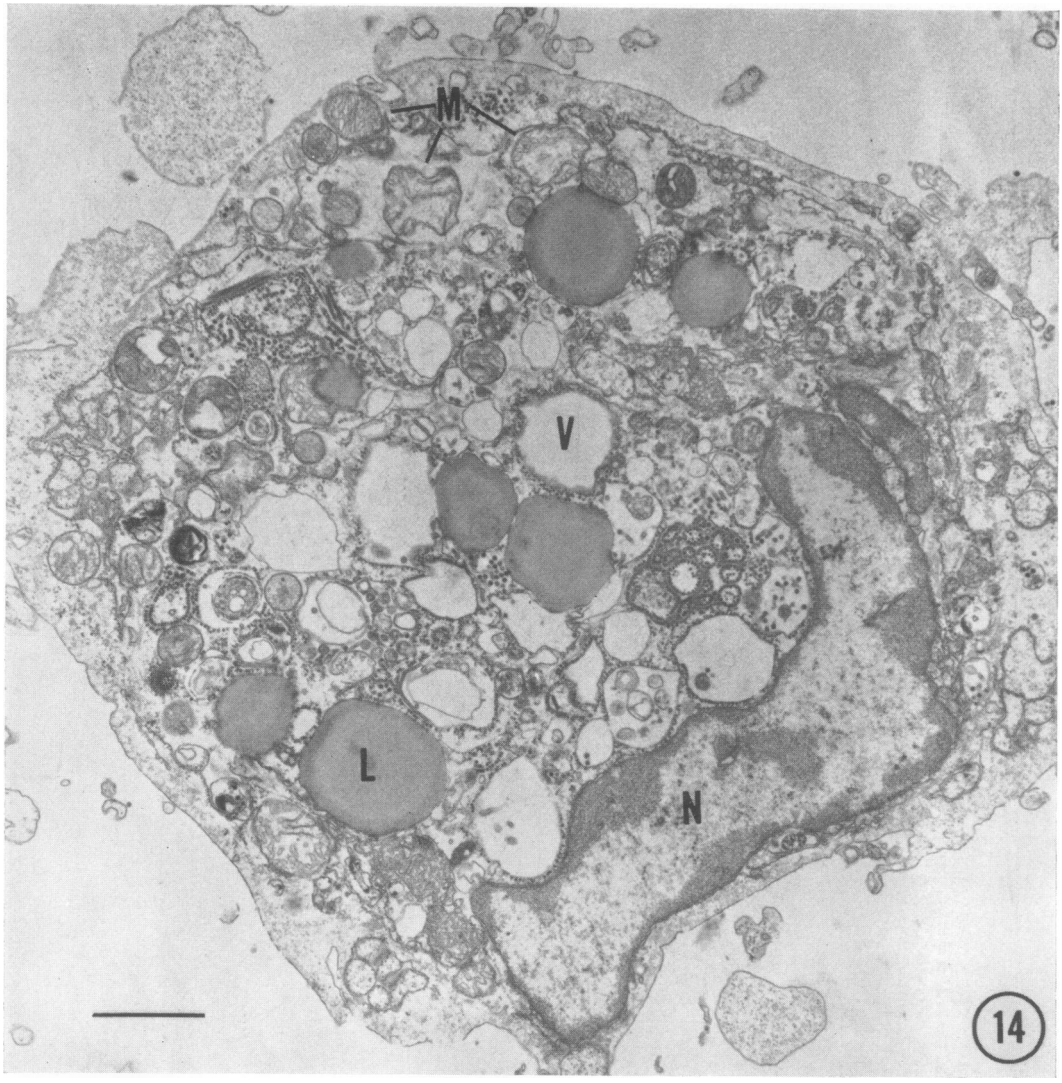


FIG. 14. *Low-power micrograph of a typical infected cell, showing numerous lipid droplets (L), vacuoles (V), distorted mitochondria (M), and an excentric nucleus (N). The cell is heavily laden with virus and has a discontinuous membrane.* × *14,700.*

by a single membrane (Fig. 7). Some of these limiting membranes were encrusted with numerous small (25 mμ), dense particles (Fig. 8).

At later time intervals, the cytoplasm of many of the cells contained a bewildering collection of linear and circular tubular structures, and small vacuoles associated with large numbers of small viral particles (Fig. 9–12). Larger (42 to 50 mμ) particles could also be seen within the small vacuoles 24 hr after infection (Fig. 10 and 13).

The 12-hr infected cultures displayed additional changes in morphology, including rounding-up of the cell, flattening and displacement of the nucleus to the cell periphery, and vacuolization of the cytoplasm. These cytopathological alterations were seen with much greater frequency in the 24- and 48-hr infected cultures. In addition to the changes described above, distorted mitochondria and numerous lipid droplets were seen in the cytoplasm (Fig. 14). These damaged cells contained vast numbers of virus particles in all stages of development.

Beyond 12 hr, tubular structures were still present, but instead of always being delineated by a relatively closely applied membrane, they were found within vacuoles. That this vacuolar mem-

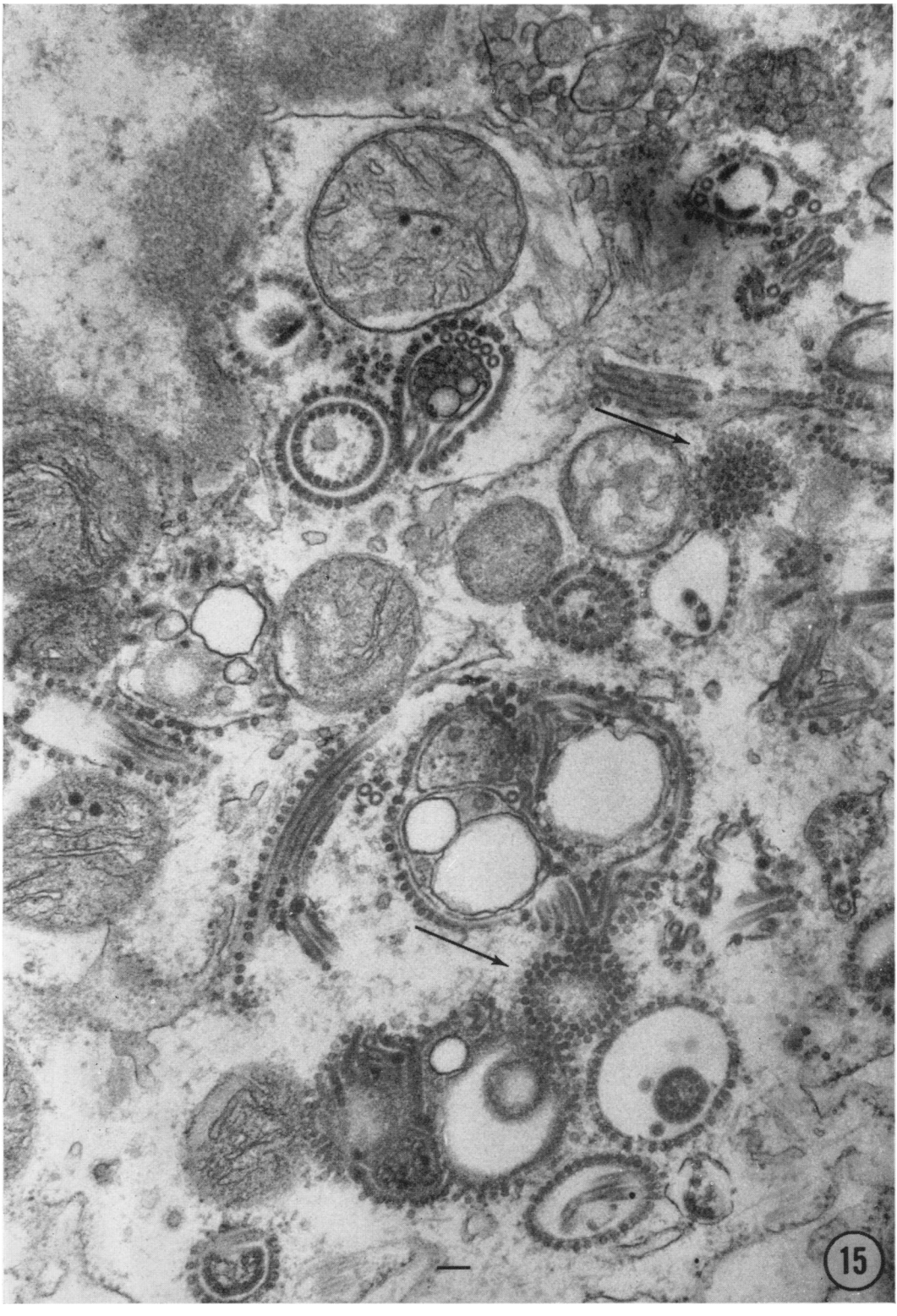
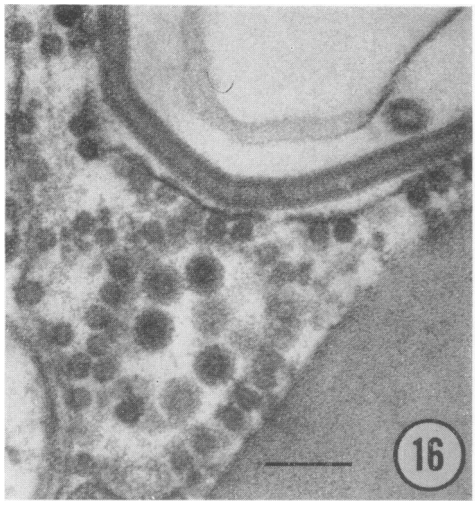
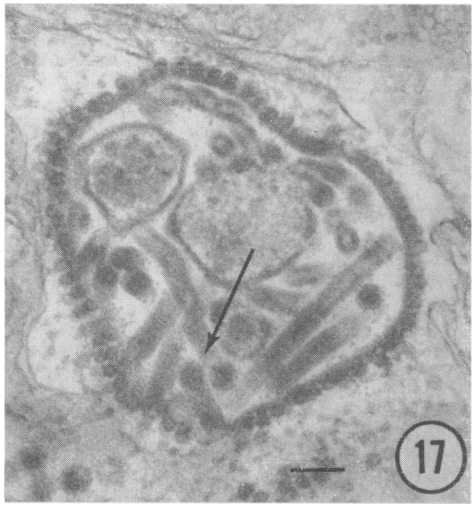
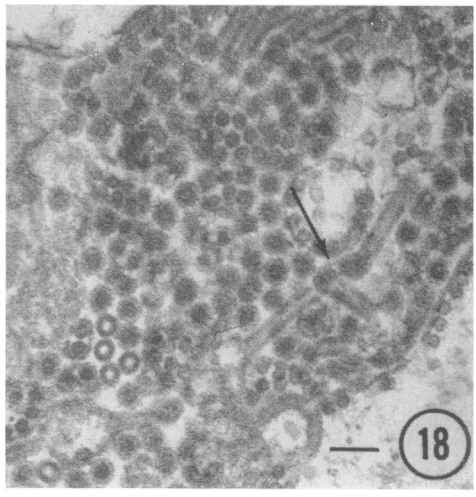


FIG. 15. *Higher-power micrograph of a portion of a cell similar to that of Fig. 14. Note the tubules in longitudinal and cross section and the numerous small particles. A few large particles are also present within vacuoles. The two clusters of small particles (arrows), which appear to be lying free in the cytoplasm, are most likely tangentially cut and attached to the vacuolar membrane.* × *49,600.*







brane is identical to the membrane surrounding the tubular structures is shown by the presence of numerous 25-mμ virus particles on its outer surface (Fig. 13, 15–17). Thus, it appears that, as the small particle-coated membrane gradually enlarged into a vacuolar entity, the tubular structures became less prominent, and the vacuole membrane often invaginated to form structures superficially resembling gastrulae (Fig. 11), but varying greatly in size and degree of invagination. Cross sections through such invaginated vacuoles give the appearance of double membranes lined outside and inside with virus particles (Fig. 15). Our interpretation of the process of development of membrane-founded tubules into the relatively large and complex vacuolar structures is illustrated diagrammatically in Fig. 25.

In addition to the structures described above, many of the vacuoles in the 24-, 48-, and 72-hr infected cultures contained varying numbers of the larger (42 to 50 mμ) particles (Fig. 10 and 13–20). These particles consisted of a dense nucleoid surrounded by an indistinct coat (capsid). They were sometimes observed within vacuoles in close proximity to, or attached to the ends of, the tubular structures (Fig. 13, 17, and 18). Frequently, they were seen attached by pedicles to the inner surface of the vacuole (Fig. 13 and 19). Occasionally, they were seen in association with and apparently developing from membranes within a vacuole (Fig. 20). Some possible mechanisms of formation of large virus particles are discussed below.

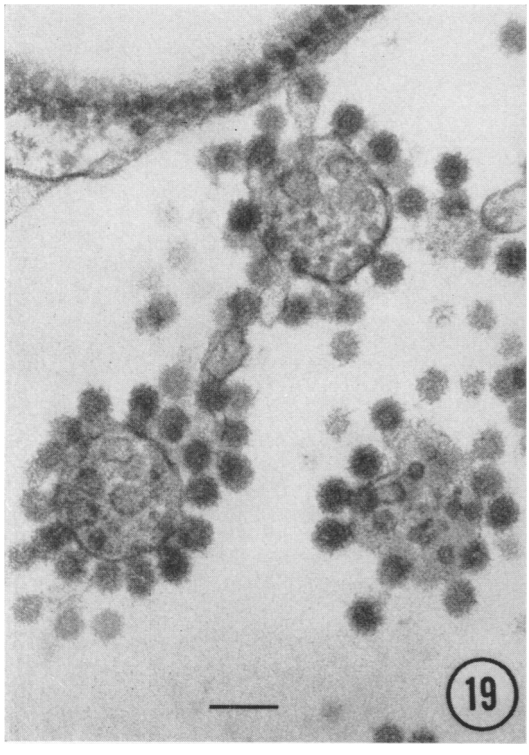
Both the small and large particles were occasionally seen in the cytoplasmic matrix of virus-damaged cells (Fig. 15 and 20).

Extracellular virus was noted 24 and 72 hr after infection—being especially numerous at the latter time. The great majority of these virus particles were the larger particles, and many appeared to be intimately associated with the cell membrane (Fig. 24). Occasionally, clusters of

FIG. 16. *In the upper half of the micrograph are two tubular structures, one in cross section (upper right). In the lower half is a portion of a vacuole which contains six or seven large particles. The vacuolar membranes are studied with small particles.* × *112,000.*

FIG. 17. *Vacuole containing numerous tubular structures. Note the close connection between the ends of two of the tubules and two of the large particles (arrow).* × *70,000.*

FIG. 18. *Portion of a vacuole similar to Fig. 17 but at a further stage of maturation since it contains large numbers of the 42 to 50-mμ particles, two of which appear to be forming at the ends of tubules (arrow).* × *66,000.*



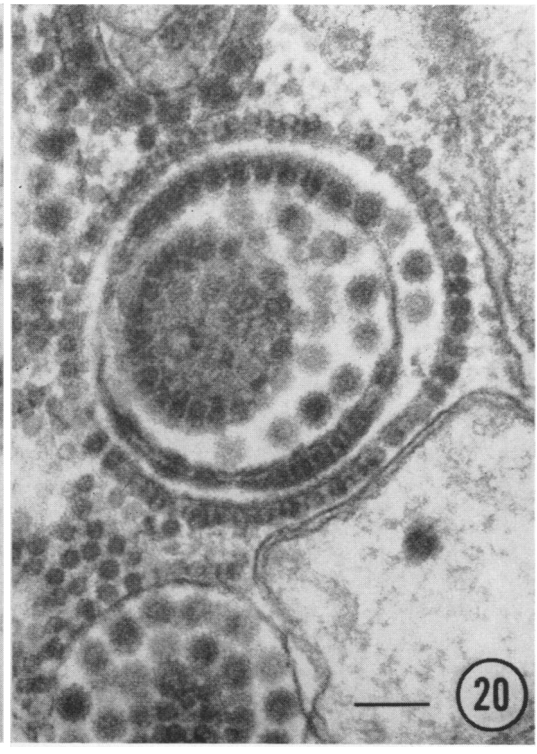


FIG. 19. *Cross sections of three vacuolar invaginations (see Fig. 25) showing numerous large particles attached to the membrane on the vacuole side (many by stalks). Some small particles can be seen on the cytoplasmic side. The upper left portion of the micrograph shows a portion of another vacuole.* × *91,000.*

FIG. 20. *The vacuole which occupies most of this photograph contains a complex membranous structure which appears to be producing large virus particles (see Discussion). The central area in this vacuole is a section through an invagination. In the lower left of the micrograph, there is another vacuole packed with large particles. Both small and large particles can be also seen in the cytoplasmic matrix.* × *100,000.*

tubules and small virus particles were observed extracellularly (Fig. 21–23).

DISCUSSION

In SFV-infected cells, the outer surface of the tubule-surrounding membrane appears to be the template site for the production of the small (25 mμ) particles, since at the early time periods these particles were always attached to this membrane. It seems likely that these small particles are the RNA cores of the larger "mature" SFV particles. The demonstration of SFV antigen by FA technique at this early time is evidence that these membranes or the associated small particles, or both, contain viral antigen. An interpretation of how these particle-studded membranes mature into the more complex cytoplasmic structures and vacuoles which were observed with the electron microscope at 24 hr and later is diagrammed in Fig 25.

Our observations suggest three processes by which the larger (42 to 50 mμ) SFV particles may develop. (These larger particles are usually found within the vacuoles but are not indicated in the diagram.) Apparently, all three processes may occur at the same time, and it is not known which one plays the major role. (i) The 25-mμ particles may cross the vacuolar membrane into the lumen, increasing in size as they do so by acquiring a peripheral coat. Figures 13 and 19 suggest this process, but if this process occurs it is probably very rapid since transitional forms were rarely encountered. (ii) The entire development of the larger SFV particles may occur within a vacuole. Such a process is suggested by Fig. 20 in which it appears that a membranous structure within a vacuole doubles and thickens on both sides, and then a dense material accumulates in the newly formed intermembranous space, increases in bulk, and eventually becomes enclosed by the

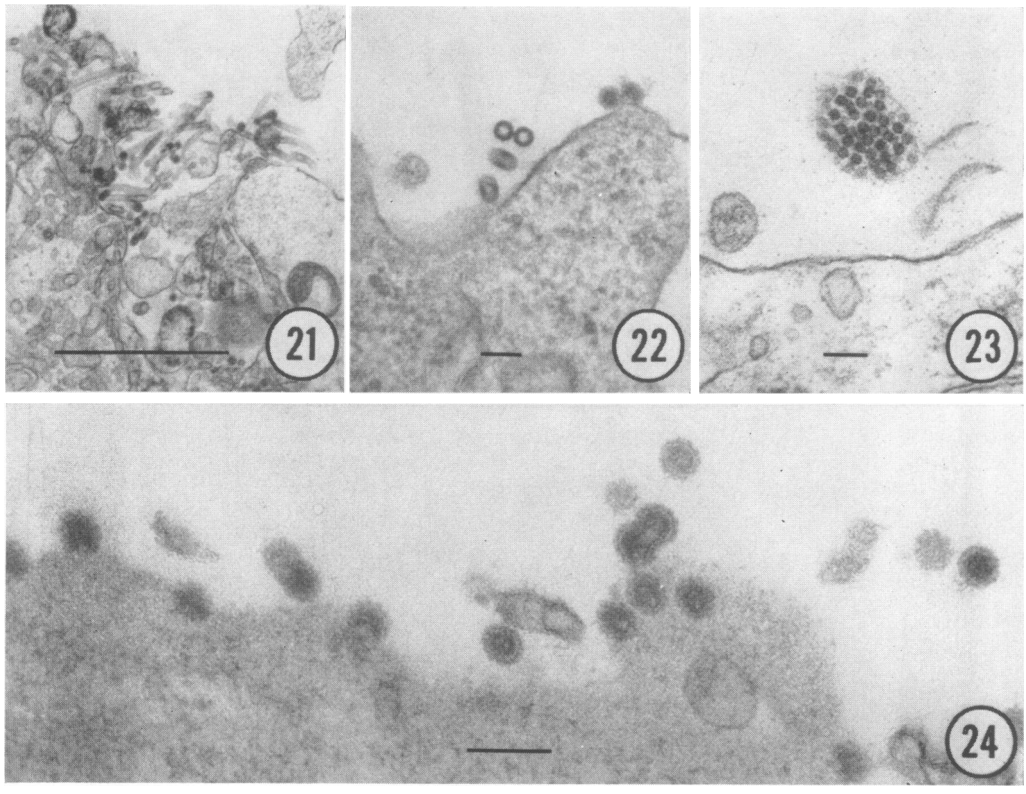


FIG. 21. *Tubular structures and some large particles, probably being liberated from the cell by rupture of a cytoplasmic vacuole at the cell surface.* × *22,800.*

FIG. 22. *Cross and tangential sections of four tubules in the extracellular space. Two large particles are attached to the cell membrane.* × *55,000.*

FIG. 23. *A cluster of small particles in the extracellular space.* × *54,000.*

FIG. 24. *A tangential section through the cell surface showing numerous large particles, some of which appear to be either entering or leaving the cell.* × *109,000.*

membrane, which probably represents the viral capsid. The origin of the intravacuolar membranous structure is unknown. (iii) The larger particles may arise from the tubules. Many of the tubules contain a dense material in their lumens (Fig. 8, 13, and 17). This material may be extruded at the ends of these structures, after being encompassed by expanded tubular wall substance (Fig. 13 and 17).

There is no previous study of SFV by electron microscopy or FA technique except Cheng's (8) electron microscopy study of purified SFV preparations which showed virus particles with a diameter of 50 mμ.

The characteristics of SFV, as revealed in the present study, are strikingly different in several respects from those of West Nile and Guaroa viruses, which we studied previously (26). Although all of the three are arboviruses, they belong to serological group A, group B, and the Bunyamwera group, respectively (5). SFV infection proceeded more rapidly, as judged by all criteria—cytopathology, infective virus production, production of antigen, and appearance of virus particles. All three viruses seemed to be exclusively cytoplasmic, but, whereas SFV was diffusely distributed in vacuolar structures throughout the cytoplasm, West Nile virus tended to concentrate in the juxtanuclear region and was closely associated with the endoplasmic reticulum, and Guaroa virus particles were formed at or near the plasma membrane. The production of infectious virus and viral antigen, and assembly into complete virus particles proceeded synchronously for SFV and West Nile virus, but the assembly of Guaroa virus particles lagged far behind the appearance of infectious virus and viral antigen. Release of West Nile virus or SFV was accompanied by disintegration of the cell, but it appeared that some cells released

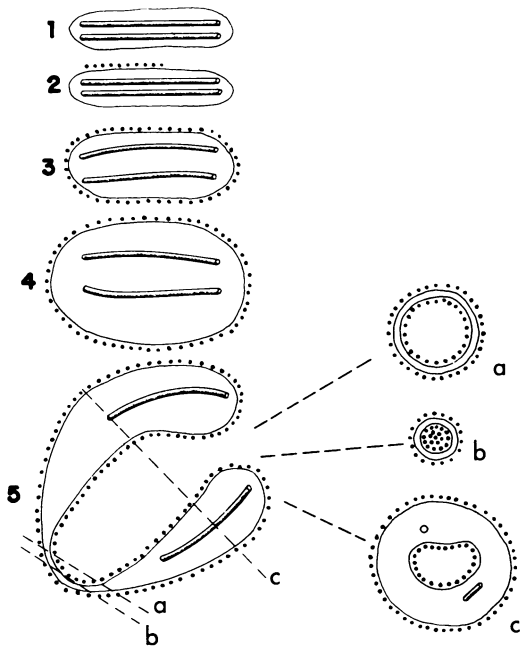


FIG. 25. *Diagrammatic reconstruction of the steps in development of the smaller (25 mμ) SFV particles around membrane-covered tubule-containing vesicles; 1, 2, 3, 4, and 5 represent successive stages in development of the vacuole; a, b, and c represent some of the relationships of particles, tubules, and membranes that are seen when the plane of section passes through various parts of invaginated vacuoles, as indicated by the dotted lines. The development of the larger particles within the vacuoles is not presented in this diagram.*

Guaroa virus particles without undergoing lysis. The three viruses also differed significantly in morphology. West Nile particles were 30-mμ spheres consisting of a nucleoid surrounded by an indistinct membrane. Guaroa particles also consisted of a nucleoid and single coat, but were slightly eliptical and much larger (70 by 90 mμ). SFV had 25-mμ dense particles with no membrane and larger 42- to 50-mμ particles consisting of a dense nucleoid (resembling the 25-mμ particles) surrounded by an indistinct capsid. The manner of formation of SFV particles is quite different from any other virus yet described, and is discussed in comparison with other arboviruses in the following paragraphs.

Other arboviruses of group A do show certain similarities to SFV, although none has been reported to show as bizarre and varied a pattern of rods, vacuoles, and large and small particles as were found in this study of SFV. Small particles of approximately the same size as the smaller SFV particles were seen on vacuolar membranes in KB cells infected with Venezuelan equine encephalitis virus (VEE) by Mussgay and Weibel (19), and in a number of tissue culture lines infected with Western equine encephalitis (WEE) by Morgan et al. (18). Some of the WEE vacuoles contained tubules, but no evaluation of their role in virus reproduction was attempted. Mayaro virus was observed within cytoplasmic vesicles of infected KB and human heart cells by Saturno (24), but no mechanism of virus formation was suggested. Chain et al. (6) studied Chikungunya virus in chick fibroblasts and reported 26- to 28-mμ "precursor particles" and membrane-covered 50- to 56-mμ particles in the cytoplasm. These particles, in contrast to other group A arboviruses, were not observed within cytoplasmic vacuoles, but the published photomicrographs are inadequate to support a conclusion that they do not occur in vacuoles. In the present study, it appeared that the smaller SFV particles formed on the membrane which was closely adherent to the tubules (Fig. 7 and 25), and that this particle-encrusted membrane then dilated to form vacuoles which resembled those described in the studies of VEE and WEE.

Virus particles of the larger size have also been described for other group A arboviruses. Morgan et al. (18) hypothesized that the larger "mature" particles of WEE virus developed as the smaller "precursor" particles passed through vacuolar membranes into the lumen of cytoplasmic vacuoles. Mussgay and Weibel (19), however, suggested that the larger virus particles of VEE formed as they passed out through the vacuolar membranes into the cytoplasm. Formation of virus particles from membranes or tubules within cytoplasmic vacuoles, as observed in the present study of SFV, has not been reported previously for any arbovirus, although in the electron micrographs of WEE (18) some "filaments" which resemble our "tubules" can be seen among the virus particles within the vacuoles. [A similar mechanism has been reported for rabbit kidney vacuolating virus. Virus particles were seen within cytoplasmic vacuoles and occasionally at the ends of tubular structures of a smaller diameter (7). This rabbit virus morphologically resembles the larger SFV particles, but it is a papova virus, not an arbovirus.]

The group B arboviruses morphologically resemble the larger mature particles of SFV and other group A arboviruses, but most of them have a different mode of formation. Thus West Nile and yellow fever viruses do not form small "precursor" particles (or naked viral cores) and the particles do not develop on the membrane of the cytoplasmic vacuoles. West Nile virus growing in HEp-2 cell cultures formed in granular foci in the cytoplasm and filled channels of the

endoplasmic reticulum (26). Similarly, yellow fever virus particles formed in cisterns and tubules of the endoplasmic reticulum in mouse brain cells (2, 4). However, in rhesus monkey liver cells (3), and in KB, HeLa, and chick embryo cells (4), yellow fever virus particles were usually found in the cytoplasmic matrix and not within tubules of the endoplasmic reticulum. Preliminary observations of Ilheus virus in HEp-2 cells suggest that the endoplasmic reticulum may be involved in the formation of this group B virus also (*personal observation*). JBE virus resembles other arboviruses of the B group in that (in porcine kidney stable cells) it lacks the small "precursor" particles, but it appears to differ developmentally in that it buds from vacuolar membranes (21). JBE particles also appeared within cytoplasmic vacuoles of mouse brain cells (28), but no "budding" of small particles into the vacuoles was reported. Powassan virus (which is a tick-borne group B arbovirus whereas all others mentioned in this discussion are mosquito-borne) had 36- to 38-mμ particles which were found along the inner surface of cytoplasmic vacuoles in monkey kidney cell cultures (1) but apparently did not develop in tubules of the endoplasmic reticulum. Thus, it seems to resemble the group A more than the other group B arboviruses.

Of the Bunyamwera group, only Guaroa has been studied by electron microscopy and FA techniques, and it differs greatly from both the A and B groups in particle size and process of development, as discussed above.

Anopheles A virus is one of the ungrouped arboviruses. It has 55- to 65-mμ particles which develop within the endoplasmic reticulum of Ehrlich ascites tumor cells (12), and thus it resembles the group B arboviruses. Rift Valley fever virus, another ungrouped arbovirus, has characteristics which differ from all other arboviruses which have been studied to date. It causes necrotizing hepatitis in mice, and in hepatic parenchymal cells of infected mice virus particles were formed within dilated endoplasmic sacs which are thought to be the Golgi apparatus (15). The virus particles had a single membrane and a diameter of about 90 mμ; no "precursor" particles or naked viral cores were observed. Unique structural alterations of the ergastoplasm were also associated with the development of this virus, as with Ilheus.

Further studies of other arboviruses from the several serological groups will be necessary before it will be known how consistently their morphological and developmental characteristics parallel their antigenic characteristics.

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