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Electron Microscopy of Cells Infected with Semliki Forest Virus Temperature-Sensitive Mutants: Correlation of Ultrastructural and Physiological Observations

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Cells infected at the permissive temperature with three temperature-sensitive mutants of Semliki Forest virus were not significantly different in appearance from cells infected, at either the permissive or nonpermissive temperature, with wild-type virus. Virus particles, nucleocapsids, spherules, and tubules were seen in the cytoplasm. But replication of the mutants was inhibited in cells infected at the nonpermissive temperature. This was evidenced by the absence of virus particles and nucleocapsids (except in one case) and the absence or limited production of spherules and tubules. These observations are discussed with reference to the physiological defects of the mutants.

Temperature-sensitive (*ts*) mutants have been isolated from Semliki Forest virus (SFV) and characterized in detail (5; Tan, *unpublished data*). These mutants replicated normally in cells incubated at 30 C (permissive temperature) but were blocked at various stages of the replication cycle at 38.5 C (nonpermissive temperature). Wild-type SFV replicated normally at both 30 and 38.5 C.

Cells infected with SFV have been studied in detail in the electron microscope (1, 2, 3). Peculiar to the infected cells were vacuoles surrounded by viral nucleocapsids, aggregates of viral nucleocapsids, virus particles, and spherular and tubular structures, all of which were seen in the cytoplasm. Virus particles were formed by nucleocapsids budding at cellular membranes to acquire an envelope. The significance of the tubular and spherular structures is not known.

As the physiological defects of the mutants are known (5), it was therefore of interest to examine in the electron microscope and then to compare the ultrastructure of cells infected with wild-type virus or with mutants. This paper reports studies with mutants which, at the nonpermissive temperature, are blocked in viral functions subsequent to viral ribonucleic acid (RNA) synthesis (5).

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MATERIALS AND METHODS

Cells. The same preparation of primary chick embryo (CE) cells, grown in monolayers, was used in all of the experiments. The preparation and maintenance of CE monolayers have been described (5).

Virus. The infection of cells with and growth of SFV and *ts* mutants were as described by Tan et al. (5), except that actinomycin D was omitted in the present study and infected cells were sampled at 14 hr after infection.

Wild-type virus and three RNA^+ mutants were investigated. These mutants, *ts*-15, *ts*-18, and *ts*-21, at 38.5 C synthesize 13, 24, and 14%, respectively, of the wild-type yield of RNA (5; Tan, *unpublished data*). All three mutants make viral membrane protein but only *ts*-15 makes nucleocapsids as well. Thus, the temperature-sensitive defect of both *ts*-18 and *ts*-21 is in the production of viral nucleocapsids and that of *ts*-15 may be maturation of virus particles (5).

Electron microscopy. Cells were scraped off the glass, pelleted, fixed with 1% gluteraldehyde [in phosphate-buffered saline (PBS)] for 5 to 10 min at room temperature, and postfixed with 1% oxmium tetroxide (in PBS) for 60 to 90 min at 4 C. After washing with PBS containing 10% sucrose, the cell pellets were dehydrated in acetone. All cell pellets were stained, during the dehydration stage, in a saturated solution of uranyl acetate in 70% acetone. After dehydration in 100% acetone, the cell pellets were embedded in arladite (as instructed, "Durcupan," ACM, Fluka). Thin sections cut with a Reichert microtome with glass knives were mounted directly on 400-mesh grids. The sections were then stained with 0.2% lead citrate in 0.1

N NaOH for 1 min, washed, and examined in a Philips EM 200 electron microscope.

RESULTS

Cells infected at 30 C. Cells infected for 14 hr at 30 C with wild-type virus or with mutants were not significantly different in appearance [*see* Acheson and Tamm (1) and Erlandson et al. (2)]. Infectious virus was still being made at this late time after infection at 30 C (5).

Several sections of each sample were examined, and representative electron micrographs of these are shown in the accompanying figures. Infected cells characteristically contained virus-associated vacuoles, viral nucleocapsids, virus particles, tubules, and spherules.

Virus particles and nucleocapsids. Infected cells were highly vacuolated compared with uninfected cells. Many of the vacuoles were associated with viral structures, being surrounded by viral nucleocapsids and frequently also containing virus particles (Fig. 1, 2a, 3a, 4a).

Nucleocapsids often occurred in aggregates but small numbers of single nucleocapsids were also observed scattered in the cytoplasm (Fig. 1, 2a, 3a, 4a). Virus particles were formed by nucleocapsids budding at the plasmalemma (not shown; 1, 2) or at the vacuolar membrane (Fig. 2a) to acquire an envelope.

Spherules and tubules. Spherules are saclike membranous structures containing an electron-dense particle in the center (Fig. 4a). They are seen only in infected cells, appearing soon after infection (1, 3). Cells infected at 30 C with wild-type virus or with mutants contained spherules in the cytoplasm (Fig. 1) and on the cell surface (Fig. 4a), frequently attached to extensive areas on the cell surface.

Tubules appeared late in infection, and in intact cells they were found in virus-associated vacuoles (Fig. 1, 2a, 3a; references 1, 2, 4). Tubules appeared to originate from the vacuolar membrane (Fig. 5a). Virus particles were often attached to the ends of tubules (Fig. 3a), and tubules may contain more than one nucleocapsid arranged in a linear order (Fig. 5a). Like spherules, tubules were absent in uninfected cells.

Cells infected at 38.5 C. Only the cells infected at 38.5 C with wild-type virus (not shown) resembled cells infected at the permissive temperature.

In cells infected at 38.5 C with either *ts*-15 (Fig. 2b), *ts*-18 (Fig. 3b), or *ts*-21 (Fig. 4b), virus particles and budding virus particles were absent, tubules were absent, and nucleocapsids, not in such massive aggregates as were present in

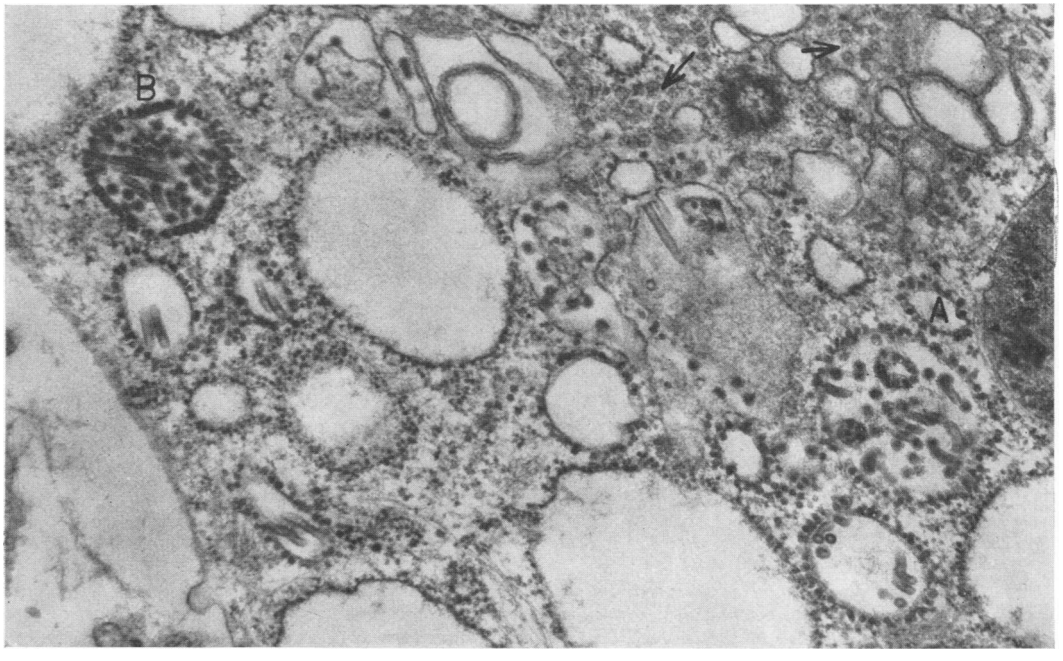

FIG. 1. *Portion of a cell infected with wild-type virus at 30 C. The cytoplasm is highly vacuolated. Some vacuoles are surrounded by viral nucleocapsids (A), and others are surrounded by nucleocapsids and contain virus particles and tubules (B). Spherules (arrows) are present in the cytoplasm.* ×34,000.

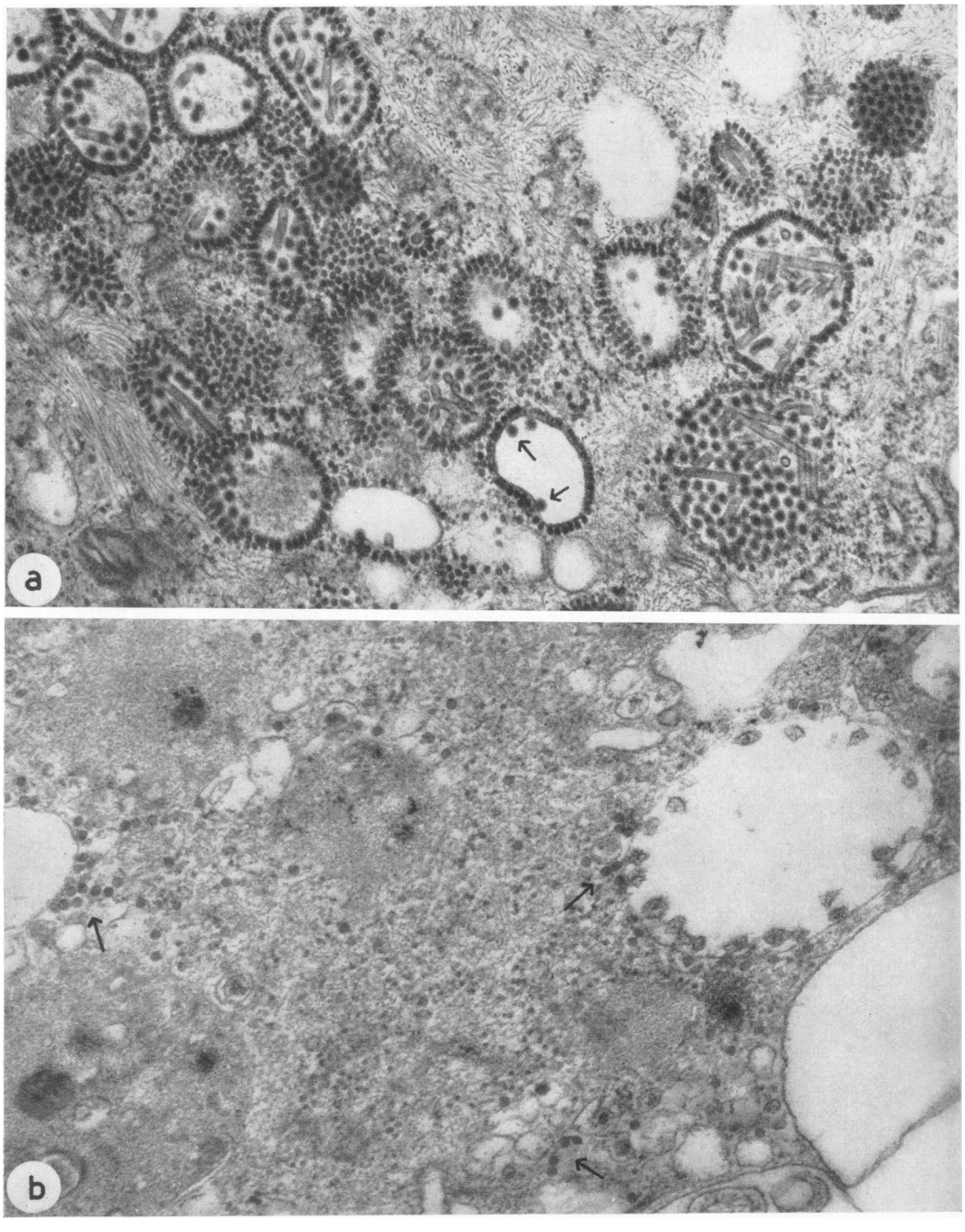

FIG. 2. *Portions of cells infected with ts-15. (a) Cell infected at 30 C showing a collection of virus-associated vacuoles and aggregates of nucleocapsids in the cytoplasm. In one vacuole, virus particles are formed by budding (arrows). ×39,000. (b) Cell infected at 38.5 C. Nucleocapsids (arrows) are scattered in the cytoplasm. One vacuole is lined on its inner edge by spherules. ×51,000.*

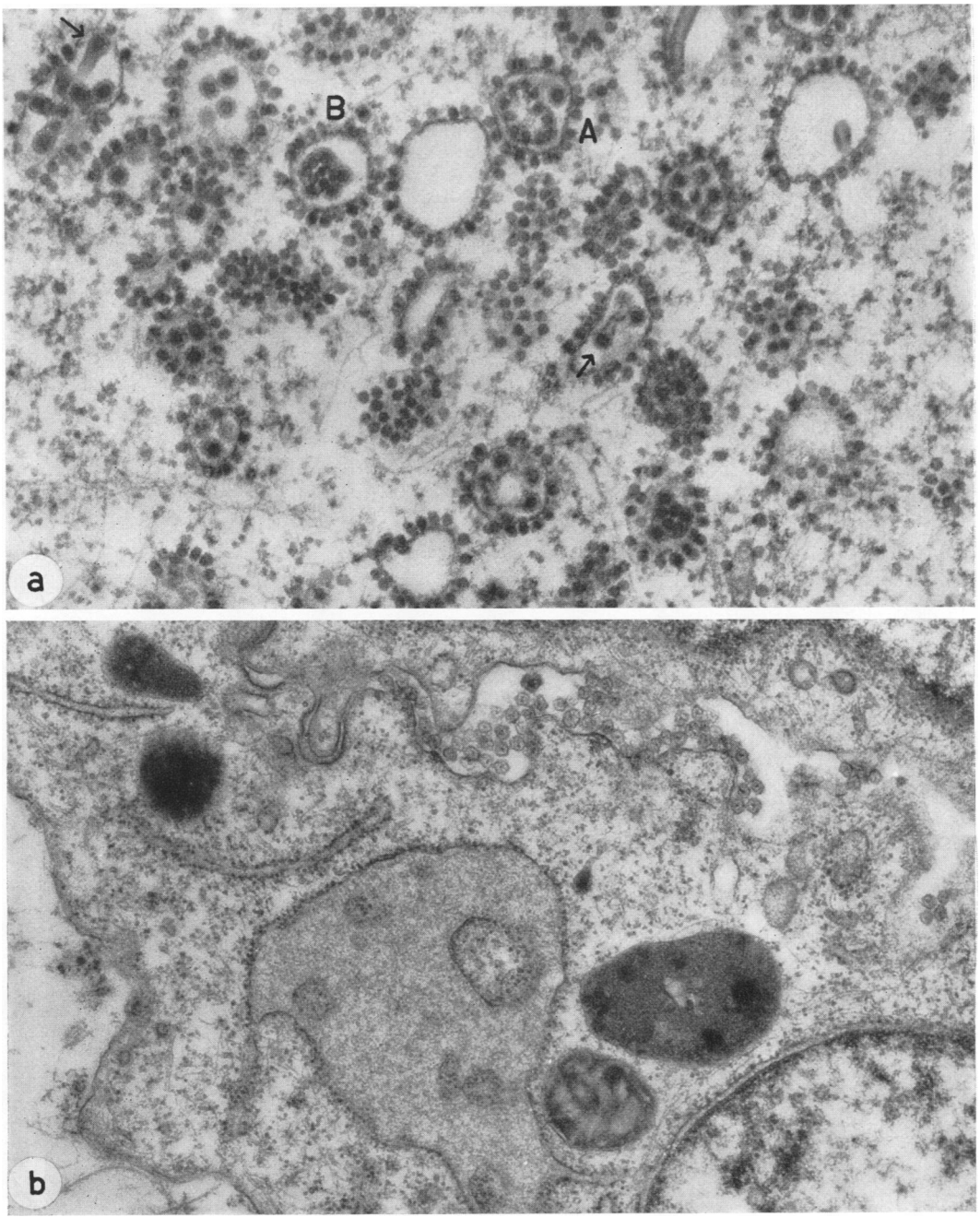

FIG. 3. *Portions of cells infected with ts-18. (a) Cell infected at 30 C. In two vacuoles, virus particles are attached to the ends of tubules (arrows). Some vacuoles (A, B) contain groups of nucleocapsids and virus particles, some of which are budding. Such vacuoles are probably invaginated [Erlandson et al. (2)]. × 64,000. (b) Portions of two cells infected at 38.5 C. Numerous spherules are present between the two cells. ×35,000.*

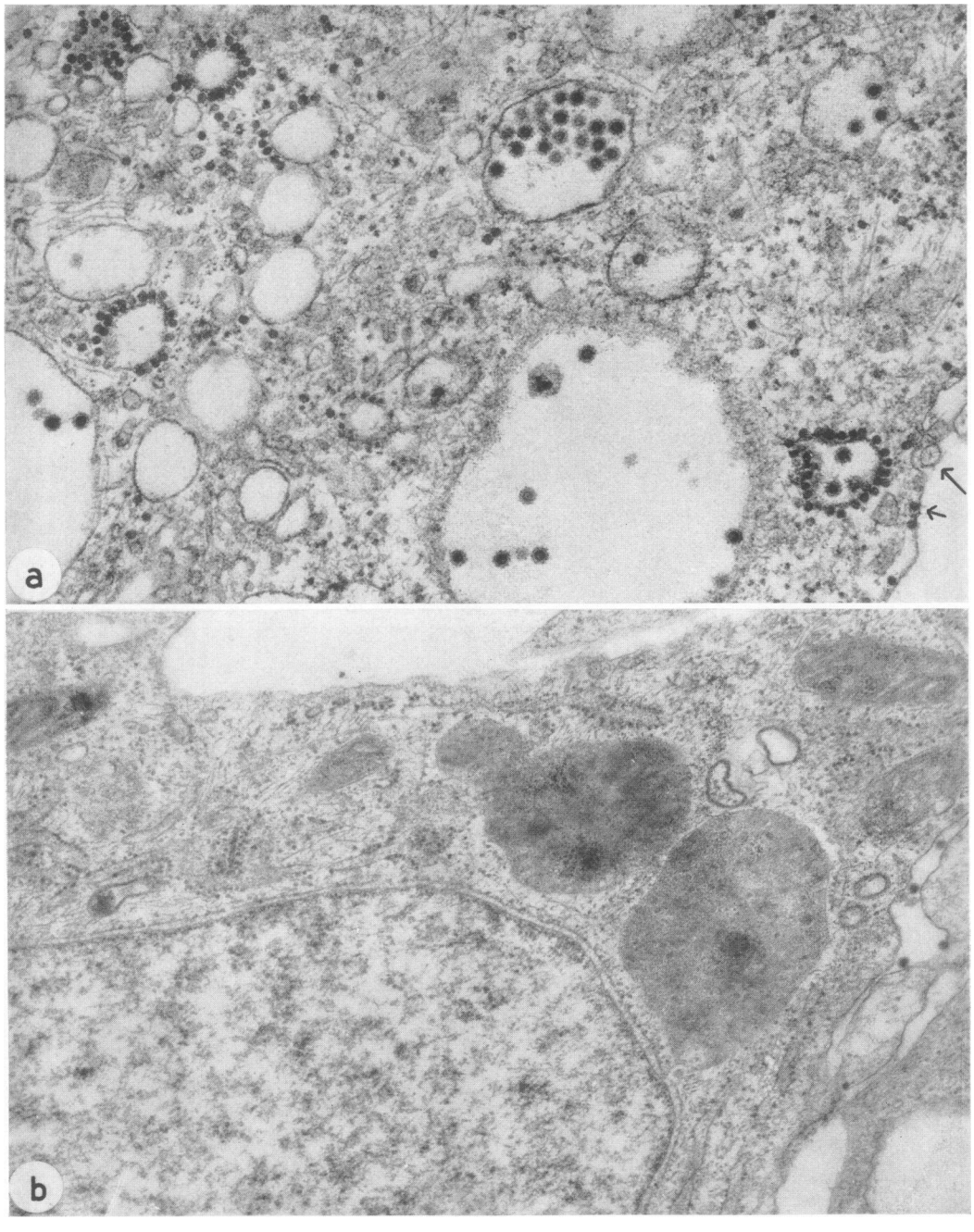

FIG. 4. *Portions of cells infected with ts-21. (a) Cell infected at 30 C. Nucleocapsids are attached to the cytoplasmic side of the plasmalemma (short arrow), and spherules are attached to the outer side (long arrow). ×47,000. (b) Cell infected at 38.5 C. Subcellular organelles are well preserved. The significance of rare extracellular virus particles is not known. ×33,000.*

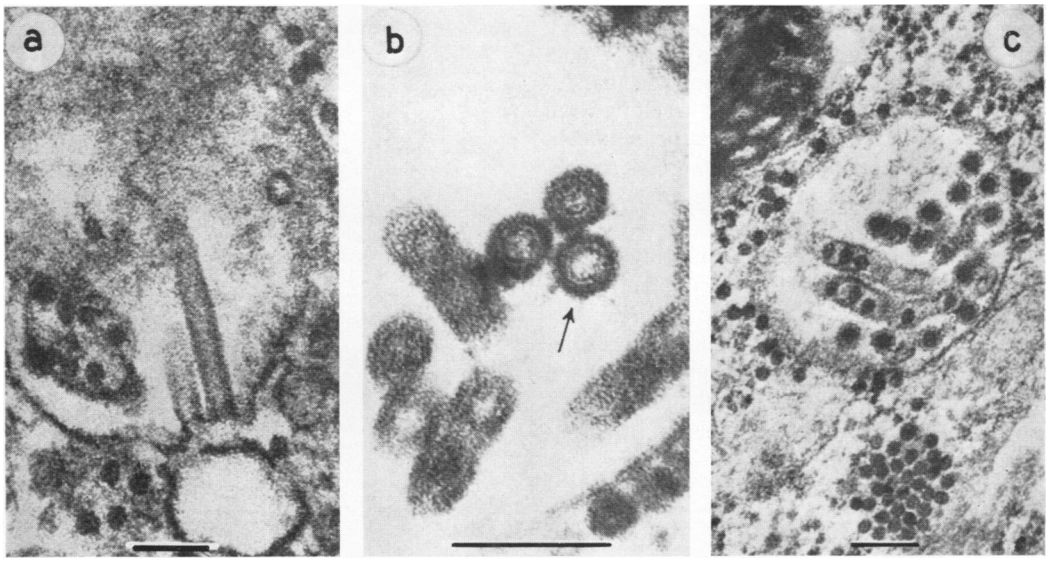

FIG. 5. *Tubules. (a) Longitudinal section of tubules attached to vacuolar membrane. (b) Transverse section of tubules (arrow). (c) Tubules containing nucleocapsids. Scale line = 100 nm.*

cells infected at 30 C, were seen only in cells infected with *ts*-15.

Spherules, fewer than in cells infected at 30 C, were observed in cells infected at 38.5 C with *ts*-15 (Fig. 2b) or *ts*-18 (Fig. 3b). Spherules were absent in all sections of cells infected at 38.5 C with *ts*-21 (Fig. 4b).

DISCUSSION

Correlation of ultrastructural and physiological observations. Wild-type SFV multiplies equally well at both 30 and 38.5 C, producing normal amounts of viral nucleocapsids, membrane protein, and virus particles, but *ts* mutants produce normal amounts of viral components only at 30 C (5). These observations were confirmed in the present study of infected cells in the electron microscope.

At 38.5 C, *ts*-15 made nucleocapsids but not complete virus particles, whereas both *ts*-18 and *ts*-21 appeared defective in the production of nucleocapsids, as investigated with the electron microscope. These findings are in complete agreement with the results obtained with physiological tests (5). In those tests, it was concluded that at 38.5 C all three mutants made viral membrane protein but only *ts*-15 made nucleocapsids as well. Morphologically, the cellular membrane and viral membrane (or envelope) are similar in appearance, except for the presence of spikes on the latter. In the maturation of the virus particle, spikes were seen only on budding virus particles (1; *unpublished data*). Thus it was not possible, in the present study, to idenfity in cells infected with the mutants at 38.5 C cellular membranes which were presumably altered biochemically by the insertion of viral membrane protein in them (5).

Spherules and tubules. One difference between the cells infected with mutants at 38.5 C and at 30 C is the reduced number of (with *ts*-15 and *ts*-18) or the absence of (with *ts*-21) spherules at 38.5 C. Mutants *ts*-15, *ts*-18, and *ts*-21 make viral RNA at 38.5 C (5), but make few or no spherules under similar conditions. This suggests that spherules are not associated with viral RNA synthesis. Spherules were always present in cells infected at 30 C, at which temperature normal viral replication occurred. Thus, spherules may be related to viral replication other than viral RNA synthesis and their nature and function remain to be elucidated.

Tubules were not detected in cells infected with mutants at 38.5 C. Thus, it seems that tubule production is associated with the ability of the virus to complete the normal growth cycle. My observations that (i) virus particles and tubules were of a similar diameter (48 and 42 nm, respectively), (ii) tubules were attached to the membrane of virus-associated vacuole, (iii) virus particles were found at the ends of tubules, and (iv) some tubules contained more than one nucleocapsid led to the interpretation that a nucleocapsid budding into a vacuole was not "nipped off" to form a virus particle; instead, the viral mem-

brane protein continued to be made, resulting in the formation of a tubule. If other nucleocapsids happen to bud at the same spot (not unlikely), then the resulting tubule will contain more than one nucleocapsid. Therefore I conclude that tubules are viral membrane protein resulting from abnormal maturation of virus particles late in infection. At such a time, the resources for normal maturation of virus particles may be restricted.

The defects of conditional lethal mutants of animal viruses have been characterized on physiological, biochemical, and genetic grounds. Detailed electron microscopic studies of cells infected with mutants have not been reported. Such studies could provide new and valuable information on the defects of the mutants and thus the replication of wild-type virus.

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