

STRUCTURAL CHANGES IN THE MEMBRANE OF VERO CELLS INFECTED WITH A PARAMYXOVIRUS

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ABSTRACT

Vero cells productively infected with the Halle strain of measles virus have been studied by means of surface replication, freeze-fracturing, and surface labeling with horseradish peroxidase-measles antibody conjugate in order to examine changes in the structure of the cell membrane during viral maturation. Early in infection, the surfaces of infected cells are embossed by scattered groups of twisted strands, and diffuse patches of label for viral antigens cover regions marked by these strands. At later stages, when numerous nucleocapsids become aligned under the plasmalemmal strands, the strands increase in number and width and become more convoluted. At this stage, label for viral antigens on the surface of the cell membrane is organized into stripes lying on the crests of strands. Finally, regions of the membrane displaying twisted strands protrude to form ridges or bulges, and the freeze-fractured membrane surrounding these protrusions is characterized by an abundance of particles smaller than those found on the rest of the cell membrane. The fractured membranes of viral buds are continuous sheets of these small particles, and the spacing between both nucleocapsids and stripes of surface antigen in buds is less than in the surrounding cell membrane. Detached virus is covered with a continuous layer of viral antigen, has unusually large but no small particles on its membrane surfaces exposed by freeze-fracturing, and no longer has nucleocapsids aligned under its surface. Thus, surface antigens, membrane particles, and nucleocapsids attached to the cell membrane are mobile within the plane of the membrane during viral maturation. All three move simultaneously in preparation for viral budding.

Subacute sclerosing panencephalitis (SSPE) virus is a measles strain that causes a rare neurological disease (SSPE) in children occurring several years after they have contracted regular measles (20). For this reason, it is considered a slow virus disease of the nervous system (17). However, once the virus is recovered by co-cultivation of the patient's brain or lymph node cells with indicator cells, it becomes able to induce productive infection in susceptible cells as a complete measles virus (14,

15, 20, 21, 35, 36). In this productive type of infection, as in ordinary measles virus infection, several changes in the plasma membrane of the infected cells can be seen in thin sections examined with the electron microscope (19, 27, 28, 34-36). These changes resemble closely those which had been described earlier in other paramyxovirus infections (11, 12, 22). Viral tubules or nucleocapsids become aligned on the inner surface of the plasmalemma in an amorphous fuzzy matrix,

while a corresponding coating of surface projections or spikes appears on the outer surface of the plasma membrane. This fuzzy material around the nucleocapsids as well as the spikes located opposite them can be labeled by conjugates of horseradish peroxidase (HRP) with antibodies from SSPE patients (14, 15). Infected cells subsequently produce membrane-limited viruses which, like other measles viruses, are pleomorphic in shape and size, covered with spikes, and contain viral nucleocapsids (28).

In the present study, freeze-fracturing and surface replication are used to define more thoroughly sequential changes in the structure of the cell membrane during productive infection with SSPE virus. The freeze-fracturing technique splits biological and viral membranes to reveal details of their internal structure (9, 10, 13, 29, 30), whereas the surface replica technique allows the study of the outer surface of the plasmalemma (5, 7, 8, 42). Furthermore, the internal structure of the plasmalemma can be correlated with its surface aspect by means of deep-etching experiments (32, 43). When these techniques are combined with immunolabeling methods, the localization, distribution, and organization of viral antigens on the surface can be precisely correlated with changes in membrane structure, and with changes in the relationship of the nucleocapsids with this membrane (6, 31, 33). Thus, our results illustrate how a complex membrane structure is put together from various components during viral maturation.

MATERIALS AND METHODS

Virus and Cells

Early passages of the Halle strain of SSPE virus were used. This strain was isolated from a lymph node of a SSPE patient (21). Primary cells were passed and co-cultivated with HeLa cells. The isolate was used after one more passage in HeLa cells or one more additional passage in Vero cells. Vero cells, which are an African Green Monkey kidney continuous cell line, were obtained commercially from Flow Laboratories, Inc., Rockville, Md. Large Falcon plastic flasks (Falcon Plastics, Div. of BioQuest, Oxnard, Calif.) were seeded with 10 ml of 3×10^5 cells/ml of suspension, whereas 25-mm plastic petri dishes received 1 ml of the same cell suspension. For the surface replica technique, cells were grown on 12-mm glass cover slips with or without collagen. The growth medium was Eagle's minimal essential medium supplemented with 5% fetal calf serum, 100 U of penicillin G, and 100 mg of streptomycin sulfate per ml. 1-3 days later, confluent monolayers were inoculated with the SSPE virus at a multiplicity of

infection of approximately one virus per cell. The virus was allowed to adsorb for 1 h at 37°C. The cultures were subsequently refed with a 2% fetal calf serum maintenance medium and kept at 37°C in an incubator with 5% CO₂ in air. Cells were fixed at 1, 2, or 3 days postinoculation. Mycoplasma detection tests on the cells used in this study were conducted at regular intervals and were all negative (15).

Freeze-Fracturing

Cells were fixed *in situ* with 1.25% purified glutaraldehyde and 1% paraformaldehyde in 0.08 M cacodylate buffer at pH 7.2 for 30 min (16, 25). The cells were then scraped off and spun down at 3,000 rpm for 30 min, onto a cushion of agar. The resulting flat sheet of cells was fixed again in 4% glutaraldehyde for 1 h, rinsed in buffer, progressively equilibrated with 25% glycerol in water, and finally cut into small squares. These were placed on gold alloy disks, rapidly frozen in monochlorodifluoromethane (Freon 22) at -146°C, and stored in liquid nitrogen. They were later fractured at -118°C in a Balzers 360 M apparatus equipped with an electron beam gun for platinum shadowing.

Immunoperoxidase Labeling of the Cell Outer Surface

Hemocyanin has been used earlier as an immunolabel for surface replicas (6, 31) whereas ferritin coupled to antibody has been used in immunofreeze-etching experiments (4, 23, 32, 33, 39, 44). However, in the present study, peroxidase-labeled antibody was used as a label in surface replicas and deep-etching experiments because of a previous familiarity with the distribution of this label in thin sections (14). In replicas, the product of the HRP reaction was easily identified as granules of various size, depending on the length of the enzymatic incubation and the amount of peroxidatic activity. High resolution of these sites could thus be achieved with high dilutions of conjugate and short incubation times.

All immunolabeling experiments were done after fixation because it is known that measles antibody can induce redistribution of surface antigens in living cells (24). Cells were rinsed several times with serum-free medium and fixed *in situ* with 1% purified glutaraldehyde in 0.1 M cacodylate buffer for 30 min. After several rinses, they were incubated for 2 h in appropriate dilutions of SSPE antibody labeled with HRP (14). This reagent typically contained 0.025 mg globulin per ml. The conjugate was prepared by Cappel Laboratories, Downingtown, Pa., using glutaraldehyde (2) to couple peroxidase with globulin from an SSPE patient showing viral titers of 1:512 or more. At the end of the incubation, cells were washed in buffer overnight and then incubated in a medium to localize peroxidatic activity (37). This medium contained 2.5 mg diaminobenzidine (DAB) in 10 ml of 0.05 M Tris HCl buffer at pH 7.6 and the incubation time was 7 min at room temperature, or 15

min at 4°C. Controls for specificity were: (a) uninfected cells treated exactly as above, (b) infected cells incubated in peroxidase labeled antibody but not incubated for peroxidatic activity, (c) infected cells incubated for peroxidatic activity. No controls showed any reaction product.

Surface Replicas

Cells were grown, fixed, and incubated on glass cover slips 12 mm in diameter. Cells not used for immunohistochemistry were postfixated in 1% osmium in 0.1 M cacodylate buffer. Osmium treatment was omitted for cells which were treated with labeled antibody and incubated for peroxidatic activity because it produced granularity difficult to distinguish from the specific label. Cover slips were then quickly passed through graded alcohols and air dried (42). A few preparations were critical-point dried in carbon dioxide (1), a procedure which permits very fine details on the membrane surface to be visualized. Cover slips were then fitted on the top of the Balzers specimen stage, and a platinum replica of the cell surface was made at -100°C in vacuum better than 10^{-6} Torr.

Deep Etching

Fixed cells were scraped from the Falcon flask so that all the incubations and washes were done on floating cells. At the end of the labeling procedure, cells were spun down 20 min at 3,000 rpm in 4% glutaraldehyde in order to obtain a tightly packed pellet which was progressively equilibrated with distilled water. The pellet of cells was then rapidly frozen with a Van Harrevel apparatus in order to avoid ice crystal formation (26, 45). In optimal preparations, good freezing was obtained in the first 20 μm at the surface of the pellet. After fracturing this superficial layer of well frozen cells, the preparation was etched for 1.5-3 min at -100°C before replication.

All replicas were cleaned in methanol and Clorox and mounted on Formvar- and carbon-coated grids. Digestion of the glass cover slip with hydrofluoric acid was avoided since it was found to alter the fine structure of the surface replica. Positive photographic prints were mounted with the origin of the platinum shadowing below or from the right unless otherwise stated.

Thin Sections

Cells treated in a manner identical to that used for freeze fracture, surface replica, or deep-etching experiments were postfixated in 1% osmium for 1 h, stained with uranyl acetate at pH 5, dehydrated in graded alcohols, and embedded in Epon. In some instances, serial sections were cut and picked up on Formvar-coated slot grids. Some grids were tilted up to 45° using the eucentric goniometer stage on a Phillips 201 electron microscope.

RESULTS

Early Stage

Specific changes were observed in the membrane of infected cells 24 h after inoculation. At that stage cell fusion was just beginning. In both replicas and thin sections, viral buds and complete virus were virtually absent, which suggests that the scattered infected cells had not yet achieved the first cycle of viral replication.

Surface replicas permitted examination of large expanses of the portion of the plasma membrane of these cells which faced the incubation medium. Uninfected cells had a rather smooth surface bearing numerous small processes which were elongated, bifid, or club shaped (Fig. 1). These villous processes actually projected from the cell surface but were flattened against the cell surface by air drying. In the infected monolayer, the outer surface of the plasmalemma of a few cells was embossed by groups of narrow serpentine strands 25-nm wide (Fig. 2, Fig. 4). They were not observed on villous processes which were identical to those seen in uninfected cells (Fig. 4). In critical-point dried preparations, these strands were found to be made of granular subunits on the cell surface (Fig. 8). Because these surface alterations were not seen in uninfected cells they were thought to be membrane changes induced by SSPE virus.

Similar numbers of cells from inoculated preparations were labeled by the immunoperoxidase technique (Fig. 3). The distribution of this label on the cell surface was similar to the distribution of strands seen in unlabeled preparations (Fig. 2). Outside of these labeled areas, no groups of strands could be identified throughout a careful search, so it seemed likely that the patches of label covered patches of strands. The label consisted of granules of various size which were assumed to be the result of reaction catalysed by peroxidase coupled to the antibody molecule, because none were found after exposure of infected cells to antibody or substrate alone. Thus, the antibody molecule itself was not detectable by the surface-replica technique, at least without critical-point drying, but its site of attachment was visualized after the HRP coupled to it interacted with the substrate. Although most of the granules of label appeared in a diffuse nonorganized pattern at this early stage of infection, small DAB granules sometimes were organized in narrow stripes (Fig. 3, arrow), suggesting that part of the viral antigen

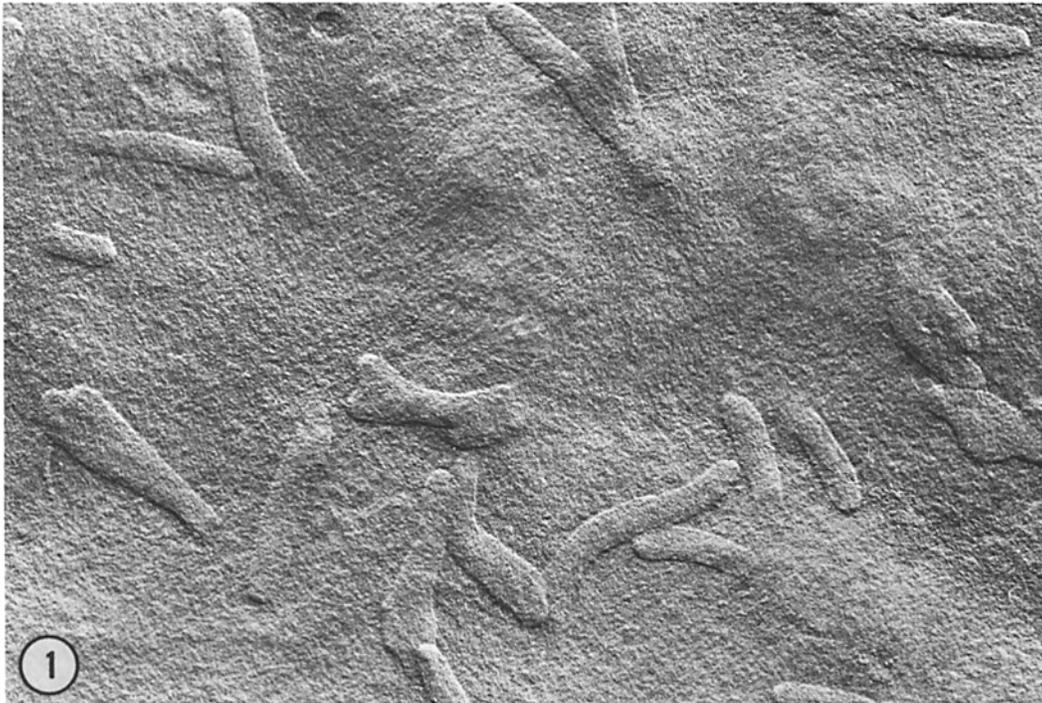


FIGURE 1 Surface replica of a portion of an uninfected cell. Cellular extensions are elongated, bifid, or club shaped. They have been flattened onto the surface by the air drying process. $\times 30,000$.

was already associated with strands. It remains possible that some reaction product diffuses from antigenic sites to adjacent sites, which would contribute to the diffuse labeling.

Both diffuse and patterned distributions of immunoperoxidase label have been observed in thin sections (14). Infected cells were identified by the presence of small groups of nucleocapsids in the cytoplasm, sometimes close to the plasmalemma. Labeling of the cell membrane was related to nucleocapsids in some places, but usually no nucleocapsids were identified under patches of membrane label.

Late Stage

All stages of virus maturation were observed at 2 and 3 days after inoculation. After 1 day, cell fusion had begun and by three days the whole monolayer was covered with multinucleated giant cells. In replicas and thin sections, various plasmalemmal changes and viral budding formations identified on giant cells were pieced together into a developmental sequence.

CELL MEMBRANE CHANGES: In surface replicas, an increase in the number and width of

the strands was observed in the infected cell membranes on the second day after inoculation (Figs. 4, 5, and 6). The spacing between the strands, however, remained constant (Table I) (Figs. 8 and 9). Both narrow and large strands were made of the same granular subunits visualized after critical point drying (Figs. 8 and 9). Wider strands assumed more convoluted shapes (Figs. 6, 9, and 17). In labeled preparations, viral antigen was observed over extensive areas of the cell surface and was often organized in stripes (Fig. 7). In thin sections through similar cells, the stripes of label appeared to lie on top of nucleocapsids apposed to the inside aspect of the plasma membrane (14), (Fig. 7, inset). In unlabeled preparations, the coincidence of stripes with underlying nucleocapsids was shown to be constant by tilting sections up to 45° to reveal nucleocapsids obscured by oblique planes of section. Also, the spacing between nucleocapsids was found not to differ significantly from the spacing of the strands seen in surface replica (Table I).

When infected cells were freeze-fractured 3 days after inoculation, serpentine ridges were identified on the inner half of the membrane which were devoid of the 8–13 nm particles present on the rest

of the membrane (Fig. 10 A). Furrows complementary to these ridges were identified on the outer half of the membrane (Fig. 10 B). The crests of ridges and the bottoms of furrows were marked by a very fine particulate material (Figs. 13 and 14). The shape, size, and spacing of the ridges seen in freeze-fracture seemed to correspond to some of

the wider type of twisted strands seen in surface replicas. Occasionally, twisted ridges established contact with each other and appeared to fuse (Fig. 12). In some instances, aggregates of the small-sized particles were found on the inner half of the membrane at places where bulges or ridges were absent (Fig. 11). These particle aggregates were

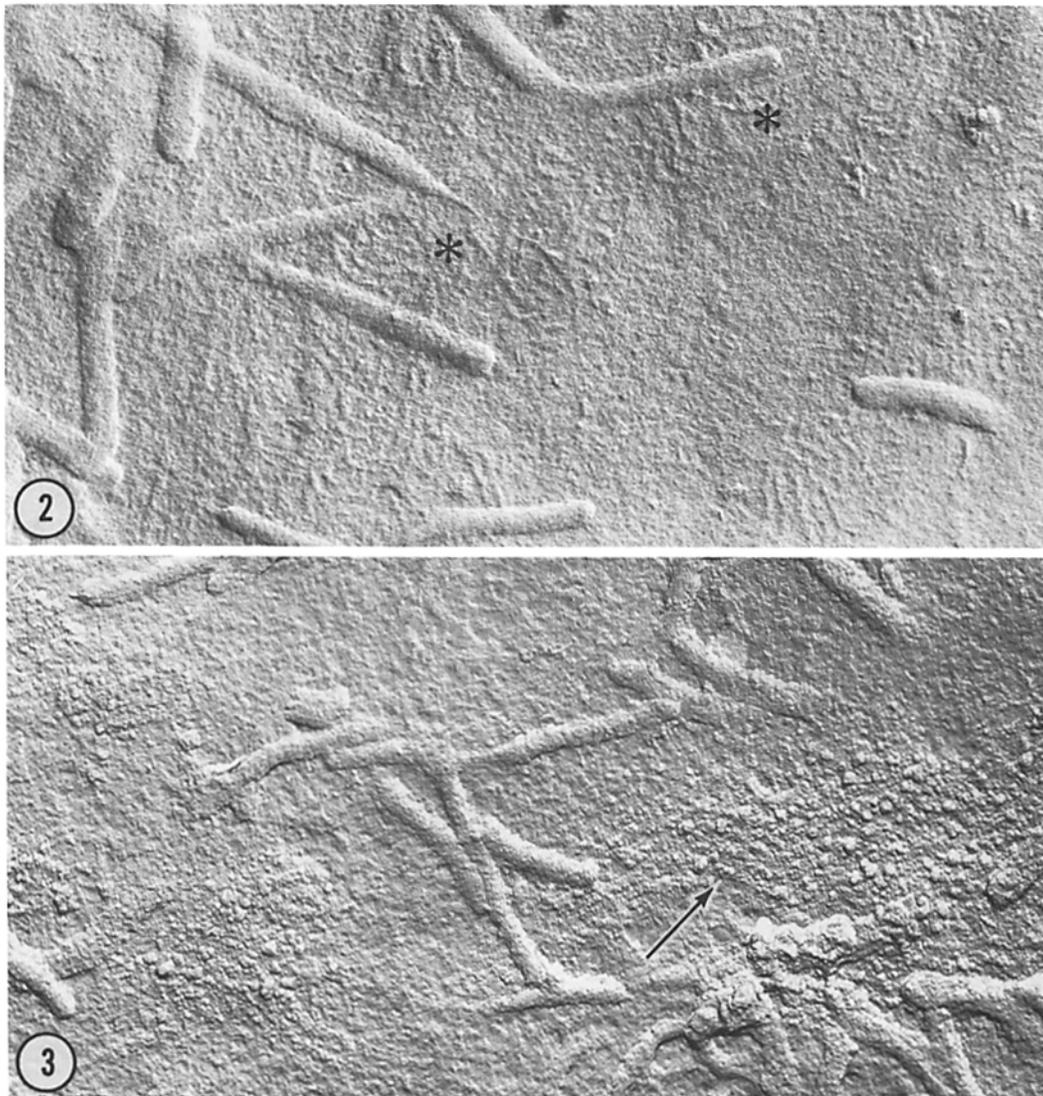


FIGURE 2 Surface replica of a portion of the plasmalemma of an infected cell 1 day after inoculation with SSPE virus. Circular or confluent regions of some cells are now embossed by twisted strands of granular material (asterisks). Cellular extensions are unaffected by these changes. $\times 30,000$.

FIGURE 3 Surface replica of an infected cell labeled by the immunoperoxidase technique 1 day after inoculation. Regions marked by granular reaction product replace the areas with granular strands seen in Fig. 2. The granules vary in size and where they are small, they are sometimes arranged in narrow stripes (arrow, right). No strands are visible. $\times 30,000$.

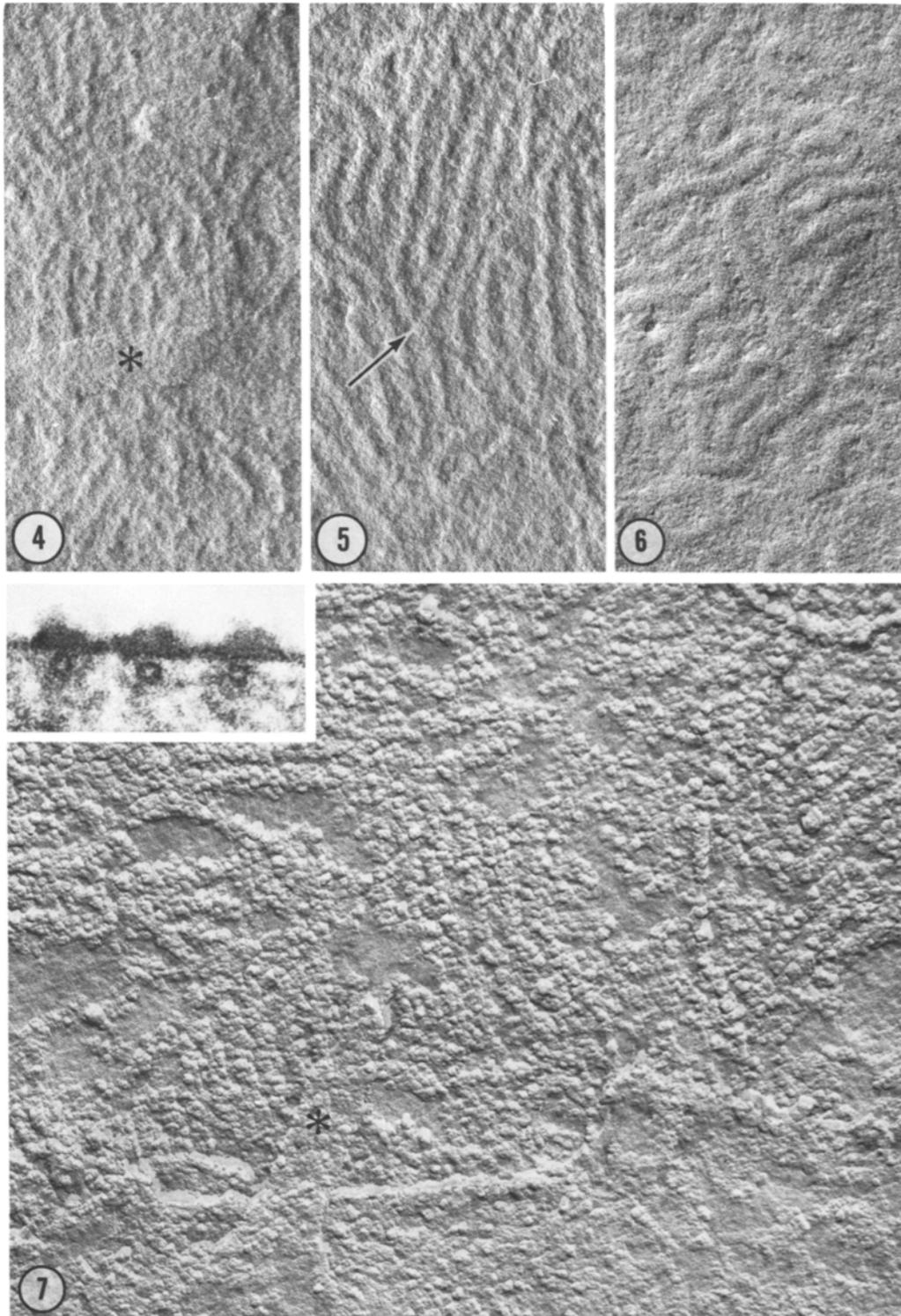


TABLE I
Spacing between Strands and between Nucleocapsids at Different Times after Virus Inoculation

Spacing	Cell membrane		Viral bud formation (2-3 days)
	1 day	2-3 days	
Strands	551 ± 71*	573 ± 75	414 ± 57‡
Nucleocapsids	563 ± 80	668 ± 78	463 ± 99‡

* Standard Deviation.

‡ Differs from cell membrane spacing at 2-3 days, $P < 0.01$.

thought to be subtle changes preceding the formation of a ridge.

Ridges were also recognized in thin sections by the fact that the plasma membrane covering the nucleocapsid protruded above the adjacent membrane (Fig. 10, inset). However, we wish to stress that there were too few ridges for them to be present at every point of nucleocapsid alignment with the cell membrane.

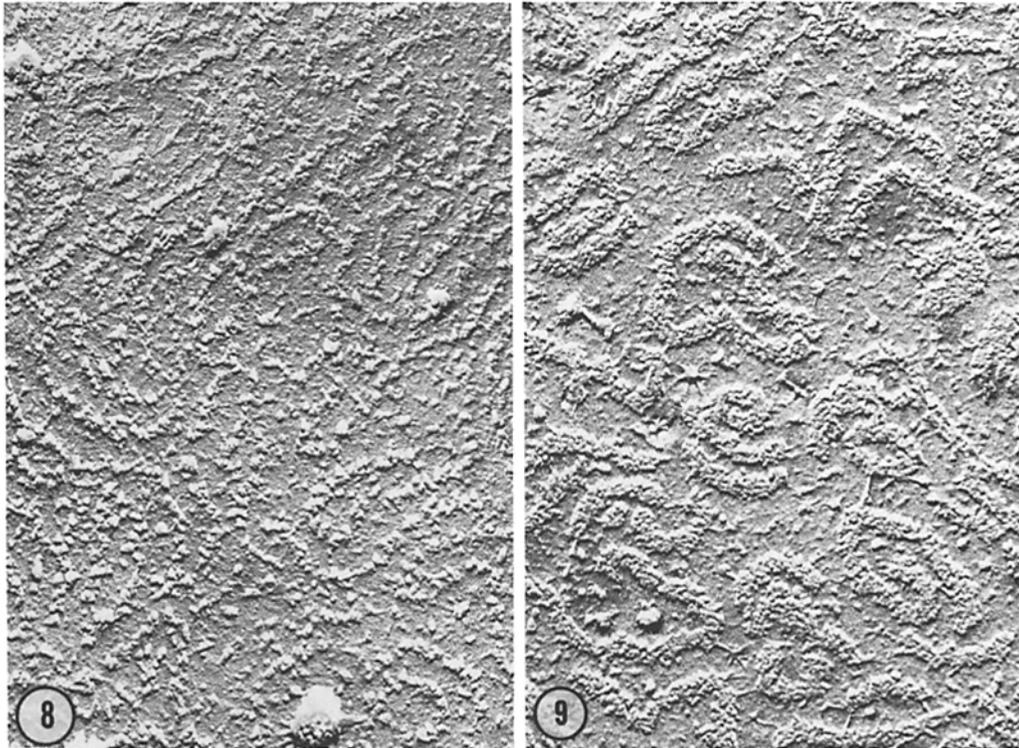
Ridges were easily recognized on fractured membranes in freeze-etching preparations immunolabeled with HRP (Fig. 15). Part of the true outer surface was uncovered by the etching process so that it could be determined that the label concentrated in stripes on the membrane surface was in register with the ridges on the inner half of the membrane (Fig. 15). As in surface replicas, the HRP reaction product appeared as granules of various sizes. In nonincubated preparations, as well as in preparations incubated in the conjugate or in the DAB substrate alone, discrete patches of finely granular material distinct from HRP reaction product were identified on the membrane surface revealed by etching and this material was thought to correspond to surface projections.

VIRAL BUD FORMATION: In surface replicas, numerous round excrescences up to 300 nm in diameter and covered with twisted strands were present in some areas of the membrane, particularly where it was covered with the wider type of strand (Figs. 16 and 17). These excrescences were single or complex, branching, and varicose in shape (Fig. 16). Some were as long as 3 μ m, whereas others consisted of a series of connected spheres of various sizes which might be viral particles ready to be pinched off (Fig. 16). Because of their size and shape, these excrescences were thought to correspond to the viral bud formations seen in thin sections (14). In some instances, their attachment to the cell surface was seen (Fig. 16), but often the attachment was not seen, particularly after critical-point drying (Fig. 17).

Strands were apparent on viral bud formations and after critical-point drying their granular subunits were seen on the viral buds as well as on the adjacent cell membrane (Fig. 17). However, the spacing between strands was smaller on viral bud formations and their necks than on the adjacent membrane (Table I, Figs. 16 and 17). After

FIGURES 4-6 Surface replicas of affected regions of the plasmalemma of cells 1 (Fig. 4) and 2 (Figs. 5 and 6) days after inoculation showing a progressive increase in the width of the strands. The periodicity of the strands, however, remains constant. Strands are absent on an overlying cell process (asterisk, Fig. 4). Strands may be branched (Fig. 5, arrow) or convoluted (Fig. 6). $\times 60,000$.

FIGURE 7 Surface replica of immunoperoxidase labeled cells two days after inoculation. The label consists of granules 15-25 nm across organized into stripes with the same spacing as the strands seen in Figs. 4-6. Between the stripes, the membrane has some diffuse label. An adjacent cell in the lower part of the picture, which extends a process (asterisk) over the heavily labeled region is lightly labeled. Inset shows a thin section through a labeled cell: each patch of label on the surface is lying over a viral tubule or nucleocapsid. $\times 60,000$ Inset, $\times 210,000$.



FIGURES 8-9 Infected cells fixed 2 days after inoculation and prepared by critical-point drying. Strands in different regions vary in width but have in common a particulate substructure. Wider strands consist of more tightly packed particles and assume various convoluted shapes (Fig. 9). $\times 60,000$.

immunolabeling, viral bud formations were covered with a continuous coat of reaction product, a finding which confirms previous conclusions based on examinations of thin sections (14). The spacing between nucleocapsids was also less in viral bud formations (Table I).

The inner and outer halves of the surface membrane of freeze-fractured bud formations were covered with small-sized particles mixed with a few larger particles 8-13 nm in diameter (Fig. 18). Thus, the whole surface of the bud formation revealed by freeze-fracturing resembled the crests of ridges. In this respect, this virus differed from other viruses where the inner half of the limiting membrane has been reported to be devoid of particles (3, 10, 39, 40).

In serial sections of infected cells covered with budding sites, the majority of spherical particles seen in the extracellular space were still attached to viral bud formations by a narrow neck. However,

approximately one-fifth of the spherical particles were close to, but not attached to, bud formations and therefore were considered to be freshly detached viruses. These had nucleocapsids aligned under their surface membrane. Detached viruses were also found in the extracellular space further from the cell. These viral particles resembled the purified virus seen by negative staining (28), because their nucleocapsids were no longer aligned under the viral envelope (Fig. 20).

The fractured membrane of some viruses had unusually large particles (12-15 nm) on both membrane halves (Fig. 19). These viruses were typically distant from viral bud formations and therefore were thought to be detached viruses. Spherical viral structures associated with bud formations did not have these large-sized particles (lower left corner in Fig. 18). After freeze-etching, detached virus, which could tentatively be identified by the presence of large size particles on their

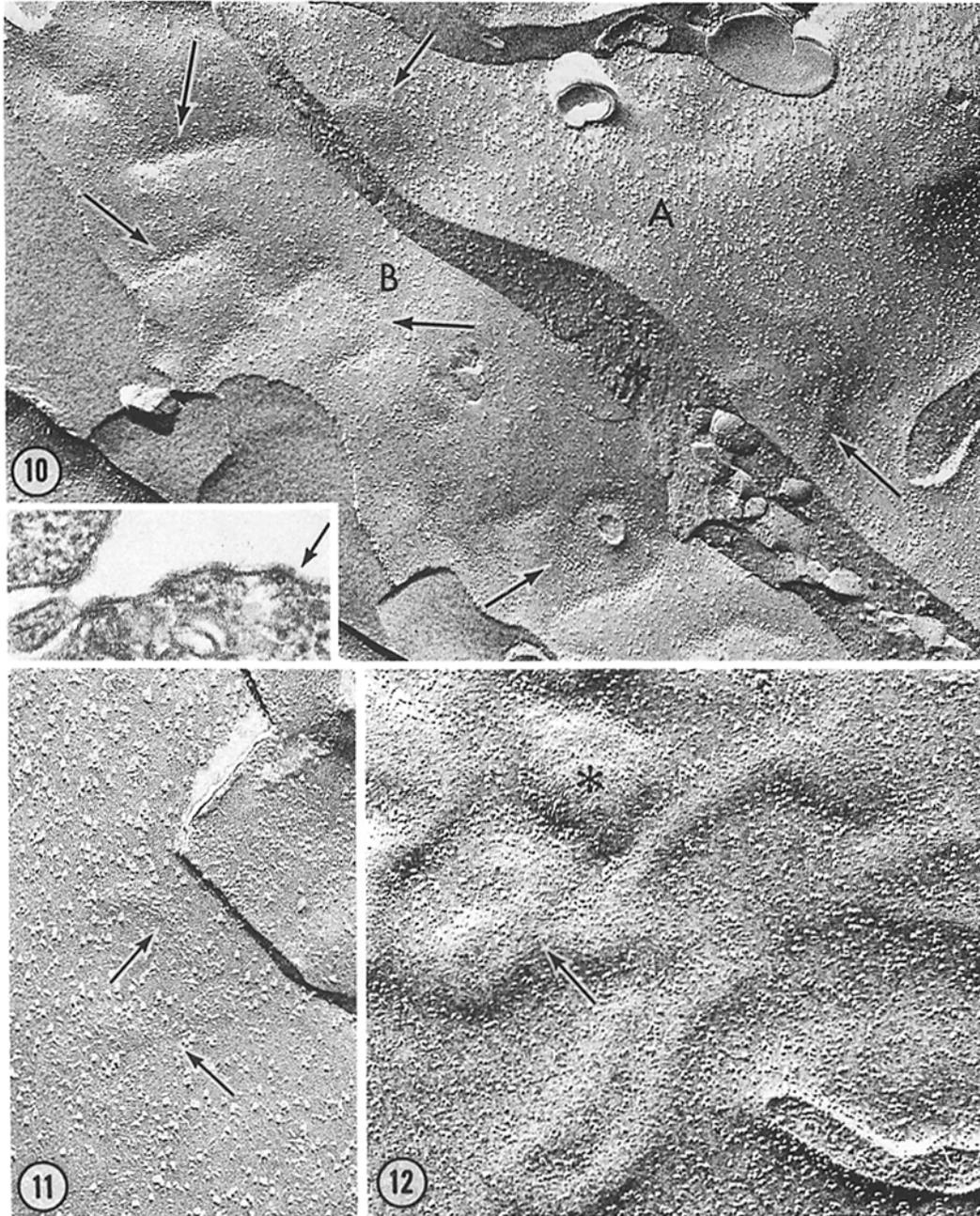


FIGURE 10 Replica of the freeze-fractured plasmalemma of infected cells three days after inoculation. The inner half of a split plasmalemma (A) and the outer half of the plasmalemma of the same cell (B) is separated by a narrow band of cross-fractured cytoplasm (asterisk). Ridges on the inner half of the membrane (arrows, right) appear to be complementary to furrows on the outer half (arrows, left). Details of the distribution of particles in ridges and furrows are shown in Figs. 13 and 14. Inset shows a section through a region of membrane which might produce ridges and furrows when freeze-fractured. The plasma membrane curves around the underlying tubules thereby protruding somewhat above the adjacent membrane (arrow). Each protrusion is crowned with tightly packed surface projections. $\times 50,000$, Inset, $\times 80,000$.

FIGURE 11 Inner half of the membrane of infected cells at 3 days after inoculation. Small intramembranous particles are grouped in a strand devoid of 8-13 nm particles (arrows) but no ridge is present. Similar small particles are found on the outer half of the adjacent membrane at right $\times 74,000$.

FIGURE 12 Spherical bulges in membrane (asterisk) which in some instances may result from fusion of ridges (arrow). These bulges may be an early stage in the development of viral bud formations. Like ridges, the surfaces of these bulges have few of the 8-13 nm particles found over the adjacent membrane. $\times 60,000$.

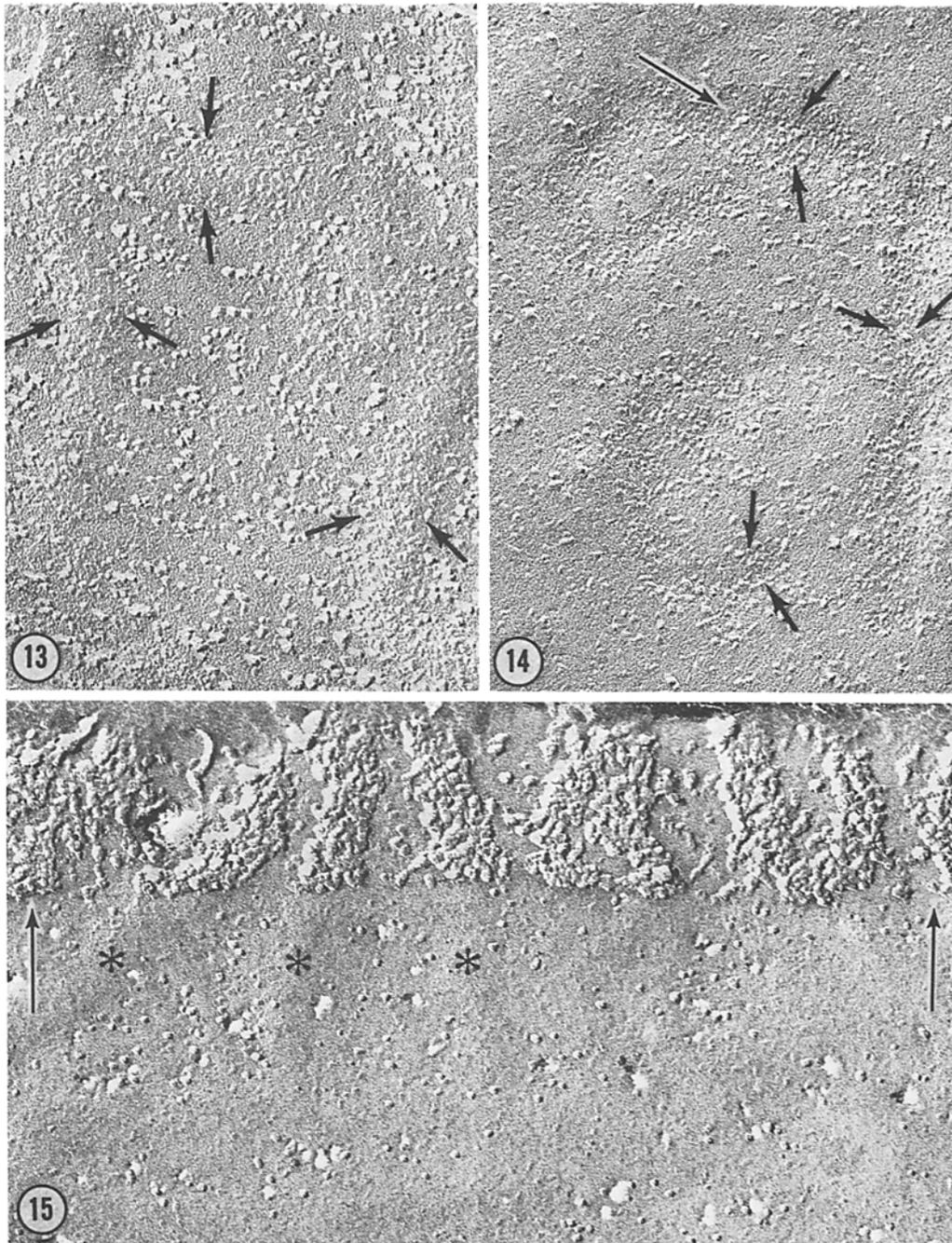


FIGURE 13 Inner half of plasmalemma of infected cell fractured along a ridge. The ridge is R shaped and its limits are indicated by pairs of arrows on its three sides. The ridge is almost devoid of particles within the range of sizes found over the rest of the plasmalemma (8-13 nm). The crest of the ridge has a granular texture due to the presence of many smaller particles. $\times 120,000$.

FIGURE 14 Outer half of plasmalemma of infected cell fractured along a ridge which, from this point of view is a furrow and has the shape of a reversed letter C. The limits of this ridge are indicated by three pairs of black arrows. The membrane at the crest of this ridge is marked by a granular texture due to the presence of many small particles and a few pits (black and white arrow). $\times 120,000$.

FIGURE 15 Immunoperoxidase labeled plasmalemma of an infected cell which was deep-etched as well as freeze-fractured in order to compare the internal structure of the plasmalemma (below) with the distribution of label on the true outer surface of the membrane (above). Arrows mark the boundary between the freeze-fractured membrane and the membrane surface revealed by etching. Ridges characteristically devoid of large intramembranous particles (asterisks) are continuous with stripes of granular reaction product on the membrane surface. The fine granularity on the tops of the ridges (shown in Fig. 13) was not resolved in this etched material. $\times 120,000$.

fractured surfaces, had a continuous layer of label on the outer surface of the membrane uncovered by the etching procedure (Fig. 20). This distribution was identical to the coat of reaction product observed in thin sections through viruses with detached nucleocapsids (Fig. 20) (14).

DISCUSSION

The development and maturation of SSPE virus, a measles variant, has been studied with surface replication, freeze-fracturing, and freeze-etching techniques. This approach has revealed fine structural changes in cell and viral membranes that would be difficult to appreciate in thin sections of embedded cells. By integrating the new observations provided by these replication techniques with data obtained from thin sections (14, 35, 36), it is possible to elaborate the sequence of structural changes in the cell membrane during maturation of SSPE virus.

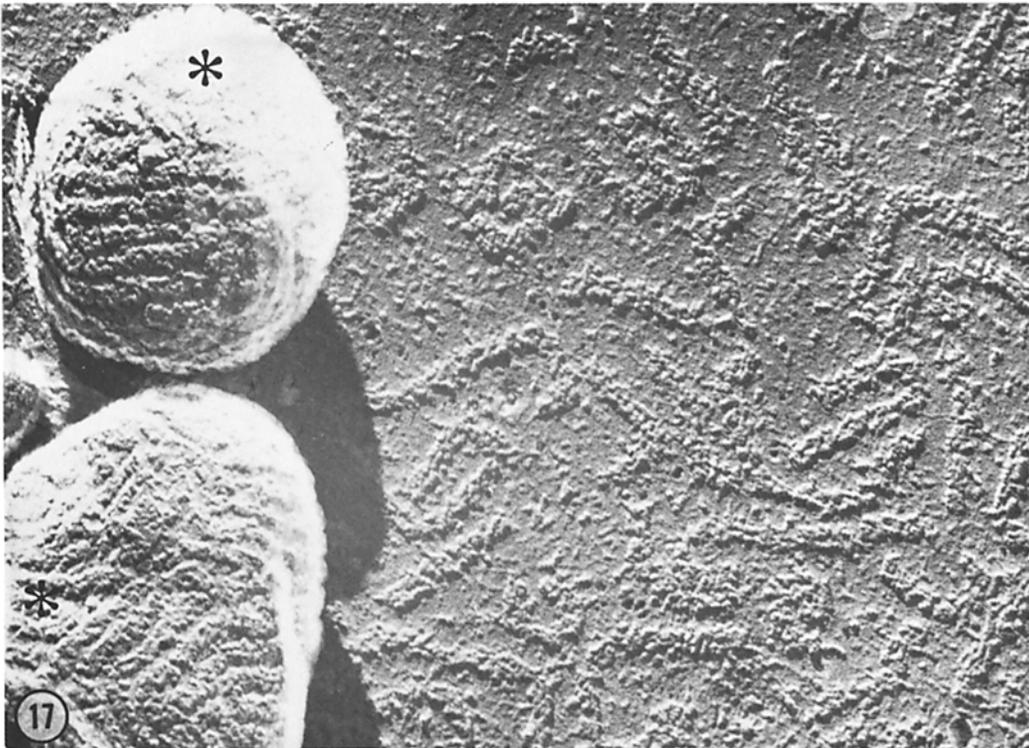
Most of the information on early stages of viral maturation was obtained with the surface replica technique which detects subtle changes in the cell surface. Circular regions of the surface of infected cells are covered with granular material which is either diffuse or organized into strands. Because the distribution of immunolabel over the regions marked by strands was diffuse, it was thought that there are diffuse as well as strand-related antigenic sites at this stage. By the comparison with data from thin sections taken at these early stages (14), it appears that alignment of nucleocapsids occurs in those regions of the plasma membrane where strands are being formed.

During the period of strand formation, cells fuse producing giant cells with large areas of their plasma membrane covered with serpentine strands. At this stage, strands are found in continuously graded widths which suggests that new viral components are continuously added to their edges. Most of the antigenic sites are now organized into serpentine stripes although regions of diffuse antigen are still present. The stripes are thought to overlay strands because their distributions are the same and no unlabeled strands are found in labeled preparations. Since the earliest localization of viral antigens is in diffuse patches, it is likely that the stripes are a later stage produced by mobility of membrane-associated antigens. Study of stripes in thin sections makes it clear that they consist of groups of labeled surface projections and that twisted nucleocapsids are aligned under them.

Another characteristic membrane change in infected cells is the formation of serpentine membrane ridges and spherical bulges over nucleocapsids, a change which can be seen in thin sections. The ridges and bulges are also striking in freeze-fracturing preparations, although no changes indicative of the earlier stages of nucleocapsid alignment or strand formation were recognized with this technique. Both the inner and outer halves of the fractured plasma membrane over ridges and bulges contain a high concentration of small membrane particles, but few particles of ordinary size. Freeze-etching and surface replication of labeled cells clearly demonstrated that ridges have stripes on their outer surface, although all stripes are not on ridges. Thus, the particles cannot be attachment sites of surface projections, although they might correspond to a later stage of attachment of nucleocapsids to the plasma membrane (38) which is instrumental in deforming it to form viral buds. Further changes, discussed below, support this conclusion.

Formation of viral buds requires continuation of the membrane deformations begun at bulges and perhaps ridges. However, additional changes in membrane organization are necessary. The spacing between twisted strands decreases so that the true outer surface of buds and freshly detached virus is covered with a continuous layer of antigen. Simultaneously, small particles inside the membrane become continuous over the aspects of viral bud membranes revealed by freeze-fracturing. Finally, twisted nucleocapsids under the membrane move closer together to coil inside the round viral bud (18). The fact that strands, nucleocapsids, and ridges have an identical twisted shape and move closer together simultaneously suggests that these structures form complexes which can move in the plane of the cell membrane. This movement could occur if the membrane matrix were sufficiently fluid (41).

The last steps in viral maturation involve further changes in the organization of membrane particles. The small particles are replaced by unusually large particles inside the viral membrane after nucleocapsids lose their membrane attachment. The fact that both the small particles and the nucleocapsid attachment to the plasma membrane disappear as soon as viral assembly is completed is further evidence that the small membrane particles are related to nucleocapsid attachment.



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FIGURES 16-17 Viral bud formations are seen 2 days after inoculation in surface replicas after air drying (Fig. 16) or critical-point drying (Fig. 17). Excrescences, thought to be the region of viral budding (asterisks) are round or varicose in shape and sometimes appear to be formed by a series of connected spheres of various sizes (Fig. 16). Critical-point drying preserves the three-dimensional shapes of these structures. Strands on the surrounding cell surface are reflected onto the surfaces of these excrescences at their attachment with the plasmalemma (lower arrow, Fig. 16). The surface of viral bud formations is marked by strands which are more narrowly spaced than those on the surrounding membrane. Strands on membranes and the viral bud formations are twisted and are composed of packed granular subunits which are clearly seen after critical-point drying. Fig. 17 is a negative image so that platinum deposits are white. Fig. 16, $\times 45,000$; Fig. 17, $\times 69,000$.

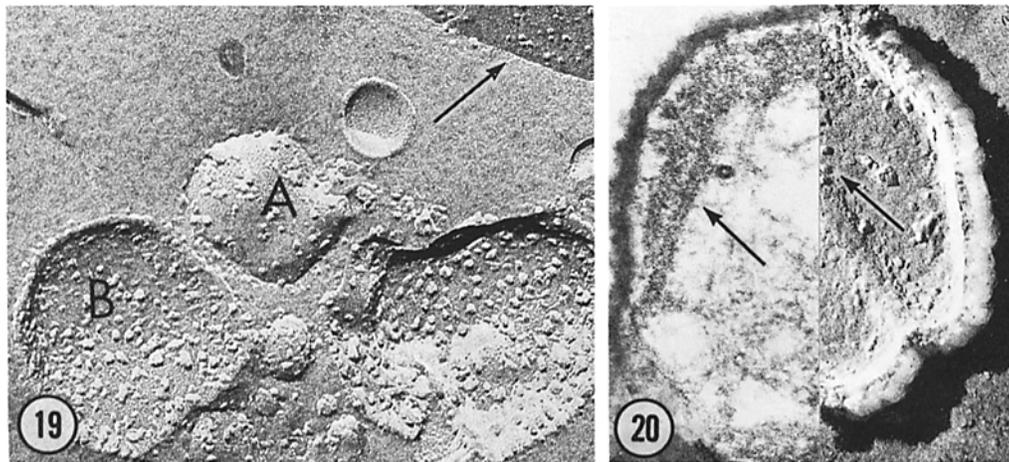
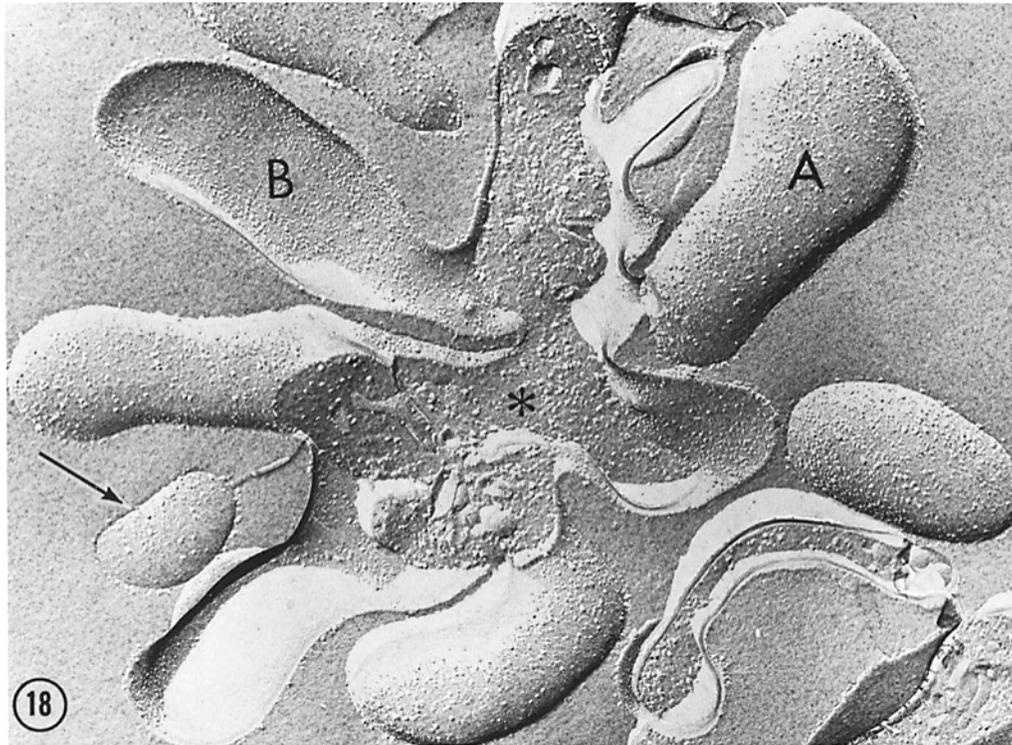


FIGURE 18 Freeze-fracture replica of budding region, such as that shown in Fig. 19. The inner (A) and outer (B) half of the plasma membrane is characterized by a continuous sheet of small particles, like those found at the crest of the ridges (Figs. 13 and 14), as well as some larger ones. The cytoplasm of this viral bud formation is cross-fractured at asterisk. Spherical regions of membrane, such as that at arrow, may be freshly detached virus or simply a fracture passing through the tip of a viral bud. This and all subsequent figures are from preparations 3 days after inoculation. $\times 60,000$.

FIGURE 19 Replica of freeze-fractured virus showing large particles on both inner (A) and outer (B) halves of the membrane. A portion of fractured plasmalemma at upper right (arrow) allows comparison of its smaller particles with the large particles associated with the viruses. $\times 90,000$.

FIGURE 20 Composite of immunoperoxidase-treated viral particles, thin sectioned (left) and freeze-etched (right). Mature viruses are characterized by detached tubules (arrow) and by very large intramembranous particles (arrow). An unbroken, dense layer of reaction product surrounds both viral particles. In the freeze-etch picture platinum deposits are white $\times 90,000$.

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