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Intramembrane Changes Occurring During Maturation of Herpes Simplex Virus Type 1: Freeze-Fracture Study

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During the maturation of two strains of herpes simplex virus type 1 (VR3 and Patton), intramembrane changes were detected with the freeze-fracture technique in the viral envelope and the infected cell plasma membrane, and these changes were compared with data obtained from thin sections. Regardless of the strain, the inner leaflet of the viral envelope of extracellular virions was characterized by a density of intramembrane particles (IMP) three times larger than the host nuclear and plasma membrane. Addition of IMP, which probably represent virus-coded proteins, was detected in the viral envelope only after budding from the nuclear membrane, whereas it occurred during envelopment of capsids at cytoplasmic vacuoles. Fused membranes also showed one of their fracture faces covered with a high density of IMP similar to that of the mature virion envelope. The internal side of the membrane leaflet bearing these numerous particles was always characterized by the presence of an electron-dense material in thin sections. In addition, the plasma membrane of fibroblasts and Vero cells showed strain-specific changes: patches of closely packed IMP were observed with the VR3 strain, whereas ridges almost devoid of IMP characterized the plasmalemma of cells infected with the Patton strain. These intramembrane changes, however, were not observed as early as herpes membrane antigens. Thus, application of the freeze-fracture technique to herpes simplex virus type 1-infected cells revealed striking structural differences between viral and uninfected cell membranes. These differences are probably related to insertion and clustering of virus-coded proteins in the hydrophobic part of the membrane bilayer.

Herpes simplex virus type 1 (HSV-1) is a DNA virus and a member of the *Herpesvirus* genus. The capsids usually acquire their envelope at the inner nuclear membrane or sometimes at cytoplasmic membranes. The enveloped virion contains at least 24 specific proteins, suggesting that assembly is a complex process (31). Once the capsid has been assembled in the nucleus, some membrane changes can be seen in thin sections examined with the electron microscope (6, 15, 18, 19, 21, 22, 28-32, 35). Nucleocapsids are seen close to protruding regions of the inner nuclear membrane which has a thin layer of electron-dense material under its inner leaflet and will form the envelope of the virus. The enveloped virus travels within a vacuole from the perinuclear space to the cell surface, where it is released into the extracellular space by reverse phagocytosis (22, 28, 31). The envelope of the extracellular virions shows a thick layer of electron-dense material on its inside and spikes on its surface. In some cell systems, the plasmalemma of infected cells has patches of electron-dense fuzz apposed to the inner leaflet of the membrane and similar to that observed on the viral envelope (31).

Biochemical studies have provided evidence that the virus buds through newly synthesized regions of the nuclear membrane containing virus-specific proteins and glycoproteins (5). In addition, new antigens that are identical to those of the viral envelopes have been detected on the plasma membrane of infected cells (24, 25). To explore whether these biochemical events result in structural changes in the membranes, the freeze-fracture (FF) technique was applied to HSV-1-infected cells. This technique splits cellular and viral membranes between the two leaflets and reveals intramembrane particles (IMP) thought to correspond to proteins and not detectable in thin sections (e.g., 3, 34). Various stages of herpesvirus maturation were recognized in FF replicas, and the structure of nuclear, cytoplasmic, and viral membranes were examined. Intramembrane alterations were detected in several viral structures and thought to be related to the insertion of virus-coded proteins inside the membrane. In addition, two different

viral strains of HSV-1 induced specific modifications of the plasma membrane.

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MATERIALS AND METHODS

Virus and cells. The Patton (14) and McIntyre (VR3) (16, 21) strains of HSV-1 were used in this study. Human embryo foreskin fibroblasts were obtained commercially from Flow Laboratories, Inc. (Rockville, Md.). Vero cells were obtained from the American Tissue Type Collection (Rockville, Md.). Cells were grown in a 5% CO_2 atmosphere at 37°C until they were confluent. The growth media was Eagle minimum essential medium supplemented with 10% heat-inactivated fetal calf serum, 100 U of penicillin G, and 100 μg of streptomycin sulfate per ml. Both strains of HSV-1 were used after two passages in human fibroblasts. Pools of virus were obtained from cells showing intense cytopathic effect. Cells were frozen and thawed three times. The fluids containing released and cell-associated virus were clarified by low-speed centrifugation and stored at −70°C. The viral titers of these pools, as measured by plaque assay (11), were 3.5×10^7 PFU/ml for the Patton strain and 2×10^6 PFU/ml for the VR3 strain. Both viral pools and cells were free of mycoplasma as tested by methods described elsewhere (27).

Infection of cell monolayers. Stationary monolayers grown in 75-cm^2 Falcon flasks (Falcon Plastics, Division of Bioquest, Oxnard, Calif.) were infected at a multiplicity of 10 PFU/cell in the case of the Patton strain and 0.3 PFU/cell in the case of the VR3 strain. The virus was adsorbed to the cells for 1 h at 4°C on a rocking machine and subsequently incubated for 30 min at 37°C to allow viral penetration.

The nonabsorbed viruses were removed, and cells were refed with minimum essential medium containing antibiotics and 2% fetal calf serum. Overlying fluids of cells infected with the Patton strain were taken at 4, 8, 12, 16, 21, 24, and 36 h postinfection (p.i.) to establish a viral growth curve. These samples were titered by the same methods as the viral pools.

Immunofluorescent staining. Cell surface and cytoplasmic-specific antigens were stained by indirect immunofluorescence techniques (20, 26) in cells infected from 4 to 48 h with the VR3 viral strain. Human hyperimmune serum with an anti-HSV-1 antibody titer of 1:1,024 by indirect hemagglutination test (17) was used and compared to human sera with no detectable herpes antibody. Anti-human immunoglobulin G coupled to fluorescein-isothiocynate was obtained commercially (Progressive Laboratory, Baltimore, Md.).

Electron microscopy. Viral maturation of two strains of HSV-1 was studied in human fibroblasts. The cells were prepared for thin section and FF study at intervals of 4, 8, 12, 16, 24, 36, and 48 h p.i. with each strain used. Also, a few experiments were done with Vero cells to determine whether some membrane changes were related to the host cell or to the viral strain used. For this purpose, cells were fixed when showing intense cytopathic effect. This happened after 21 h p.i. with the VR3 strain and after 36 h p.i. with the Patton strain. The monolayers were fixed in situ for 45 min at 4°C with a mixture of 1.25% glutaraldehyde and 1% paraformaldehyde in 0.06 M cacodylate buffer at pH 7.2 (13). The latter fixative was then replaced by a solution of 4% glutaraldehyde, and the cells were detached from the culture surface with a rubber policeman. The cells were divided into two aliquots and spun down in fixative at 2,000 rpm for 30 min. Cells to be processed later for thin sections were pelleted in conical centrifuge tubes, whereas those for the FF study were spun down onto a cushion of agar. Further treatment of the cells to obtain thin sections and FF replicas was described in detail previously (13). Briefly, cells to be thin sectioned were postfixed in osmic acid, dehydrated in alcohol, and embedded in Epon. Standard and serial thin sections were cut with a diamond knife and stained with uranyl acetate and lead citrate. Cells to be freeze-fractured were equilibrated with 25% glycerol in water, frozen in Freon 22, stored in liquid nitrogen, and freeze-fractured at −118°C in a Balzers 360 M apparatus. Electron micrographs were taken with a Phillips 201 electron microscope. The measurements of structures observed in those micrographs were done with a Hewlett-Packard Digitizer. Student's t test was used for statistical analysis.

Nomenclature. The nomenclature proposed by Branton et al. (7) was adopted to describe the changes observed in cellular membranes. Briefly, the membrane leaflets in contact with the nucleoplasm or cytoplasm were designated P faces (from protoplasmic), whereas those closest to the perinuclear or extracellular space and the vacuolar lumen were designated E faces (from exoplasmic).

RESULTS

Virus growth curve. Using a multiplicity of infection of 10 PFU/cell, all cells were synchronously infected with the Patton strain of HSV-1 (Fig. 1). After infection, the eclipse period of the virus lasted approximately 8 h. Yield of infectious virus increased 3,500-fold between 8 and 21 h p.i. and thereafter remained stationary.

Immunofluorescent staining. Four to 8 h p.i. with the VR3 strain, approximately 10% of the fibroblasts showed punctuate fluorescence on their surface and diffuse staining of the protoplasm. The proportion of these positive cells increased with time, and by 24 h p.i. all cells were positive. Controls were negative.

Electron microscopy. Observations made with the FF technique on viral morphology, viral assembly, and cell membrane changes occurring during HSV-1 infection were analyzed successively and compared to data obtained from thin sections. All of these results are schematized in the last figure (see Fig. 16).

Viral morphology. In thin sections, typical

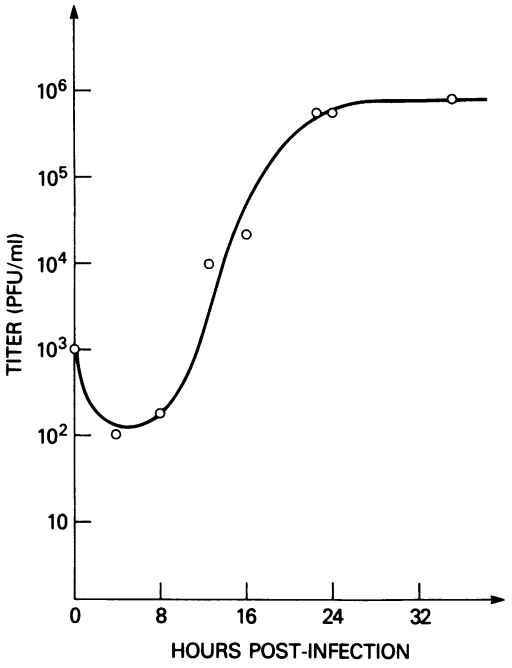


FIG. 1. *Growth curve showing the titers of released virus for the Patton strain of HSV-1 in human fibroblasts. Multiplicity of infection = 10 PFU/cell.*

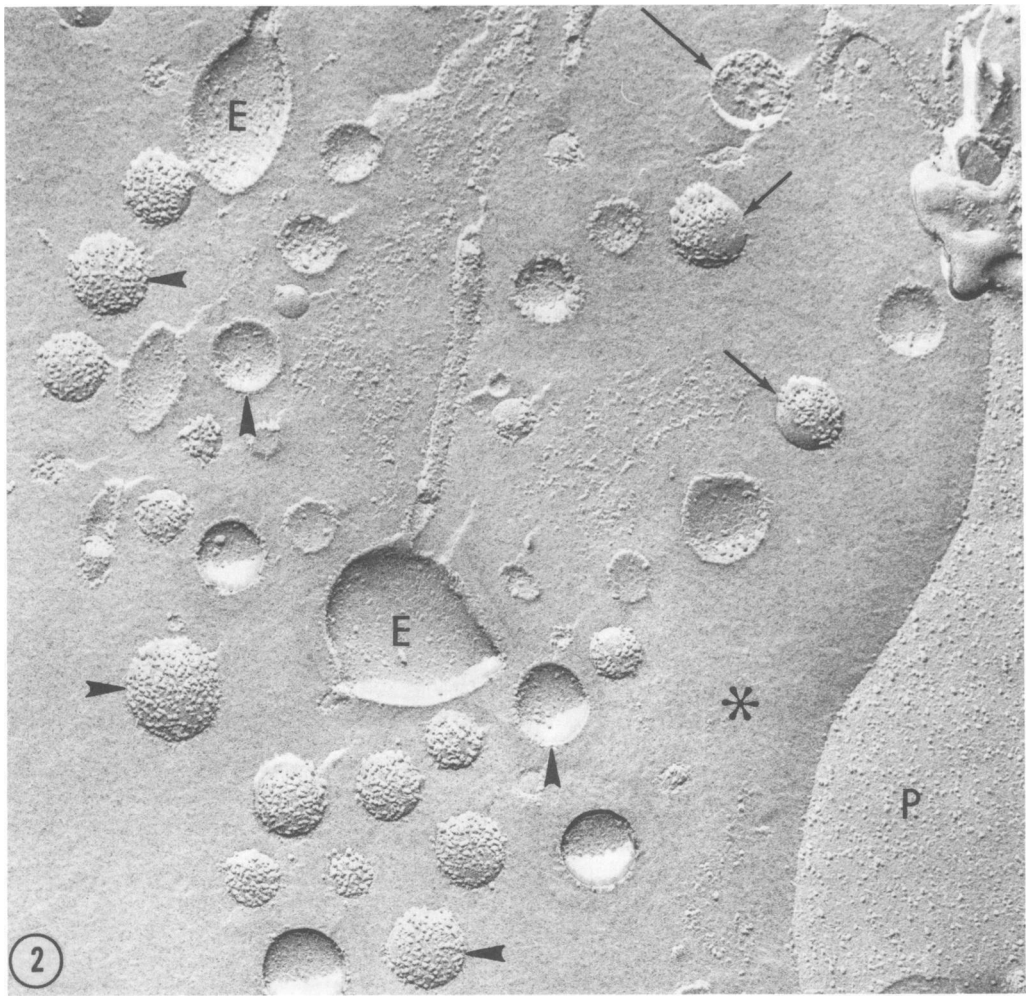
herpesvirus (27) was first seen in the extracellular space at 8 h p.i. with the Patton strain and at 12 h p.i. with the VR3 strain. In the platinum replicas obtained after freeze-fracturing infected cells at 12 to 48 h p.i., hemispherical membranes with mean diameters of 164 ± 12.7 nm (standard deviation) were observed in the extracellular space (Fig. 2). These concave and convex membranes were identified, respectively, as the outer and inner leaflets of free viruses because of their size and shape as well as their absence around uninfected control cells. Histograms of the diameters of these hemispherical structures and those of virions observed in thin sections showed a similar distribution. However, the diameters of virions that had been embedded in Epon were consistently smaller than those that had been frozen in glycerol and fractured (Fig. 4). This difference in size was probably due to the shrinkage of the virus during the dehydration process in cells embedded for thin sectioning.

A striking feature of the inner leaflet of the viral envelope in the FF replicas was the increased number of IMP per square micrometer. The size of IMP on the virion was, however, similar to that of host cell IMP. The mean density of IMP per square micrometer on the inner leaflet was significantly higher than that of the P face of the inner nuclear membrane surrounding budding sites (2,815 ± 673 versus 1,064 ± 217, $P < 0.01$) and than the density in IMP of the P face on control cell plasmalemma (2,815 ± 673 versus 1,021 ± 300, $P < 0.01$). In contrast, the outer leaflet of the viral envelope did not show more IMP than the E face of infected cell plasmalemma (Fig. 2). In cross-fracture, the virions contained a circular arrangement of granules of the size of a capsid as observed in thin sections (Fig. 2).

A number of virions did not have the same structural features all over their envelope. In the FF replicas, IMP were absent on one pole of the inner half of the envelope of some viruses (Fig. 2 and 3d, and see E in Fig. 16). The density of IMP on these inner leaflets was 2,329 ± 870 IMP/μm^2 when areas without IMP were considered in the measurement. On the other hand, the density at the poles covered with IMP was similar to that of virions without a pole. Similarly, in thin sections, one region of the envelope sometimes lacked the dense fuzzy material normally seen on the inside of the viral membrane (18, 28) (Fig. 3a, and see E in Fig. 16). The percentages of virions showing heterogeneity of their envelopes were closely similar both in FF replicas and in thin sections and almost doubled between 12 and 48 h p.i. (Table 1). This suggests that some structural modification of the viral membrane progressively developed after the virus was released from the cell. This modification of the virion envelope might be related to degradatory changes related to aging of the virus.

Viral assembly. In thin sections, viral capsids were first seen in the nucleoplasm at 8 h p.i. with each viral strain used. They were either scattered or in groups, sometimes forming crystalline arrays (27, 28, 31). These capsids were readily identified in the FF replicas of cells infected for 8 h or more (Fig. 5) (23). A cluster of granules frequently present in the center of these capsids probably corresponds to the viral core.

Protrusions of the inner nuclear membrane over a viral capsid were observed in thin sections at 12 and 16 h p.i. in cells infected with the Patton strain and at 16 to 48 h p.i. in cells infected with the VR3 strain. In cells freeze-fractured at similar times p.i., scattered protrusions with a well defined circular base were detected on the P face of the inner nuclear membrane and had a mean diameter of 170 nm ± 37.2 nm (Fig. 6, and see A in Fig. 16). These protrusions were interpreted as viral budding sites because of their size and shape and their absence in control nuclear membranes. Similar numbers of elevations on the E face of the outer nuclear membrane were slightly bigger and had less defined contours than those at the P face (23) (Fig. 6). When the fracture plane jumped from one nuclear membrane to another, it be-



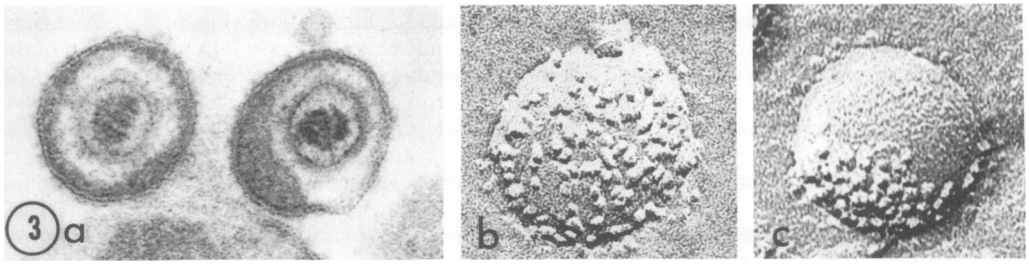


FIG. 2. *FF replica of virions released in the extracellular space (asterisk) at 36 h p.i. with the VR3 strain. This and all subsequent figures are of human fibroblasts. The fracture frequently revealed the inner half of the viral envelopes (horizontal arrowheads) which is covered with IMP in greater density than that on the P face of the adjacent cell membrane. On the right, two virions have a pole devoid of IMP (short arrows). The outer half of the viral envelope (vertical arrowheads) has as few IMP as fragments of the E face of the plasma membrane of cellular fragments (E). Upper right corner, one virion has been cross-fractured and contains a circular structure of the size of a capsid (long arrow) as observed in thin sections (see Fig. 3a).* ×60,000.

FIG. 3. *Details of the viral envelope in thin sections (a) and FF replicas (b and c). (a) Two virions are shown which have different characteristics. In the left one, the capsid is separated from the envelope by an electron-lucent space and an electron-dense fuzz apposed to the inner side of the unit membrane which constitutes the envelope. This dense material is present only at one pole of the virion shown on the right and is considerably thicker than on the virus shown on the left. Similarly, IMP cover the whole surface of the inner leaflet shown in (b), whereas they are clustered at one pole of the virus shown in (c).* ×150,000.

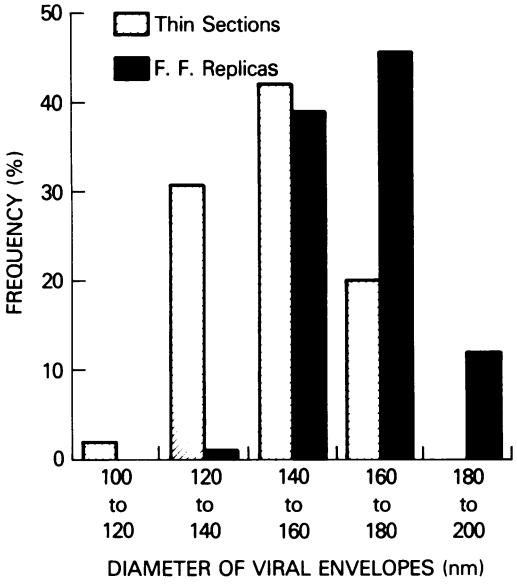


FIG. 4. *Histograms of the diameters of viral envelopes observed in thin sections (oblique lines) and FF replicas (in black). Only virions which showed a capsid in thin sections and rounded membranes that produced a shadow in FF replicas were measured. Both histograms have a similar pattern, but a difference of 20 nm exists between the most frequent diameters.*

came clear that protrusions on both nuclear membranes sometimes pertained to the same budding site (Fig. 6). These nuclear budding sites were covered with IMP similar in size and density to those of the adjacent nuclear membrane.

Changes in the distribution of IMP on the inner nuclear membrane were observed in cells infected with the VR3 strain and late in infection (36 and 48 h p.i.). Round areas lacking IMP were seen scattered on the P face and measured 200 ± 12 nm in diameter. They were sometimes seen close to budding sites (Fig. 6) (23). In addition, clusters of IMP on discrete protrusions of the P face of the inner nuclear membrane were seen occasionally (Fig. 7). These protrusions did not have the size and shape of the much more frequent budding sites and might be related to discrete areas of thickening of the nuclear membrane seen in thin sections (29).

Individual virions released in the perinuclear space were observed at the same time that the nuclear budding sites were identified in cells infected with the Patton strain (12 and 16 h p.i.). In the replicas, the inner leaflet of the viral envelope was characterized by a population of IMP similar to that of the nuclear membrane at the budding site (Fig. 8, and see B in Fig. 16). These virions were often partly engulfed in a depression of the outer nuclear membrane (Fig. 8). Much later in infection (48 h p.i.), groups of virions were occasionally seen in the perinuclear cisternae, although nuclear budding sites were rarely detected after 16 h p.i. (Fig. 9). Their membrane was strikingly different from the early virions, since they had much more IMP per square micrometer as did the extracellular virion. This suggests that some virions can stay for a prolonged period of time in the perinuclear cisternae where their envelope can progressively enrich in IMP.

At 12 and 16 h p.i. with the Patton strain, serial sections revealed complete virions in membrane-bound vacuoles localized at various distances between the perinuclear cistern and the cell surface. Most likely, the membrane limiting these vacuoles is derived from the outer nuclear membrane, which probably engulfs the virus in a process similar to phagocytosis at the cell surface (Fig. 8) (28). The inner leaflet of virions lying inside vacuoles was rarely uncovered by the fracture process so that no quantitative appreciation of the IMP density could be obtained for the extracellular virions. The inner leaflet of two virions observed inside vacuoles at 12 h p.i. appeared similar to that of virions detected in the perinuclear cisternae at that time.

Vacuoles connected to the plasma membranes were identified in serial sections, and their limiting membrane differed from the unopen ones. Indeed, at the site where a vacuole had probably fused with the cell surface, the plasmalemma was often invaginated into vesicles similar in size and shape to pinocytotic vesicles (Fig. 10a, and see B3 in Fig. 16). These were normally scattered all over the cell surface in uninfected cells. In contrast, when the E face of the plasma membrane of infected cells was revealed by the fracture, vesicles were seen to form clusters around depressions of the plasma membrane containing released virions (Fig. 10b and c). These membrane infoldings might result from addition to the plasma membrane of the limiting membrane of the vacuole containing the virions.

Unenveloped capsids lying in the cytoplasm and cytoplasmic envelopment were observed only late in infection (36 and 48 h p.i.) with both strains of HSV-1. They were, however, more

TABLE 1. *Proportion of virions with heterogeneity in their envelopes at different times after viral infection*

Type of virions	Virions (%) showing heterogeneity at:	
	12 h p.i.	48 h p.i.
Virions with an excentric fuzz (thin sections)	34	66
Virions with a pole devoid of IMP (FF replicas)	38	58

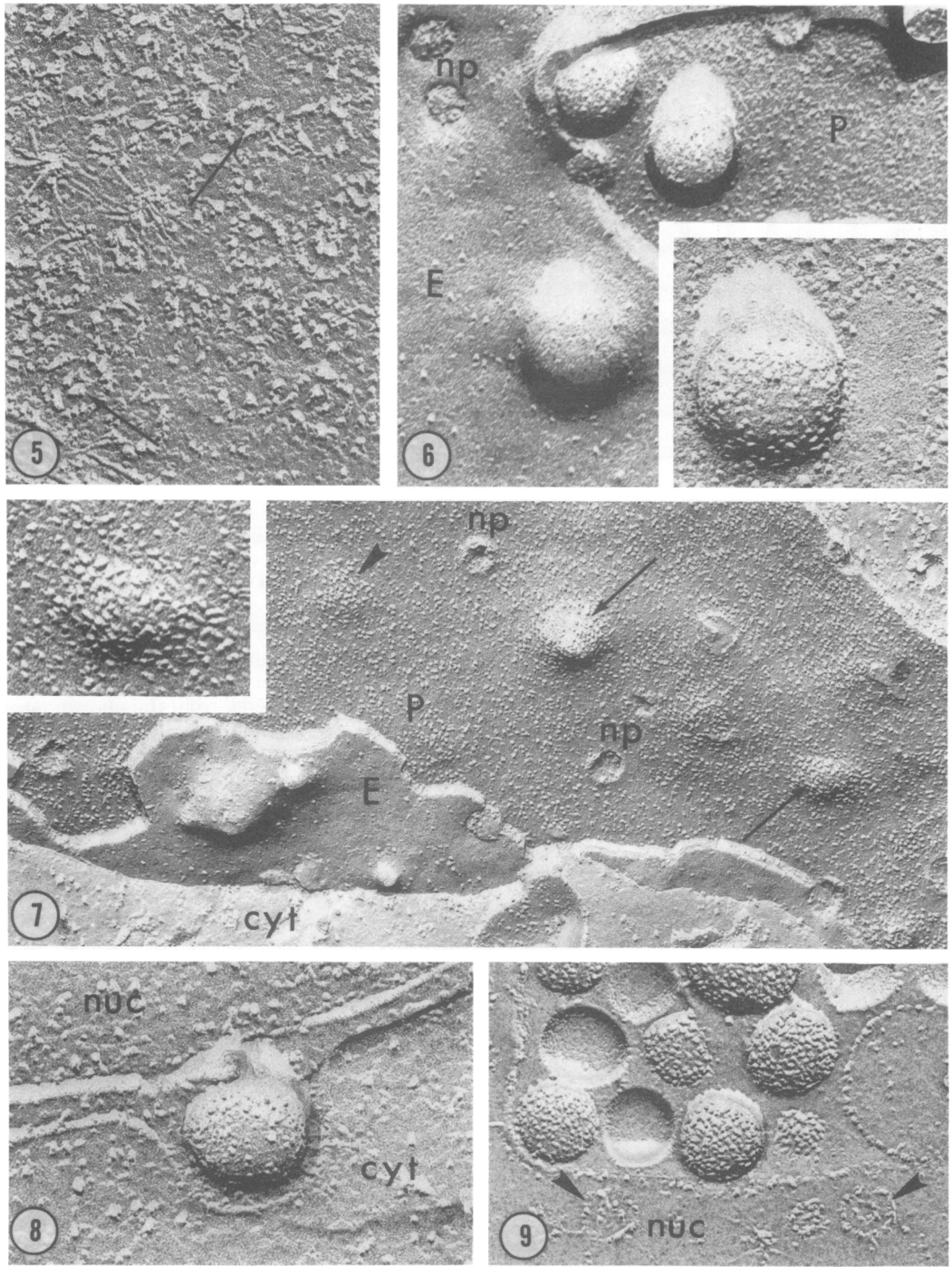


FIG. 5. *Portion of the nucleoplasm of an infected cell that was cross-fractured. Viral capsids appear as granules arranged in a circle, sometimes enclosing a group of central granules probably corresponding to the viral core (arrows).* ×80,000.

FIG. 6 and 7. *Aspect of nuclear membranes at 16 h p.i. with the Patton strain (Fig. 6) and at 36 h p.i. with the VR3 strain (Fig. 6 inset and Fig. 7). In Fig. 6 two protrusions are present on the P face of the inner nuclear membrane (P) and another on the E face of the outer nuclear membrane (E). The number of IMP on the protrusions is similar to that of the adjacent nuclear membrane. The inset in Fig. 6 shows, at higher*

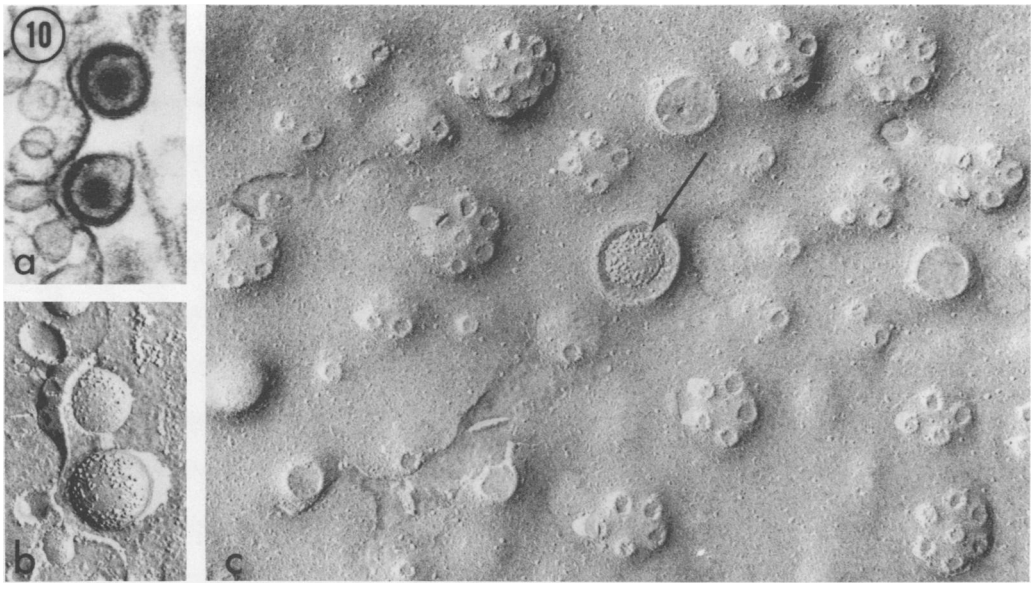


FIG. 10. *Interaction between extracellular virus and the plasma membrane early after release from cells infected for 12 h with the Patton strain of HSV-1. Free viruses sitting in the extracellular space (on the right) are in close contact with depressions of the plasma membrane in thin section (a) and FF (b). Vesicles, which are formed by plasma membrane invagination, appeared to be clustered around these depressions. This is more clearly seen when the E face of such plasma membrane has been largely uncovered during the fracture process (c). The vesicles have been cross-fractured, and groups of them, arranged in a geometrical pattern, are associated with depressions probably related to extracellular viral particles. In one instance, a cross-fracture through a depression has indeed uncovered an extracellular virion (arrow). ×60,000.*

frequently observed in cells infected with the VR3 strain. The virus was seen budding from the cytoplasm into vacuoles which often contained complete virions (Fig. 11 inset). In the FF replicas, elevations of various altitudes protruding into the vacuolar lumen were identified as budding sites on the vacuolar P face (Fig. 11). In contrast to the budding sites at the nuclear membrane, the P face of these protrusions was covered with a higher density of IMP than the adjacent vacuole membrane and resembled the inner leaflet of extracellular virions (Fig. 11, and see C in Fig. 16).

Membrane changes not associated with viral maturation. Membrane changes not associated with viral maturation were all observed late in infection (36 and 48 h p.i.). Fused membranes, often arranged in a concentric way, were observed in the cytoplasm of infected cells and consisted of pairs of unit membranes which are probably extensions of the nuclear membrane (Fig. 12a) (6, 29, 30). Pairs of membranes were separated by an electron-dense material similar to the one observed at the inner leaflet of the viral envelope (Fig. 13a). These concentric fused membranes were readily identified in FF replicas. The fracture faces of the leaflets apposed to the electron-dense material were covered with numerous and closely packed IMP of a density similar to that of the inner leaflet of extracellular virions (3,078 ± 783 IMP/μm^2) (Fig. 12b and 13b, and see F in Fig. 16). Leaflets in contact

magnification, one of these protrusions on the P face on the left of a flat area devoid of IMP. In contrast to the elevations shown in Fig. 6, more discrete protrusions seen in Fig. 7 (arrows) are covered with clusters of IMP. On the left, this clustering is even detected on a flat area of the inner nuclear membrane (arrowhead). The protrusion on the right is shown at higher magnification in the inset. Abbreviations: np, nuclear pores; cyt, cytoplasm. Figure 6, ×80,000; Fig. 7, ×60,000; insets, ×120,000.

FIG. 8 and 9. *Comparison between virions within the perinuclear cisternae at 12 (Fig. 8) and 48 h (Fig. 9) p.i. with the Patton strain of HSV-1. Figure 8 shows a single virion in which the inner leaflet is covered with a density of IMP similar to that of the P face of the inner nuclear membrane surrounding the budding sites (see Fig. 6). In contrast, the inner leaflets of the virions shown in Fig. 9 are covered with closely packed IMP as seen on the extracellular virions (see Fig. 2). Note the presence of nuclear capsids aligned along the inner nuclear membrane (arrowheads). Abbreviations: nuc, nucleoplasm; cyt, cytoplasm. Figure 8, ×120,000; Fig. 9, ×80,000.*

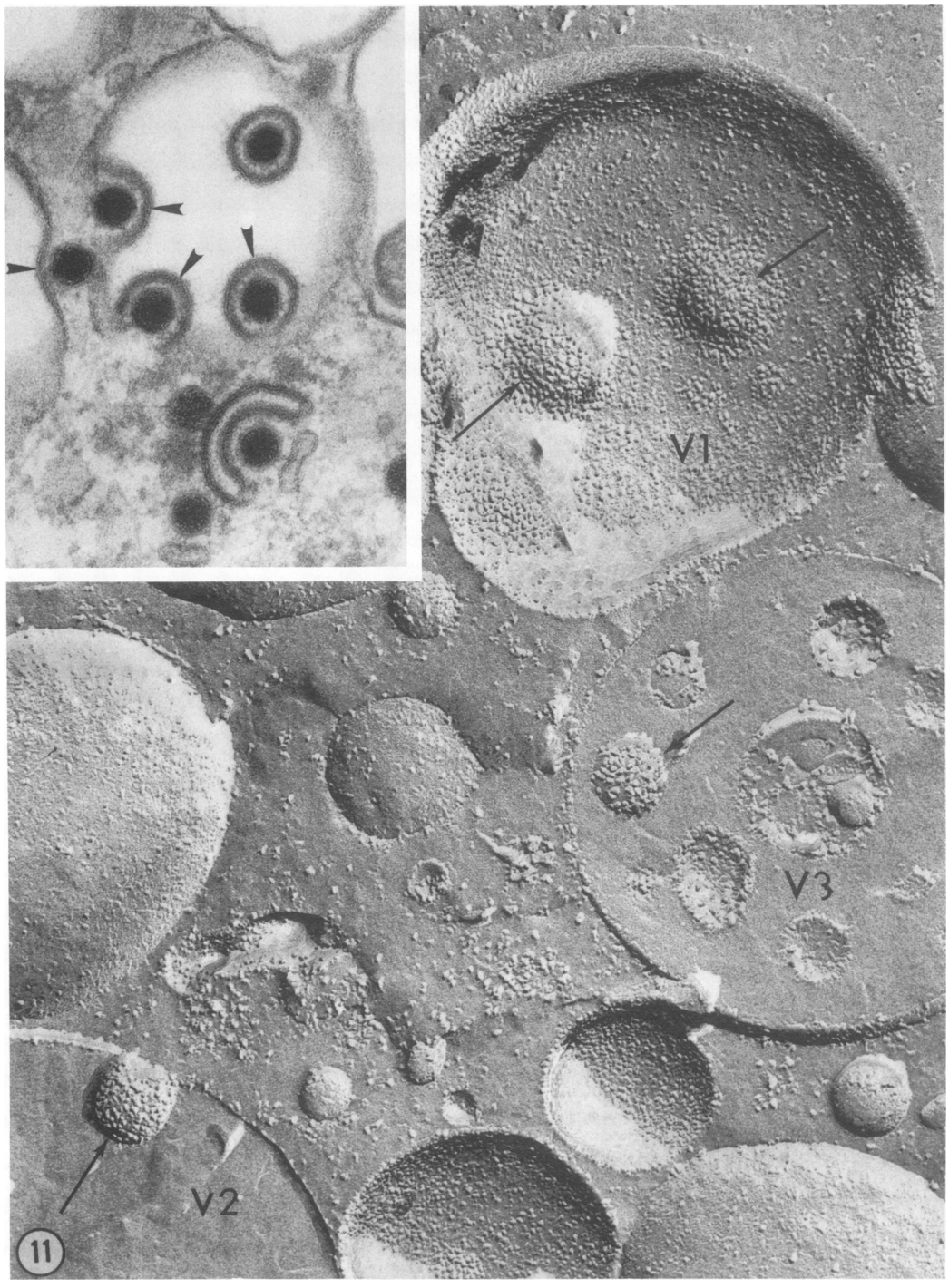


FIG. 11. *Cytoplasmic envelopment at 48 h p.i. with the Patton strain of HSV-1. Different stages of budding are detected in these three cytoplasmic vacuoles and can be compared with their aspect in thin sections (inset). In V1, protrusions interpreted as early buds show clustering of IMP on their P face (arrows); in V2, a bud is starting to detach from the vacuole membrane and is probably completely detached in V3 (arrows). The inset shows unenveloped capsids lying in the cytoplasm and different stages of cytoplasmic envelopment (arrowheads).* ×*80,000.*

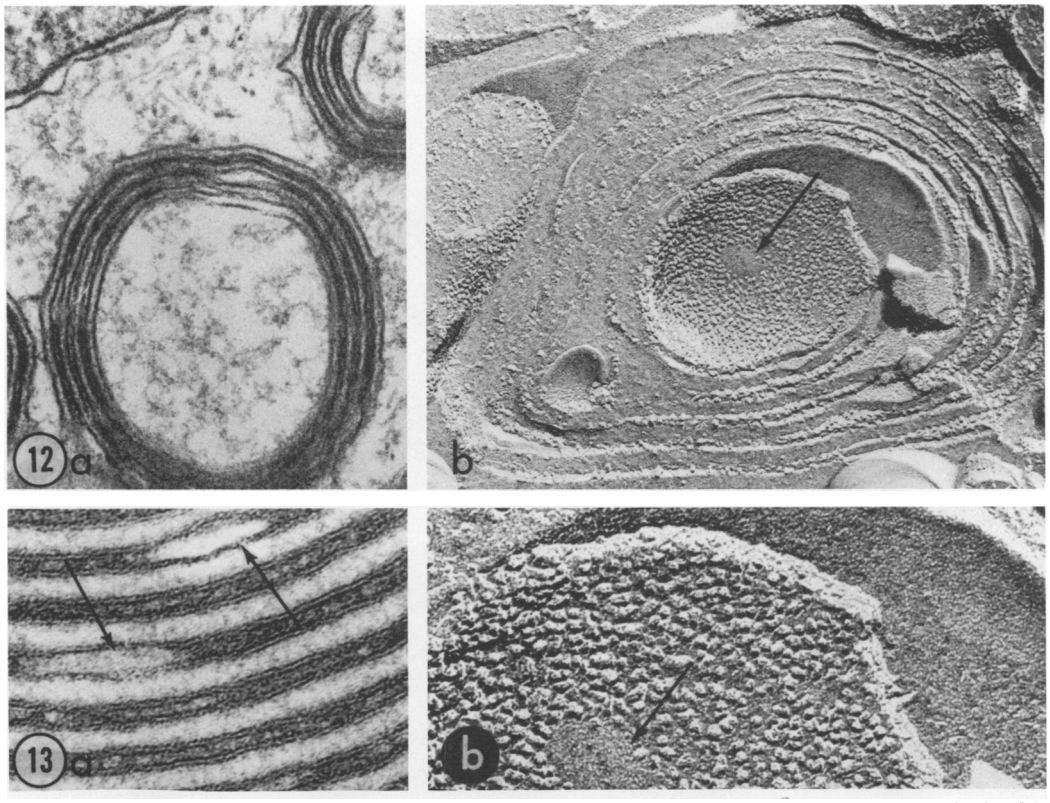


FIG. 12 and 13. *Aspect of concentrically arranged membranes at 48 h p.i. with the VR3 strain in thin sections (Fig. 12a and 13a) and in FF replica (Fig. 12b and 13b). The most external membrane is directly in contact with the cytoplasm and, inside this first concentric membrane, every other space between two membranes is separated by an electron-dense material. After FF, the leaflet apposed to the fuzz in thin sections shows IMP in high density as the inner leaflet of the extracellular virions (see Fig. 2). A central smooth area (arrows in Fig. 12b and 13b) might correspond to the absence of fuzz in some places of the concentric layers (arrow in Fig. 13a). Figure 12, ×60,000; Fig. 13, ×150,000.*

with the clear space in thin sections showed a population of particles similar to that of the E face of the nuclear membrane.

The P face of the plasma membrane of fibroblasts and Vero cells occasionally showed structural membrane changes that appeared to be strain specific. Indeed, they were independent of the host cell used since each strain produced identical changes in the two types of cells studied. The Patton strain induced, in 20% of each cell type, the formation of ridges of various lengths, 175 to 200 nm in width (Fig. 14, and see G in Fig. 16). These ridges were almost devoid of IMP, but particles sometimes formed small clusters at the periphery of the ridge. The ridges were covered with fine filamentous material running along the axis. Cells infected with the VR3 strain did not show any ridge on their plasma membrane P face, but 20% of Vero cells and 5% of fibroblasts had scattered areas of various shape and size where IMP were densely packed together (Fig. 15, and see I in Fig. 16). The distribution and density of IMP of the membrane surrounding these clusters, however, did not appear to differ from that of control cell membrane. Thin sections of identical cells did not reveal changes in structure and shape of the membrane which could correspond to the modifications detected in FF replicas.

DISCUSSION

The maturation of HSV-1 (strains Patton and VR3) and the structure of the infected cell membranes have been studied with the FF technique and compared with data obtained from thin sections. A distinction was made between early and late events related to viral maturation and alterations of cellular membranes at sites where no envelopment occurred (Fig. 16). The most striking observation made in this study was that the envelope of HSV-1 shows numerous IMP after FF. Thus the intramembrane structure of HSV-1, which assembles at nuclear and cytoplasmic membranes, is strikingly different from

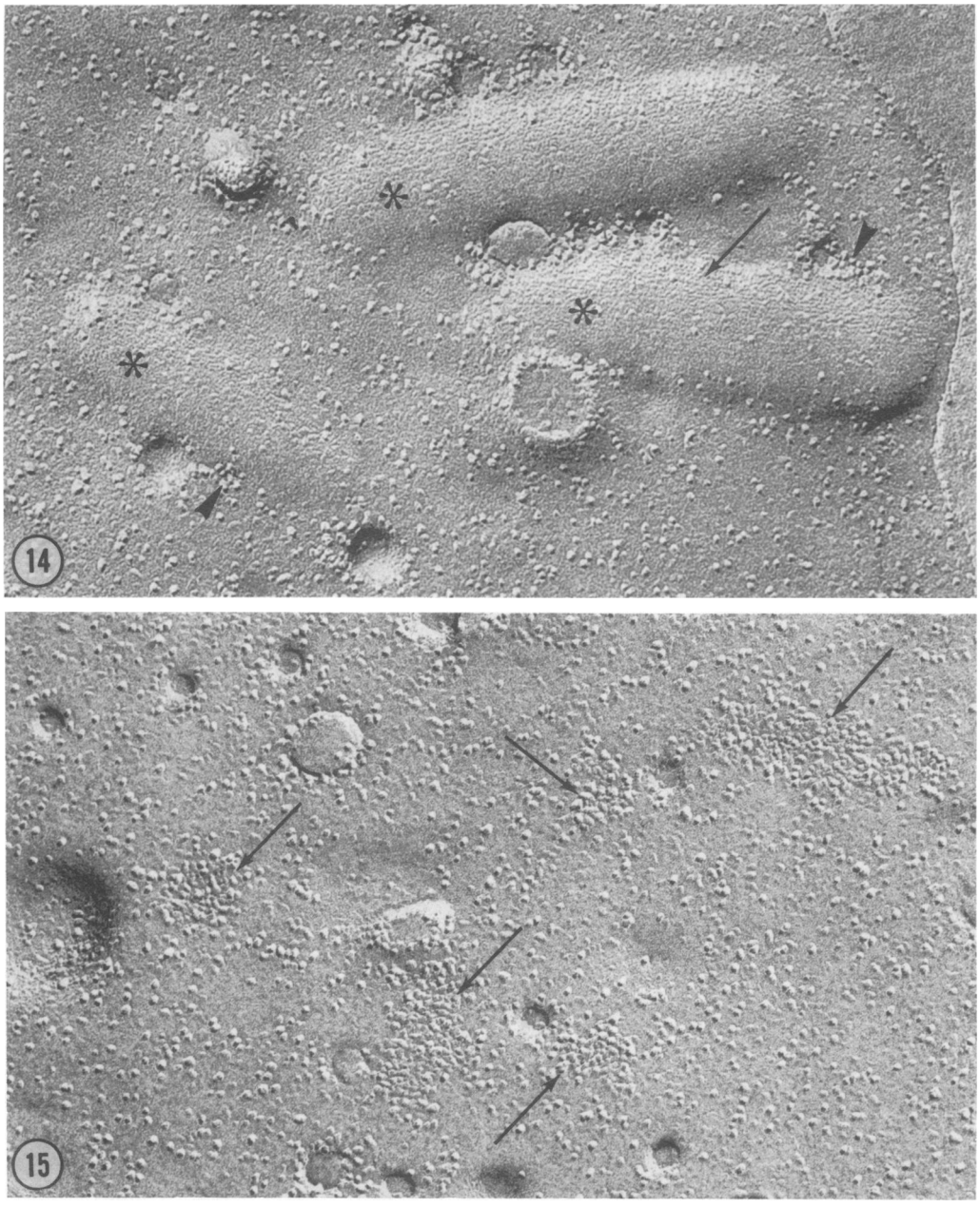


FIG. 14 and 15. *Comparison between plasmalemmas of fibroblasts infected for 48 h with the Patton strain (Fig. 14) and the VR3 strain (Fig. 15). In Fig. 14, ridges 175 to 200 mm in width (asterisk) are covered with fewer IMP than the surrounding P face. Their surface shows fine filaments parallel to the major axis of the ridge (arrow). Small clusters of IMP around the ridges are seen at arrowheads. In Fig. 15, five patches of various sizes and shapes can be detected at arrows. They are made of closely packed IMP. In both Fig. 14 and 15, the rest of the plasmalemma shows a slightly uneven distribution of IMP on its P face.* ×*100,000.*

that of various enveloped RNA viruses (influenza, Sindbis, visna, and vesicular stomatitis viruses) budding from the plasma membranes (1, 9, 10, 13, 33). In all of these cases, exclusion of IMP probably representing host proteins was observed at the site of virus budding as if the proteins of these viruses did not penetrate into the hydrophobic part of the bilayer. However, in measles infection, numerous IMP, smaller than most host cell IMP, have been observed at

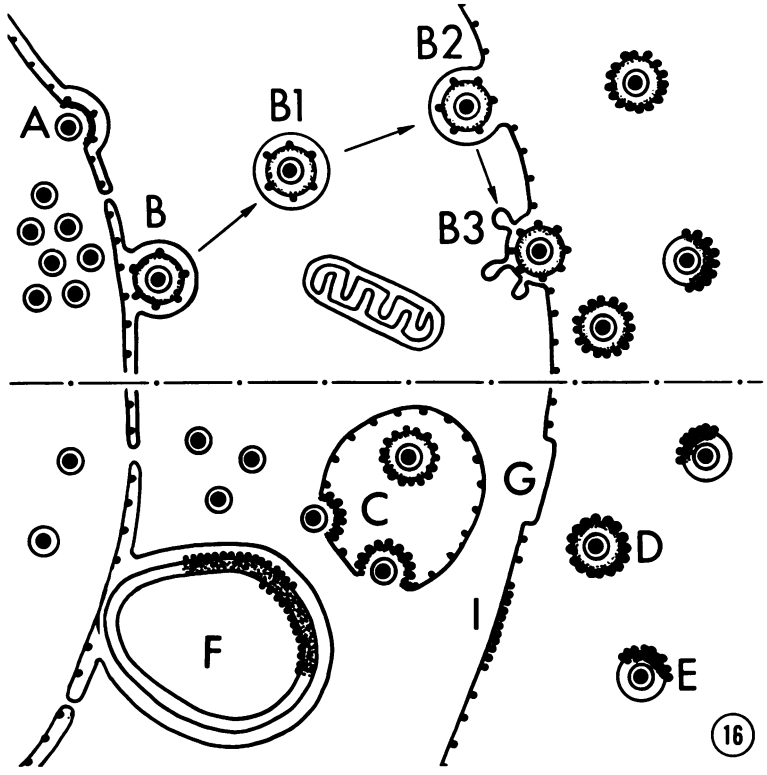


FIG. 16. *This diagram summarizes FF observations on HSV-1-infected cells. The black dots apposed to cellular and viral membranes represent the intramembranous particles (IMP) observed on the P face of cellular membranes and the inner leaflets of viral envelopes. Since nothing remarkable was observed on the outer leaflets of those membranes, they have been omitted from this scheme. The virus budding at the nuclear membrane (A) as well as the virion released in the perinuclear space (B) are covered with a similar number of IMP as the nuclear membrane. In contrast, virus budding in cytoplasmic vacuoles (C) are covered with IMP with a density three times higher than the rest of the vacuolar membrane. Virions enveloped at the nuclear membrane travel through the cytoplasm within smooth vacuoles (B1) and are released at the cell surface by a process of reverse phagocytosis (B2). The membrane around the released virus is folded into vesicles (B3). Extracellular virions are covered with three times more IMP than the nuclear and plasma membrane (D). Some of these virions lack IMP at a pole (E). Similarly, fused membranes (F) have fracture faces covered with three times more IMP than the nuclear membrane. At the plasma membrane, ridges almost devoid of IMP (G) were specific of the Patton strain, whereas clusters of IMP (I) were specific of the VR3 strain.*

the sites of attachment of the nucleocapsid to the modified plasma membrane as well as on the viral buds (12).

Another remarkable membrane feature of herpes infection is that the number of IMP per square micrometer in the inner leaflet of the extracellular virion envelope, on the budding sites at cytoplasmic vacuoles, and on the fused membranes was three times higher than that of the host cell nuclear and plasma membrane. In contrast, the density of IMP in the outer leaflet of the viral envelope was similar to that of the E face of the host cell membranes. Thin sections revealed a thick, electron-dense material under the membranes bearing a high density of IMP. This observation and the high density of IMP on the inner leaflet of the viral envelope suggest that viral proteins are more strongly anchored in the inner leaflet of the viral envelope and most likely to the electron-dense material seen on the inside of viral membrane.

It has been shown that new nuclear membrane is synthesized during herpes infection and that the proteins used to build the nascent nuclear membrane are virus coded exclusively (4, 5). However, most of the nuclear membrane appeared normal in FF replicas, whereas it did tag specific antibodies conjugated to ferritin in thin section studies (30). It is possible that viral proteins are evenly dispersed in the nuclear membranes of infected cells and thus not distinguishable from host cell proteins in FF replicas. In

contrast, IMP in increased density might represent a specific organization of membrane protein exclusively of viral origin.

Virions freshly released in the perinuclear cistern did not show as many IMP as did extracellular virions. Thus, a progressive enrichment in IMP seems to occur in the viral envelope during the passage of the virus through the cytoplasm, very early after release, or more rarely within the perinuclear cistern. It might be that some proteins that were integrated into the virion during nuclear envelopment move progressively from an internal position within the envelope or from the viroplasm toward the hydrophobic layer of the viral envelope. Interestingly, viral particles isolated from the nucleus, which probably consist mostly of unenveloped capsids, have been shown to contain viral glycoproteins similar to those associated with the viral envelope (4).

The difference in structure of the virus-modified membrane at nuclear and cytoplasmic budding sites might be related to differences in nature between nuclear and cytoplasmic membranes. This was suggested earlier in thin-section studies on other members of the *Herpesvirus* genus which revealed differences between envelopes of virions observed in the perinuclear cistern and in the cytoplasm (18, 19, 25). The present study of two strains of HSV-1 seems to confirm these differences even though they were seen only in FF replicas. The definite structural characteristics of the inner leaflet of the viral envelope were indeed acquired after envelopment at the nuclear membrane, whereas they were acquired during budding at cytoplasmic membranes. It is possible that viral proteins are inserted directly at the site of envelopment. The fate of the host cell proteins in that case is not clear.

Although the maturation steps of the two strains of HSV-1 studied were closely similar, different structures were seen in the P face of the plasmalemma of cells infected with different viral strains. The Patton strain induced large ridges almost devoid of IMP but covered with filamentous material. These filaments might be attached on the inner side of the membrane face (3) and be uncovered during the clearing of IMP observed on virus-induced ridges. The second strain studied (VR3) produced clusters of IMP which might contain some of the virus-specific proteins identified earlier in the plasma membranes of infected cells (24–26). However, fibroblasts infected with the VR3 strain manifested membrane fluorescence much earlier and in many more cells than the membrane alterations detected by FF. The clusters seen in FF replicas resemble the aggregates of globular particles seen at gap junctions between normal ependymal cells (8). In gap junctions as well as in HSV-1-induced clusters, the clustering of IMP may be more or less marked, but the virus-induced clusters on the P face did not have any complementary pits on the E face as did gap junctions.

In conclusion, a characteristic feature of HSV-1 infection is that, in contrast to other budding viruses, viral proteins in the mature virion can be identified inside the envelope bilayer as IMP with a characteristic density much higher than that of the nuclear and plasma membrane of the host cell. When a technique combining FF and immunolabeling of intramembrane structures becomes available, it will be possible to better define the nature of virus-induced intramembrane changes.

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