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Labile Coat: Reason for Noninfectious Cell-free Varicella-Zoster Virus in Culture[1]

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Experiments designed to determine why cell-free varicella-zoster virus replicated in cell culture is noninfectious were performed. Electron micrographs in which varicella-zoster virus (a herpesvirus) was compared to herpes simplex virus in primary human amnion cell cultures showed that the viruses were morphologically indistinguishable inside the nucleus. However, extranuclear varicella-zoster viruses were distinguished from herpes simplex virus by the presence of pleomorphism, incomplete coats, and a resultant loss of central dense cores. This result indicates that varicella-zoster virus possesses a labile coat which is degraded outside the nucleus. It is suggested that the labile coat is a principal reason for the lack of cell-free infectious virus in this system.

Cell-free varicella-zoster (V-Z) virus obtained from vesicular fluid is infectious (14). In cell cultures, on the other hand, even though the virus produces a progressive cytopathic effect involving adjacent cells, cell-free virus generally cannot be obtained (18). Thus, serial passage of the virus is usually accomplished by the use of infected human diploid cells. The mechanism responsible for this alteration in infectivity in vitro is not readily apparent, since morphologically intact viral particles have been seen in electron micrographs of infected cells in culture (1, 15).

We attempted to delineate the mechanism(s) responsible for the noninfectiousness of cell-free supernatant fluids in V-Z virus infected cell cultures by comparing the morphogenesis of V-Z virus with the related herpes simplex virus (HSV) in cultured primary human amnion cells. As expected, cell-free supernatant fluids from cells infected with V-Z virus were noninfectious, whereas HSV efficiently produced cell-free virus in this system. It will be shown that V-Z virus envelopes and capsids (collectively called the "coat" in this paper) are degraded outside the nucleus with a resultant loss of central dense cores and, therefore, infectivity. Although other causes of noninfectiousness are possible, these results suggest that it is principally, if not exclusively, the result of cytoplasmic degradation of V-Z virions.

[1] A preliminary report of this investigation was presented at the Annual Meeting of the American Society for Microbiology, Detroit, Mich., 1968.

MATERIALS AND METHODS

Cells and media. Primary human amnion cells obtained from cesarean sections were used in all experiments. They were prepared by standard methods and planted in 200-ml milk dilution bottles or 2-oz (59 ml) French square bottles in a growth medium consisting of Hanks' balanced salt solution, supplemented with newborn, hypogammaglobulinemic calf serum (30%), lactalbumin hydrolysate (0.5%), penicillin (100 units/ml), streptomycin (0.1 mg/ml), and mycostatin (50 units/ml). After 2 days of incubation at 37 C, a monolayer had formed, and the medium was replaced by a maintenance medium of Eagle's basal medium containing 10% serum, penicillin (200 units/ml), streptomycin (0.2 mg/ml), and mycostatin (50 units/ml). This medium was also used for virus replication.

V-Z virus. V-Z virus was isolated from vesicular fluid taken on the third day of rash from a child with chicken pox. Foci of infection were evident 8 days after monolayers of human amnion cells had been infected, and the isolate was subsequently maintained through 38 serial passages.

Numerous attempts to pass the virus with supernatant fluids from infected cultures only confirmed earlier reports that this method is ineffective. Therefore, the virus was passed through the use of infected cells. Foci of such cells were removed from the glass with an antibiotic trypsin-ethylenediaminetetraacetate solution (7) centrifuged at 500 × *g* for 5 min, resuspended, and placed on uninfected monolayers. These infected cells attached to the monolayers, and new foci of infection were initiated.

HSV. The macroplaque strain (3) of herpes simplex virus (HSV-MP) was kindly supplied by Bernard Roizman. Stocks of the virus were obtained by infect-

ing monolayers of human amnion cells which were maintained as were the V-Z virus infected cultures. When cytopathic effects were complete, supernatant fluids were removed, clarified at 500 × *g* for 5 min, and frozen at −90 C.

Electron microscopy. Since amnion cell preservation was extremely poor if the cells were pelleted either before or after fixation, a procedure similar to that described by Howatson and Almeida (4) was developed. A light carbon film was evaporated on one side of clean glass slides, and these were placed in 2-oz (59 ml) French square bottles and autoclaved. After cells were grown on them and had been infected, these slides were removed and the cells were processed for electron microscopy as a cell sheet. Initial fixation was in 0.25 to 1% glutaraldehyde (12) in phosphate buffer (8) at *p*H 7.4 to 7.6 for 15 min to 1 hr at 4 or 37 C. Postfixation in 1% osmium tetroxide in the same buffer at 4 C for 0.5 hr was followed by rapid dehydration in graded alcohols and propylene oxide. Final embedding in Epon 812 (6) was accomplished by inverting gelatin capsules filled with the embedding mixture on the slides. Polymerization was at 60 C for 3 to 4 days. After removal of the capsule, the cell sheet (now embedded on the flat surface) was examined by light microscopy, and an area was chosen for sectioning. Thus, sections were cut in a plane roughly parallel to the direction of growth. Thin sections were made on an MT-1, Sorvall, Porter-Blum ultramicrotome using a diamond knife. They were double-stained with uranyl acetate (17) and either lead oxide (5) or lead citrate (11, 16), and they were examined in a Siemens Elmiskop I.

Viral particle counts. Counts made of virus particles were done on pictures of both V-Z and HSV-MP infected primary human amnion cells. Particles with a central dense core versus particles with a partial or empty core were counted from electron micrographs with a final magnification of 20,000 times or more. Nuclear, cytoplasmic, vacuolar, and extracellular areas were examined for each virus. A total of 120 micrographs were employed, some of which contained particles in more than one cellular region. The 82 pictures of V-Z virus infected cells used in this study were made from a variety of specimens obtained from two different electron microscopic embeddings, one in an early (2nd) and one a later (15th) passage. The 38 pictures used for HSV-MP infected cell assays were made from several specimens of two separate embeddings of this virus in different passages (4th and 6th).

RESULTS

Morphological comparisons. Morphological studies of the maturation of herpesviruses have shown a general similarity in development (2). In summary, intranuclear immature particles first appear as capsids which may be either "empty" or contain a morphological variety of components, the most mature of which is considered to be the "central dense core." Such capsids become morphologically complete virions after they derive an envelope from the inner nuclear membrane or extensions from it. They are next released into cytoplasmic vacuoles that migrate to the plasma membrane. Fusion of the walls of vacuoles containing viruses with the cell membrane followed by rupture of the membranes is postulated as the major route for passage to the extracellular space.

An example depicting the intranuclear development of HSV-MP in human amnion cells is shown in Fig. 1. Immature particles are seen surrounding dense bodies (DB), clustered in the membrane-bound group on the left near the nuclear membrane (NM), or free in the nucleoplasm on the right. These viral forms either contain central dense cores or "ring-shaped" internal components, and are occasionally associated with filamentous material. Crystals composed of capsids were not observed. A group of mature virions (V) with central dense cores and envelopes is visible in the center.

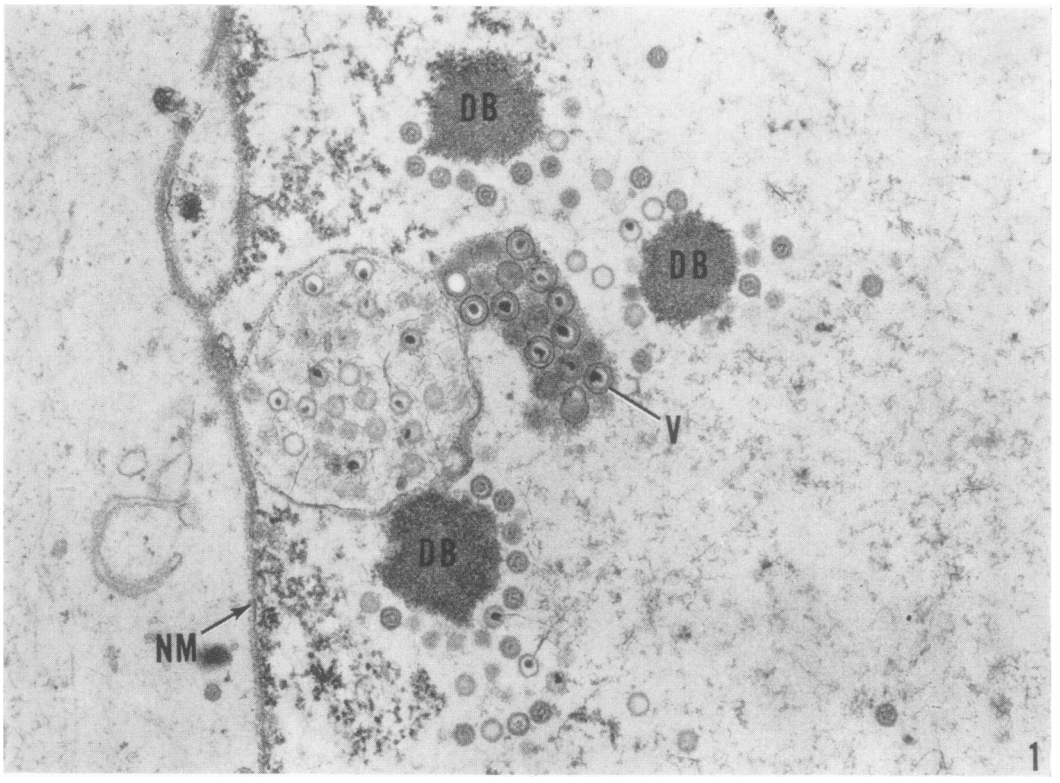
Intranuclear V-Z viral forms are evident in Fig. 2. Capsids without an envelope are seen free in the nucleoplasm (extreme right) or in clusters as shown on the right of a nucleolus-like structure (Nu). The clusters of capsids were occasionally observed in crystalline array. These immature forms, as in the case of HSV-MP, contain either central dense cores or ring-shaped internal components. Filamentous material was also observed to be associated with these forms, although this is not illustrated here. Six mature V-Z virions (V) with central dense cores and envelopes are seen in the lower left of the nucleus, near the nuclear membrane (NM).

The appearance of HSV-MP inside cytoplasmic vacuoles is observed in Fig. 3. Almost all viruses are enveloped, although some "coreless" particles are pictured.

When V-Z virus in cytoplasmic vacuoles is compared to HSV-MP (Fig. 4), striking differences in viral structure are noted. V-Z viruses are covered with a dense material, which is also seen between the envelope and the capsid in some instances. In addition, viral pleomorphism, open envelopes, disrupted capsids, and a paucity of central dense cores are clearly evident.

In Fig. 5, HSV-MP is seen in the extracellular space. Virus morphology similar to that in Fig. 3 is obvious, although several examples of multiple nucleocapsids contained inside a single membrane are pictured. This observation was also made with V-Z viruses. Most viruses are mature, and close examination reveals that those lacking a central dense core have no obvious breaks in either envelope or capsid. This phenomenon suggests that they were assembled without a central dense core.

The typical appearance of V-Z virus in the



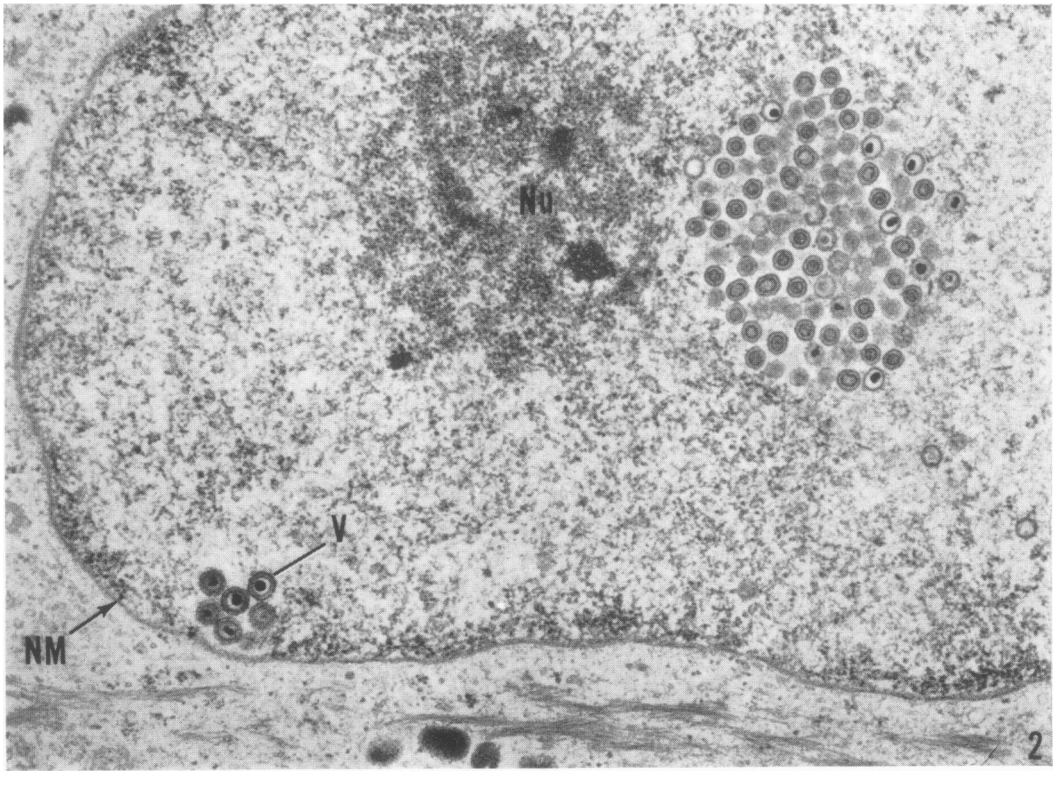
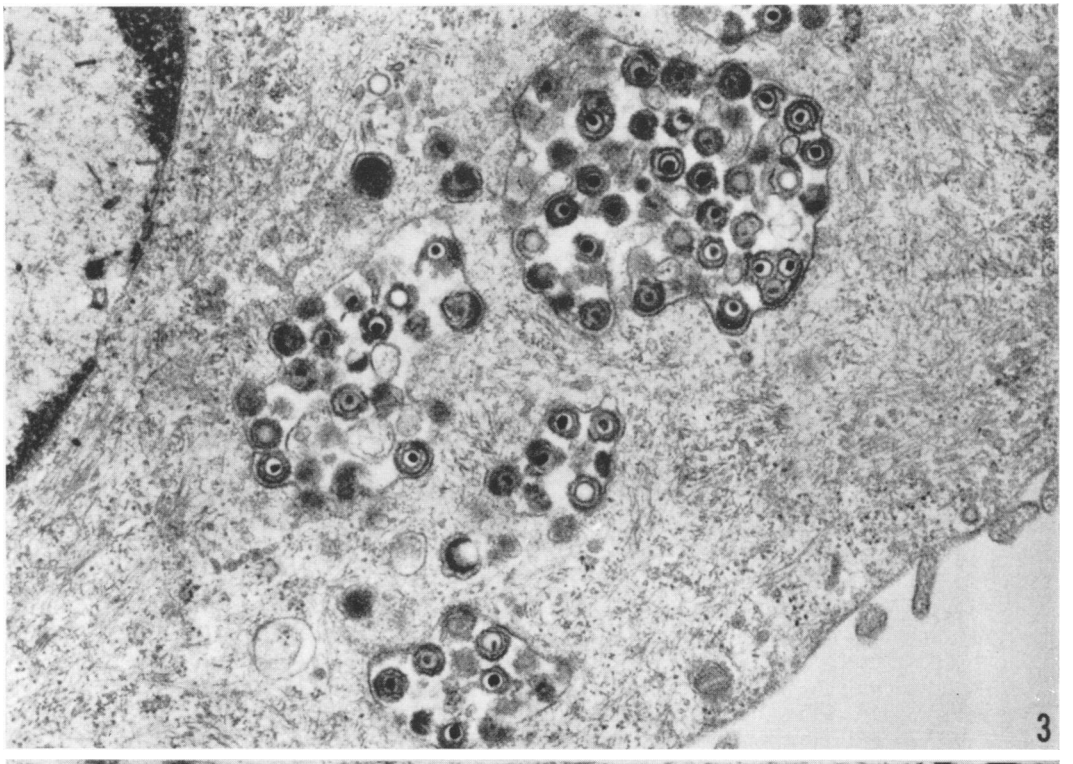


FIG. 1. *HSV-MP in the nucleus of an infected human amnion cell. Immature viral forms are seen surrounding dense bodies (DB), free in the nucleoplasm, and membrane-bound near the nuclear membrane (NM). Mature virions (V) are also seen.* × *31,000.*

FIG. 2. *V-Z virus in the nucleus of an infected human amnion cell. Immature viral forms are seen clustered near a nucleolus-like structure (Nu) and free in the nucleoplasm. Mature virions (V) are seen near the nuclear membrane (NM).* × *31,000.*



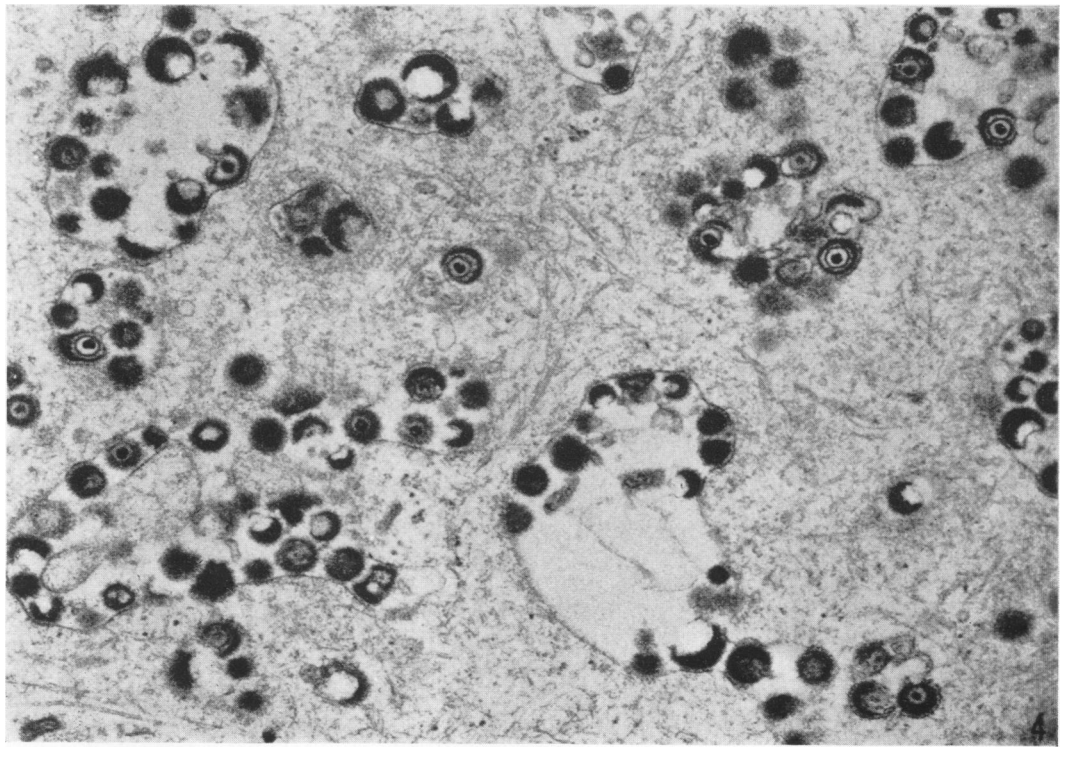


FIG. 3. *HSV-MP in cytoplasmic vacuoles.* × *35,000.*
FIG. 4. *V-Z virus in cytoplasmic vacuoles.* × *35,000.*

extracellular space is seen in Fig. 6. Bizarre forms are abundant, and the relative lack of central dense cores is clearly evident. These striking observations of incomplete, extranuclear V-Z viruses compared to HSV-MP suggested that the particles were being degraded in extranuclear areas, and were thus rendered noninfectious. This suggestion led to the quantitative studies presented in the next section.

Particle counts of V-Z virus and HSV. By using absence of a central dense core as an index of degradation, counts were made of particles from electron micrographs taken for the morphological studies of HSV-MP and V-Z virus infected amnion cells. Particles with a central dense core compared to particles with a partial or no core were tabulated in four cellular compartments and contrasted for each virus (Table 1).

It is clear from these results that there is a marked difference in the morphological integrity of V-Z viruses compared to HSV-MP in the passage to the outside of the cell. By using disappearance of the central dense core as an index of degradation, V-Z virus is obviously degraded during this passage.

Several features of this quantitative investigation deserve comment. First, the procedures used in preparing infected cultures for electron microscopy probably removed severely damaged and therefore less adhesive cells. This no doubt resulted in the elimination of cells in the late stages of infection when immature viral forms are thought to be released into the cytoplasm as a result of nuclear membrane breakage (2). This explains the low total number of either virus found free in the cytoplasm, since in this investigation the usual method of passage from the nucleus to the extracellular space was found to be in membrane-bound vesicles. That the percentage of "cored" HSV-MP in the vacuolar and extracellular regions is identical is probably caused by the efficiency of cellular release for this virus.

TABLE 1. *Total counts and percentage of cored HSV-MP and V-Z virus in four regions of human amnion cells*

Region of cell	HSV-MP		V-Z virus	
	Total	Cored	Total	Cored
		%		%
Nuclear	738	36	364	26
Cytoplasmic	44	46	128	28
Vacuolar	218	75	735	14
Extracellular	865	76	1,445	9
Total	1,865		2,672	

The HSV-MP counted as "noncored" in these areas were almost all enveloped capsids without a central dense core. This can be most logically explained by the exportation from the nucleus of coreless, enveloped capsids. Such particles were observed in nuclei infected with HSV-MP but not with V-Z virus.

Numerous extranuclear dense particles and membranous fragments of probable V-Z viral origin were not counted unless definitive identification was possible. This no doubt resulted in higher percentages of cored V-Z viruses being scored in these areas. Also, it is important to note that we used a "drastic" alteration in morphology as our criterion of virion destruction. It is probable that the more subtle changes in the coat which precede loss of cores are sufficient to cause inactivation of the particle. From this, it is concluded that the 9% of cored particles noted must be a high estimate of the actual number of intact infectious virions.

Finally, it might be argued that the low percentage of cored V-Z viruses in the extracellular space is not due to cytoplasmic destruction but is the result of exportation from the nucleus of complete but coreless particles. This explanation is untenable for the following reasons.

(i) There is a lower percentage of cored V-Z virus particles found in the vacuolar and extracellular regions of the cell than that found in the nucleus. Also, there is a graded diminution of cored particles as they pass from the cytoplasm to the extracellular space.

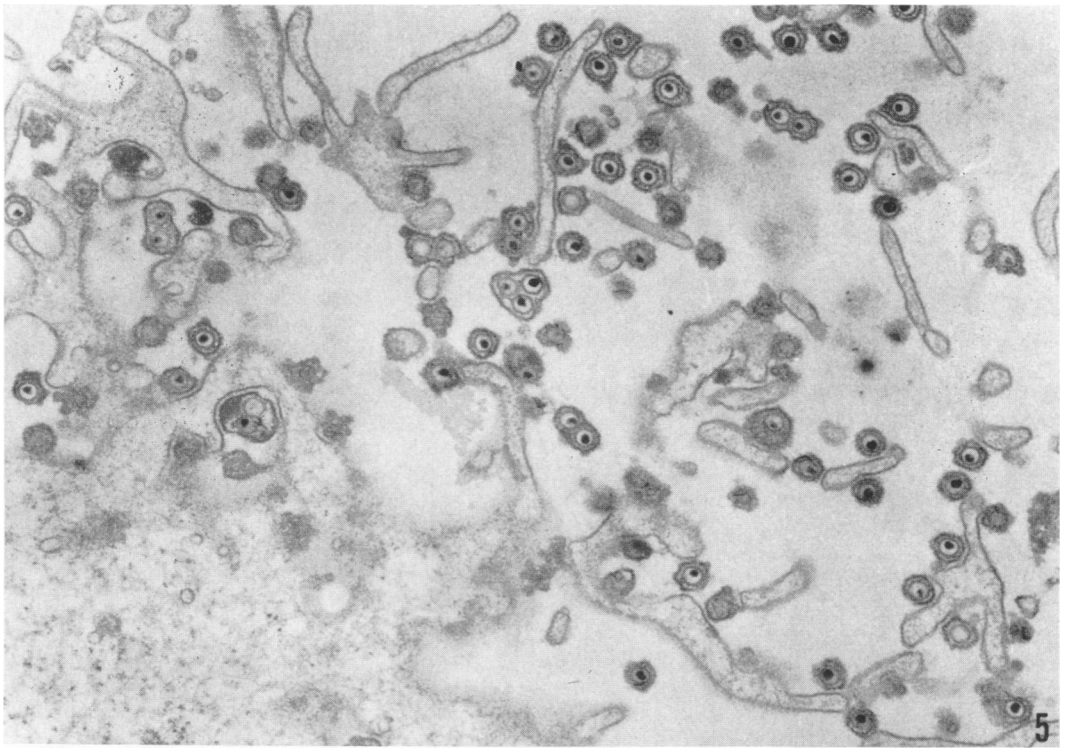
(ii) Those extranuclear particles which are noncored exhibit degradative signs such as open envelopes and bizarre forms.

(iii) Contrary to the picture seen in HSV-MP infected nuclei, V-Z virus particles with envelopes but without a central dense core were almost never seen in V-Z virus infected cell nuclei.

From these considerations, it is concluded that V-Z virus possesses a labile coat which is broken down outside the nucleus.

DISCUSSION

The comparison of intranuclear forms of HSV-MP and V-Z viruses emphasizes their similarity. Immature (naked) capsids of both viruses contain either ring-shaped internal components or dense cores. Filamentous material was associated with these nonenveloped forms in the nuclei of cells infected with either virus, although this phenomenon was observed less frequently in V-Z virus infection. Inside the nucleus, mature virions of the two viruses were also morphologically identical. The dense bodies surrounded by immature viral capsids in the HSV-



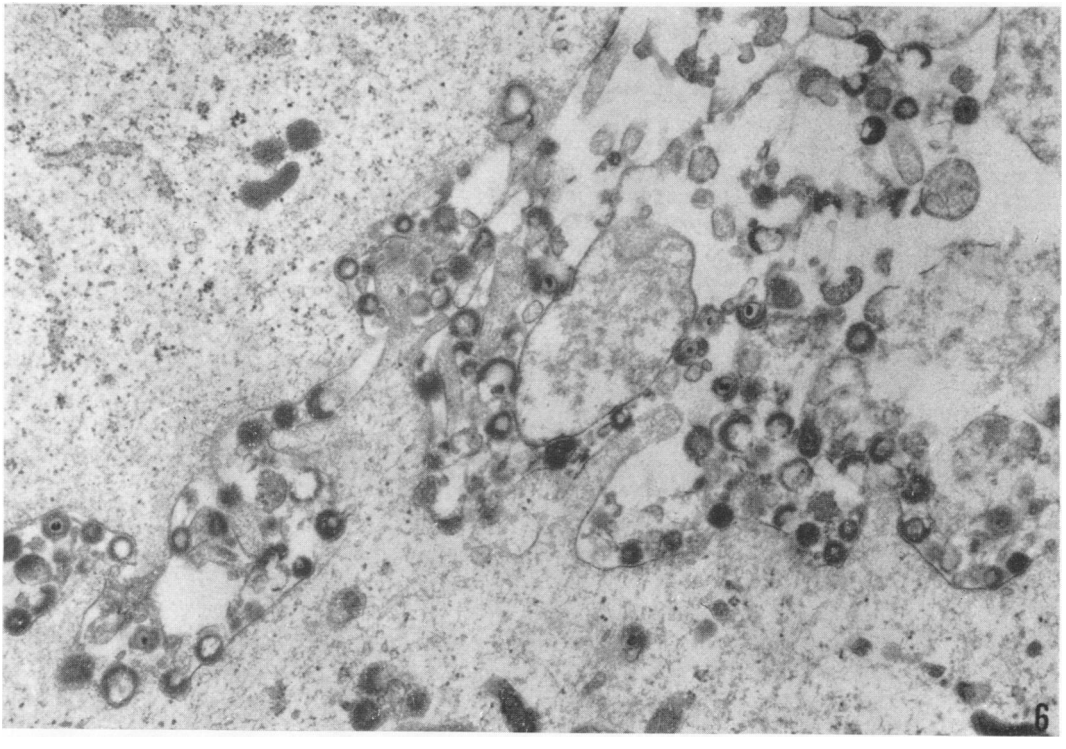


FIG. 5. *HSV-MP in the extracellular space.* × *28,000.*
FIG. 6. *V-Z virus in the extracellular space.* × *28,000.*

MP infected nuclei were not seen in V-Z virus infected cells. However, these bodies are often lacking in descriptions of HSV maturation (9, 13), and may be peculiar to HSV-MP. The clusters of capsids (occasionally in crystalline array) seen in V-Z virus infected nuclei were not observed in the HSV-MP infected nuclei, but they have been described in other strains of HSV (10). With these two exceptions, the intranuclear development of the two viruses appears to be identical.

The principal difference between the viruses becomes obvious in extranuclear areas. V-Z virus is seen to be associated with densely stained material and exhibits pleomorphism, loss of central dense cores, envelope breakage, and other signs of degradation not seen with HSV-MP.

The particle count studies confirmed the impression that, compared to the relatively efficient HSV-MP infection, few V-Z virions reach the extracellular space in a morphologically intact form. This leads to the conclusion that this virus is vulnerable to extranuclear degradation.

Destruction of the viral coat could be the result of one of two phenomena. Either the viral genetic code is not read correctly by the amnion cell and "faulty" coat proteins are made, or the amnion cell contains enzymes that specifically attack V-Z virus coats. At present, we cannot differentiate between these two alternatives.

Finally, it is interesting to speculate on the possible function of the envelope in insuring the stability of virions in the cytoplasm. It may well be that the envelope is the primary site of the lesion and that the "naked" particles of any herpesvirus are relatively unstable cutside the nucleus.

Because of the technical difficulties inherent in studying this system, we are unable to adequately assess all possible causes of noninfectiousness. However, since a major proportion of V-Z virions are degraded outside the nucleus, it can be concluded that the vulnerable coat is a significant, if not the only, reason for noninfectiousness.

Acknowledgments

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