Electron Microscopy of Herpes Simplex Virus

I. Entry

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Although capsids of herpes simplex virus were encountered within phagocytic vesicles, they were more commonly observed free within the cytoplasm. Stages in the release of virus from vesicles were not seen. There appeared to be five distinct steps in the process whereby the virus initiates infection: attachment, digestion of the viral envelope, digestion of the cell wall, passage of the capsid directly into the cytoplasm, and digestion of the capsid with release of the core. Antibody probably interferes with the first two stages.

This paper describes and illustrates the probable manner in which herpes simplex virus (*Herpesvirus hominis*) initiates cellular infection. The three ensuing papers are concerned with the sequential stages of viral development, the effects of blocking DNA synthesis, and the antigenic alteration of host cell components.

Since the initial examination of herpes simplex virus in thin sections (21, 22) a confusing nomenclature has arisen, including such descriptive terms as internal body, nucleoid, central body, internal membrane, peripheral coat, second membrane, single membrane form, naked particle, double membrane form, and complete virus. ["Nucleoid" was originally coined to describe the dense, eccentrically placed structure in immature forms of vaccinia and fowl pox viruses (23). It has become an unfortunate misnomer when applied to the dense cores characterictic of the majority of viruses.] Application of the negative staining technique, however, has now provided more precise information about the structure of the virus (33), which indicates that it consists of a core surrounded by a capsid. The capsid is icosahedral in shape and is composed of 162 capsomeres arranged in 5:3:2 axial symmetry. Capsids may or may not be enclosed within an envelope that is devoid of clearly defined subunits. Applying this nomenclature (19) to the appearance of the virus in thin sections, one can recognize a clearly defined core, a capsid (the first or inner membrane), and an envelope (the second or peripheral membrane). These terms will be used henceforth.

Electron microscopic examination of reovirus (9), vaccinia virus (5, 1, 7, 10, 25), adenovirus (5), Newcastle disease virus (24, 31), parainfluenza virus SV5 (4), and influenza virus (8) suggests that viral particles gain entry into the host cell by a process of phagocytosis or "viropexis," as originally suggested by Fazekas de St. Groth (12). Studies of herpes simplex virus also have yielded results that have been interpreted as being con-

sistent with this concept (11, 13, 30). Dissenting opinions, however, have been voiced. Hoyle and Finter (16) [*see also* Wecker and Schäfer (32)] concluded from an investigation of influenza virus labeled with radioactive sulfur "that on entry into the cell the virus nucleoprotein is hydrolyzed with release of amino-acid and free nucleic acid, while the virus envelope protein and haemagglutinin remain on the cell surface." Subsequently, Zhdanov et al. (35) reached a similar conclusion based on autoradiographic studies of Sendai virus. Rubin and Franklin (28) suggested that, in the case of Newcastle disease virus, "it is unlikely that engulfment (phagocytosis) plays an important part," for, they reasoned, the minute amounts of antibody apparently needed to prevent penetration of the virus into the host cell could "hardly be expected to interfere with engulfment." Then Cohen (3), in reviewing mechanisms of penetration, called attention to two micrographs published 6 years previously by Adams and Prince (1). These authors described their observation in a caption as follows: "In some cases the section plane passes through the adsorption site (of Newcastle disease virus) suggesting disappearance of the external lamella of the limiting membrane of the particle as well as the subjacent plasma membrane of the (Ehrlich ascites) cell at the point of contact." Unfortunately, this was the extent of their comment, and the observation was nowhere alluded to in the main text of their paper. Nevertheless, Cohen (3) suggested that "this observation provides a clue to a hypothesis, admittedly speculative, of myxovirus penetration which has the advantage of being compatible with all the experimental observations relevant to penetration. This is that on close

apposition of virus and cell surfaces there is a fusion of the opposing lipoprotein membranes with establishment of continuity. In this way, the virus-membrane envelope becomes incorporated in the cell membrane, and the internal constituents of the virus become extruded into the cytoplasm." Finally, in a recent electron microscopic study of the penetration of Newcastle disease virus, Meiselman et al. (20) noted fusion of the virus to cell membranes within 10 min and commented that "one must consider the possibility of virus entry by fusion of the viral envelope with the cell membrane; in this case, the viral nucleoprotein would be directly released into the cytoplasm." They were unable, however, actually to observe stages in the passage of nucleoprotein into the cytoplasm of the host cell. [Since submission of this manuscript, Philipson, in a study of adenovirus eclipse, suggested that the virus may be uncoated at the cell surface (J. Virol. 1: 868–875, 1967).]

The purpose of this communication is to illustate and describe (i) fusion of herpes simplex virus with the host cell; (ii) the process whereby the capsid enters the cytoplasm; and (iii) release of the core, which is presumed to contain the infectious nucleic acid.

MATERIALS AND METHODS

Virus. The Miyama strain (26) of herpes simplex virus was grown on monolayers of HeLa cells in Eagle's medium supplemented with 10% fetal calf serum. High-titer stocks were obtained by sonically treating infected cultures and using the supernatant fluid after it had been cleared by low-speed centrifugation.

Inoculation. Plastic 30-ml flasks were seeded with 10^5 HeLa cells. Sparse cell layers (before there was appreciable contact inhibition) were washed once with Earle's solution and inoculated with 0.5, 1.0, or 1.5 ml of prewarmed, undiluted stock virus suspension containing approximately 10^7 $TCID_{50}$/ml. In one experiment, the stock had been frozen and thawed, but the results did not differ from those obtained with fresh virus suspensions. At intervals of 10, 20, and 40 min, during which time the flasks were rocked gently at 37 C, the cells were harvested for electron microscopy. In other experiments, the cells were washed with Earle's solution after 1 hr of incubation with the virus inoculum. Fresh prewarmed Eagle's medium supplemented with 5% fetal calf serum was then added, and the cells were incubated for an additional 1 to 3 hr.

Neutralization. Concentrated virus inoculum was mixed with herpes simplex rabbit antibody globulin conjugated with ferritin by the method of Rifkind et al. (27). (The antibody was kindly provided by R. A. Malmgren, National Cancer Institute.) The mixture was added to the cultured HeLa cells and incubated for 2 to 4 hr at 37 C. After the cells were washed in Earle's solution, they were processed for electron

microscopy. Rabbit antibody to normal, uninfected FL cells was applied in a similar manner.

Preparation for electron microscopy. The cells were washed with Tyrode's solution, scraped, centrifuged gently into a pellet, fixed for 30 min in 1% glutaraldehyde, washed, fixed for 30 min in 1% osmium tetroxide, dehydrated, and embedded in epoxy resin (Epon 812). The sections were stained with uranyl acetate and lead citrate before examination in a Phillips E. M. 200 electron microscope.

RESULTS

Figures 1–14 and 17–20 are reproduced at the same magnification (180,000 ×). In Fig. 1 (10 min after addition of virus), the viral envelope adjacent to the cell wall on the left is flattened and appears thin. Figure 2 illustrates a particle whose envelope has been more extensively damaged. There are also regions of low density between envelope and capsid at sites removed from the cell wall. In Fig. 3, the process of digestion is still further advanced, and part of the cell wall appears indistinct. Figure 4 shows beginning fusion of the viral envelope with the cell. As in previous micrographs, the envelope adjacent to the cell had disintegrated. In Fig. 5, fusion seems to have been completed, and nothing intervenes between capsid and cytoplasm; the cell wall appears to have broken down in the upper portion of the field. Figures 6–8 illustrate a similar sequence of events, but with the interesting appearance of a projection of the virus extending toward, and fusing with, the cell wall. This phenomenon suggests that attachment of the viral capsid to the cell is so firm as to resist forces which seek to dislodge the virus (such as washing the preparation). Thus, the envelope at the site of attachment is stretched or pulled out. Figures 9 and 10, taken 20 min after addition of the virus, illustrate fusion of a double capsid particle to the cell (which occupies the lower portion of the field) and the presence of two capsids in the cytoplasm just beneath the cell wall. Note in the latter instance the dense granular material, which presumably represents part of the viral envelope close to the site of penetration. In Fig. 11, the lower particle has fused with the cell while the upper one is intact. It may be supposed either that the latter lodged at this site just before the preparation was fixed and hence digestion had not begun or that the plane of section does not pass through the site of attachment. Figure 12 shows a viral particle which had been treated with ferritin-conjugated antibody to the virus before addition to the tissue culture. Although the cell was not fixed until 4 hr after application of the virus, there is no clear digestion of the envelope adjacent to the cell on the left. (The probable reason for the somewhat "moth eaten" appearance of the envelope will be discussed at the conclusion of the
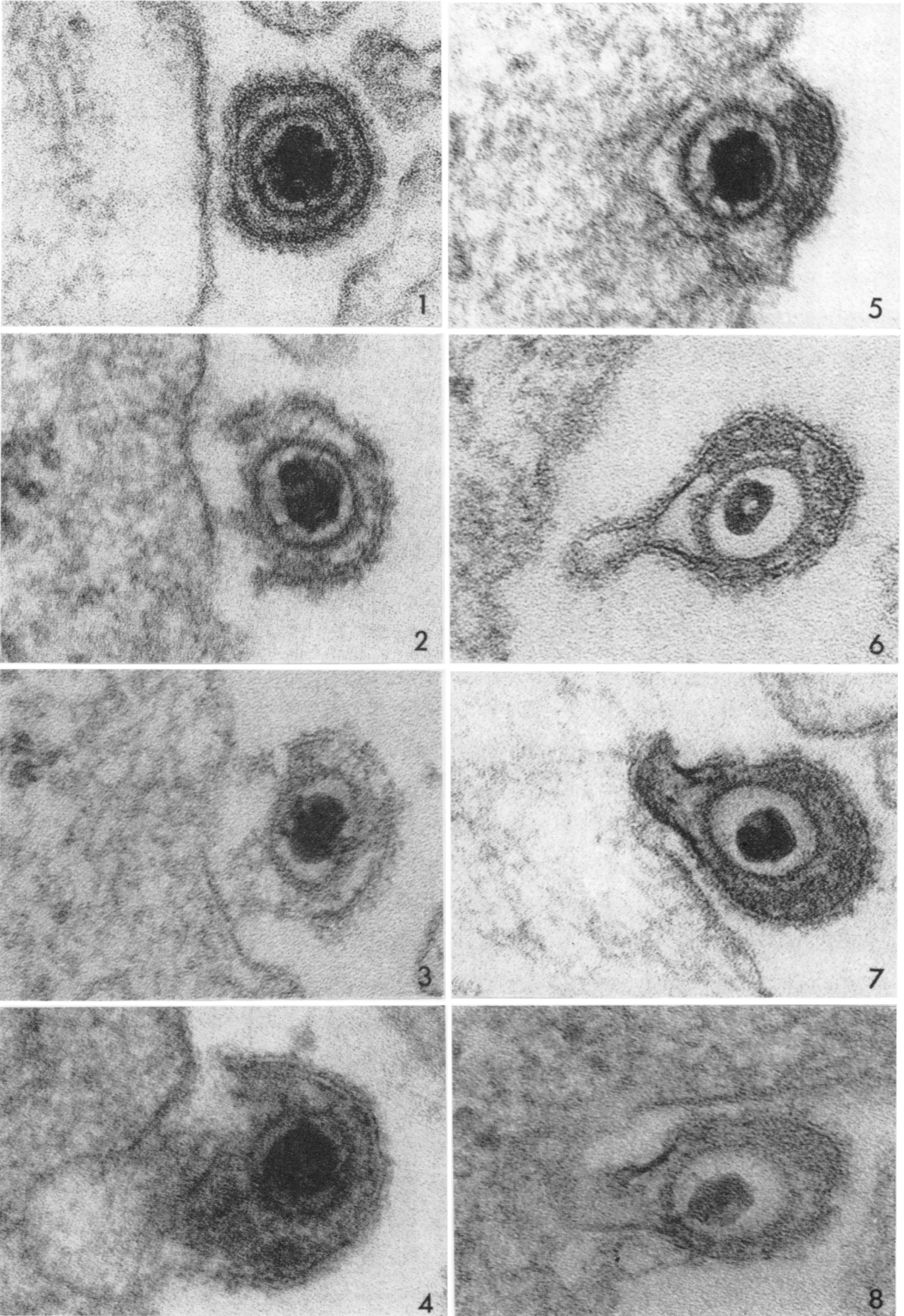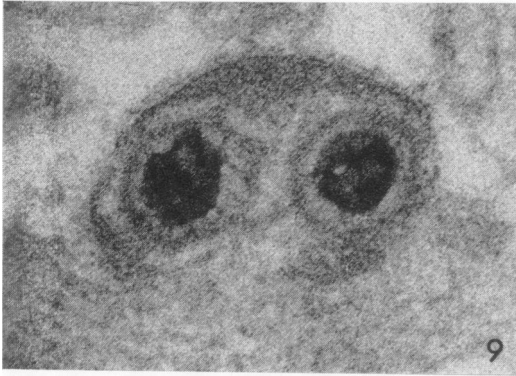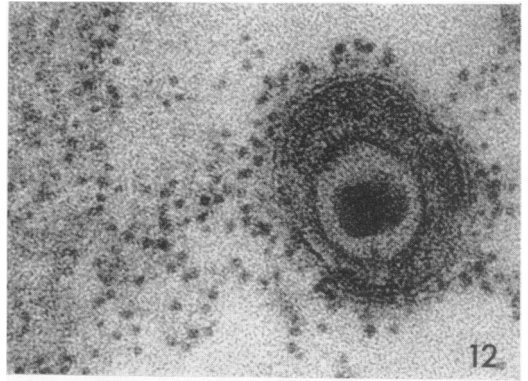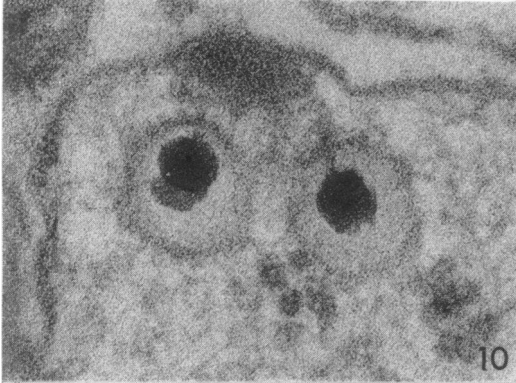
FIG. 1–5. *Stages in the attachment and digestion of the viral envelope at the cell surface.* $\times$ *180,000.*
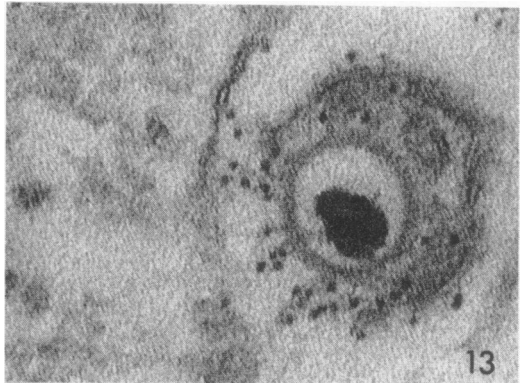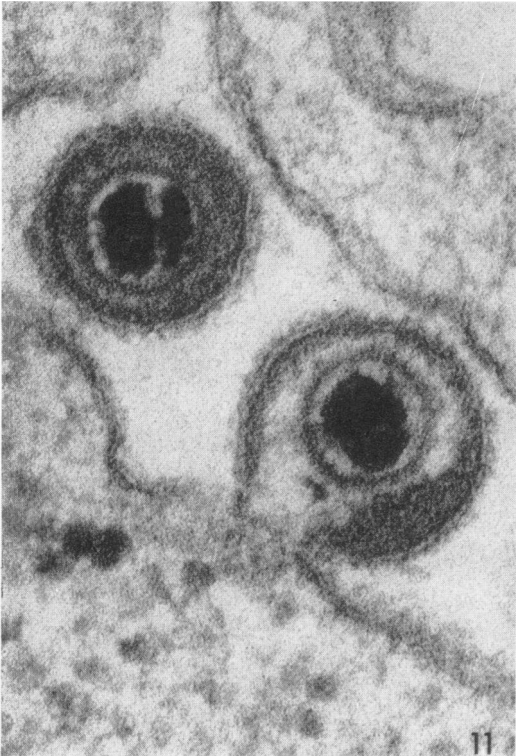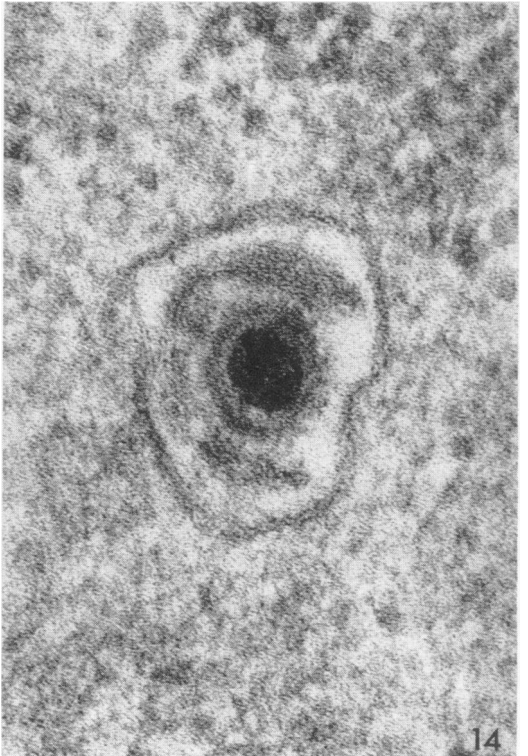FIG. 6–8. *Distorted viral envelopes at the cell surface.* $\times$ *180,000.*

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Fig. 9 and 10. *Fusion and entry of double capsid forms of the virus.* × 180,000.
Fig. 11. *Fusion of virus to the cell wall.* × 180,000.
Fig. 12. *Virus with ferritin-conjugated neutralizing antibody.* × 180,000.
Fig. 13. *Virus with ferritin-conjugated antibody specific for FL cells.* × 180,000.
Fig. 14. *Virus within a phagocytic vacuole 2 hr after adding virus to the tissue culture.* × 180,000.

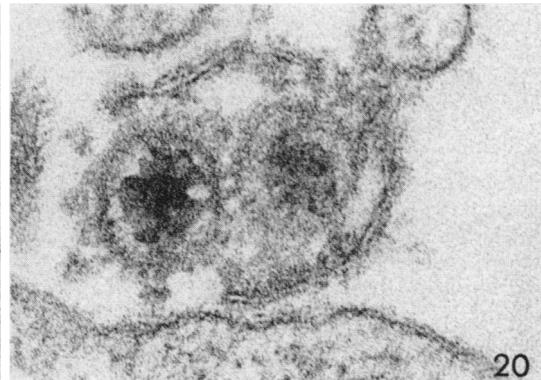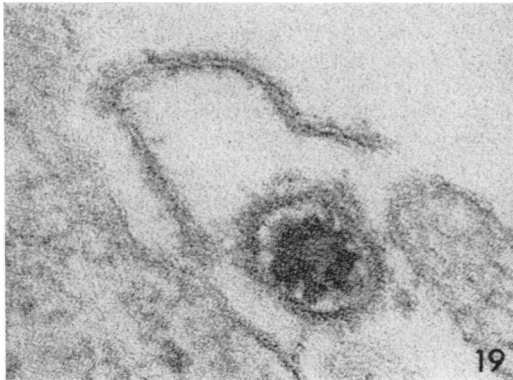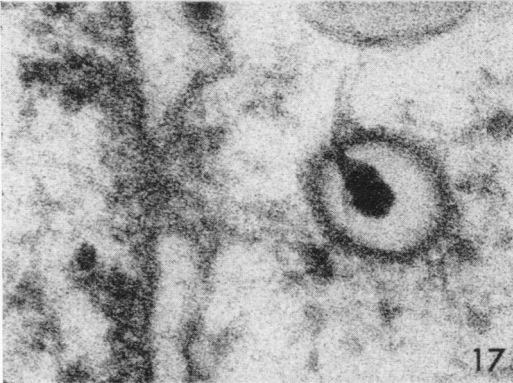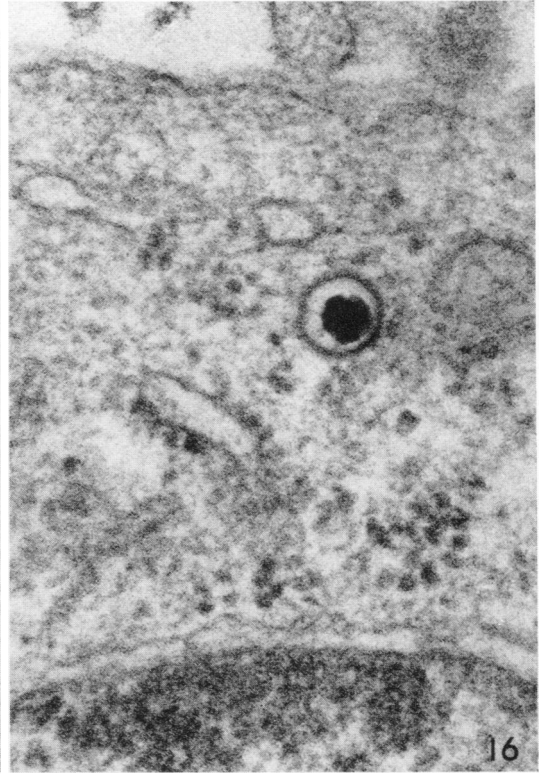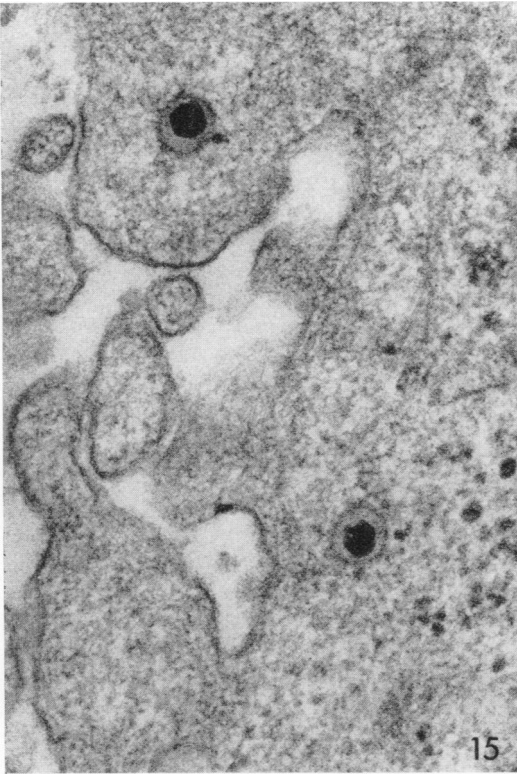Fig. 15. *Two capsids within the cytoplasm near the cell surface.* × 85,000.

Fig. 16. *A capsid within the cytoplasm about half way between the cell surface at the top and the nucleus at the bottom.* × 110,000.

Fig. 17. *A capsid close to the nucleus at the left. There appears to be continuity between the cytoplasm and the nuclear matrix.* × 180,000.

Fig. 18-20. *Stages in the disintegration of capsids at the cell surface. Two micrographs illustrate twin capsids. In each case the cell wall has remained intact.* × 180,000.

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next paper in this series.) It should be emphasized at this point that very little virus treated with antibody was encountered at the cell surface, and a prolonged search was necessary to find five such particles. Figure 13 illustrates a particle after a large excess of ferritin-conjugated antibody to FL cells had been added to the virus at the time of inoculation. Digestion of the envelope and cell wall is evident. It is of interest that ferritin is scattered between the capsid and envelope, indicating that this portion of the virus has become permeable, as might have been suspected from examination of Fig. 2 and 3.

Figure 14 illustrates virus within a phagocytic vacuole, 2 hr after addition of virus and 1 hr after the cells had been washed. The viral envelope has been partially digested but the capsid and wall of the vacuole appear to be intact.

In Fig. 15, two intact capsids enclosing dense cores lie within the cytoplasm just beneath the irregular cell surface, which is evident on the right. Figure 16 shows an intact capsid half-way between the surface of the cell at the top and the nucleus at the bottom. These two micrographs illustrate cells fixed 20 min after inoculation of virus. In Fig. 17, at 40 min, a capsid is in close proximity to the nucleus at the left. The nuclear membrane has disrupted and there seems to be continuity at this site between the cytoplasm and the nuclear matrix. Although no clear break in the capsid is evident, the elongation of the core and its fusion with the capsid at the upper left suggest that release was about to occur. (It is of interest in this connection that a small percentage of cores appear to be released without obvious disruption of the capsid. This phenomenon is currently under investigation.)

Figures 18–20 are believed to illustrate examples of envelope digestion without capsid penetration. The cells were fixed 2 hr after inoculation. Figure 19 and more particularly Figure 20 reveal stages in the disintegration of capsid and core at the surface of the cell. There is every reason to suppose that enzymatic digestion of the envelope would not always be accompanied by fusion with the cell. Under such circumstances, the capsid, devoid of its protective envelope, might be expected to disintegrate.

Figures 21 and 22, both taken at 40 min, show stages in digestion of the capsid. In the former, the envelope of a viral particle within a crypt appears to be undergoing digestion at one site. To the right is an intact capsid with attached dense material similar to that noted in the description of Fig. 10. Near the bottom of the field, the capsid has nearly disappeared, leaving the core free within the cytoplasm. Figure 22 shows an intact capsid, a capsid which has largely disinte-grated, and a free viral core. Capsids and cores were not found within the endoplasmic reticulum, suggesting that passage to the nucleus is haphazard and not the result of an ordered, channelled system of transport.

DISCUSSION

With regard to phagocytosis, cells would be expected to imbibe virus, since the process is nonspecific and acts on any particulate matter. Indeed, thorotrast introduced into the medium has been shown to be taken up by the same vesicles as the virus (13). Although phagocytosis cannot be excluded as the process whereby herpes simplex virus initiates infection, three observations would seem contrary to this concept. First, phagocytosis cannot explain the formation of the polykaryocytes, which result from cell fusion and will be discussed in detail subsequently. Second, it was far more common to encounter virus in process of digestion at the cell surface than to find it within phagocytic vacuoles. Third, whereas capsids and cores were repeatedly found free in the cytoplasm, stages in their release from vacuoles were not observed. This discrepancy was also noted by Holmes and Watson (13) as well as by Epstein et al. (11). The latter authors commented that "the stripped, naked, central portion of the virus was occasionally found in vacuoles but was more commonly free in the cytoplasmic matrix; the mode of transition between these sites could not be determined."

The first stage of entry would appear to be attachment of the viral envelope to the protein coating the surface of the host cell. Once this has occurred, antibody presumably cannot intervene. Attachment must occur rapidly, for the inability of antibody to prevent infection when added after adsorption of the virus (2) becomes evident within 10 min (17, 29). As previously noted, the scarcity at the cell surface of virus complexed with antibody is consistent with the generally accepted view that antibody interferes with attachment. The second stage, the beginning of which is evident by the 10th min, is characterized by disintegration of the viral envelope adjacent to the cell wall. The appearance of this phenomenon in the electron microscope and the probable temperature dependence of the reaction (17) are in accord with the concept that enzymatic digestion is responsible. The fact that the envelope of particles coated with specific antibody was not digested by the 4th hr indicates that antibody can act to block this second stage of entry. [Studies of Newcastle disease virus (28) suggest that the role of antibodies can be separated into two phases: the first acting on attachment, the second
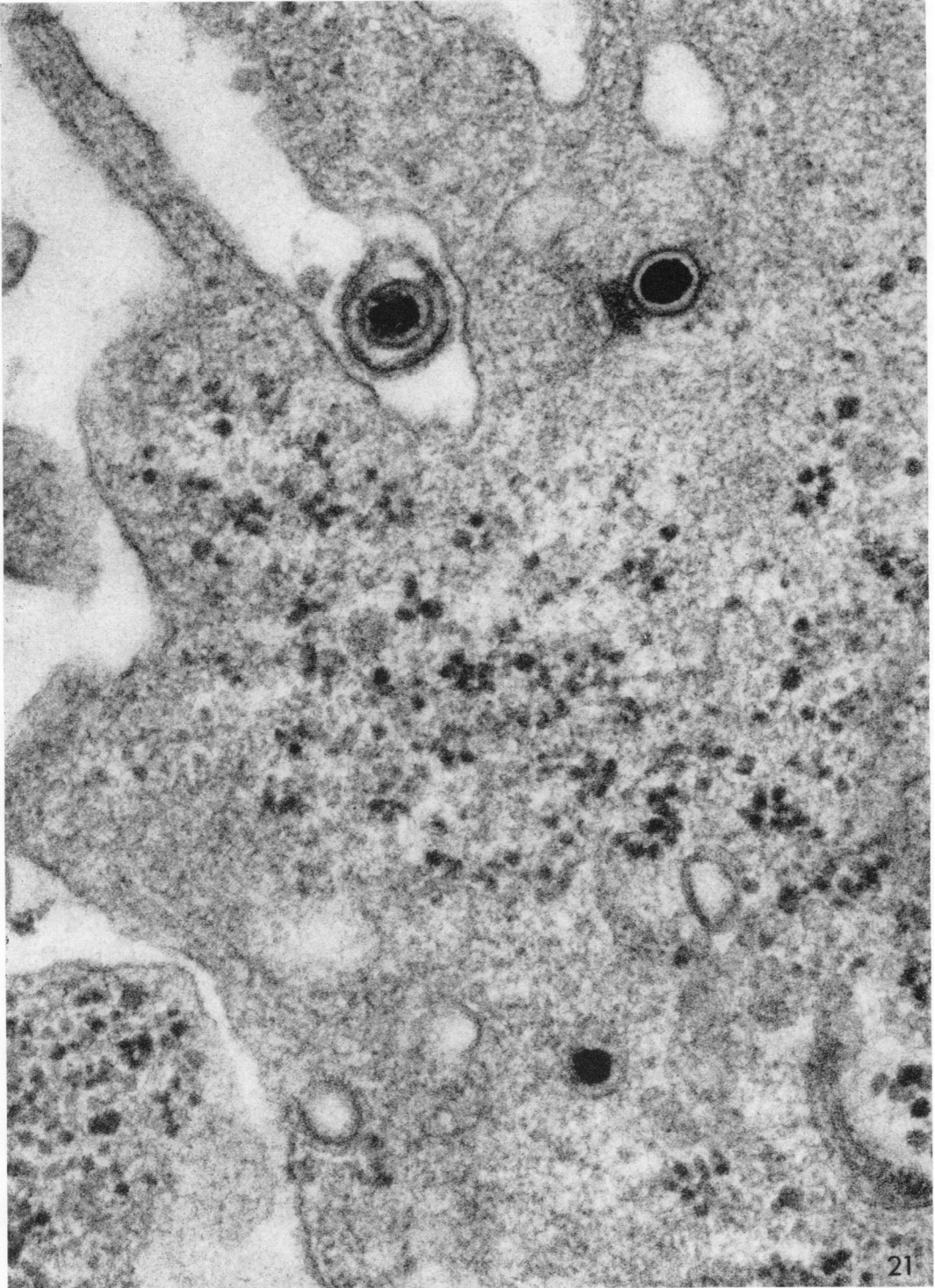
FIG. 21. *Three stages observed at 40 min. (a) Digestion of the envelope. (b) Presence of the capsid in the cytoplasm. (c) Digestion of the capsid.* × *110,000.*
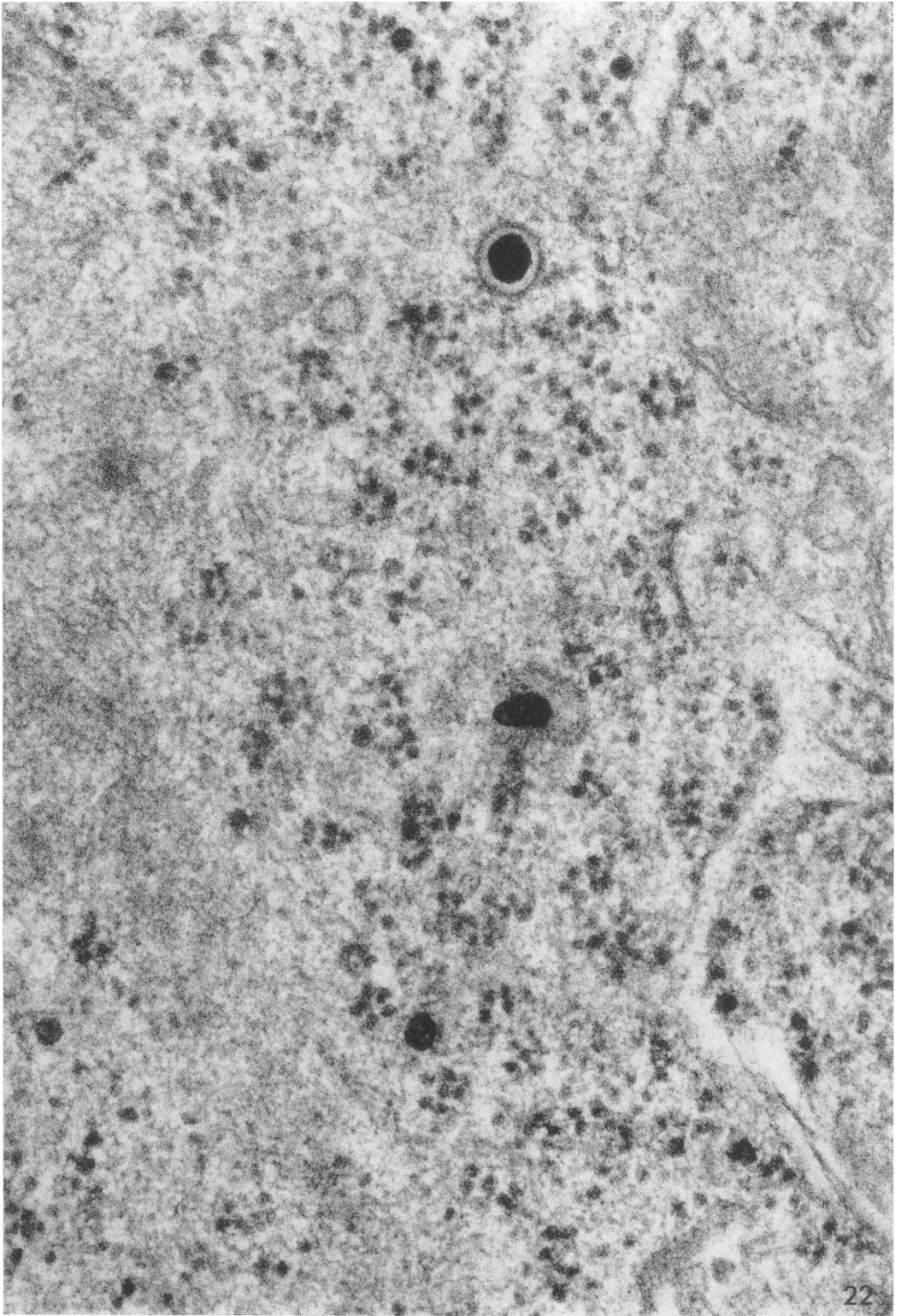
FIG. 22. *Three stages in release of the core from the capsid.* $\times$ *110,000.*

on penetration. Of these, the authors concluded that the second is "the basic mechanism of neutralization."] As digestion proceeds, the remnants of the viral envelope fuse with the cell wall. Subsequently, the wall of the cell also disintegrates, probably through the action of a second enzyme. Were such an enzyme a component of the virus, as suggested by Zhdanov and Bukrinskaya (34), one might expect that it would also be operative on the invaginated cell walls composing the phagocytic vacuoles. Such, however, was not the case, for, as noted above, the vacuoles containing virus exhibited intact walls. Presumably, then, an enzyme capable of digesting the cell wall is activated by the virus at the site of attachment. Finally, it is necessary to postulate the existence of a third enzyme capable of attacking the capsid within the cytoplasm. This enzyme probably appears between 20 and 40 min after inoculation of virus, since capsids were first encountered in process of disintegration in the third sample (taken at 40 min). From this point on, the core is lost to view because it becomes indistinguishable from other dense granules often present in the cytoplasm and nuclei. Extensive search failed to reveal any cores enclosed by capsids within nuclei. It should be pointed out that a variety of stages in the process of entry and release of deoxyribonucleic acid was encountered, even up to the 2nd hr, suggesting that attachment and penetration may continue for a considerable period of time under the conditions of these experiments.

The foregoing sequence of events is not dissimilar in broad outline to that found to occur in the case of poxviruses. Joklik (18) reported that there are two stages in the uncoating of the viral genome. There is immediate removal of coat protein, together with breakdown of the phospholipid. Protein synthesis was not found to be necessary for this first stage. After a lag of about 1 hr (the duration depending partly upon the multiplicity of infection), the deoxyribonucleic acid is liberated from the viral core. Joklik postulated "that a certain pox-virus protein molecule (the viral inducer protein) causes the host cell genome to code for a protein (the uncoating protein) which liberates the viral genome."

The question naturally arises as to why others have not observed the process of herpes viral entry described in this communication. One explanation may be that the event occurs quickly and thus can be easily missed. (A laborious and extensive search was necessary to record the sequential stages reported here.) Another explanation may be that it has been seen but not recognized. For example, particle i in Fig. 4 and the unmarked particle in Fig. 7 of the micrographs illus-

trating the paper by Epstein et al. (11) show viral envelopes which clearly seem to be undergoing disruption at the cell surface.

If herpes simplex virus initiates infection by the process herein described, it might be expected that other viruses, particularly those with ether-sensitive, lipoprotein coats might act in a similar manner. The work of Hoyle and Finter (16) on influenza virus and of Zhdanov et al. on Sendai virus (35) has been mentioned. More recently, negative staining and analysis of ^{32}P-labeled virus were employed to define the reaction of influenza virus with fragments of particles obtained from normal chorioallantoic membranes (14). In 1962, Hoyle (15) summarized the results of these studies as follows: "Immediately after adsorption of virus to the N.C.P. (normal cell particle) surface an interaction takes place between the lipoprotein envelope of the virus and that of the normal cell particle. The experiments with ^{32}P-labeled virus indicate that phospholipid becomes displaced from the virus lipoprotein envelope. In the electron microscope, the virus envelope shows a patchy disintegration, and many of the particles appear to burst and release their inner component. The normal cell membrane may also show disintegration as a result of contact with the virus." As was noted previously, the electron microscopic studies of Newcastle disease virus by Adams and Prince (1) and by Meiselman et al. (20) are entirely consistent with his hypothesis.

In conclusion, it should be emphasized that the purpose of this paper is not to deny the possibility that phagocytosis may play a role in the process whereby certain viruses gain entry and initiate infection, but rather to propose an alternative mechanism. Electron microscopic examination alone cannot define which of two viral particles entering by two different mechanisms actually infects the host cell. Studies employing other methods are now in progress in the attempt to isolate and characterize the enzymes involved and thus shed further light on the process of entry.

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