

STRUCTURE AND DEVELOPMENT OF VIRUSES AS OBSERVED
IN THE ELECTRON MICROSCOPE

VII. INCOMPLETE INFLUENZA VIRUS*

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Previous electron microscopic studies have revealed stages in the differentiation of influenza virus at the surface of the host cell (1). Since the viral particles appear to acquire their peripheral coat during the process of emergence, it might be expected that viral antigen would be present on the cellular surface. Such, indeed, is the case. Hoyle (2), employing the darkfield microscope, reported that cytoplasmic protrusions were shed from the surface of infected chorioallantoic membranes and suggested that these detached fragments could adsorb to the surface of red blood cells. Watson and Coons (3), studying amniotic cells of chicken embryos treated with fluorescent antibodies to influenza virus, noted that "staining of the cytoplasm appeared to be brightest along the cell walls in direct contact with the infected extraembryonic fluid." Liu (4), using fluorescent antibody absorbed with soluble antigen, observed fluorescence of the free border of infected ferret nasal epithelium. Holtermann *et al.* (5), after fluorescent staining of infected beef embryo kidney tissue cultures, saw an "accumulation along the periphery of the cells." Hotchin *et al.* (6), in an electron microscopic study of hemadsorption (7), encountered erythrocytes in direct contact with the surface of infected monkey kidney cells, and proposed that "after infection with influenza virus the host cell surface undergoes a change in its immunologically specific structure, rendering the surface capable of forming an attachment to red cell membranes analogous to that shown by mature infectious viral particles."

The preceding observations have been confirmed and extended by the use of ferritin-conjugated antibody (8), which permits the location of individual antibody molecules to be visualized by electron microscopy. Antigen was found to be present not only at sites where characteristic virus was in process

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of development and release, but also in regions of the cellular surface that were devoid of viral particles. The hypothesis was presented that detached cytoplasmic fragments coated with viral antigen might constitute the incomplete virus which results from the use of concentrated inocula (9-11). In order further to explore this possibility, chorioallantoic membranes infected with undiluted inocula in serial passage according to the method of von Magnus (12) were treated with ferritin-conjugated antibody and examined in the electron microscope. The results of these experiments are reported and discussed in this paper.

Materials and Methods

The initial inoculum of undiluted chorioallantoic fluid was obtained from 13-day-old chicken embryos infected 48 hours previously with approximately 1000 EID₅₀ of influenza virus, strain PR8. 0.2 ml of this undiluted fluid was inoculated into each of five 11-day-old embryos by the chorioallantoic route and aliquots of the fluid were removed and pooled after 24 hours' incubation at 37°C. The pooled fluid was found to have an EID₅₀ of 10^{8.9} and a hemagglutinin titer of 10^{8.3} (I/HA = 5.6). After two additional serial passages in the same manner the EID₅₀ of the chorioallantoic fluid had fallen to 10^{6.8} and the hemagglutinin titer was 10^{2.7} (I/HA = 4.1). For electron microscopic examination the chorioallantoic membranes were harvested after the first and third passages of undiluted virus. The membranes, treated by immersion in ferritin-conjugated antibody globulin, were washed, fixed in osmium tetroxide, dehydrated, and embedded in methacrylate, according to methods previously described (8).

For the examination of concentrated virus a portion of the initial inoculum was centrifuged at low speed for 30 minutes and the supernate was then centrifuged at 100,000 × G for 1 hour. Microdrops of the pellet suspended in distilled water were placed on formvar-coated screens. A dilute solution of phosphotungstic acid was added and the preparations were allowed to dry. Negative staining of sections of chorioallantoic membrane was accomplished by putting a drop of phosphotungstic acid solution on sections which had previously been mounted on formvar-coated screens and cleared of methacrylate by immersion in amyl acetate.

RESULTS

Figs. 1 to 3 illustrate chorioallantoic membranes harvested after the first passage of undiluted virus. Fig. 1¹ shows part of the surface of two contiguous cells. The intercellular boundary is marked by the parallel membranes traversing the lower half of the field diagonally. On each side are deep indentations of the cellular surfaces extending toward the lower right corner. Viral particles at various stages of differentiation and release are scattered among cytoplasmic fragments, some of which appear to be in process of detachment. Although the virus and the globules of cytoplasm possess similar surface configuration, namely a sharply defined limiting membrane and diffuse outer coat, they differ in other structural features. The viral spheres are generally smaller and more uniform in shape and they contain dense internal components. The three particles at the top of the picture are probably cross-sectioned viral filaments

¹ Figs. 1 to 4 and 6 are reproduced at the same magnification to facilitate comparison.

with interiors of low density. The viral particles vary in the extent to which they have been tagged with ferritin-conjugated antibody, those associated with the cell on the right generally having more ferritin granules on their surface than do those on the left. Some of the cytoplasmic fragments are not tagged, but close inspection reveals that others do exhibit small numbers of ferritin granules at their surface. Viral antigen on the cellular surface is largely confined to sites where virus is emerging. Near the top of the picture, however, ferritin is adherent to portions of the surface which are devoid of viral particles.

In Fig. 2 the surface of the cell traverses the left border and the extracellular space is on the right. There are numerous typical viral particles tagged with ferritin-conjugated antibody. The cytoplasmic fragments are pleomorphic and differ in the number of ferritin granules attached to their surface. Serial sections would be necessary to determine whether any given fragment were connected to the cell at a site removed from the plane of this section, but one can assume, in view of the average length of the cytoplasmic protrusions, that those at some distance from the cell have become detached. It is of interest that three such fragments (see arrows) exhibit viral particles which appear to be in the process of emergence.

In Fig. 3 the extracellular space is at the top and to the right. Absolute identification of individual viral particles is difficult because they lie at various levels within the section, but four probable viral particles are indicated by arrows. Examination of the cellular wall reveals globules of cytoplasm, many of which appear to be at different stages of formation and release. It is evident that much of the surface of the cell and of the cytoplasmic globules contains viral antigen, as denoted by the presence of ferritin-conjugated antibody.

Figs. 4 and 5 illustrate cells from chorioallantoic membranes harvested after the third serial passage of undiluted virus. In Fig. 4 the undulant surface of the cell traverses the lower portion of the field. One probable viral particle is indicated by the arrow. At the lower right where the cellular wall is thick there is a suggestion of a double membrane and ferritin granules reveal the presence of viral antigen. To the left the cellular surface has been cut obliquely and consequently appears ill-defined. There are numerous cytoplasmic fragments, the majority of which are heavily tagged with ferritin. Ferritin granules, which appear to be unassociated with structural components of the cell, are evident along the left border. Although displacement of the granules during preparation of the specimen could account for this phenomenon, the accurate localization generally encountered on the surface of the virus makes such an explanation unlikely. Rather, it appears that homogeneous material, which has low density and hence is difficult to see in thin sections, may accumulate on the surface of infected cells. Such material, clearly visible in very thick sections from which the methacrylate has been removed (unpublished data) as well as in some thinner sections with the methacrylate in place (see reference

13), is presumably permeable to the ferritin but viscous enough to hold detached cytoplasmic fragments and viral particles in place at some distance from the cellular surface. Scattered accumulations of antigen, adherent to this substance, would account for the presence of the ferritin-conjugated antibody at sites removed from any visible structure.

In Fig. 5 the surface of a cell is shown at higher magnification than in the preceding micrographs. No virus is seen but there are numerous particles containing material that closely resembles the cytoplasm of the cell. These particles exhibit a sharply defined limiting membrane and diffuse peripheral coat, in and on which the ferritin-conjugated antibody is bound. Two small intracytoplasmic vesicles are indicated by arrows. Although vesicles of this sort generally possess a limiting membrane, their random distribution and failure to accumulate at the free cellular border makes it unlikely that their extrusion accounts for an appreciable number of the extracellular particles.

Fig. 6 illustrates a negatively stained preparation of the inoculum used for the initial passage. The relatively uniform, spherical particles are presumably virus, their large size probably resulting from suspension in distilled water and flattening during the process of drying. Interspersed among the viral particles are larger, pleomorphic forms. In Fig. 7, which shows part of the preceding micrograph at twice the magnification, the radial projections of the surface component described by Horne *et al.* (14) and Hoyle *et al.* (15) are seen on most of the particles, even those which clearly are not typical of virus. Unconjugated ferritin granules, consisting of a dense iron core and protein coat,² are scattered through the field. These were added to the suspension before drying in order to compare the size of the ferritin with the surface units of the virus. The inset shows part of a section stained with phosphotungstic acid. The surface of the cell passes horizontally across the bottom. Removal of the methacrylate has resulted in distortion of fine structure, but part of a viral sphere is evident at the left. Three filamentous forms seem to be in process of emergence, the cytoplasm of the cell being continuous with the filament on the right. The disposition of the stain at the surface of the virus suggests the presence of radial projections.

DISCUSSION

Examination of Figs. 1 to 5 reveals that the cytoplasmic fragments or globules on the cellular surface vary in size and shape but that the majority of them have similar structure, which is characterized by an interior of low density, a sharply defined limiting membrane, and a diffuse peripheral coat containing viral antigen. Such particles, devoid of recognizable internal viral components but with surface characteristics closely resembling the virus, might be expected

² Only the core is visible in sections. The protein coat accounts for the encapsulated appearance of the granules in Fig. 7.

to exhibit the properties of incomplete virus, namely, to possess relatively little soluble antigen (16-18) and ribonucleic acid (19, 20); to be largely, if not entirely, non-infectious (9-12); to have a high lipid content (17, 21); to adsorb to and elute from erythrocytes (22); and to provoke neutralizing and hemagglutinin-inhibiting antibodies (22). It is of interest in this regard that morphologic evidence supporting the similarity between the cytoplasmic fragments and incomplete virus is provided by electron microscopic examination of purified preparations. Dried-down suspensions of incomplete virus contain particles which are pleomorphic, generally larger than infectious virus and flattened in appearance, often resembling empty sacs (18, 23-27). Moreover, many of the forms observed in sectioned pellets (28) closely resemble the particles illustrated in this paper. Finally, in an electron microscopic study of purified influenza virus stained with phosphotungstic acid, Horne *et al.* (14) reported that in the case of incomplete virus "there was great variety in shape and size of the structures seen, but all retained the outer membrane characteristic of the virus." The foregoing considerations lead us to the conclusion that the particles or globules of cytoplasm which are coated with viral antigen but which exhibit centers of low density are in fact incomplete virus, and they will be so designated throughout the remainder of the discussion. It should be emphasized at this point, however, that the term "incomplete virus," which has been adopted here in order to conform with current usage, does not imply that the particles necessarily are precursors of the virus.

Although the limited data obtainable from electron microscopic examination of thin sections preclude quantitative comparison, the results are in consonance with those of von Magnus (12), since incomplete forms varied considerably in proportion from cell to cell after the first passage of undiluted virus (Figs. 1 to 3), whereas they were encountered almost exclusively after the third passage (Figs. 4 and 5).

Regarding the intracellular events which lead to the formation of incomplete virus, Lief and Henle (29) found that soluble antigen was synthesized but did not appear subsequently to be incorporated into the particles. This observation is in agreement with the results of immunofluorescence studies by Breitenfeld and Schäfer (30), who examined cells at sequential stages after infection with fowl plague virus, which closely resembles influenza virus in its mode of development (31). Under conditions in which infectious virus was being formed they found that the soluble antigen appeared first in the nucleus and later in the cytoplasm, whereas the hemagglutinin was confined to the cytoplasm. This led them to suggest that soluble antigen diffuses from the nucleus into the cytoplasm where it combines with hemagglutinin during differentiation of infectious virus. The same sequence of events was seen in the case of influenza virus by Holtermann *et al.* (5) and Traver *et al.* (32). However, in cells producing incomplete fowl plague virus, which also appears to be similar to incomplete influenza virus (33, 34), Franklin and Breitenfeld (35) reported that "the soluble antigen remains confined to the nucleus during the latter stages of infection and, hence, cannot unite with hemagglutinin to form infectious particles." Hillis *et al.* (36) ob-

served the same phenomenon in a study of influenza virus-infected HeLa cells, which, as Henle *et al.* (37) have shown, release incomplete virus. The question arises as to whether the incomplete particles are viral precursors which accumulate as a result of a block at some stage in the synthesis of infectious particles. This seems not to be the case, however, for as Burnet (38) has pointed out, there is no change in the proportion of infectious virus to hemagglutinin at various times after infection (12), incomplete and complete virus being liberated synchronously (39). Moreover, it is difficult to believe, when the morphology of the two forms is compared, that incomplete virus was at a stage of transition into the complete particle. One can only conclude, therefore, that incomplete virus is produced by a mechanism of synthesis which differs in some way from that of the infectious virus.

As has been noted, the mechanism whereby incomplete virus is formed appears to be detachment of cytoplasmic fragments. The shedding of fragments or "blisters" of variable size from the surface of cells is a common phenomenon (40) quite unrelated to viral infection *per se* (41). Rupture of the cell does not necessarily result, because the stalk of cytoplasm connecting the bleb to the cell pinches or closes off at the site of detachment. Globules of membrane-bound cytoplasm are thus cast off into the extracellular fluid. When, as a result of infection, the surface of the cell is transformed so as to contain viral antigen, such detached fragments of cytoplasm will exhibit the characteristics of incomplete virus. Hoyle (2), by means of the darkfield microscope, observed cytoplasmic fragments budding off normal chorioallantoic membranes and noted that after infection with influenza virus the process became more pronounced but did not otherwise differ. One should expect, therefore, the formation of incomplete virus, even under conditions optimal for the synthesis of infectious virus, and this seems actually to occur (42-46, 8). In view of the foregoing, we wondered whether the uniformity of particle size repeatedly seen in published electron micrographs might not reflect either loss of the larger component during the procedure of purification or judicious selection of the picture to be reproduced. Accordingly, it was decided to search a purified preparation of the initial inoculum, which had been obtained by the passage of dilute seed virus, in order to ascertain whether there were particles of incomplete virus. As can be seen in Fig. 6, large, pleomorphic forms, which appear to be detached fragments of cytoplasm, were found scattered among the viral particles. The presence of surface structure (Fig. 7) indistinguishable from that acquired by the virus (14) during the process of release (inset to Fig. 7) strongly suggests that the large particles are incomplete virus, presumably formed by the same mechanism of cytoplasmic detachment that appears to operate in the presence of undiluted inocula.

The problem arises as to how the undiluted inoculum exerts a twofold effect, namely, to suppress the formation of infectious virus without appreciably altering the release of incomplete virus. A partial answer, at least, may be provided by the following hypothesis. As Horsfall has suggested (47), the concentrated inoculum is toxic and causes progressive damage to many of the cells. Consequently, although the early stages of viral synthesis relating to the production of soluble antigen and hemagglutinin are initiated, the final assembly of components into infectious particles cannot be carried out due to increasing impairment of the metabolic functions of the cell. On the other hand, the toxic action of the inoculum does not inhibit the protrusion and

detachment of cytoplasmic fragments. Indeed, under a variety of circumstances cellular injury appears to stimulate this process (2, 40, 41). If, as seems likely, the formation of hemagglutinin is accompanied by its accumulation at the surface of the cell, then the shedding of membrane-bound cytoplasmic fragments will result in the continuing production of incomplete virus. The preceding explanation does not, of course, exclude the possibility that other factors, such as interference (48), are also operating. Whether there are different grades of infectivity, as Burnet (38), Paucker and Henle (48), Burnet, Lind, and Stevens (49), and Barry (50) have suggested, remains to be proved, but it is not unlikely that some soluble antigen containing infectious ribonucleic acid could reach the cytoplasm near the surface of the cell and thus become incorporated into the protrusions. The filamentous forms of the virus may also be a type of incomplete particle because they lack internal structure (1), exhibit characteristic surface configuration (51, 52), and have viral antigen on their surface (evident in Fig. 1 and noted in a previous publication, reference 8). Filaments, however, do not appear to constitute the incomplete forms associated with undiluted inocula for they were not especially numerous in the thin sections examined, nor do they appear to make up a significant proportion of the particles seen in purified preparations (18, 23-28).

If the hypothesis regarding toxic action of the inoculum on the formation of incomplete virus is correct, it should apply to a variety of cell systems, and such appears to be the case. Henle *et al.* (37) found that the concentrated inocula used to initiate formation of incomplete influenza virus by HeLa cells were injurious to the cells and that hemagglutinin could be detected within these cells early in the course of infection, although it did not appear extracellularly until after cytopathic changes had become apparent in the light microscope. In a study of incomplete virus resulting from the injection of mouse brains with non-neurotropic strains of influenza virus, Schlesinger (53) commented on the toxic effects of the inoculum, and Werner and Schlesinger (23) found by electron microscopic examination that the resulting non-infectious particles "consisted largely of pleomorphic, flattened bodies which varied widely in diameter" and "were strikingly similar to virus recovered from allantoic membranes of 'undiluted passages' in eggs." Moreover, Mims (54) using fluorescent antibodies, also observed a toxic action of the inocula and reported that there was a single growth cycle during which cytoplasmic fluorescence tended to accumulate at the free surface of the ependymal and meningeal cells. Ginsberg (55), investigating the effects of unadapted strains of influenza virus on mouse lungs, discovered that the extent of pulmonary consolidation was related to the formation of incomplete virus and concluded that "host cell injury was essential to the development of non-infectious viral particles."

As previously mentioned, incomplete fowl plague virus closely resembles incomplete influenza virus, and recent evidence suggests that the same may be true for Newcastle disease virus (56). It is tempting to speculate that incom-

plete virus may be associated not only with other myxoviruses but also with those viruses which, although unrelated to influenza virus, appear to share in common a mechanism whereby surface components are acquired during differentiation at, or passage through, the cellular wall. The Rous sarcoma virus (57), the virus encountered in studies of spontaneous leukemia in mice (58), the virus of erythroblastosis of chickens (59), and Western equine encephalomyelitis virus (60) can be cited as examples of this group.

In conclusion, we suggest that the term "incomplete virus" as applied to the antigenically altered cytoplasmic fragments described in this report is probably a misnomer, especially because these particulate extrusions do not appear actually to be the precursors of virus, but rather to form in a somewhat different or aberrant manner.

SUMMARY

Chicken embryos were infected by the chorioallantoic route with influenza virus, PR8 strain, in the form of undiluted chorioallantoic fluid. Electron microscopic examination 24 hours after infection revealed that membrane-bound fragments of cytoplasm appeared to be in process of release from endodermal cells of the chorioallantois. The number of such fragments was greatly increased in proportion to the number of typical viral particles after the third serial passage, which was accompanied by a reduction of the infectivity-hemagglutinin ratio (von Magnus effect). The lack of recognizable internal components, together with the presence of surface structure which closely resembled that of the virus and frequently contained viral antigen, suggested that many of these fragments were incomplete viral particles. It is proposed that concentrated inocula damage the cells and interfere with differentiation of the virus, but do not inhibit formation and detachment of cytoplasmic processes. Under these circumstances the accumulation of viral antigen at the surface of the cell will result in the predominant formation of incomplete virus.

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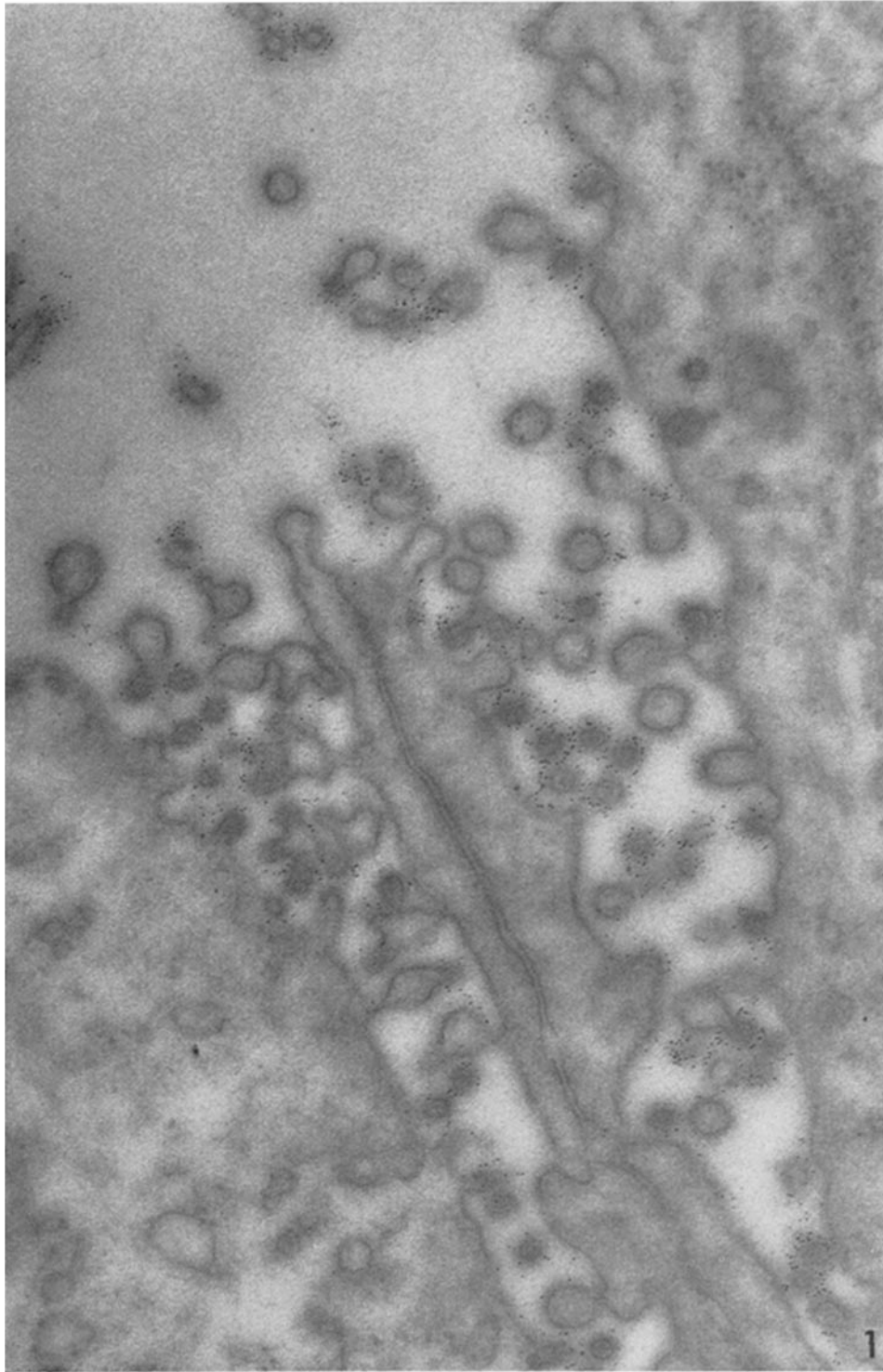
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EXPLANATION OF PLATES

PLATE 76

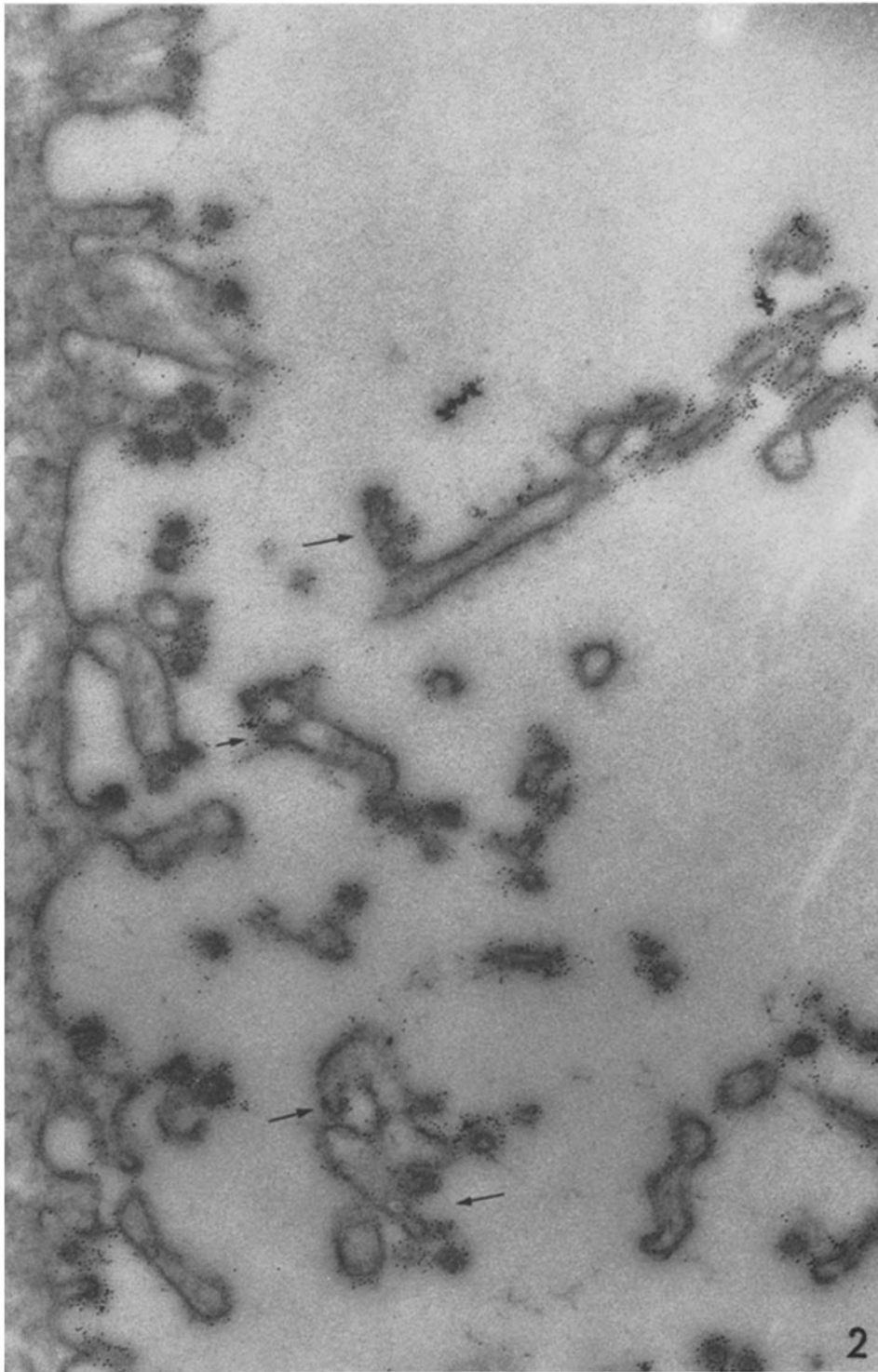
FIG. 1. The indented surface of two contiguous entodermal cells. The intercellular boundary is marked by the closely approximated, parallel membranes traversing the field diagonally. The small, dense, spherical, viral particles are tagged with ferritin-conjugated antibody. Three cross-sectioned filaments appear at the upper margin. The large, irregularly shaped particles of low density are believed to be cytoplasmic fragments. The surfaces of some contain small amounts of antigen as indicated by the presence of ferritin granules. This and the following two micrographs illustrate cells infected with first passage concentrated inoculum. $\times 63,000$.



(Morgan *et al.*: Studies of incomplete influenza virus)

PLATE 77

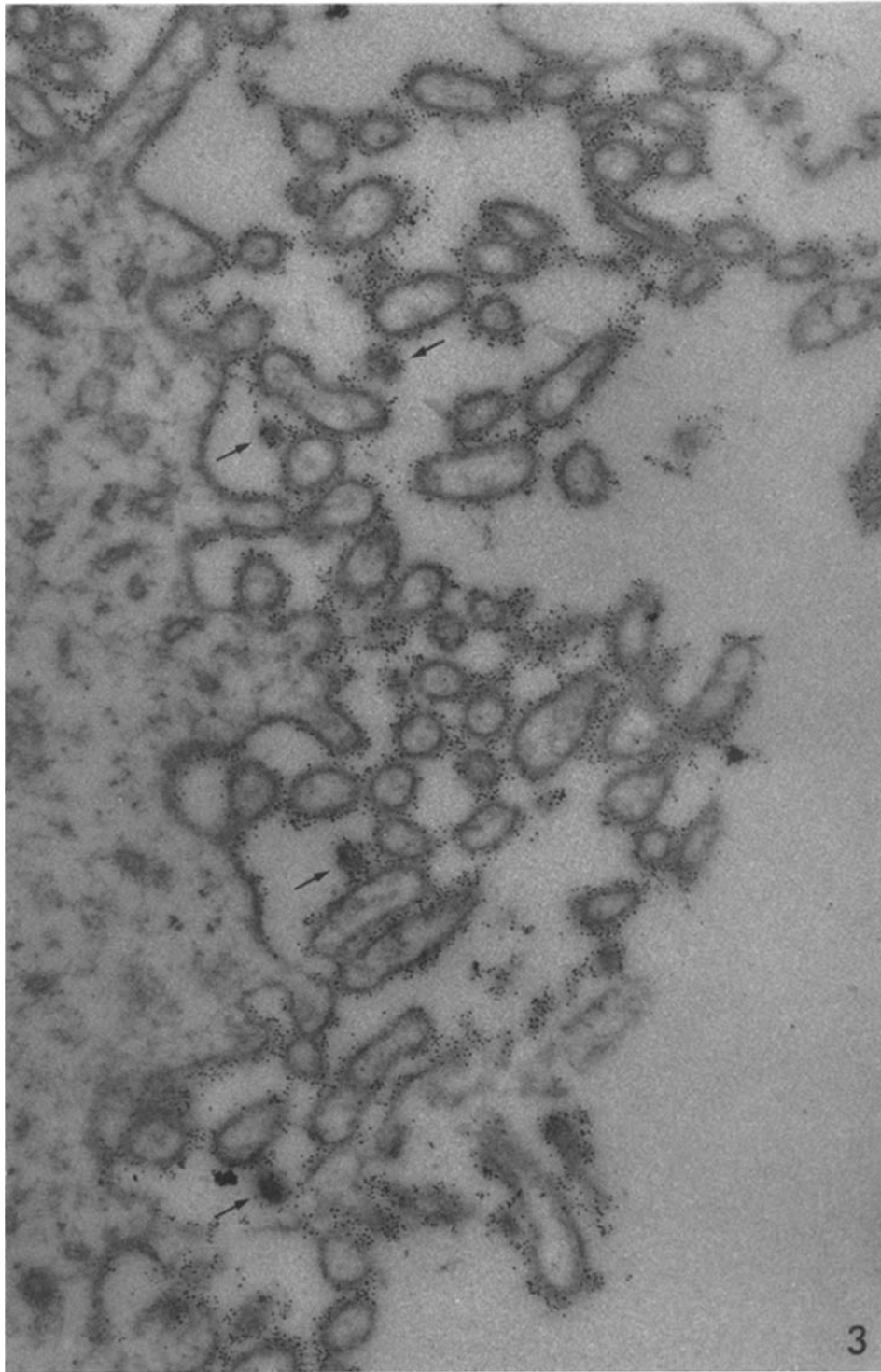
FIG. 2. The surface of a cell with many viral particles and cytoplasmic protrusions, which appear to be in process of release. The arrows indicate virus in differing stages of development at the surface of cytoplasmic fragments which presumably have become detached from the host cell. Ferritin reveals the presence of variable amounts of viral antigen on the cytoplasmic fragments. $\times 63,000$.



(Morgan *et al.*: Studies of incomplete influenza virus)

PLATE 78

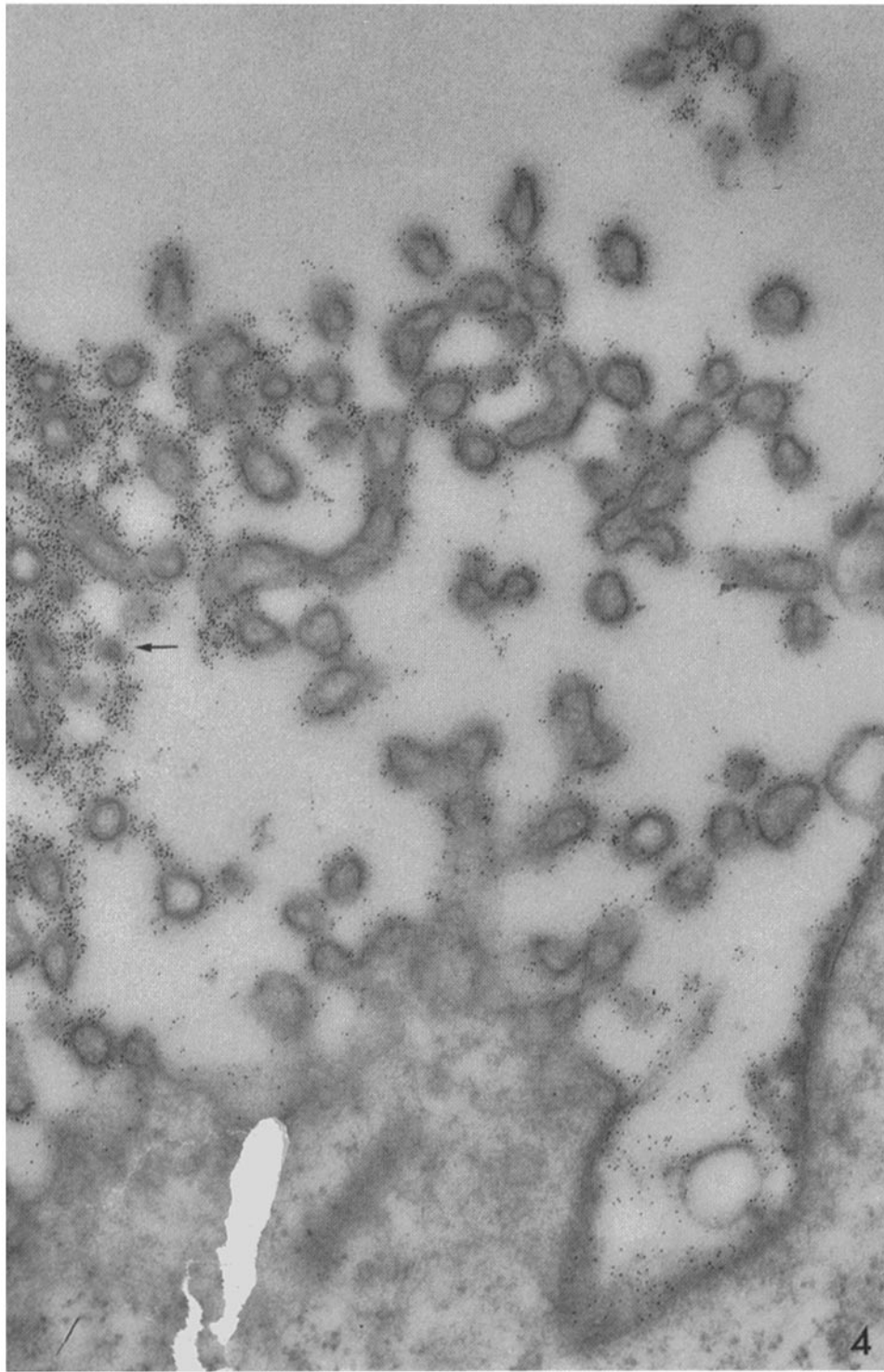
FIG. 3. Numerous cytoplasmic fragments, some of which seem to be in process of detachment. Much of the cellular membrane, as well as most of the particles, are heavily tagged with ferritin. Four probable viral particles are indicated by arrows. $\times 63,000$.



(Morgan *et al.*: Studies of incomplete influenza virus)

PLATE 79

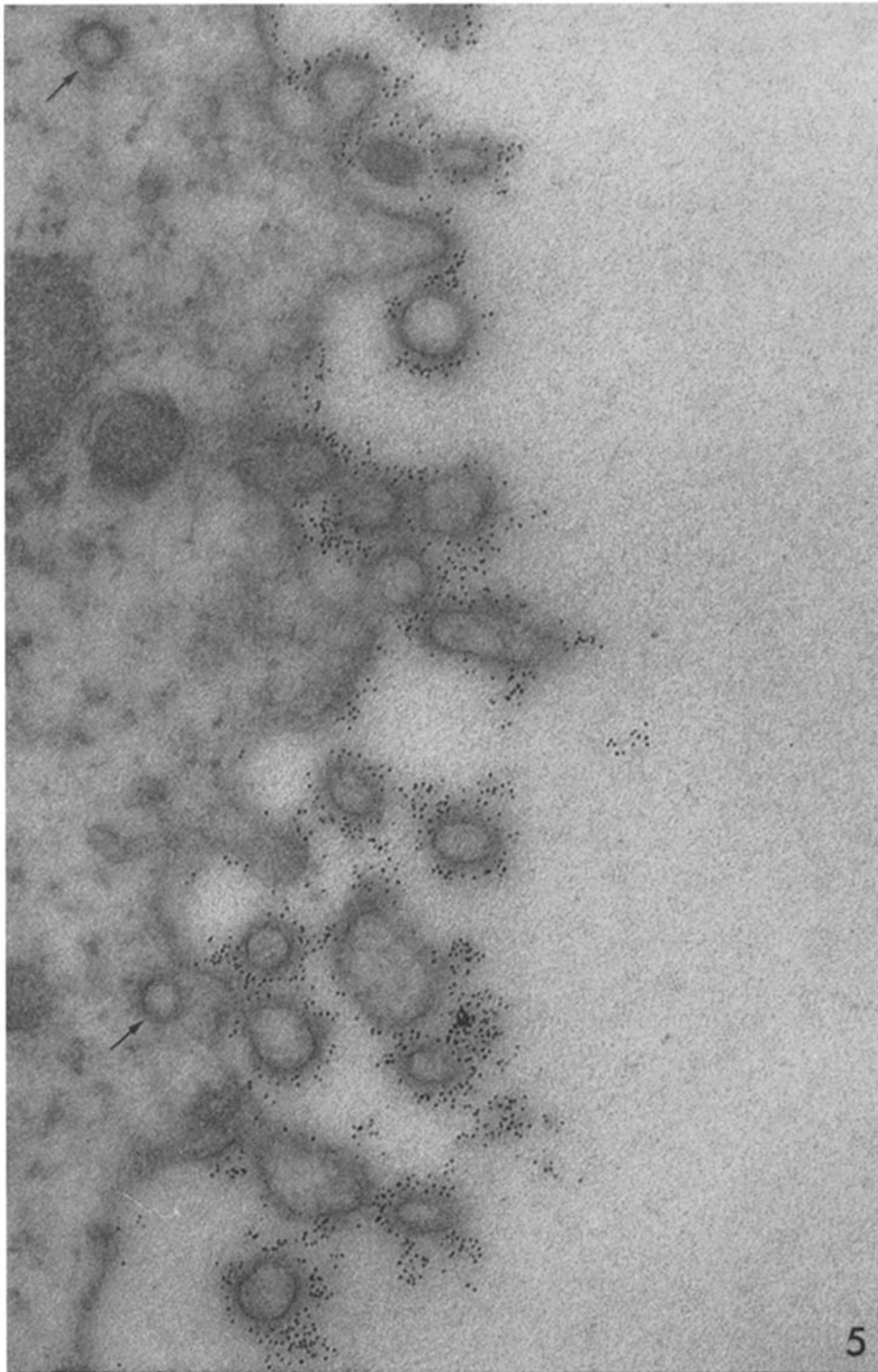
FIG. 4. Cytoplasmic fragments coated with viral antigen. The irregular surface of the cell appears to have been sectioned obliquely on the left and is thus poorly defined. The structure of one particle (see arrow) is typical of the complete virus. This and the next picture illustrate cells infected with third passage inoculum. $\times 63,000$.



(Morgan *et al.*. Studies of incomplete influenza virus)

PLATE 80

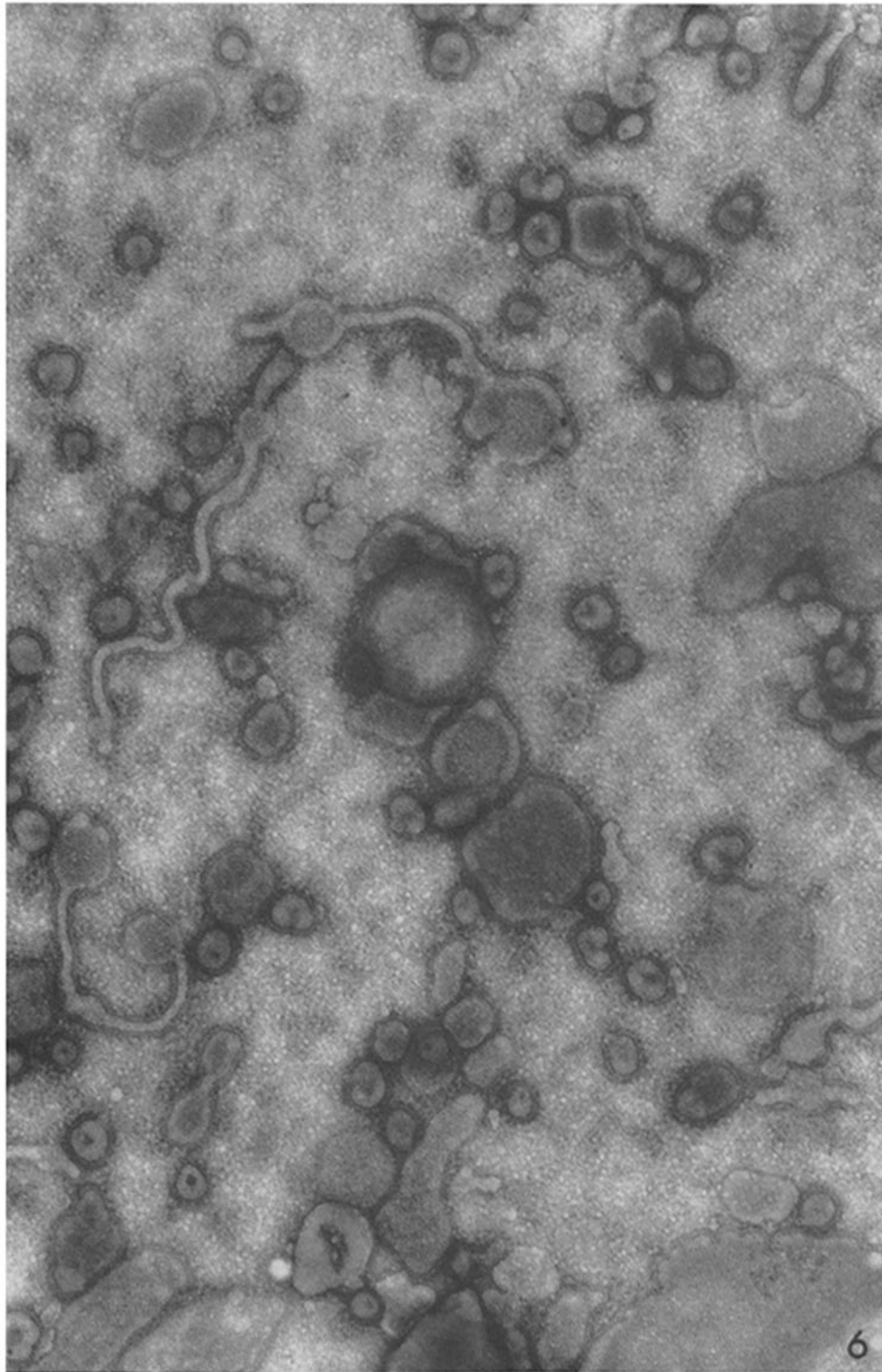
FIG. 5. Particles with diffuse coats containing viral antigen, sharply defined peripheral membranes and interiors of low density closely resembling the cytoplasm of the host cell. There are no viral particles. Two intracytoplasmic vesicles are indicated by arrows. $\times 95,000$.



(Morgan *et al.*: Studies of incomplete influenza virus)

PLATE 81

FIG. 6. A dried-down, negatively stained preparation of the original inoculum. Particles, presumed to be virus, are scattered among pleomorphic structures. $\times 63,000$.

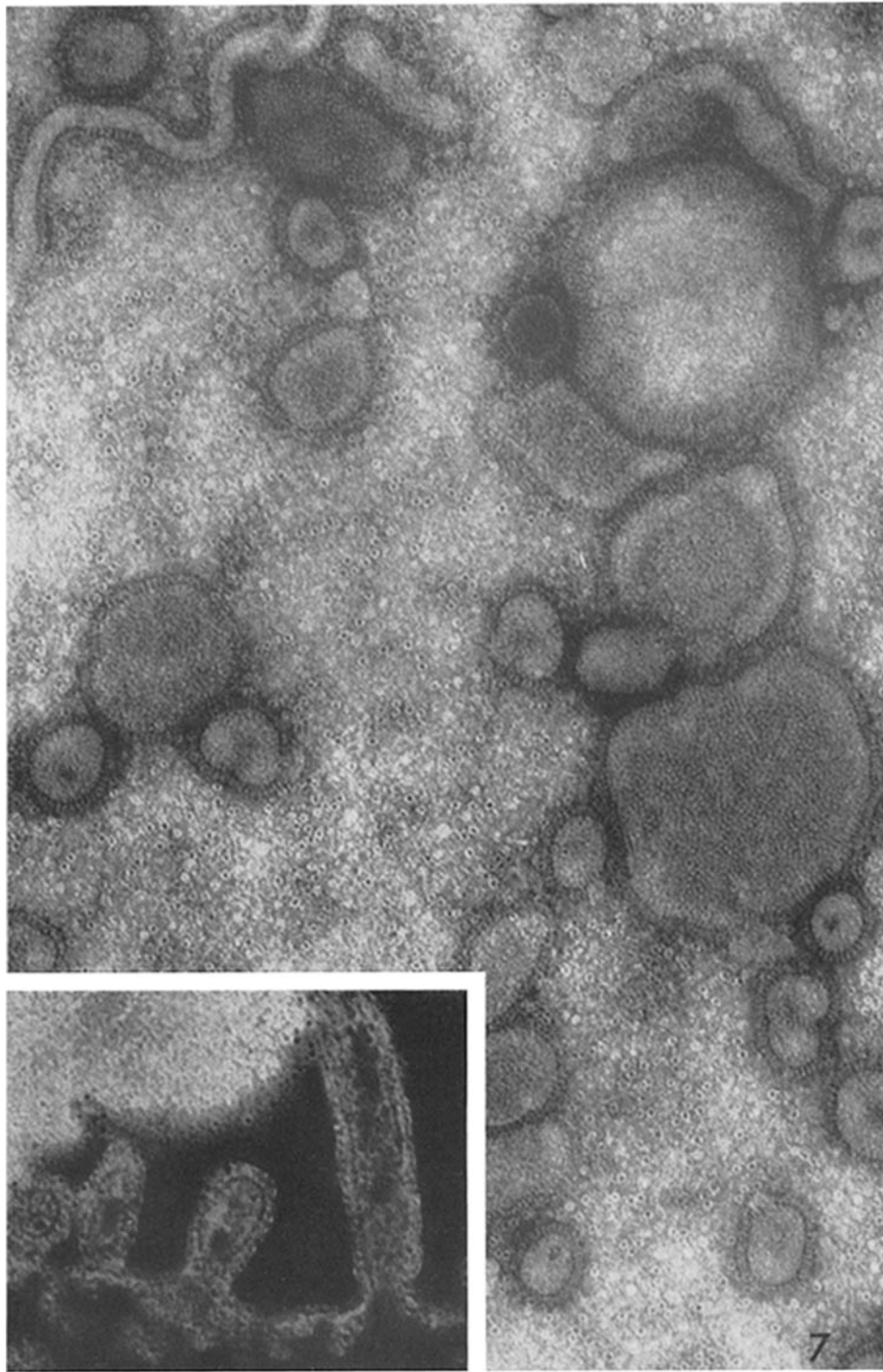


(Morgan *et al.*: Studies of incomplete influenza virus)

PLATE 82

FIG. 7. Part of the preceding micrograph reproduced at higher magnification. Most of the particles can be seen to exhibit the surface projections characteristic of the virus. Unconjugated ferritin granules, added to the suspension before drying, are scattered through the field. The protein, which coats the iron core of the ferritin, is rendered visible by the phosphotungstic acid stain. $\times 126,000$.

The inset shows a relatively thick section stained with phosphotungstic acid after dissolving out the methacrylate. Four viral particles are visible at the surface of the cell, which traverses the lower portion of the field horizontally. The interior of the viral filament on the right appears to be continuous with the cytoplasm of the cell. Although removal of the embedding plastic has caused considerable distortion of fine structure, surface projections are visible. $\times 115,000$.



(Morgan *et al.*: Studies of incomplete influenza virus)