# THE APPLICATION OF FERRITIN-CONJUGATED ANTIBODY TO ELECTRON MICROSCOPIC STUDIES OF INFLUENZA VIRUS IN INFECTED CELLS

## I. The Cellular Surface*

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It has been demonstrated (1) that ferritin conjugated with specific antibody globulin by the method of Singer (2) attaches to particles of influenza virus. In view of the fact that the viral spheres and filaments form at the surface of the host cell (3) immersion of infected chorioallantoic membranes in ferritin-conjugated antibody prior to fixation should permit visualization of the sites on the cellular membrane at which antigen appears during viral differentiation and release. The purpose of this paper is to illustrate and describe the use of this technique, to show that antigen is present at the surface of infected cells, and to propose an hypothesis regarding the nature of incomplete virus.

### *Materials and Methods*

*Virus.*—Eleven-day-old chicken embryos were inoculated by the allantoic route with approximately 1000 $EID_{50}$ of PR8 or Lee strains of influenza virus and were then incubated at 35°C for 48 hours.

*Antibody.*—Chickens were immunized by inoculating 5.0 ml intraperitoneally and 5.0 ml intravenously of chorioallantoic fluid from infected chicken embryos. Two weeks later the animals were exsanguinated and the blood serums were collected. Each serum was assayed for antibody by the hemagglutination-inhibition test, using 0.75 per cent chicken erythrocytes and 4 hemagglutinating units of virus.

*Ferritin conjugation.*—The globulin fraction was separated from the antisera by sodium sulfate precipitation (4) and was conjugated with ferritin by the method of Singer (2), employing *m*-xylylene diisocyanate, without appreciably altering the hemagglutination-inhibition titer. Before use the ferritin-conjugated globulin was absorbed overnight with acetone-extracted chicken embryo powder and clarified by low speed centrifugation. In some instances ferritin-conjugated globulin was separated from unreacted globulin by centrifugation at

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100,000 *G* for 2 hours and resuspension of the resulting pellet in a small volume of phosphate buffered saline at pH 7.5. This procedure was suggested in a personal communication by Dr. S. J. Singer.

*Application of Ferritin-Conjugated Antibody to the Chorioallantoic Membrane.*—Small pieces of shell with attached infected chorioallantoic membrane were excised, washed briefly in Tyrode's solution, and immersed in ferritin-conjugated specific antibody globulin for 2 to 60 minutes.[1] The membranes were then peeled off the shell and washed in Tyrode's solution. Blocking experiments were carried out by placing the membranes in unconjugated antibody globulin for 1 hour, followed by ferritin-conjugated globulin for 2 minutes. Controls for the blocking experiments were exposed to non-specific antibody globulins before tagging with ferritin-conjugated specific globulin.

*Preparation for the Electron Microscope.*—After washing in Tyrode's solution the membranes were fixed for 20 minutes in buffered, isotonic osmium tetroxide, dehydrated in graded dilutions of ethyl alcohol, embedded in methacrylate, and sectioned on a Porter-Blum type microtome.

## RESULTS

Fig. 1 shows two contiguous areas at the surface of a cell infected with the PR8 strain of influenza virus and exposed to ferritin-conjugated antibody globulin. The cytoplasm of the cell borders the left margin of the field; the extracellular space is on the right. Two viral particles at the bottom left are shown again at the upper right. The relative density and definition of the cellular membrane reflects the angle at which it has been cut. The cytoplasmic protrusions obviously vary with respect to their orientation in the section, the base of one appearing at the upper left, part of another being visible in the upper right. At the lower right several irregular protrusions appear to have been transected at differing angles. Virus at various stages of development and release is scattered at the cellular surface. Serial sections have shown that the density and definition of the viral particles depend upon the level at which they lie within the section (3), those of greatest density and with a clearly defined internal membrane being central to the section. Ferritin granules are visible on the virus. The illusion that some granules are within the viral particles probably results from superimposition of ferritin on the surface of virus which has been cut eccentrically. It is noteworthy that the cellular membrane is largely free of ferritin. At the upper right, for example, a viral particle appears to be in process of budding from the tip of a cytoplasmic extension. The external surface of the virus has been tagged but the adjacent cellular membrane is devoid of ferritin granules. Little or no ferritin has penetrated the cell.

Fig. 2 illustrates the surface of a cell at higher magnification. The diffuse appearance of the cellular membrane probably reflects the oblique angle at which it has been sectioned. Several viral particles in the mid-third of the field

[1] Treatment for 1 hour was employed in the initial experiments (illustrated by Figs. 1 and 2). Subsequently, however, it was found that immersion for 2 minutes resulted in equally effective tagging.

seem to have been fixed during differentiation and emergence. The dense, sharply defined membrane of one particle passes downward toward the cell, an appearance consistent with a process of budding. Ferritin granules have tagged fully formed virus as well as two particles which appear to be at early stages of differentiation. It should be emphasized at this point that although characteristic filaments of PR8 virus are not illustrated they were repeatedly encountered and were found to be tagged with ferritin in the same manner as the spheres.

In Fig. 3 two viral particles with ferritin granules on their surfaces are visible at the tip of a cytoplasmic protrusion near the right margin. The particle farthest to the right is slightly removed from the host cell and ferritin is evident on the nearby cellular membrane. Toward the center of the field ferritin is distributed on the distal portion of another cytoplasmic protrusion, which appears to be free of virus. On the left are two small clusters of ferritin granules. Fig. 4 illustrates part of a cellular surface, most of which has been tagged with ferritin. On the right several cytoplasmic protrusions, also tagged with ferritin, appear to be in process of detachment. No characteristic viral particles are evident.[2]

Fig. 5 shows the result of a blocking experiment. No tagging of the virus on the surface of the cell has occurred. Fig. 6 illustrates a control for the foregoing experiment. The membrane was treated with non-specific globulin for 1 hour before immersion in ferritin-conjugated specific antibody globulin. There is characteristic localization of ferritin on the free surface of the virus, although very little ferritin has penetrated between contiguous particles. Fig. 7 shows the PR8 strain of influenza virus after immersion in ferritin-conjugated antibody globulin to the Lee strain, and Fig. 8 illustrates the reverse procedure, namely, Lee virus treated with ferritin-conjugated antibody globulin to PR8 virus. In the latter picture two filaments appear to be in process of budding from the tips of cytoplasmic projections. Two other filaments toward the center have been obliquely sectioned. No appreciable tagging with ferritin is evident.

In Fig. 9 a large vacuole lies within the cytoplasm of an infected cell. The nucleus occupies the lower left and an adjacent entodermal cell the lower right. On the free surface bordering the allantoic cavity at the upper left are scattered viral particles which, at higher magnification, were seen to be tagged with ferritin. Fig. 10 pictures the upper right margin of the vacuole at higher magnification and reveals that the viral particles in the lumen and in process of budding from the surface of the cytoplasmic projection (at the left) are not tagged with ferritin.

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[2] The presence of antigen in cellular walls devoid of recognizable viral particles explains the adsorption of erythrocytes directly to the surface of infected cells, as described by Hotchin *et al.* (5) in the case of monkey kidney cell cultures and as confirmed by us in studies of chorioallantoic membranes.

## DISCUSSION

The specificity of the antigen-antibody reaction between influenza virus and ferritin-conjugated homologous antibody seems to have been adequately established by successful blocking experiments (Fig. 5) and by the failure of antibody against one type of influenza virus to tag the virus of another type (Figs. 7 and 8). Moreover, ferritin alone failed to adhere to the viral particles.

It has not been possible to determine the absolute thickness of any given section and thus to calculate the number of conjugated antibody molecules attached to the virus, but inspection of Figs. 1 and 2 leaves the impression that relatively few of the available antigenic sites have been tagged. Presumably, this is due in part to the presence of unconjugated antibody and in part to the size of the globulin-ferritin complex.[3] Superimposition within the section makes the precise location of ferritin on the virus difficult to ascertain. Examination of viral particles believed to be central to the plane of section reveals that most of the ferritin is attached to the outer coat about 150 A from the dense viral membrane. This is well demonstrated on one particle to the right of center near the top of Fig. 6.

A study of cells such as those illustrated in Figs. 1 to 4 suggests that viral antigen first appears on the cellular surface at sites where viral particles are differentiating; that during emergence the virus is coated with components of the altered cellular membrane; and that as infection proceeds the antigen continues to accumulate until most of the cytoplasmic membrane contains antigen. It is also possible, of course, that cells differ in their response to infection, some producing a sufficient number of viral particles to utilize the available surface antigen, others piling up this antigen in excess.

Hoyle, employing dark-field microscopy, observed that protrusions of cytoplasm were constantly shed from the surface of infected membranes (7). Noting that these fragments adsorbed to the surface of red blood cells, he suggested that bits of cytoplasm should be present in specimens of purified virus obtained by the technique of adsorption and elution, the size of the particles reflecting the degree of differential centrifugation employed. The electron microscope has provided confirmation of these observations. Cytoplasmic protrusions are encountered at the surface of normal cells but appear to become more numerous after infection. In Fig. 1 of a previous publication (3), for example, many membrane-bound protrusions of cytoplasm were shown at the surface of an infected cell. Fig. 4 of the present paper reveals that such protrusions are enclosed by a dense membrane and a diffuse outer coat, which contains viral antigen. When the protrusions detach, as appears to have occurred at the right of Fig. 4, cytoplasmic fragments are formed with a surface

[3] Ferritin is composed of a protein shell about 95 A in diameter enclosing an iron micelle, which ranges up to 55 A in diameter (6). Only the latter is visible in sections.

closely resembling the virus but with an interior lacking characteristic viral components. The presence of such structures in "purified" preparations of virus is strongly suggested by the examination of sectioned pellets (8). Moreover, "purified" suspensions dried and negatively stained with phosphotungstic acid have been found to contain particles with "marked pleomorphism," ranging up to 1800 A in diameter and differing in shape, but with a surface composed of a limiting membrane and peripheral, radiating rods[4] virtually indistinguishable from the virus (10). It is of interest that both of the latter papers call attention to the striking increase in percentage of these particles when incomplete virus was prepared from chorioallantoic membranes infected with undiluted inocula according to the method of von Magnus (11). An increase in the number of filaments was not observed.

One is led by the foregoing observations to the conclusion that infection causes part or all of the surface of the host cell to be transformed in such a manner that it becomes identical *both* antigenically and structurally with the surface of the virus; that the constant detachment of cytoplasmic fragments, in itself not an abnormal process, results during infection in the presence in chorioallantoic fluid of particles, variable in size and shape, which are capable of hemadsorption but incapable of initiating infection; and that these particles constitute "incomplete virus" of the von Magnus type (11). It is tempting to postulate that in cells damaged by concentrated inocula the initial synthesis of antigen, together with its localization in the cellular membrane, may proceed, although subsequent differentiation of viral particles, especially during the later stages of infection, may be impaired. Under these circumstances there is reason to suppose, in view of the numerous cytoplasmic processes frequently observed at the surface of necrotic cells (12), that release of cytoplasmic fragments, *i.e.* incomplete virus, would continue unchanged or even be augmented. Presumably, then, the chorioallantoic fluid would exhibit the maintenance of hemagglutinin titer, diminution in infectivity (11), and reduction in ribonucleic acid (13), which characterize the presence of incomplete virus. In this regard it is of interest that Watson and Coons (14), using the technique of immunofluorescence, noted that infected cells undergoing necrosis liberated "specifically stained cell debris."

Fig. 9 shows that influenza virus can form at the surface of intracytoplasmic vacuoles as well as at the cellular surface. Although this phenomenon had been suspected in previous studies, it was hitherto not possible to obtain a sufficient number of serial sections to prove that the presumed vacuole did not represent a cross-section through an infolding of the undulant surface of the cell; but the absence of ferritin within the vacuole described herein reveals that it does not communicate with the extracellular space. The ability of influenza virus to

[4] Negatively stained fragments of uninfected chorioallantoic membranes show a smooth surface (9).

differentiate at membranes both on the cellular surface and within the cytoplasm appears to be shared by Western equine encephalomyelitis virus (15). It should be emphasized, however, that in contrast to Western equine encephalomyelitis virus the occurrence of influenza virus within intracellular vacuoles is the exception rather than the rule.

SUMMARY

Immersion of chorioallantoic membranes infected by influenza virus in ferritin-conjugated antibody globulin prior to fixation permits recognition of viral antigen at the cellular surface. In some cases the antigen is confined to sites at which viral particles appear to be in process of release, whereas in others it is present along the entire cellular surface. During infection part or all of the cellular membrane presumably is transformed so that it contains the antigenic and structural components which coat the virus during emergence. It is suggested that detached fragments of cytoplasm possessing surface hemagglutinin but incapable of initiating infection constitute at least one form of "incomplete virus."

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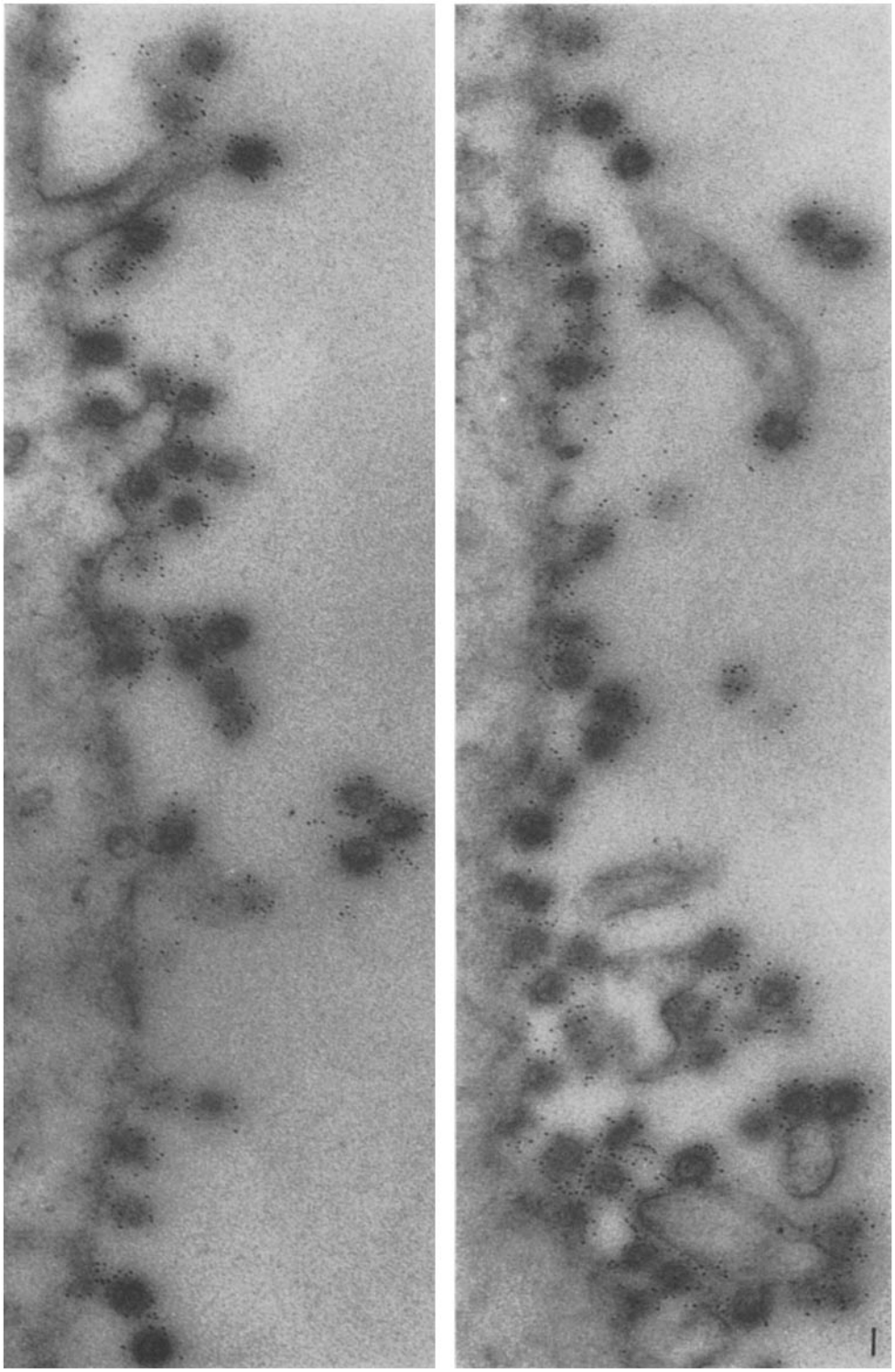
## EXPLANATION OF PLATES

### PLATE 81

FIG. 1. Two contiguous parts of the surface of a cell infected with PR8 influenza virus and immersed in ferritin-conjugated antibody before fixation. Cytoplasm is on the left; the extracellular space on the right. Ferritin is evident on the viral particles, several of which appear to be in process of emergence. The cellular membrane is largely devoid of ferritin. × 78,000.



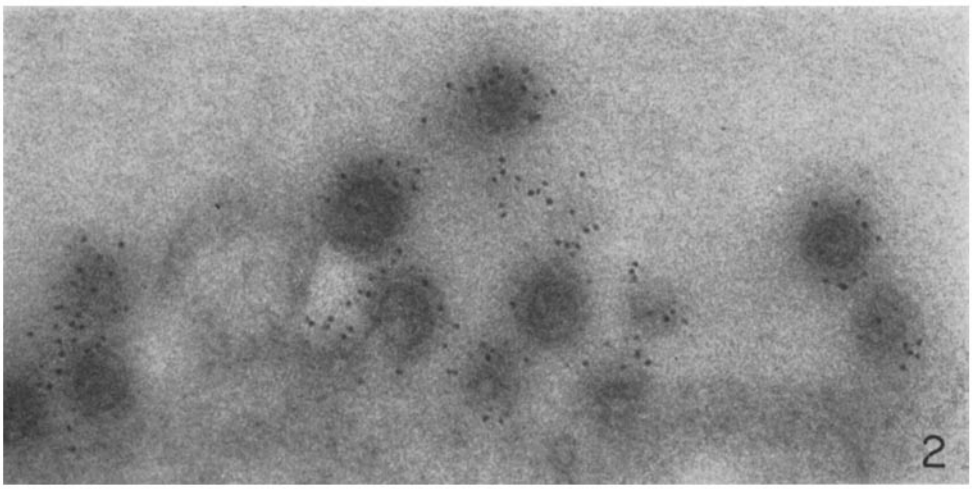
(Morgan *et al.*: Ferritin-conjugated antibody and cell surface)

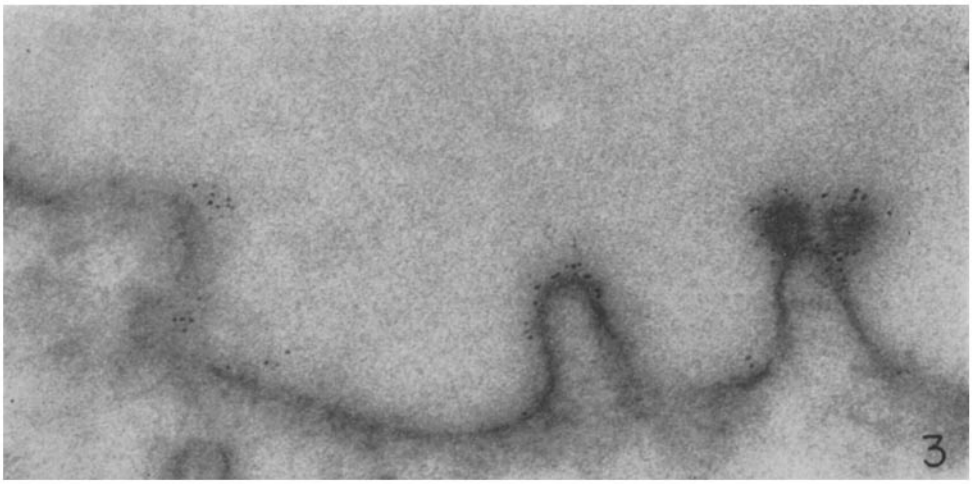
PLATE 82

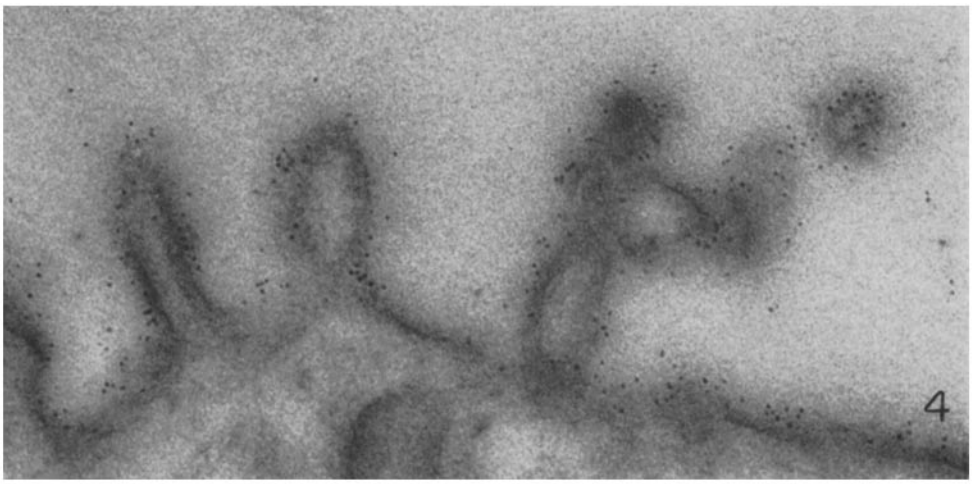
FIG. 2. Virus at the cellular surface. The membrane of the cell has been cut obliquely and cytoplasm occupies the lower border of the field. It is of interest that, in addition to fully formed virus, ferritin has tagged one particle (to the left of center) presumed to be in process of budding and two others (below and to the right) probably at early stages of differentiation. The ferritin apparently lying free may actually be attached to virus which has been cut eccentrically. × 140,000.

FIG. 3. The surface of an infected cell. On the right is a cytoplasmic protrusion with two ferritin-tagged viral particles. Near the center the distal portion of another protrusion is lined with ferritin and at the left are small clusters of ferritin granules. × 98,000.

FIG. 4. The surface of a cell, with several cytoplasmic extensions, largely lined by ferritin granules. No virus is evident. At the right cytoplasmic fragments, also tagged with ferritin, appear to have become detached. × 98,000.




(Morgan *et al.*: Ferritin-conjugated antibody and cell surface)

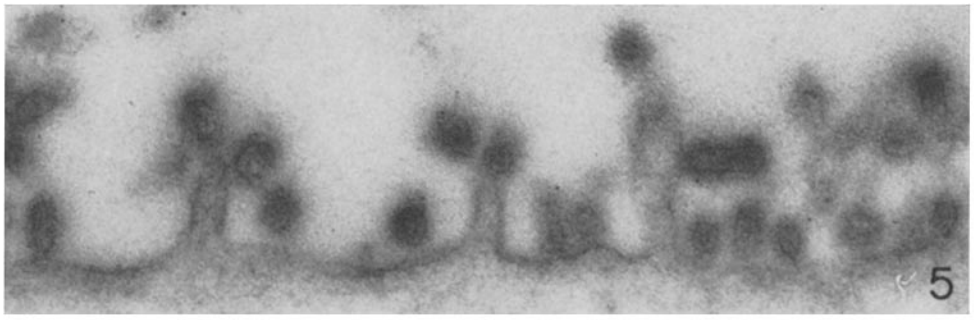
PLATE 83

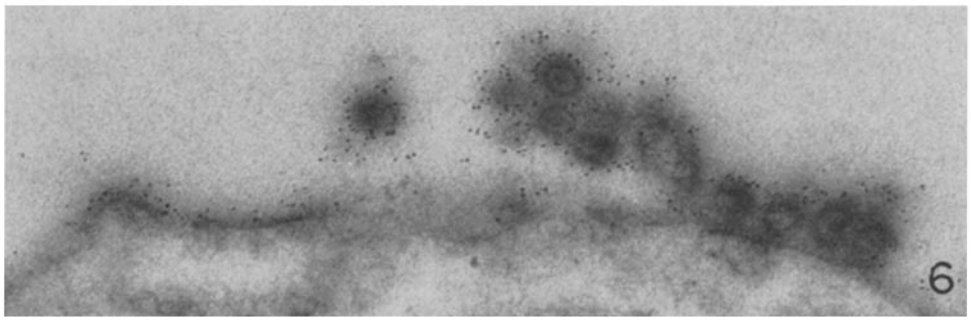
FIG. 5. The results of a blocking experiment. Unconjugated specific antibody globulin was applied to the cell before ferritin-conjugated globulin. Few ferritin granules are seen. × 79,000.

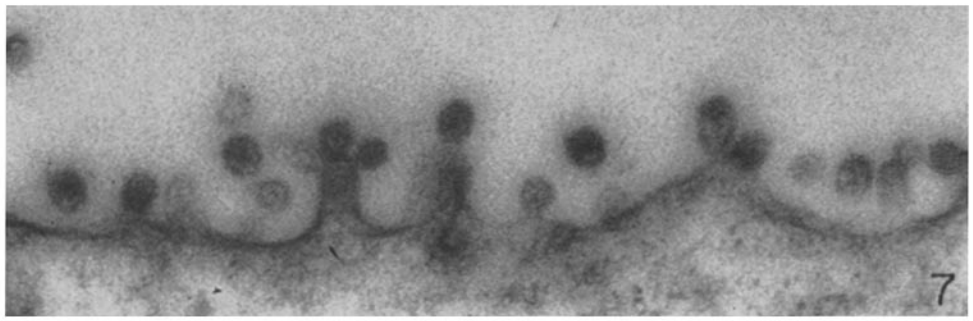
FIG. 6. A control for the foregoing. Non-specific antibody globulin has not prevented subsequent tagging by ferritin conjugated with specific antibody globulin. × 79,000.

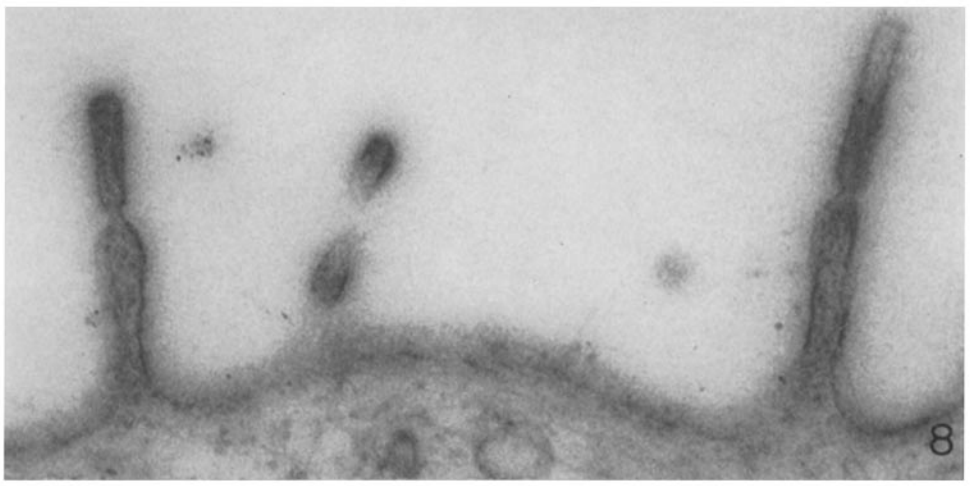
FIG. 7. A PR8 strain of influenza virus at the surface of a cell that had been immersed in ferritin-conjugated antibody to a Lee strain. × 79,000.

FIG. 8. A Lee strain of virus treated with ferritin-conjugated antibody to a PR8 strain. The continuity of two filaments with cytoplasmic extensions of the cell is clearly shown. Two other filaments, which have been obliquely sectioned, are evident to the left of center. × 79,000.



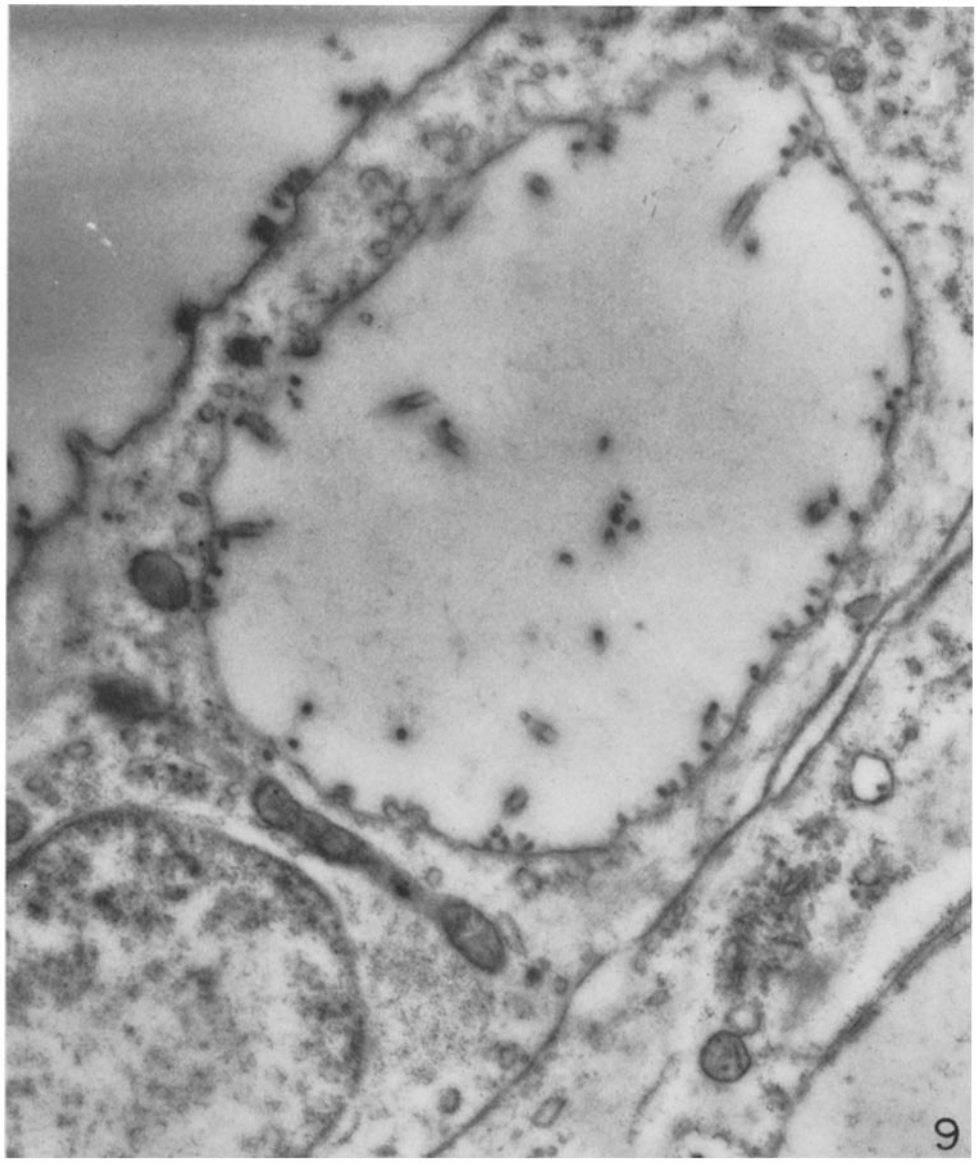



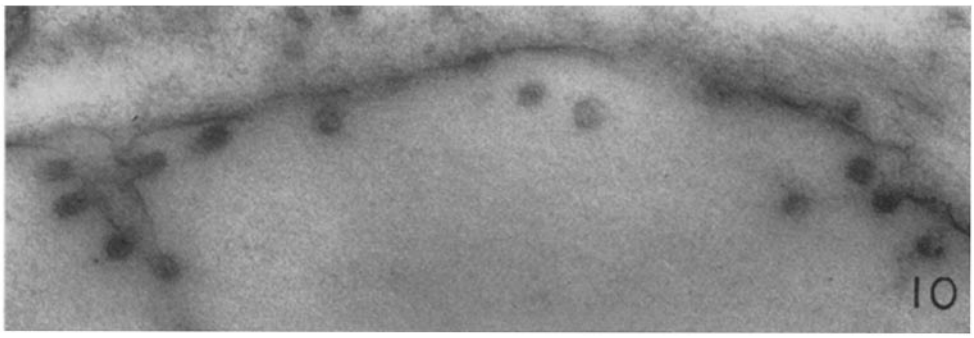

(Morgan *et al.*: Ferritin-conjugated antibody and cell surface)

PLATE 84

FIG. 9. A cytoplasmic vacuole containing viral particles both free and in process of formation at its margin. When examined at sufficient magnification virus on the surface of the cell, at the upper left, was seen to be tagged with ferritin. The nucleus of the cell occupies the lower left corner. × 20,000.

FIG. 10. Part of the limiting membrane (upper right) of the vacuole in Fig. 9 viewed at higher magnification. The absence of ferritin granules on the virus reveals that the vacuole does not communicate with the surface of the cell. × 58,000.




(Morgan *et al.*: Ferritin-conjugated antibody and cell surface)