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Structure and Development of Viruses as Observed in the Electron Microscope

VIII. Entry of Influenza Virus

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It is suggested that, after attachment of influenza virus to the cell, the viral coat disintegrates. With fusion of virus to the cytoplasmic membrane, the latter undergoes dissolution. Rupture of the viral core permits release of nucleoprotein directly into the cytoplasm. Stages in the process are illustrated.

It has been reported that entry of herpes simplex virus can be divided into stages, which begin by the tenth minute after cells have been exposed to the virus. These are, first, dissolution of the viral envelope in proximity to the cell surface; second, fusion of the envelope to the cytoplasmic membrane; third, dissolution of the cell membrane; fourth, entry of the capsid into the cytoplasm; fifth, release of the core from the capsid (6). In the light of these observations, it was decided to examine influenza virus, which also is inactivated by ether, in order to determine whether penetration occurred in a similar manner. Previous experiments employing ferritin had revealed that the entodermal cells of the chorioallantoic membrane (CAM) of chick embryos showed considerably less phagocytic activity than did cells in tissue culture. Accordingly, the CAM was selected for examination of viral entry. By allowing the cells to adsorb virus at 4 C and then rapidly warming the preparation to 37 C, an attempt was made to synchronize events sufficiently so as to determine the rate at which entry occurred. In addition, procedures using ferritin-conjugated antibodies and negative staining were carried out in order to gain further insight into the process. The results of these studies constitute the basis of this report.

MATERIALS AND METHODS

Eleven-day-old chick embryos were infected by the allantoic route with 10^5 EID_{50} of influenza virus, PR8 strain, and the chorioallantoic (CA) fluids were harvested and pooled after 48 hr. The virus was sedimented by ultracentrifugation and resuspended in one-tenth the original volume of CA fluid. Amounts (1 ml) of this suspension were placed in watch glasses and cooled to 4 C. Small fragments of shell with attached CAM from 11-day-old chick embryos were immersed in the viral suspension. After 1 hr at 4 C, the membranes were removed, washed, and transferred to beakers containing Earle's solution warmed to 37 C. After incubation for 5, 8, 10, 15, 20, 30, 45, 50, 60, and 90 min, the membranes were stripped from the shell, fixed for 1 hr in 1% glutaraldehyde, washed, fixed for 30 min in 1% osmium tetroxide, dehydrated in ethyl acohol, and embedded for cross-sectioning in epoxy resin (Epon 812).

In an effort to slow the process of penetration, CAM was also incubated with virus for 20 or 45 min and then cooled to 4 C by floating the watch glass on ice water for 15 min before fixation and embedding. (In these experiments the virus was not adsorbed at 4 C.)

A solution of ferritin-conjugated antibodies specific for the V antigen and prepared as previously described (2) was placed in watch glasses, and CAM attached to the shell was transferred to it at the conclusion of the 1-hr adsorption period at 4 C. After incubation at 37 C for 20 and 60 min, the CAM was washed, removed from the shell, fixed, and embedded.

The sections, stained with uranyl acetate and lead citrate, were examined in a Phillips EM 200 electron microscope. All micrographs were taken at an initial magnification of 25,000 times and are reproduced at a magnification of 200,000 times.

For negative staining, CAM attached to the shell was immersed in virus suspension for 1 hr at 4 C and then warmed to 37 C for 1 or 10 min. Formvar-carbon coated grids were pressed gently aganst the entoderm, withdrawn, and immediately stained with a drop of 1% phosphotungstic acid, buffered at *p*H 7.0.

RESULTS

Figures 1-6 illustrate virus examined 5 min after the preparation had been warmed to 37 C. The characteristic core of the virus (Fig. 1 and 2) shows irregularly arranged dense regions (presumably transected coils of nucleoprotein) and a thin limiting membrane. The cores differ in density and in the clarity with which the internal

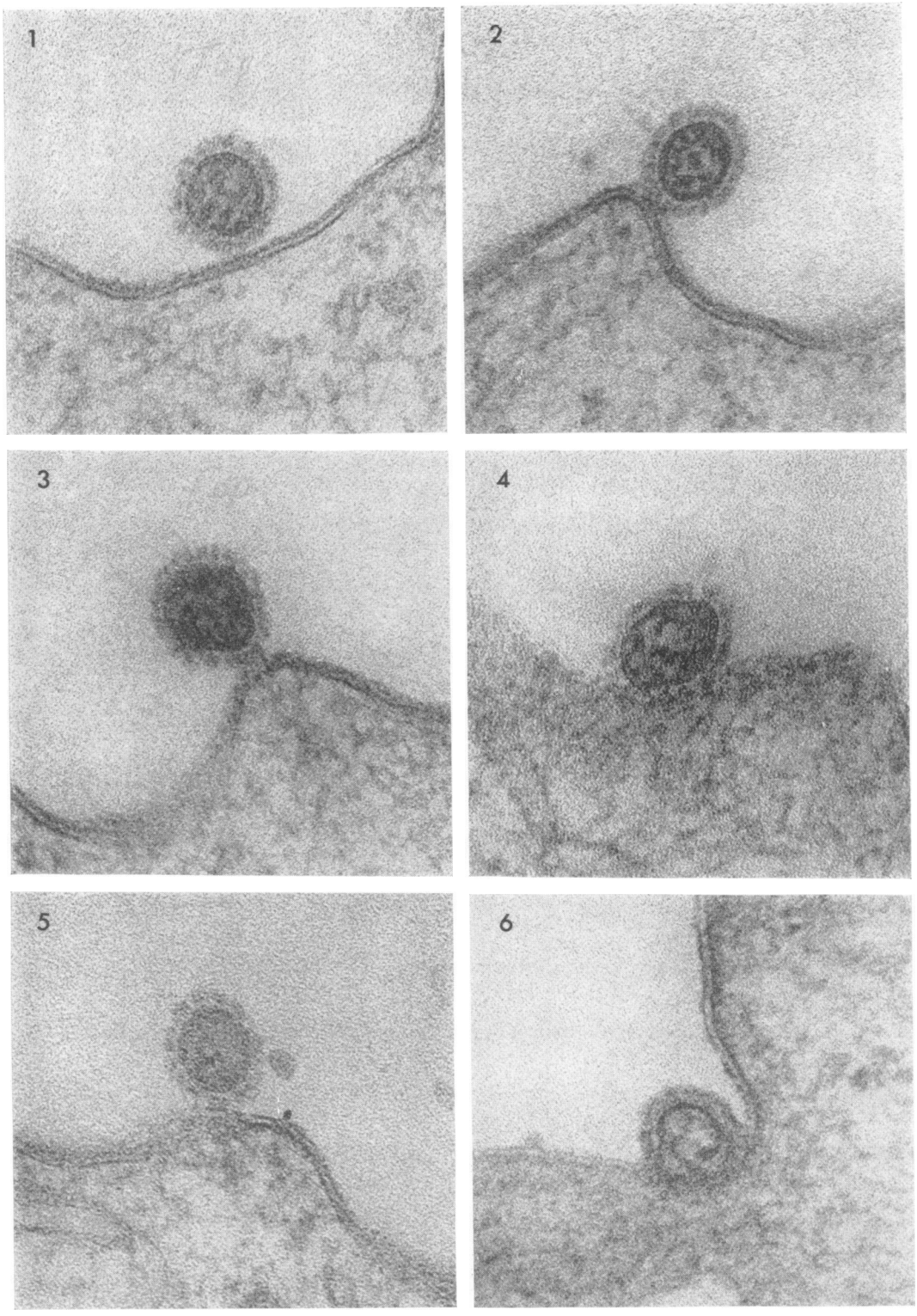


FIG. 1–6. Virus adsorbed to CAM for 1 hr at 4 C and then kept at 37 C for 5 min. ×200,000

components can be visualized (*see* Fig. 3, 4, and 5), so that considerable caution must be exercised in attempting to interpret structural changes in the nucleoprotein component during penetration into the cell. The diffuse external coat of the virus comprises the radial spikes observed in negatively stained preparations. The definition of these projections varies, depending on the thickness of the section and whether the particle is central to the plane of section. There is some variation in size of the viral particles as can be observed by comparing Fig. 1 and 2 with Fig. 3 and 4. It will be noted that small, discrete droplets occasionally are attached to the virus (Fig. 5) or the cytoplasmic membrane (Fig. 6 and 8). These may be the globules of protein or lipoprotein commonly found in CA fluid. In Fig. 1, the virus is in close proximity to the cell, whereas in Fig. 2 it appears attached to the bilamellar cell membrane, which seems to be intact. In Fig. 3, there seems to be a connecting bridge of material between the viral envelope and the cell membrane. In Fig. 5, neither the cell membrane nor the lower aspect of the viral envelope can be distinguished. It should be emphasized at this point that the state of the cytoplasmic membrane at any given site is very difficult to determine with certainty because the surface of the cell follows an undulant course. As a general rule, however, the membrane seldom bends sharply so that there is a transition zone from areas in which the membrane is cross-sectioned and thus sharply defined to areas where it lies obliquely and is thus ill defined (*see* Fig. 2). At sites of presumed viral penetration, the cell membrane was repeatedly found to be indistinct, and in such instances, the transition from sharply defined to diffuse structure was usually abrupt (Fig. 5 and 12). In Fig. 6, the envelope of the virus appears to have merged with the cell, and the limiting membrane of the core is disrupted, suggesting that the nucleoprotein has obtained access to the cytoplasm of the cell.

These first six micrographs serve to illustrate the stages by which it is believed influenza virus attaches to the cytoplasmic membrane and the viral nucleic acid gains entry to the host cell. After 15 min of incubation at 37 C, about 85% of the virus had disappeared (when compared with the number of particles seen at 5 min). This proportion increased to 95% after 30 min, and at the end of 1 hr virtually all viral particles had vanished. Although the preparations were warmed quickly to 37 C, there was considerable asynchrony even by the fifth minute; this became more striking with the passage of time. Accordingly, reference will not be made in the text to each time interval in discussing the ensuing micrographs, which have been chosen to illustrate details in the process of viral penetration. (The reader is referred to the legends for information regarding the time intervals.)

Virus was often observed at some distance from the cell (Fig. 7). As mentioned in a preceding communication (5), it is possible in thick sections to see a layer, presumed to be mucoprotein, covering the surface of the entoderm. Since this layer is of low density and does not pick up the uranyl or lead stains to any great extent, it is virtually invisible in the thin sections usually employed. The virus appears first to become attached to this layer and, in fixed specimens, seems to be suspended in space. Penetration to the surface of the cell proceeds rapidly. Initially, even though virus appears to be in contact with the cell, both layers of the cytoplasmic membrane remain clearly defined (Fig. 8). The site of contact between viral coat and cytoplasmic membrane broadens (Fig. 9), and fusion of the viral coat with the external layer of the membrane may occur (Fig. 10). Next, there seems to be disintegration of the cytoplasmic membrane in proximity to the virus (Fig. 11). With fusion of the virus to the cell and underlying dissolution of the membrane, direct continuity between the viral core and the cytoplasm is established (Fig. 12).

Stages in rupture of the core with release of nucleoprotein were so rarely observed that even after prolonged search only a few examples could be found. It was concluded that entry of the nucleoprotein must occur rapidly and, hence, be rarely encountered in the usual type of preparation. Assuming that the process of entry resembles an enzymatic reaction, which is inhibited at 4 C, it seemed reasonable to suppose that cooling the membranes from 37 to 4 C might slow up the reaction and enable it to be visualized at various stages. Such proved to be the case, and micrographs were obtained which clearly seemed to demonstrate the process of entry (Fig. 13–17). Despite careful study, the migration of individual nucleoprotein strands into the cytoplasm could not be followed, presumably because dispersion of the strands after their release from the viral core takes place quickly even at low temperatures. The final stages in disappearance of the virus were not easy to document because identification of the viral particles became increasingly difficult. Figure 18 illustrates a typical example of what appears to be a poorly defined viral particle. Note that the cytoplasmic membrane is indistinct both directly beneath and in the immediate vicinity of the particle. A study of serial sections showed that such particles were not eccentric to the plane of section since they were repeatedly found in only one section of a series.

In the case of herpes simplex virus, it was pointed out that occasionally the process appeared to misfire, and the capsid was divested of

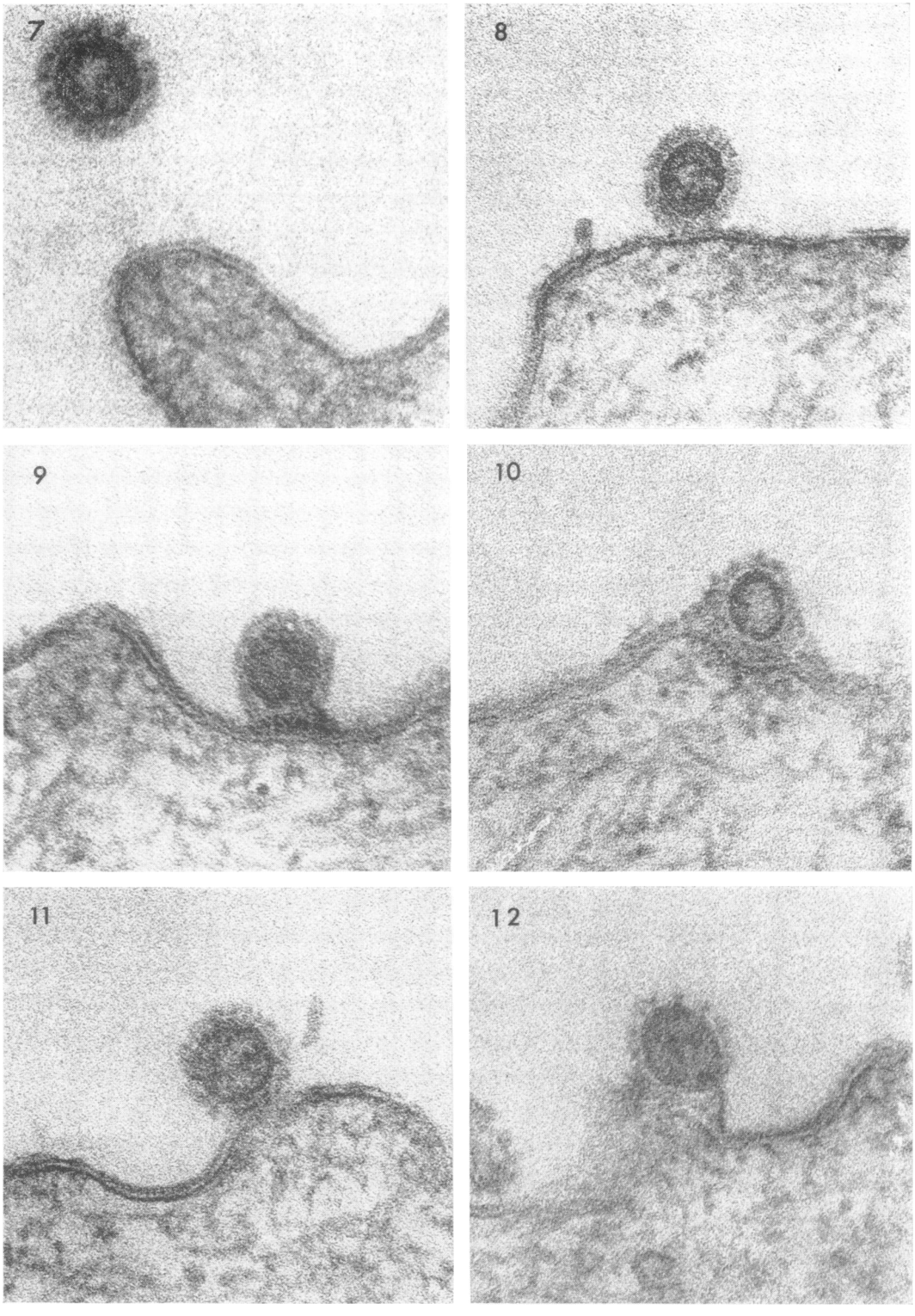


FIG. 7. Virus adsorbed to CAM for 1 hr at 4 C and then exposed to 37 C for 45 min followed by 15 min at 4 C. × 200,000.

FIG. 8–12. Virus adsorbed to CAM for 1 hr at 4 C and then kept at 37 C for 10 min (Fig. 8–11) or for 15 min (Fig. 9 and 12). × 200,000.

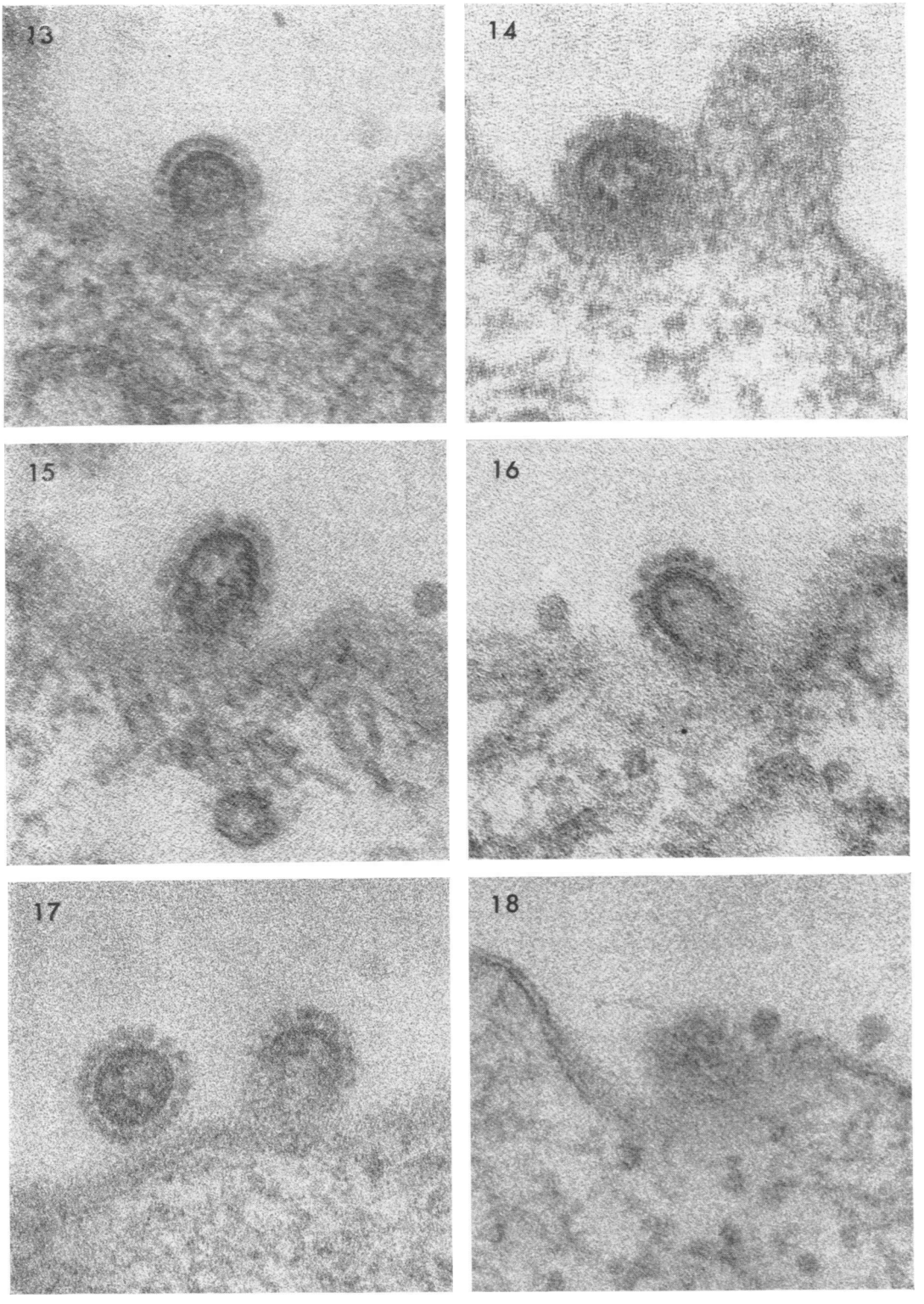


FIG. 13–18. Virus adsorbed to CAM for 1 hr at 4 C and then exposed to 37 C for 45 min followed by 15 min at 4 C. × 200,000.

its envelope before a passageway into the cytoplasm had been established (6). A similar event seems to occur with respect to influenza virus. Figures 19–23 illustrate examples of viral disintegration under circumstances which would not appear conducive to penetration of the nucleoprotein into the cytoplasm. The linkage between virus and cell, illustrated in Fig. 22, is reminiscent of the protrusions associated with herpes simplex virus (6).

In rare instances, there appeared to be a break in the cytoplasmic membrane that permitted penetration of a viral particle with an intact core and a discernible envelope. In Fig. 24, for example, the cytoplasmic membrane close to the junction with an adjacent cell has parted, and a viral particle is observed within the cytoplasm. Another uncommon observation was the attachment of viral filaments to the cell surface (Fig. 25). The scarcity of filamentous forms probably reflects their relative instability.

Although virtually no virus remained on the cell surface after 1 hr at 37 C, an interesting exception was the particle shown in Fig. 26. The cell had undergone lysis so that very little remained of the cytoplasm. Presumably the irregular, rumpled wall of the cell permitted attachment of the virus but penetration could not take place.

As noted earlier, the CAM exhibits relatively little phagocytic activity, and, therefore, virus was rarely encountered within cytoplasmic vacuoles. In most such instances, the virus appeared to be intact (Fig. 27), which was obtained from a preparation incubated for 1 hr. The wall of this vacuole is poorly defined and probably oblique to the plane of section. Virus was never observed at stages of transit from a vacuole into the cytoplasm. It thus appeared, in contrast to the concept of penetration by phagocytosis, that the virus actually may be protected and remain intact for a considerable length of time after being incorporated in vacuoles.

In a further effort to identify virus at terminal stages of entry, it was decided to add ferritin-conjugated antibody to the preparation when incubation at 37 C was begun. It was hoped that virus, such as the particle illustrated in Fig. 18, would be labeled and thus definitively identified. To our surprise, however, ferritin rarely attached to the virus once uncoating had begun. This suggested that during entry the antibody-combining sites at the surface of the virus distal to the cell become altered. The presence of ferritin-conjugated antibody on the surface of the virus did not interfere with attachment (Fig. 28) but did prevent penetration. Moderate numbers of particles (considerably more than in preparations devoid of antibody) were adherent to the cytoplasmic membrane after 1 hr of incubation at 37 C. Note in Fig. 29 that ferritin is not evident between the cell surface and the virus particle, which probably means that tagging occurred after attachment had taken place. Nevertheless, the cell wall just beneath the virus, as well as the viral core, appears to be intact. Of particular interest was the observation that very few antibody molecules were necessary to prevent penetration. On the viral particle (Fig. 30), for example, only two ferritin particles can be identified within the plane of section. Antibody did not prevent phagocytosis as illustrated by Fig. 31, which shows virus within a vacuole.

One confusing type of structure often observed within the cytoplasm is shown in Fig. 32. At first, it was believed that intact virus had penetrated the cytoplasm, where it was uncoated with release of nucleoprotein (Fig. 33). Since such structures were also present in uninfected cells, it soon became apparent that small phagocytic vesicles either with (Fig. 34) or without (Fig. 35) dense contents formed at the free margins of the cell and migrated into the cytoplasm where, on occasion, they ruptured. The dark zone, so strongly suggestive of the viral coat in section, was commonly present on the surface of the vacuole and apparently formed on the interior of the cytoplasmic membrane at the moment of budding inwards. From observations such as these, it can be inferred that the thickening of the cytoplasmic membrane just beneath the particle (Fig. 36) indicates that phagocytosis of the virus was about to occur.

It seemed of interest to observe by negative staining the appearance of virus on the surface of cells. Figure 37 shows virus after 1 min and Fig. 38 after 10 min of incubation at 37 C, following adsorption at 4 C. It is evident in the latter micrograph that the coat of the virus has been altered. Where the wall of the virus has not disintegrated, the surface spikes are still visible, but they are poorly defined and lack the orderly arrangement evident in the upper picture. Such changes are consistent with the suggestion made above that the antigenic sites of the viral coat at the time of entry become altered so that antibody fails to attach. The increased density of the interior of the virus (Fig. 38) may be due to greater permeability of the coat to the stain.

DISCUSSION

One of the most striking aspects of these observations is the rapidity with which uncoating and entry of the virus appears to occur. Virtually all stages of the process were encountered within 5 min (Fig. 1–6), and there is reason to suppose

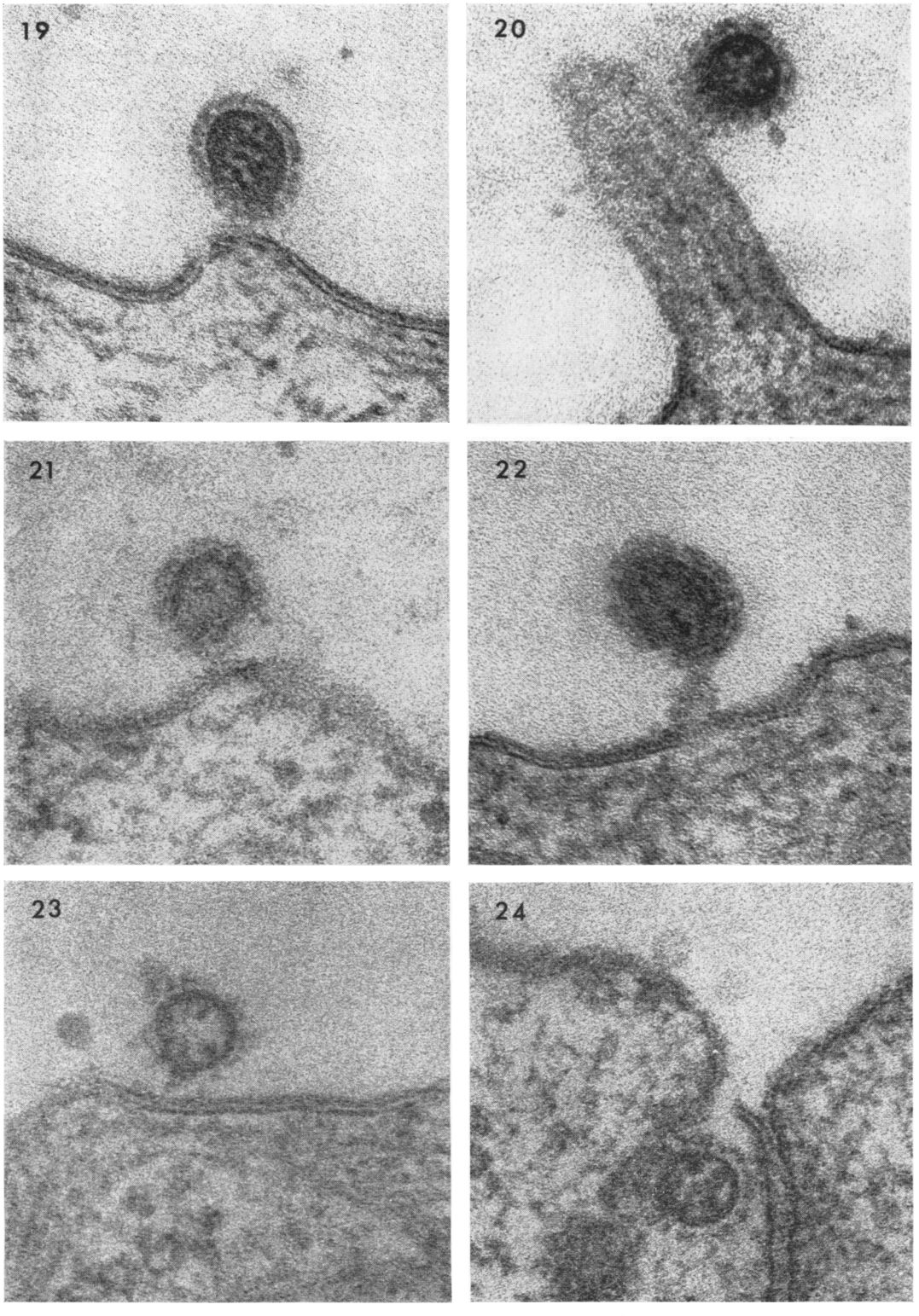


FIG. 19–24. Virus adsorbed to CAM for 1 hr at 4 C and then kept at 37 C for 5 min (Fig. 19–21) or exposed to 37 C for 45 min (Fig. 22–24) followed by 15 min at 4 C. × 200,000.

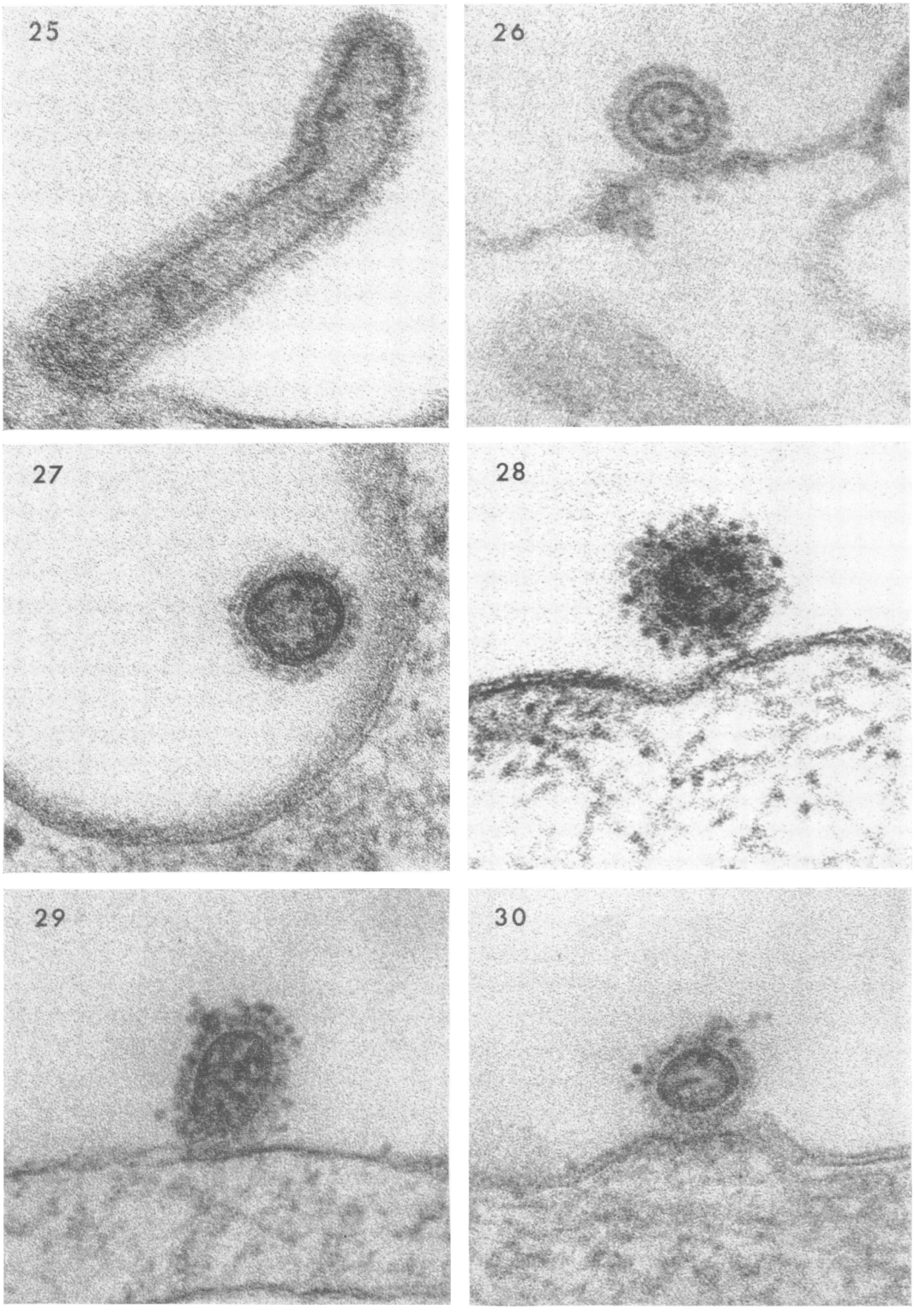


FIG. 25. Virus adsorbed to CAM for 1 hr at 4 C and then exposed to 37 C for 45 min followed by 15 min at 4 C. × 200,000.

FIG. 26–27. Virus adsorbed to CAM for 1 hr at 4 C and then kept at 37 C for 1 hr. ×200,000.

FIG. 28–30. Virus adsorbed to CAM for 1 hr at 4 C and then exposed to 37 C for 1 hr. Ferritin-conjugated antibody was added after adsorption at 4 C. × 200,000.

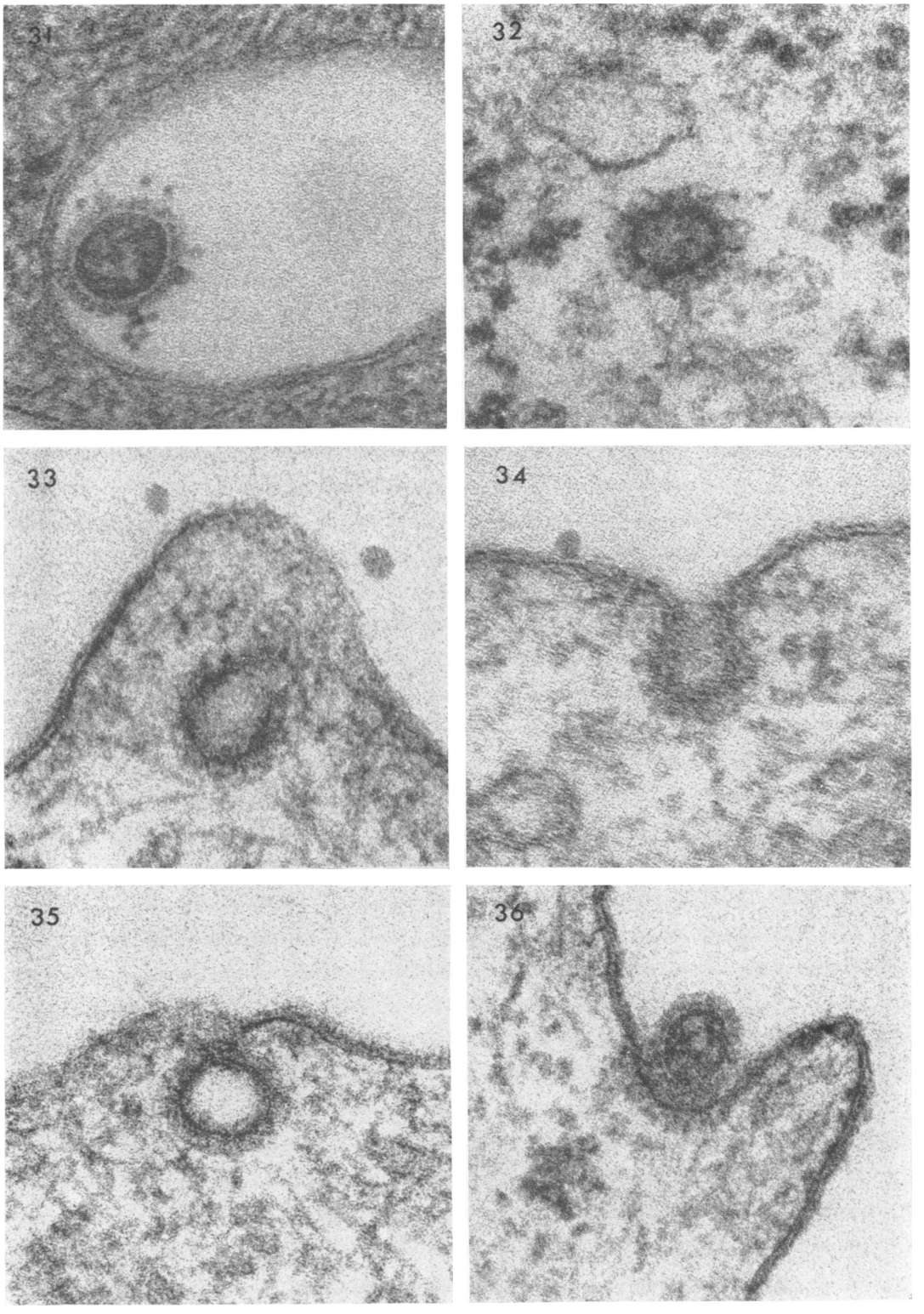
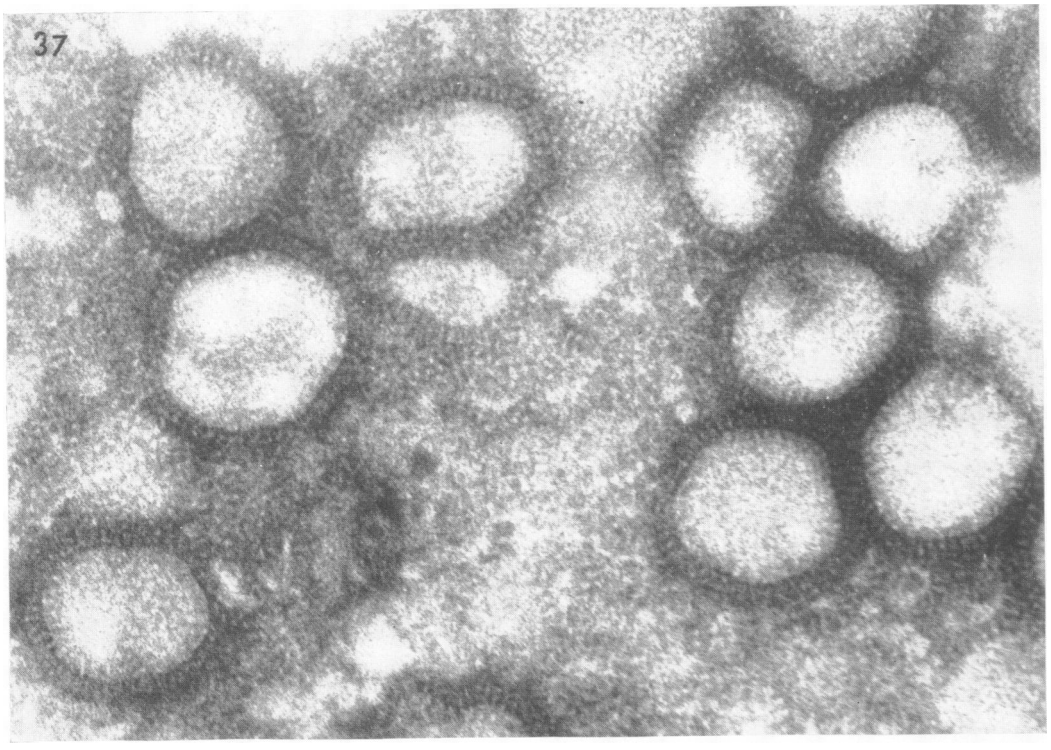


FIG. 31. Virus adsorbed to CAM for 1 hr at 4 C and then exposed to 37 C for 1 hr. Ferritin-conjugated antibody was added after adsorption at 4 C. × 200,000.

FIG. 32–36. Virus adsorbed to CAM for 1 hr at 4 C and then kept at 37 C for 30 min (Fig. 32–35) or for 10 min (Fig. 36). × 200,000.



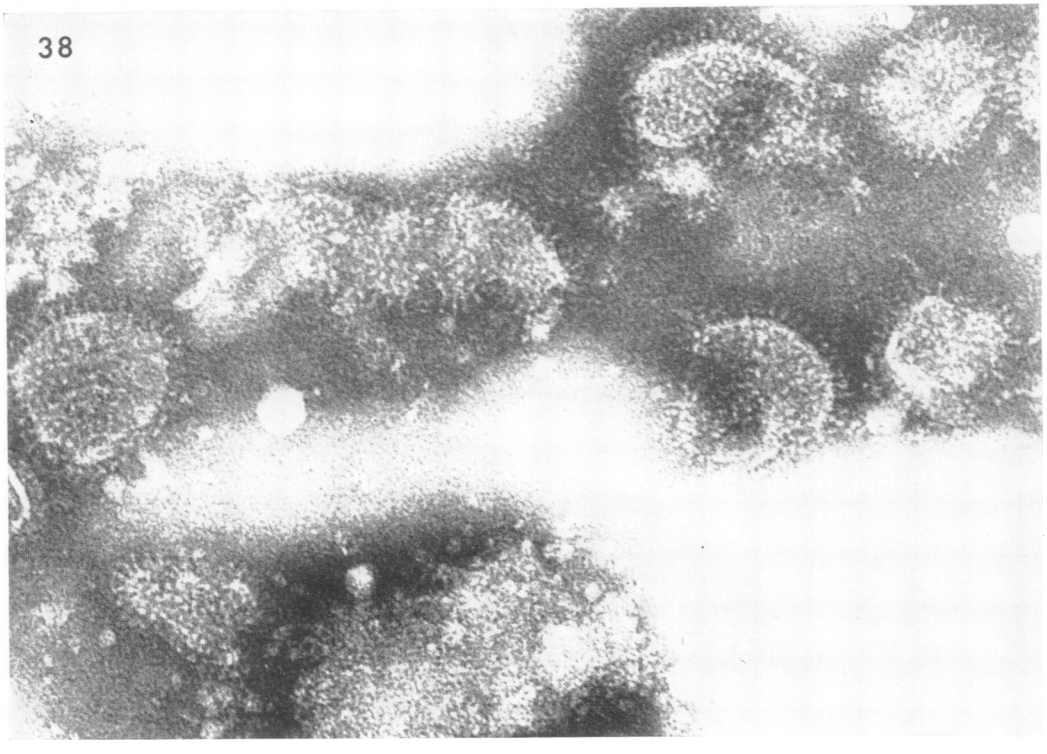


FIG. 37–38. The surface of an entodermal cell with attached virus. Negative stain. Virus adsorption to CAM was for 1 hr at 4 C. Interval after adsorption was at 37 C for 1 min (Fig. 37) or for 10 min (Fig. 38). × 200,000.

that the uncoating of any given particle occurs in even less time. As has been noted, approximately 85% of the virus had disappeared by 15 min. Considerable asynchrony was encountered so that a variety of stages were always observed in any preparation. This may reflect, in part at least, the time necessary for the virus to penetrate the surface coating of the cells (Fig. 7) and become closely attached to the cytoplasmic membrane (Fig. 8). In some cases, it would appear that a bond develops between the envelope of the virus and the outer layer of the cell wall (Fig. 9 and 10), thus providing a channel through which the nucleoprotein can enter the cytoplasm (Fig. 12). In other cases, the core is in close proximity to the cell (Fig. 6 and 11). The cytoplasmic membrane initially shows dissolution at a small locus just beneath the virus (Fig. 11). This process often extends so that at the moment of entry a larger portion of the cell membrane becomes indistinct (Fig. 13–17). It can, of course, be argued that in any given micrograph the membrane of the cell may have been cut obliquely and, for this reason, is poorly defined. The micrographs cited above, however, are representative of a large number obtained over months of study, and it would seem unlikely that in each instance the cell membrane in the vicinity of the virus was oblique to the plane of section. The extensive dissolution of the cell membrane occasionally encountered could result from the attachment of a cluster of viral particles (Fig. 37), only a few of which appear within the plane of section. Disintegration of the cytoplasmic membrane was not accompanied by loss of cytoplasmic components, and one can only surmise that attachment of the virus in some manner prevents outward movement of cell contents. In addition, repair of the cell membrane appeared to be almost instantaneous after disappearance of the virus. Cells, which must have received many viral particles, showed no visible alteration after the process of entry had been completed. It is of interest that there was great variation in the number of viral particles attached to any given cell. Whether cells differ in the numbers which attach or in the rapidity of entry, or both, could not be determined, nor could the appearance of the mitochondria, endoplasmic reticulum, ribosomes, or nucleus be related to the amount of virus at the cell surface.

If the stages of penetration are (i) attachment, (ii) dissolution of the viral coat, (iii) dissolution of the cytoplasmic membrane, (iv) rupture of the core, and (v) entry of nucleoprotein into the cytoplasm, it is not surprising, considering the rapidity with which these events are occurring, that the precise sequence might occasionally go awry. Thus, dissolution of the viral coat and core might occur before firm attachment of the virus or before an opening of the cytoplasmic membrane in close proximity to the virus is achieved, such as would seem to be the case in Fig. 19–23. Conversely, one can suppose that the cytoplasmic membrane could give way before dissolution of the viral coat, as may have occurred in Fig. 25. Examples of the former situation greatly outnumbered the latter.

Phagocytosis of virus was observed (Fig. 27), but it was uncommon when compared to the large numbers of viral particles encountered in process of entry at the cell surface. It is noteworthy that very little phagocytosis of the virus had occurred by the tenth minute, at which time uncoating and entry at the cell surface were most active. Moreover, stages in passage of the virus or of viral components through the wall of vacuoles into the cytoplasm were not observed. Indeed, the vacuoles actually appeared to protect virus from the events which had overtaken particles attached to the cytoplasmic membrane. Such observations lead us to conclude that infection is initiated by entry at the cell surface rather than by phagocytosis.

It is fitting at this point to inquire why uncoating and entry of the virus at the cell surface had previously escaped our attention, as well as that of others (1), who have used the thin-section technique for studying virus replication. The answer undoubtedly lies in the rapidity with which the process occurs. If one examines cells that have been incubated in the presence of virus, only a very small proportion of the particles is encountered at a definable stage of entry. In the present study, two different methods were used to circumvent this problem. First, some degree of synchrony was obtained by permitting attachment to proceed in the cold and then rapidly warming the specimens. Second, entry was slowed down by cooling the membranes after incubation. It would not otherwise have been possible to dissect sequential stages in the process. In the usual course of examination, after failing to observe entry, one is naturally inclined to examine cells at progressively longer periods of incubation. Since phagocytosis of virus is taking place at a slow but constant rate, and since, as has been mentioned, the virus appears to remain within vacuoles for a considerable time, more and more viral particles are observed within phagocytic vacuoles as the time of incubation is lengthened. Hence, it becomes natural to conclude that it is by this mechanism, rather than by the rapid and illusive process of uncoating at the cell surface, that infectious nucleic acid obtains entry to the cytoplasm.

It should be emphasized that uncoating of influenza virus at the cell surface with penetration of nucleoprotein is not a new concept. As early as 1957, Hoyle and Finter (4), using virus labeled

with radioactive isotopes, concluded "that on entry into the cell the virus nucleoprotein is hydrolyzed with release of amino-acid, while the virus envelope protein and haemagglutinin remain on the cell surface." More recently, Hoyle (3) again studied uncoating by examining in the electron microscope fragments of cytoplasm which had been allowed to react with suspensions of concentrated influenza virus. He reported disintegration of the virus lipoprotein layer, which "may result in rupture of the virus envelope with escape of the inner component." (Actually Fig. 4 in his paper resembles Fig. 38 of the present communication.) He makes it clear, however, that the eclipse phenomenon with disappearance of the virus does not occur in his system for it is "dependent on the active metabolic processes of the living cell." Presumably, he did observe attachment and the initial stages of viral uncoating, although he was not able to follow events to completion.

Perhaps the most perplexing problem which is raised by these observations is the precise mechanism responsible for the uncoating process. If mediated by an enzyme, which would seem at first glance to be the most likely explanation, then the enzyme must be present in situ, for it could hardly be synthesized in the minute, or very few minutes, necessary for uncoating of the virus to occur. But if one assumes that some enzyme actually attacks the coat of the virus, then how is it that very few antibody molecules, and these not interposed between the virus and the cell, can prevent the action from occurring? Until additional information is obtained it would seem idle to speculate on these questions.

ACKNOWLEDGMENTS

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