JOURNAL OF VIROLOGY, Mar. 1971, p. 395-406
Vol. 7, No. 3

Electron Microscopic Studies of Tumor Viruses

I. Entry of Murine Leukemia Virus into Mouse Embryo Fibroblasts

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Received for publication 30 November 1970

Entry of Rauscher leukemia virus into mouse embryo fibroblasts was studied by electron microscopy. The polycation diethylaminoethyl-dextran enhanced viral attachment and subsequent entry. At the site of viral attachment to the cell membrane, three distinct interactions occurred between the viral envelope and cell membrane, namely, (i) dissolution of viral envelopes on the cell membrane, which itself remained unaltered; (ii) simultaneous dissolution of both the envelope and cell membrane, resulting in passage of viral nucleoids directly into the cytoplasm; and (iii) dissolution of the cell membrane with direct penetration of intact enveloped particles into the cytoplasm, followed by intracytoplasmic disruption of the envelope, resulting in release of nucleoids into the cytoplasm. These interactions occurred with both mature and immature C-type particles. At no time was fusion of viral envelopes with the cell membrane observed. The mechanism of these interactions is discussed.

Little is known about the early events in murine leukemia virus (MuLV) infection. Virus entry and subsequent events before viral differentiation have not as yet been determined. One reason has been the difficulty in obtaining cell cultures which are susceptible to MuLV but do not contain latent virus morphologically similar to MuLV. Recently, Hartley et al. (11) have reported that National Institutes of Health (NIH) Swiss mice are free from latent virus, whereas their embryo tissue cultures are susceptible to most strains of MuLV. Such cell cultures have great advantages for time sequence studies of MuLV infection.

The present studies deal with the entry mechanism of Rauscher leukemia virus (RLV; reference 19) as the first step to understanding the early events in MuLV infection. Earlier chemical and electron microscopic studies have suggested that animal viruses enter cells by viropexis (1, 10). During the past few years, however, the early interaction between virus and host cells has been re-examined. Morgan and his colleagues have reported that viruses with envelopes enter cells by fusion of their envelopes with the cell membrane (13–15), whereas viruses without envelopes enter cells directly through the cell membrane (16). Entry by membrane fusion has been suggested to be closely related to the cell fusion which is produced soon after addition of inoculum virus (13, 17). Thus, the fact that MuLV has an ether-sensitive envelope (5, 6, 20, 22) but does not produce cell fusion suggests that MuLV may enter cells in a different manner.

The results obtained from the time sequence experiments reported here indicate that after viral attachment three distinct interactions between the viral envelope and cell membrane can occur, two of which appear conducive to virus entry.

MATERIALS AND METHODS

Cells and culture media. Primary NIH Swiss mouse embryo tissue cultures (NIH-METC) were prepared by trypsinization of minced NIH Swiss mouse embryos (11). One-day-old secondary cultures of NIH-METC were used for the present experiments. Eagle's minimal essential medium (MEM) was obtained from Flow Laboratories, Inc., Rockville, Md., and supplemented with 10% fetal bovine serum. For infected cells, MEM was supplemented with 5% heat-inactivated calf serum.

Virus. RLV (19) was originally obtained from Janet Hartley, National Institutes of Health, and was propagated in NIH-METC. In our laboratory, a cell line of NIH-METC persistently infected with the virus has been established.

Preparation of inocula. The persistently infected NIH-METC was seeded in 32-oz (ca. 900 ml) culture bottles, and 5 days later the virus-containing medium was collected from the bottles. Cellular debris was removed by low-speed centrifugation for 10 min. The virus-containing supernatant fluid (approximately 2 liters) was then centrifuged for 90 min at 19,000 rev/min in a Spinco model L-2 ultracentrifuge with a no. 19 rotor, and the resulting virus pellet was resuspended in 10 ml of Earle's solution. This concentrated virus suspension was used immediately as inoculum virus.

Virus infection. Sparse monolayers (3×10^5 cells per dish) in plastic 60-mm petri dishes (Falcon Plastics, Los Angeles, Calif.) were exposed at 37 C for

30 min to 25 μg of the polycation diethylaminoethyl-dextran (DEAE-D) per ml (Pharmacia, Uppsala, Sweden; molecular weight, 2.0×10^6), by the method of Duc-Nguyen (7). After washing the monolayers twice with Earle's solution, the cultures were cooled to 4 C in an ice bath for 15 min and were inoculated with 0.4 ml of cold, concentrated virus suspension. Viral adsorption was carried out at 4 C for 1 hr with occasional swirling. Cultures were then washed, warmed quickly to 37 C by addition of prewarmed MEM, and incubated at that temperature for 2, 5, 10, 20, 30, 45, 60, 120, and 180 min.

Preparation of cells for electron microscopy. After adsorption for 1 hr at 4 C and at the end of each incubation period at 37 C, the cultures were cooled to 4 C in an ice bath, fixed for 30 min in situ with cold 1% glutaraldehyde buffered at *p*H 7.2, scraped, and pelleted. The cell pellets were thoroughly washed with Sorenson's phosphate buffer (*p*H 7.2), fixed for 30 min at 4 C with 1% osmium tetroxide, dehydrated with ethanol, and embedded in epoxy resin (Epon 812). The sections were stained with uranyl acetate followed by lead citrate and were examined in a Hitachi HU-11E electron microscope.

RESULTS

One of the problems encountered in initial experiments was the fact that only a few virus particles attached to the cell surface. Duc-Nguyen (7) reported that pretreatment of cells with the polycation DEAE-D greatly enhances not only the focus-forming titer of murine sarcoma virus but also viral attachment to cells. When cells were pretreated with this drug, by the method of Duc-Nguyen, virus particles on the cell surface increased in number more than 20 times and more particles were encountered within cells, as compared to untreated cells. The results with pretreated and untreated cells were similar, except for the appearance of the cell surface which will be described below. Therefore, all observations, except for those shown in Fig. 8, were made with DEAE-D-treated cells. All micrographs are reproduced at the same magnification (150,000 ×).

Initial events in attachment of virus to the cell surface are shown in Fig. 1 to 4. Figure 1, which was taken at the end of 1 hr at 4 C (time 0), illustrates a mature C-type particle (21) localized at some distance from the cell membrane, which itself is covered with amorphous material of low density. Of particular interest is the fact that such amorphous material was observed frequently on the DEAE-D-treated cells but not on untreated cells. Figure 2, which was taken at 10 min after warming the preparations to 37 C, shows two mature C-type particles localized at a similar distance from the cell membrane. The envelope of the particle on the right appears stretched on the surface facing the cell membrane and is clearly attached to dense amorphous material localized on the cell membrane. This phenomenon suggests that attachment of the viral envelope to the amorphous material is so firm as to resist being dislodged during the fixation procedure. Similar distortion of virus particles on the cell surface was reported for herpes simplex virus (15) and parainfluenza virus (Sendai; reference 13). However, the particle on the left appears to be suspended in the extracellular space at a site where the cell membrane is not covered with the amorphous material. Such "suspended" particles were encountered more frequently than those attached to the amorphous material (this point will be discussed later). The filaments seen in the cytoplasm were often encountered in uninfected cells (also see Fig. 5, 7, 14). It should be emphasized that the nucleoid of mature C-type particles greatly varies in shape and density (compare Fig. 1–3). In Fig. 3, which was taken at time 0, a mature C-type particle is localized in the immediate vicinity of the cell membrane, suggesting that attachment of the viral envelope to the cell membrane occurs at 4 C. Figure 4, which was taken at 10 min, illustrates an immature C-type particle (21) in close proximity to the cell membrane. The intermediate membrane is clearly visible in the space between the envelope and the nucleoid with its translucent center. In contrast to the mature C-type particle, the immature C-type particle was always uniform in appearance. The virus which attached to the cell surface at time 0 consisted of more than 99% mature C-type and less than 1% immature C-type particles; particles without envelopes were not observed.

After warming the preparations to 37 C, three distinct interactions between the viral envelope and cell membrane appeared to occur at the sites of attachment. These were observed most frequently in the samples taken at 30 and 45 min but less frequently at 20 and later than 60 min. Each of these interactions will be shown in a logical order in the following micrographs (Fig. 5 to 25).

Figures 5 to 8 show dissolution of the viral envelope on the intact cell membrane. In Fig. 5, a mature particle can be seen in close proximity to the cell membrane, which does not appear to be altered. However, the envelope shows disintegration at the immediate vicinity of the cell membrane, which suggests the initiation of envelope dissolution at the site of attachment to the cell membrane. Part of the viral envelope at the right is pulled out and appears to be continuous with the dense amorphous material on the cell membrane, suggesting firm attachment of the envelope to the dense material, as noted in Fig. 2. There is also a dense region between the intact cell membrane and disintegrated envelope. Figure 6 illustrates an immature particle where both the

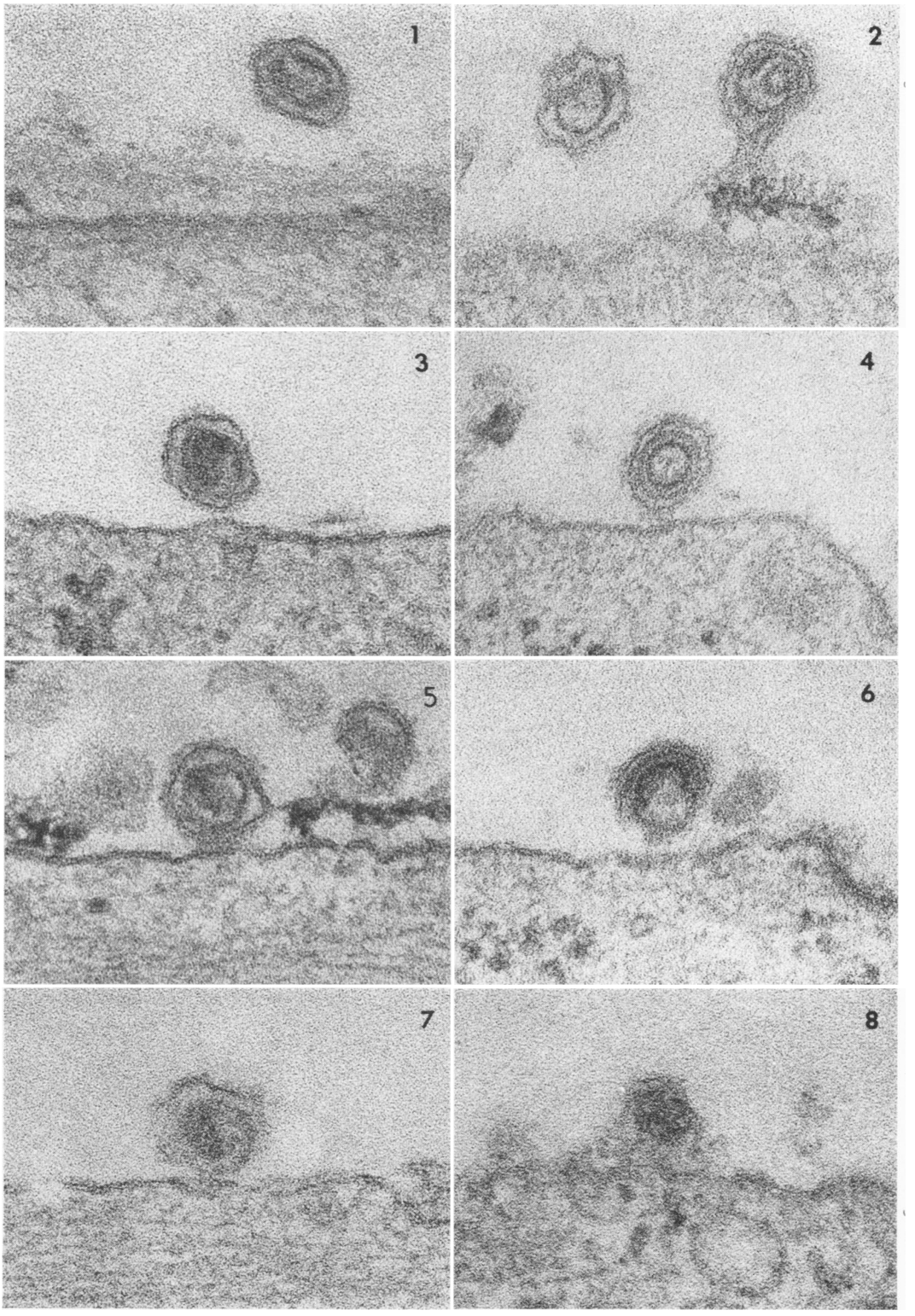

FIG. 1 and 2. *Viral attachment to the mucoprotein layer covering the cell membrane, which is modified to different degrees by DEAE-D. Adsorption at 4 C for 1 hr (Fig. 1). Incubation at 37 C for 10 min after adsorption at 4 C for 1 hr (Fig. 2).* × *150,000.*

FIG. 3 and 4. *Mature (Fig. 3) and immature (Fig. 4) particles in the immediate vicinity of the cell membrane. Adsorption at 4 C for 1 hr (Fig. 3). Incubation at 37 C for 10 min after adsorption at 4 C for 1 hr (Fig. 4).* × *150,000.*

FIG. 5–8. *Stages in the dissolution of the viral envelope at the site of attachment on the intact cell membrane. Incubation at 37 C for 30 (Fig. 6 to 8) and 60 (Fig. 5) min after adsorption at 4 C for 1 hr.* × *150,000.*

envelope and intermediate membrane are disintegrated in the area proximal to the cell. In Fig. 7, the process of envelope dissolution is still further advanced. Part of the envelope distal to the cell remains unaltered, and the nucleoid appears to attach directly to the intact cell membrane. Figure 8 (without pretreatment with DEAE-D) shows a terminal stage of envelope dissolution on the intact cell membrane. Although the envelope is disintegrated entirely, the nucleoid is still bound to the intact cell membrane by dense material surrounding it. The fate of such nucleoids was not determined.

Figures 9 to 16 illustrate simultaneous dissolution of both the viral envelope and cell membrane. Figure 9 shows two mature particles. The one on the left is attached directly to the cell membrane where both the envelope and cell membrane appear indistinct, suggesting the initiation of their simultaneous dissolution at the site of attachment. The particle on the right appears to be undergoing envelope dissolution on the intact cell membrane, as noted in Fig. 5. In Fig. 10, only the limited portion of the envelope is disrupted at the site of attachment where the cell membrane appears indistinct. In Fig. 11, the envelope of the mature particle on the left appears disintegrated in the region proximal to the cell where the cell membrane is undergoing dissolution (arrow). Figure 12 illustrates rupture of both the envelope (of the particle at the center) and the cell membrane at the site where the particle is believed to have attached. The disrupted cell membrane has not fused with the viral envelope. The undisrupted portion of the envelope appears thin and irregular in shape, suggesting that envelope dissolution would be expanded to the portion distal to the cell. In Fig. 13, dissolution of both the envelope and cell membrane is further advanced, resulting in widening rupture of the cell membrane. Once again no fusion is seen. Figures 14 and 15 show nucleoids of the mature and immature particles, respectively, which are localized at the opening of the cell membrane and in direct contact with the cytoplasm. The absence of their envelopes and the intermediate membrane of the immature particle presumably resulted from completion of envelope dissolution. Figure 16 illustrates a nucleoid of low density from a mature particle which lies free in the cytoplasm beneath the cell opening.

Figures 17 to 25 show dissolution of the cell membrane without any alteration of the viral envelope. In Fig. 17, the cell membrane appears thin at a limited portion in close proximity to a mature particle at the center (arrow). The viral envelope does not appear to be altered. Figure 18 shows a mature particle with a well-defined envelope and rupture of the cell membrane at the site where the particle is believed to have attached. Figures 19 to 21 illustrate stages of direct penetration of intact enveloped particles into the cytoplasm through the disrupted cell membrane. Note in these micrographs that the rupture of the cell membrane becomes widened as viral penetration proceeds but is limited to a length, corresponding to the approximate diameter of the enveloped particle. Such limited rupture of the cell membrane is similar to that shown in Fig. 12 to 16. Figures 22 and 23 show immature and mature particles with intact envelopes, respectively, free in the cytoplasm just beneath the cell opening. Figure 22 shows the loss of cytoplasmic components at the site of the cell opening. In Fig. 23, the cell opening (arrow) appears indistinct, suggesting that the disrupted cell membrane is in the process of repair after virus penetration. Figure 24 illustrates an unusual instance of the interaction of a mature particle lodged between two different cells. The cell membrane at the bottom is disrupted, whereas the envelope is not altered, as noted in Fig. 18. However, the upper cell membrane appears indistinct, whereas the envelope also appears to be disintegrated, as noted in Fig. 11. Figure 25 shows an immature particle localized at the cell opening which is presumably produced by the mature particle present free in the cytoplasm. Here is seen a streak of an area of low density in the cytoplasm between the immature particle and the lower left corner of the micrograph. Such areas of low density may have resulted from loss of cytoplasmic components through the cell opening before the immature particle filled it. The cell membrane (arrows) is covered with the amorphous material of low density, similar to that noted in Fig. 1, which is discontinuous at the site of viral penetration. An oval structure at the immediate lower right of the immature particle is a pinocytic vesicle which has just formed from the cell membrane.

Figures 26 to 30 illustrate stages in intracytoplasmic envelope dissolution after the penetration of the intact virus. Figure 26, which was taken at 20 min, shows partial envelope dissolution (arrow) of a mature particle free in the cytoplasm of a cell process. In Fig. 27, an immature particle lies just beneath the cell membrane, whose envelope is thin and disrupted at the upper left portion (arrow). However, its intermediate membrane and nucleoid remain unaltered. In Fig. 28, the envelope of a mature particle in the middle part of the cytoplasm has nearly disappeared, leaving its dense nucleoid free within the cytoplasm. Figure 29 is believed to illustrate a nucleoid of an immature particle near the nucleus (at the top). The envelope is extensively disintegrated,

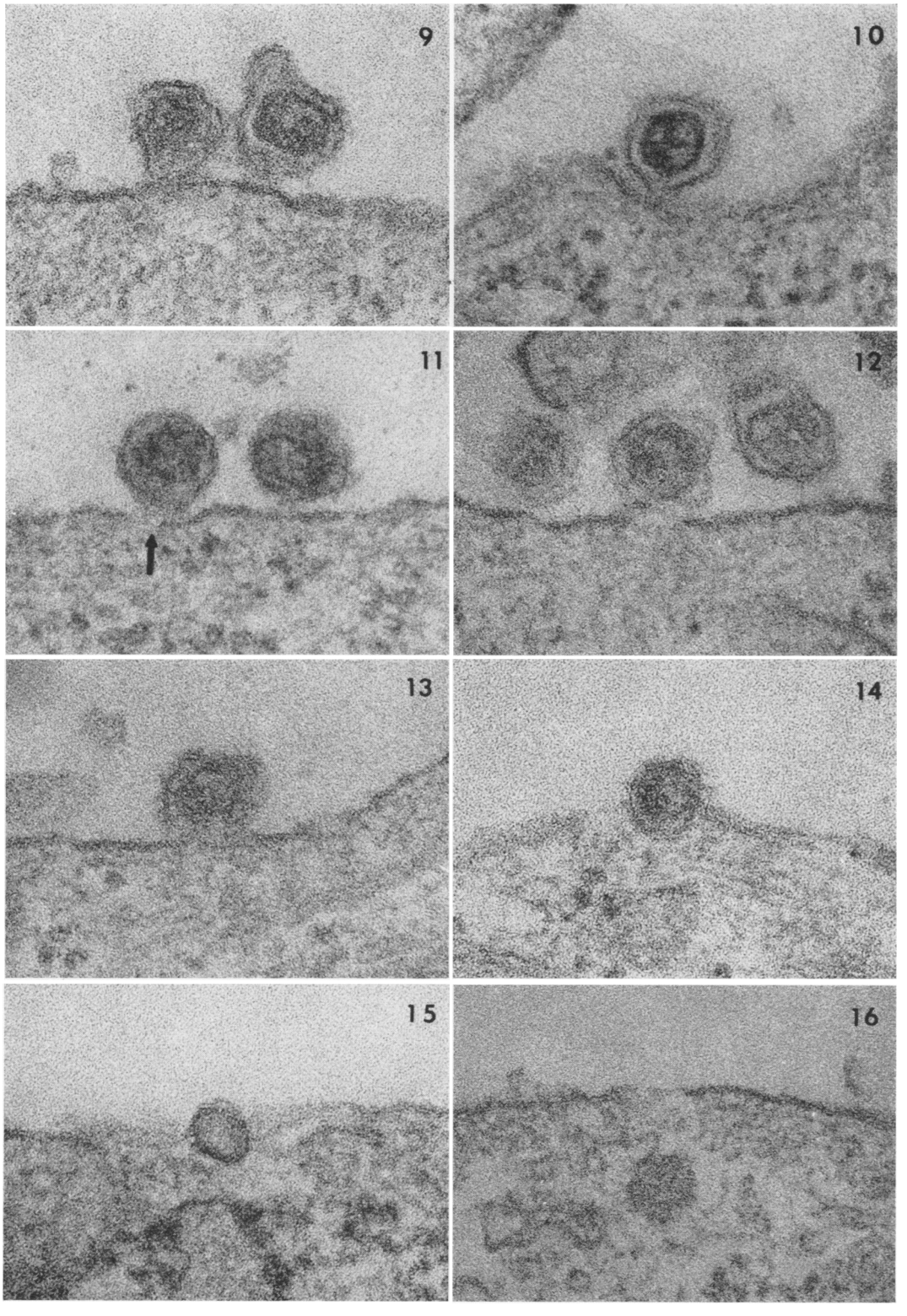

FIG. 9–16. *Stages in the dissolution of both the viral envelope and the cell membrane at the site of attachment and penetration of viral nucleoids into the cytoplasm. An arrow in Fig. 11 shows dissolution of the cell membrane. Incubation at 37 C for 30 (Fig. 11, 13, 16), 45 (Fig. 9, 12, 14, 15), and 60 (Fig. 10) min after adsorption at 4 C for 1 hr. × 150,000.*

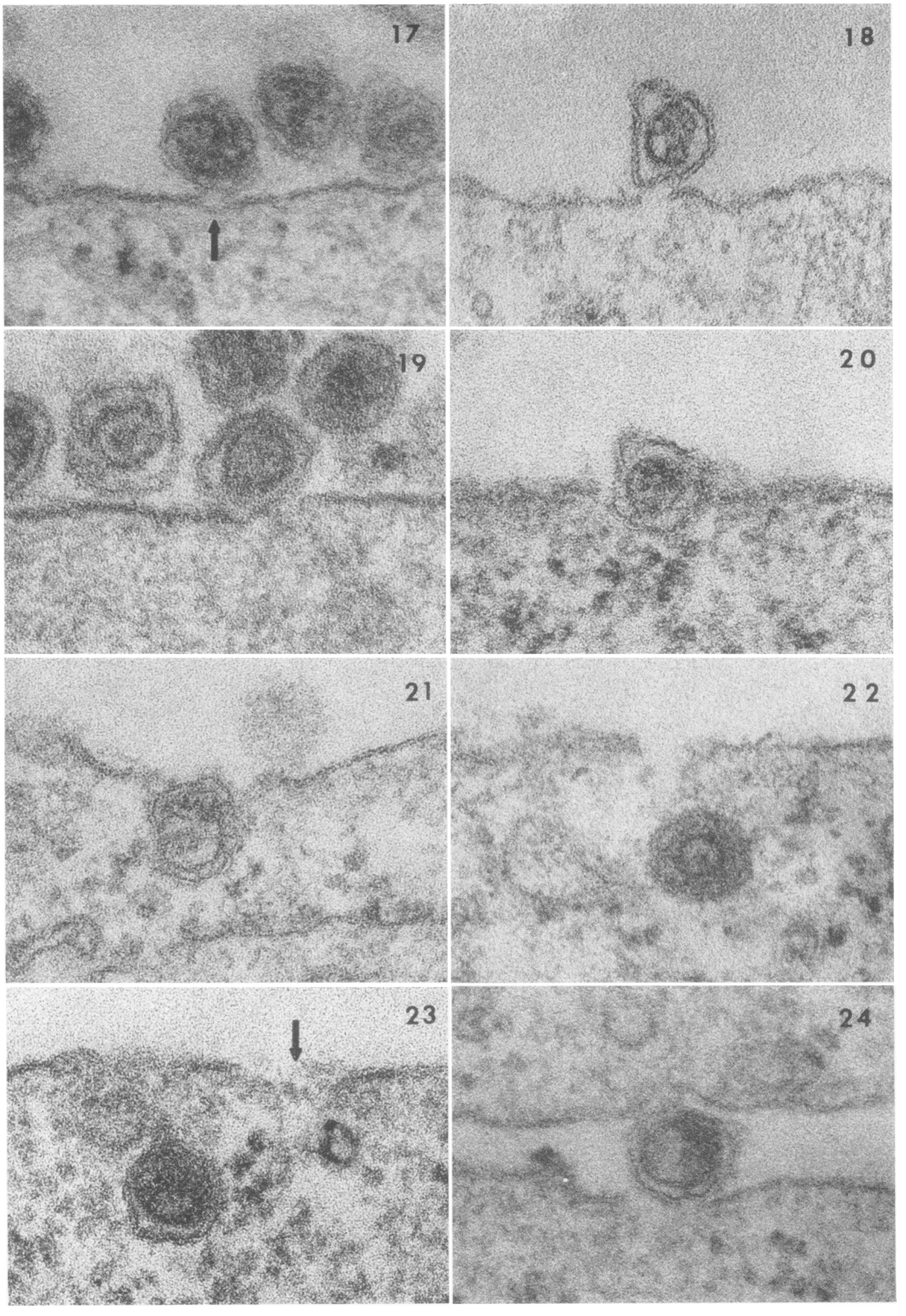

FIG. 17–23. *Stages in the dissolution of the cell membrane at the site of attachment and penetration of the intact enveloped particles into the cytoplasm. An arrow in Fig. 17 shows the initiation of disruption of the cell membrane, and an arrow in Fig. 23 shows the cell opening in the process of repair. Incubation at 37 C for 30 (Fig. 22, 23), 45 (Fig. 17, 18, 20, 21), and 120 (Fig. 19) min after adsorption at 4 C for 1 hr. × 150,000.*

FIG. 24. *A mature particle shows two different interactions between its envelope and two different cells. Incubation at 37 C for 45 min after adsorption at 4 C for 1 hr. × 150,000.*

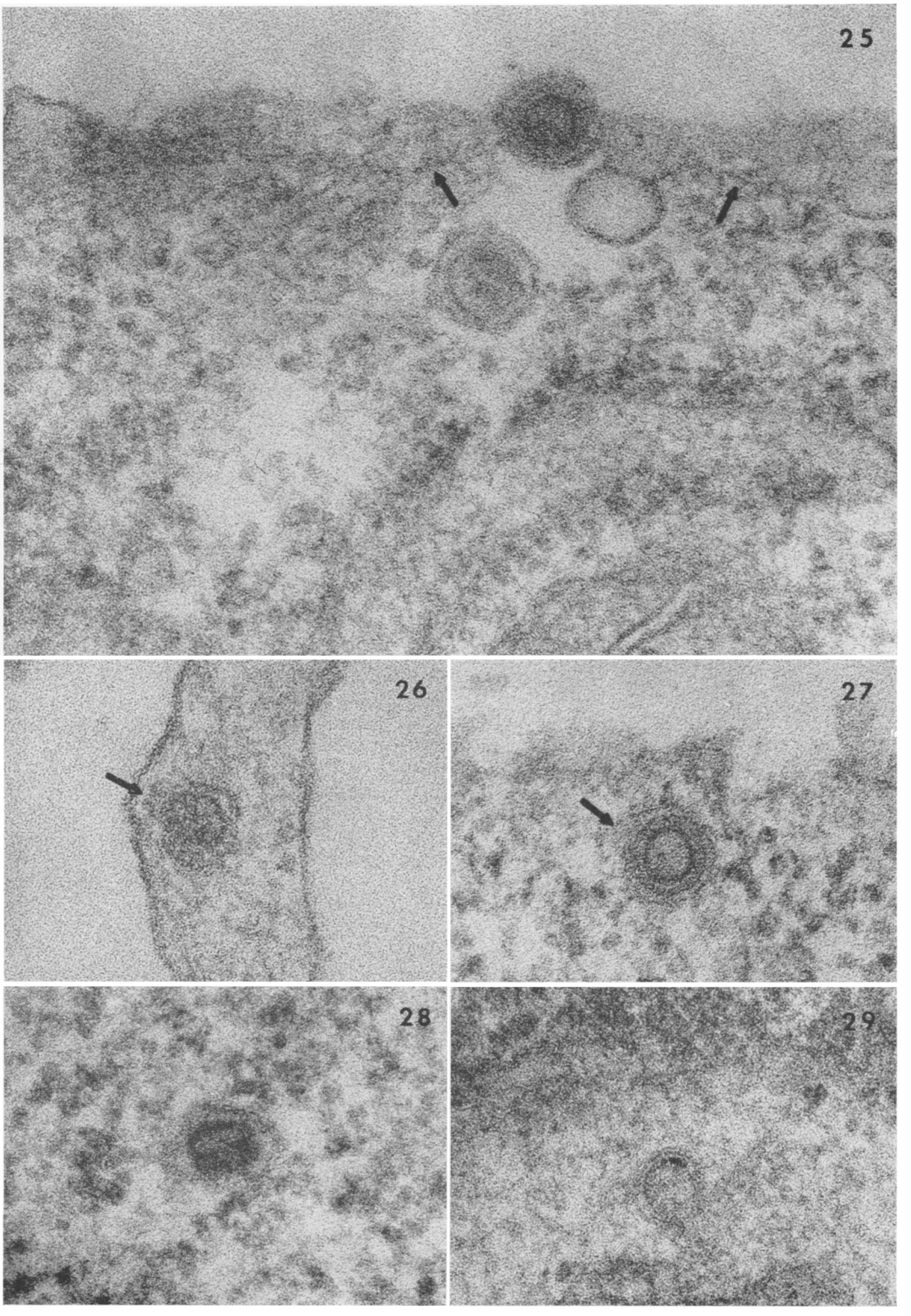

FIG. 25. *An immature particle falls in the cell opening which is produced by the mature particle present in the cytoplasm. Arrows show the cell membrane covered with the modified mucoprotein layer of low density. Incubation at 37 C for 30 min after adsorption at 4 C for 1 hr.* × *150,000.*

FIG. 26–29. *Stages in the dissolution of the viral envelope in the cytoplasm. Arrows in Fig. 26 and 27 show disrupted portions of the envelope. Incubation at 37 C for 20 (Fig. 26), 45 (Fig. 28), and 60 (Fig. 27, 29) min after adsorption at 4 C for 1 hr.* × *150,000.*

except for the portion proximal to the nucleus, and both the intermediate membrane and nucleoid with translucent center appear to be disintegrated at its lower portion. Figure 30 shows an immature particle and a nucleoid of low density from a mature particle, both of which are localized free in the cytoplasm. Although the two cell openings (arrows) appear to be in the process of repair, here are also seen the areas of low density in the cytoplasm, as noted in Fig. 25.

Phagocytosis of virus particles was already evident in the samples taken at 5 min after warming the preparations to 37 C. However, the majority of the particles in the phagocytic vesicles in the samples taken later than 45 min showed disintegration to various extents. In Fig. 31, the envelope is apparently disintegrated at its left portion without any contact with the vesicular membrane. In Fig. 32, disintegration of the particle is further advanced. Figures 33 and 34 illustrate unusual particles in the phagocytic vesicles. In Fig. 33, both the envelope of a mature particle and vesicular membrane appear indistinct at the site of attachment. In Fig. 34, the vesicular membrane appears to be disintegrated at the upper portion, and the intact envelope at this site appears to be localized free in the cytoplasm (arrow).

Figure 35 shows a hypothetical schema to explain the mechanism of the three distinct interactions described above between the viral envelope and cell membrane. The details are discussed below.

DISCUSSION

Morgan et al. (15) and Morgan and Rose (14) have suggested that virus attaches first to the mucoprotein layer covering the cell membrane, which is invisible in the thin sections usually employed, and then the virus penetrates into the layer before direct attachment to the cell membrane. It is of interest, in this connection, that the cell membrane of DEAE-D-treated cells is frequently covered with amorphous material of increased density. Such material, which is not seen on untreated cells, may result from modification of the mucoprotein layer by DEAE-D. At the sites where the amorphous material is absent, virus particles appear to be suspended in the extracellular space at some distance from the cell membrane, which corresponds approximately to the thickness of the amorphous material. Such particles, however, were encountered more frequently than those attached to the amorphous material on the cell membrane. Therefore, it is not unreasonable to assume that the mucoprotein layer, whether visible or invisible, may increase in affinity for virus particles after DEAE-D treatment. Viral attachment and penetration into the mucoprotein layer and subsequent attachment to the cell membrane may proceed at 4 C. It was undetermined, however, whether the mucoprotein layer which is extensively modified by DEAE-D (and hence which appears very dense) would allow virus penetration, whereas the less dense visible mucoprotein layer was penetrated by the virus.

After warming the preparations to 37 C, three distinct interactions appear to be initiated at the sites of viral attachment to the cell membrane. One is the entire dissolution of viral envelopes on the intact cell membrane, which would not appear conducive to penetration of viral nucleoids into the cytoplasm. Similar evidence of viral disintegration at the intact cell membrane has been reported for herpes simplex virus (15) and influenza virus (14). However, the other two interactions, simultaneous dissolution of both the envelope and cell membrane and dissolution of the cell membrane without alteration of the envelope, apparently result in direct penetration of viral nucleoids or enveloped particles into the cytoplasm through the disrupted cell membrane. Recently, Morgan (Bacteriol. Proc., p. 203, 1970) reported similar observations in reovirus entry. The frequency with which these three distinct interactions occurred was not determined. The initiation of the virus-cell interactions was not synchronized, and the various interactions appeared to occur quickly as relatively few particles were encountered in the process of interaction, as compared to finding the enveloped particles free in the cytoplasm. Also, the electron microscopic observations were limited to only an instant during a dynamic process; thus hundreds of micrographs were required to follow the whole process of the interactions, with samples taken at selected postinfection periods. The plane of the individual sections was a very important factor in determining the sites of the interactions and deciding their type. For example, if particles like those shown in Fig. 12 and 13 were sectioned obliquely, including the intact cell membrane apart from the disrupted portion, the micrographs obtained could resemble that shown in Fig. 5. Furthermore, when particles like those shown in Fig. 18 and 19 were sectioned obliquely through the intact cell membrane, the micrographs would illustrate these particles as though they were intact particles attached to the intact cell membrane. To avoid such misjudgments resulting from the section plane, only those micrographs were selected for presentation where the sites of the interactions were sectioned tangentially. For the above reasons, frequency

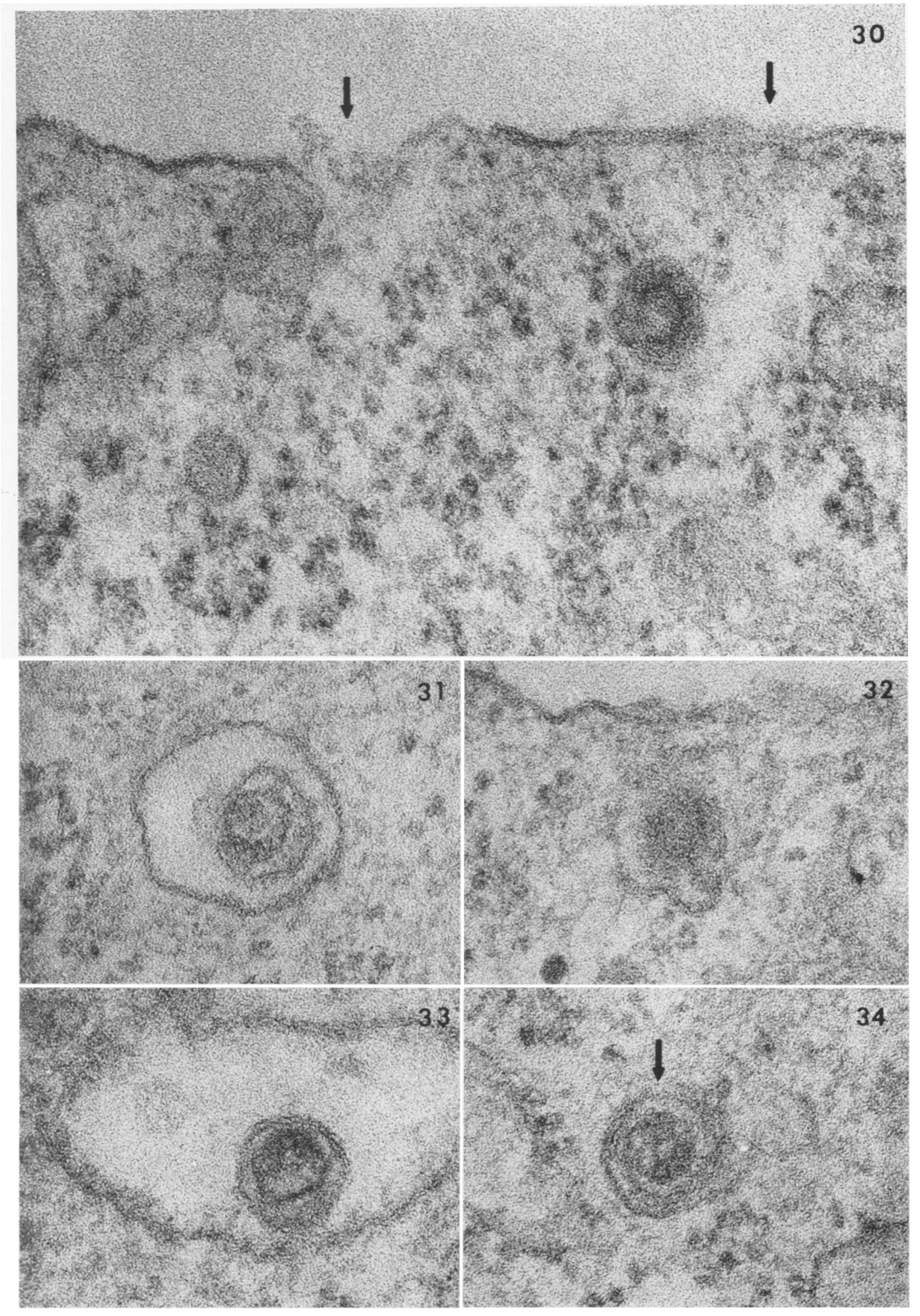

FIG. 30. *An immature particle and a nucleoid of the mature particle in the cytoplasm beneath the cell openings (arrows). Incubation at 37 C for 45 min after adsorption at 4 C for 1 hr. × 150,000.*

FIG. 31 and 32. *Stages in the disintegration of virus particles in phagocytic vesicles. In Fig. 32, the cell membrane is at the top. Incubation at 37 C for 30 (Fig. 31) and 45 (Fig. 32) min after adsorption at 4 C for 1 hr. × 150,000.*

FIG. 33. *Viral attachment to, and disintegration of, the vesicular membrane. Incubation at 37 C for 30 min after adsorption at 4 C for 1 hr. × 150,000.*

FIG. 34. *Disruption of the phagocytic vesicle (arrow) containing an intact mature particle. Incubation at 37 C for 20 min after adsorption at 4 C for 1 hr. × 150,000.*

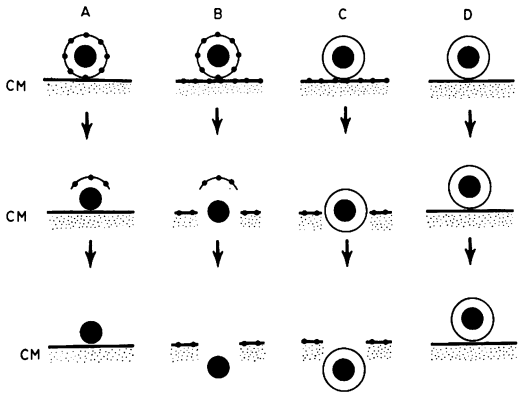

FIG. 35. *Hypothetical mechanism of the interactions between the viral envelope and cell membrane. Each of the lines (A, B, C, and D) illustrates the interactions after direct attachment of the viral envelope to the cell membrane (CM). Solid circles of the envelopes or cell membrane, or both, represent the presence of the hypothetical inactive enzymes responsible for the membrane dissolution, which are activated after attachment of the envelope to the cell membrane. Line A is summarized from Fig. 5 to 8, line B from Fig. 9 to 16, and line C from Fig. 17 to 23.*

distribution of the three distinct interactions could not be determined and the micrographs were presented in a "logical order." The enveloped particles, which entered the cells directly through the disrupted cell membrane, undergo envelope dissolution in the cytoplasm, resulting in release of their nucleoids. A similar release mechanism of viral nucleic acid has been reported for herpes simplex virus where the viral core is released into the cytoplasm by rupture of the capsid (15), presumably mediated by host cell enzymes (*unpublished data*). The viral nucleoids which enter the cytoplasm directly or by intracytoplasmic envelope dissolution are thereafter lost to view because they are undistinguishable from the dense granules present in the cytoplasm and nucleus of both infected and uninfected cells.

As described earlier, viruses with ether-sensitive lipoprotein coats (envelopes), such as herpes simplex virus (15), influenza virus (14), parainfluenza virus (Sendai; reference 13), and vesicular stomatitis virus (12), have been demonstrated to enter cells by fusion of the viral envelope with the cell membrane. Nii et al. (17) and Morgan and Howe (13) have suggested that this entry mechanism may play a role in the process whereby cell fusion occurs soon after addition of virus. However, RLV, which also has an ether-sensitive envelope (5, 20, 22), does not produce such cell fusion. This may result from the fact that the envelope of RLV does not fuse with the cell membrane throughout the entry process. Rupture of the cell membrane without fusion of the envelope is accompanied by loss of cytoplasmic components. Repair of the cell membrane does not appear to be instantaneous after viral penetration, but rapidity of the repair may depend upon the length of the disrupted portion of the cell membrane, since loss of cytoplasmic components is not evident during adenovirus entry (16). An unusual example was found (Fig. 25) of a second virus entering into the cell opening produced by another virus.

The question arises as to what mechanism is involved in the various interactions described above. The following considerations lead us to suggest that autolysis carried out by similar enzymes located in the viral envelope and the cell membrane may be responsible for the variability in the virus-cell surface interactions. First, the temperature dependency of the interactions suggests that enzyme action is required for the membrane dissolution. The observation that the membrane dissolution is initiated exactly at the site of attachment suggests that such an enzyme may be localized at the site of attachment in the viral envelope or in the cell membrane of susceptible cells, or in both. The hypothetical enzymes are presumed present in inactive form and only activated upon direct contact of the viral envelope with the cell membrane. This would be necessary to prevent nonspecific digestion of both cell and virus. The lag phase between viral attachment and the initiation of membrane dissolution (less than 20 min, since the first virus free in the cytoplasm was encountered in the samples taken at 20 min) may represent the period required for enzyme activation in the present experimental condition. Secondly, the suggestion that similar enzymes may be involved in both viral envelope and cell membrane dissolution is prompted by a number of observations showing the presence of cell-derived enzymes on the virion surface. For example, Novikoff et al. (18) and de-Thé et al. (3) have demonstrated by ultracytochemical studies that avian myeloblastosis virus acquires adenosine triphosphatase activity in its envelope from the host cell membrane during the budding process of the virus. Similarly, alkaline phosphatase activity on the envelope of Moloney leukemia virus has been suggested to have its origin in the alkaline phosphatase-positive leukemic cells (2). Concerning adenosine triphosphatase activity, moreover, it has been suggested that the surface enzymatic activity, which shows heterogeneity at different portions of the cellular membrane, does not differ between

cells in normal and leukemic mice (2, 4) or between uninfected and herpes simplex virus-infected HeLa cells (8, 9). This must mean that some surface enzymatic activity is retained in the host cell membrane even after virus infection. Thus, RLV virions which bud from the host cell membrane where the inactive enzyme is present could carry the enzyme in their envelopes, whereas virus which buds from the sites where the enzyme is absent would not carry the enzyme. Although the localization in RLV-infected cells of the nucleotide phosphatase and alkaline phosphatase activity was examined by de-Thé et al. (4) and de-Thé (2), there was no enzymatic correlation between the viral envelope and leukemic cell membrane. This observation, however, does not appear inconsistent with the present discussion, as the present interest centers on the hypothetical inactive enzyme. On the basis of the above considerations, a hypothetical mechanism of the interactions is illustrated in Fig. 35, where the inactive enzyme present in the viral envelope and host cell membrane is represented by solid circles. Viruses, regardless of the possession of the inactive enzyme in their envelopes, can penetrate into cells only when they attach to the cell membrane at the portions where the inactive enzyme is present (B and C). Rupture of the cell membrane is limited to only the length required to allow penetration of enveloped particles. This may result from enzyme activation in the cell membrane limited to the sites in immediate proximity to where the envelope becomes attached. However, the enzyme activation in the envelope (A and B) may be expanded from the site of attachment to the portion distal to the cell. It is of interest, in this connection, that Morgan and Rose (14) suggested that "during entry the antibody-combining sites at the surface of influenza virus distal to the cell become altered." As seen in Fig. 19, intact virus particles were frequently encountered attached to the intact cell membrane even at 2 and 3 hr. The hypothetical interactions of these viruses with the cell surface are shown in Fig. 35D.

Thus, the present observations have suggested that not only the viral envelope (6, 20, 22) but also the specificity of the host cell membrane is closely related to the initiation of MuLV infection. However, whether the limited host cell range in MuLV infection is due to differences in the surface enzymatic specificity of the unsusceptible cells or whether events subsequent to viral penetration do not proceed in unsusceptible cells must be determined by further study.

The majority of the phagocytosed virus particles appear to be disintegrated in the vesicles without contact with the vesicular membrane. Only two particles throughout the present examination appeared to undergo any interaction between the viral envelope and vesicular membrane. These interactions appeared to occur at the time when the interactions between the viral envelope and cell membrane were evident. Therefore, the possibility could not be excluded that a few phagocytosed viruses would penetrate into the cytoplasm through the vesicular membrane.

The cells employed here (11) provide an excellent tool for further studies of the early events following MuLV infection. Although several thousand cell profiles were examined in the present study, no budding particles similar to MuLV were encountered either in infected or in uninfected cells at early times after infection. Budding particles resulting from RLV infection were found in the cells which were infected in the same manner as in the present study and fixed later than 5 days after infection (*unpublished data*). The possibility of detecting cellular alterations induced by MuLV, which are evident before viral differentiation, is currently under investigation.

ACKNOWLEDGMENTS

This investigation was supported by contract NIH69-97 from the Special Virus Cancer Program, National Cancer Institute.

The excellent technical assistance of Yong Ki Lee, Carol Owen, and Diane Krell is gratefully acknowledged.

LITERATURE CITED

1. Dales, S. 1965. Penetration of animal viruses into cells. Progr. Med. Virol. 7:1–43.
2. de-Thé, G. 1966. Association of enzymes: adenosine triphosphatase and alkaline phosphatase with the virions of murine leukemias. Nat. Cancer Inst. Monograph 22:169–189.
3. de-Thé, G., C. Becker, and J. W. Beard. 1964. Virus of avian myeloblastosis (BAI strain A). XXV. Ultracytochemical study of virus and myeloblast phosphatase activity. J. Nat. Cancer Inst. 32:201–235.
4. de-Thé, G., C. Becker, and J. W. Beard. 1966. Ultrastructural cytochemistry of enzymes associated with murine leukemia viruses. I. Adenosine triphosphatase. Int. J. Cancer 1:119–138.
5. de-Thé, G., and T. E. O'Connor. 1966. Structure of a murine leukemia virus after disruption with Tween-ether and comparison with two myxoviruses. Virology 28:713–728.
6. de Tkaczevski, L., E. de Harven, and C. Friend. 1968. Structure and leukemogenic activity of a murine leukemia virus. J. Virol. 2:365–375.
7. Duc-Nguyen, H. 1968. Enhancing effect of diethylaminoethyldextran on the focus-forming titer of a murine sarcoma virus (Harvey strain). J. Virol. 2:643–644.
8. Epstein, M. A., and S. J. Holt. 1963. The localization by electron microscopy of HeLa cell surface enzymes splitting adenosine triphosphatase. J. Cell Biol. 19:325–336.
9. Epstein, M. A., and S. J. Holt. 1963. Electron microscopic observations on the surface adenosine triphosphatase-like enzymes of HeLa cells infected with herpes virus. J. Cell Biol. 19:337–347.
10. Fazekas de St. Groth, S. 1948. Viropexis, the mechanism of influenza virus infection. Nature (London) 162:294–295.
11. Hartley, J. W., W. P. Rowe, W. I. Capps, and R. J. Huebner. 1969. Isolation of naturally occurring viruses of the murine leukemia virus group in tissue culture. J. Virol. 3:126–132.

12. Heine, J. W., and C. A. Schnaitman. 1969. Fusion of vesicular stomatitis virus with the cytoplasmic membrane of L cells. J. Virol. 3:619–622.
13. Morgan, C., and C. Howe. 1968. Structure and development of viruses as observed in the electron microscope. IX. Entry of parainfluenza I (Sendai) virus. J. Virol. 2:1122–1132.
14. Morgan, C., and H. M. Rose. 1968. Structure and development of viruses as observed in the electron microscope. VIII. Entry of influenza virus. J. Virol. 2:925–936.
15. Morgan, C., H. M. Rose, and B. Mednis. 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2:507–516.
16. Morgan, C., H. S. Rosenkranz, and B. Mednis. 1969. Structure and development of viruses as observed in the electron microscope. X. Entry and uncoating of adenovirus. J. Virol. 4:777–796.
17. Nii, S., C. Morgan, H. M. Rose, and K. C. Hsu. 1968. Electron microscopy of herpes simplex virus. IV. Studies with ferritin-conjugated antibodies. J. Virol. 2:1172–1184.
18. Novikoff, A. B., G. de-Thé, D. Beard, and J. W. Beard. 1962. Electron microscopic study of the ATPase activity of the BAI strain A (myeloblastosis) avian tumor virus. J. Cell Biol. 15:451–462.
19. Rauscher, F. J. 1962. A virus induced disease of mice characterized by erythrocytopoiesis and lymphoid leukemia. J. Nat. Cancer Inst. 29:515–543.
20. Shibley, G. P., F. J. Carleton, B. S. Wright, G. Schidlovsky, J. H. Monroe, and S. A. Mayyasi. 1969. Comparison of the biologic and biophysical properties of the progeny of intact and ether-extracted Rauscher leukemia viruses. Cancer Res. 29:905–911.
21. Suggestions for the classification of oncogenic RNA viruses. 1966. J. Nat. Cancer Inst. 37:395–397.
22. Zeigel, R. F., and F. J. Rauscher. 1964. Electron microscopic and bioassay studies on a murine leukemia virus (Rauscher). I. Effects of physicochemical treatments on the morphology and biological activity of the virus. J. Nat. Cancer Inst. 32:1277–1307.