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Electron Microscopic Study of the Development of Simian Virus 40 by Use of Ferritin-labeled Antibodies

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An electron microscopic study of simian virus 40 has revealed a number of structural changes that are related to the development of the virus. The presence of viral antigens in both the nucleus and the cytoplasm has been demonstrated by means of ferritin-labeled antibodies. Although cytoplasmic virions are readily tagged, the lack of tagging of nuclear particles presents a perplexing problem. Presumably, the virus, after release from the nucleus, acquires a new antigenic reactivity in the cytoplasm.

Electron microscopic observations on the development of simian virus 40 (SV40) have been described by Gaylord and Hsiung (7), Mayor et al. (12, 13), and Granboulan et al. (8). In addition, studies of the viral structure with the negative stain technique have been reported by Bernhard et al. (4), Mayor et al. (11), and Black et al. (5). Since the application of ferritin-labeled antibodies has been shown to be an excellent method of localizing viral antigens at the ultrastructural level, this method was used in the present study to examine SV40 viral antigen in infected BS-C-1 cells.

MATERIALS AND METHODS

Virus. The VA45-54 strain of SV40, obtained from M. R. Hilleman, Merck Institute for Therapeutic Research, was used throughout these studies.

Cell cultures. Hopps' BS-C-1 continuous line of African green monkey cells was supplied by E. L. Buescher, Walter Reed Army Institute of Research. The cells were grown in medium 199 supplemented with 20% fetal calf serum. Infected cells were maintained in Eagle-Hanks' medium with 2% fetal calf serum.

Ferritin conjugation. Monkey anti-SV40 serum was provided by Fred Rapp, Baylor University. Sodium sulfate-precipitated γ-globulin was conjugated to ferritin with *meta*-xylylene diisocyanate, by use of the method of Singer (17) as modified by Rifkind et al. (16).

Procedure. Monolayers of BS-C-1 cells were inoculated with 0.5 ml of undiluted stock virus from frozen and thawed infected tissue culture fluid. After an adsorption period of 4 hr, the inoculum was replaced with Eagle-Hanks' medium containing 2% fetal calf serum. At 2, 3, and 4 days after infection, the cells were scraped from the glass, fixed in glutaraldehyde with osmium tetroxide added afterwards, dehydrated in graded dilutions of ethyl alcohol, and embedded in Epon 812. The ferritin-labeled antibodies were applied as previously described by Morgan et al. (15). In essence, the infected monolayers were briefly fixed in Formalin, treated with 10% dimethyl sulfoxide in 0.1 M phosphate buffer, frozen in a CO_2-ethyl alcohol bath, thawed, immersed in the ferritin-labeled antibody for 1 hr, and washed in phosphate buffer. The cells were then pelleted, fixed in glutaraldehyde, post-fixed in osmium tetroxide, dehydrated in graded ethyl alcohol, and embedded in Epon 812. Thin sections were stained with uranyl acetate and lead citrate, and were examined in either an RCA 3G or a Philips 200 electron microscope.

RESULTS

Thin sections showed only intranuclear virus after 2 days and few intracytoplasmic viral particles by the third day. Four days after infection, however, the cells exhibited widely differing stages of viral development, and many contained viral particles in the cytoplasm. Consequently, subsequent studies on the development of SV40 were largely based on the examination of BS-C-1 cells 4 days after infection.

Figure 1 illustrates intranuclear crystals composed of SV40 virus. The nuclear membrane and part of the cytoplasm are evident on the right.

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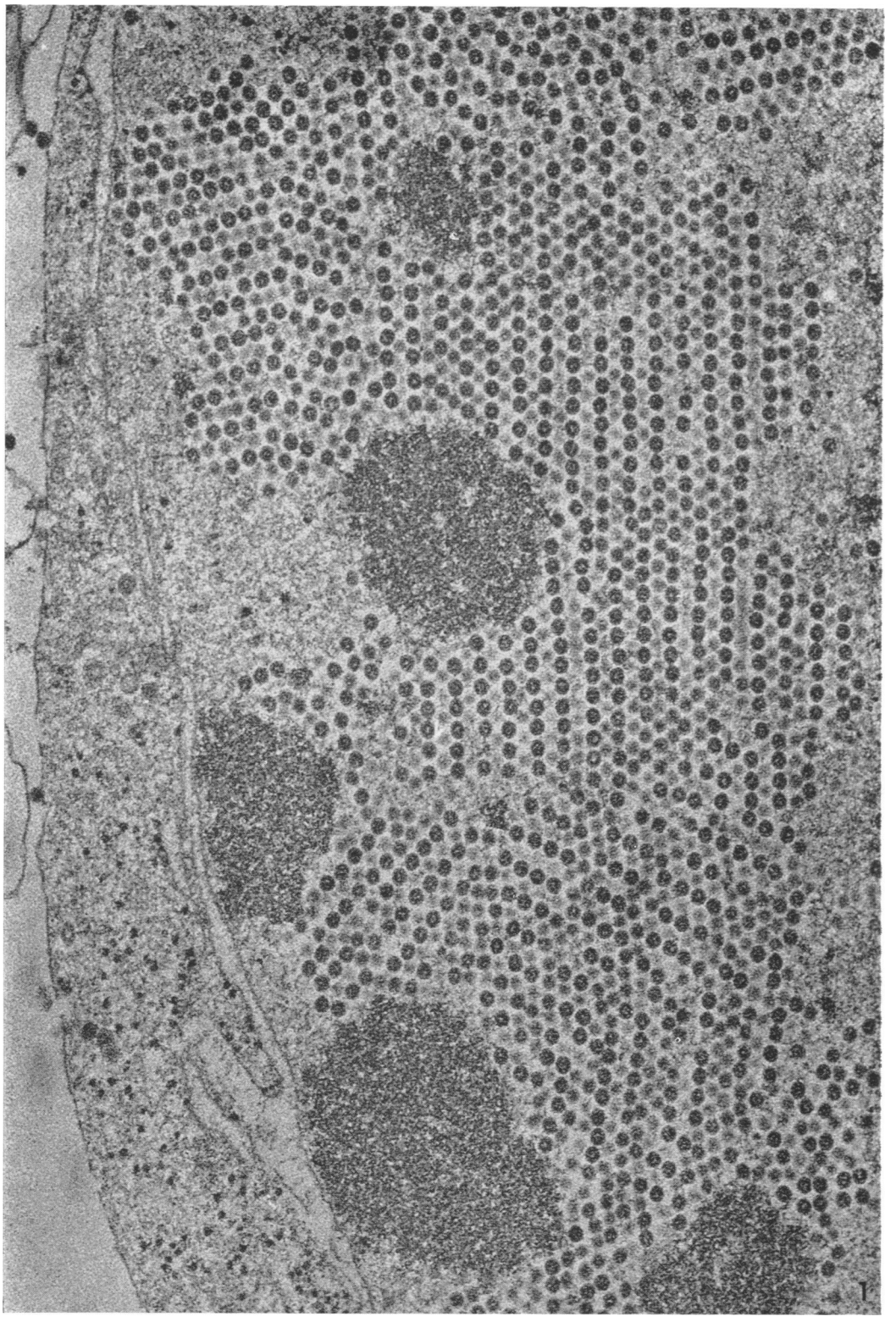


FIG. 1. *Intranuclear crystalline arrays of virus. Nuclear membrane and cellular surface are on the right side of the picture.* × *58,000.*

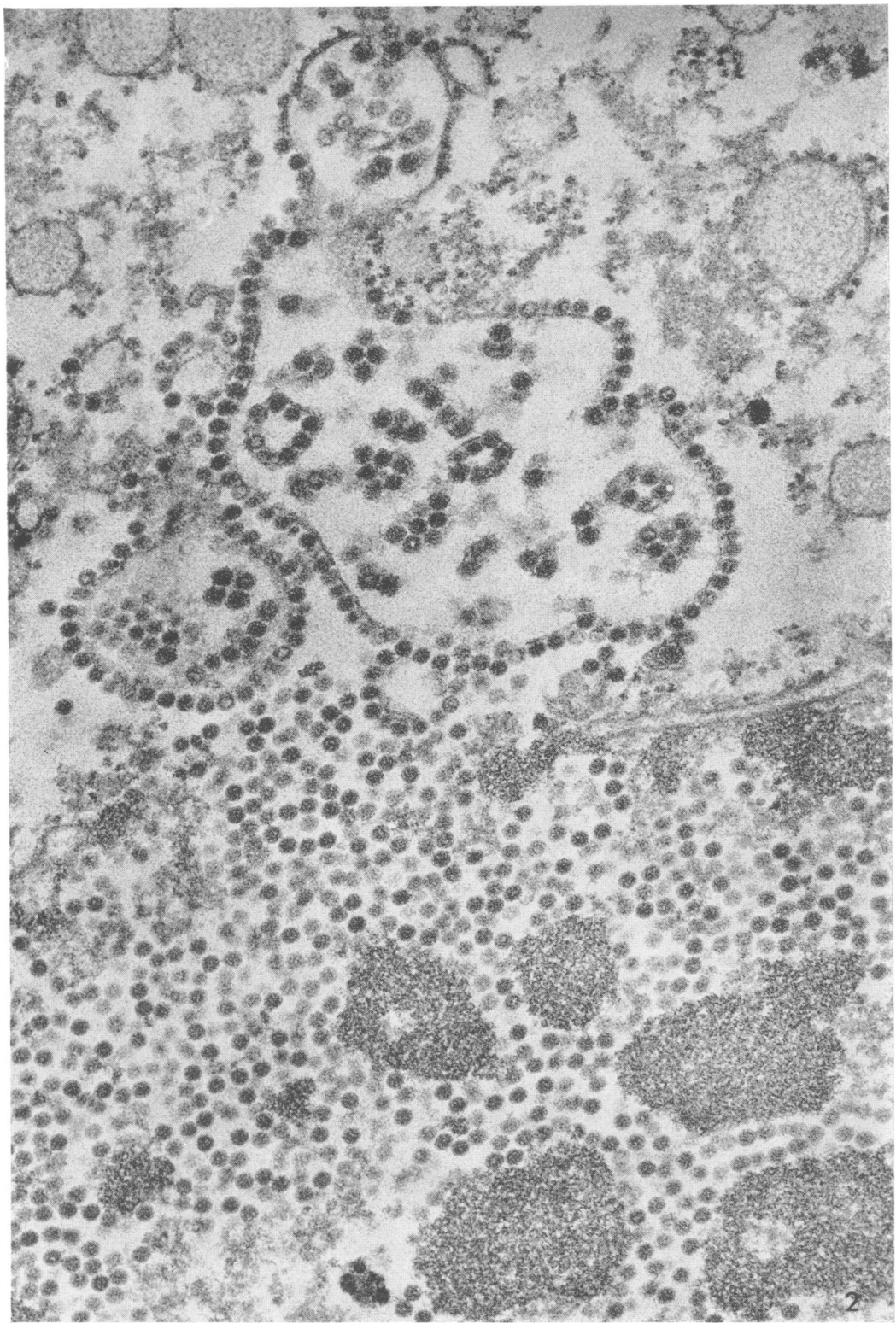


FIG. 2. *Viral particles emerging through a break in the nuclear membrane and aligning on membranous structures in the cytoplasm. As in Fig. 1, patches of dense chromatin are scattered in the nucleus.* × *66,000.*

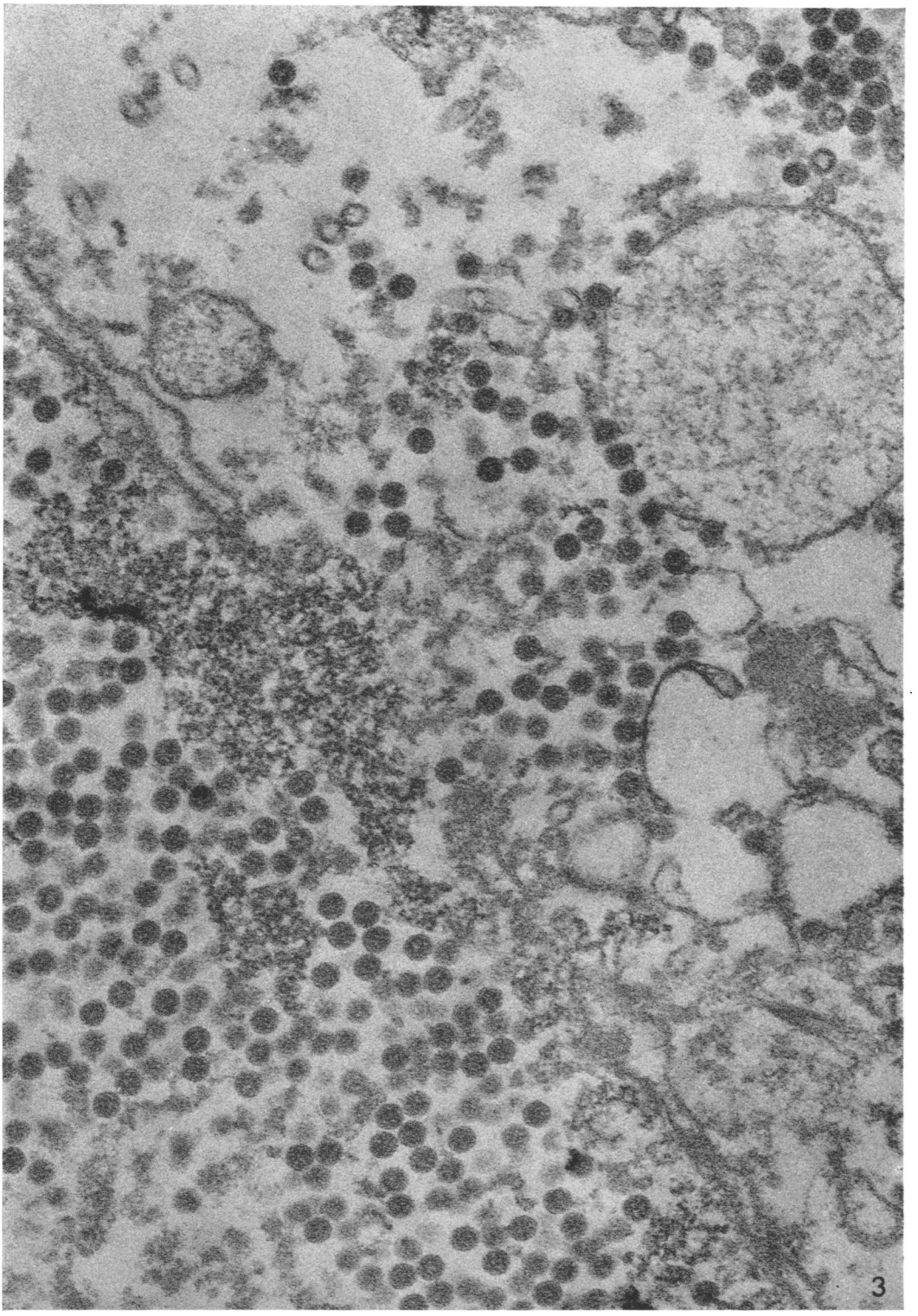
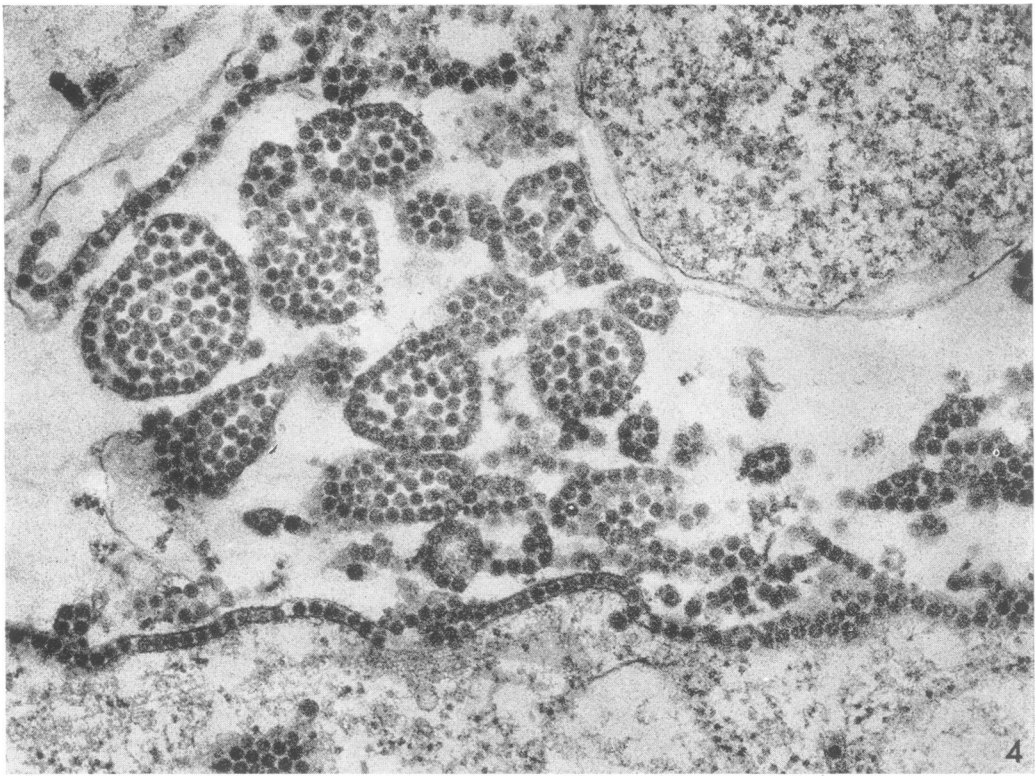


FIG. 3. *Viral particles emerging through a break in the nuclear membrane and aligning on membranous structures in the cytoplasm. As in Fig. 1, patches of dense chromatin are scattered in the nucleus.* × *91,000.*



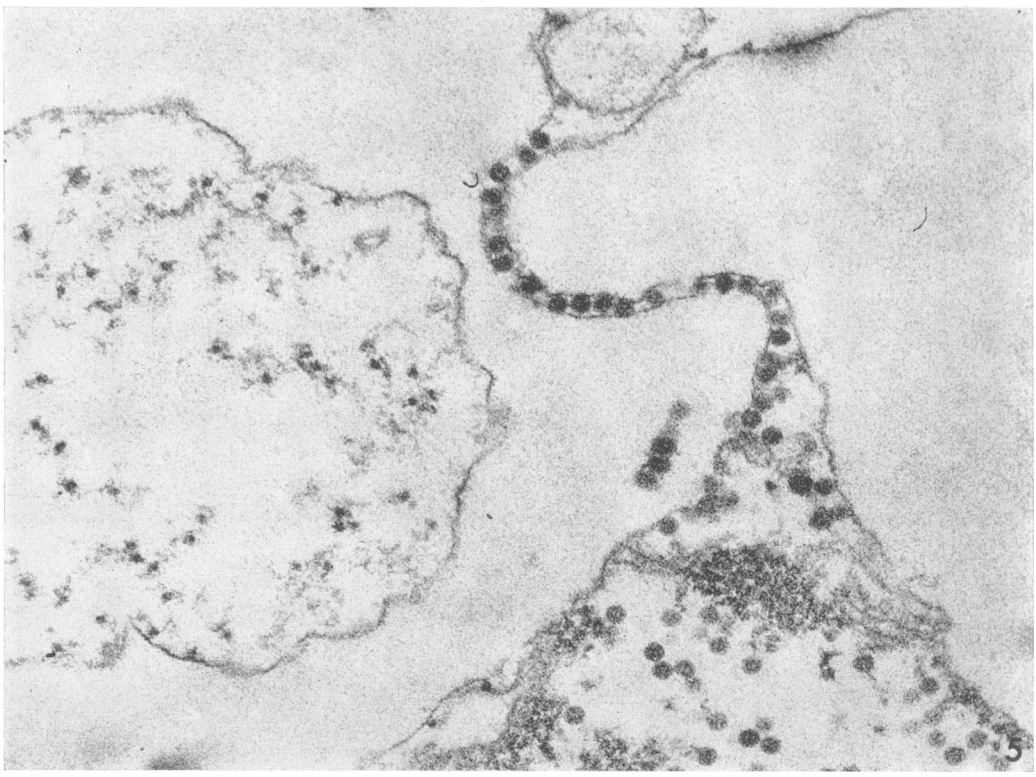


FIG. 4. *Groups of cytoplasmic viral particles contained within membranes. There are also rows of particles enclosed by two membranes.* × *45,000.*

FIG. 5. *Viral particles emerging from the nucleus into the cytoplasm between membranes of the endoplasmic reticulum.* × *63,000.*

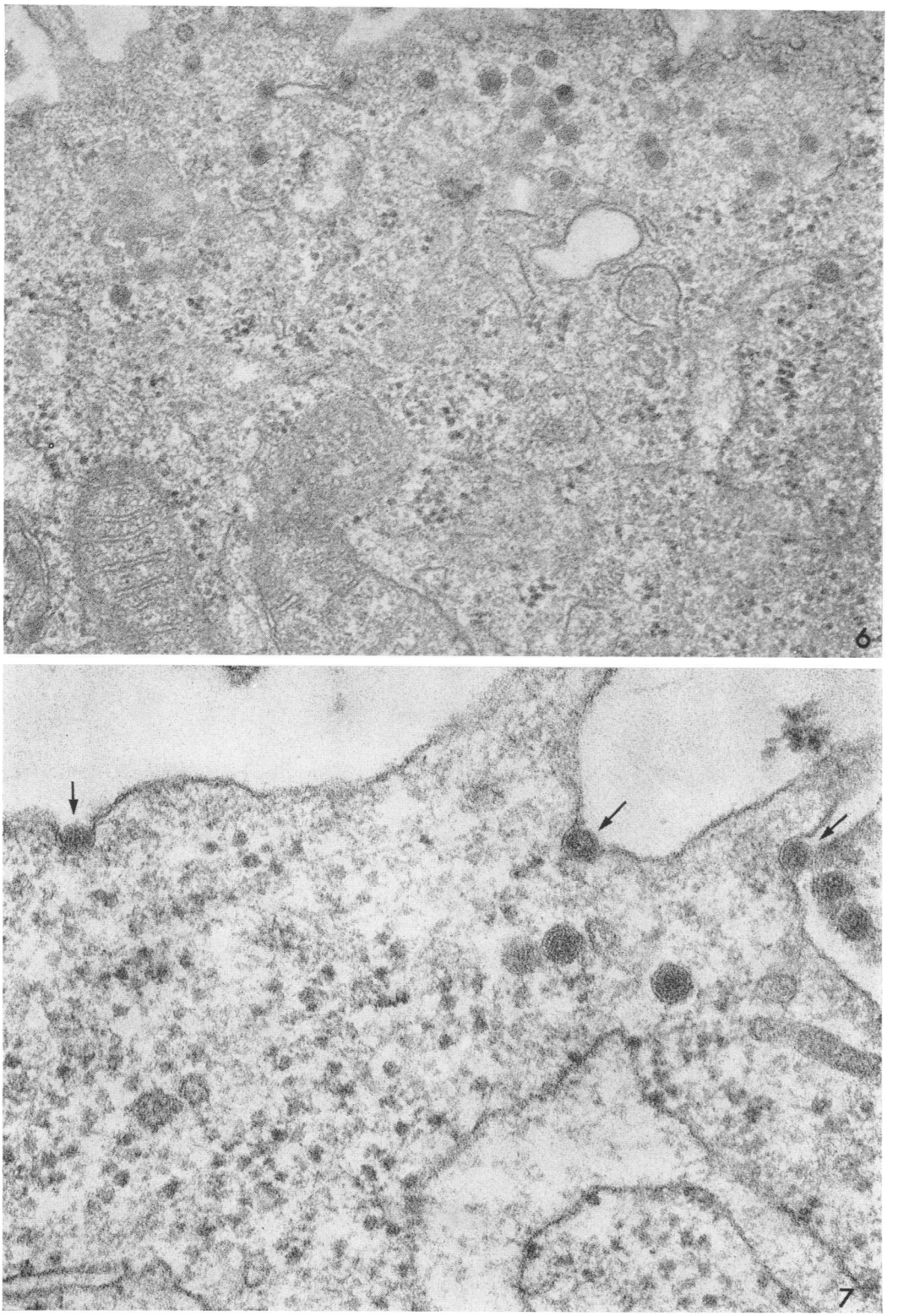


FIG. 6. *Cytoplasm of a cell showing many enveloped forms of the virus. Cellular surface traverses the top of the field.* × *55,000.*

FIG. 7. *Three viral particles in process of ingestion. Two enveloped particles are visible just below the surface membrane.* × *106,000.*

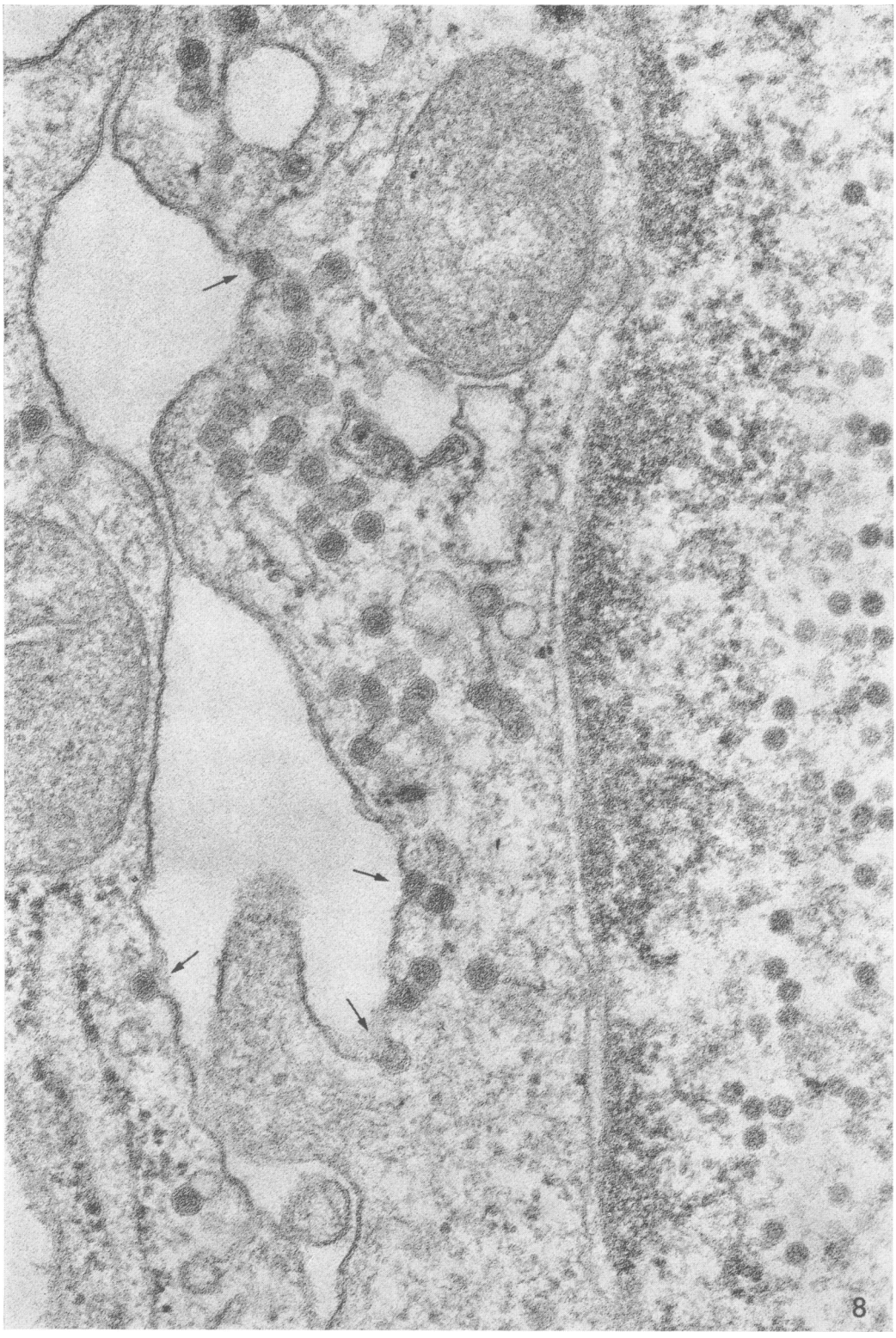


FIG. 8. *Infected cell showing intranuclear particles, which are smaller than the enveloped particles in the cytoplasm to the left. Arrows indicate several particles in the process of being ingested. The surface of an adjacent cell is seen on the left.* × *90,000.*

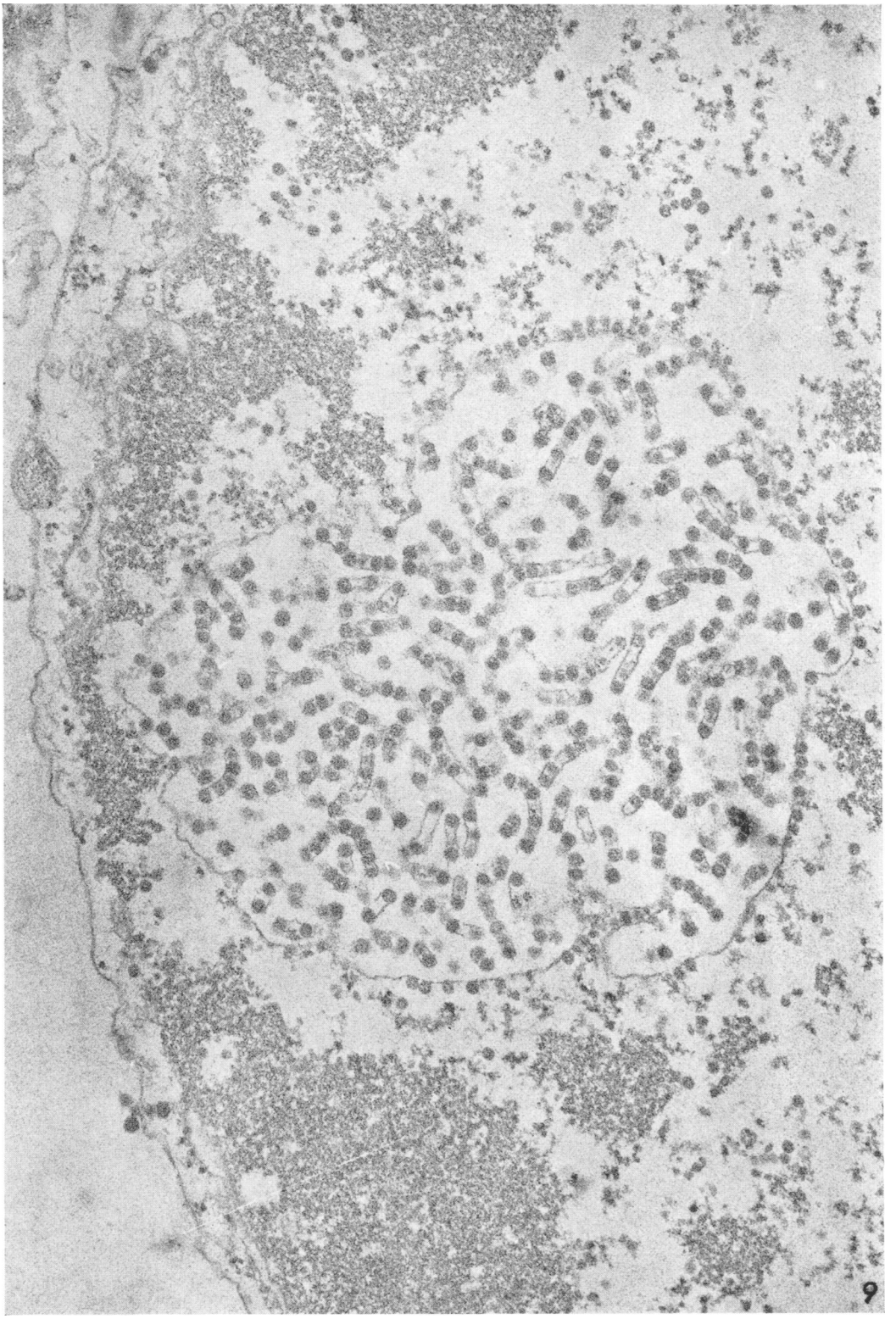


FIG. 9. *Nuclear inclusions containing viruses between membranes.* × *46,000.*

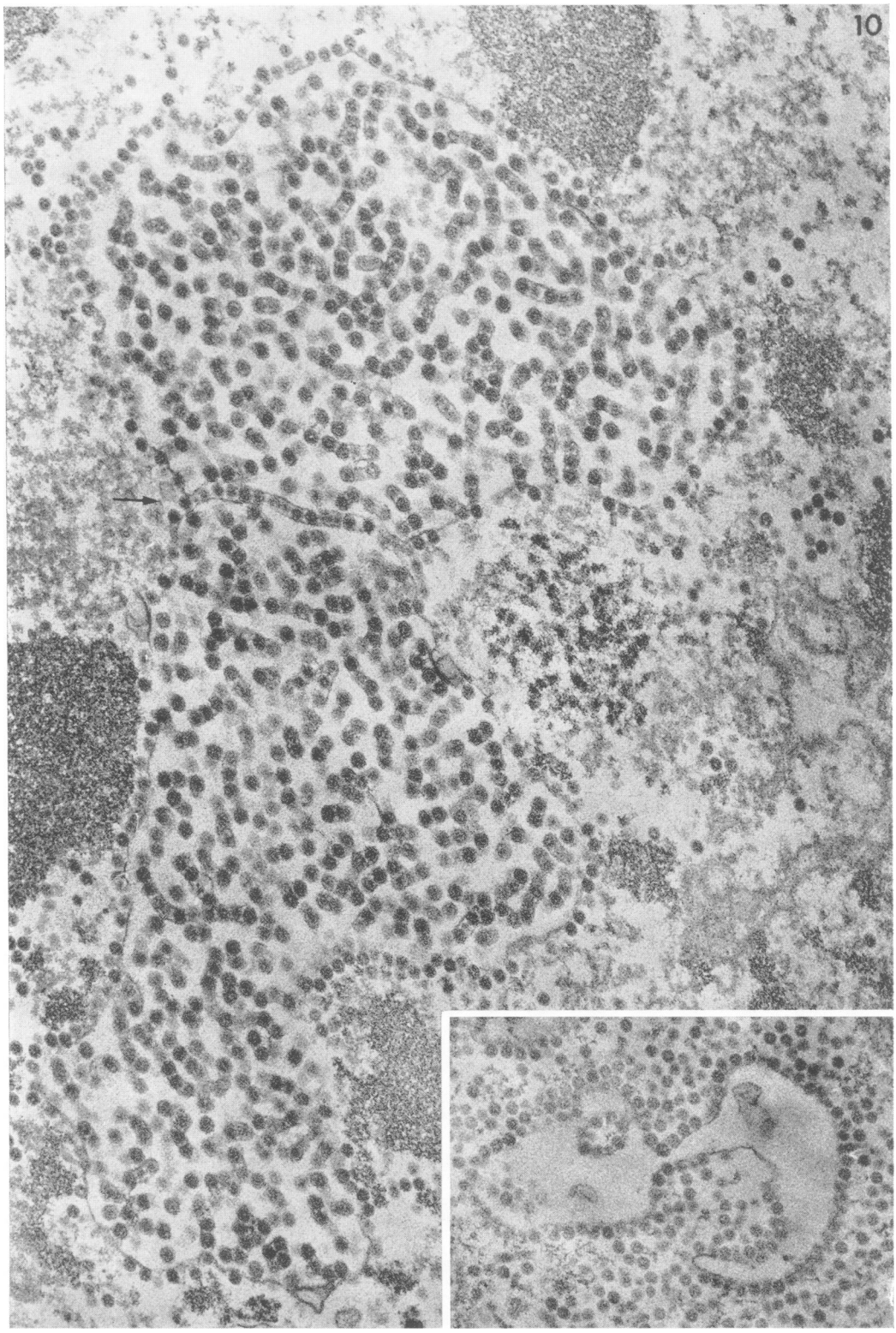


FIG. 10. *Nuclear inclusions containing viruses between membranes. Arrow indicates a row of viral particles between the delimiting membranes of two adjacent inclusions. Inset illustrates viral particles budding into and pinching off within a nuclear vacuole.* × *50,000; inset,* × *39,000.*

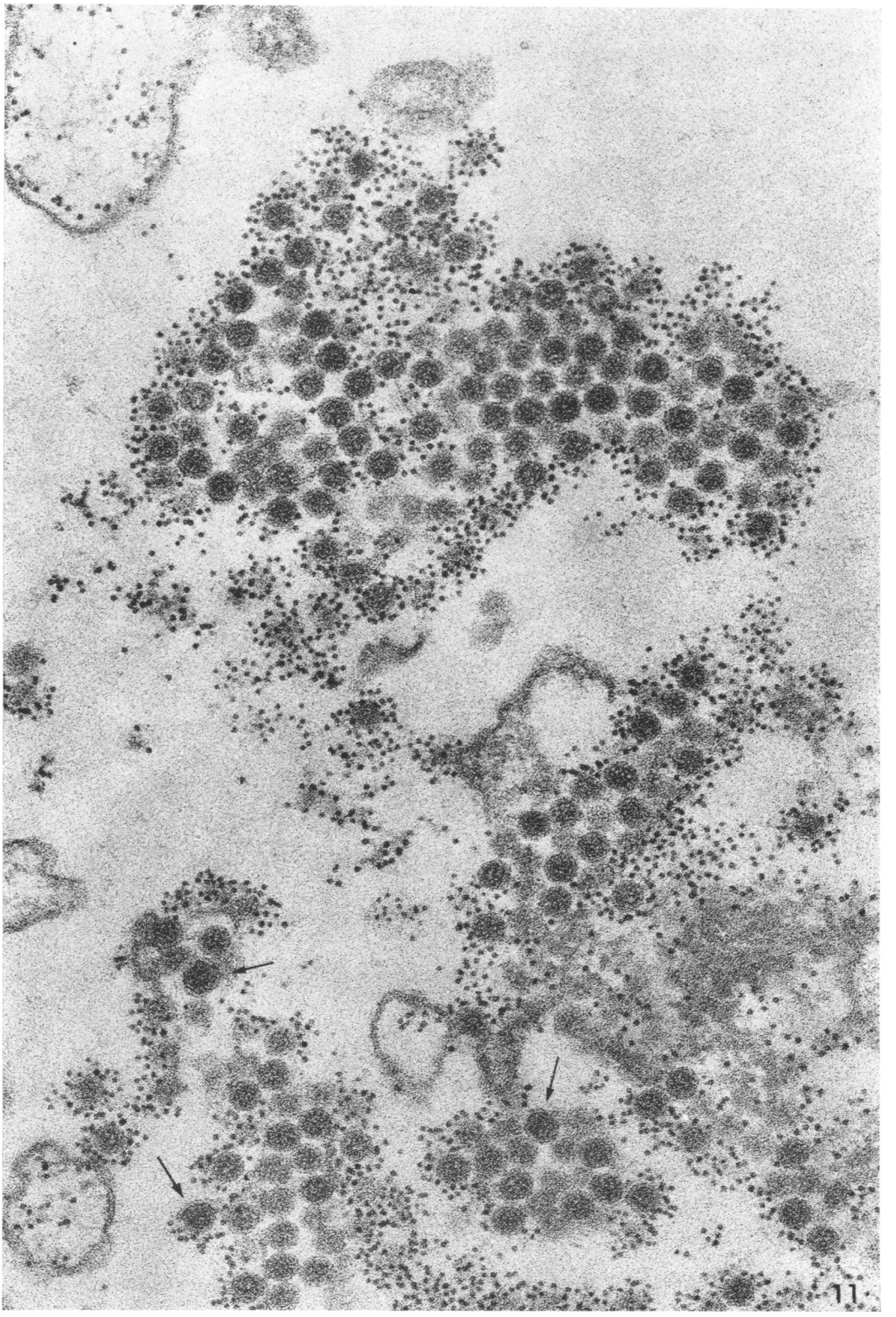


FIG. 11. *Intracytoplasmic viral particles heavily tagged with ferritin-labeled antibodies. Arrows point to particles which are protected by a cellular membrane.* × *120,000.*

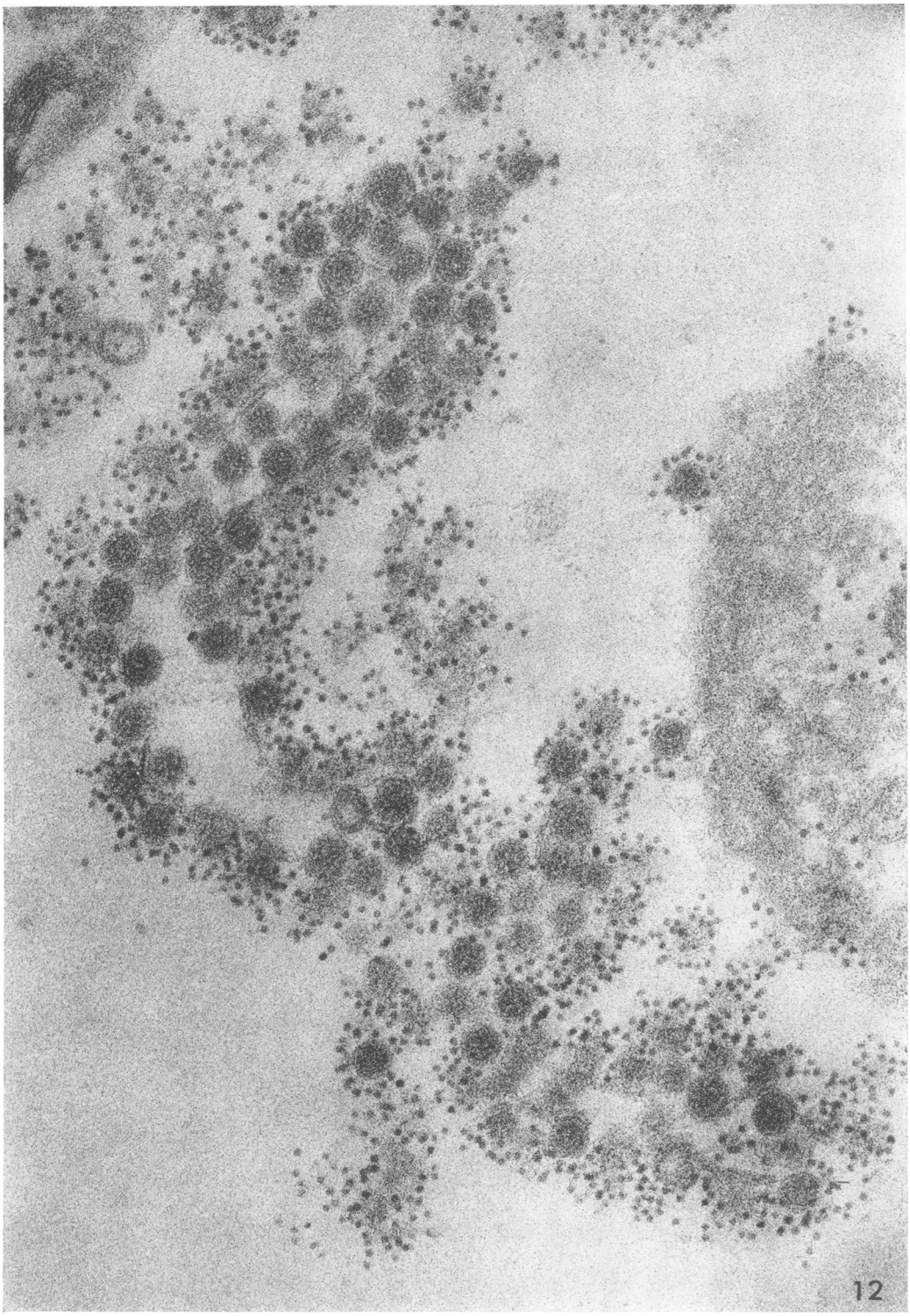


FIG. 12. *Intracytoplasmic viral particles heavily tagged with ferritin-labeled antibodies.* × *150,000.*

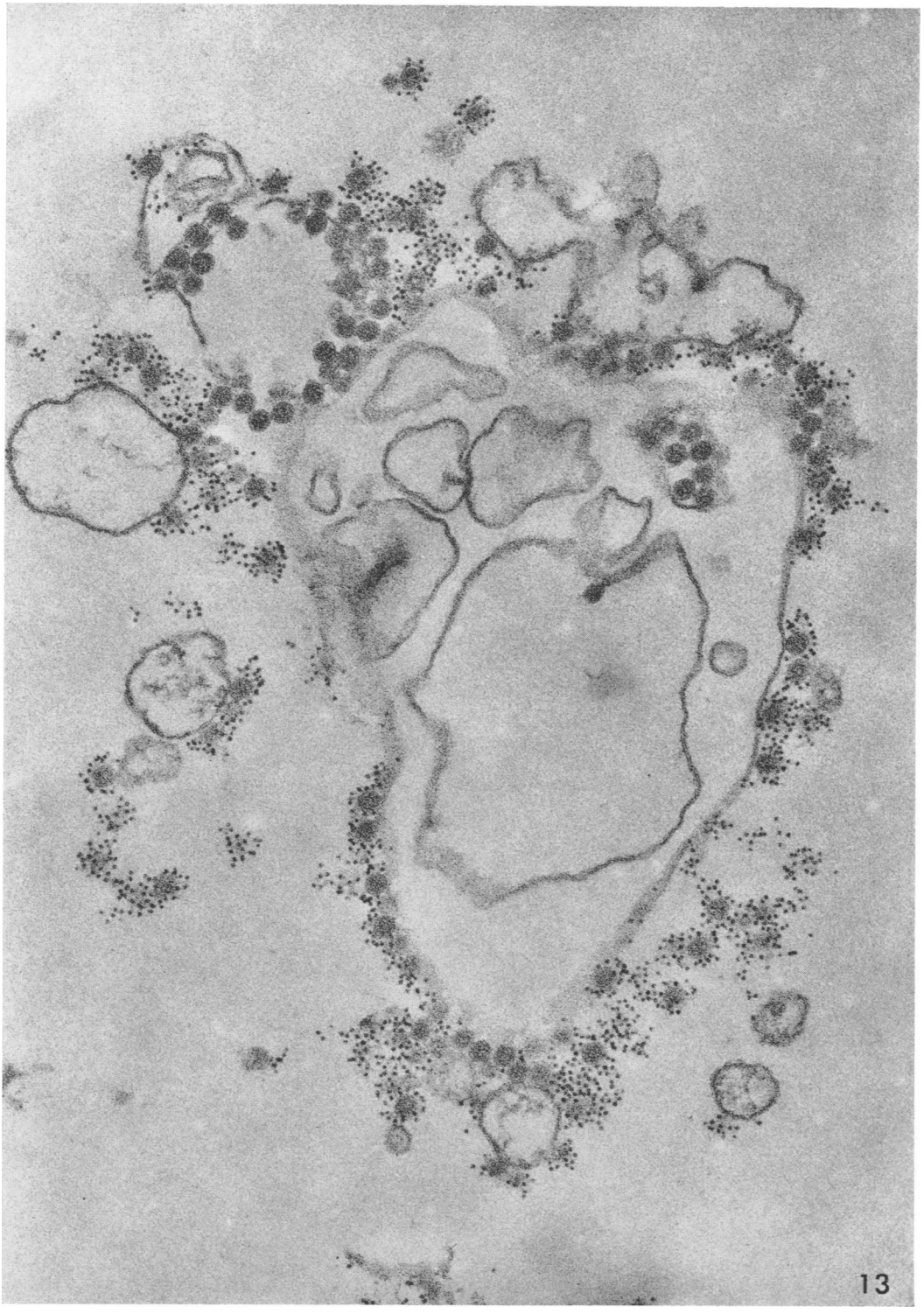


FIG. 13. *Intracytoplasmic viral particles heavily tagged with ferritin-labeled antibodies.* × *80,000.*

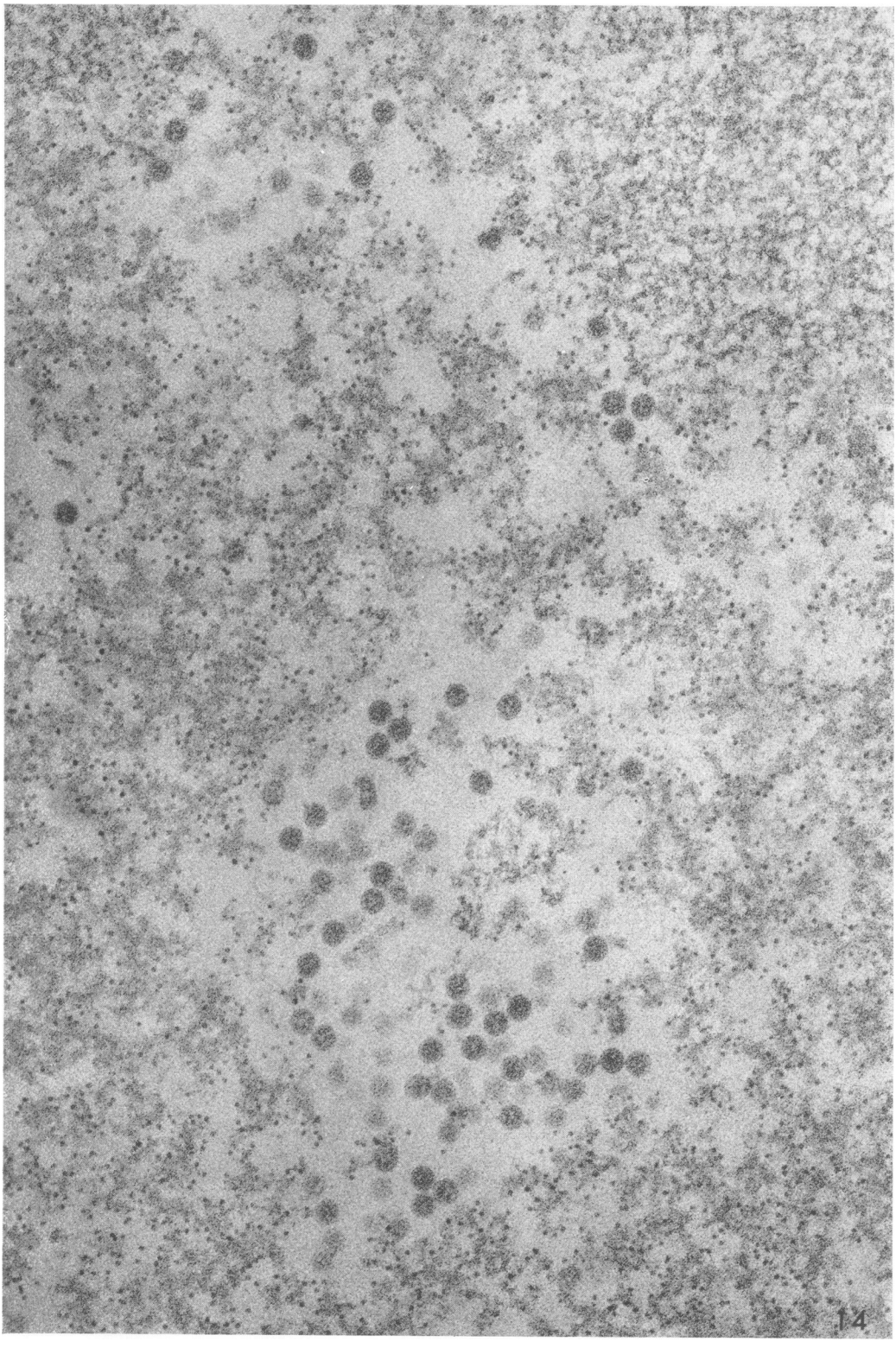
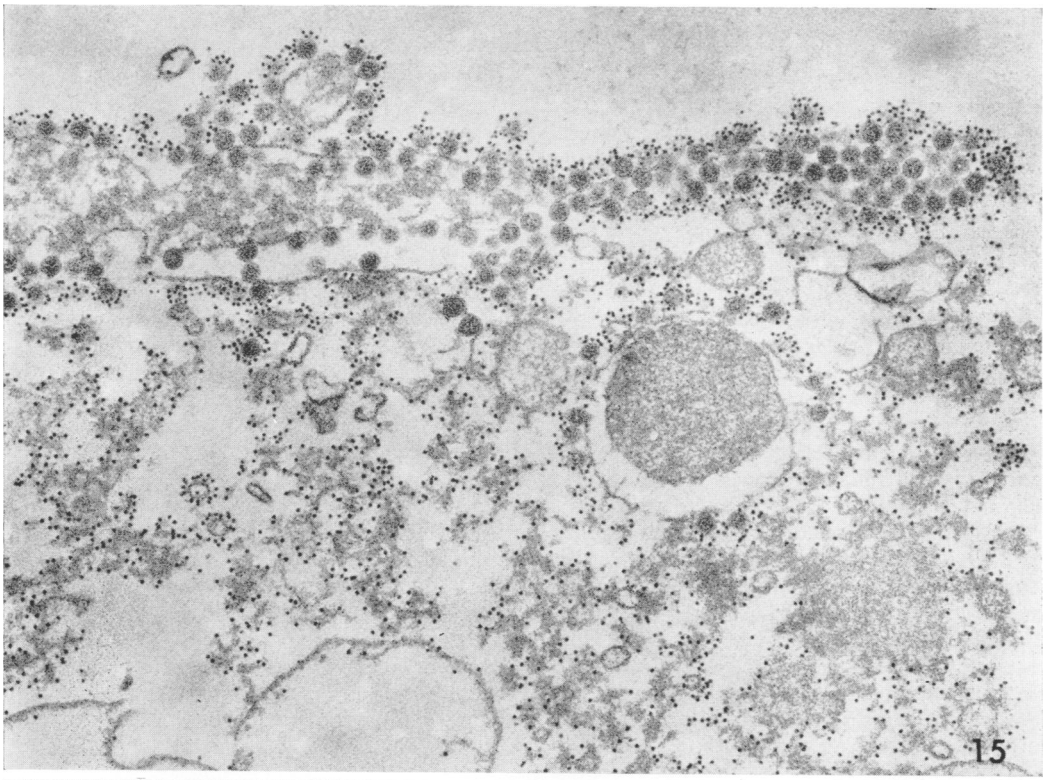


FIG. 14. *Nuclear viral particles untagged by ferritin-labeled antibodies. Dense amorphous material is heavily tagged, indicating its antigenic nature. Nuclear chromatin at the upper right corner is not tagged.* × *97,000.*



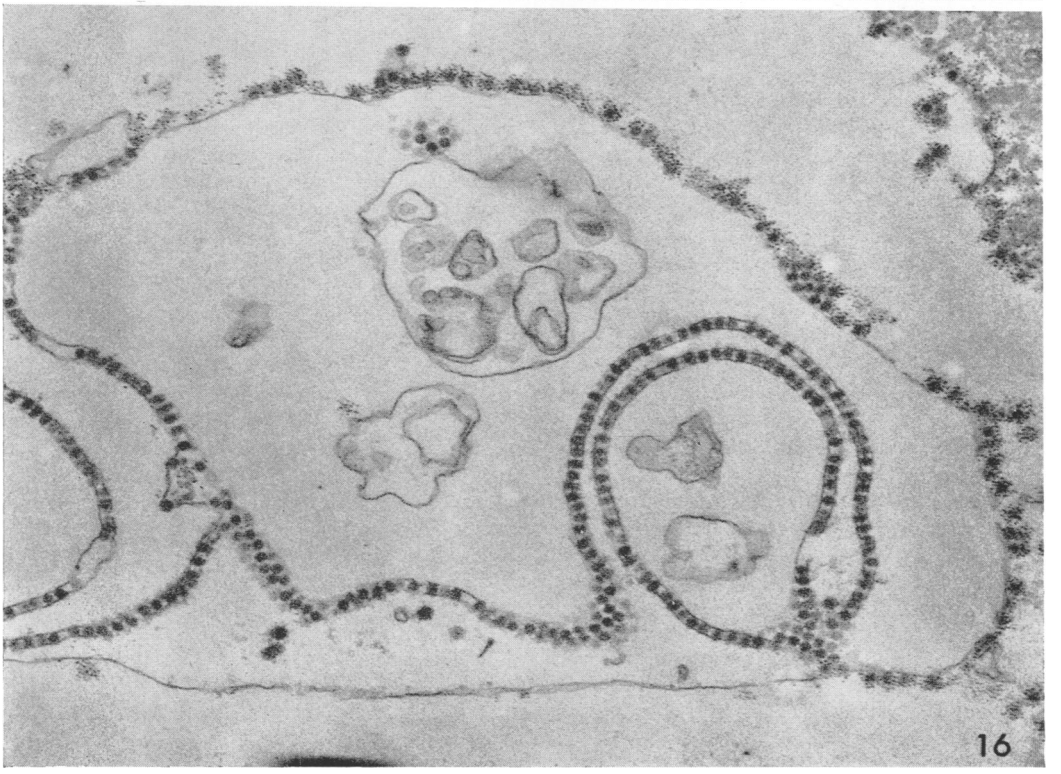


FIG. 15. *Cytoplasm of an infected cell treated with ferritin-labeled antibodies, showing tagging of viral particles and amorphous antigenic material.* × *62,000.*

FIG. 16. *Cytoplasm of a cell at an advanced stage of infection. Viral particles which are enclosed between two membranes are not tagged. Particles which are lined against a single membrane are heavily tagged on the exposed surface. Membranes are devoid of ferritin.* × *37,000.*

Clumps of chromatin are scattered between the viral crystals. Figures 2 and 3 show one of several ways in which virions may enter the cytoplasm: there are breaks in the nuclear membrane with escape of virus. Many of the particles appear to be attached to a membranous structure within the cytoplasm.

Not infrequently intracytoplasmic virus was found to be contained between two continuous membranes of varying lengths (Fig. 4, 5, and 6). Figure 4 illustrates a portion of the cytoplasm at an advanced stage of infection. In the bottom and upper left portion of Fig. 4, one can readily see long rows of parictles enclosed between two membranes. The way in which such enclosure may occur is illustrated in Fig. 5. The nucleus is at the bottom. The viral particles, which have emerged into the cytoplasm through a break in the inner nuclear membrane, are caught between membranes of the endoplasmic reticulum.

Figure 6 illustrates enveloped forms of the virus located in the cytoplasm just beneath the surface of a cell. Presumably these particles have been phagocytized. This phenomenon is seen more clearly in Fig. 7, which shows a cell in the process of ingesting three particles (see arrows). Probably the two viral particles which appear just below the surface near the center of the field have just been phagocytized. In Fig. 8, a portion of the nucleus containing virus is on the right of the micrograph. The cytoplasm occupies the middle of the picture, and the cell membrane borders the surface of an adjacent cell to the left. It can be seen that the virus is in different stages of ingestion (arrows). One may argue that the virus was leaving the cell, but a study of this and many other similar cells revealed no virus that appeared to be in the process of extrusion from the cytoplasm, suggesting that the intracytoplasmic virus is not being released.

Figure 9 illustrates another feature of virus development which was occasionally observed. Within the nucleus is a walled vacuole containing rows of virus enclosed by membranes. The inset in Fig. 10 shows a similar region within a nucleus and illustrates the probable mechanism whereby the particles are enclosed by membranes. The virions seem to bud into the vacuole and are pinched off within membranes. Figure 10 reveals a row of intranuclear viral particles (see arrow) contained within a membranous structure. Rupture of the nucleus with release of virus enclosed by membranes resulted in the pattern of Fig. 4.

Results of the application of ferritin-labeled SV40 antibodies to infected cells are illustrated in Fig. 11 to 16. In Fig. 11 to 13, which show intracytoplasmic viral particles heavily tagged with ferritin, the cells are at a relatively advanced stage of infection with consequent loss of cytoplasmic matrix. In Fig. 11 and 12, it is evident that ferritin granules do not penetrate areas where the virus is closely packed. In Fig. 13, virus within membranes remains untagged since the methods of preparation do not render these membranous structures permeable. Figure 14 shows that intranuclear viral particles are not tagged in spite of the presence of numerous ferritin granules attached to amorphous aggregates of viral antigen scattered in the nuclear matrix. The filamentous material at the upper right corner, which is devoid of ferritin, is nuclear chromatin. Not infrequently, after rupture of the nucleus, viral antigen accumulates in the cytoplasm. In Fig. 15, the illustration of the surface of a disrupted cell, ferritin has tagged this antigen as well as the virus. As in Fig. 13, ferritin has not penetrated the membrane-lined vesicle containing virus. Figure 16 shows virus within membrane-lined channels. Ferritin has tagged the viral particles only in regions where the membrane has been disrupted. The fact that the membranes are not labeled would indicate that they are composed of host-cell protein.

DISCUSSION

Previous studies of the development of SV40 by Mayor et al. (12, 13), Granboulan et al. (8), and Black et al. (5) have demonstrated many of the structures shown in the present investigation. We have attempted to ascertain the probable manner in which these structures evolve. We have also sought to demonstrate the presence of viral antigen, both in association with the actual viral particle and elsewhere within the host cell.

The long arrays of viral particles lying between two membranes (8, 13), which are illustrated in Fig. 4 and 16, could be formed in any of three different ways. In the first, the viral particles break through the inner nuclear membrane and emerge into the cytoplasm between the outer nuclear membranes of the endoplasmic reticulum (Fig. 5). In the second, virions which leave the nucleus by rupture of the nuclear membrane are enclosed within vacuoles and frequently become attached to the membranes lining such vacuoles (Fig. 2 to 4). Lastly, the virus may be enclosed within membranes in the process of protrusion into intranuclear vacuoles (Fig. 9 and 10).

Enveloped viral particles, which were approximately 10 mμ larger than nuclear particles, were observed in the studies of Granboulan et al. (8) and Black et al. (5). It appears that these particles (Fig. 6) acquire the membrane during phagocytosis (Fig. 7 and 8) in a manner analogous to that observed for adenoviruses (6). It is interesting to note, as others have done, that many of the

structures which are observed in SV40 infections resemble those seen in polyoma-infected cells (1, 2, 9, 14).

The lack of tagging of nuclear virus with ferritin-labeled antibodies is a perplexing problem in view of the excellent tagging that is observed in the case of intracytoplasmic virions. If this were in fact a reflection of what takes place in serum neutralization of SV40, one could surmise that nuclear particles are devoid of a protein coat. This can be discounted, however, for Bernhard and Tournier (3) have demonstrated in thin sections a protein coat which is digested by pepsin. It is possible that host-cell nucleic acid could coat intranuclear virus, as suggested by Mayor (10, 13), thus preventing antibody from reacting with the antigenic sites on the surface. If such were the case, however, one would expect to see an amorphous coat on the surface of the intranuclear particles. Neither in sections nor after negative staining of virus released from infected cells by sonic treatment has such coating been observed. It appears more likely that the viral particles acquire a new surface antigenic reactivity in the cytoplasm after release from the nucleus.

Similar observations on the lack of surface tagging of nuclear adenoviruses with ferritin-labeled antibodies have been made in this laboratory and elsewhere (Levinthal et al., *in press*). If one accepts the possibility that neutralizing antibody does not react with intranuclear particles of SV40 and adenovirus, then this form of the virus should be noninfectious. However, the possibility that technical difficulties impede the attachment of ferritin-labeled antibodies to the surface of intranuclear virus cannot be entirely excluded, and further studies along these lines are being conducted.

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