JOURNAL OF VIROLOGY, Nov. 1969, p. 777-796

Vol. 4, No. 5
Printed in U.S.A.

Structure and Development of Viruses as Observed in the Electron Microscope

X. Entry and Uncoating of Adenovirus

COUNCILMAN MORGAN, HERBERT S. ROSENKRANZ, AND BAIBA MEDNIS

Department of Microbiology, College of Physicians and Surgeons, Columbia University, New York, New York 10032

Received for publication 10 July 1969

Stages in the direct penetration of adenovirus through the cell membrane are illustrated. Phagocytosis with rupture of the vacuole and release of virus into the cytoplasm may also account for entry of some particles. Uncoating by digestion within phagosomes was not observed. Rather, alteration of capsid and core occurred to virions free in the cytoplasm. Nucleoprotein released from virus close to the nucleus was transported to the nuclear matrix by a unique mechanism. These events were not prevented by puromycin and hence were not dependent upon the synthesis of new enzymes. They were, however, energy-dependent.

A previous electron microscopic study of type 7 adenovirus (2) suggested that phagocytosis is the mechanism by which adenovirus gains entry to cells. It was stated, however, that the presence of free virus within the cytoplasm raises the possibility that "a fraction of the adsorbed virus penetrates directly through the (cell) membrane," a phenomenon that could not be documented at the time intervals (1 and 2 hr) chosen for examination. The present study, which examines the entry of adenovirus in detail, differs from the preceding communication in four particulars. First, adsorption and penetration of the virus took place in monolayer cell cultures while the cells were still in situ. Second, the virus inoculum was purified by banding in cesium chloride. Third, the initiation of viral entry was synchronized by quickly warming the cells to 37 C after a period of viral attachment in the cold. Fourth, and perhaps most important, major attention was devoted to the events occurring within the first 45 min after warming the specimens, because it was during this period of time that Lawrence and Ginsberg (5) and Philipson (9) reported that the viral DNA becomes sensitive to deoxyribonuclease. The results to be reported show that virus is phagocytosed but that direct entry to the cytoplasm also occurs. Stages in the uncoating of the virus and entry into the nucleus of nucleoprotein are illustrated and described.

MATERIALS AND METHODS

Virus. The stock of type 7 adenovirus was kindly supplied by H. M. Rose.

Tissue culture. HeLa cells were grown in Eagle minimal essential medium (MEM) with 10% fetal calf serum.

Preparation of inocula. The cells were infected with a 10^{-1} dilution of stock virus. After incubation for 3 days, the cells were scraped into MEM and exposed to sonic vibration for 5 min; cellular debris was removed by low-speed centrifugation for 10 min. The supernatant fluid was then centrifuged for 3 hr at 35,400 $\times$ g in a Spinco model L ultracentrifuge, and the resulting pellet was resuspended in 3 ml of Earle solution by exposure to sonic vibrations for 5 min.

Further purification of the virus was achieved by centrifugation in gradients of cesium chloride. For this purpose, the virus was prepared as described above, except that the pellet was resuspended in 10 ml of Earle solution. To 3.5-ml portions of this suspension, 1.86 g of CsCl (Penn Rare Metals) was added and the samples were spun for 24 hr at 30,000 rev/min in a SW 50 rotor of a Spinco model L-2 ultracentrifuge. The viral bands were collected as described previously (3), and the CsCl was removed before use by dialysis against several changes of MEM without serum.

General experimental method for attachment and entry. After washing the tissue culture cells with MEM devoid of serum, 0.3 ml of the inoculum was added to sparse monolayers in 30-ml Falcon plastic flasks. These flasks were maintained at 4 C for 30 to 60 min; then they were quickly warmed by replacing the inoculum with MEM at 37 C and incubated at that temperature for 1 to 180 min. In an attempt to slow down processes which might occur rapidly at 37 C, some samples were initially incubated at 37 C for 20 or 40 min and then were held at room temperature for 30 min before fixation.

Preparation of cells for electron microscopy. After

cooling the tissue culture to 4 C for 5 min, the cells were fixed for 20 min in situ with 1% glutaraldehyde buffered at *p*H 7.2. They were then thoroughly washed in Sorenson buffer, scraped off the plastic, pelleted gently, fixed for 30 min in osmium tetroxide, dehydrated in ethyl alcohol, and embedded in epoxy resin (Epon 812). The sections were stained with uranyl acetate followed by lead hydroxide and were examined in a Philips 200 electron microscope.

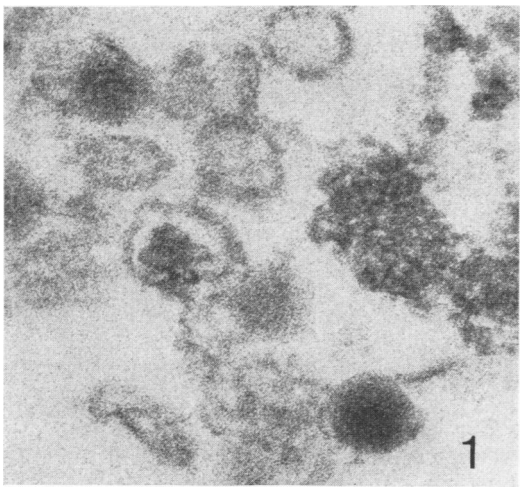
Metabolic experiments. The ability of metabolic inhibitors to interfere with protein synthesis was monitored by supplementing bottles containing HeLa cells (5×10^6) with ^{14}C-algal protein hydrolysate (New England Nuclear Corp); final concentration, 0.48 μg in 3 ml of MEM, 0.22 μc/ml. The cells were exposed to the radioactive precursors for 30 min, and then the cell sheets were washed thoroughly with chilled 0.15 M NaCl and removed from the plastic surface by trypsinization (0.025% trypsin in ethylenediaminetetraacetic acid, 1:5,000). The detached cells were resuspended in 5% trichloroacetic acid, and, after standing in the cold, the insoluble residues were extracted at 90 C for 40 min and collected on Munktell no. 1F filter discs. The residues were washed thoroughly with cold 5% trichloroacetic acid, and the dried filter discs were placed in vials containing 10 ml of omnifluor (4 g per liter of toluene). The retained radioactivity was determined in a liquid scintillation spectrometer.

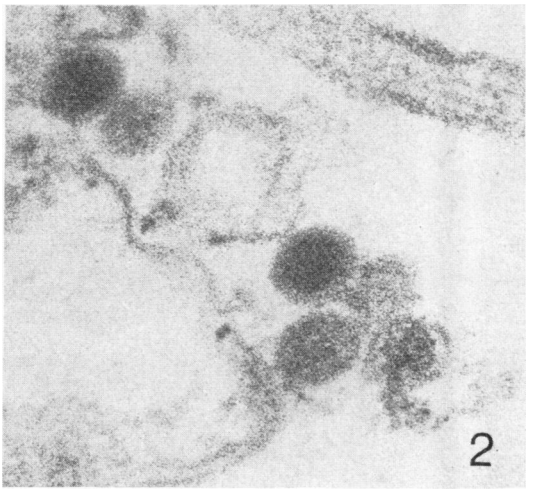
RESULTS

Viral entry. In the initial experiments, tissue cultures were inoculated with suspensions of pellets, which had been obtained by ultracentrifuging the sonic-treated material of infected cells after first clarifying it by low-speed centrifugation. It soon became apparent that such preparations were of little use for the study of viral uncoating, since viral particles, even before entry at the surface of cells, were found in various stages of nonspecific disintegration (Fig. 1 and 2). Moreover, massive clumping of virus was common. Virus obtained by banding in cesium chloride, however, presented an entirely different picture (Fig. 3). The virions exhibited remarkable uniformity in size, shape, and density and were not aggregated. Accordingly, fresh, unfrozen inocula of this type were used for all subsequent experiments. After an adsorption period at 4 C of 30 to 60 min, the cells were incubated at 37 C for 0, 1, 5, 10, 20, 30, 40, 45, 60, 120, and 180 min. At time 0, no intracellular virions were encountered. (It might be noted that there was remarkable variation from one cell to the next in the number of virions attached to the surface. The reason for this is not known.) By the 5th min, a few virions were observed within phagocytic vacuoles near the surface. The first free virus in the cytoplasm was found at 10 min (Fig. 10). Although at later times the entry of virus was not synchronous (a point that will be discussed below), the sequence of events could be arranged in what seems to be a logical order. Figures 4 to 6 show initial stages of viral penetration. (The difference in appearance of the virus is due to the marked contrast in section thickness, the section illustrated by Fig. 5 being unusually thin.) The fine fibrils in the cytoplasm coursing just beneath the surface were commonly encountered and are unrelated to entry of the virus.

Viral "uncoating." Figures 7 to 13 illustrate virions which have passed further into the cytoplasm. It is important to note that in Fig. 7 to 9 and 11 the core of the virion within the cell appears more granular than that of the virion on the surface. The clarity with which this change could be observed depended upon the resolution of the micrograph and the thickness of the section. In thicker sections (Fig. 12 or 13), the granularity of the core was less clearly visualized. Another interesting change in the virion, which occurred at about the same time as the increased granularity of the core, was that the surface became indistinct or fuzzy and the crystalline faces were lost, the particle thus appearing to be spherical. To illustrate this clearly, section thickness is again important, but, in addition, the level of the virus in the plane of section also plays a role. Thus, the alteration of the capsid is difficult to ascertain with certainty for any given particle, but, when viewed repeatedly and carefully compared with virus on the surface, the phenomenon is unmistakable. The change is less obvious at 10 min (Fig. 10). It might be noted how close the nucleus at the left margin of Fig. 11 is to the surface of the cell. It was not unusual for this distance to measure less than 200 nm (Fig. 33). Figures 14 to 16 show three serial sections. When central to the plane of section, the two virions on the surface are sharply defined and exhibit (particularly in Fig. 14) crystalline faces, whereas the virion in the cytoplasm (Fig. 15) is poorly defined with a diffuse peripheral coat. The same phenomenon can be seen very clearly in the serial sections illustrated by Fig. 17 to 19. In Fig. 20, it will be noted that virions nearest to the cell surface (on the right) generally appear less altered than those further in. The foregoing subtle, yet extraordinary, changes in the virion have been illustrated and discussed in some detail simply because their recognition is essential for an understanding of the initial stages in the uncoating process.

Phagocytosis of virus. It was hardly surprising to find virus within vacuoles, since phagocytosis is an active mechanism by which cells ingest





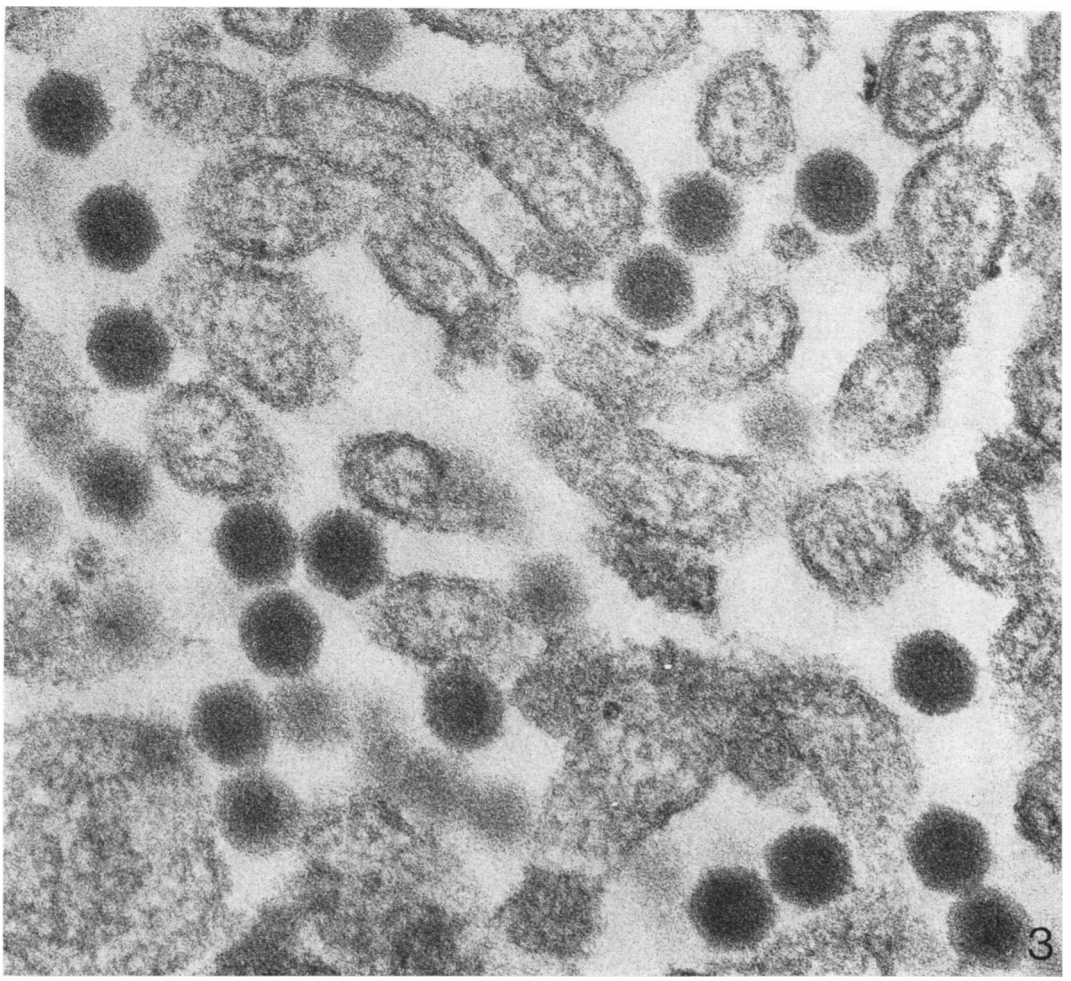
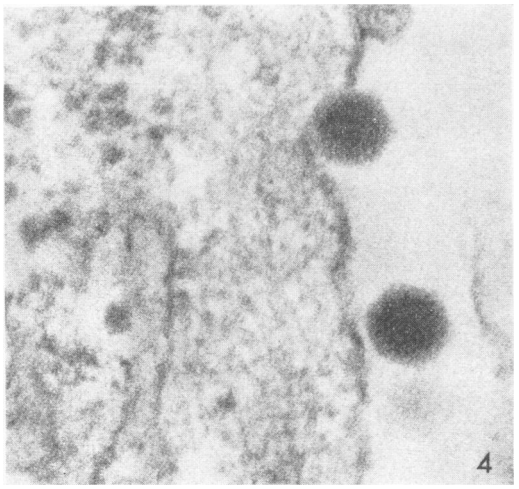
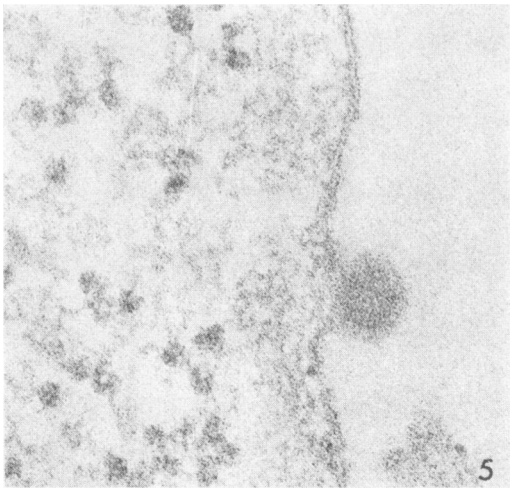
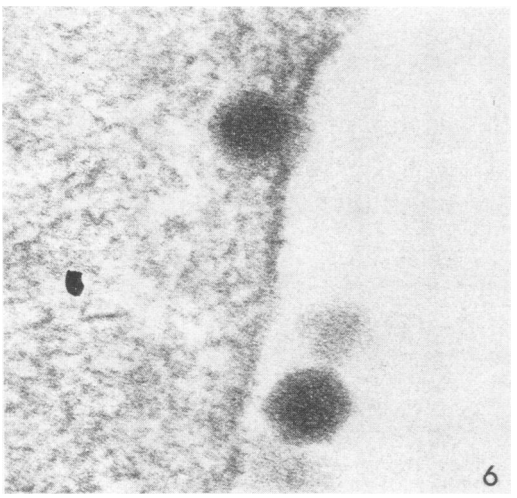
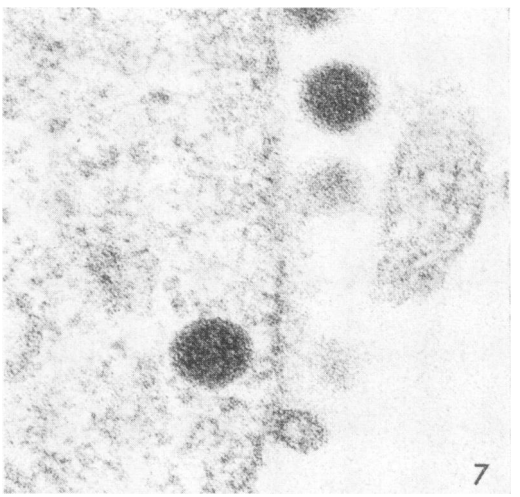


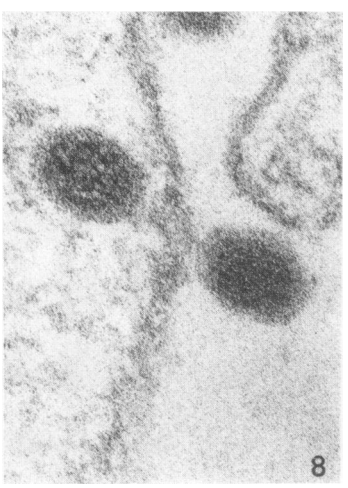
FIG. 1. *Virus from resuspended pellet; 60 min.* × *150,000.*
FIG. 2. *Virus from resuspended pellet; 10 min.* × *150,000.*
FIG. 3. *Virus after banding in CsCl. Virions are trapped among cytoplasmic extensions of a disintegrating cell; 40 min.* × *150,000.*











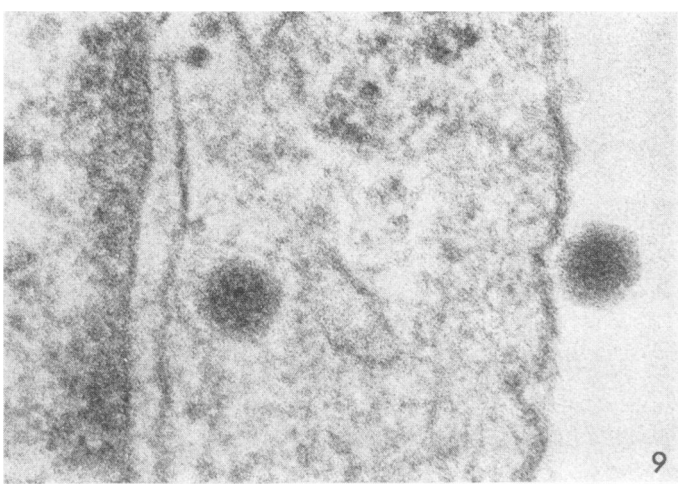


FIG. 4–6. *Passage of virus into the cytoplasm. Note that the extracellular particles in these and other micrographs are seldom in apposition with the cell membrane. Figure 5 is a very thin section.* × *150,000.*

FIG. 7. *Entry with alteration of the core.* × *150,000.*

FIG. 8. *Entry with alteration of the core.* × *200,000.*

FIG. 9. *Virus close to the nucleus (at the left margin). Note the granular core and poorly demarcated periphery compared to the extracellular virion on the right.* × *150,000.*

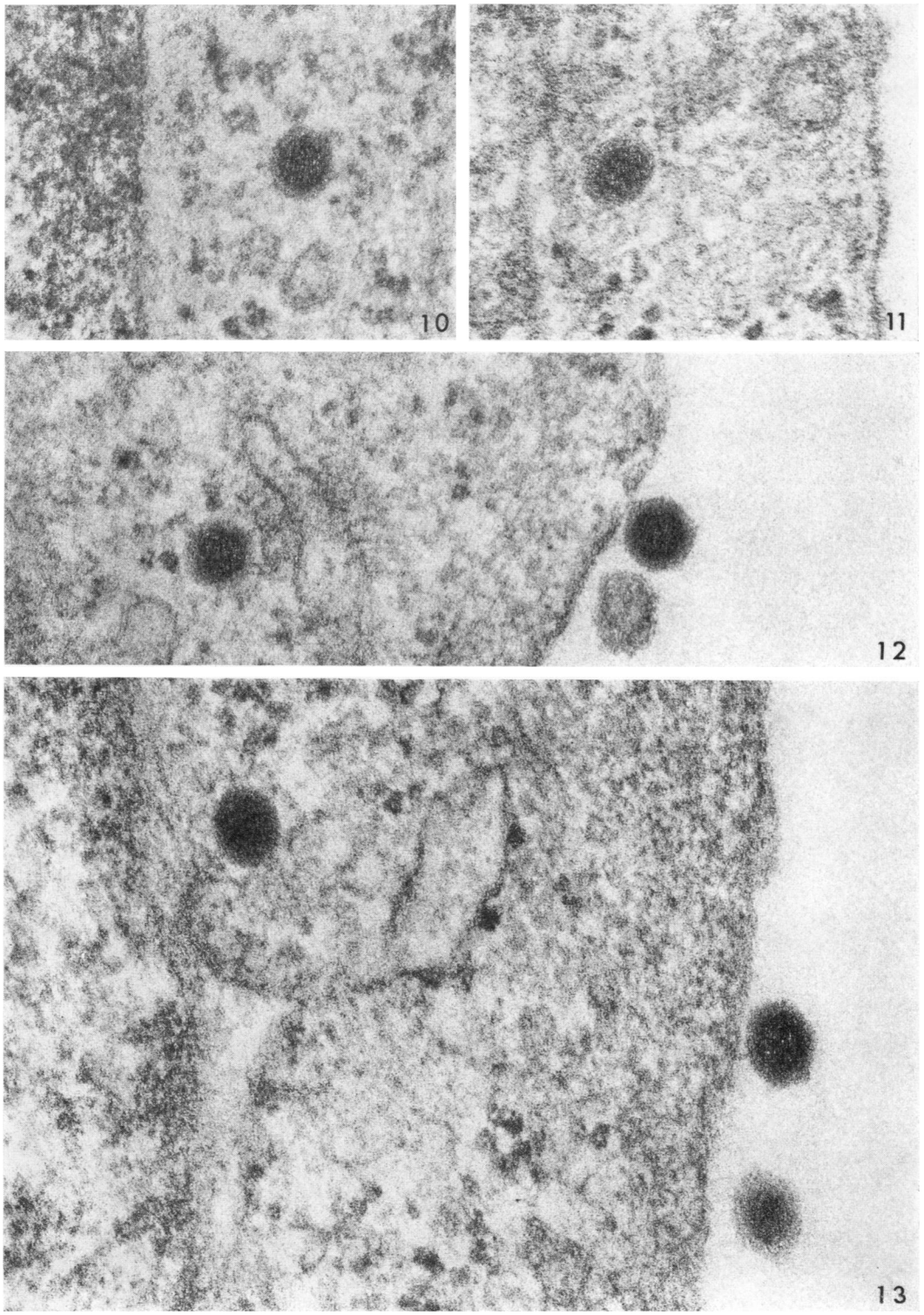


FIG. 10. *Virus close to the nucleus; 10 min.* × *150,000.*
FIG. 11–13. *Intracytoplasmic virions with altered cores and capsids; 20 min.* × *150,000.*

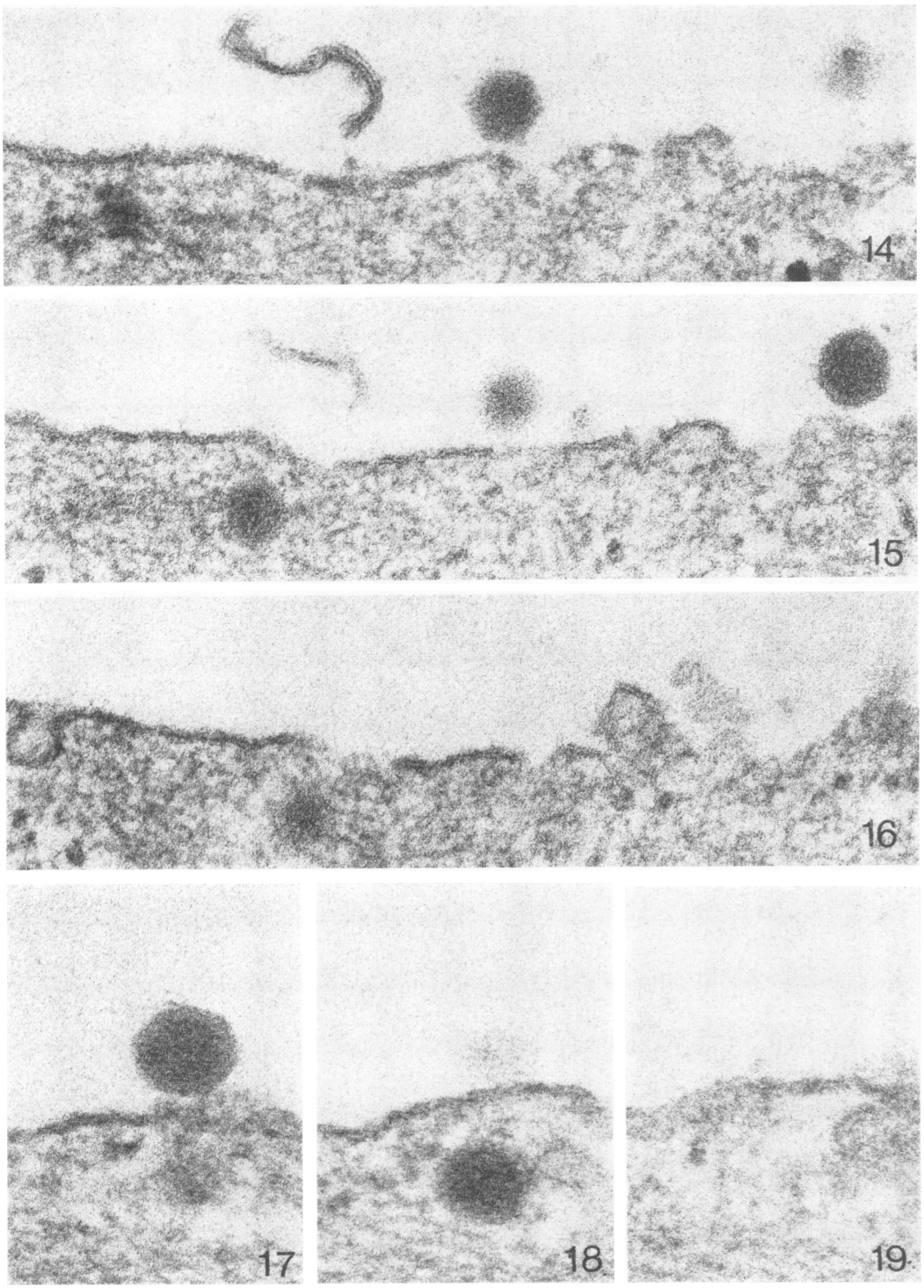


FIG. 14–16. *Serial sections. Note the diffuse appearance of the intracytoplasmic virion on the left of Fig. 15. This particle must be central to the plane of section since it is only seen once. A different virion begins to appear in Fig. 16.* × *150,000.*

FIG. 17–19. *Serial sections demonstrating that, although the virion in Fig. 18 is nearly central to the plane of section, the capsid is diffuse in appearance.* × *200,000.*

large amounts of particulate material, viral or otherwise. It was significant, however, that virions within phagocytic vacuoles were generally unaltered, neither appearing granular nor exhibiting diffuse margins. In Fig. 21, the virion at the upper right is extracellular, whereas the one below lies within a vacuole. They are similar in appearance and do not show the changes visible in the virion free within the cytoplasm at the lower left. Other examples of unaltered virions in vacuoles are shown in Fig. 22 and 23.

Occasionally, ruptured vacuoles were seen with virus in the process of release to the cytoplasm (Fig. 24–27). There did not seem to be a purposeful migration of vacuoles, since they never appeared in ordered arrays as though marching from the cell surface to the nucleus. Rather, discharge of virus from vacuoles appeared to be at random, occurring at any distance from the cell surface to the nucleus. It should be emphasized that not only was it unusual to encounter virions in the process of release from vacuoles but also that the phenomenon, with rare exceptions (Fig. 24), was observed only after incubation for 60 min.

Sequestration of virus. A significant proportion of the virus became sequestered within vacuoles. Thus, at 3 hr, although most of the free virus within the cytoplasm had disappeared, structurally intact virions were found within vacuoles enclosing matrix material of low (Fig. 28) or high (Fig. 29) density. The relative proportion of free virus, in comparison with that in vacuoles, at different intervals after infection can best be illustrated at low magnification. In Fig. 30, which shows a cell after 1 hr of incubation at 37 C, there are numerous free virions (marked by arrows). In Fig. 31, which shows a cell 2 hr later, all of the remaining intracellular virus is present within vacuoles and none is lying free within the cytoplasm. A few particles were always found attached to the surface. Presumably such virions had defective capsids which prevented penetration (see also under Formalin treatment).

Entry of nucleoprotein into the nucleus. Ultimately, much of the free virus came to lie in close proximity to the outer nuclear membrane. It is of interest in this connection that the virus could traverse a considerable distance through the cytoplasm, not infrequently coming to rest on that portion of the nucleus opposite the free surface of the cell. The association of virus with nuclear pores seemed to be quite random. In Fig. 32, for example, one virion (on the right) is close to a pore, whereas the other two are not. (The dense intranuclear body at the lower right cannot be identified as altered virus or viral nucleoprotein since similar structures also occur in control cells.) In some instances, viral nucleic acid appeared to be discharged from the virus directly through a pore into the nucleus (Fig. 33 and 34). This phenomenon was sufficiently rare, however, as to make documentation exceedingly difficult. The events to be described below were much more commonly encountered, reaching their greatest frequency at about 45 min.

Figure 35 illustrates a virion within a cytoplasmic protrusion, which indents a nucleus. Several nuclear pores are evident, but there is no abnormal alteration of either nuclear or cytoplasmic membrane. Figure 36, however, shows the cytoplasmic membrane in proximity to the virus bulging toward the nucleus, while in Fig. 37 a small channel appears to connect the cytoplasm with the nuclear matrix. In Fig. 38, dense material resembling chromatin has collected so as to form a pocket extending into the nucleus, and the virus has undergone a striking change. The core is contracted, the diffuse outer coat has disappeared, and a sharply defined membrane-like structure is apparent. This membrane may be composed of viral protein(s) (P antigen) thought to be present within the virus but in a masked form (11) and hence not clearly observed heretofore. In Fig. 39, the cytoplasmic membrane curves inward to the nuclear pocket where it vanishes. The pocket is demarcated by granular, chromatin-like material, and the viral membrane proximal to the pocket appears to have ruptured. One even gains the impression that the dense viral core material may have begun to escape. Figures 40 and 41 strengthen this impression. Occasionally, the entire virion seemed to disintegrate (Fig. 42).

Usually, after discharge of nucleoprotein, the viral remnant could not be identified, but in rare instances the ruptured, empty, viral membrane remained within the pocket, as seen in the exceedingly thin section illustrated by Fig. 43. The fate of the pocket varied. The side contiguous to the cytoplasm was generally closed off by a rim of chromatin, the nuclear and cytoplasmic membranes re-forming to close the nuclear defect. The opposite side of the pocket then opened so that, in a manner analogous to locks in a canal, the viral nucleic acid passed into the nuclear matrix. Stages in this process are shown in Fig. 44 and 45. Sometimes the pocket detached and migrated from the margin of the nucleus, presumably opening with release of contents at a later time. In other instances, the pocket opened so that direct continuity was established between the cytoplasm and the nuclear matrix. Figure 46 is an example of this.

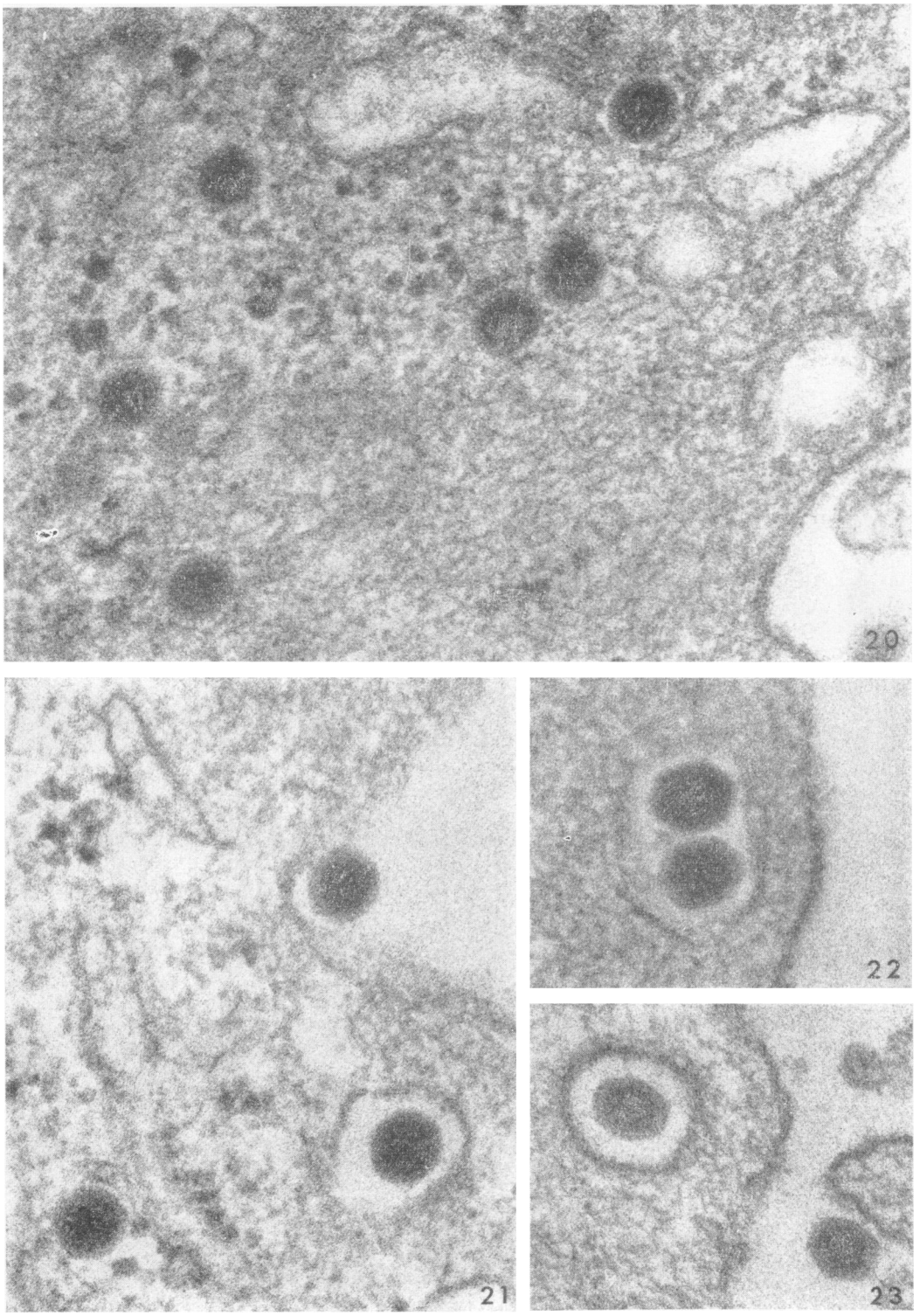


FIG. 20. *Intracytoplasmc virions at 20 min. Those to the left are nearer the nucleus and appear more altered.* × *150,000.*

FIG. 21. *Virus on the surface (upper right), within a vacuole (lower right), and free within the cytoplasm (lower left); 40 min.* × *150,000.*

FIG. 22 and 23. *Virions within vacuoles.* × *150,000.*

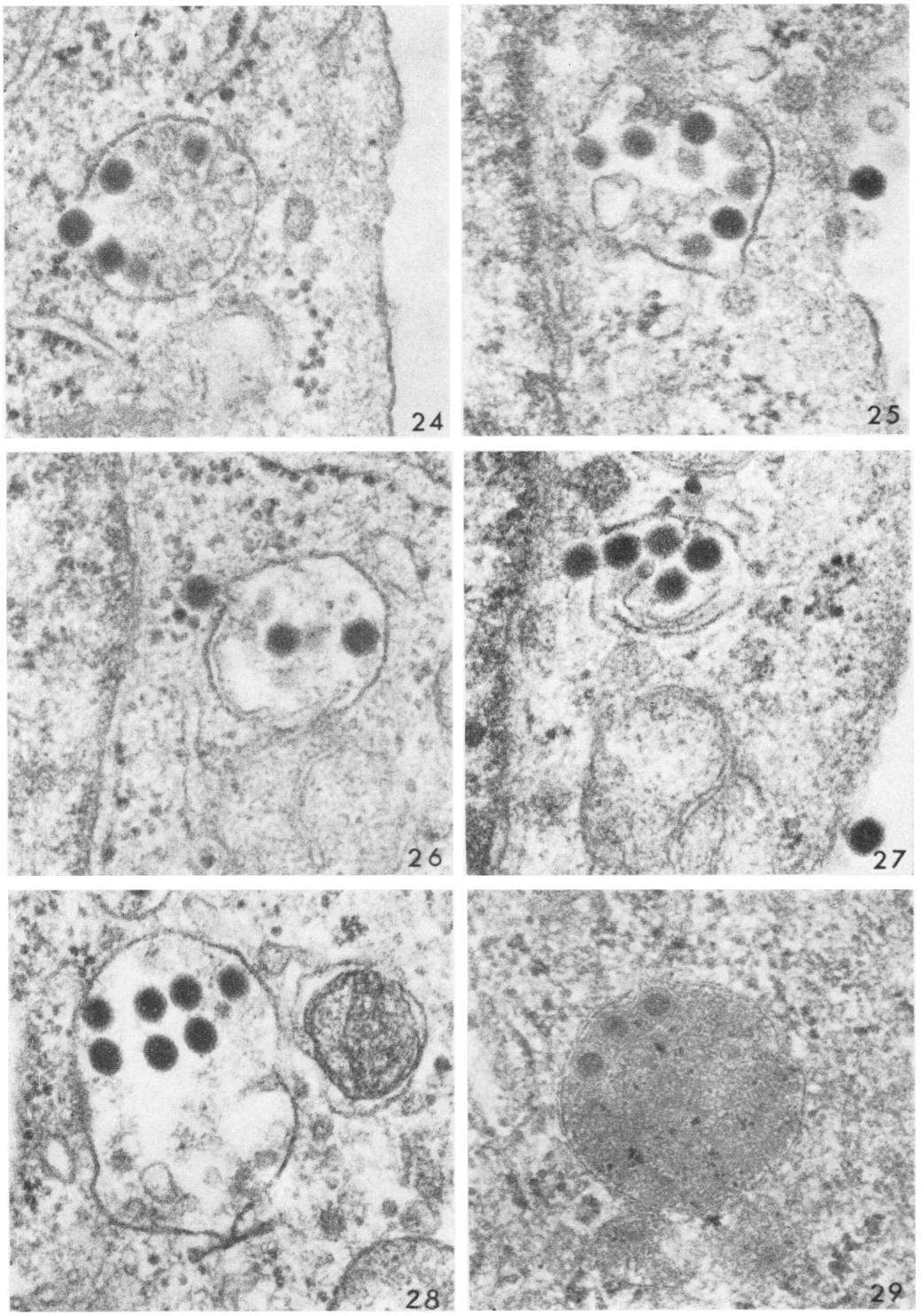


FIG. 24. *Virion in the process of release from a vacuole; 45 min.* × *75,000.*

FIG. 25–27. *Virus at stages of release from vacuoles near the nucleus (visible on the left in each case); 60 min.* × *75,000.*

FIG. 28. *Intact virus within a vacuole after incubation for 3 hr.* × *75,000.*

FIG. 29. *Unusually thin section showing apparently intact virus within a lysosome after incubation for 2 hr.* × *75,000.*

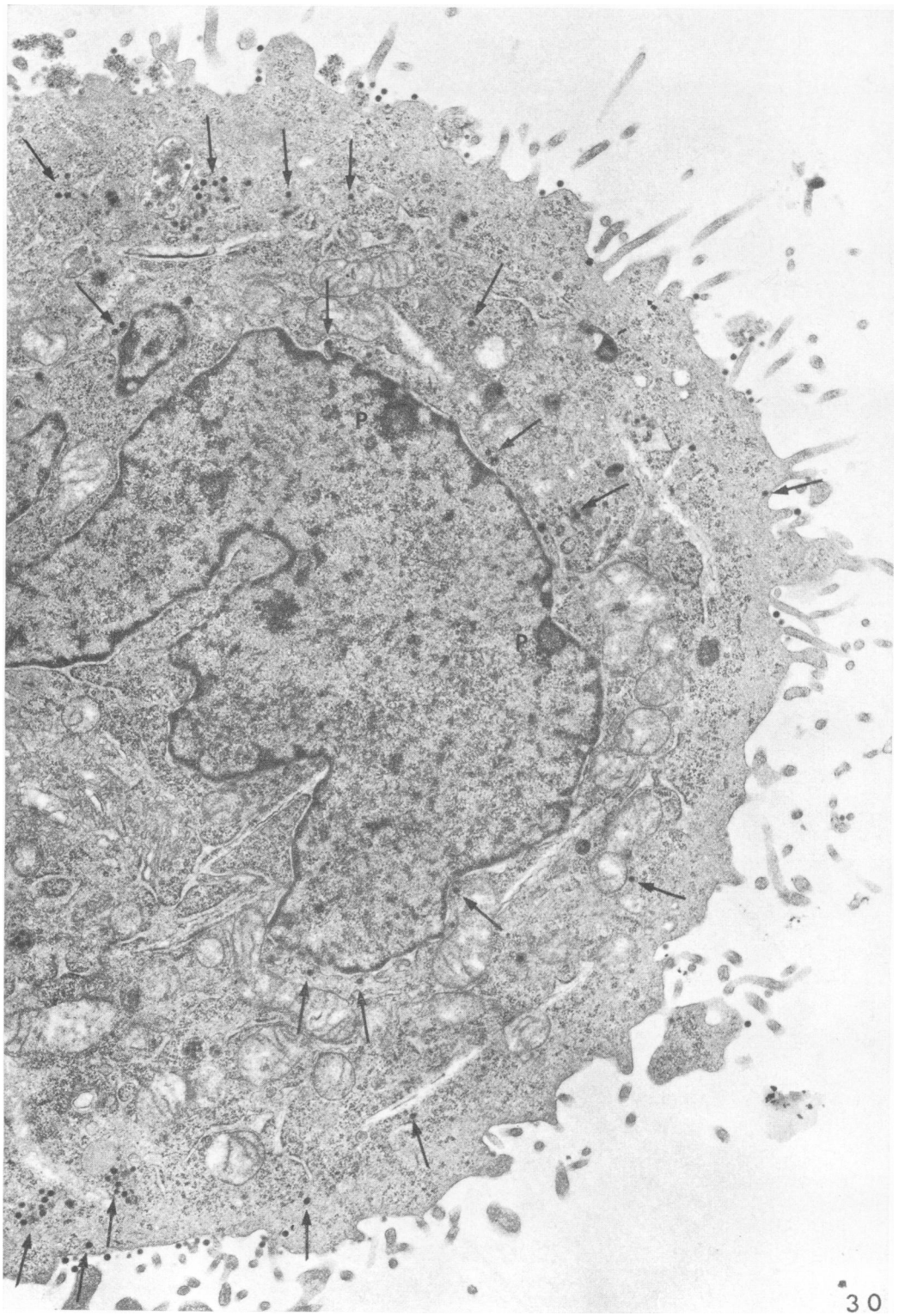


FIG. 30. *Free virus (marked by arrows) within the cytoplasm at 1 hr. Note two nuclear pockets (P). (See also Fig. 44 and 45.)* × *15,000.*

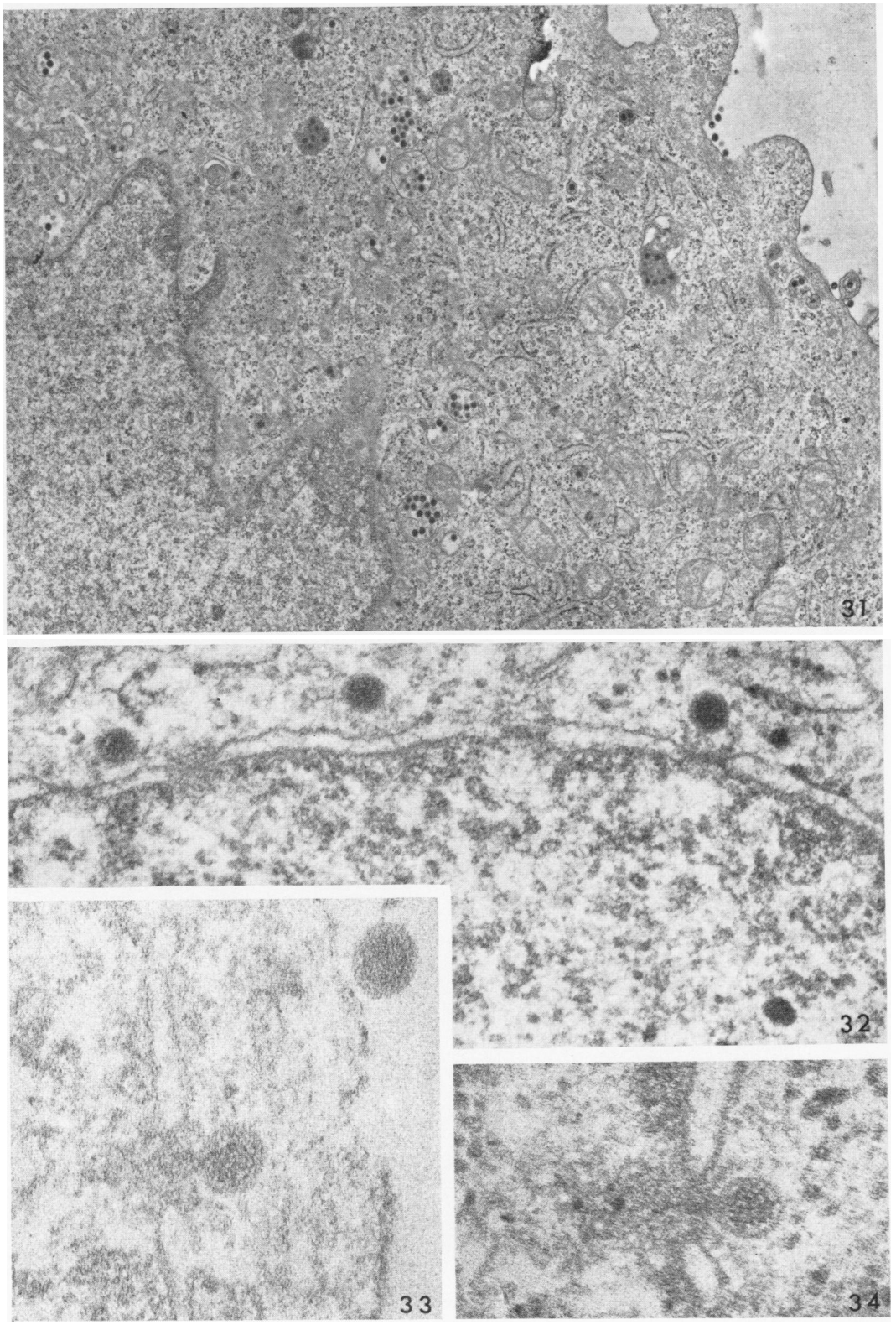


FIG. 31. *Virus still sequestered within vacuoles at 3 hr. No free intracellular virus remains.* × *15,000.*
FIG. 32. *Virus near the nucleus; 45 min.* × *150,000.*
FIG. 33 and 34. *Very thin sections showing probable release of viral nucleoprotein through a pore into the nucleus; 45 min.* × *150,000.*

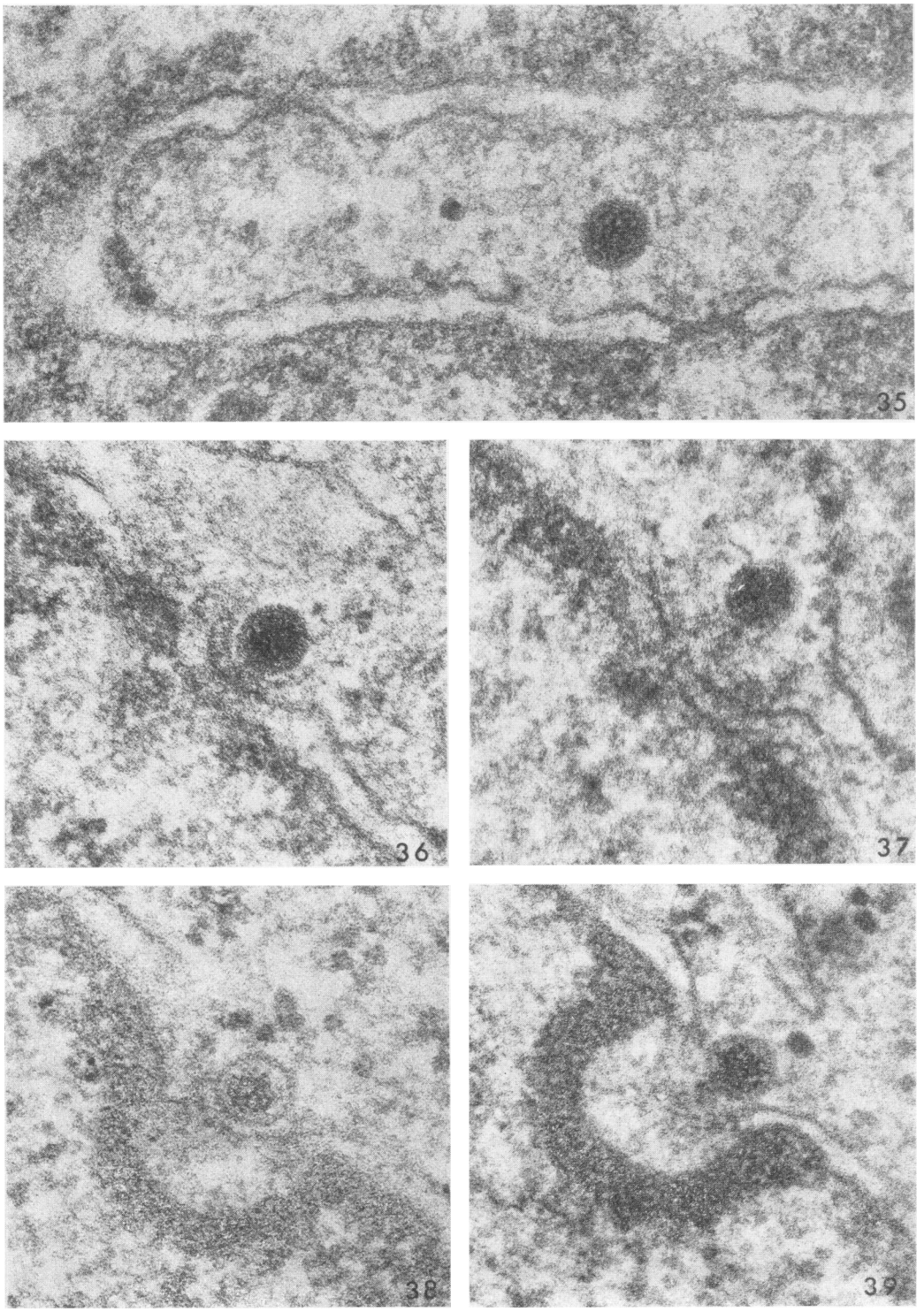
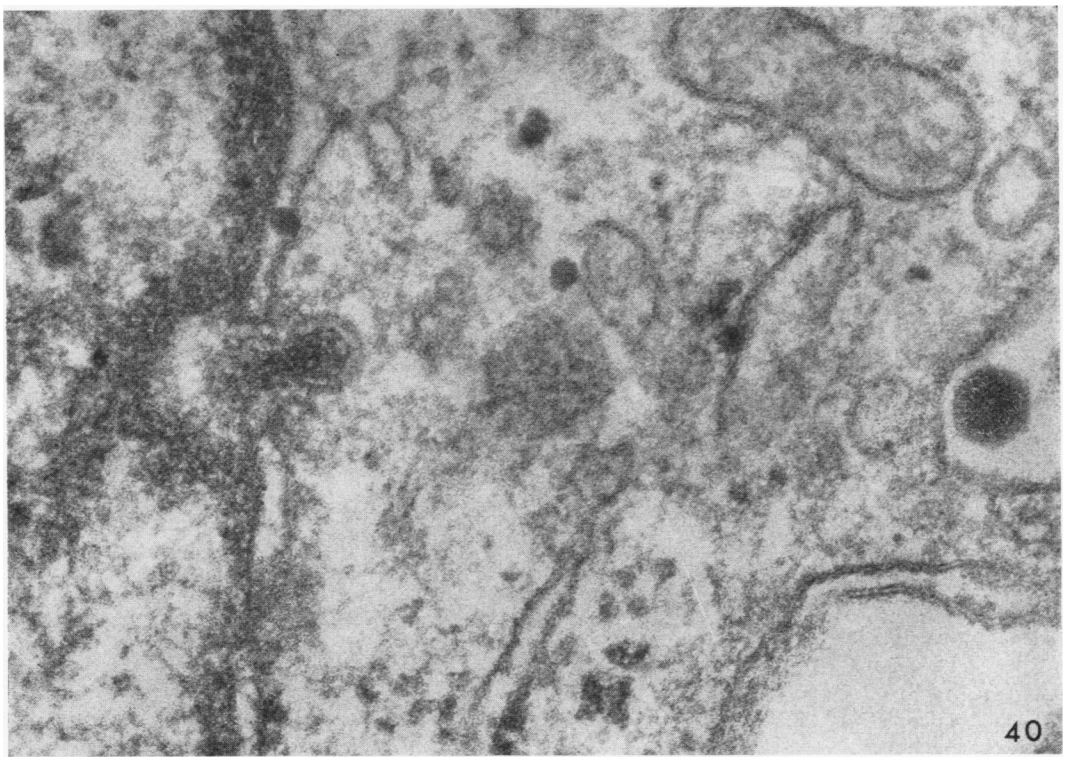


FIG. 35. *Virion lying within a finger of cytoplasm, which has indented the nucleus. Nuclear pores are evident; 20 min.* × *150,000.*

FIG. 36–39. *Early stages in the release of nucleoprotein. Fig. 36, 20 min; Fig. 37–39, 40 or 45 min.* × *150,000.*



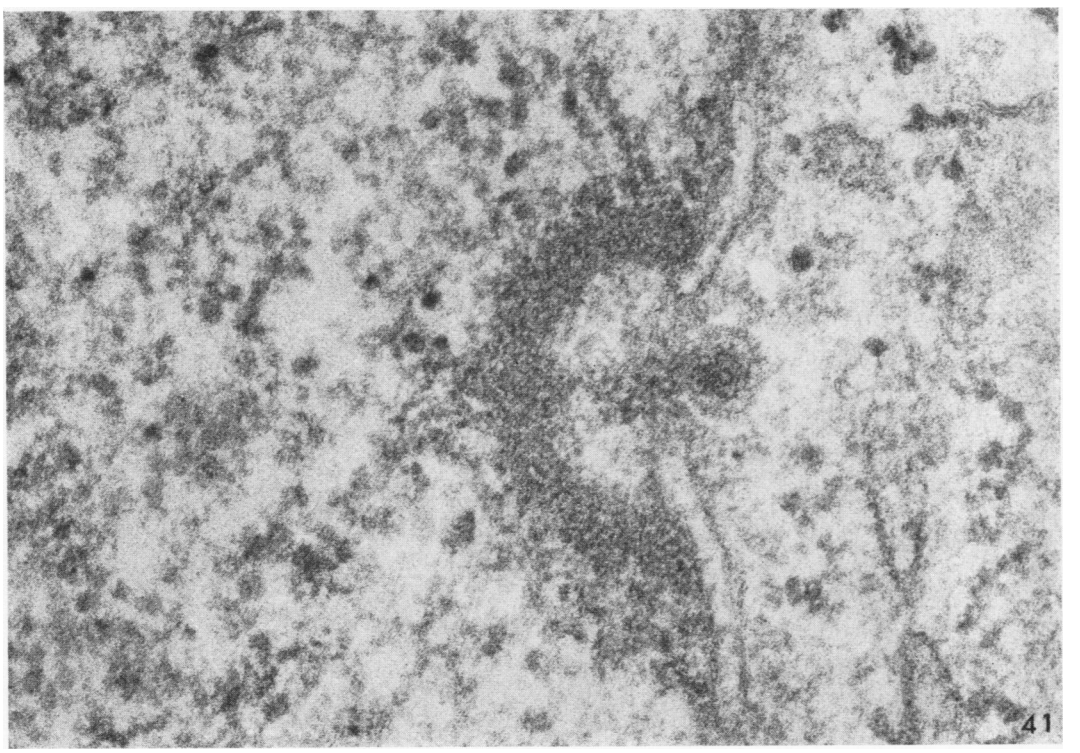
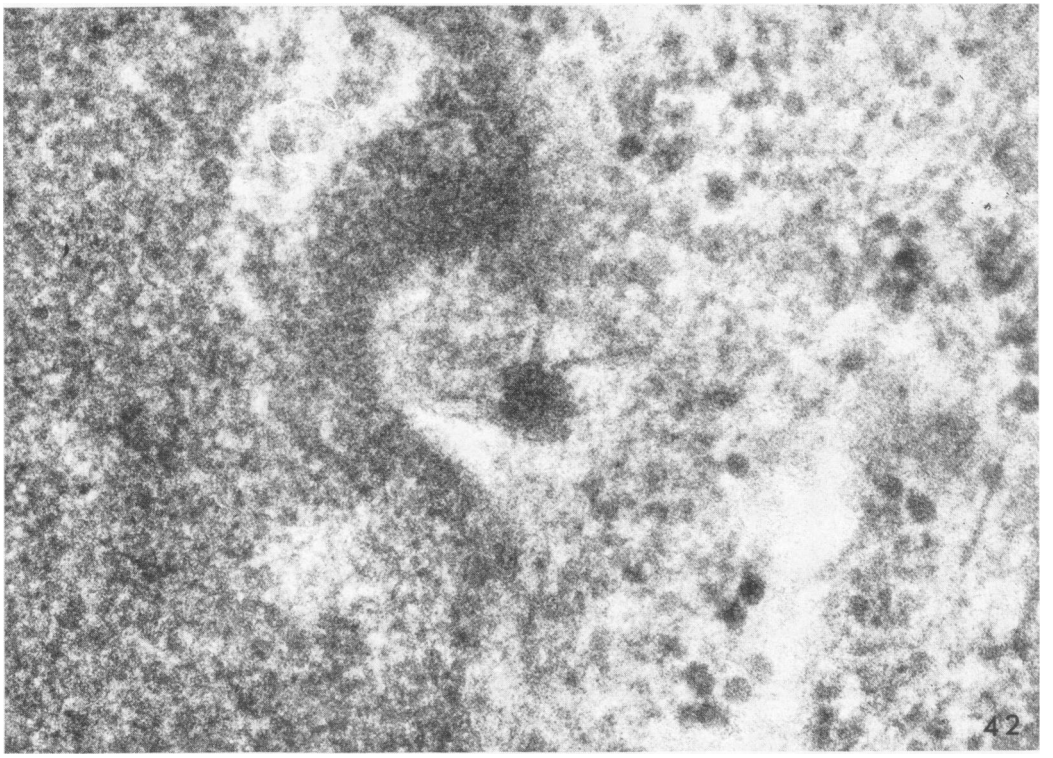


FIG. 40. *Core material (nucleoprotein) in the process of release to a nuclear pocket. For comparison, an unaltered virion is shown on the surface of the cell; 40 min.* × *150,000.*

FIG. 41. *Thinner section showing core material (nucleoprotein) in the process of release to a nuclear pocket; 40 min.* × *150,000.*



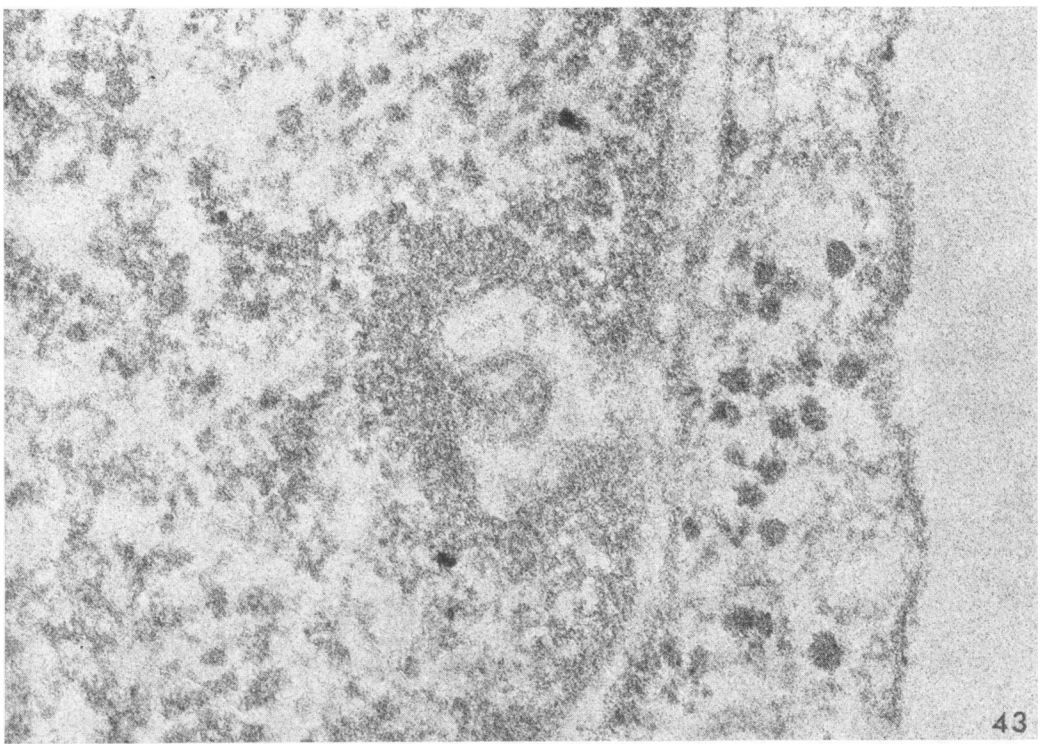


FIG. 42. *Probable altered virion at the nuclear margin; 30 min.* × *150,000.*

FIG. 43. *Unusually thin section showing an empty viral membrane within a nuclear pocket. Note how close the nucleus is to the surface of the cell; 40 min.* × *150,000.*

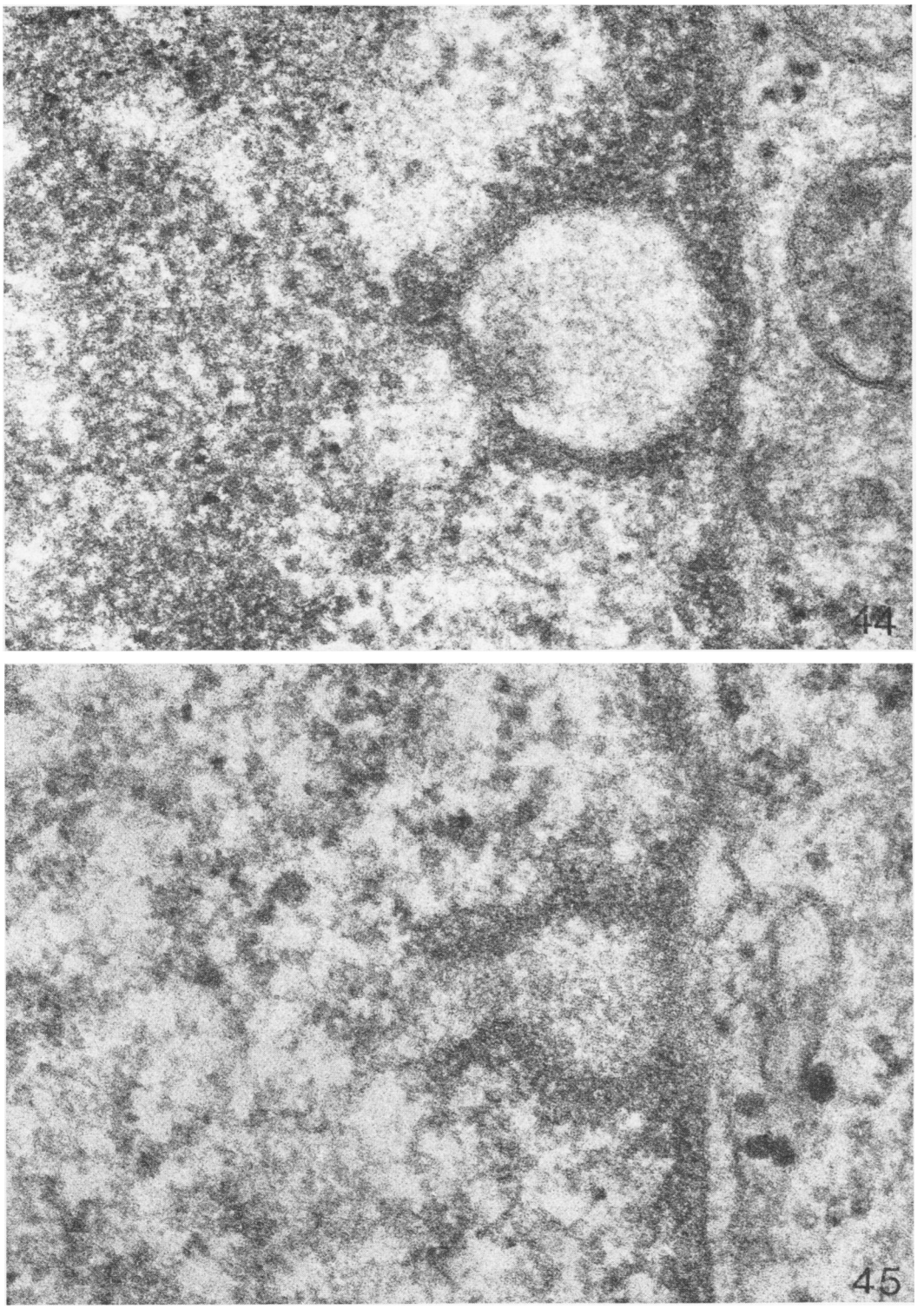


FIG. 44 and 45. *Nuclear pockets similar to those shown at low magnification in Fig. 30; 40 min.* × *150,000.*

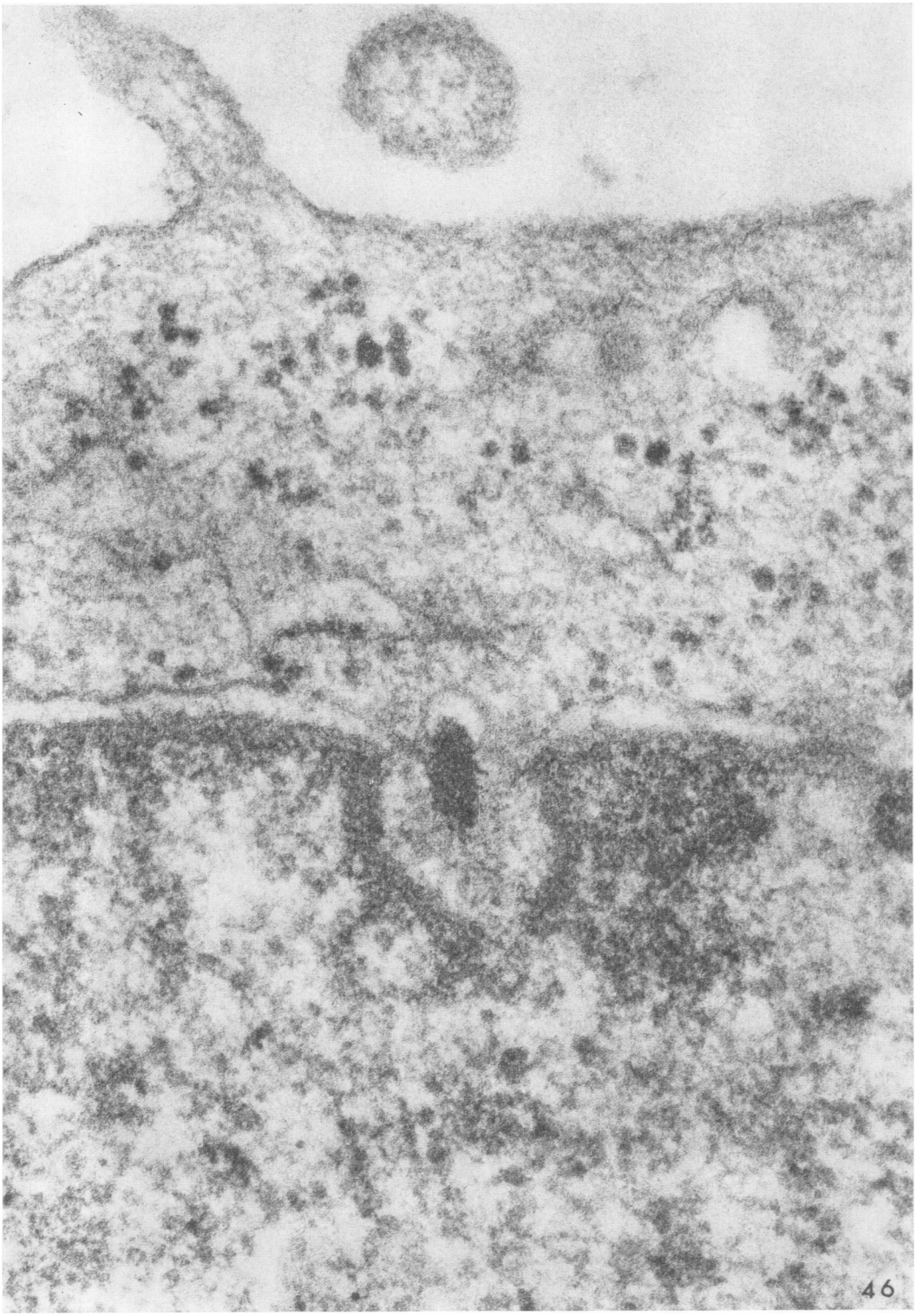


FIG. 46. *Nucleoprotein at a stage in passage from a viral remnant through a nuclear pocket into the nuclear matrix; 40 min.* × *150,000.*

The nucleoprotein is observed just as it escapes from the viral shell. The presence of virions within nuclei (4) could not be confirmed.

Formalin treatment of the inoculum. In an effort to determine whether alteration of the capsid interfered with penetration of virus through the cell membrane, resuspended pellets of virus were incubated with 1% Formalin for 1 hr at 37 C before banding on CsCl. After dialysis, the virus was applied to the cells, which were held at 4 C for 30 min. The preparations were then warmed to 37 C and fixed 1 hr later. There were considerably fewer attached viral particles at the cell surface and only moderate numbers within phagocytic vacuoles. Virtually no virus was found free in the cytoplasm.

Attempts to prevent phagocytosis. Pretreatment with India ink in an effort to blockade the cells was unsuccessful; 0.5% India ink in MEM was added to the tissue cultures 19 hr before addition of virus. It was of interest that there was great variability in the amount of ink taken up by different cells. However, cells that were actively phagocytic invariably engulfed some viral particles.

Metabolic inhibition with colchicine, puromycin, dinitrophenol (DNP), and arsenate was also studied for the effects of these agents on phagocytosis and viral entry. Cells were pretreated for 19 hr with 5 μg of colchicine per ml of medium. Even though viral adsorption in the cold and incubation at 37 C for 1 hr were carried out in the presence of the drug, phagocytosis still took place. Pretreatment with 50 μg of puromycin per ml for 2 hr, with continued treatment during viral adsorption and incubation, reduced, but failed to prevent, phagocytosis. In the presence of puromycin, occasional virions were found free in the cytoplasm, and virus in the process of discharging nucleoprotein into nuclear pockets was seen.

DNP (2×10^{-3} M) was applied to the cells for 2 hr, and treatment was continued during viral adsorption in the cold for 30 min and incubation at 37 C for 45 or 60 min. Although the cells showed a striking loss of polyribosomes, phagocytosis of virus occurred. Moreover, free virus was found in the cytoplasm, and, in a few instances, viral nucleoprotein was observed at stages of passage to the nuclear pockets. In view of the likelihood that cells in the presence of DNP could utilize energy obtained by glycolysis, 10^{-2} M arsenate, an inhibitor of both oxidative and fermentative energy metabolism (1, 16; see also references 10, 14, 15), was applied to the cells 1 hr before and during the period of viral adsorption in the cold for 30 min and incubation for 1 hr at 37 C. This agent caused severe damage to the cells, as manifested by beginning autolysis with loss of ribosomes, discontinuity of endoplasmic reticulum, and fragmentation of mitochondrial cristae. Yet, even under these circumstances, some phagocytosis of virus resulted. No free virus was encountered in the cytoplasm. In another series of experiments, the cells were not pretreated, but 10^{-2} M arsenate was added with the viral inocula. After a 30-min period of adsorption at 4 C, the preparations were warmed to 37 C for 1 hr. Although moderate phagocytosis and occasional free virions in the cytoplasm were observed, the viral particles were not altered in appearance and nuclear pockets were not formed. Attempts to revive the cells by removing the arsenate after the period of incubation, with the hope of initiating viral uncoating, were unsuccessful, the cells appearing to be too badly damaged to recover.

To monitor the effectiveness of these agents on HeLa cells, their ability to interfere with protein synthesis was determined. It was found that 50 μg of puromycin per ml prevented protein synthesis, whereas 2×10^{-3} M DNP allowed some synthesis to occur (12% of the control; Table 1). Arsenate (10^{-2} M) inhibited metabolism completely, provided that the cells were pretreated for at least 30 min (Table 2).

TABLE 1. *Effect of puromycin and DNP on the metabolism of HeLa cells*[a]

Addition	Radioactivity incorporated (counts/5 $\times 10^6$ cells)
None	21,531
Puromycin (50 μg/ml)	0
DNP (2×10^{-3}M)	2,561

[a] Monolayers of HeLa cells (5×10^6/bottle) were exposed to the puromycin or DNP in 3 ml of Earle solution for 2 hr at 37 C and then were washed with cold Earle solution also containing the agent. After maintaining the cells at 4 C for 30 min, they were incubated in the presence of the inhibitors for 20 min at 37 C, whereupon radioactive amino acids were added and the preparations were incubated for another 30 min. The incorporated radioactivity was determined as described. The general procedure was similar to the one used when cells were actually infected with virus. The results were corrected for the nonspecific adherence of radioactivity to the cells. This was calculated in a control experiment, in which radioactive amino acids were added to a chilled culture and the cells were processed immediately for measurement of the retained radioactivity.

TABLE 2. *Effect of arsenate on the metabolism of HeLa cells*[a]

Part	Conditions	Radioactive pulse (min)	Radioactivity incorporated (counts/5 × 10^6 cells)
I	Preincubated with 0.1 M arsenate for 1 hr at 37 C	0–30	0
II	As in I	30–60	0
III	No pretreatment; arsenate added at $T = 0$	0–30	2,682
IV	As in III	30–60	0
V	Untreated cells	0–30	8,258
VI	Untreated cells	30–60	10,875

[a] Before adding the pulse of radioactive amino acids, the cells were held at 4 C for 30 min and then were quickly warmed to 37 C in a manner identical to that employed for the viral adsorption and entry experiments. In parts I, III, and V, the radioactive label was added immediately upon warming the cells ($T = 0$) and the preparation was harvested at 30 min, whereas in parts II, IV, and VI the radioactive label was added after 30 min of incubation at 37 C and the cells were harvested 30 min later.

DISCUSSION

This study has shown that unlike ether-sensitive viruses, such as herpes simplex (8), influenza (7), and Sendai (6), which appear to fuse with the cell membrane and release their contents into the cytoplasm, adenovirus can pass directly through the cell membrane. Presumably this mode of entry requires an unaltered capsid, since it was not observed to occur after treatment of the virion with Formalin. It should be emphasized that virus in passage through the cell membrane was far less commonly encountered than was virus within phagocytic vacuoles. However, in attempting to assess the relative importance of two differing mechanisms from micrographs of fixed specimens, it does not necessarily follow that an event which is seldom seen rarely occurs. Stages in the direct penetration of virus were not often encountered, but this can be explained by assuming that the process is rapid rather than uncommon. Since nonenveloped virus was found deep within the cytoplasm as early as 10 min after the initiation of entry (Fig. 10), it is not unreasonable to conclude that the actual passage of the virion through the cell membrane might occur in a small fraction of this time. On the other hand, phagocytosis is a constant process resulting in the slow, progressive accumulation of large numbers of particles within vacuoles, such as were observed in the studies with India ink. It is hardly surprising then that many virions were found within phagocytic vacuoles. Philipson (9) alluded to this situation by making the following statements. "The present results are, however, not entirely compatible with electron microscopic data on adenovirus penetration and eclipse (2). Thus the rapid disintegration of 65 to 85% of the virions observed in this study is in contrast to the slow penetration and accumulation of intact particles within the cell observed by electron microscopy."

No evidence was found to support the hypothesis that uncoating occurs within phagosomes. Virus sequestered in vacuoles actually appeared to be protected, since structurally intact virions were still present even after 2 to 3 hr of incubation at 37 C. In rare instances, vacuoles containing virus were encountered at stages of rupture with release of virus into the cytoplasm. Whether such rupture is fortuitous or whether virus can penetrate the limiting membrane of a vacuole in the same way as the surface of the cell is not clear. However this may be, some virus undoubtedly does reach the cytoplasm upon the disruption of phagocytic vacuoles. In view of the difficulty encountered in blocking phagocytosis, it is not possible to determine the proportion of virus released from vacuoles as opposed to that entering directly through the cell membrane. The question naturally arises as to why others have failed to observe direct entry of virus into the cytoplasm (2, 4). Presumably, as stated above, this phenomenon occurs so quickly that the event could easily escape detection if some degree of synchrony were not attained by rapidly warming the cells after adsorption of virus in the cold, and if intensive study were not devoted to the ensuing 30 min. An interesting question raised by the present investigation is why all of the attached virus does not enter simultaneously within, say, the first 10 min. One can only surmise that for admission to occur the capsid and the cell membrane must be contiguous. As was mentioned in the study of influenza virus (7) and as is evident from examination of the accompanying micrographs, the virions may lodge in the proteinaceous coating of the cell at some distance from the cell membrane. Presumably a variable interval of time ensues before virus and cell achieve actual contact and penetration results.

Once within the cytoplasm, the virion very quickly underwent subtle but significant alteration. The core became granular while the surface assumed a diffuse appearance, and the crystalline faces, so commonly exhibited by extracellular virus, were no longer seen. One can con-

clude that both the configuration of the capsid as well as that of the core changed. It is of interest in this connection that Lawrence and Ginsberg found that "though the viral capsid was altered to expose the viral DNA to deoxyribonuclease activity, the capsid proteins were not degraded by the process" (5). They commented on the rapidity of this event by stating: "Uncoating followed penetration closely; both processes occurred at similar rates, and both were complete by 60 min." [Philipson (9) reported completion within 30 min.] In addition, they noted that "the uncoated viral DNA was stable within the cell; ie.. it was not degraded to acid-soluble material." One is led to suggest, therefore, that the aforementioned changes in structure of the viral capsid and core probably correspond to uncoating. In other words, "uncoating" is not the actual removal of the coat but refers "to the intracellular alterations of the viral capsid which render parental DNA accessible to the action of deoxyribonuclease," to quote Lawrence and Ginsberg again. Perhaps the alteration is analogous to that observed upon heating, wherein the pentons and neighboring five hexons are lost with accompanying development of susceptibility of deoxyribonucleic acid to deoxyribonuclease (12). Such a change, or one similar to it, in configuration of the capsid may account for the diffuse appearance of the viral surface seen in thin section.

The events occurring at the nuclear membrane were truly remarkable. After passing a variable distance through the cytoplasm, the virus came to lodge in proximity to the nucleus. The capsid then was either markedly altered or actually stripped off, leaving a thin, well-defined membrane enclosing the nucleoprotein. Quite possibly these changes are, in fact, the "further alterations" which Lawrence and Ginsberg deduced must occur "before free viral DNA is liberated" (5). At approximately the same time, chromatin accumulated in the nucleus adjacent to the virus so that a pocket was formed; the cytoplasmic and nuclear membranes bulged inward toward the nucleus and gave way. The viral membrane ruptured and the nucleoprotein passed directly from the virus into the pocket. The latter, either at the same time or shortly thereafter, opened and the nucleoprotein passed to the nuclear matrix. [Dales (2) seems to have encountered this phenomenon, as illustrated in Fig. 22 of his paper, but he interpreted the micrograph as probably showing a stage in the actual entry of the virion into the nucleus.]

As was noted in the description of the micrographs, viral particles were occasionally found near nuclear pores, and in rare instances discharge of nucleoprotein seemed to occur through these structures. There was no evidence, however, of any predilection by the virus to lodge close to pores and little to indicate that they served as the main portal of entry, a suggestion which occurs in reports of other systems [see the review by Stevens (13)].

In view of the morphological observations recorded above, the effects of metabolic inhibitors were investigated. It was thus found (Table 1) that, although puromycin blocked protein synthesis, this property was not shared by DNP. Presumably cells exposed to DNP are able to obtain energy for protein synthesis through fermentative processes. Arsenate, on the other hand, halted protein synthesis completely, provided that the cells were pretreated for at least 30 min (Table 2). Entry of virus, alteration of the virion, and discharge of nucleoprotein to the nucleus were not prevented by pretreatment of the cells with puromycin, indicating, as observed by Lawrence and Ginsberg (5) and by Philipson (9), that uncoating of the virus is not dependent upon the synthesis of new enzymes. When cell energy was blocked by arsenate, however, entry of small numbers of virions into the cytoplasm was seen but alteration of virions, formation of nuclear pockets, and release of nucleoprotein did not ensue. Phagocytosis would seem to be one of the last manifestations of cellular activity that was lost, since, in cells which had been treated with arsenate and were obviously undergoing autolysis, a few vacuoles were found to contain structurally intact virions.

The purpose of this communication has not been to exclude phagocytosis as a means of viral entry but rather to indicate that direct penetration can also occur. It was repeatedly observed that phagocytosis tended to sequester structurally intact virions, often for considerable periods of time, a phenomenon which could be interpreted as suggesting a defensive mechanism of the cell rather than a specific mode of viral entry. However this may be, only virus free in the cytoplasm was found to become altered and to release nucleoprotein to the nucleus. Penetration of recognizable virions into the nuclear matrix was never encountered.

Illustrations. To facilitate direct comparison most of the micrographs are reproduced at × 150,000. In each figure, with the exception of the six serials (Fig. 14 to 19) and the last micrograph (Fig. 46), the free surface of the cell is to the right and the nucleus to the left, whether these structures do or do not appear in the field chosen for reproduction. When significant, the time of incubation at 37 C is indicated.

ACKNOWLEDGMENTS

This investigation was supported by Public Health Service grant AI-06814 from the National Institute of Allergy and Infectious Diseases and grant GB-6013 from the National Science Foundation. One of the authors (H.S.R.) is a Research Career Development Awardee of the Division of General Medical Sciences, United States Public Health Service (5K3-GM29, 024). This investigation was conducted under the sponsorship of the Commission on Influenza, Armed Forces Epidemiological Board, and was supported by the U.S. Army Medical Research and Development Command, Department of the Army, under research contract no. DADA 17-67-C-7141.

ADDENDUM

After submission of this report we were informed of the study of Karl Lonberg-Holm and Lennart Philipson entitled "Early Events of Virus-Cell Interaction in an Adenovirus System," which was published in vol. 4, p. 323–338, Oct. 1969, of this journal. The results appear to be comparable, in spite of the fact that different types of adenovirus were used. Presumably, their B component corresponds to the structures shown in Fig. 4, 5, and 6 of this paper. Their C component is probably the spherical form with the granular core illustrated in Fig. 7 through 21; their D component may be the dense core visible in the particles of Fig. 38 through 41. Whether the E component has been visualized is uncertain. It seems likely that forms C and D, rather than being intranuclear as they suggest, were adherent to the nuclear membrane and thus appeared in the nuclear fraction.

LITERATURE CITED

1. Clifton, C. E. 1946. Microbial assimilation. Advan. Enzymol. 6:269–308.
2. Dales, S. 1962. An electron microscope study of the early association between two mammalian viruses and their hosts. J. Cell Biol. 13:303–322.
3. Ellison, S. A., and H. S. Rosenkranz. 1963. An apparatus for sampling after cesium chloride density gradient centrifugation in the preparative ultracentrifuge. Anal. Biochem. 5:263–265.
4. Fong, C. K. Y., K. G. Bensch, and G. D. Hsiung. 1968. Productive and abortive infections of simian and nonsimian cells with a simian adenovirus SV15. 1. Microscopic observations. Virology 35:297–310.
5. Lawrence, W. C., and H. S. Ginsberg. 1967. Intracellular uncoating of type 5 adenovirus deoxyribonucleic acid. J. Virol. 1:851–867.
6. 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.
7. 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.
8. Morgan, C., H. M. Rose, and B. Mednis. 1968. Electron microscopy of herpes simplex virus. I. Entry. J. Virol. 2:507–516.
9. Philipson, L. 1967. Attachment and eclipse of adenovirus. J. Virol. 1:868–875.
10. Reiner, J. M. 1948. The inhibition of enzyme formation and nitrogen assimilation by arsenate. Arch. Biochem. 19:218–228.
11. Russell, W. C., and B. E. Knight. 1967. Evidence for a new antigen within the adenovirus capsid. J. Gen. Virol. 1:523–528.
12. Russell, W. C., R. C. Valentine, and H. G. Pereira. 1967. The effect of heat on the anatomy of the adenovirus. J. Gen. Virol. 1:509–522.
13. Stevens, A. R. 1967. Machinery of exchange across the nuclear envelope, p. 189–271. *In* L. Goldstein (ed.), The control of nuclear activity. Prentice-Hall, Inc., Englewood Cliffs, N.J.
14. Sussman, M., and S. Spiegelman. 1950. The use of arsenate in the analysis of energy relations amongst synthetic reactions. Arch. Biochem. 29:85–100.
15. Ter Welle, H. F., and E. C. Slater. 1964. Uncoupling of respiratory-chain phosphorylation by arsenate and evidence for the existence of a stable X $\sim$ P intermediate of oxidative phosphorylation. Biochim. Biophys. Acta 89: 385–388.
16. Warburg, O., and W. Christian. 1939. Isolierung und Kristallization des Proteins des oxydierenden Gärungsferments. Biochem. Z. 303:40–68.