ELECTRON MICROSCOPIC OBSERVATIONS ON THE DEVELOPMENT OF HERPES SIMPLEX VIRUS*

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Electron microscopic study of the H.R. strain of herpes simplex virus in cells of the chicken chorioallantoic membrane (1) suggested that the virus differentiates within the nucleus, where particles possessing a central core and a single limiting membrane are randomly dispersed. In this earlier work the finding that ruptured nuclei were associated with large numbers of intracytoplasmic particles possessing double membranes appeared to be consistent with the hypothesis that the virus acquired a second membrane in the cytoplasm after release from the nucleus. However, cells in tissue culture infected with the HFEM strain of herpes simplex virus and examined by Stoker, Smith, and Ross (2) and herpes B virus examined by Reissig and Melnick (3) subsequently revealed intranuclear particles with double as well as single membranes, thus raising the possibility that development could be completed in the nucleus.

Recently, a strain (J.M.) of herpes simplex virus was found to crystallize (4) in a manner analogous to the adenoviruses (5–9). Moreover, the nuclear membranes of cells infected by this strain frequently showed remarkable proliferation. The purposes of this paper are to illustrate and describe the viral crystals as well as the morphology of the cellular response to infection, and to propose an hypothesis concerning the manner of development and the mechanisms whereby virus may gain egress from intact cells.

Materials and Methods

HeLa cells were cultured in a medium consisting of Earle's balanced salt solution which contained 0.5 per cent lactalbumin hydrolysate, 0.25 per cent glucose, 0.1 per cent yeast extract, 0.3 per cent tris(hydroxymethyl)-aminomethane and 20 per cent horse serum. Stable human amnionic cells were cultivated in Eagle's medium to which 20 per cent horse serum

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was added. For transfer to tube cultures the cells were removed with 0.1 per cent solution of trypsin and each tube was inoculated with approximately 100,000 cells suspended in 1 ml. of fresh nutrient medium. The tubes were incubated at 37°C. for 2 to 3 days and then were inoculated with 0.1 ml. of appropriate dilutions of the J.M. strain (4) of herpes simplex virus. After reincubation for intervals of 48 to 96 hours following inoculation, the cell sheets were washed with balanced salt solution, freed from the glass by 0.1 per cent trypsin, centrifuged to form pellets, fixed in osmium tetroxide, dehydrated in ethyl alcohol, embedded in methacrylate, and sectioned according to methods previously described (7). In addition, several preparations were fixed for 1 hour in isotonic formalin buffered at pH 7.4. Sections of these blocks were examined either directly or after exposure to fumes of osmium tetroxide for 18 hours. An RCA type EMU-2E electron microscope was used.

RESULTS

Fig. 1 shows a nucleus with two nucleoli and a finely granular matrix. The double nuclear membranes, forming characteristic indentations, can be differentiated where they lie perpendicular to the plane of section. Below the nucleoli is a small collection of dense, oval granules. Fig. 2 illustrates, in a considerably thicker section, a nucleus containing two aggregates of large granules which resemble in density and general appearance the aforementioned smaller granules. The matrix of this nucleus is not uniformly distributed, but shows irregular clumps, which are somewhat more closely packed adjacent to the nuclear membrane. Fig. 3 reveals the characteristic pattern formed by margination of the nuclear chromatin. Although virus is present on the surface of the cell, intracellular viral particles cannot be identified in this or the preceding pictures.

Fig. 4 shows part of a nucleus containing a few viral particles which on higher magnification appear to be composed of a single membrane enclosing a central body. Margination of chromatin is apparent and at the upper right there is a cluster of dense granules resembling those illustrated in Fig. 2. The extensive convolutions of the nuclear membrane, which have been repeatedly encountered in cells containing virus, prevent identification of the multinucleated cells seen by light microscopy, since it is impossible, without numerous serial sections, to determine whether portions of nuclei are in fact separate or connected at a site removed from the plane of section.

Fig. 5 shows part of a nucleus with clumps of marginated chromatin as well as small, dense granules and filaments, which vary in thickness and are located in a broad peripheral zone. Near the nuclear membrane at the top of the field is a large aggregate of granules which differ in appearance from those previously mentioned. Although the cytoplasm contains no visible virus, a row of viral particles is present in the extracellular space separating the two cells at the right. In Fig. 6, which illustrates the same nucleus cut at a slightly different level and viewed at four times the magnification, the granules appear to be relatively uniform in size and shape and often form short, straight or curved rows. Here and there groups of adjacent rows are parallel. Viral particles scattered among the granules are characterized by a spherical core, *ca* 40 mμ in diameter, enclosed by a single membrane, 70 to 80 mμ in diameter. Fig. 7[1] illustrates part of the same field at double the magnification. Re-

[1] This print is of a different negative from that used in Fig. 6. Comparison of the pictures thus permits details of fine structure to be distinguished from defects in the photographic emulsion.

markable structural variation of the virus is evident. Two particles exhibit incomplete membranes. In the viral aggregate at the lower right many of the central cores are small, approaching in size the 20 mμ dimensions of the granules. Moreover, some cores are uniformly dense, whereas others have a central region of diminished opacity, resembling the larger internal bodies of the scattered viral particles. A number of viral particles appear to be empty. Although the nuclear membranes traversing the upper right corner vary in orientation with respect to the plane of section and are thus difficult to trace, they seem to be reduplicated.

Fig. 8 shows an unusually thin section through another aggregate of granules at the periphery of a nucleus. In the lower portion, small groups of viral particles exhibit a spatial arrangement which suggests that they are oriented in crystalline arrays. At the left and lower borders are clusters of very small granules and, as in the previous picture, segmental reduplication of the nuclear membrane appears to have taken place. In Fig. 9 a protrusion of the nucleus contains granules as well as a crystal composed of viral particles. The pattern of distinct and indistinct particles undoubtedly reflects orientation of the crystalline lattice with respect to the plane of section in a manner analogous to that observed with the adenoviruses (10). The viral crystal extends well into the aggregate of granular material and a row of viral particles is lodged between the granules and the nuclear membranes. These membranes are clearly quadruple where they are sharply defined. Near the top two viral particles with opaque central bodies and triple coats are contiguous to the nuclear membranes. The small, dense granules and filaments on the left resemble those illustrated in Figs. 5 and 6. Figs. 10 and 11 show an hexagonal, viral crystal in thick and thin sections, respectively. In the former, most of the viral particles are relatively well defined, whereas in the latter those eccentric to the plane of section are indistinct and less dense.[2] The three viral particles with double coats at the upper left of Fig. 11 lie between the nuclear membranes. It may be noted here that double coated particles have not been encountered in crystalline array.

Figs. 12 and 13 illustrate consecutive serial sections of a crystal. The rows of particles have been lettered, the capitals signifying those which lie nearly central to the plane of section. The pattern formed by sharply defined virus reflects the level as well as the angle at which the crystalline lattice has been cut. It is evident that when the virus is sufficiently eccentric to the section, superimposition causes the peripheral membrane to appear indistinct. In several particles the inner cores are located eccentrically. Presumably, this accounts for the fact that whereas row *G* (Fig. 12) contains well defined particles devoid of internal bodies, the same row in the adjacent

[2] By superimposing transparent prints of serial sections, one can observe the effect of section thickness on the appearance of the crystals. Although variable displacement and compression of the particles by impact of the microtome knife confine accurate superimposition to relatively small areas, it has been possible to determine that the cumulative image of three thin sections (such as the one shown in Fig. 11) will result in the appearance recorded by Fig. 10. Moreover, the hexagonal outline, which some viral particles exhibit in Fig. 10, can be demonstrated to result from the optical effect produced by overlap of the limiting membranes. At this angle of cutting most particles which seem to be contiguous in the thicker section lie at different levels and therefore in the thinner section the virus seldom appears in apposition.

section (Fig. 13) exhibits distinct cores surrounded by diffuse coats. In addition to the virus characterizing row *G*, there are scattered particles with interiors of such low density to the electron beam as to suggest that the central portion has been dislodged during sectioning. Whether such an event reflects insufficient fixation, incomplete dehydration, or inadequate penetration by methacrylate is not known. Fig. 14 shows an unusually large intranuclear viral collection composed of several crystals in apposition. The viral particles vary somewhat in shape and the crystals exhibit localized defects which probably result from distortion introduced during preparation of the specimen.

In Fig. 15 the nuclear membrane traverses the upper portion of the field, separating the cytoplasm above from the nuclear matrix below. Although some viral particles are spatially oriented, the majority are scattered among granules which resemble in size and shape those illustrated in Figs. 7 to 9. In this picture, however, the granules are relatively few in number compared to the virus. Near the upper right corner a particle with a double coat lies adjacent to several membranes, one of which appears partially to enclose it.

In addition to the multiple membranes at the surface of nuclei (shown in Figs. 7 to 11 and 15), membranes of variable length may be scattered through the nuclear matrix, as illustrated by Fig. 16. Near the lower border of the intranuclear crystalline virus on the right is one viral particle with three membranes, of which the outermost is incomplete. Triple membranes are most frequently encountered in association with virus at the periphery of the nucleus. Fig. 17, for example, shows two intranuclear viral particles with three membranes. The external membrane partially enclosing the particle on the right appears to connect not only with the fragment between both particles, but also with the multiple limiting nuclear membranes above. The external membrane of the particle on the left is open at the bottom, where it assumes an hourglass shape. In Fig. 18 a viral particle is partially enclosed by a lamellar structure, which is clearly continuous with the limiting nuclear membranes. Below and to the left in the nuclear matrix are other membranes, one of which lies near two contiguous viral particles with single coats. Figs. 19 and 20 illustrate consecutive sections. Nuclear matrix occupies the left border, while at the lower right is a nuclear pouch containing granules and virus with single coats. This pouch is separated by a zone of cytoplasm from the main body of the nucleus on the left. The reduplicated nuclear membranes, which traverse the field vertically, are discontinuous. Some exhibit closed ends similar to those observed in normal cells (11), whereas others become indistinct, suggesting that their orientation in the section varies. At the top of Fig. 19 a viral particle with a double coat is contained within the nuclear membranes. Near the center of the field another particle lies between two membranes which either connect with the nucleus at another level or form a sack within the cytoplasm. In Fig. 20 these particles are eccentric with respect to the plane of section and, hence, exhibit diffuse margins with little internal structure. The indistinct appearance of membranes adjacent to the virus emphasizes the difficulty encountered in ascertaining the integrity or tracing the course of lamellar structures which change orientation at different levels.

Complex ramification of lamellae in the cytoplasm may accompany viral infection. Occasionally those portions of the membranes which seem to be tangential to the

section contain spherical structures similar in shape and dimensions to the dense outlines of nuclear "pores" observed in thin sections of mammalian cells by Rhodin (12) and others (11, 13). In Fig. 21 part of a nucleus containing a small cluster of viral particles with single coats occupies the lower left portion of the field. In the right upper half, near the free surface of the cell, is an oval structure composed of lamellae surrounding characteristic granules and scattered virus. Presumably this is a nuclear diverticulum in cross-section. Above and to the left is a group of roughly parallel double membranes separated by small dense granules. At the lower margin of this group, where the outer membranes appear to expand or alter orientation in the section, several oval pore-like structures are visible. In the lower third of the micrograph there are numerous, similar pore-like components.

Fig. 22 illustrates the cytoplasm of a cell with large, apparently empty, vacuoles, as well as walled vacuoles containing one or more viral particles. In the upper half of the field groups of characteristic pore-like structures are scattered among mitochondria, which are well preserved. At the lower border may be seen the irregular margin of the nucleus, which exhibits reduplication of its membranes. Fig. 23 shows the cytoplasm and free surface of a cell. As in the preceding micrograph, virus with double membranes[3] lies within walled vacuoles of variable shape. In some instances (see inset from another field) the outer viral membrane is irregular and "balloons out" on one aspect. The surface of the cell exhibits numerous cytoplasmic extensions which have been cut at differing angles and at the upper border of the main picture two vacuoles, each enclosing one particle, are separated from the extracellular space by a thin rim of cytoplasm.

Fig. 24 shows particles of virus with double peripheral membranes clustered on the surface of a cell. The inset shows the upper right portion of the field in the next contiguous section. Particles *A* to *C* are central to the plane of section of the inset, particles *D* and *E* are similarly placed with respect to the larger picture. The outer membrane not infrequently protrudes on one aspect (see particles *C* and *D*, among others). Near the lower left border of the figure, two viral cores with single membranes are enclosed by a single coat.[4] Another example of this compound form may be particle *F*, which appears to exhibit a well defined central body in both sections. As many as four particles with single membranes have been observed within one peripheral coat. The diameter of viral particles with double membranes varies considerably, but the majority approximate 120 to 130 mμ.

Cells with disrupted nuclei are also encountered. Fig. 25, for example, shows cytoplasm containing granules which resemble in size, shape, and density those illustrated in Figs. 6 to 9 and 15. Part of the ruptured nucleus occupies the lower right corner. A viral particle with a single membrane is visible near the left border, in the middle third of the picture. Altered mitochondria, vacuoles and bundles of fine filaments are scattered through the field. Extracellular forms of the virus lie near the surface at the top. In Fig. 26, only fragments of the nuclear membrane are visible with clumps of chromatin lying adjacent to cytoplasmic components. At the left, an aggregate of

[3] Unless the virus is central to the section, overlap of the double membranes will produce the appearance of a dense, homogeneous, peripheral coat.

[4] A similar type of compound viral particle can also be seen in a cytoplasmic vacuole near the left lower margin of Fig. 22.

virus appears to be in process of extrusion from the nucleus. The arrangement of particles in distorted arrays suggests that the virus had been in crystalline form but that dissolution was occurring at the time of fixation. Several viral particles with double membranes are free, whereas others are contained within walled vacuoles.

An attempt to elucidate further details of viral structure by formalin fixation resulted in several surprising observations, examples of which are shown in Figs. 27 to 29.

In the first place, no intranuclear virus could be identified. Secondly, extranuclear virus contained a dense internal body, which frequently was oval and exhibited a central area of low density, and a rather diffuse peripheral coat. In Fig. 27, the nucleus occupies the lower portion of the field. There are three viral particles in the cytoplasm and one (at the upper left corner) in the extracellular space. The inset illustrates two extracellular particles at higher magnification to show the oval form of internal body on the left and a stellate form, which was less commonly encountered, on the right. The outer dense zone is contiguous to the central body and is not separated from it by a clear area, as is the case with osmium-fixed virus.

It seemed possible that the intranuclear formalin-fixed virus was unstable under impact of the electron beam, in a manner analogous to that observed in studies of vaccinia virus (14). Accordingly, sections of the formalin-fixed blocks were placed in fumes of osmium tetroxide before examination. Characteristic micrographs of the nuclei in such sections are shown in Figs. 28 and 29. The single membranes, both scattered and in crystalline arrays, are clearly visualized, but the central bodies are missing. Extranuclear virus (see top of Fig. 28) resembles in appearance the untreated formalin-fixed particles.

It is clearly important to determine whether crystallization of the virus and reduplication of the nuclear membranes is associated with the strain of virus or results from the reaction of the particular cells in which the virus was propagated. Accordingly, the H.R. (1) and C.G.[5] strains were examined under similar conditions and in the same cell lines as those described above for study of the J.M. strain. Aggregates of intranuclear granules were seen in close proximity to virus, but viral crystals were not encountered. Although there was reduplication of the nuclear membranes, virus with double coats was infrequently observed in the nuclear matrix and between the membranes.

Fig. 30 shows part of the nucleus of a HeLa cell infected with the C.G. strain. Cytoplasm occupies the left border. Granules, morphologically indistinguishable from those illustrated in Figs. 7 to 9, are aggregated at the nuclear margin, which traverses the field nearly vertically. Among these granules are several small collections of membranes varying in shape, length, and degree of definition. To the right, viral particles with single membranes are scattered in the nuclear matrix.

[5] C.G. is a strain of herpes simplex virus isolated from a case of herpetic keratitis by Dr. S. A. Ellison.

DISCUSSION

Any hypothesis derived from electron microscopic studies concerning the development and release of herpes simplex virus must be advanced with caution. Several technical factors which make acquisition of data difficult have been described elsewhere (15) and need not be enumerated. Problems presented by the interpretation of thin sections, however, while common to similar investigations, are particularly disturbing in the case of herpes simplex virus and deserve comment. Considering the probability that the sections average 40 mμ in thickness (16), approximately 300 would be necessary to examine the entire contents of a normal nucleus. If the virus were dispersed throughout the nucleus and if the nuclear response were relatively uniform, the appearance of one section might be considered as representative. Nuclei infected with herpes simplex virus, however, become swollen and frequently assume irregular shapes, as illustrated by Fig. 4, while the events related to viral differentiation generally seem to become manifest in relatively small foci at the nuclear periphery. Examination of Fig. 5, for example, suggests that the nucleus therein illustrated could be cut in such a way that no granules or virus would be contained within the section. Actually, the cluster of viral particles on the right of Fig. 6 is not present in Fig. 5. Under these circumstances one cannot assume that the structures encountered at one level necessarily reflect the state of an entire nucleus. Figs. 19, 20, and 24 reveal that particles must be nearly central to the plane of section for the structural components to be well demarcated. Since, within any single section relatively few of the randomly distributed particles are cut at a level such as to permit their identification, a generalized process which involves large numbers of viral particles may actually appear as a series of isolated phenomena. Lastly, it should be remembered that the clarity with which cellular membranes can be visualized varies according to the angle at which they have been cut. If a membrane pursues a variable course, it may appear narrow and sharply defined in one region, broad with diffuse margins in another, and nearly invisible (depending on the relative background density) in a third. Consequently, it is generally impossible to determine either the precise number of membranes in a given field or the integrity of any single membrane, as may be seen by comparing Figs. 19 and 20.[6]

Despite the foregoing problems of interpretation, spatial orientations of the virus with respect to certain components of cellular architecture occur with sufficient frequency as to suggest more than fortuitous association. For example, aggregates of relatively uniform granules ~20 mμ in diameter (Figs. 7 to 9), which can be readily distinguished from the reticular, marginated

[6] The complexity is compounded by changes in shape and orientation of some structures resulting from sublimation and probably slight flow of the methacrylate which occurs under impact of the electron beam (14).

chromatin, are generally associated with the presence of virus; these granules have not been encountered in either control nuclei or nuclei infected with adenoviruses. Moreover, the crystals of virus may extend into the granular aggregates in such a manner as to suggest that the former are replacing the latter at a template site. The concept that the granules may represent a template site for the developing virus is reinforced by the presence of rows of viral particles adjacent to nuclear membranes, as shown in Figs. 8 and 9, for this would be an unusual place for them to lodge if they differentiated in some other portion of the nucleus. It appears likely, then, that the granular areas represent foci in which the various components of the viral particle become spatially arranged so as to present the electron microscopic appearance of a central body and a single outer membrane. Such a mechanism would probably entail the progressive development of a membrane enclosing the central body and, therefore, particles viewed at a stage when the process was incomplete would exhibit only partially formed membranes.[7] The question naturally arises as to why, if virus is developing within the clusters of granules, the process cannot be demonstrated with greater clarity for the J.M. strain. In Figs. 7 and 9, for example, incomplete membranes can be identified, but they are scattered at random and are not concentrated at the margins of crystals, where differentiation presumably should be most active. The situation may be similar to that postulated to occur in the case of crystallizing strains of adenovirus, where there is also a paucity of developmental forms (21). Presumably, crystallization *in vivo* requires rapid differentiation of virus at a given locus in order to achieve sufficient purity for operation of the electrostatic forces carried by the particles. To put it another way, development must progress with enough synchronism in a region of the template site so that relatively large numbers of particles form during brief intervals of time and crystallize before dispersal can occur. The presence of contiguous crystals, which exhibit differing orientations in the section (see Figs. 9 and 14), suggests that viral differentiation can be initiated either simultaneously or sequentially at multiple, separate foci within the template area. If the foregoing hypotheses are correct, one might expect that strains of virus less prone to crystallize would be more apt to reveal the process of development, not because the process differs basically, but merely because it occurs more slowly, thus providing a greater opportunity to visualize different stages. Such, indeed, appears to be the case, as shown in Fig. 30. Study of this field leads to the inescapable impression that membranes are actually in process of formation at many sites in the aggregate of granules. On the right, some viral particles with single membranes lie close to the presumably active template areas, whereas others are scattered in the nuclear matrix. This spatial orientation is

[7] An analogous phenomenon in the cytoplasm has been noted in the case of influenza (17), vaccinia (18), fowl pox (18), Shope fibroma (19), and molluscum contagiosum (20) viruses.

consistent with the concept that when particles differentiate individually and asynchronously they tend to become dislodged before sufficient numbers can accumulate at one locus to crystallize.

In view of the fact that viral particles with double membranes were repeatedly observed within infected nuclei and considering the propensity of such nuclei to form membranes, one is led to conclude that the viral particles become enclosed by a second membrane before release from the nucleus. Consistent with this concept is the presence of twin forms of the virus (evident in Figs. 22 and 24) which would result if a membrane were to wrap around two contiguous particles, such as those illustrated on the left of Fig. 18. Virus with double membranes has not been encountered either within the aggregates of granules or within the crystalline arrays. This suggests that the second, outer membrane forms at an intranuclear site removed from the primary template area where the internal body and initial membrane are believed to differentiate. At the developmental stage characterized by the formation of a second membrane the central body appears to become altered, since the great majority of these bodies enclosed by single membranes have a central region of low density, whereas those surrounded by double membranes exhibit uniform opacity after fixation in osmium tetroxide. Formalin, moreover, fixes the latter but does not preserve the former (see Figs. 27 to 29). Although differences in permeability of the viral membranes could explain these observations, it is not illogical to assume that there is some change in the chemical composition or physical state of the central body which is reflected in the altered morphology and reaction to formalin.

The dense, irregular filaments seen in Figs. 5, 6, and 9 and the granules of variable shape shown in Figs. 1, 2, and 4 exhibit no consistent spatial orientation to the virus. Since such structures were not found in control nuclei, however, they would appear to reflect some abnormal reaction of the cell. It is of interest that intranuclear granules resembling those illustrated in Figs. 1, 2, and 4 have been observed in the nuclei of cells infected with adenovirus (22) and influenza virus (23). Whether such morphologic similarity also denotes a chemical relationship remains to be determined.

Virus may enter the cytoplasm upon disruption of the nucleus (Figs. 25 and 26), but, in contrast to the adenoviruses (7), this does not appear to be the sole mechanism of egress. Reduplication of the nuclear membranes probably enables the virus to gain release into the cytoplasm without disruption of the nucleus. Figs. 15, and 17 to 19 are believed to illustrate different stages in the process whereby new membranes are laid down behind the virus as it passes into the cytoplasm. Within the cytoplasm this strain of virus is generally lodged in walled vacuoles, as shown in Figs. 22 and 23. It is unlikely that the vacuoles containing virus are in reality cross-sections of finger-like projections from the nucleus, simply because such projections would appear as tubules

when cut longitudinally and their connections with the nucleus would sometimes be encountered. If the walls of the vacuoles enclose the virus after release, one would expect to see the process taking place in the cytoplasm near the nucleus. Such a phenomenon has not been observed. Moreover, particles may be surrounded by a third membrane while still within the nucleus, as shown in Figs. 9, 16, and 17. The foregoing observations suggest that the walls of the intracytoplasmic vacuoles probably represent nuclear membranes which have surrounded viral particles, either singly or in clusters, just prior to or during release of virus from the nucleus. The three particles at the upper right of Fig. 11 may have been fixed during a stage in this process.

Examination of the cytoplasm of infected cells raises the possibility that when vacuoles reach the surface the walls of both vacuole and cell open with release of virus into the extracellular space, in a manner closely similar to that suggested for the egestion of phagocytized particles by leukocytes (24). Under such circumstances the cellular membrane could close over the site of rupture. The process would morphologically resemble, but functionally be the reverse of, phagocytosis and pinocytosis (25–30). Indeed, by study of a single picture, such as Fig. 23, one cannot determine whether virus is entering the cell or being released. If ingress were taking place generally, however, one would expect to see the phenomenon occurring during the early stages of infection. This was not found to be the case. Cells devoid of intracytoplasmic virus usually exhibited normal nuclei or minimal nuclear changes (Figs. 1 to 4), whereas cells containing the largest amount of virus in the cytoplasm showed marked nuclear alteration.

Cells with ruptured nuclei might be expected to contain large numbers of intracytoplasmic viral particles possessing single membranes. Actually, however, relatively few were seen, and these generally lay in the vicinity of the nucleus. This observation, together with the fact that virus with single membranes has never been encountered in the extracellular space, leads to the conclusion that this form of the virus is unstable and disintegrates in the cytoplasm. Presumably, then, only particles with double membranes survive outside the nucleus and thus constitute infectious virus.

In a previous publication on the growth of the H.R. strain of herpes simplex virus in chicken embryo chorioallantoic membranes (1), the apparent absence of intranuclear viral particles with double membranes led the authors to suggest that the second membrane may be acquired within the cytoplasm. Subsequently, Reissig and Melnick (3), in a study of herpes B virus grown in tissue cultures of monkey kidney cells, noted occasional intranuclear particles with two membranes as well as "within the double membrane surrounding the infected nucleus." Stoker, Smith, and Ross (2) confirmed the presence of intranuclear particles with double membranes in tissue cultures infected with the HFEM strain. Similar observations have been made during electron microscopic examination of two viruses which appear morphologically to resemble

herpes simplex virus, *viz.*, the virus associated with renal adenocarcinoma of frogs (31) and varicella virus (32). In the last two instances, as well as in a study of salivary gland virus (33), collections of intracytoplasmic viral particles were found within vacuoles. However, in none of the instances cited above was reduplication of the nuclear membranes or the presence of intranuclear lamellae noted.

In the light of these reports and the results of the current studies, the micrographs of the H.R. strain of herpes simplex virus in chicken embryo chorioallantoic cells were reviewed. A few well defined particles with double membranes were discovered in nuclei, and reduplication of nuclear membranes, although less striking than in the present instance, was observed. In addition, the J.M. strain of herpes simplex virus was transferred from tissue cultures directly to the chicken embryo chorioallantois. 3 and 5 days after inoculation sections of the resulting focal lesions were examined in the electron microscope. Although virus with double membranes was found, this form seldom appeared within nuclei. Intranuclear lamellae and reduplication of nuclear membranes were observed only rarely. The preceding observations suggest that the behavior of the nucleus with respect to the formation of membranes differs in a quantitative rather than a qualitative manner. In other words, the nuclear membranes can undergo reduplication both in chorioallantoic cells and in HeLa or amnionic cells, but, under the conditions of the present experiments at least, the phenomenon occurs more frequently and to a greater degree in the latter cell systems. The data are consistent with the concept that the basic mechanisms of viral development and release are not affected by the type of host cell.

Except for the profusion of lamellae, many of which exhibited pore-like structures, and the presence of walled vacuoles containing virus, changes observed in the cytoplasm of infected cells could not be distinguished from the alterations which accompany "non-specific degeneration" (34) and, therefore, could not be attributed to any unique action of the virus. The viral forms with one membrane which were found in the cytoplasm exhibited no consistent orientation to any components and probably had been released prematurely from ruptured nuclei. As already indicated, the scarcity of these forms near the cell surface and their absence from the extracellular space suggest that such forms probably disintegrate in the cytoplasm. Since the formation of membranes apparently can occur in infected nuclei and since no evidence has been obtained to confirm our initial hypothesis that the virus acquires its outer coat in the cytoplasm, we are inclined toward the belief that the development of herpes simplex virus is completed within the nucleus.

SUMMARY

Study of the J.M. strain of herpes simplex virus in human amnionic and HeLa cell tissue cultures revealed the presence of intranuclear crystals composed of viral particles with a single membrane enclosing a central body.

Randomly dispersed virus with double coats was seen in the nuclear matrix and between multiple membranes at the nuclear periphery. The majority of intracytoplasmic viral particles were within walled vacuoles. It is suggested that this strain of virus differentiates and frequently crystallizes at template sites which are characterized by aggregates of granules near the nuclear margin; that particles, either singly or occasionally in small groups, become enclosed by a second peripheral membrane while still within the nucleus; that the virus can pass into the cytoplasm through reduplications of the nuclear membrane which are deposited behind the virus in such a manner as to prevent rupture of the nucleus; that most of the intracytoplasmic virus is contained within sacks formed by nuclear membranes; and that rupture of these sacks at the cell surface results in extrusion of virus without disruption of the cell. No evidence was obtained to support the hypothesis that virus develops in the cytoplasm. Examination of the H.R. and C.G. strains of herpes simplex virus in identical cell lines grown under similar conditions failed to show viral crystals, but reduplication of the nuclear membranes was evident. Study of the J.M. strain in cells of chicken embryo chorioallantoic membranes indicated that the basic mechanisms of viral development and release did not differ from those operating in HeLa and human amnionic cells.

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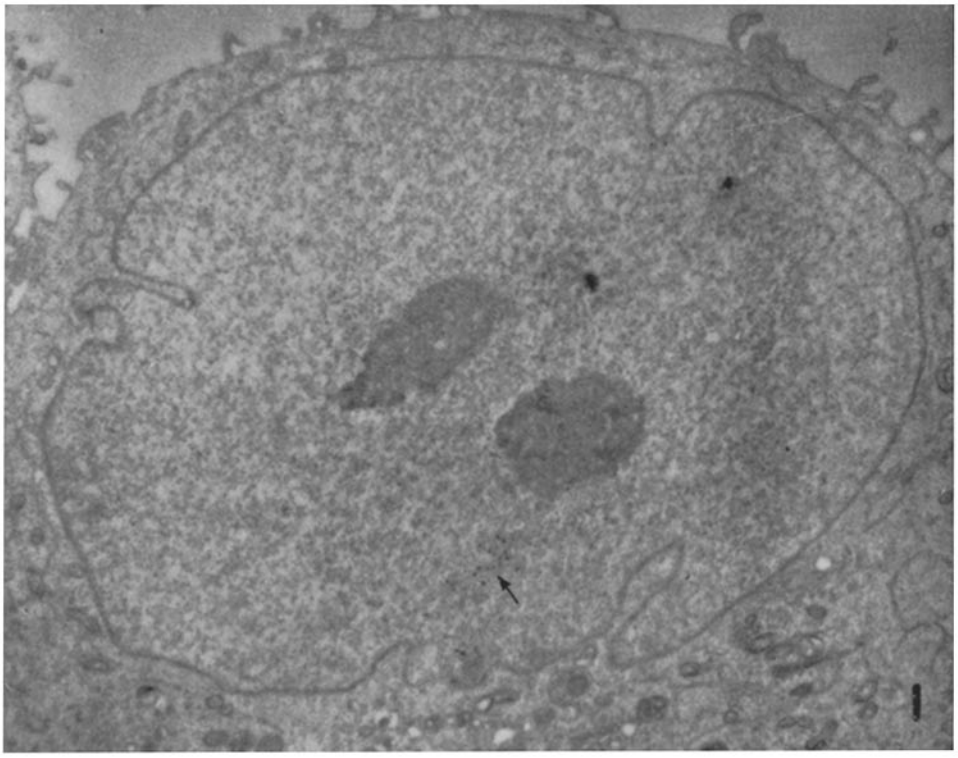
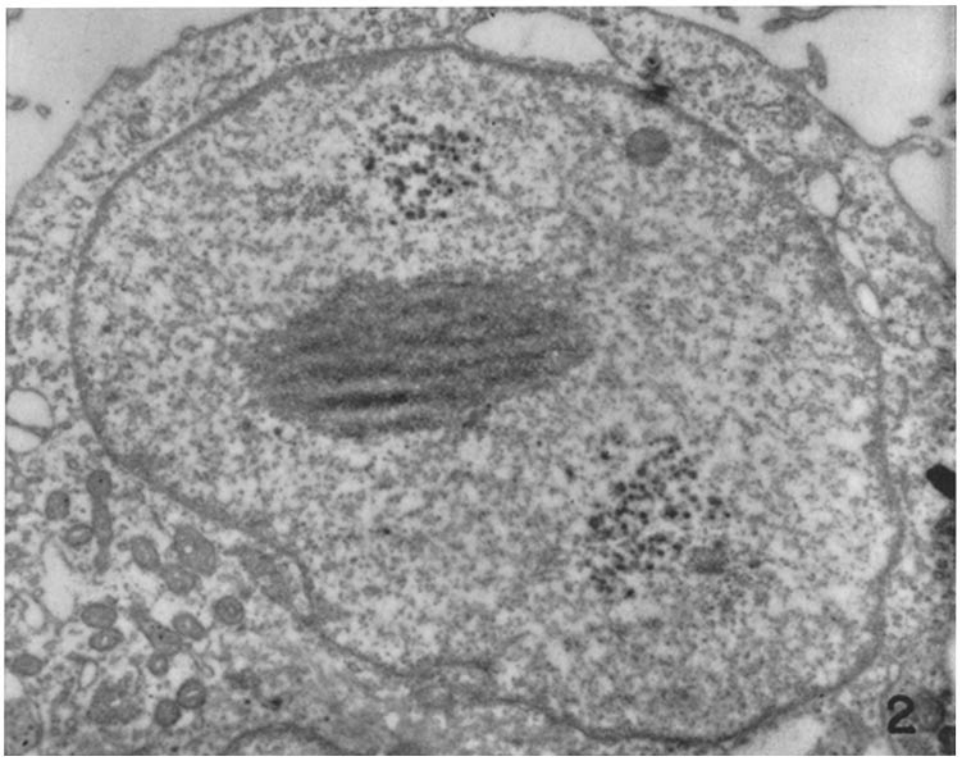
EXPLANATION OF PLATES

PLATE 43

FIG. 1. A nucleus containing two nucleoli and, in the lower portion, a cluster of small, dense granules (arrow). Two fragments of dirt are apparent in the upper half. × 9,000.

FIG. 2. A nucleus with two clusters of dense granules, which vary in size and shape. The nuclear matrix is irregularly clumped. × 13,000.



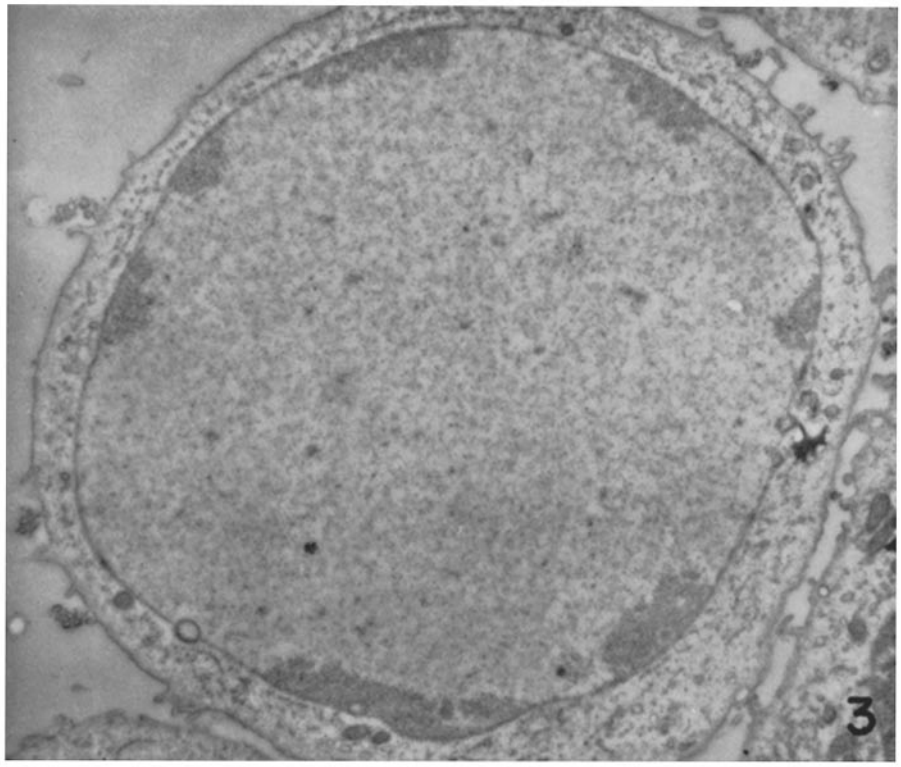


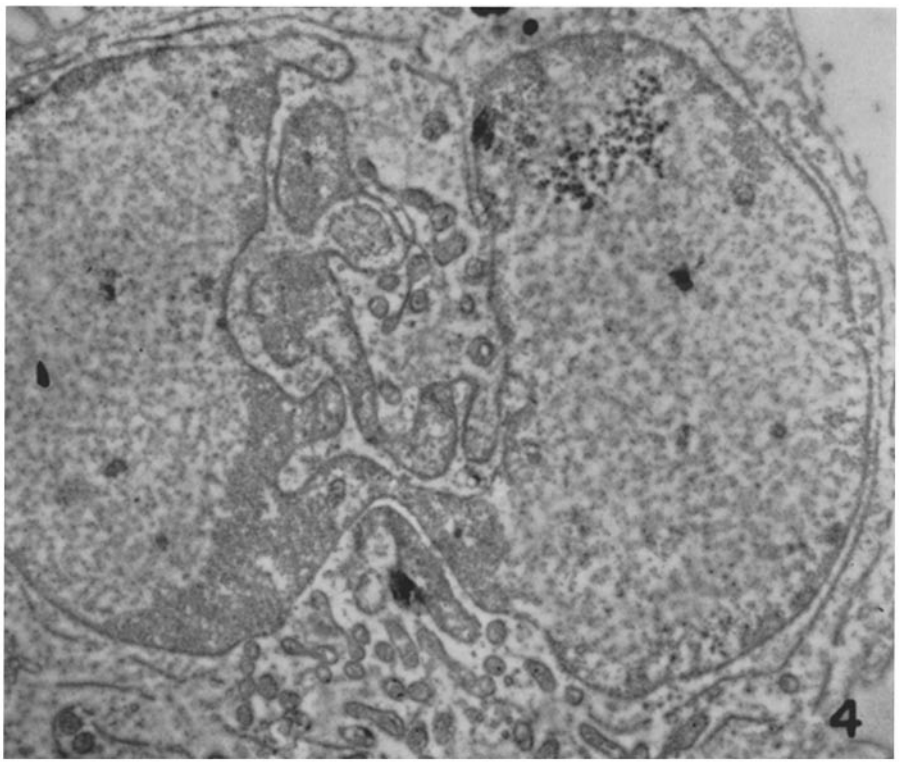
(Morgan *et al.*: Herpes simplex virus)

PLATE 44

FIG. 3. A swollen nucleus with marginated chromatin. No intracellular virus can be identified, although there are groups of viral particles on the free surface. × 7,800.

FIG. 4. A lobulated nucleus containing marginated chromatin and a few viral particles with single limiting membranes. Dense granules are visible at the upper right. × 11,000.



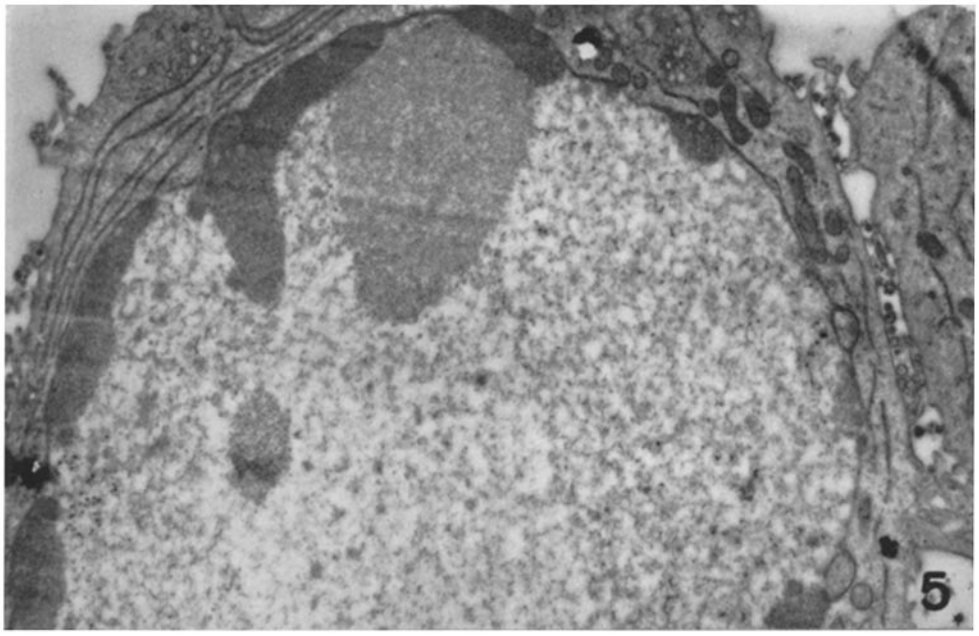


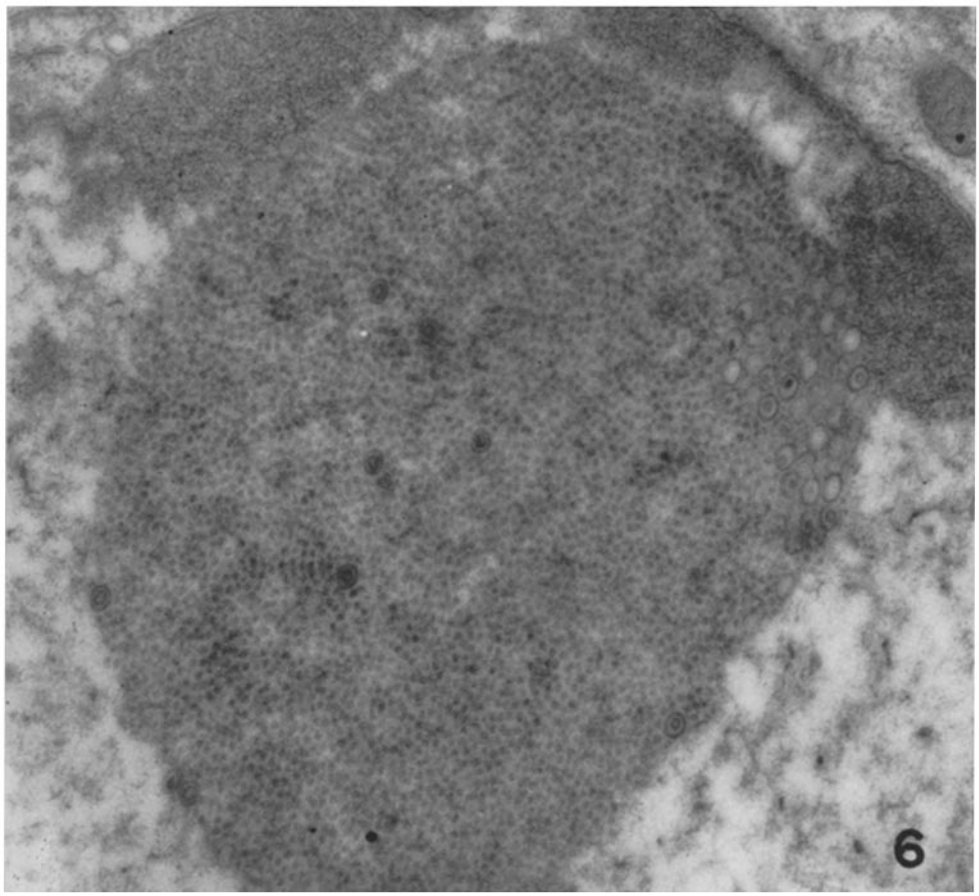
(Morgan *et al.*: Herpes simplex virus)

PLATE 45

FIG. 5. Part of a nucleus in which there is marginated chromatin, dense irregular granules and filaments laterally, and an aggregate of small regular granules at the top. × 10,000.

FIG. 6. The granular aggregate in Fig. 5 sectioned at a slightly different level. The granules are nearly uniform in size and occasionally form short rows. Scattered among the granules are viral particles with an internal body and a single limiting membrane. × 40,000.

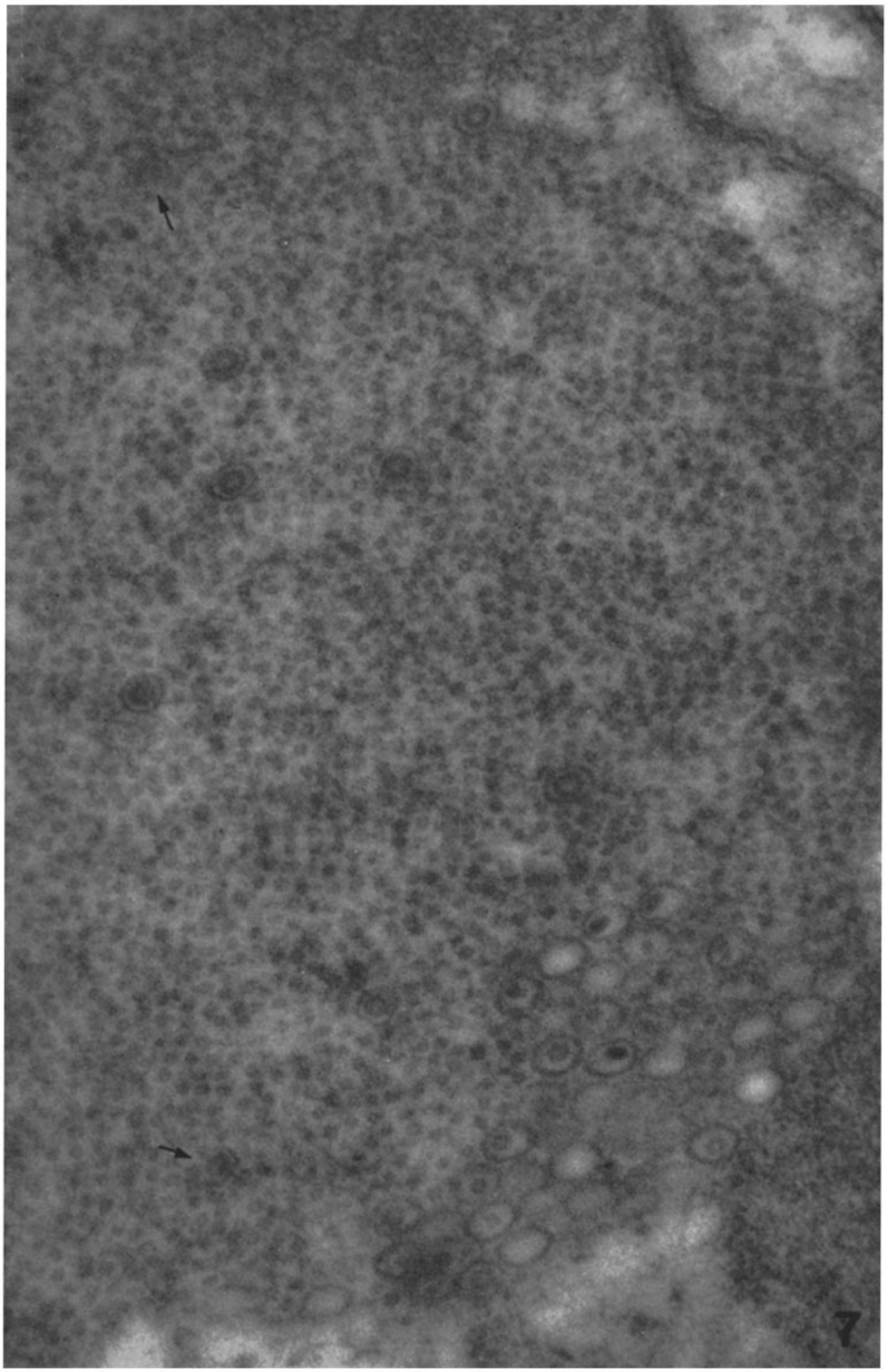




(Morgan *et al.*: Herpes simplex virus)

PLATE 46

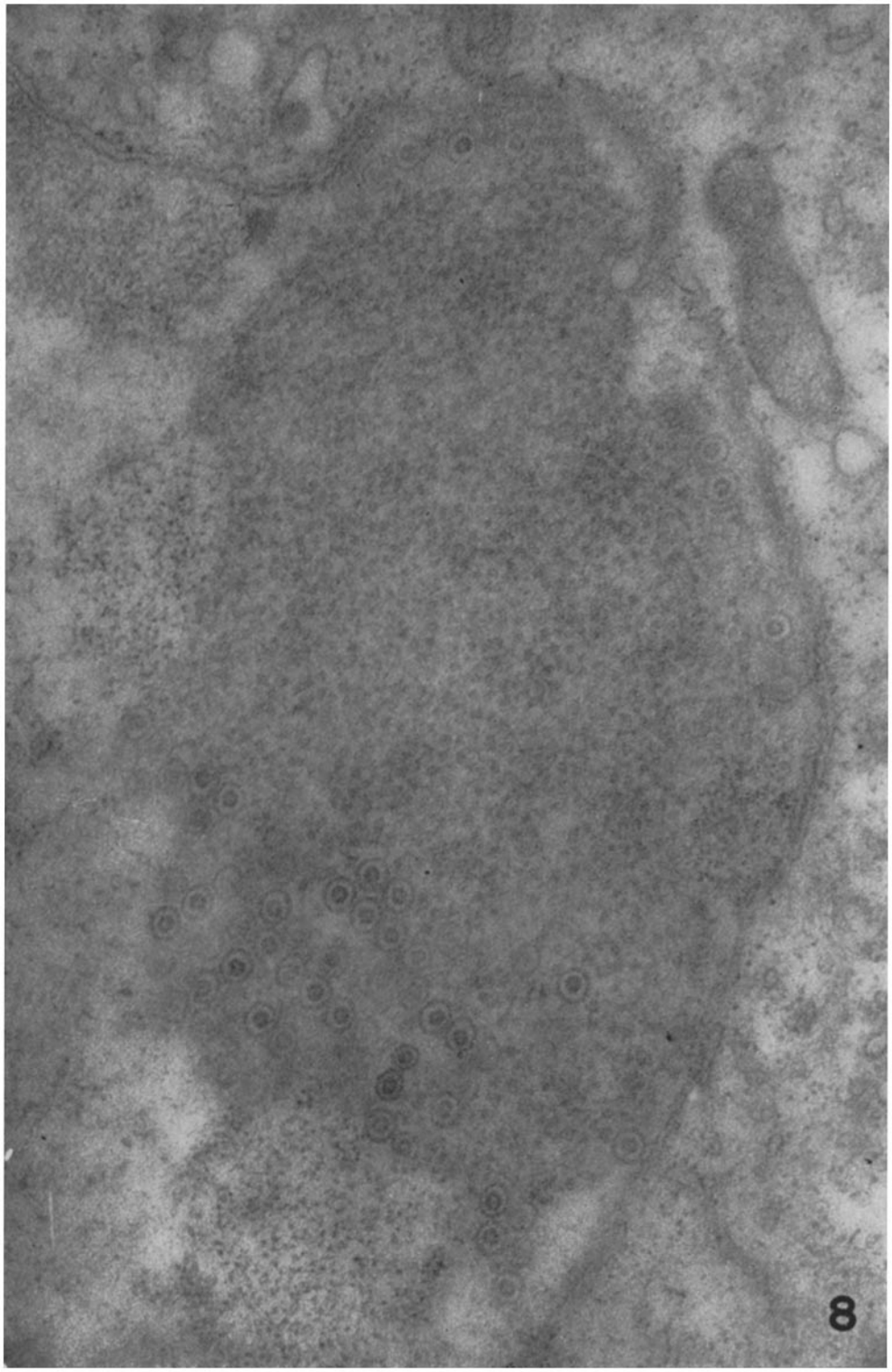
FIG. 7. Part of Fig. 6, printed at twice the magnification from a different negative. Two discontinuous viral membranes are visible (arrows). The cores of the viral particles clustered in the lower right vary in size, shape, and density and differ from the internal bodies in the scattered virus, which are larger and contain a central region of diminished density. × 80,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 47

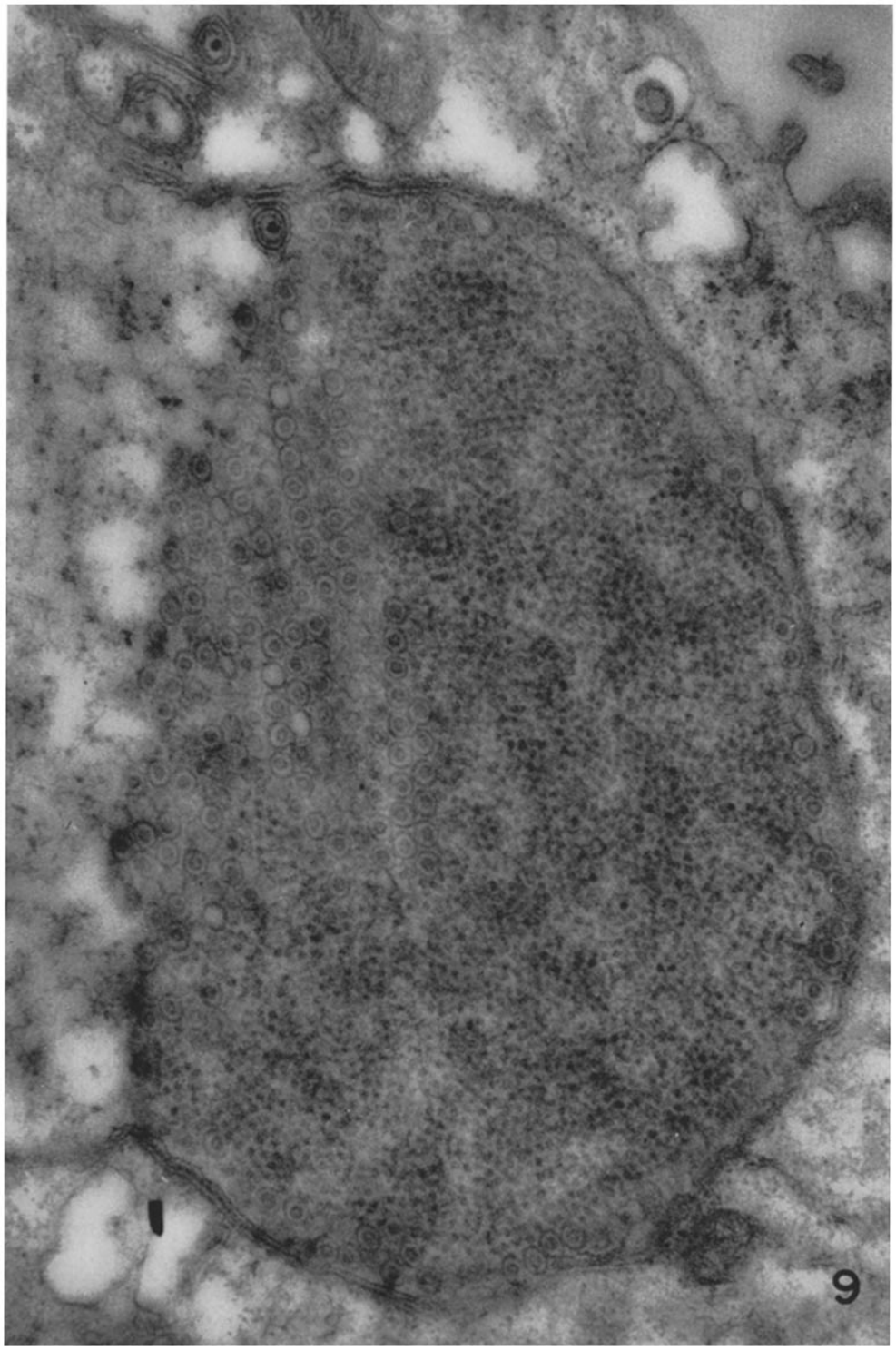
FIG. 8. An unusually thin section through the periphery of a nucleus. Viral particles with single membranes exhibit crystalline array. Several particles lie between the aggregate of granules and the nuclear membrane, which traverses the right and top margins of the picture. × 52,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 48

FIG. 9. A collection of granules and viral crystals within a nuclear protrusion. A row of viral particles lies adjacent to the nuclear membrane, which is reduplicated. At the top two intranuclear particles possess triple membranes. × 44,000.

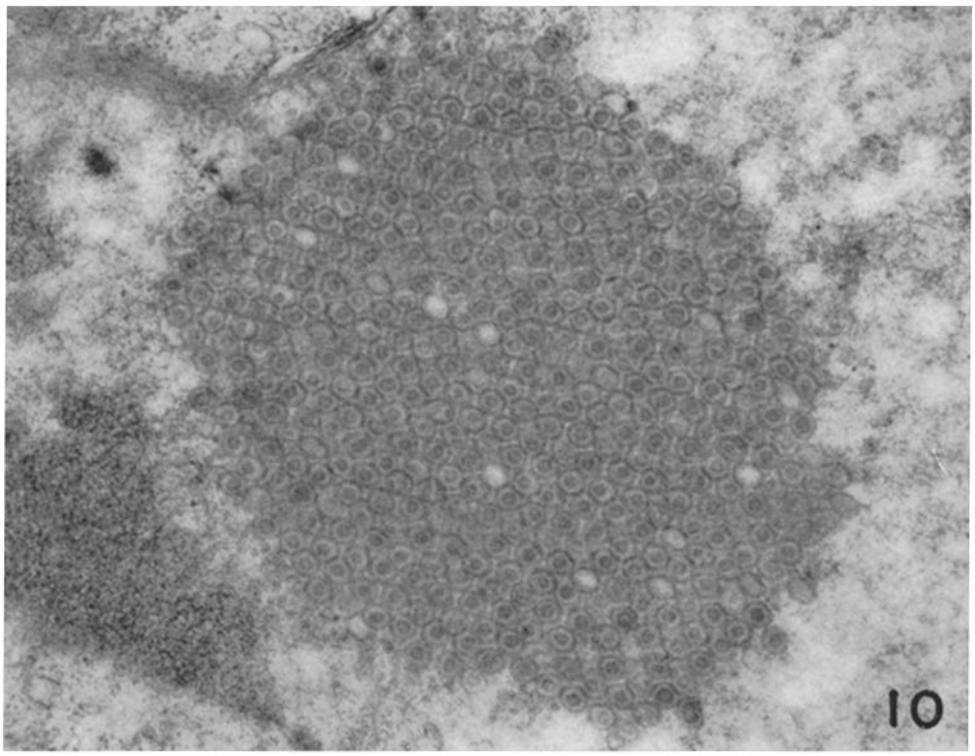


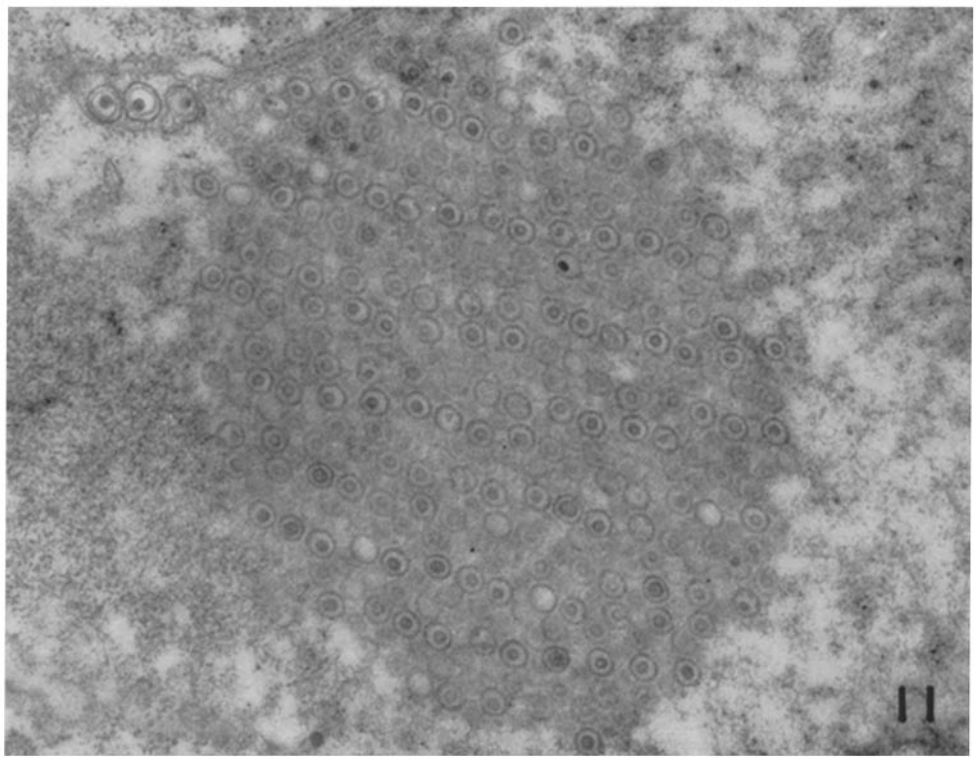
(Morgan *et al.*: Herpes simplex virus)

PLATE 49

FIG. 10. A moderately thick section through a hexagonal shaped viral crystal. At the upper left, several free viral particles border the multiple nuclear membranes. × 45,000.

FIG. 11. The same crystal as it appears in a thinner section. Virus central to the plane of section is sharply defined. At the upper left, three viral particles with double coats lie between the nuclear membranes. × 45,000.

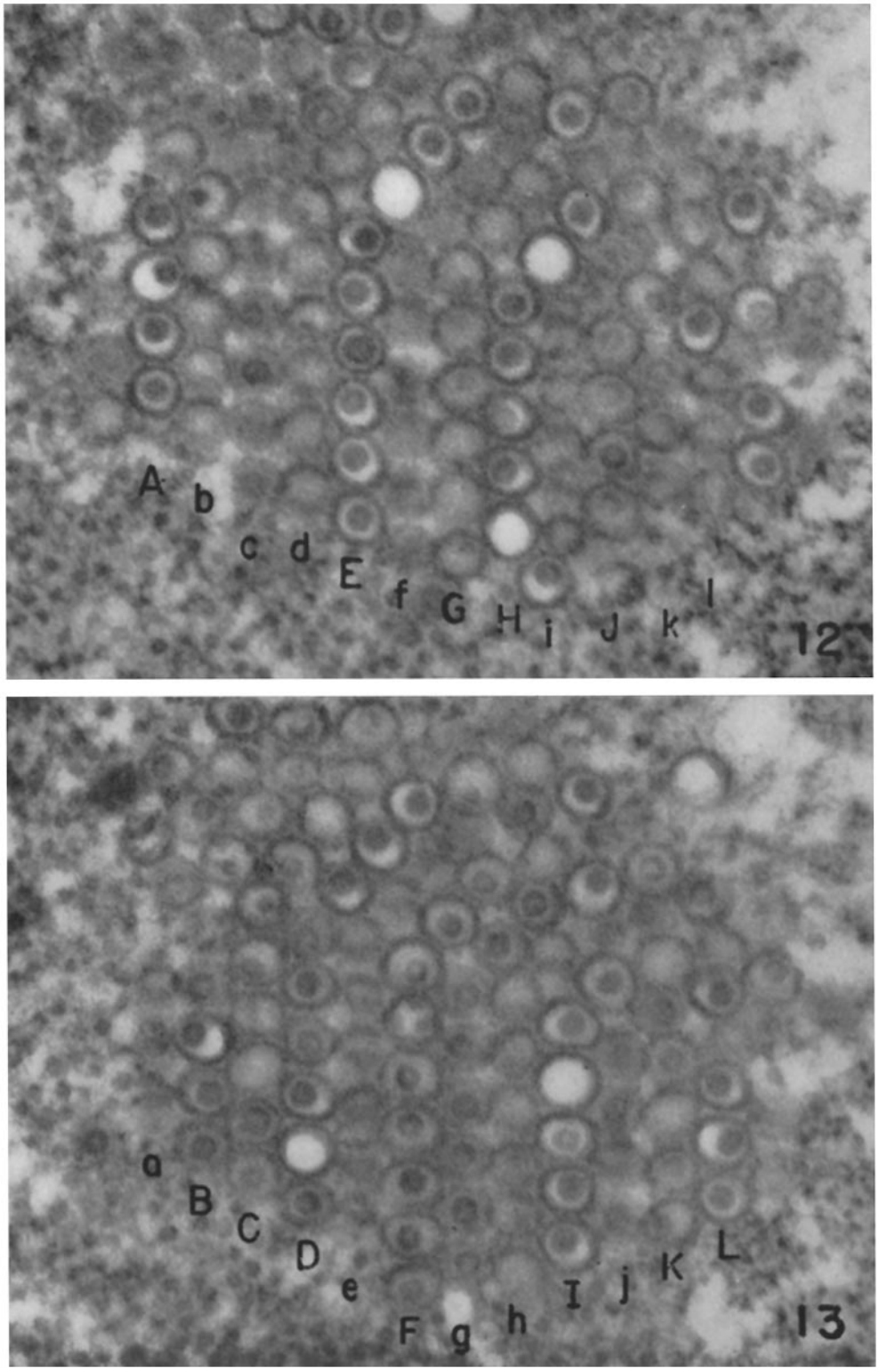




(Morgan *et al.*: Herpes simplex virus)

PLATE 50

FIGS. 12 and 13. A viral crystal in two consecutive serial sections. The rows indicated by capital letters are believed to be nearly central to the plane of section and, hence, exhibit sharply defined membranes. Some particles are devoid of internal structure. × 97,000.

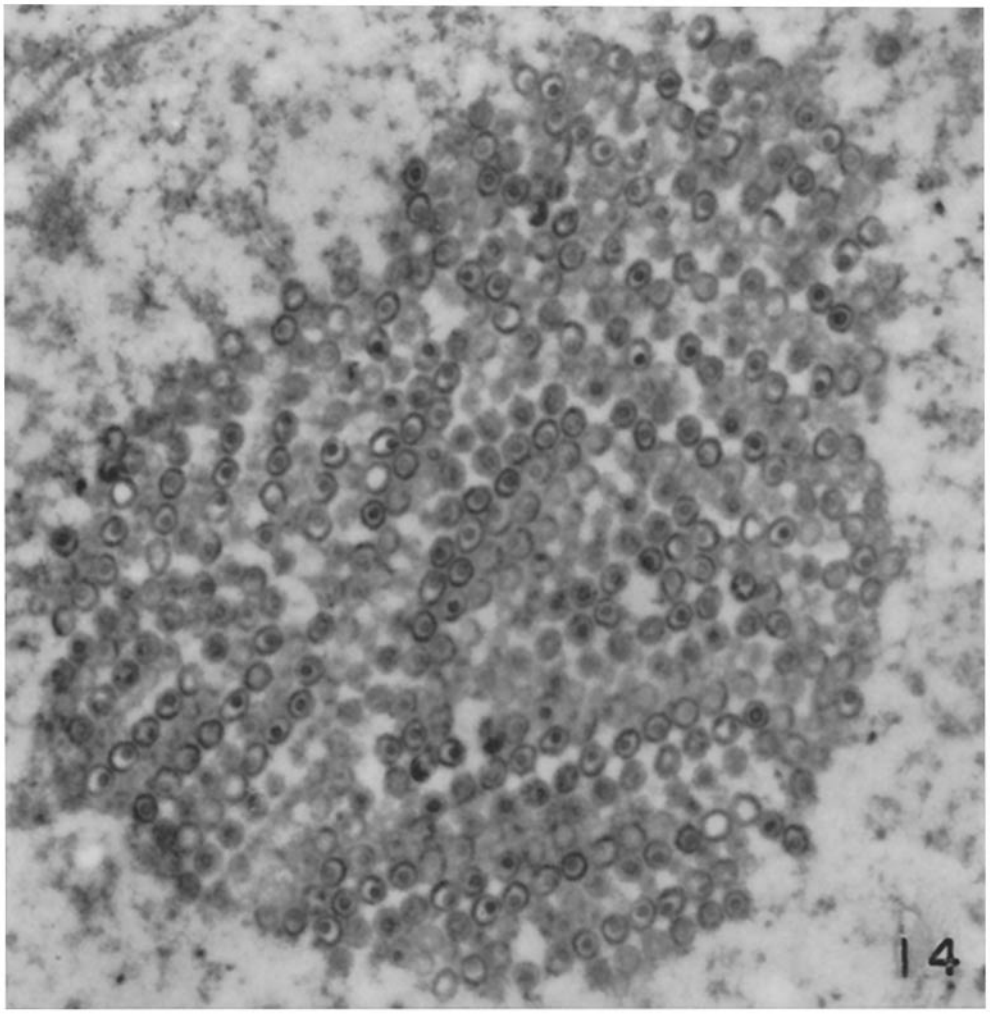


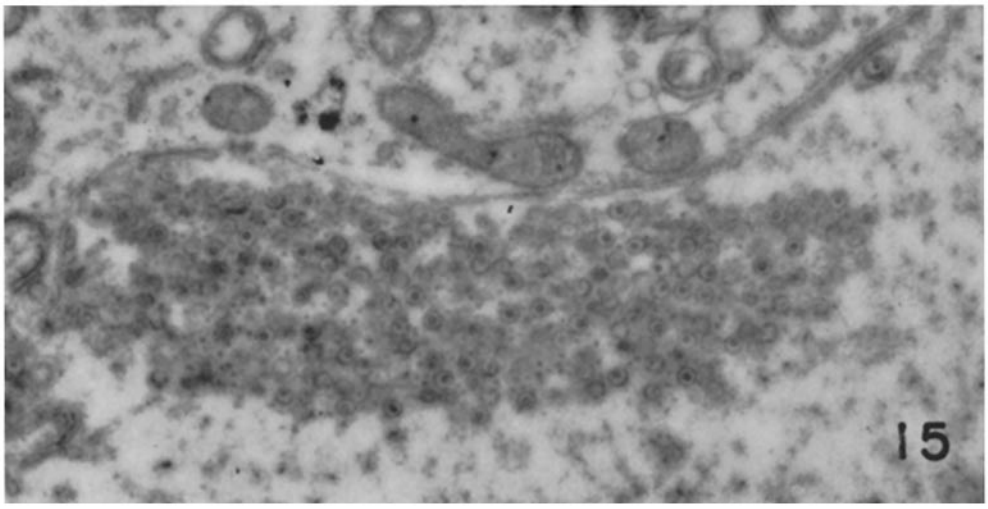
(Morgan *et al.*: Herpes simplex virus)

PLATE 51

FIG. 14. Several viral crystals in apposition. The internal bodies of the virus differ in density. × 42,000.

FIG. 15. Viral particles among scattered granules. The nuclear membrane traverses the upper portion of the field. At the top right a double coated particle is near the multiple nuclear membranes. × 28,000.





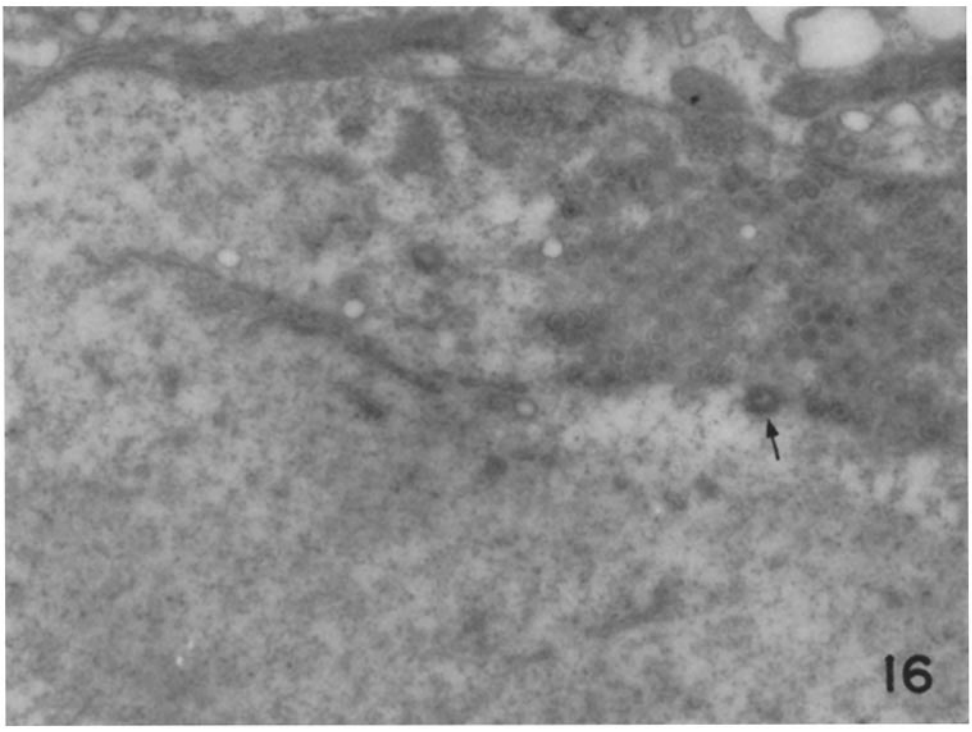
(Morgan *et al.*: Herpes simplex virus)

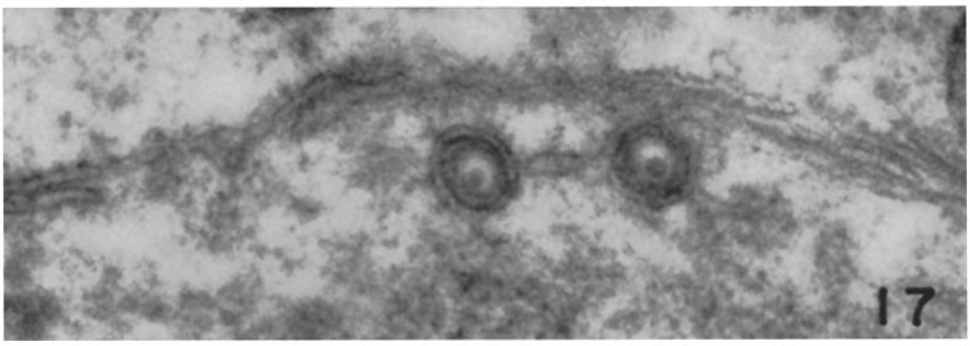
PLATE 52

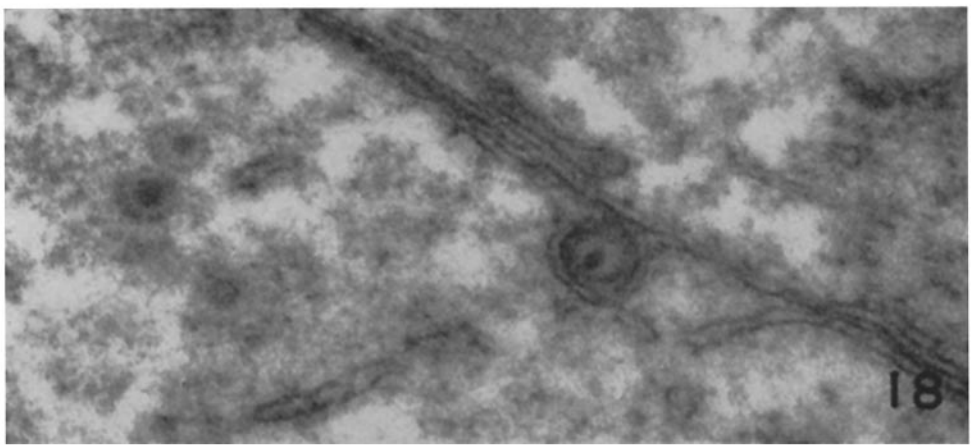
FIG. 16. A nucleus containing viral crystals and scattered double lamellae of variable length. At the upper left there is reduplication of the nuclear membranes. One viral particle exhibits three membranes, of which the outermost is incomplete (arrow). × 28,000.

FIG. 17. Viral particles with triple membranes. The outermost membrane partially encloses the virus and appears on the right to be continuous with the nuclear membranes. Cytoplasm traverses the top of the field. × 87,000.

FIG. 18. Nuclear membranes partly surrounding one viral particle. On the left, two contiguous particles with single membranes lie near a lamellar fragment in the nuclear matrix. × 87,000.



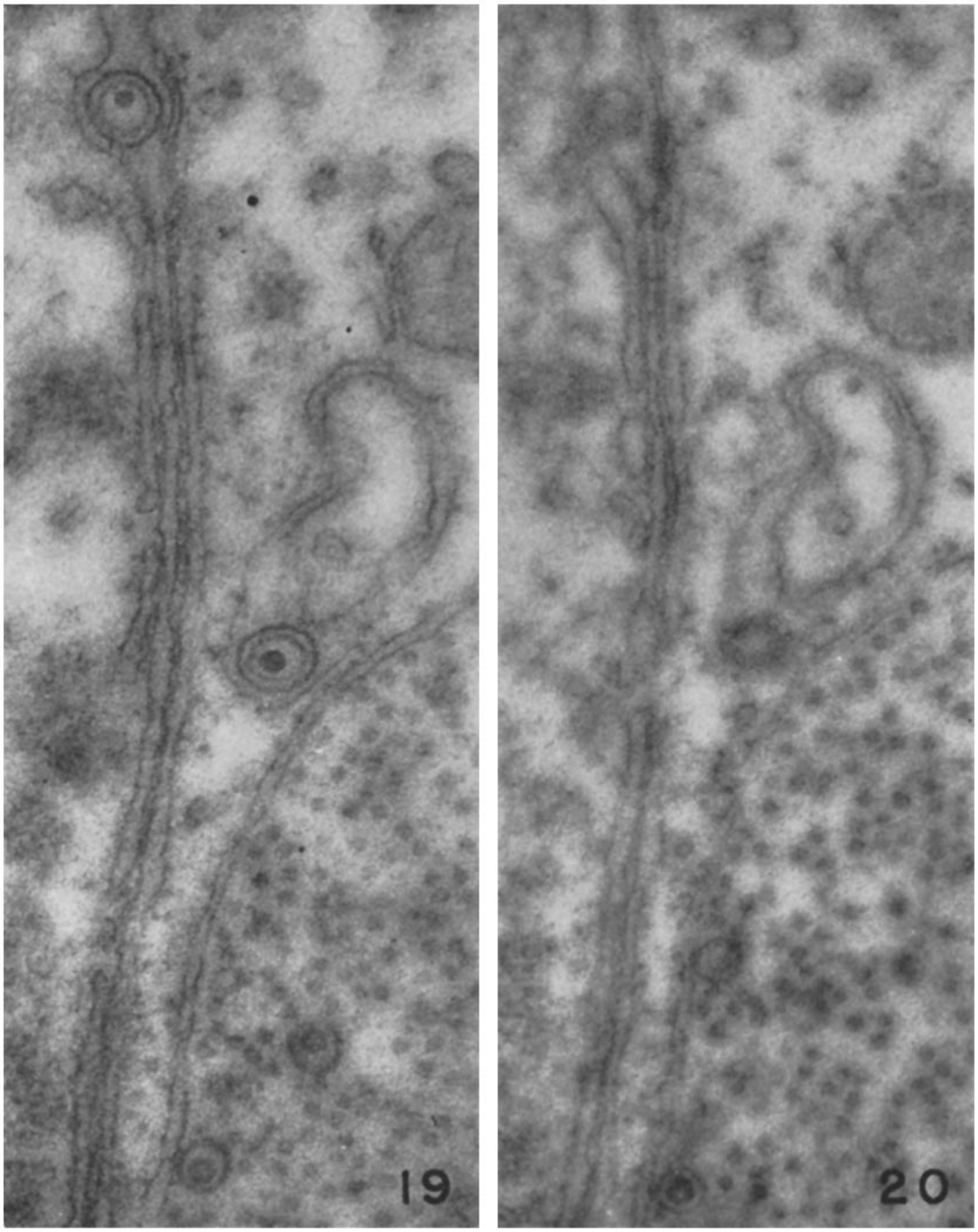




(Morgan *et al.*: Herpes simplex virus)

PLATE 53

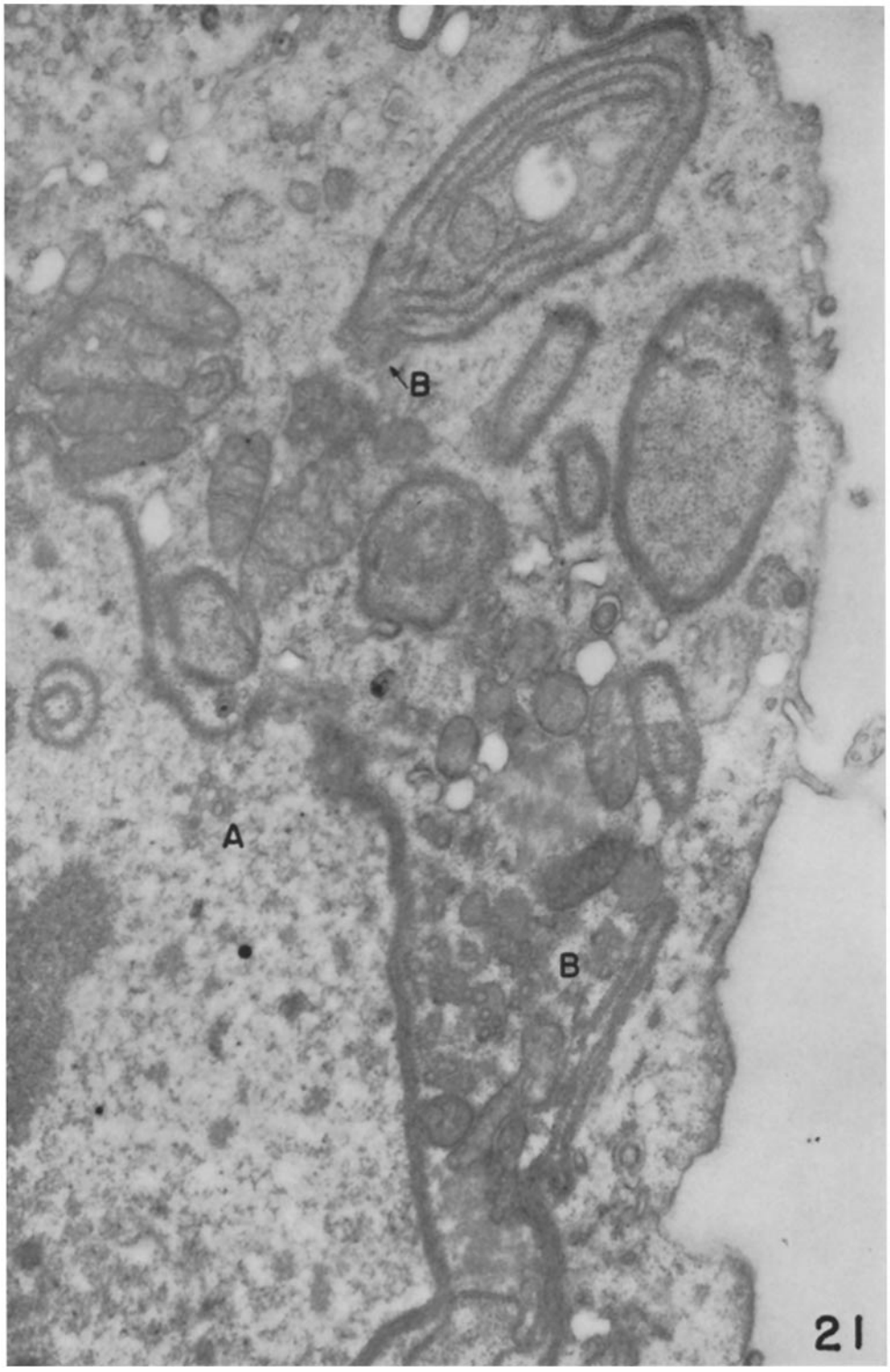
FIGS. 19 and 20. Two serial sections showing (at the top) a viral particle between multiple nuclear membranes, which traverse the field vertically and separate the nucleus on the left from the cytoplasm on the right. Near the center of the field a viral particle lies within a cytoplasmic vacuole. In the lower right a nuclear protrusion contains granules and single-membrane forms of the virus. × 87,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 54

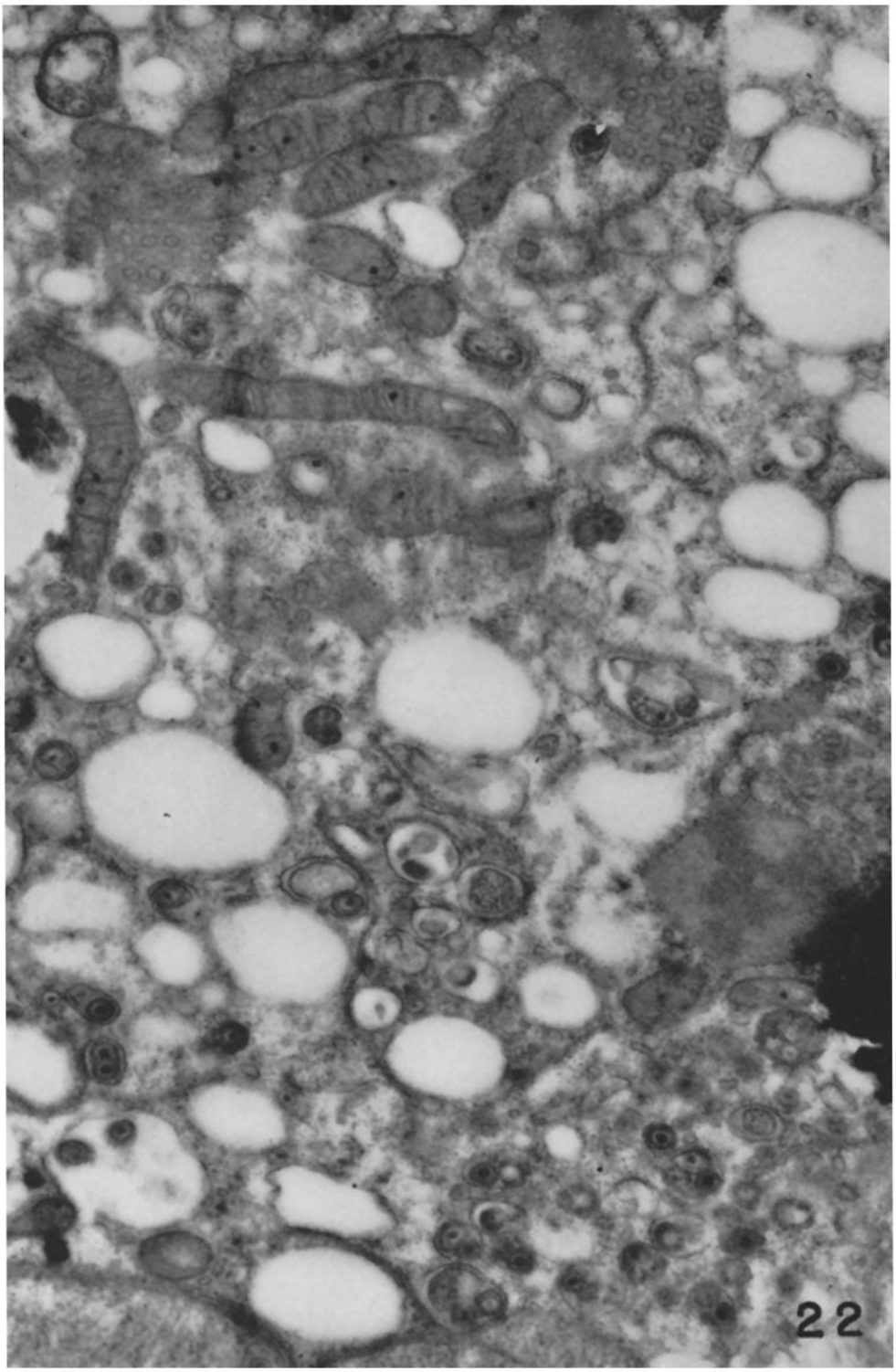
FIG. 21. Part of the cytoplasm and, at the lower left, a portion of the nucleus of an infected cell. In the nucleus there is a small cluster of viral particles with single membranes (A). Numerous membranes traverse the cytoplasm. In several regions (best seen in the lower third) oval, pore-like structures are visible where the membranes are tangential to the section (B). × 25,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 55

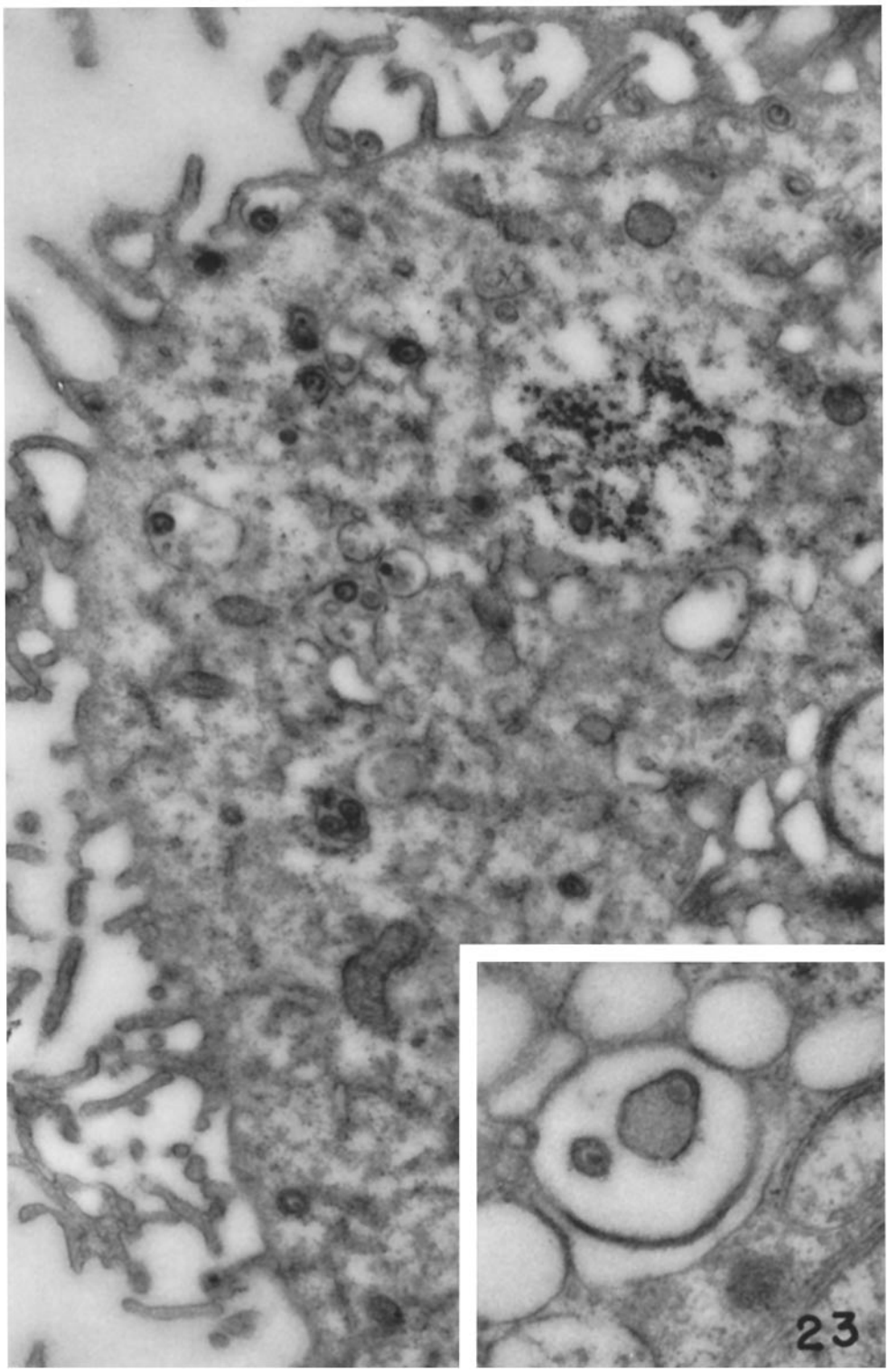
FIG. 22. The cytoplasm of a cell containing viral particles, the majority of which lie within vacuoles. Mitochondria and pore-like structures are visible in the upper portion of the field. × 34,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 56

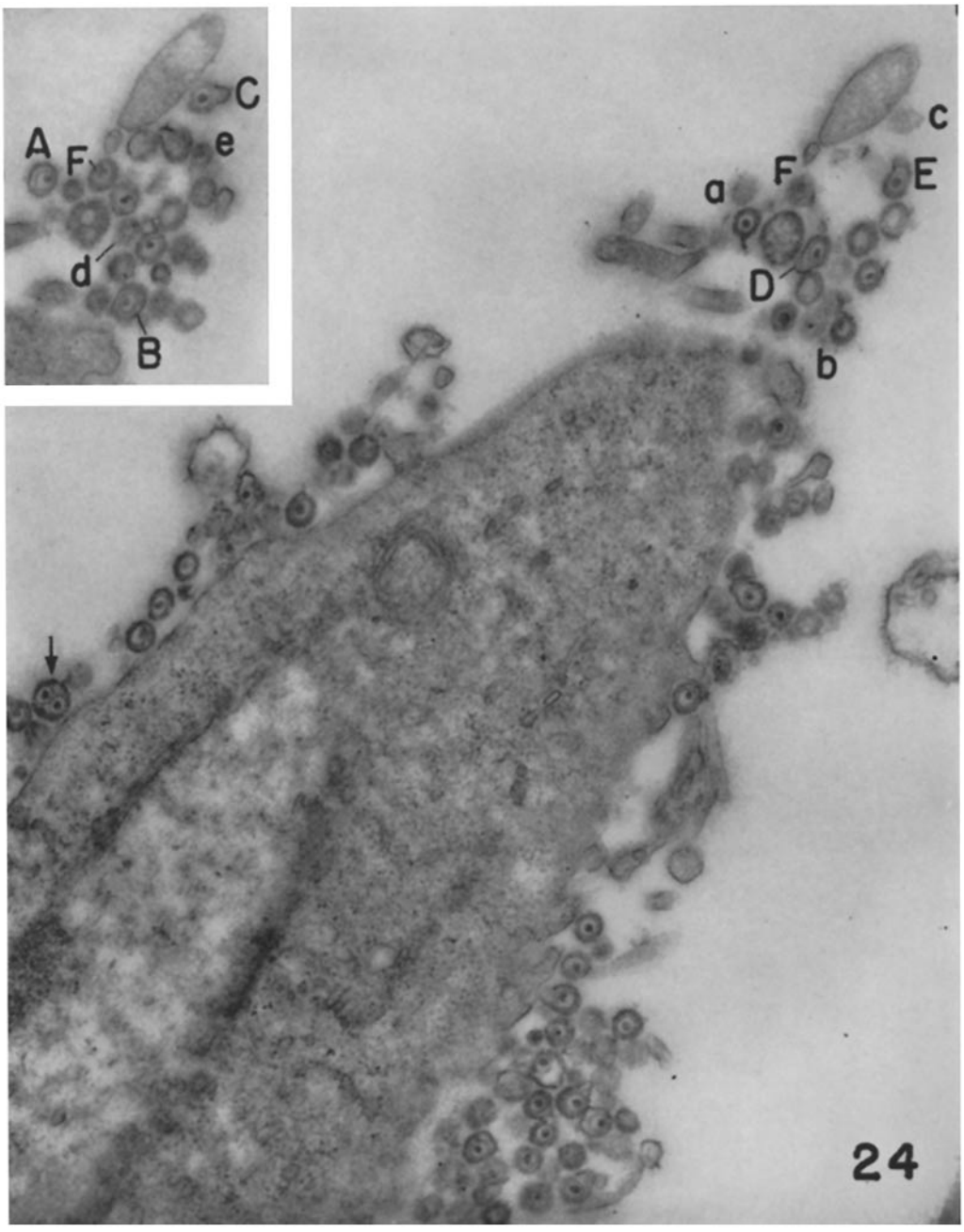
FIG. 23. The cytoplasm and surface of a cell. Characteristic, occasionally branched, cytoplasmic processes extend into the extracellular space. Near the top, vacuoles containing virus are separated from the exterior by a narrow zone of cytoplasm. × 28,000. The inset shows a vacuole containing two viral particles, one of which exhibits an irregularly shaped peripheral membrane. × 49,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 57

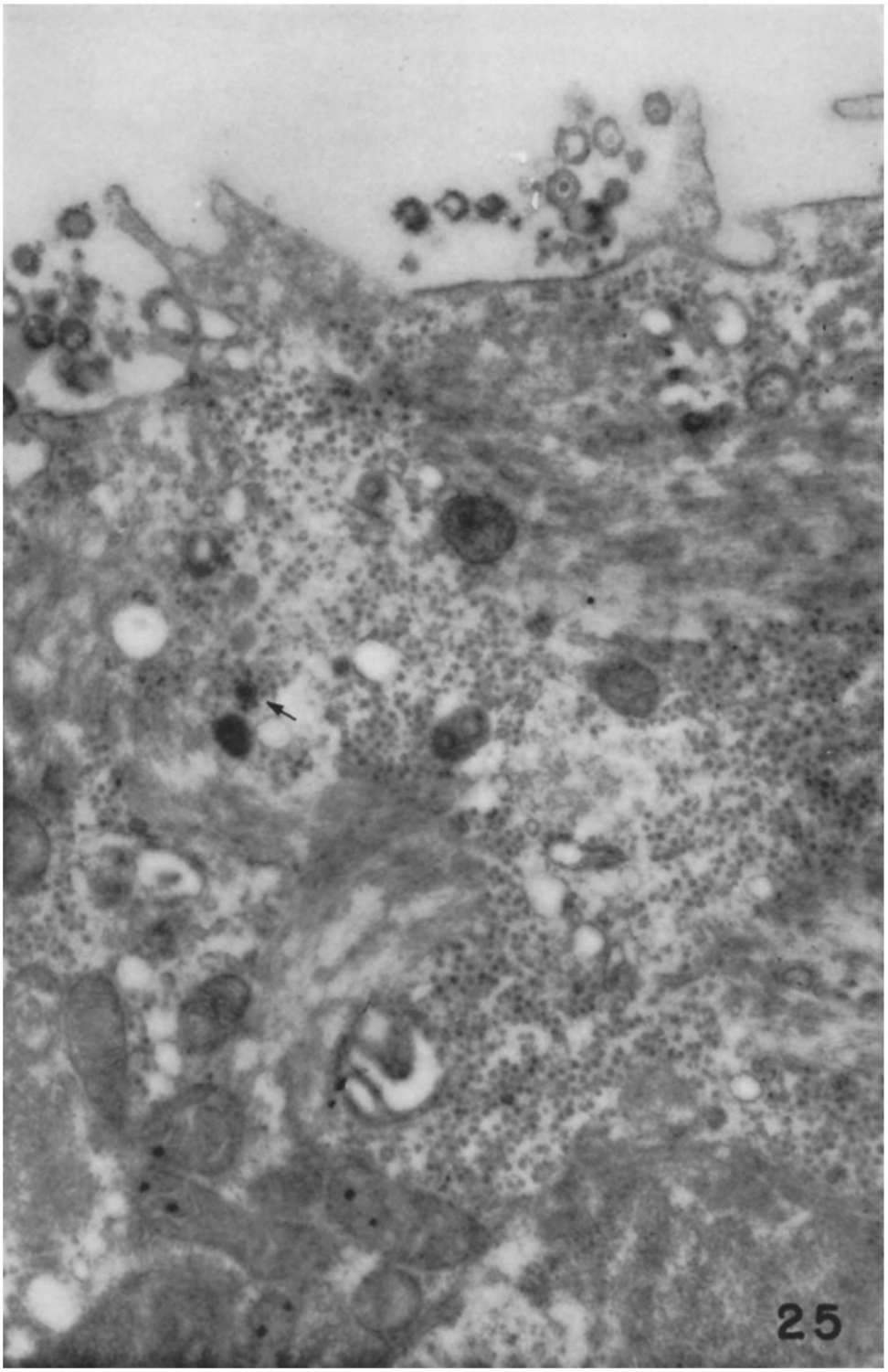
FIG. 24. Virus on the surface of a cell. Many particles have distorted outer membranes. Near the left border, two viral particles with single membranes possess a common external coat (arrow). The inset shows the upper portion of the field in a contiguous section. The capital letters indicate virus which has been cut centrally. Particle F probably represents the twin form with well defined internal bodies in each section. × 34,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 58

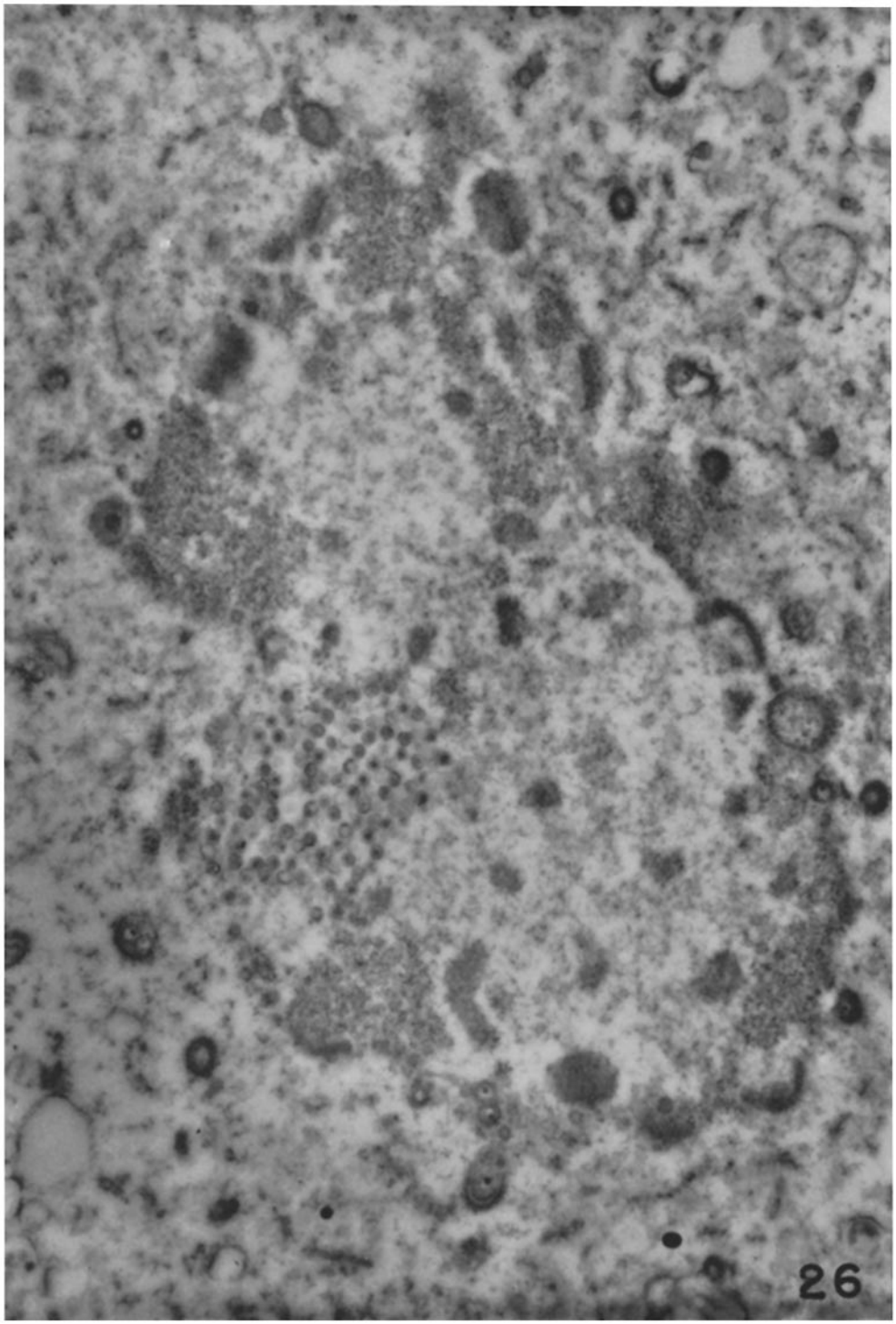
FIG. 25. Intracytoplasmic granules, resembling those previously illustrated in nuclei. One clearly defined viral particle with a single membrane is visible on the left in the mid-third of the field (arrow). There are several bundles of fine filaments and a number of mitochondria, some of which appear abnormal. Part of the ruptured nucleus is visible at the bottom. × 39,000.



(Morgan *et al.*: Herpes simplex virus)

PLATE 59

FIG. 26. A disrupted nucleus. The viral crystal in process of release into the cytoplasm appears to be undergoing dissolution. Some double coated viral particles are free, whereas others lie within vacuoles. The degenerative changes of the cytoplasm are non-specific in appearance. × 25,000.

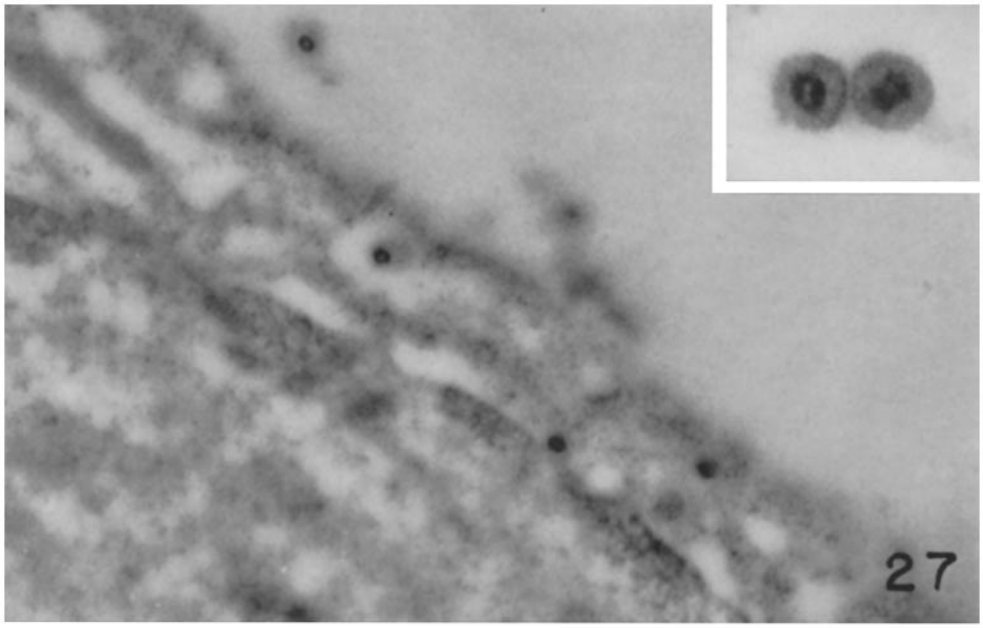


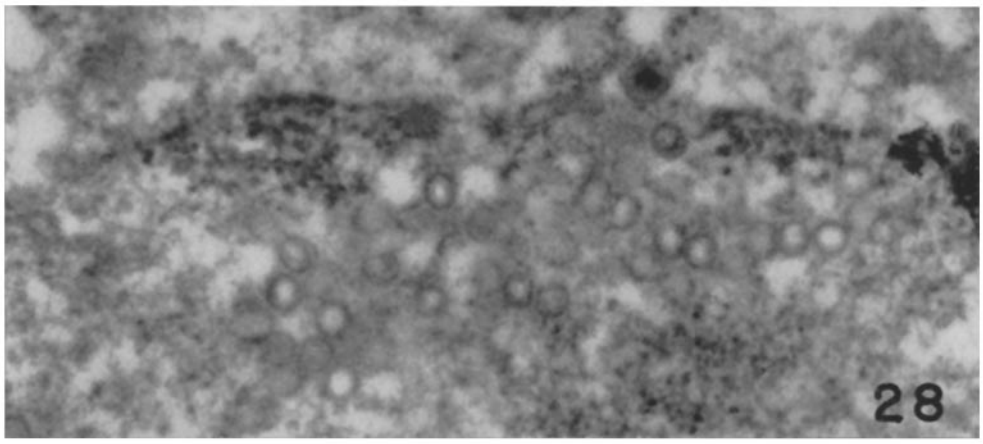
(Morgan *et al.*: Herpes simplex virus)

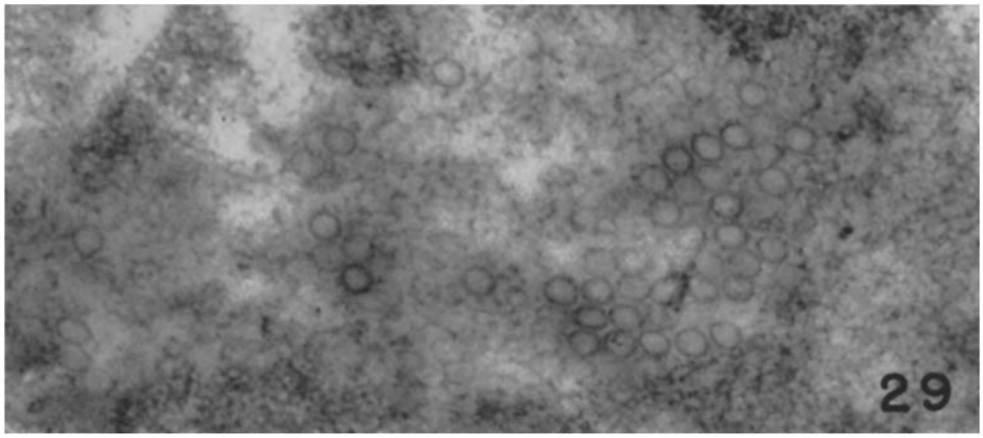
PLATE 60

FIG. 27. Part of the cytoplasm and nucleus of a formalin-fixed cell. Intranuclear virus cannot be identified. The intracytoplasmic and extracellular viral particles exhibit a dense inner core and a diffuse coat. × 36,000.

FIGS. 28 and 29. Infected nuclei after formalin fixation of the tissue and exposure of the sections to fumes of osmium tetroxide. The intranuclear virus has a clearly defined single membrane, but lacks internal structure. Crystalline arrays are well preserved in the lower picture. × 53,000 and × 45,000.



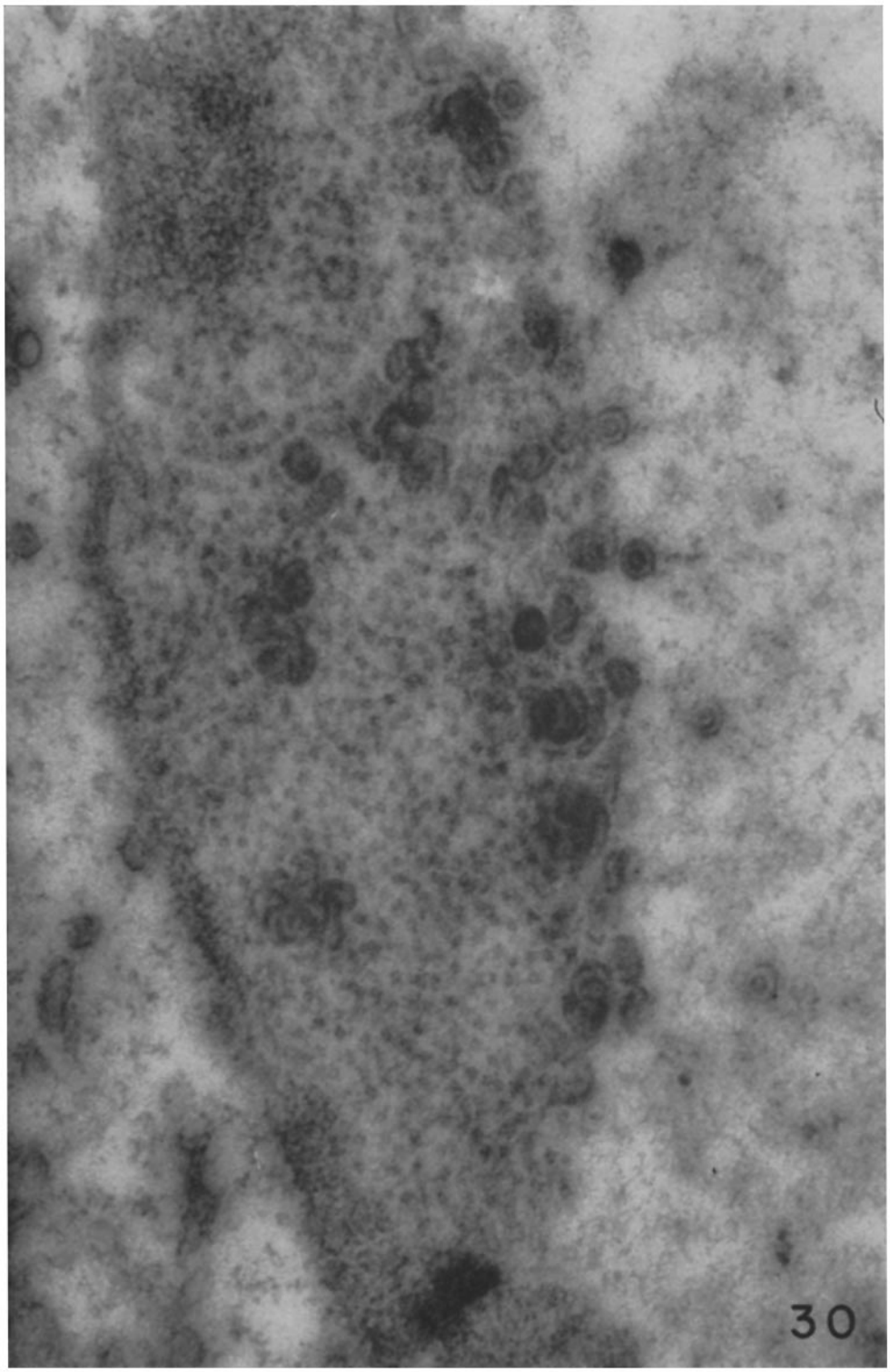




(Morgan *et al.*: Herpes simplex virus)

PLATE 61

FIG. 30. The margin of a nucleus infected with the C. G. strain of herpes simplex virus. Scattered among the granules are aggregates of membranes, which vary in length and definition and are believed to be in process of formation. To the right are dispersed viral particles with a central body and a single membrane. The cytoplasm is on the left. × 60,000.



(Morgan *et al.*: Herpes simplex virus)