STRUCTURE AND DEVELOPMENT OF VIRUSES OBSERVED IN THE ELECTRON MICROSCOPE

IV. Viruses of the RI-APC Group*

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Plates 72 to 85

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A new group of viruses which are responsible for acute infections of the respiratory tract has been discovered in the past few years. These agents were independently recognized, by virtue of their cytopathogenic effects, in cultures of human adenoidal tissue from apparently healthy children (1) and in cultures of HeLa cells inoculated with throat washings from cases of acute respiratory illness (2). Further studies have since shown not only that they share the capacity to induce degenerative changes in HeLa cells and possess group-specific antigens which evoke complement-fixing antibodies, but also that they can be subdivided into at least fourteen types on the basis of cross-neutralization tests (3). Some of these types have not yet been definitively related to clinical syndromes, but others have been shown to be associated etiologically with a variety of disease states, including acute respiratory disease (ARD), primary atypical pneumonia, pharyngoconjunctival fever, infectious exanthemata, mesenteric lymphadenitis, and epidemic keratoconjunctivitis (2, 4–13). In default of an as yet generally accepted nomenclature, they will be referred to in this paper as viruses of the RI-APC[1] group.

Antecedent electronmicroscopic studies of two members of this group in thin sections of infected HeLa cells have revealed intranuclear particles, approximately 40 to 50 mμ in diameter, sometimes arranged in "crystalline-like patterns" (14) or "in rows as if in crystal formation" (15). However, the examination of purified suspensions of the virus after these were air-dried and shadowed with uranium has yielded somewhat different results in that

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[1] RI-APC, respiratory illness–adenoidal pharyngeal conjunctival.

the average diameter of free particles was found to be 90 mμ (80 mμ center to center spacing in close packed aggregates) (16). These earlier findings can now be greatly amplified, and from the observations about to be described it is possible to offer a fairly extensive morphologic description of these viral agents, as seen in infected cells, and to propose an hypothesis concerning their manner of development. Moreover, the probable structure of the viral crystal can be deduced.

Materials and Methods

HeLa cells were cultured at 37°C. for 4 to 5 days in bottles containing a nutrient medium which consisted of a mixture either of 75 per cent tris[2] solution, 15 per cent human serum, and 10 per cent horse serum, or of 85 per cent tris solution, 10 per cent human serum, and 5 per cent horse serum. For transfer to tube cultures the cells were removed with a 0.01 per cent solution of trypsin and were then washed once in balanced salt solution. Each tube was inoculated with approximately 30,000 cells suspended in 0.4 ml. of maintenance solution consisting of a mixture of 85 per cent tris solution and 15 per cent horse serum. The tubes were incubated at 37°C. for 2 to 3 days and then, after microscopic examination to insure that good cell mono-layers were present, received 0.1 ml. of virus inoculum. RI-APC virus types 3, 4, and 7 were used[3]; the figures herein illustrate type 3. The tubes were reincubated and, at intervals from 18 to 96 hours following inoculation, cells which had become detached spontaneously from the glass surface, or which had been freed by 0.01 per cent trypsin, were washed twice in balanced salt solution and then were centrifuged to form small pellets. The pellets were fixed for 20 minutes at room temperature in 1 per cent osmium tetroxide in 0.34 osmolar buffer at pH 7.2, according to the method of Rhodin (18). The intact pellet was dehydrated in graded solutions of ethyl alcohol and embedded in partially polymerized butyl methacrylate. Polymerization was completed at 80°C. Control preparations of uninfected cells were prepared in an identical manner. Sections were cut on a thermal expansion Porter-Blum type microtome (19) equipped with glass knives (20). The sections were floated onto the surface of an acetone-water mixture, picked up on formvar-coated copper grids and examined in an RCA type EMU 2E electron microscope.

RESULTS

Fig. 1 shows part of a HeLa cell. Most of the field is occupied by the nucleus, near the upper margin of which is a characteristic nucleolus composed of dense granular material. Elsewhere in the nucleus are scattered irregular condensations of apparently similar material. No particles considered to be virus are visible in the cell. The cytoplasms of two adjacent cells occupy the upper and lower left corners of the micrograph. Fig. 2 illustrates intranuclear condensations resembling those shown above. Adjacent to them are clusters of spherical particles which are believed to be the virus. In areas of greatest viral concentration the nuclear matrix has been replaced or displaced. Al-

[2] The tris solution contained 0.5 per cent lactalbumin hydrolysate, 0.25 per cent dextrose, and 0.1 per cent yeast extract, with 0.3 per cent tris(hydroxymethyl)aminomethane as the buffer (17), in Earle's balanced salt solution. It was adjusted to pH 7.7 with hydrochloric acid.

[3] The authors wish to thank Dr. Maurice R. Hilleman, Army Medical Service Graduate School, Washington, D. C., for supplying the strains of virus.

though most of the particles exhibit random orientation, some have formed parallel rows. The nuclear membrane appears to be intact and no virus is visible in the cytoplasm.

Fig. 3 shows several intranuclear condensations with adjacent viral particles at higher magnification. Within the largest condensation can be seen small dense particles, many of which are poorly defined. They are believed to be virus at an early stage of development. Near the top of the field, rudimentary order of some viral particles is suggested by linear arrays. As indicated by the arrows there are three laminated structures. In other nuclei an ordered orientation of viral particles was encountered. For example, close to the right margin of Fig. 4, 29 particles are disposed in a crystalline array. At the left and lower borders of the micrograph the spatial relationships of the particles are irregular. Within the crystal the granular and fibrillar components of the nuclear matrix are not visible. Fig. 5 shows at lower magnification the ordered array of more numerous viral particles in another nucleus. Here also the nuclear matrix has been displaced or replaced by the crystals. Fig. 6 illustrates part of a nucleus containing larger crystals. Several are in apposition, whereas others are discrete. The apparent differences in arrangement of the viral particles are believed to reflect the orientation of the crystals with respect to the plane of section. This will be discussed below. The nuclear membrane traversing the upper portion of the field has been transected obliquely. Fig. 7 shows at higher magnification two contiguous crystals with flat, angulated faces. At the right border of the micrograph is a membranous structure similar to those illustrated in Fig. 3. Fig. 8 shows a discrete intranuclear crystal composed of viral particles, some of which even at this magnification can be seen to contain a central body of low density. The nuclear membrane in the upper left corner shows discontinuities which may represent pores or may reflect early stages in nuclear disintegration. Some nuclei contained relatively large crystals, as shown in Fig. 9, wherein a single crystal occupies most of the nucleus at the level of this section. Although the majority of the viral particles exhibit characteristic orientation, those at the margins of the crystal are randomly disposed. No virus is visible in the cytoplasm.

Figs. 10 and 11 show parts of viral crystals at higher magnification. In Fig. 10 the contiguous faces of two crystals (indicated by an arrow) traverse the right third of the field in a nearly vertical direction. The three crystals illustrated in this plate contain parallel zones in which particles of relatively high density and sharp definition alternate with those of low density and poor definition. The particles are ellipsoidal. Because the long axis of the ellipsoids was invariably parallel to the knife edge, it appeared likely that the virus is actually spherical, its shape in the sections reflecting distortion caused by impact of the knife similar to that encountered for other viruses (21–23). The diameter, calculated from the average of the major and minor axes,

approximates 60 mμ. Many of the particles have a central body which is enclosed by a sharply defined membrane and averages 24 mμ in diameter. In Fig. 12, four faces of a crystal traverse the corners of the field. Some viral particles appear to have been displaced with resulting discontinuity of the ordered array. The particles of greatest density exhibit no internal structure, whereas those of lesser density frequently contain an inner body.

Swollen nuclei were repeatedly observed to exhibit a central region containing crystals and a peripheral zone in which the viral particles were dispersed at random. Fig. 13 shows part of one such nucleus. In the central region viral aggregates, several of which show crystalline structure, lie adjacent to dense material. The peripheral zone is composed of fine granules and contains scattered viral particles. It is less dense than the matrix of normal nuclei and resembles the material encountered at certain stages in the development of herpes simplex virus (21). The nuclear membrane is intact and no virus can be identified in the cytoplasm. Crystals encountered in the peripheral zone frequently exhibited irregular margins and were surrounded by scattered viral particles, as shown in Fig. 14. In many areas of this figure, where the matrix of low density extends into the central zone, the crystalline array is discontinuous and viral particles are dispersed at random. Although at several sites particles lie close to defects in the nuclear membrane, no recognizable virus is visible in the cytoplasm. Fig. 15 illustrates part of a nucleus containing small crystals embedded in moderately dense material. In the less dense peripheral zone, viral particles are scattered at random and near the upper and lower borders of the field they lie adjacent to defects in the nuclear membrane. In Fig. 16 the virus is dispersed throughout the nucleus. Near the right border the nuclear membrane has disrupted and viral particles are visible in the adjacent cytoplasm. Vacuolization of the cytoplasm is evident, one vacuole (at the lower left corner) lying between the double membrane enclosing the nucleus.

Release of virus from the nucleus appeared to occur at any stage of development. Fig. 17 is believed to represent a stage comparable to that illustrated in Figs. 2 and 3. The particles, many of which are adjacent to condensations of material, are not aligned in crystalline array. The nuclear membrane has ruptured and virus is present in the cytoplasm. At the left margin are characteristic, non-specific, cytoplasmic extensions which were encountered in normal as well as infected cells. Fig. 18 illustrates a stage probably similar to that shown in Figs. 4 or 5. There are small viral clusters, many of which show crystalline arrangement. Only remnants of the nuclear membrane are visible. Aggregates of virus extend into the cytoplasm and in several regions particles are close to the cell surface. Cells with no recognizable nuclei and exhibiting extensive necrosis frequently contained numerous scattered viral particles as well as occasional crystals randomly distributed in the cytoplasm.

Intranuclear laminated structures, usually tubular in form, were repeatedly observed in cells infected with type 3 virus. Fig. 19 shows part of a nucleus containing these structures. They vary in orientation with respect to the plane of section, some having been transected obliquely and others perpendicularly. The walls of the tubules are composed of single or multiple membranes arranged in spiral form or in concentric layers. Two tubules contain viral particles. Elsewhere the virus is scattered in relatively clear zones in the nuclear matrix, the reticular and granular components of which were rarely observed within the lumen of the tubules.

Recently it has been possible to examine the same viral crystal, as well as the same non-crystalline viral aggregate, in both the electron and light microscope.[4] This was accomplished by cutting serial sections in which thin sections for electron microscopy alternated with thicker sections for light microscopy. The thicker sections were used for histochemical study. The crystals, as well as the non-crystalline aggregates, were strongly Feulgen-positive, indicating that the virus contains deoxyribosenucleic acid.

DISCUSSION

Sections of tissue infected with RI-APC viruses revealed that the viral particles centrally located within the plane of section appeared more dense and more sharply defined than those located at one or the other surface, as has also been noted with influenza virus (24). This is reflected in the crystals by parallel zones, wherein particles of relatively high density and sharp definition alternate with particles of low density and poor definition. These zones are believed to result from orientation of the lattice to the plane of section, a conclusion supported by the observation that in serial sections the periodicity, width, and orientation of the zones remained relatively constant within each crystal.

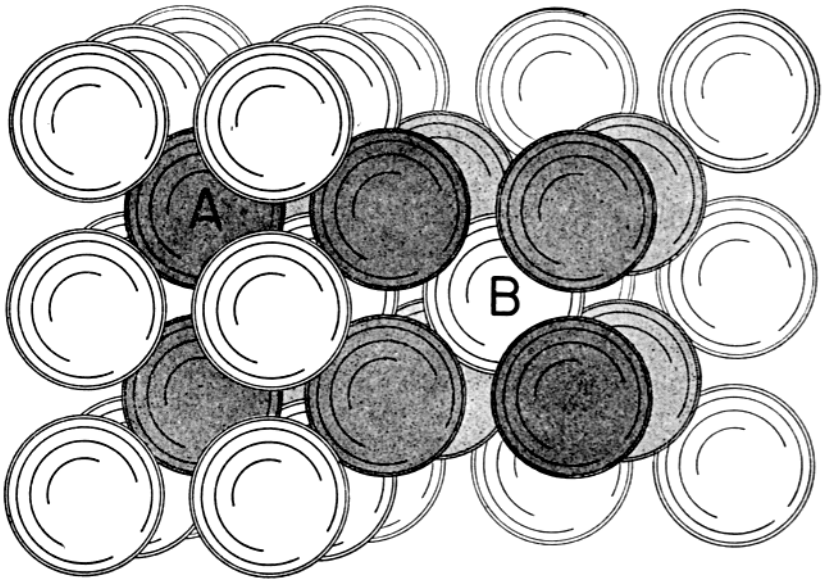
Study of the electron micrographs suggests that the viral particles are packed in a cubic body-centered lattice.[5] A three dimensional lattice is an array of points such that each point has the same environment as every other point. In the cubic body-centered lattice each point has eight nearest neighbors. Thus, in Text-fig. 1 the gray sphere at A is surrounded by eight equidistant white spheres situated at the corners of a unit cube and the white sphere at B is similarly surrounded by eight equidistant gray spheres. Thus the choice of a lattice origin is purely arbitrary and there is no real distinction between a sphere at the center and one at the corner of a cube. Different layers of

[4] This work was done in collaboration with Dr. D. P. Bloch and Dr. G. C. Godman, Department of Surgical Pathology, College of Physicians and Surgeons, Columbia University, and will be reported in detail elsewhere.

[5] A detailed account of this study by Dr. B. W. Low and Dr. P. R. Pinnock, Department of Biological Chemistry, Harvard Medical School, will appear subsequently in this journal.

spheres in the text-figure were given different tones in order to show more clearly the packing arrangement around a single sphere. This device does not imply any difference whatsoever between layers. The electron micrographs show sections parallel to many different crystalline planes through this lattice.

As was previously noted, viral particles central to the plane of section appeared more dense and more sharply defined than those located eccentrically. Since the average thickness of the sections was less than the diameter of the virus, the internal structure should also be more clearly visualized in centrally



TEXT-FIG. 1. A drawing which shows the crystalline arrangement of the viral particles. The lattice is the cubic body-centered packing of identical spheres. The two tones of gray were used to give perspective.

placed particles. However, very dense particles without evidence of internal structure were repeatedly encountered, and particles in which an internal body could be identified were generally less dense, as illustrated in Figs. 8, 10, 11, and 12. Moreover, as was shown in Figs. 6 and 7, the dense particles occurred at random within the crystalline lattice. These observations indicate that the viral particles differ in density as well as in structure. The fact that Hilleman *et al.* (16) found low infectivity titers in viral preparations with high particle counts suggests the possibility that the observed morphologic differences may be related to function. For example, if the possession of an internal body were essential for infectivity, only a proportion of the particles encountered in sections could be considered to be infectious units.

Measurement of viral size was complicated by the relatively indistinct margins of the particles. On the assumption that certain sections were oriented with respect to the crystalline array in such a manner as to reveal close packing (Figs. 10 and 12), the center to center spacing should reflect the actual diameter of the virus. This spacing approximated 65 mμ. The somewhat larger size of the virus dried down from suspension (16) probably reflects the flattening which results from forces exerted by surface tension. There is little doubt that these same forces may also disrupt particles and produce "doughnuts" (16) resembling the ring or empty shell forms of herpes simplex virus (21).

The following hypothesis concerning the development and release of the virus is proposed. Dense granular and reticular material condenses at multiple foci within the nucleus. That these condensations do not necessarily represent fragmented nucleoli is suggested by the occasional presence of apparently intact nucleoli (Fig. 1) in cells believed to be in an early stage of infection. Within or contiguous to this material the viral particles differentiate (Figs. 2 and 3). When the concentration of virus becomes sufficiently high at any site, with replacement of the nuclear matrix, crystallization occurs (Figs. 4 and 5). Depending on unknown factors of host cell response, the crystals may continue to increase in size (Figs. 6 to 9) or they may disintegrate (Figs. 13 and 14). Disintegration usually occurs in a zone at the periphery of the nucleus while at the center differentiation of virus continues. With the production of large amounts of virus the nucleus enlarges. If the nuclear membrane remains intact the dense material is progressively utilized by the developing virus (Figs. 13 to 15) until the nucleus resembles a sack filled with viral particles (Fig. 16). On the other hand, the nuclear membrane may disrupt at any stage of viral development releasing particles into the cytoplasm (Figs. 17, 18, 16).

The possibility arises that crystals assumed to be undergoing disintegration may in reality be forming by accretion of the dispersed viral particles. Several observations, however, suggest that this is not the case. Cells containing intact crystals with smooth faces (Figs. 6 to 8) generally showed little evidence of disintegration, whereas the majority of cells containing dispersed viral particles had damaged cytoplasm and disrupted nuclear membranes. Moreover, crystals encountered in the cytoplasm invariably showed irregular faces and discontinuity of particulate array. If crystallization occurred in the cytoplasm, one should expect to see crystals with smooth faces and intact arrays at some stage of development. Unlike herpes simplex virus (21), the intracytoplasmic particles of RI-APC virus were indistinguishable from those observed in the nucleus, suggesting that further differentiation of particles in the cytoplasm does not occur after their release from the nucleus. Degeneration of the cytoplasm, characterized by vacuolization, fragmentation of endoplasmic reticulum, disruption of mitochondria, disintegration of mito-

chondrial cristae, and aggregation of particulate components, was repeatedly encountered in necrotic cells of control cultures and therefore cannot be considered as a specific response to viral infection.

The intranuclear tubules composed of laminated membranes (Figs. 3, 7, 19) were observed only in cells infected with type 3 virus and were not consistently encountered. They appeared at various stages in the evolution of virus and exhibited no spatial relationships to the clusters and crystals, suggesting that they are not directly related to viral development. Their function is unknown. The three types of RI-APC virus studied to date were similar with respect to their morphology, crystalline structure, and apparent mode of development.

SUMMARY

Representative viruses of the RI-APC group were observed with the electron microscope in thin sections of infected HeLa cells. The viral particles varied in density, were approximately 60 mμ in diameter and had a center to center spacing when close packed of about 65 mμ. Many of the less dense particles exhibited an internal body averaging 24 mμ in diameter. It was suggested that within the nucleus the virus differentiated from dense granular and reticular material and formed crystals. Disintegration of the crystals and disruption of the nuclear membrane with release of virus into the cytoplasm appeared to occur at any stage. No evidence to suggest development of the virus in the cytoplasm was obtained.

It was possible to deduce the structure of the viral crystal from the electron micrographs. The viral particles are packed in a cubic body–centered lattice.

Correlative histochemical observations in the light microscope which are now in progress revealed that the crystals and non-crystalline aggregates of virus were strongly Feulgen-positive.

The authors gratefully acknowledge the technical assistance of Miss Dorothy Gelber, Miss Ermalee Grant, and Miss Regina Weiss.

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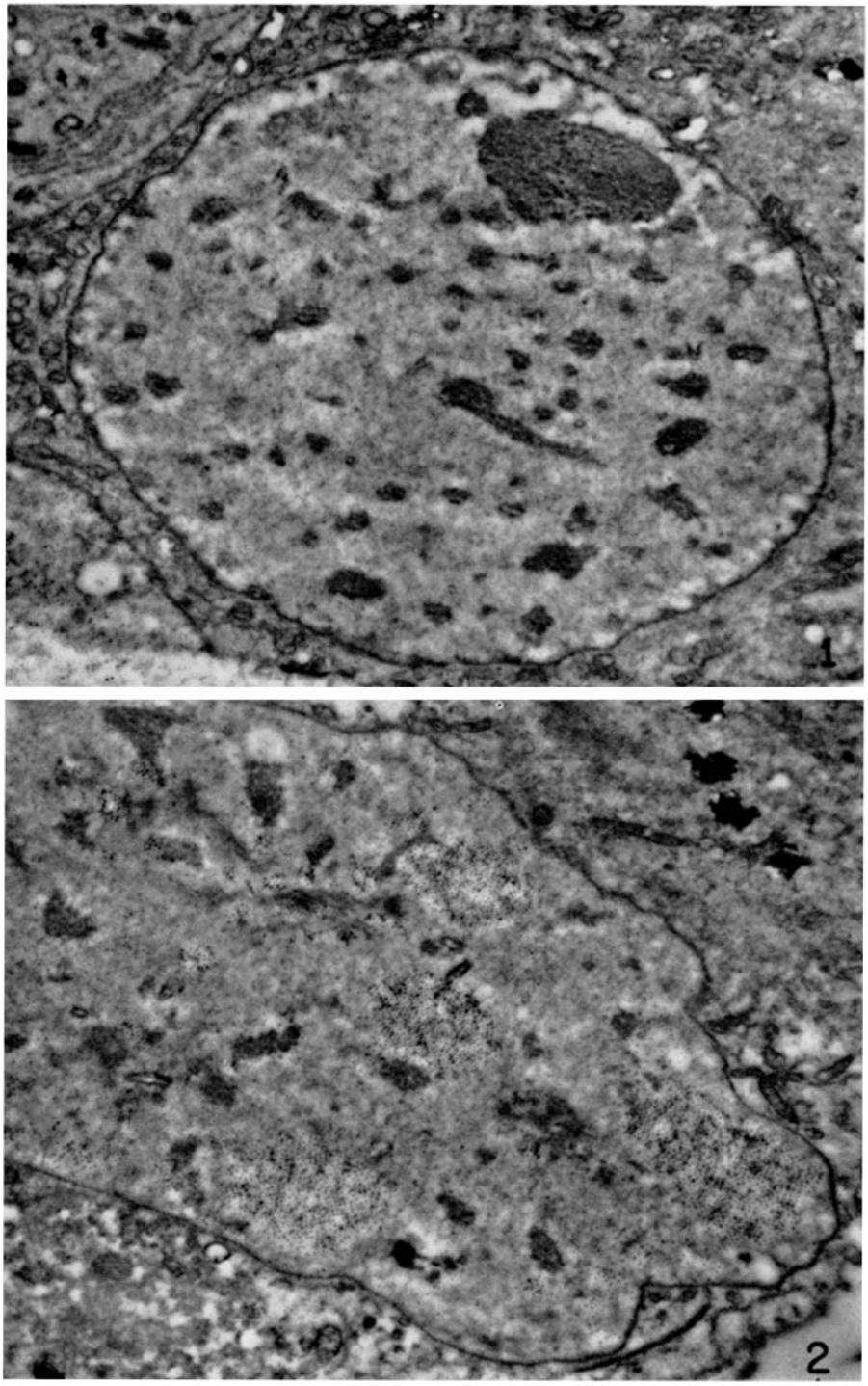
EXPLANATION OF PLATES

PLATE 72

FIG. 1. A nucleus with condensations of dense granular and reticular material. No virus is visible in this cell. The cytoplasm of two adjacent cells occupies the upper and lower left corners of the field. × 9,600.

FIG. 2. Clusters of virus adjacent to condensations of dense material. Some particles are arranged in rows. × 9,600.



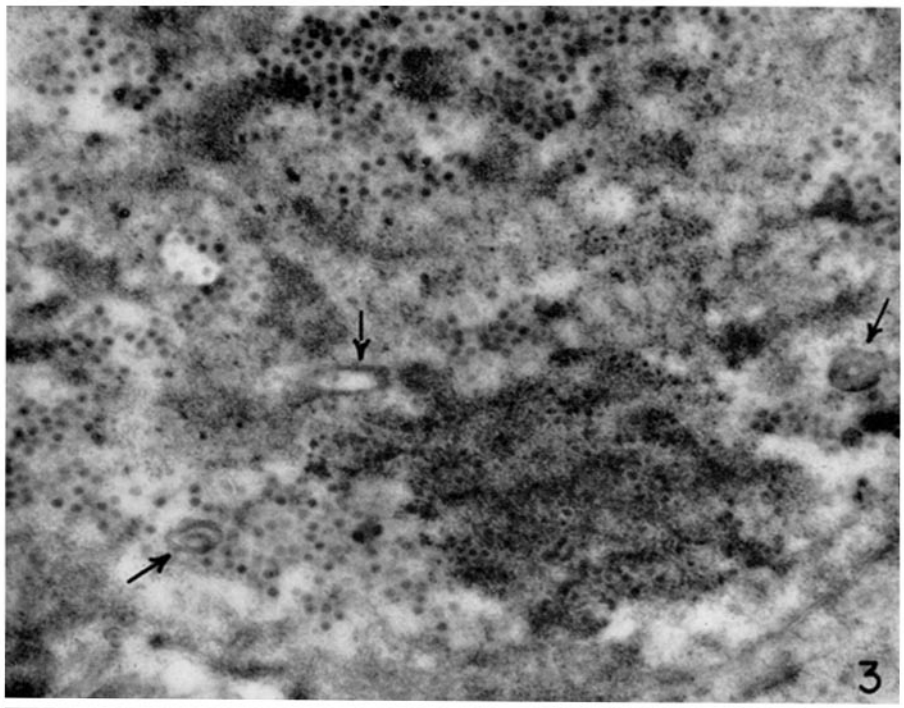
(Morgan *et al.*: Structure and development of viruses. IV)

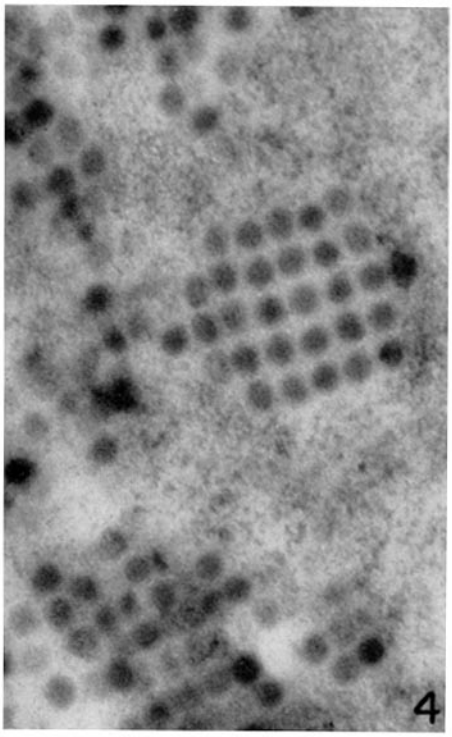
PLATE 73

FIG. 3. Intranuclear condensations with adjacent viral particles viewed at higher magnification. Within the dense material in the lower half of the field are small particles, many of which are poorly defined. At sites of greatest viral concentration the nuclear matrix has been replaced. Near the upper border rows of particles suggest rudimentary order. ×28,000.

FIG. 4. Viral particles, some of which are in crystalline array. ×74,000.

FIG. 5. Numerous viral particles exhibiting crystalline arrangement. These small contiguous crystals, enclosed by dense nuclear matrix and possessing irregular margins, are believed to be in process of formation. × 23,000.





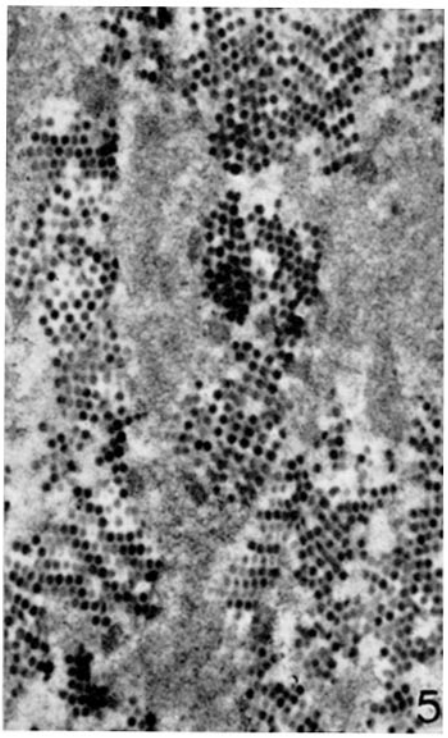


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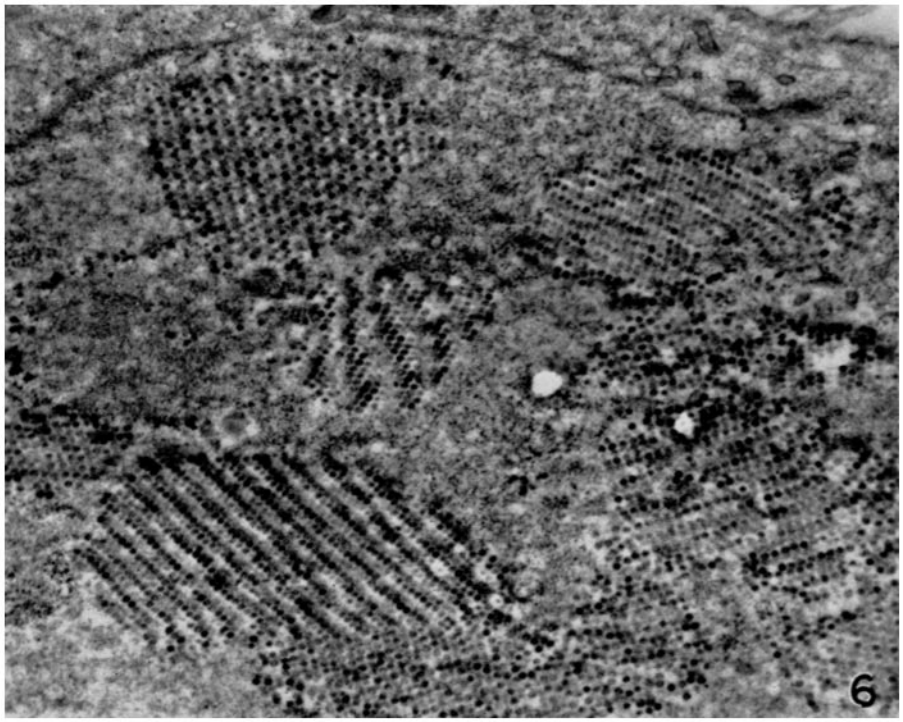
PLATE 74

FIG. 6. Larger crystals, several of which are in apposition. The spacing and definition of the viral particles reflects the orientation of the crystalline array with respect to the section. Very dense particles, however, are scattered within the crystalline lattice. Three localized defects in the section are evident. × 16,800.

FIG. 7. Two contiguous crystals with flat, angulated faces. In the lower third of the field the irregular zone containing indistinct particles probably reflects damage to the crystal during preparation of the specimen. × 30,700.



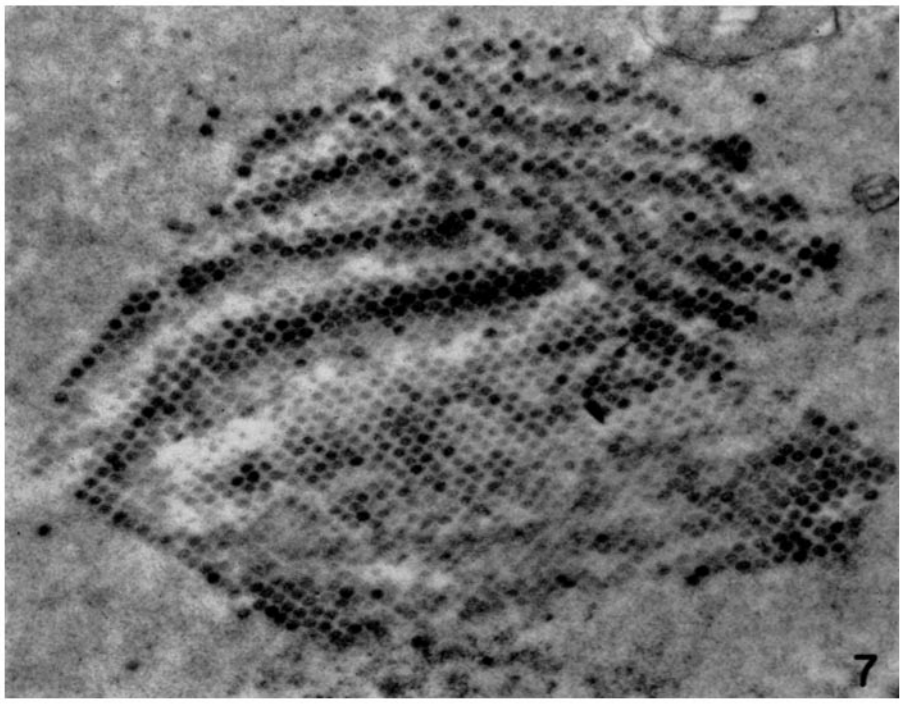


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PLATE 75

FIG. 8. A discrete crystal. Many of the particles exhibit a central body of low density (examples indicated by arrows). The nuclear membrane traversing the upper left corner is discontinuous. × 36,200.

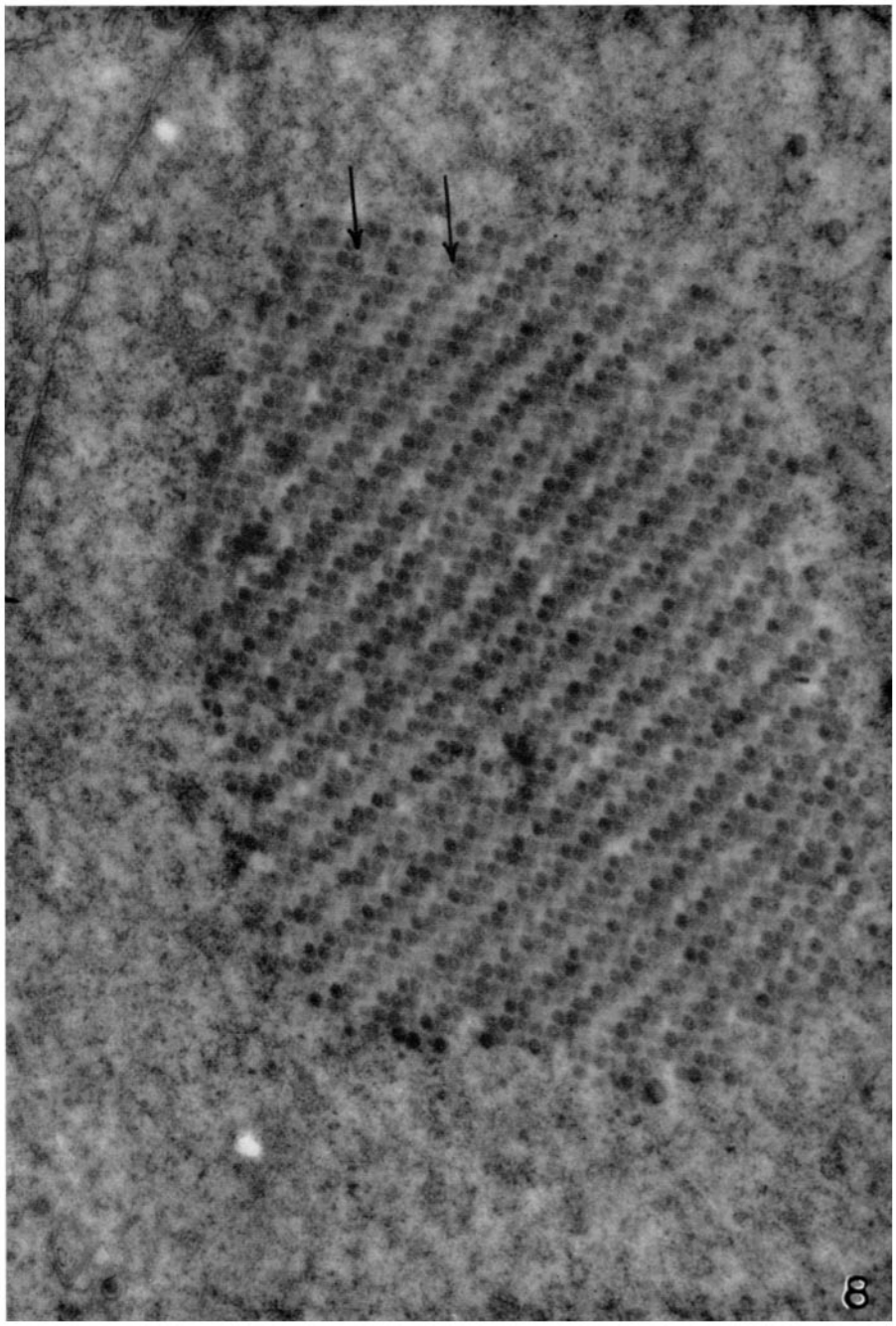

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PLATE 76

FIG. 9. A large crystal with particles randomly distributed at the periphery. The localized defects in the particulate array near the center were frequently encountered in such crystals and are believed to be artifacts. No virus can be identified in the cytoplasm. × 16,000.

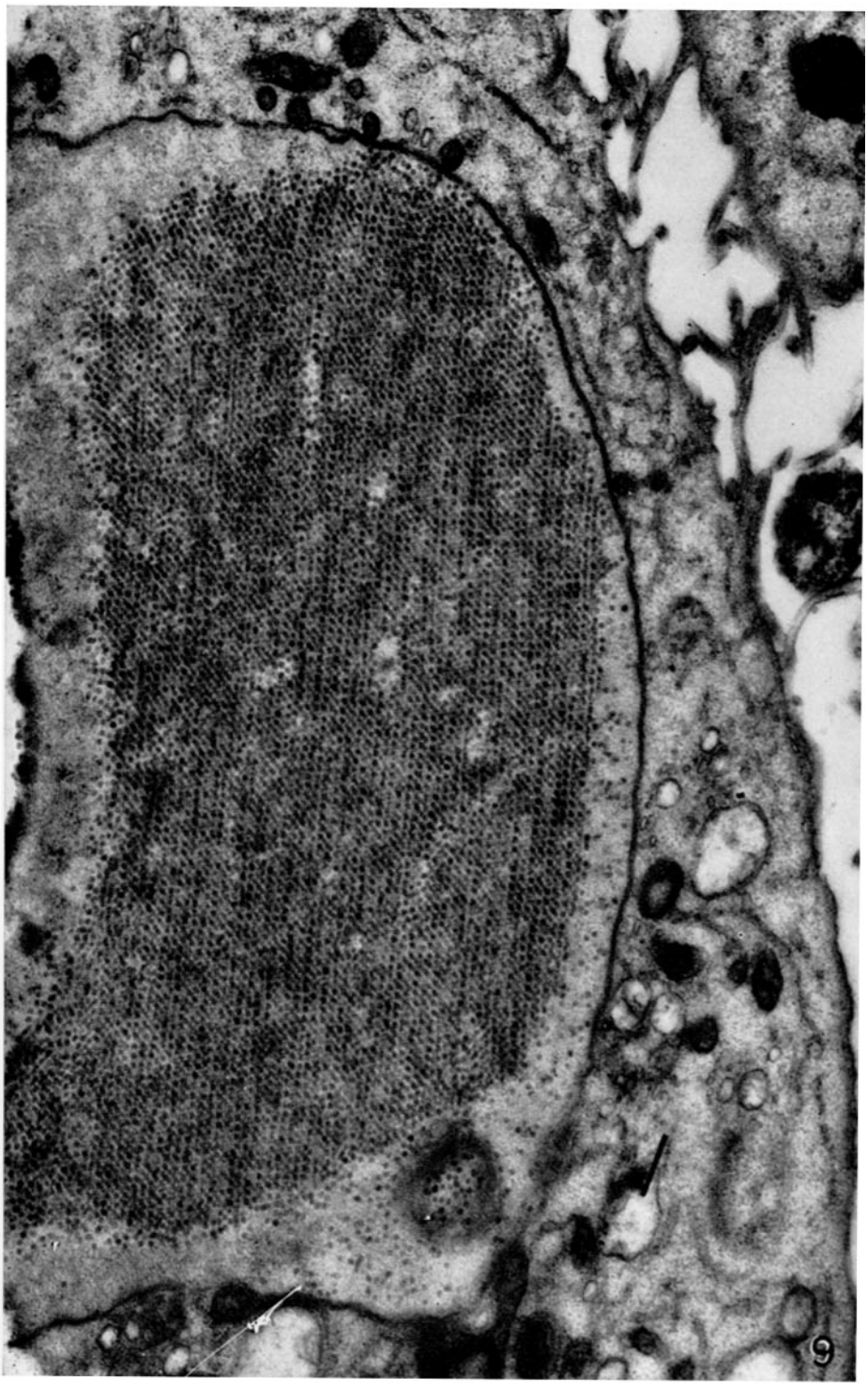

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PLATE 77

FIG. 10. Part of two crystals. The contiguous margins traverse the right half of the field in a nearly vertical direction. In this and the following figure many of the particles exhibit an internal body. × 76,000.

FIG. 11. Part of a crystal cut at such an angle that parallel zones of dense, well defined particles alternate with zones of less dense, poorly defined particles. In this and the preceding figure the knife passed vertically, producing characteristic distortion of the viral particles. × 97,000.

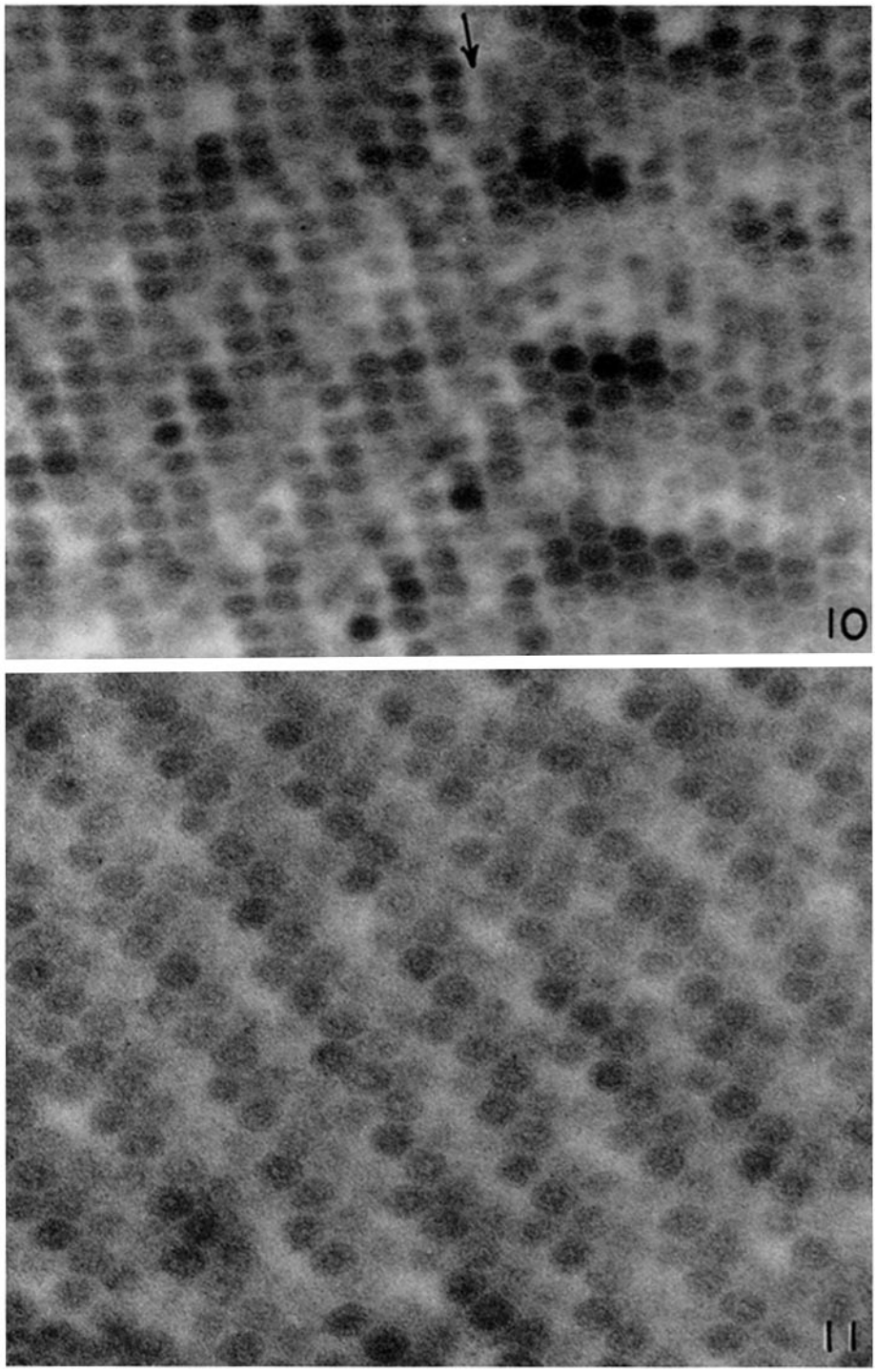

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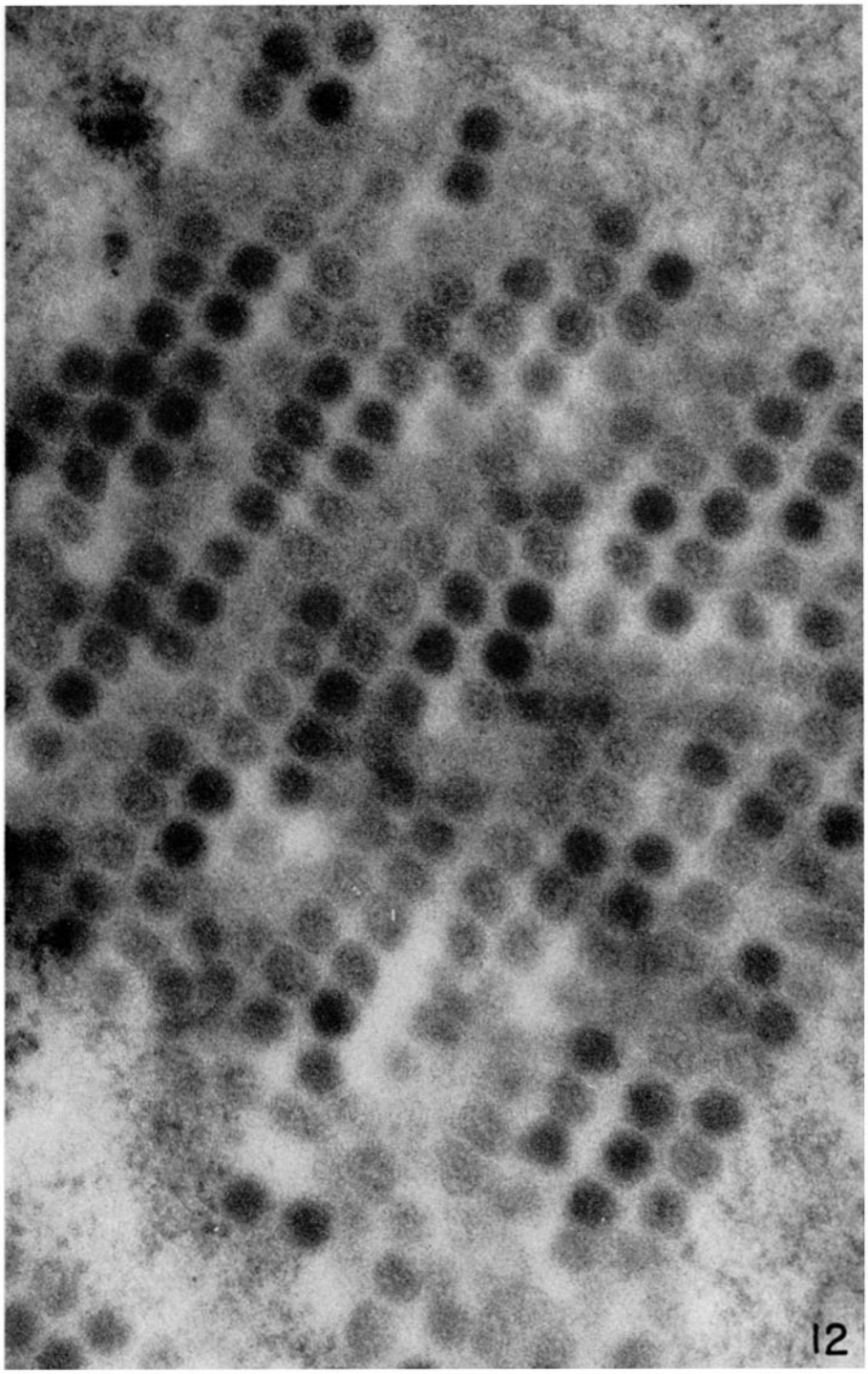
PLATE 78

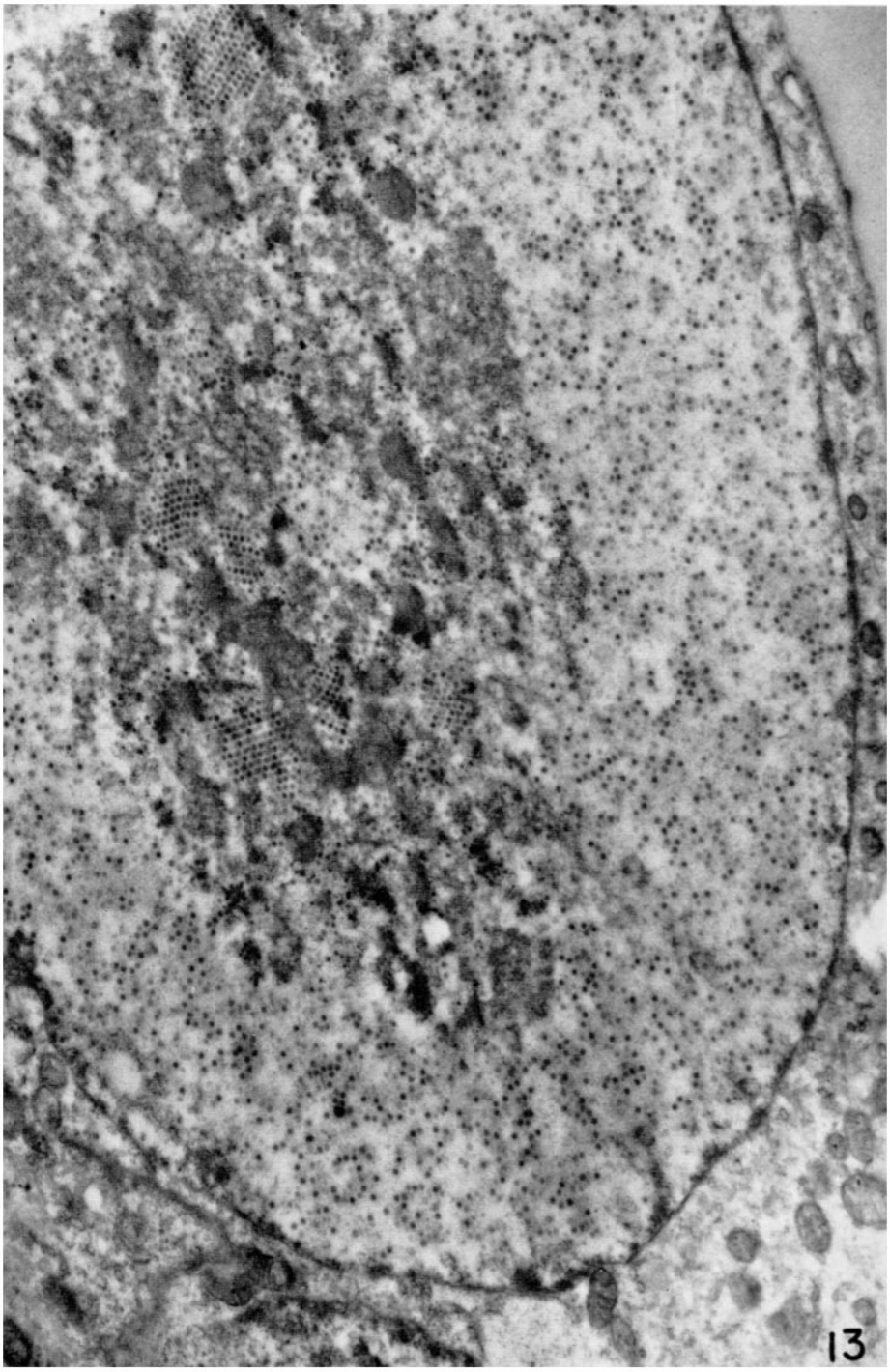
FIG. 12. A crystal viewed at sufficient magnification to distinguish clearly two types of particles—dense particles with no apparent internal structure, and less dense particles with a central body. The array in this crystal has been distorted. × 123,000.



(Morgan *et al.*: Structure and development of viruses. IV)

PLATE 79

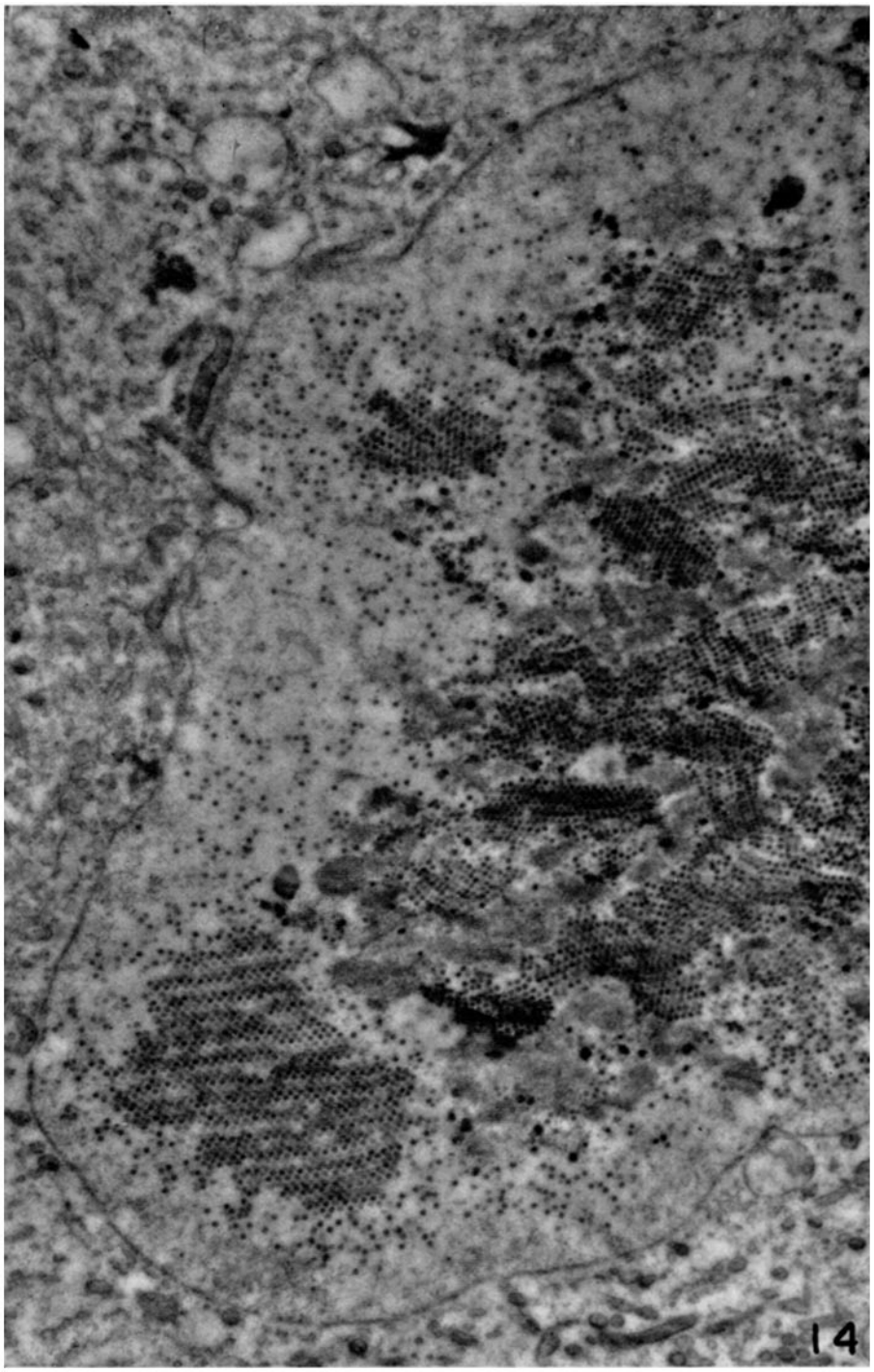
FIG. 13. A nucleus containing a central zone of dense matrix with clusters and crystals of virus and a peripheral zone of less dense material with dispersed viral particles. × 15,500.



(Morgan *et al.*: Structure and development of viruses. IV)

PLATE 80

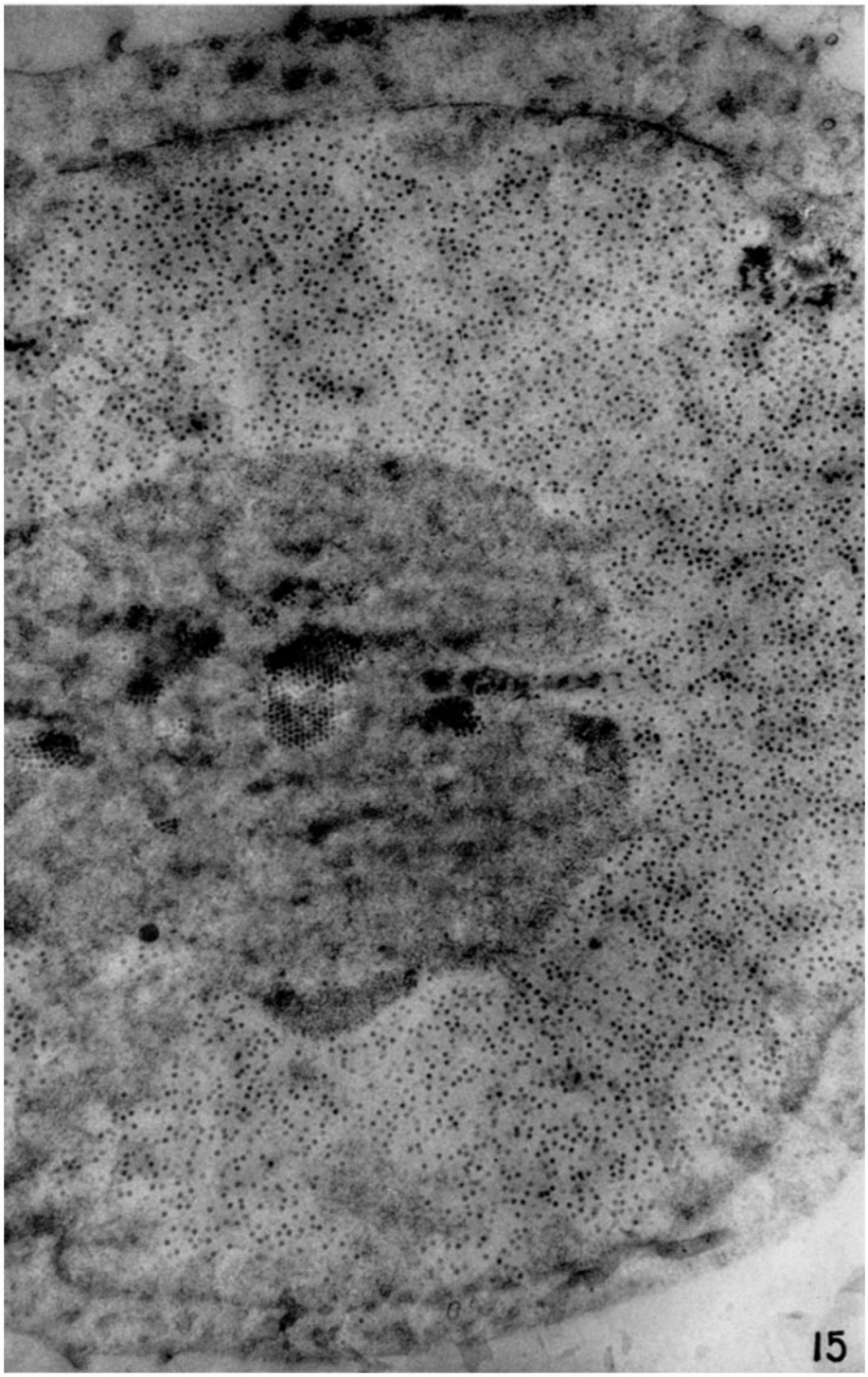
FIG. 14. Two discrete crystals with irregular faces which are believed to be in process of disintegration. At numerous sites the peripheral zone of low density extends into the central zone. × 18,700.



(Morgan *et al.*: Structure and development of viruses. IV)

PLATE 81

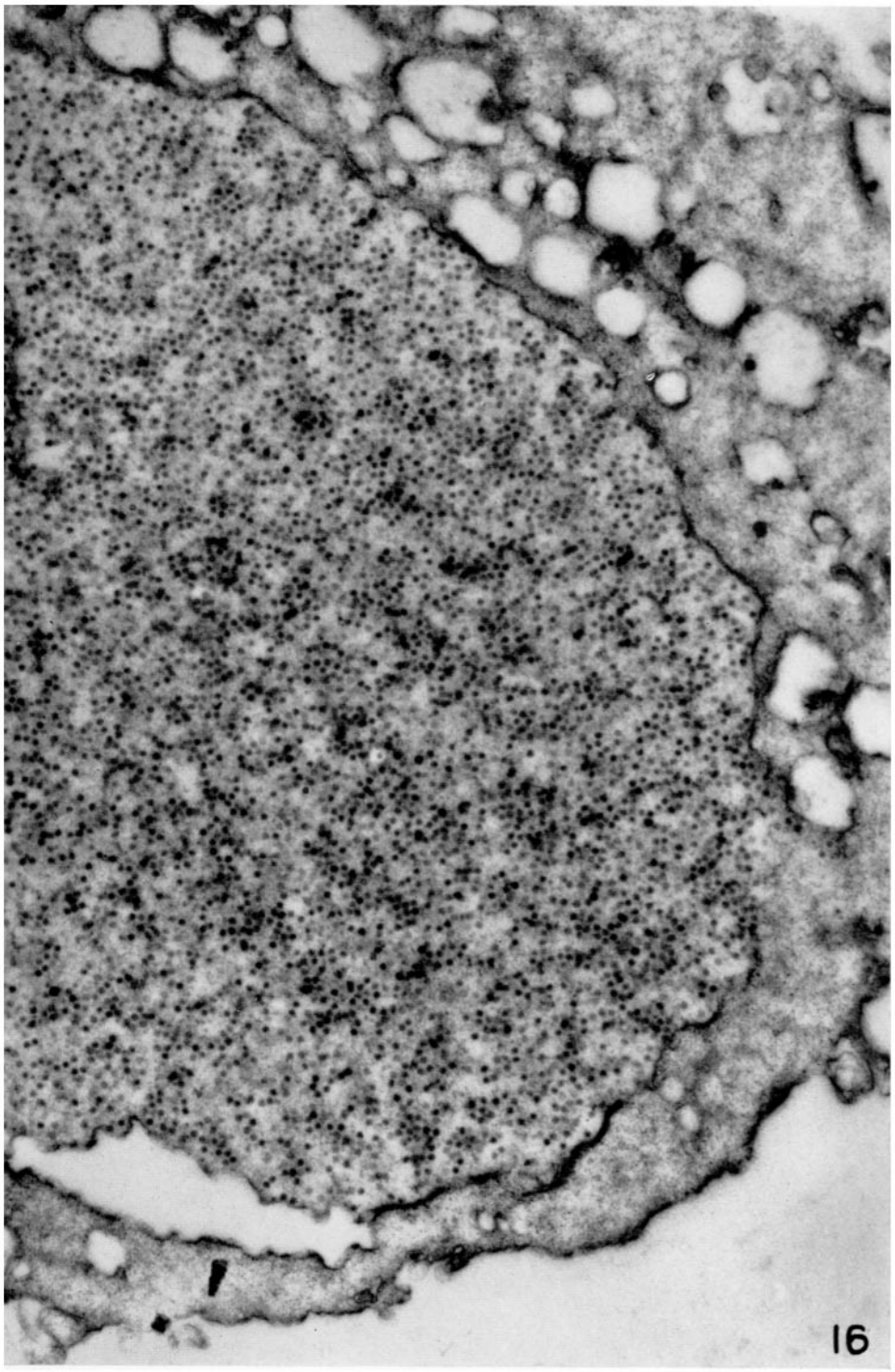
FIG. 15. An enlarged nucleus containing a wide peripheral zone of scattered particles and a small central zone of dense matrix enclosing several crystals. At several sites the particles are adjacent to defects in the nuclear membrane. × 13,500.



(Morgan *et al.*: Structure and development of viruses. IV)

PLATE 82

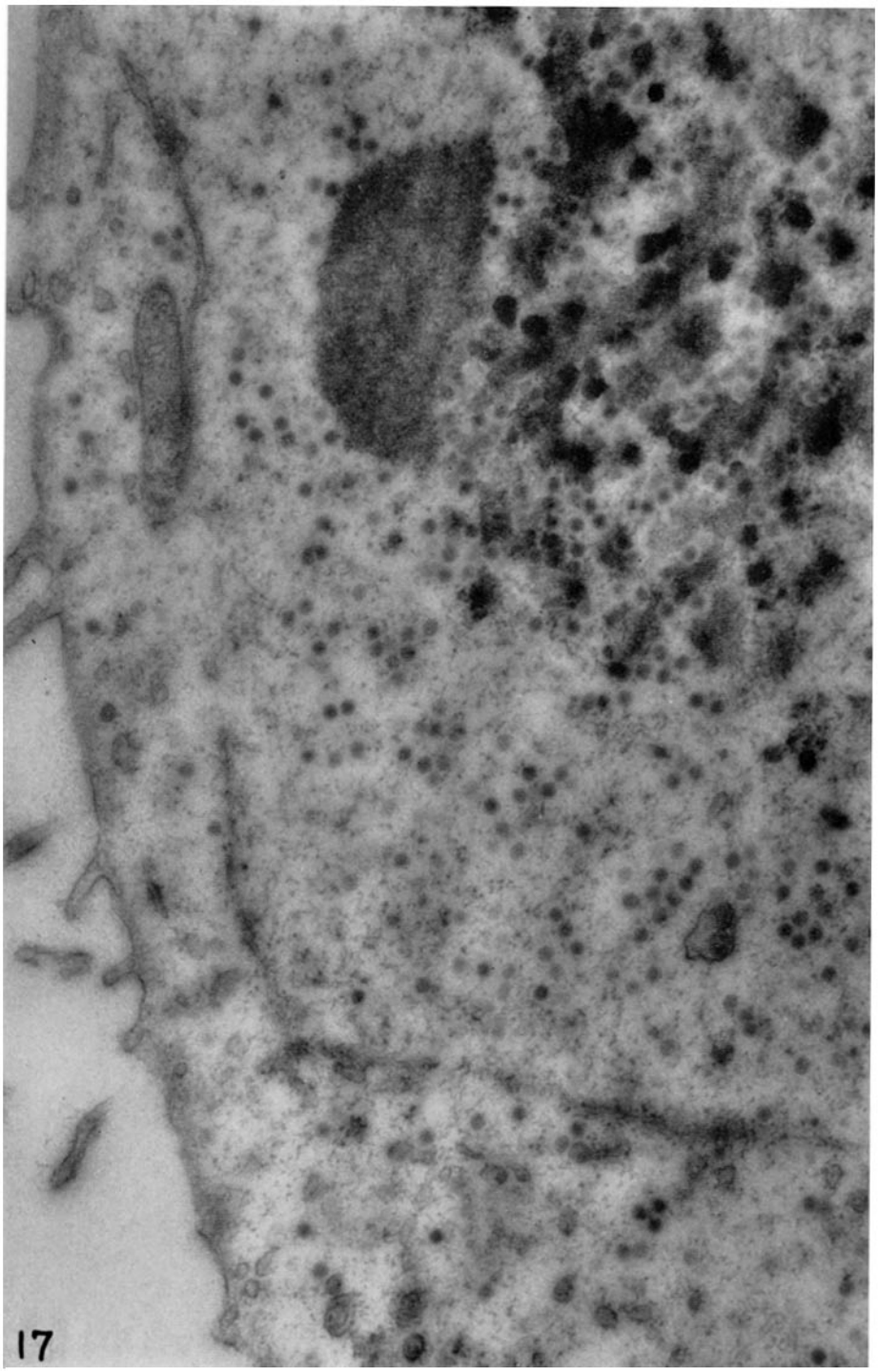
FIG. 16. A nucleus filled with dispersed virus. On the right the nuclear membrane has ruptured and particles are present in adjacent cytoplasm. Mitochondria have disintegrated and vacuolization is prominent. × 18,000.



(Morgan *et al.*: Structure and development of viruses. IV)

PLATE 83

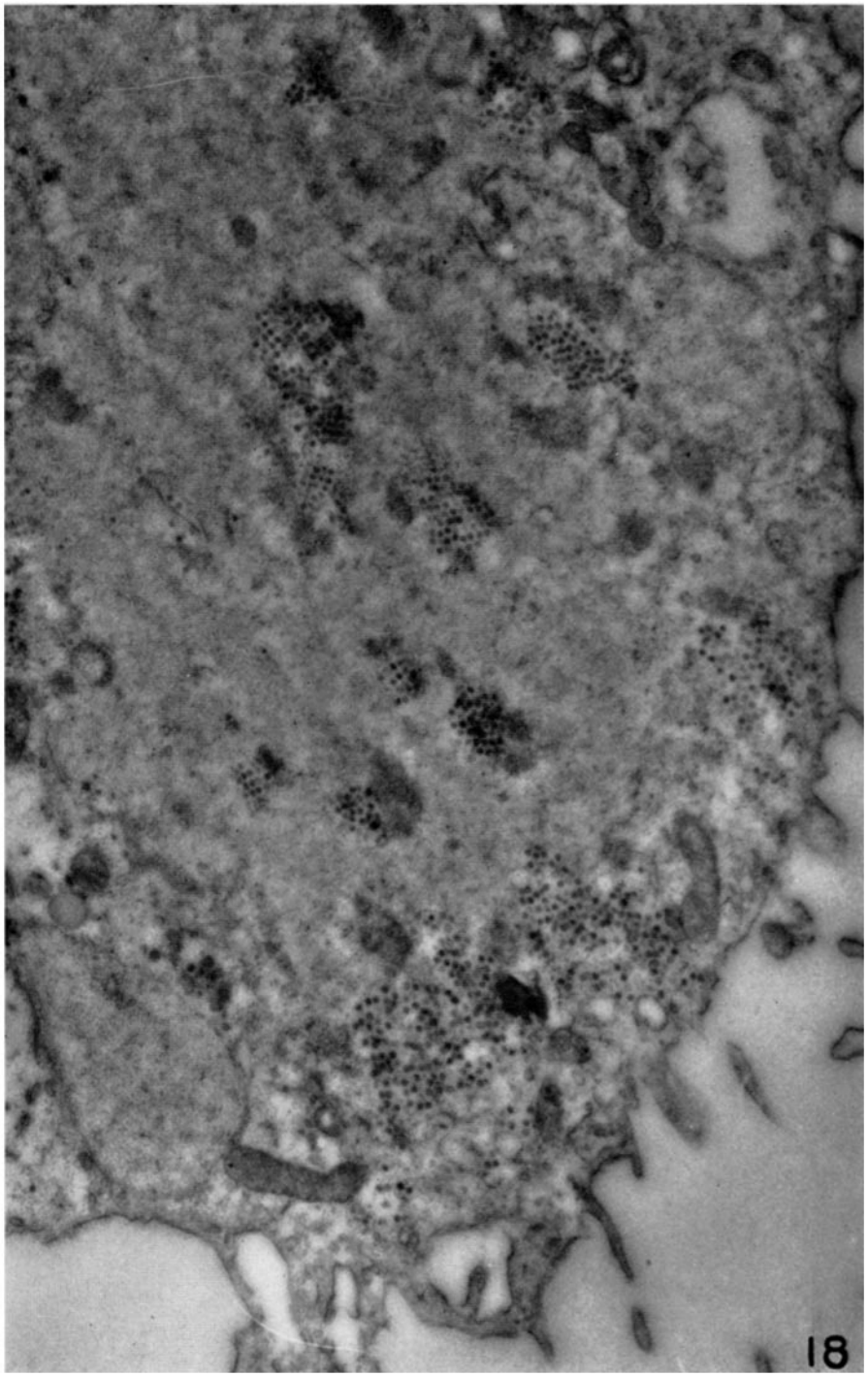
FIG. 17. Viral particles adjacent to dense condensations of nuclear matrix resembling those illustrated in Fig. 3. The nuclear membrane has disrupted and virus is scattered in the cytoplasm. The fragmented cristae in the mitochondrion probably reflect beginning disintegration. × 39,000.



(Morgan *et al.*: Structure and development of viruses. IV)

PLATE 84

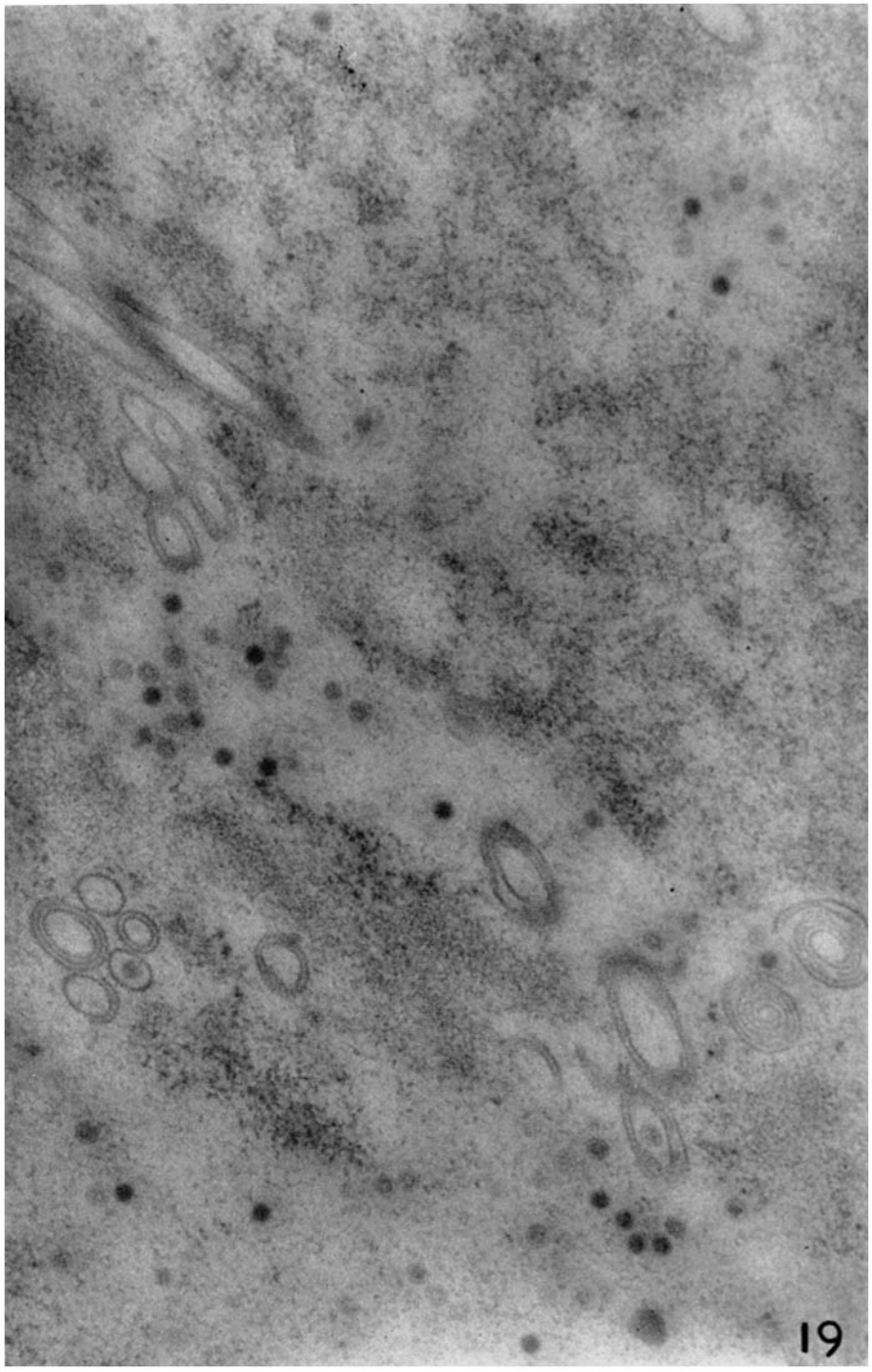
FIG. 18. Clusters and crystals of virus adjacent to condensations of nuclear matrix. Only remnants of the nuclear membrane remain. Viral particles appear to be in process of release into the cytoplasm and are close to the cell surface at several sites. × 18,500.



(Morgan *et al.*: Structure and development of viruses. IV)

PLATE 85

FIG. 19. One of a series of serial sections which reveal the tubular form of these intranuclear structures. The tubules vary in orientation with respect to the section. The enclosing membranes are single or multiple and are arranged either in spiral form or in concentric layers. Two tubules contain viral particles. × 53,000.



(Morgan *et al.*: Structure and development of viruses. IV)