STRUCTURE AND DEVELOPMENT OF VIRUSES AS OBSERVED IN THE ELECTRON MICROSCOPE

I. Herpes Simplex Virus*

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Plates 7 to 17

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The mode of development of herpes simplex virus has been the object of periodic study ever since Lipschütz (1) discovered that intranuclear inclusion bodies characteristically appear in the cells of tissues infected by this agent. Interest has centered chiefly on attempts to determine the nature of the nuclear changes but the results have not been concordant. Some observers have concluded that the inclusions actually represent a stage in viral multiplication and are composed at least in part of viral material. Others have inferred that they consist solely of abnormal products of cellular metabolism. The earlier work bearing on this subject has been well reviewed by Van Rooyen and Rhodes (2). More recently, Crouse *et al.* (3) carried out histochemical studies which revealed a distinct accumulation of desoxyribose nucleoprotein in early nuclear inclusions. This work was confirmed by Wolman and Behar (4) who considered the findings to be corroborative of the hypothesis that the herpetic inclusion body represents a colony of virus inside a cellular matrix. Scott *et al.* (5) have extended these observations by a careful study wherein the development of infectious virus was followed in temporal relation to the cytologic changes. These authors concluded that the nucleus probably contains virus during the early formation of the inclusion body. In contrast, Francis and Kurtz (6) and Ackermann and Kurtz (7) reported data on the infectivity of cellular fractions obtained by differential centrifugation which they interpreted as indicating that herpes simplex virus is not associated with the nucleus.

A more direct approach to this problem can be made by examining infected tissues in the electron microscope. Morgan *et al.* (8) have previously reported that altered nuclei could easily be identified by means of the electron micro-

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scope in sections of chorioallantoic membrane from infected chicken embryos. Such altered nuclei contained numerous particles 100 to 130 mμ in diameter, which were believed to be virus. Larger particles also considered to be virus were seen in the cytoplasm and at the surface of cells. In this earlier work the sections were relatively thick and the embedding substance was removed before examination. Subsequently, as noted elsewhere in a preliminary communication (9), these observations were confirmed and an inner structure of the viral particle was revealed by studying ultrathin sections of infected chorioallantoic membrane with the embedding substance in place. It is the purpose of this paper to present these findings in greater detail, to illustrate more completely the inner structure of herpes simplex virus, and to advance a theory concerning developmental stages of the virus.

Methods and Materials

The HRE strain of herpes simplex virus was used (10). Dropped chorioallantoic membranes of 11-day-old chicken embryos were inoculated with 0.2 ml. of a suspension of infected passage membrane containing approximately 300 ID_{50} of the virus. After 24 to 72 hours of incubation at 35°C. the membranes were removed and immediately fixed for 20 minutes in 1.0 per cent osmium tetroxide buffered at pH 7.4, according to the method of Palade (11). Small pieces were excised from the fixed membrane, dehydrated in graded dilutions of ethyl alcohol, and embedded in methacrylate.

Method 1.—In Part I of the results described below the embedded tissue was sectioned by the method of Newman, Borysko, and Swerdlow (12), using a glass knife (13). The sections were transferred from the knife to the surface of a 50 per cent aqueous solution of dioxane and were floated onto stainless steel grids coated with formvar. They were then immersed in amyl acetate to remove the methacrylate and lightly shadowed with palladium at an angle of 26°.

Method 2.—In Part II of the results the sections were cut on a microtome similar to that described by Porter and Blum (14). With this instrument sections 0.1 mμ, or less, in thickness could be obtained. The sections were floated onto the surface of a 30 per cent acetone–70 per cent water mixture, transferred to formvar-coated grids, and examined directly. The embedding substance was not removed and the sections were not shadowed.

All the sections were examined in an RCA type EMU electron microscope.

RESULTS

Part I.—Characteristic cellular changes and particles presumed to be virus were easily and repeatedly observed in infected tissues, whereas none were seen in control material prepared from normal chorioallantoic membranes. The most striking changes were observed in cells of the ectodermal portion of the membranes.

In many cells the nuclei were swollen and dense reticular material had accumulated on the inner surface of the nuclear membrane. Although the nature of such reticulum is not known, the term "marginated chromatin" will be employed to describe it, in conformity with the terminology of previous investigators who have observed the same phenomenon by light microscopy.

Coincident with the appearance of marginated chromatin, structures considered to be nucleoli were commonly found at the periphery of the nucleus. Such nucleoli were either fragmented or flattened against the nuclear membrane in many instances.

Within or adjacent to the marginated chromatin one or more clusters of dense, spherical particles, measuring 40 to 60 mμ in diameter, were frequently seen. Where large numbers of particles were present the clusters appeared to have expanded and to have replaced the reticular ground substance of the nucleus as well as the coarser reticulum characterizing the marginated chromatin. Fig. 1[1] shows such a cluster and suggests on close examination that the particles may have formed by segmentation of the solid strands composing the marginated chromatin. Only a small part of the nucleus is shown and the margin which separates it from the cytoplasm can be seen near the right border of the photograph. A larger cluster of particles, most of them measuring 40 to 50 mμ in diameter, is illustrated by Fig. 2. Much of the marginated chromatin appears to have been replaced by particles, further suggesting that this reticular material may have contributed to their formation. Other nuclei exhibited centrally located clusters in addition to diffusely scattered viral particles. Fig. 3 shows a nucleus containing four such clusters with a particle size ranging from 50 to 60 mμ. The finely granular oval body at the lower right margin of the nucleus may represent a nucleolus which has not marginated. The nuclear membrane has disrupted at numerous points and the location of many viral particles suggests that they are in process of release into the adjacent cytoplasm.

Rare nuclei also were found in which unusually large numbers of viral particles were present; these nuclei appeared to have undergone marked degenerative changes with disruption of the nuclear membrane. Fig. 4 illustrates such a nucleus in which the particles range from 70 to 100 mμ in diameter. When these larger particles were numerous the smaller ones could no longer be seen. Other nuclei contained still larger particles (100 to 130 mμ), possibly representing later stages of infection. It is of interest that particles considered to be virus exceeding 130 mμ in diameter were never observed in nuclei.

Within the cytoplasm of infected cells the viral particles were generally larger than those seen within nuclei, attaining diameters up to 200 mμ. Intracytoplasmic particles measuring 60 to 100 mμ were uncommon except in the immediate vicinity of infected nuclei which had apparently undergone dissolution. An aggregation of particles within a cytoplasmic vacuole is illustrated by Fig. 5. The clearly demarcated particles measure 100 to 190 mμ in diameter. Fragmented and shell forms are visible. At the lower left hand corner of the picture part of a viral shell has been destroyed, revealing a

[1] Figs. 1 to 6 are negative prints.

spherical inner body 40 mμ in diameter. At the lower right corner a particle has apparently disrupted with release of the 40 mμ inner body. Examination at high magnification of the intranuclear virus also showed shell forms of particles larger than 60 mμ which may be seen on close inspection of Figs. 3 and 4.

The viral particles tended to aggregate within spaces between cells. Such spaces were found to communicate directly with areas which presumably had contained extracellular fluid, thus allowing release of virus into the mesoderm. The extracellular viral particles did not exceed 200 mμ in diameter. They were found within and adjacent to clumps of amorphous debris and collagen fibers or were clustered on the surface of mesodermal cells. The nuclei of many mesodermal cells contained the characteristic smaller forms of the virus, those nuclei nearest the ectoderm appearing in a more advanced stage of infection than others further removed. Within the mesoderm there usually existed a sharply defined zone which ran roughly parallel to the ectodermal cells and beyond which no viral particles were observed. The distance of this zone from the ectoderm varied in each membrane examined. It is uncertain whether the virus may be dispersed at a uniform rate by the extracellular fluid or whether the host develops some defensive mechanism at such a zone.

Anderson (15) found that virus was disseminated by the blood stream in the chicken embryo and noted inclusions within occasional endothelial cells. Viral invasion of the endothelium was also noted in the present studies, although it appeared to be uncommon. Occasional endothelial nuclei contained characteristic viral particles and showed margination of chromatin. In Fig. 6 the cytoplasm of an endothelial cell runs from left to right. At the top is the capillary lumen, at the bottom a few collagen fibers. Scattered between these collagen fibers are several particles and at the upper left corner, apparently about to be released into the capillary lumen, is a cluster of particles which closely resemble and are believed to be herpes simplex virus.

Part II.—Despite the resulting loss of contrast, high resolution is obtainable in ultrathin sections examined without removal of the embedding plastic. Although fewer viral particles are contained within such sections each particle is transected, thus permitting detailed study of the internal structure. Moreover distortion of the fine components of cellular architecture caused by removal of the methacrylate is avoided.

Fig. 7 shows a nucleus considered to represent an early stage of infection preceding characteristic margination of chromatin. The nucleoplasm has formed small, irregular aggregates and the nucleolus at the lower right appears to be in process of dispersion. Scattered through the nucleoplasm are particles resembling the primary, viral bodies to be shown at higher magnification below. Fig. 8 illustrates a nucleus considered to represent a later

stage of infection. It is markedly enlarged and possesses a small amount of marginated chromatin. The nuclear membrane has disrupted at one point. Viral particles are dispersed through the nucleoplasm, which has become finely granular. Within and adjacent to the inner aspect of the marginated chromatin in such nuclei aggregates of small, dense particles, 30 to 40 mμ in diameter, were frequently encountered. Fig. 9 shows such a collection. A partially disrupted nuclear membrane separates the nucleus from the cytoplasm at the right border of the illustration. One particle is partially enclosed by a single membrane. At higher magnification, in Fig. 10, four particles of nearly uniform density and 30 to 40 mμ in diameter, are seen. These are thought to represent the first recognizable stage of development. Immediately adjacent are two larger particles which consist of a primary or central body and an outer membrane. It will be observed that the outer membranes are incomplete, suggesting that they are being laid down by a progressive process. The central bodies are 40 to 50 mμ in diameter and are less dense centrally than at the periphery. Fig. 11 shows an aggregate of particles composed of a central body and an outer membrane. The outer membranes of some well visualized particles are incomplete, as noted in Fig. 10; the indistinct appearance of other particles probably reflects the level of transection. The elliptical shape of the particles and the uniform orientation of their long axes are thought to result from compression of the specimen by impact of the knife. The average of the major and minor diameters (used in all subsequent measurements) of the central bodies is 40 to 50 mμ. The outer membranes average 70 to 80 mμ in diameter and are 6 to 8 mμ thick. Examination of many particles within infected nuclei showed the diameters of the single membranes to vary from 70 to 100 mμ. Such aggregates of particles generally lay adjacent to or appeared to replace the marginated chromatin. Segmentation of the reticular material into small, primary bodies (as illustrated by Fig. 1) was not observed.

Within the cytoplasm of the host cell as well as in the extracellular space most of the viral particles exhibited a double membrane enclosing the central body, as illustrated by Figs. 12 to 15. The variation in their appearance probably resulted from differences in the plane of transection. The central body of some particles did not lie in the plane of section. The double membranes of other particles apparently overlapped when cut at a tangent, thus producing a poorly defined, dense structure. The diameter of the intracytoplasmic particles varied from 110 to 130 mμ.

Fig. 16 shows a nucleus in the lower half of the illustration containing several central bodies, partially or completely enclosed by a single membrane. The nuclear membrane, irregularly lined by marginated chromatin, has disrupted at several points. The cytoplasm of the cell runs horizontally across

the illustration. A particle with a double membrane, characteristic of the *intracytoplasmic* form of the virus, is apparently in process of release into the extracellular space through a break in the cell membrane.

DISCUSSION

The discrepancies in size between the virus examined in sections after removal of the methacrylate and the virus studied in the embedding plastic can probably be accounted for by the action of the solvent and subsequent effects of drying. When methacrylate is removed from thin sections containing transected viral particles the outer membranes are often ruptured and the central bodies are frequently lost. It is suggested that distortion of the virus in drying may also account for the larger particle size of the virus observed by Coriell *et al.* (16)—average 175 mμ—and by Evans and Melnick (17)—average 213 mμ—in specimens of vesicular fluid from human herpetic lesions. Munk and Ackermann (18) recently reported that herpes simplex virus recovered by centrifugation from the allantoic and amniotic fluids of infected chicken embryos had a predominating particle size of about 116 mμ in the electron microscope, which is well in line with our findings. Moreover, these authors noted in unshadowed preparations that the particles were not homogenous and appeared to contain a central structure.

Within infected cells of the chorioallantoic membrane herpes simplex virus appeared to develop in the following manner. Margination of fine, dense, reticular material constituted the first apparent change in infected nuclei. At scattered foci adjacent to or within this reticulum small, dense primary or central bodies, 30 to 40 mμ in diameter, differentiated. The reticular material appeared to contribute in some manner to the development of the virus, but actual segmentation of the reticulum was not observed in sections from which the methacrylate had not been removed. A membrane, 6 to 8 mμ in thickness and 70 to 100 mμ in diameter, formed and enclosed the central body, which increased to 40 to 50 mμ in diameter and exhibited a less dense center. Some nuclei contained multiple foci of development where large numbers of viral particles formed as aggregates. The nuclear membrane appeared to disrupt at any stage of recognizable infection with liberation of virus into the cytoplasm. Within the cytoplasm a second membrane was formed, having a diameter of 120 to 130 mμ. The mature viral particle, possessing a central body and a double membrane, was released into the extracellular fluid. Many mitochondria of infected cells showed swelling, vacuolization, and various degrees of disintegration, but bore no apparent morphologic relation to the virus at any stage of its development. The material examined to date has not revealed significant evidence concerning the earliest phase of infection as the virus penetrates the cell.

In an earlier report (8) it was stated that inclusion bodies were readily identified by the electron microscope in thin sections of chorioallantoic membrane infected with herpes simplex virus. Further study has indicated that these so called inclusion bodies were, in fact, altered nuclei in which the chromatin was marginated and viral particles were present. These structures probably correspond to those which other investigators have considered to be stages in the development of the typical type A inclusion body (3–5). Actually it has proved impossible with the electron microscope to visualize an intranuclear structure resembling the type A inclusion body as seen in the light microscope. It should be pointed out, however, that for light microscopy the sections are generally fixed by a different method, are cut much thicker, and are differentially stained.

It should be emphasized that no information is yet available concerning the stage of viral development at which the attribute of infectivity is acquired. Since evidence obtained thus far indicates that the viral particles do not become morphologically complete until they possess a double membrane, there is a possibility that the intranuclear forms with a single limiting membrane are non-infective. This problem is currently under investigation with the objective of determining the relation of the developmental structure of herpes simplex virus to its antigenic and infective properties.

SUMMARY

Herpes simplex virus was visualized by the electron microscope in sections of infected chorioallantoic membrane of chicken embryos. Removal of the embedding methacrylate from relatively thick sections permitted large numbers of viral particles to be seen but caused extensive alteration of cellular components as well as variable distortion of viral structure. This distortion was characterized by disruption of particles and loss of central bodies, resulting in ring or empty shell forms. An inner structure of the virus was revealed in ultrathin sections from which the embedding plastic was not removed. The nuclei of infected cells contained small, dense, primary bodies (30 to 40 mμ in diameter) as well as slightly larger and less dense particles (40 to 50 mμ in diameter) surrounded by a single membrane (70 to 100 mμ in diameter). In the cytoplasm most of the particles possessed a double outer membrane (120 to 130 mμ in diameter). It is suggested that the initial site of viral development is restricted to the nucleus where primary bodies form and become enclosed by a single outer membrane. Upon release into the cytoplasm these particles appear to acquire a second outer membrane and presumably represent the mature virus.

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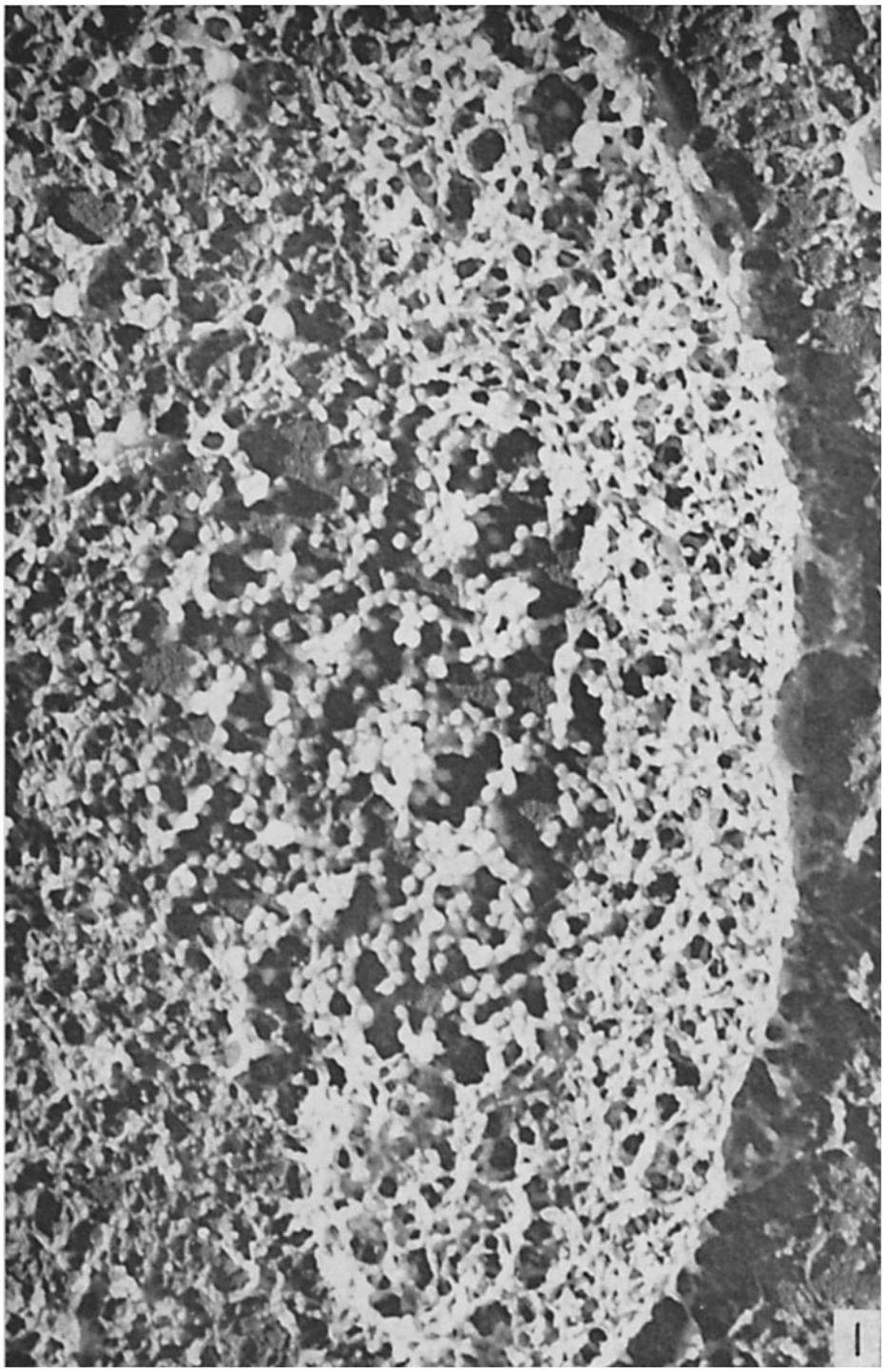
EXPLANATION OF PLATES

PLATE 7

FIG. 1. A portion of the nucleus and cytoplasm of an infected cell. A cluster of particles is seen in association with dense reticulum (marginated chromatin) in the nucleus. The cytoplasm occupies the right border of the picture. This and the following five figures are negative prints of sections shadowed after removal of the embedding plastic. Particle size 40 to 60 mμ. × 45,000.

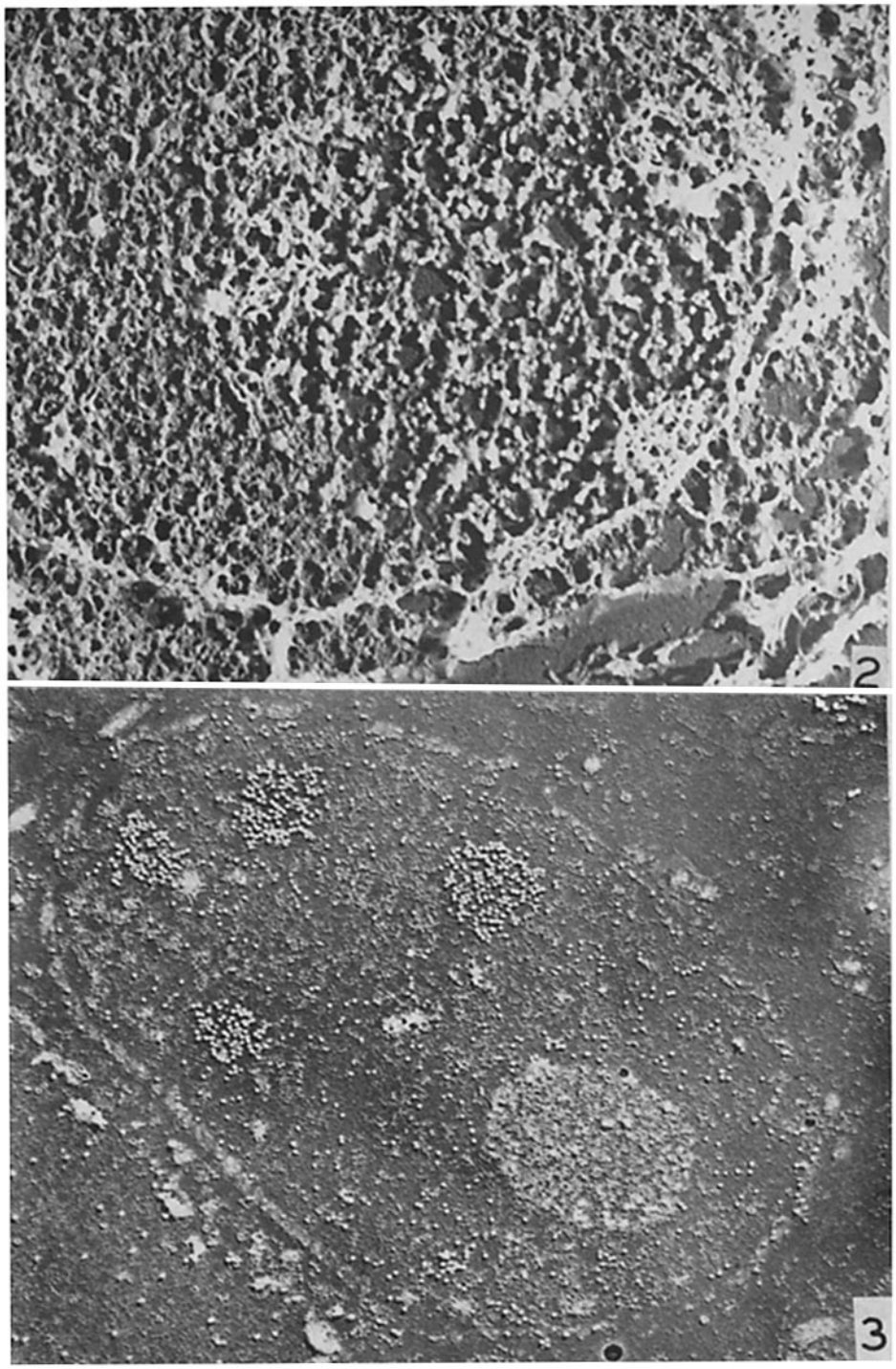


(Morgan *et al.*: Structure and development of viruses. I)

PLATE 8

FIG. 2. A larger cluster of intranuclear particles with little marginated chromatin. Particle size 40 to 50 mμ. × 23,500.

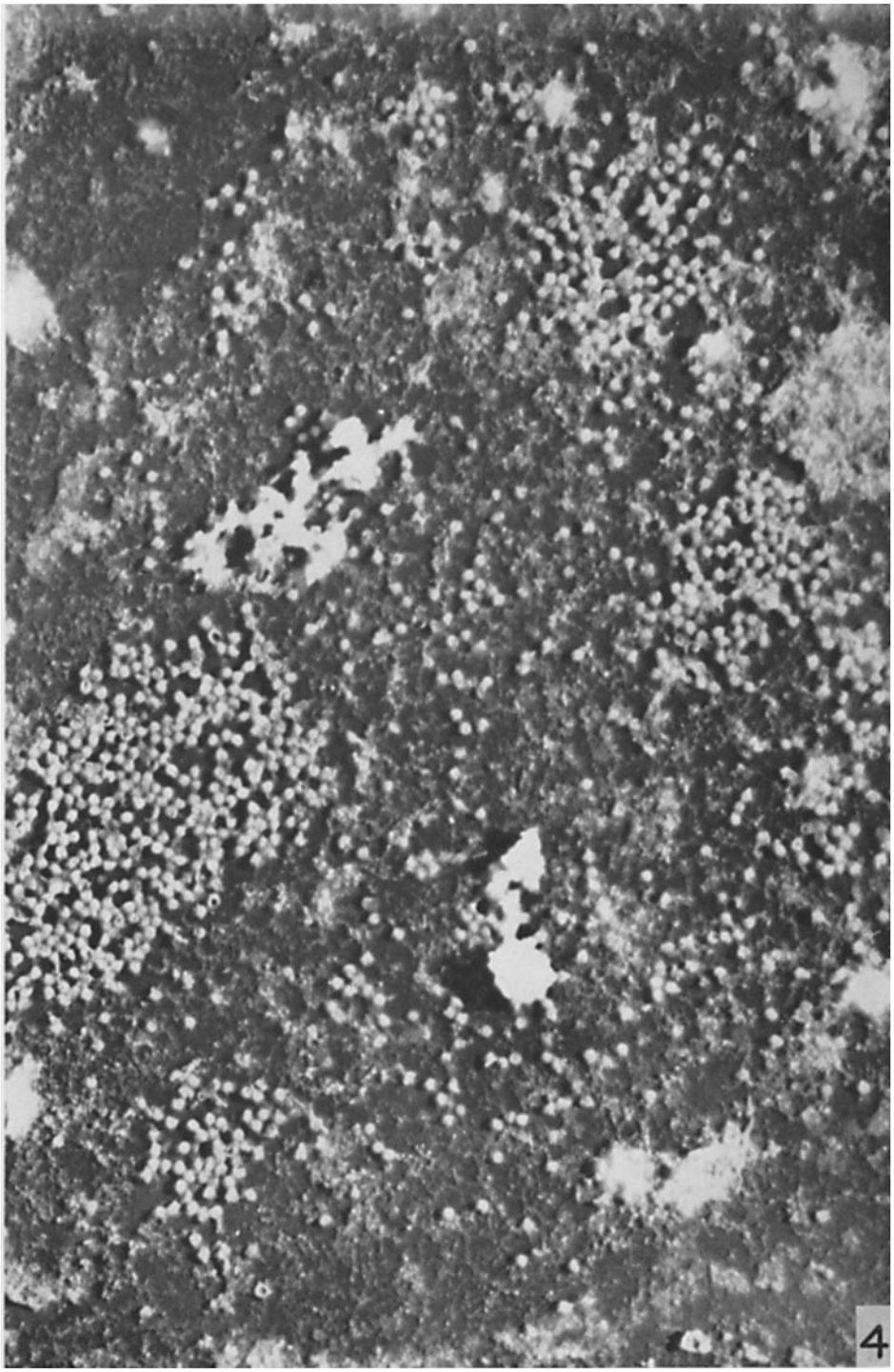
FIG. 3. A nucleus containing four non-marginal clusters of viral particles and an aggregate of reticular material, probably representing a nucleolus. Particle size 50 to 60 mμ. × 10,000.



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

PLATE 9

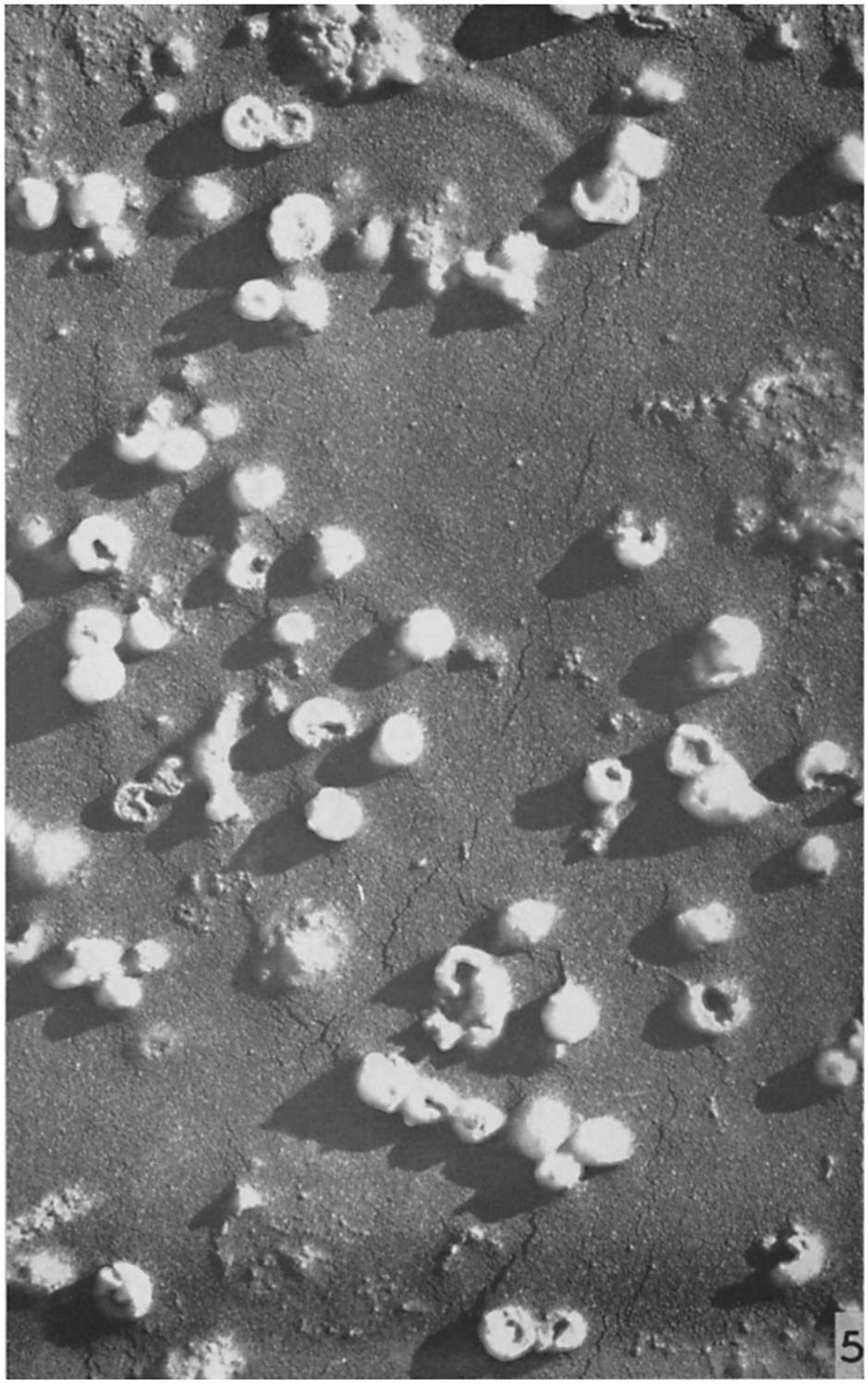
FIG. 4. Particles of virus within a disintegrating nucleus. Some empty ring forms as well as rings enclosing a central body may be seen. Particle size 70 to 100 mμ × 16,000.



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

PLATE 10

FIG. 5. An aggregate of intracytoplasmic viral particles. Some particles appear to be intact while others show various types of distortion produced by removal of the methacrylate with subsequent drying. At the lower left corner a particle appears to have lost part of its capsule revealing the inner body, while at the lower right a particle has been disrupted with release of its inner body. × 47,000.

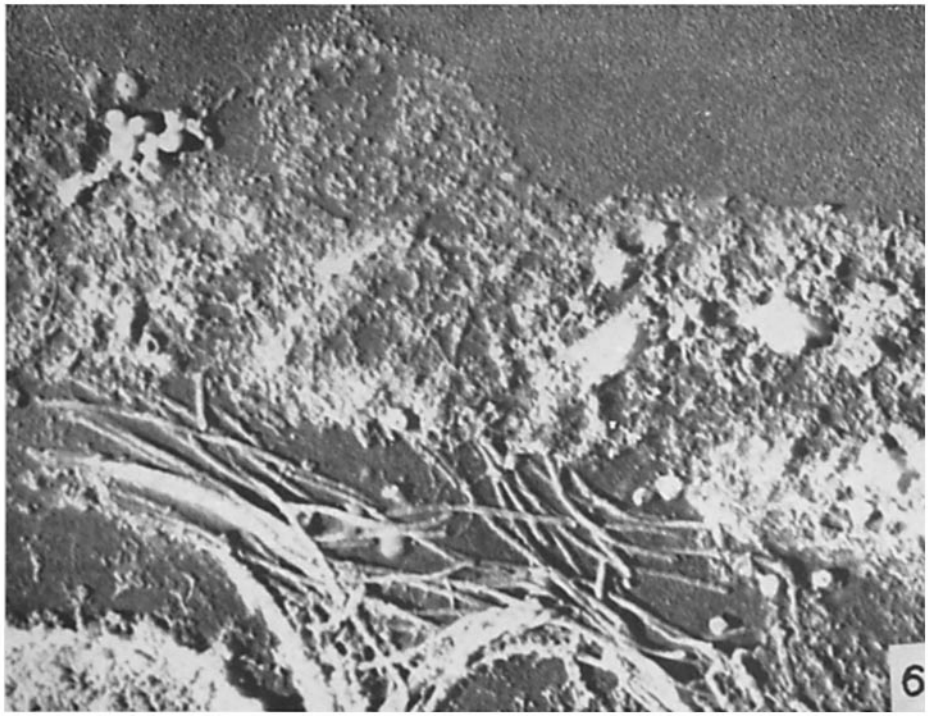


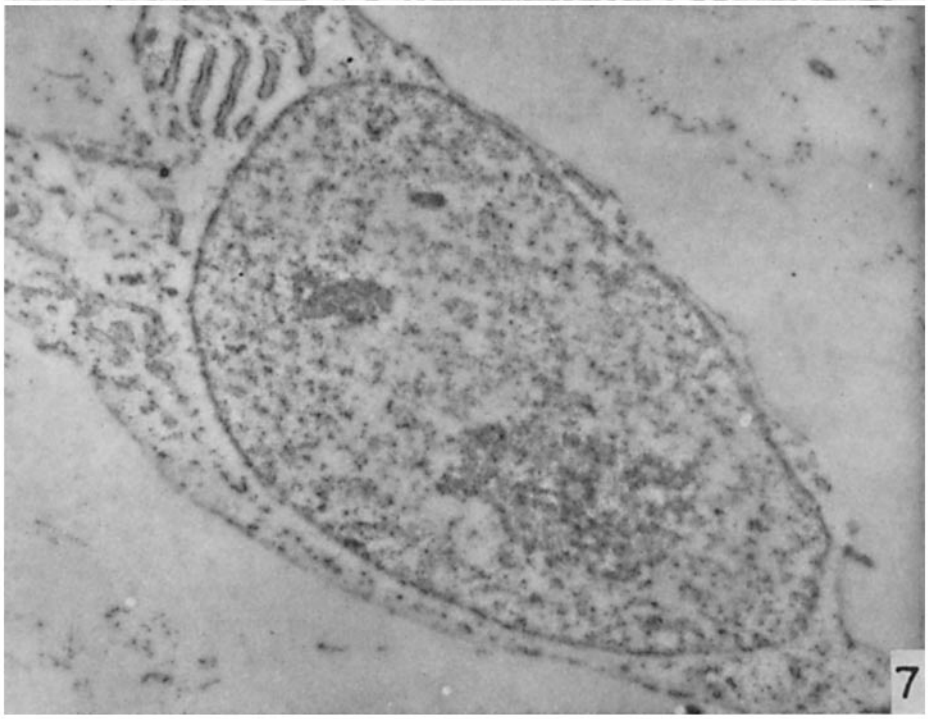
(Morgan *et al.*: Structure and development of viruses. I)

PLATE 11

FIG. 6. The wall of a blood vessel within the mesoderm. The lumen of the vessel occupies the upper border of the picture. The cytoplasm of an endothelial cell extends horizontally across the illustration. Just below it are seen particles scattered among collagen fibers. At the upper left corner is a cluster of viral particles extending into the lumen of the vessel. × 23,500.

FIG. 7. A nucleus considered to represent an early stage of infection. Although margination of chromatin is not apparent the nucleoplasm has formed small aggregates, the nucleoli are poorly demarcated and, at higher magnification, clusters of particles resembling the unencapsulated, primary bodies of virus may be seen. This and the following figures illustrate sections from which the methacrylate has not been removed. × 9,000.

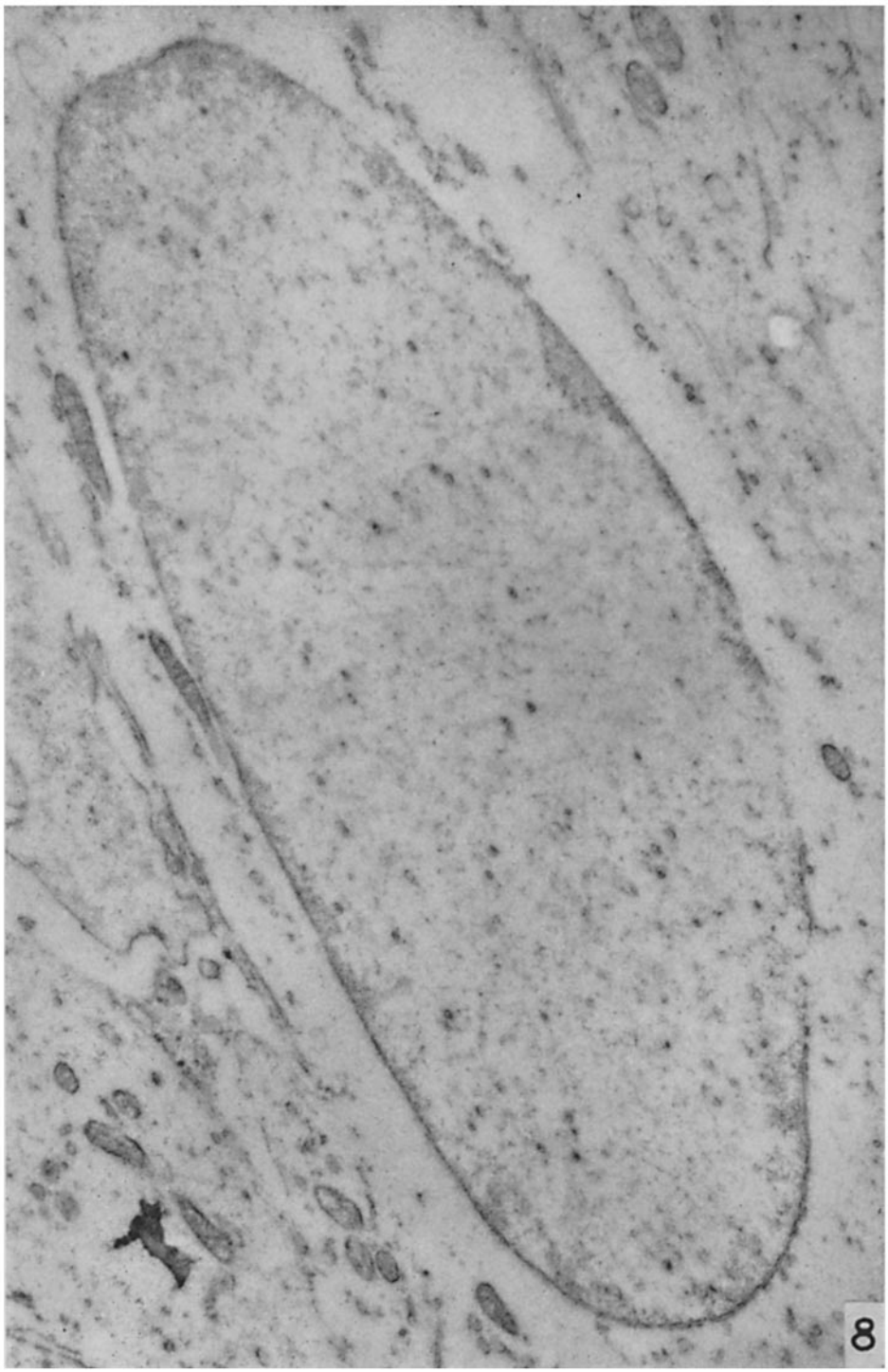




(Morgan *et al.*: Structure and development of viruses. I)

PLATE 12

FIG. 8. A nucleus considered to represent an advanced stage of infection. It is swollen, shows irregularly marginated chromatin, and contains dispersed viral particles with single membranes. Few particles suggestive of the primary form can be found. The double nuclear membrane has disrupted near the right border of the figure. × 15,000.



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

PLATE 13

FIG. 9. Peripheral area of an infected nucleus. A partially disrupted nuclear membrane separates it from the adjacent cytoplasm at the right border of the figure. Primary, viral bodies may be seen in the lower third of the illustration. They lie within and adjacent to marginated chromatin. One body is partially enclosed by an incomplete membrane. × 76,000.

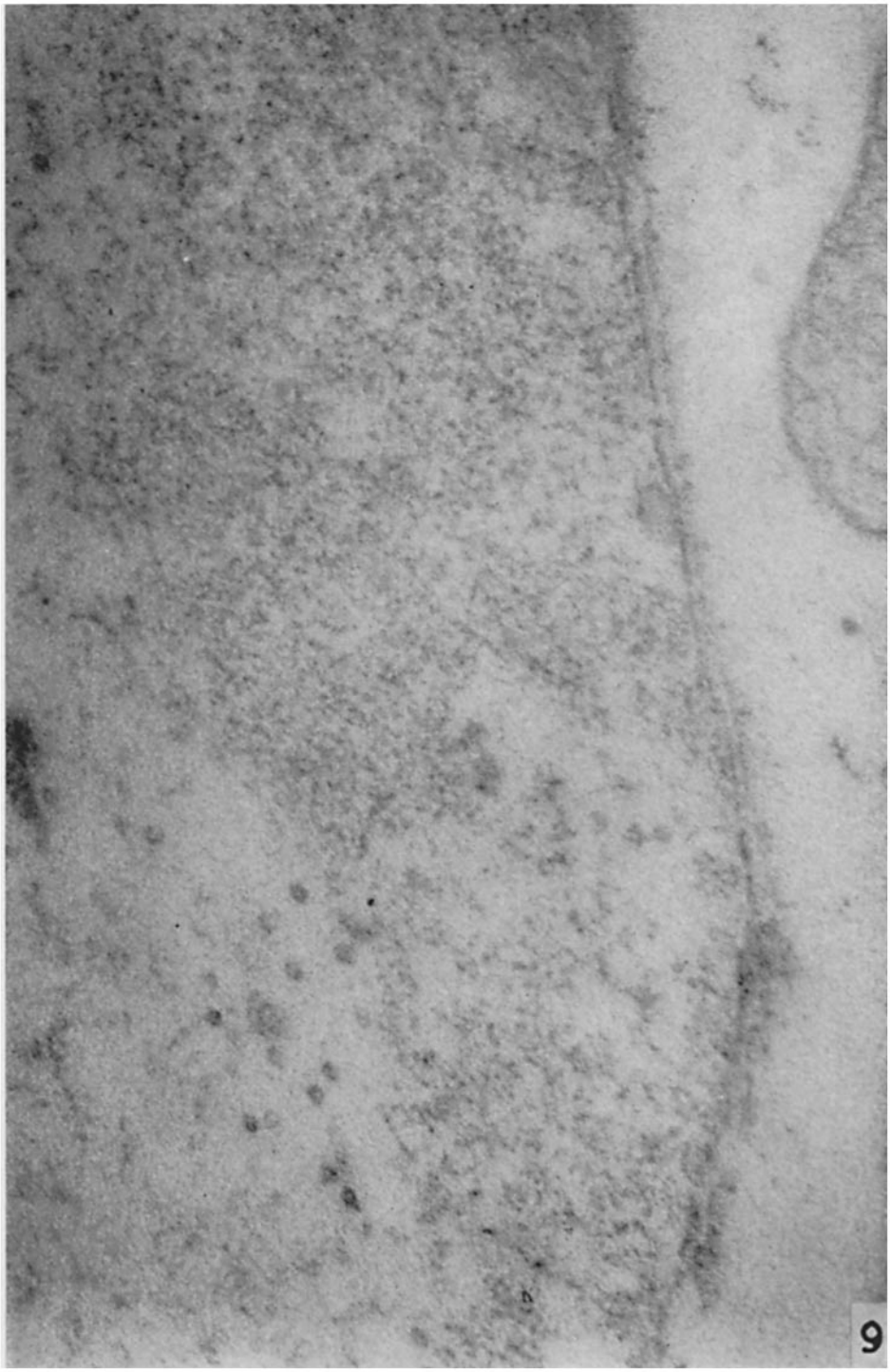


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PLATE 14

FIG. 10. Four unencapsulated, primary, viral bodies adjacent to marginated chromatin within a nucleus. Single membranes, apparently in process of formation, partially enclose two larger bodies. × 131,000.

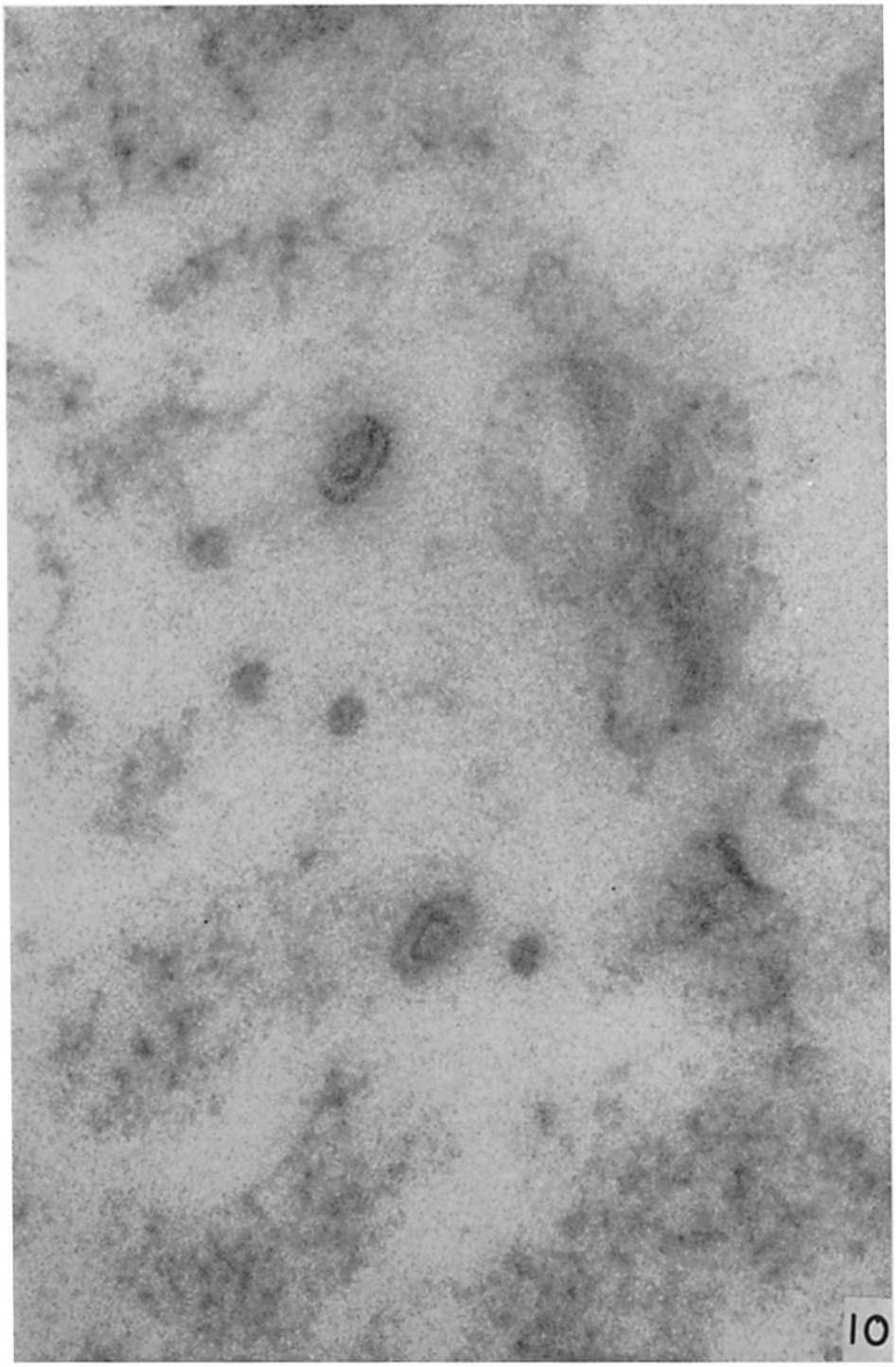

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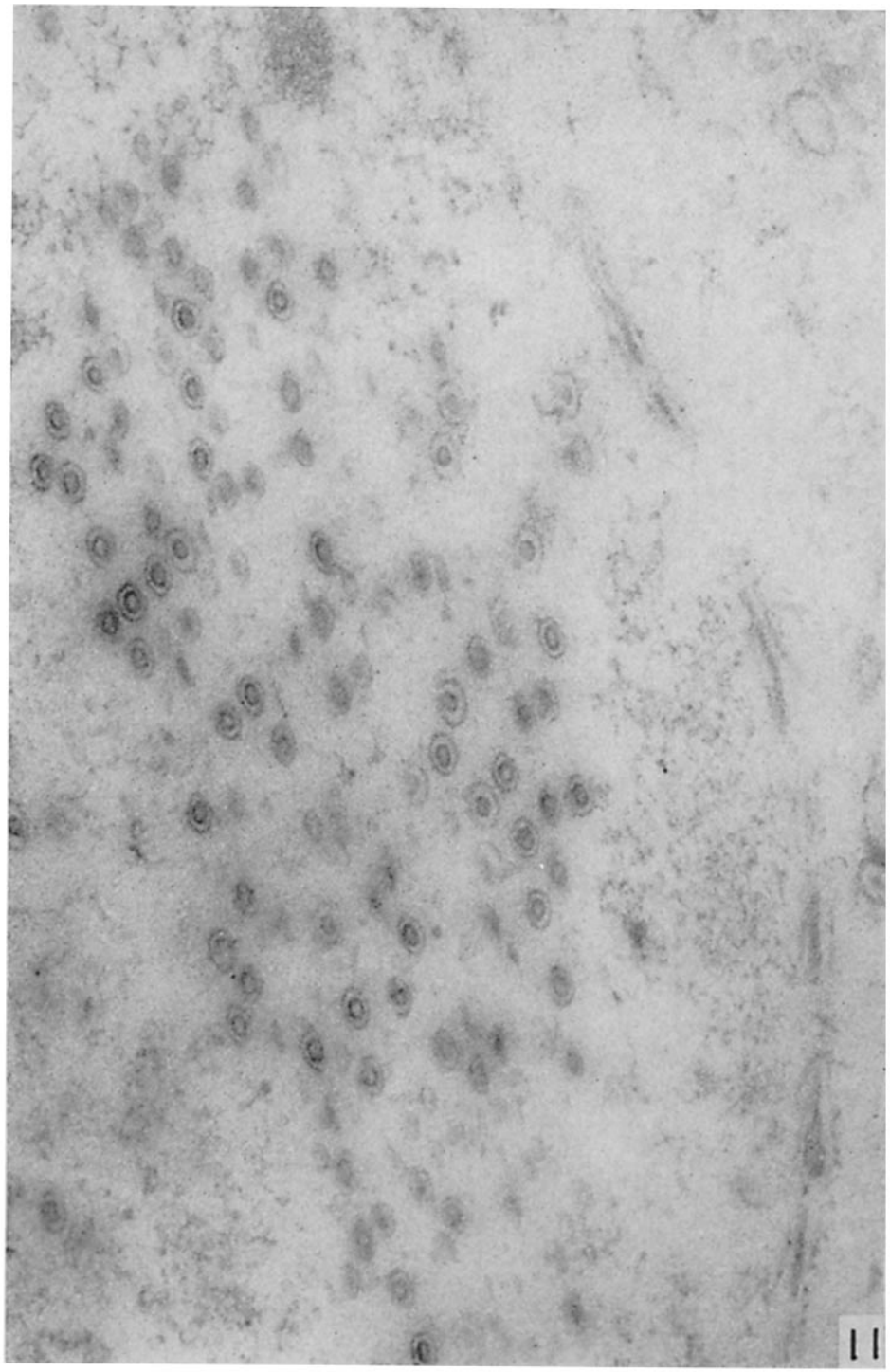
PLATE 15

FIG. 11. An aggregate of intranuclear particles believed to represent a developmental focus within marginated chromatin. The inner bodies contain central zones of lesser density. Some viral membranes appear to be in process of formation, while others are complete. Numerous indistinct particles probably represent virus sectioned at one margin with loss of density and overlapping of structures. The disrupted nuclear membrane is visible near the right border of the figure. × 65,000.



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

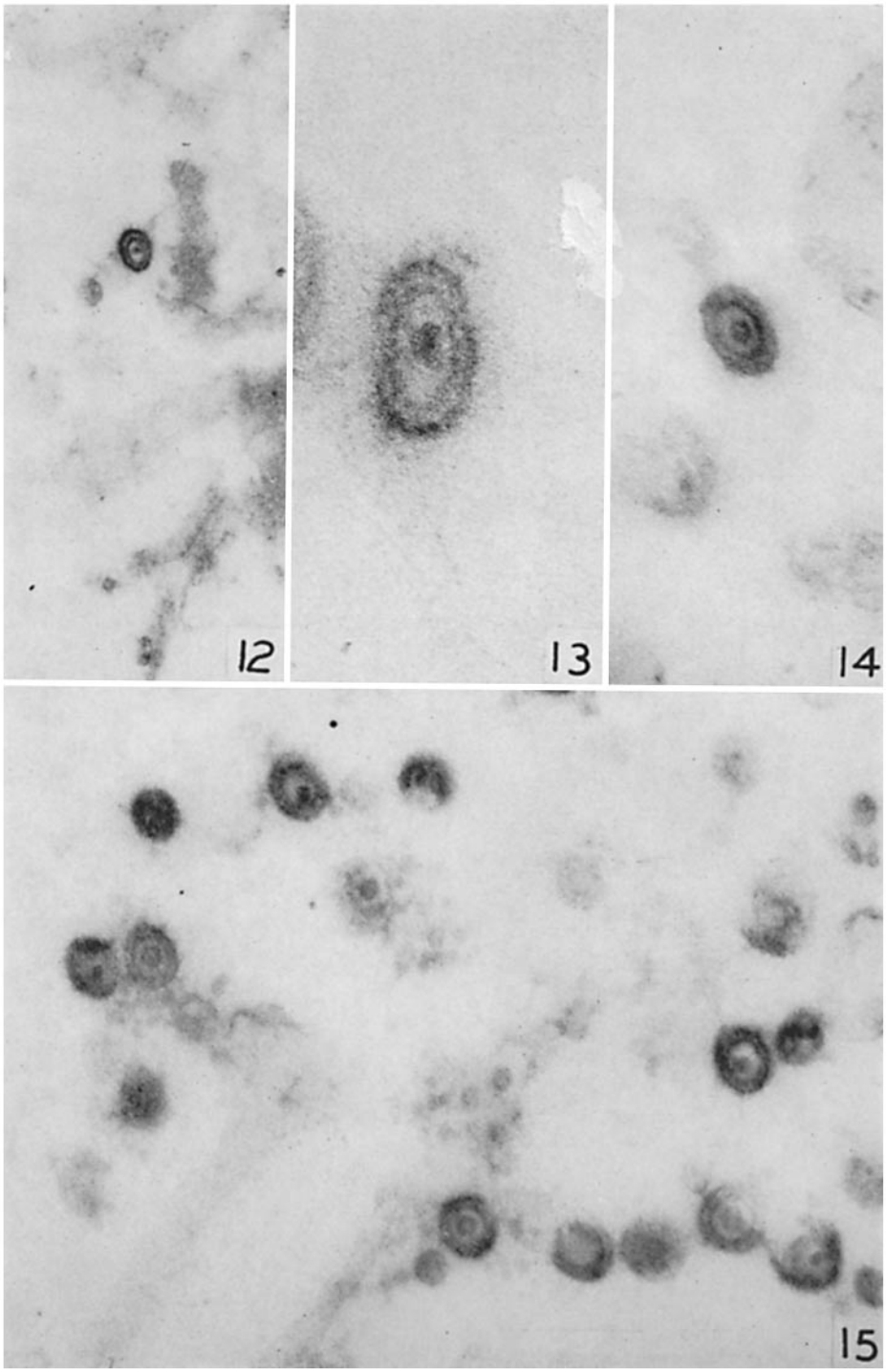
PLATE 16

FIG. 12. A viral particle with a central body and double membrane characteristic of the intracytoplasmic and extracellular forms of the virus. × 46,000.

FIG. 13. A viral particle within the cytoplasm. The uniform density of the central body may represent a tangential section through it. The double membranes are clearly separate. × 166,000.

FIG. 14. A viral particle in the extracellular space. The outer membrane is indistinct. × 80,000.

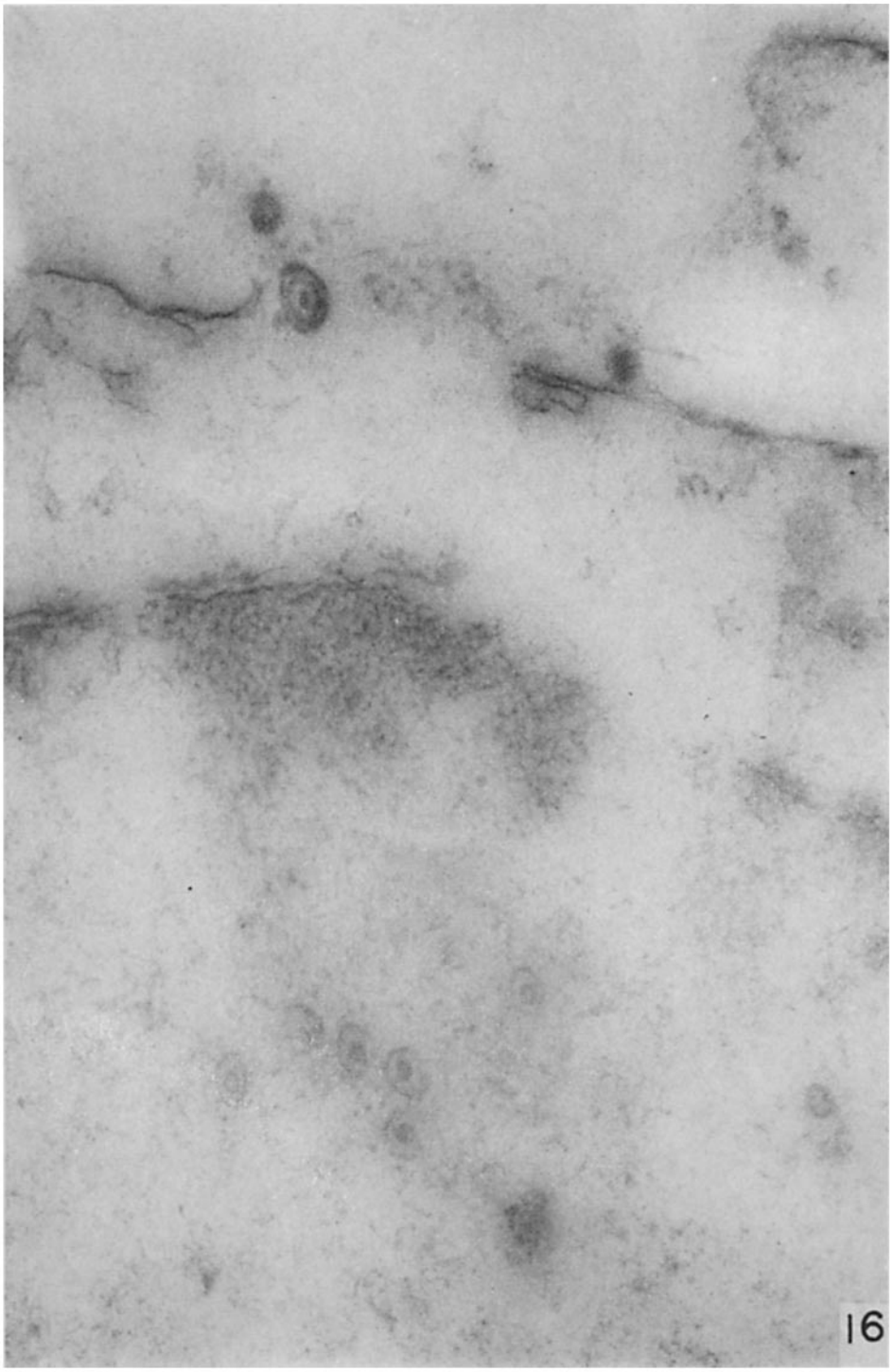
FIG. 15. An aggregate of intracytoplasmic viral particles. The variations in density of the central bodies and enclosing membranes may represent variation in viral morphology or overlapping of structures sectioned at different levels. × 71,000.



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

PLATE 17

FIG. 16. A collection of intranuclear viral particles, four of which show an incomplete membrane. The nuclear membrane with aggregates of marginated chromatin, extending horizontally across the middle third of the figure, has disrupted at several points. Near the top of the picture a viral particle possessing a double membrane appears in process of release from the cytoplasm of the host cell into the extracellular space through a break in the cell wall. × 67,500.



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