

STRUCTURE AND DEVELOPMENT OF VIRUSES OBSERVED IN THE ELECTRON MICROSCOPE

II. VACCINIA AND FOWL POX VIRUSES*

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PLATES 31 TO 39

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Examination in the electron microscope of ultracentrifuged preparations of vaccinia and fowl pox viruses has shown the particles generally to be brick-shaped with dimensions about $210 \times 210 \times 260 \mu\mu$ and $265 \times 265 \times 330 \mu\mu$, respectively (1).

In 1942, Green, Anderson, and Smadel (2) noted that vaccinia virus appeared to have a limiting membrane and an internal structure composed of a central zone of greater density, often surrounded by four small bodies. The presence of an inner body was confirmed by Sharp *et al.* (3). Dawson and McFarlane (4) observed that the central body resisted pepsin digestion, whereas the peripheral area became less opaque to the electron beam. They noted a limiting membrane but suggested that the satellite bodies, described by Green *et al.*, were artefacts. Internal structure has also been seen by others (5-7), and Peters and Nasemann (8) have illustrated and described a small, dense body located at the periphery of some viral particles, which they consider to represent developmental forms. Bang *et al.* (9) observed a dense, central body in fowl pox virus and also noted a decrease in density at the periphery of the virus particle after treatment with pepsin.

Although vaccinia (10, 11) and fowl pox (12) viruses have previously been identified in sectioned tissues, the relatively thick sections employed and the distortion produced by removal of the embedding plastic with solvents have tended to obscure details of viral structure. More recently, Gaylord and Melnick (13), employing thinner sections without removal of methacrylate, noted several forms of vaccinia virus, including "empty circles" and "circles filled with homogeneous material of low electron density," as well as internal structures described as granules, bars, and dumbbells.

In order to obtain further information concerning the structure and development of vaccinia and fowl pox viruses, ultrathin sections of infected

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chicken embryo chorioallantoic membranes were studied by high resolution electron microscopy. The results of the study are presented in this communication.

Materials and Methods

Vaccinia.—The vaccinia virus was a stock laboratory strain originally obtained a number of years ago from the New York City Department of Health. The virus was adapted subsequently to the chicken embryo and had been passaged many times on the chorioallantoic membrane. Dropped chorioallantoic membranes of 11-day-old chicken embryos were inoculated with 50 to 100 ID₅₀ of virus and the embryos were incubated for 48 hours at 35°C. Infected membranes were fixed in osmium tetroxide, dehydrated in alcohols, embedded in methacrylate, and sectioned according to the methods previously described (14).

Fowl Pox.—Fowl pox virus¹ was passaged on the chorioallantois of 11-day-old chicken embryos, the inoculum containing approximately 100 ID₅₀ of virus. Membranes were harvested after incubation for 3 to 5 days at 35°C. and were prepared for microscopic examination in a manner identical with that used with membranes infected with vaccinia virus.

RESULTS

Vaccinia.—Sections through focal areas of hyperplasia revealed particles believed to be the virus within the cytoplasm of ectodermal cells. Fig. 1 shows a group of such particles. They are composed of moderately dense, granular material, enclosed by a single limiting membrane. Three particles contain a denser but also granular eccentric body, which is separated from adjacent structures by a variable zone of lesser density. This structure superficially resembles the nucleus of a cell and will be referred to hereafter as a nucleoid; the remainder of the contents of the viral particles will be arbitrarily called the viroplasm. The particles in Fig. 1 have major and minor axes measuring about 300 and 180 m μ , respectively, their elliptical shape probably reflecting compression of the section by impact of the knife. Although these represent average dimensions, considerable variation in size was observed. The nucleoids also varied in size, probably reflecting in part the level of transection. The nucleoid in the particle at the left of Fig. 1 measures 110 \times 150 m μ , whereas the nucleoid of the particle at the upper right has dimensions of 70 \times 135 m μ . At higher magnification, Fig. 2, the granular character of the viroplasm and nucleoid can be seen more clearly. These particles measure approximately 160 \times 260 m μ . The nucleoid of the lower particle measures 55 \times 125 m μ . It will be noted that the membrane of the upper particle in Fig. 2 is not clearly demarcated; and in Fig. 1 a poorly defined structure is present near the upper right corner. These variations in appearance could be accounted for by differences in plane of section. In an eccentric section the membrane would be cut obliquely and thus appear diffuse.

In order to clarify this point, serial sections were made. It was hoped, in

¹ Obtained through the courtesy of Dr. Hilary Koprowski, Lederle Laboratories Division, American Cyanamid Company.

addition, to ascertain whether the sections were thin enough to permit transection of the viroplasm without including the nucleoid. If such were the case, the apparent absence of nucleoids in the two particles near the lower border of Fig. 1 could be accounted for. Figs. 3 and 4 illustrate consecutive serial sections. It will be noted that some particles which have been transected in one figure lie outside the plane of section in the other. Certain particles appear smaller and less distinct at one level of section but are recognizable as virus in the other, indicating that a peripheral section results in loss of density and superimposition of the viroplasm and limiting membrane. Other particles exhibit nucleoids in one section but not in the other. In one particle (*Y*) the nucleoid has been transected twice, the indistinct limiting membrane in Fig. 4 indicating a peripheral section. In other particles only the viroplasm has been sectioned. The evidence obtained to date suggests that nucleoids vary in size and shape and that each viral particle at this stage of development probably contains one, although it may lie outside the plane of any given section. A particle in Fig. 1 suggests by its appearance that it has been transected at the level of the clear zone separating the nucleoid from the viroplasm.

Incomplete limiting membranes were encountered at multiple foci within the cytoplasm of infected cells. Fig. 5 shows viral particles similar to those illustrated in the earlier figures as well as membranes which only partially enclose fragments of granular material resembling viroplasm.

Within the cytoplasm near the surface of the host cell, and in the extracellular space, the structure of the virus was characterized by an inner body of variable shape and density, enclosed by a double limiting membrane. The membranes were each 3 to 4 $m\mu$ thick and were separated by a zone of lesser density 8 to 12 $m\mu$ wide. Fig. 6 illustrates several viral particles in an extracellular space between four cells. The appearance of the inner body can be explained by assuming that it is a disk with a center of lessened density and that it has been cut at different angles. The inner body of the particle near the upper right corner would presumably represent a cross-section of a disk. The particle at the lower left contains an inner body which may have been cut through the central area of lesser density in a plane parallel to the flat surface of the disk. The appearance of the inner body nearest the right margin of the figure could be accounted for by assuming transection nearly parallel to the flat surface, only part of the internal zone of lesser density entering the plane of section. It should be emphasized, however, that variations in appearance caused by irregular shrinkage during preparation of the tissues cannot at present be excluded. Serial sections similar to those illustrating cytoplasmic particles, showed that an eccentric section apparently resulted in overlapping of membranes with consequent poor demarcation of structure. Examples of this may be seen near the center of Fig. 6.

Adjacent to extracellular viral particles the cell membrane was frequently

indented and presented a greatly increased opacity to the electron beam. It is not yet clear whether such an appearance actually represented a focal increase in density or whether at such zones the cell membrane was more nearly perpendicular to the plane of section. Fig. 7 shows at higher magnification two viral particles illustrated in the preceding figure. Although the particles appear quite dense, the extreme thinness of this section can be judged by the dearth of material constituting the cellular cytoplasm.

Because removal of methacrylate from sections was found to cause variable distortion of herpes simplex virus (14), it was decided to determine the effect of such a procedure on vaccinia virus. Accordingly, sections were immersed briefly in amyl acetate and were allowed to dry before examination in the electron microscope. When ultrathin sections, such as those illustrated above, were treated in this manner, the viral membranes were generally disrupted and the internal structure was distorted beyond recognition. However, when relatively thick sections were employed, the viral particles frequently assumed a brick-like shape resembling their appearance in dried, purified preparations. Fig. 8² shows typical viral particles after removal of methacrylate from a thick section. The cytoplasm of the host cell has undergone fragmentation and aggregation. The nucleus is not shown. The more clearly defined, brick-shaped particles have average dimensions of $240 \times 320 \mu\mu$. Lying below a characteristic viral particle near the left border in the lower third of the illustration may be seen a small, spherical body partially enclosed by a membrane-like structure. This may represent the remnant of a viral particle sectioned eccentrically. The small, dense structures dispersed among the viral particles may be fragments of virus or aggregated cytoplasmic components.

Fowl Pox.—Sections through lesions of the chorioallantoic membrane apparent in the gross revealed particles believed to be virus within the cytoplasm of ectodermal as well as mesodermal cells. The particles resembled those of vaccinia virus in size and structure. Fig. 9 shows incomplete limiting membranes partially enclosing finely granular material which is similar in appearance to, but less dense than, the material constituting the viroplasm. This granular material has displaced or replaced the characteristic cytoplasmic structures. Near the right border of the illustration is a mitochondrion which is nearly the same size as the virus at this level of section but can be identified by cristae (15) and a typical, small, dense, internal particle.

Fig. 10 illustrates another aggregate of fowl pox virus within the cytoplasm of a host cell. The border of the cell is seen near the upper and lower margins of the illustration. The nucleus is not shown. Several mitochondria, possibly in various stages of disintegration, occupy the lower third of the figure. In the upper third of the illustration is a focus of granular material displacing or replacing the characteristic cytoplasmic components. Bordering it are several

* This section was shadowed with palladium at an angle of 26° and is printed as a negative.

membranes which are incomplete on the side contiguous to the granular area. Slightly removed from this focus are characteristic viral particles. The viroplasm of the particles nearest the focus is generally less dense than that of particles somewhat removed. Such areas of granular material bordered by incomplete membranes and lying near typical viral particles containing nucleoids were repeatedly encountered in cells infected with fowl pox virus. Often multiple foci were present within the cytoplasm of a single cell, as illustrated by Fig. 11. Replacing the cytoplasm to the right of the mitochondrion are several foci of granular material partially enclosed by incomplete membranes. A nearby section of this area, Fig. 12, shows the same mitochondrion but probably different viral particles. It is a thinner section and hence exhibits less contrast. Slightly to the right and above the center is a small, oval aggregate of dense granular material resembling a nucleoid situated within one of the foci adjacent to incomplete membranes.

Fig. 13 illustrates a zone similar to the zones described above; it also shows several aggregates of a substance resembling in structure and in density to the electron beam the material constituting the viral nucleoid. Near the right border of the photograph, two viral particles containing nucleoids may be seen. Just above and to the left of these particles is situated a sharply demarcated, oval mass of nucleoid material within a poorly defined viral particle. Part of a mitochondrion occupies the upper right corner of the illustration. Fig. 14 shows extracellular particles of fowl pox virus. These particles have double membranes and inner bodies and closely resemble, both in size and structure, the forms of vaccinia virus seen in Figs. 6 and 7.

Two strikingly different forms of the virus were illustrated and described above. The intracytoplasmic virus, at one stage of its development, was shown to be composed of a nucleus-like structure, a granular "viroplasm," and a single limiting membrane. But near the surface of the host cell and in the extracellular space the virus was seen to have a dense, possibly disk-shaped, inner body enclosed by a double limiting membrane. Other viral particles were encountered which are believed to represent transitional forms.

DISCUSSION

Evidence obtained from sectioned tissues suggests that vaccinia and fowl pox viruses are spherical at the stage of their development when they contain nucleoids. The long axis of the elliptical form they exhibited in sections was perpendicular to knife scratches, indicating compression by the knife. The degree of compression was variable in different sections. Viral particles possessing a central body and a double limiting membrane sometimes appeared to have been compressed along an axis but often assumed a variable shape. Such variation could be accounted for by assuming that the virus was elongated, or egg-shaped. The observed shape would then result, in part, from the plane of

transection. Moreover, that segment of the limiting membrane which lay perpendicular to the section would show clear definition, whereas the remainder of the membrane would appear indistinct.

Removal of the methacrylate from thick sections with subsequent drying produced brick-shaped particles resembling those frequently encountered in preparations purified by differential centrifugation and dried down on plastic screens. This suggested that certain viral particles were deformed by surface forces which acted during the drying process. It is tempting to conjecture that collapse or shrinkage of the limiting membrane onto an inner body, with the shape illustrated at the left of Fig. 7, could result in a brick-shaped form, but the data obtained so far are inadequate to substantiate such an assumption.

On the basis of evidence obtained thus far, the following hypothesis may be offered concerning the development of fowl pox virus. At one or more foci an aggregate of fine granular material replaces or displaces the cytoplasm of the host cell. Membranes form at the periphery of such areas and progressively enclose portions of the granular material, which will constitute the viroplasm. At other foci, either within or adjacent to this fine granular region, very dense material appears and fragments into small units, which are incorporated into the virus with the viroplasmic component and will constitute the nucleoids (see Fig. 13). At this stage of development the virus is composed of a nucleoid separated from the viroplasm by a narrow zone of lesser density and the particle is enclosed by a single limiting membrane. The nucleoid then loses its granular appearance, develops an inner zone of lesser density, increases in size, and assumes a central position. A double limiting membrane appears. It is not clear whether the single membrane splits in two or whether a second, separate membrane develops. One can speculate that the former mechanism is operative, since developmental stages in the formation of a separate membrane have not been recognized. When the above changes have been completed in the host cell the viral particle is released into the extracellular space. The data are insufficient to warrant any definite assertions concerning the structure of the inner body of extracellular viral particles. Until more extensive study of serial sections has been completed it can only be suggested tentatively that a disk could explain the various shapes encountered.

It is also possible that the foci of fine granular material repeatedly observed in infected cells are small areas of degeneration rather than zones where viroplasm is being generated. The invariable association of incomplete viral membranes with such foci might further suggest that they represent sites where viral particles are undergoing disintegration. However, if this were the case some alternative mechanism of development would have to be proposed for which there is as yet no evidence.

The present studies indicate that vaccinia virus develops in a manner similar to that in which fowl pox virus develops but that the aggregates of granular

material at presumed foci of development are generally smaller. In cells infected by fowl pox virus, relatively large amounts of this material were repeatedly encountered (as illustrated by Figs. 9 to 13), whereas relatively little was seen in association with vaccinia virus (as illustrated by Fig. 5). In the latter instance, viral particles often appeared to differentiate as individual units scattered at random through the cytoplasm of the host cell. It is of interest in this connection that Beveridge and Burnet (16) rarely found classical inclusions in cells of the chorioallantoic membrane infected by vaccinia virus, whereas Woodruff and Goodpasture (17) noted numerous, typical, intracytoplasmic inclusions in similar cells infected by fowl pox virus. On the basis of such indirect evidence, it seems possible that the aggregates of granular material and incomplete viral particles observed in tissues infected by fowl pox virus actually represent the inclusion bodies seen in the light microscope. However, using morphologic appearance as the sole criterion, intracytoplasmic inclusions could not be identified in thin sections examined in the electron microscope.³

The development of fowl pox and vaccinia viruses appeared to resemble in certain aspects the development of herpes simplex virus (14, 18). In each case a membrane seemed progressively to enclose a pre-existing component of viral structure. The primary body of herpes simplex and the nucleoids of fowl pox and vaccinia were altered in some way during the formation of the complete viral particle, both structures becoming less dense at their centers. Finally, all three viruses possessed a double limiting membrane before release from the host cell. It should be noted, however, in striking contrast to herpes simplex virus, that the viruses of vaccinia and fowl pox appeared to develop entirely within the cytoplasm, the nucleus of the host cell exhibiting no specific morphologic alteration. The nuclear membranes were generally intact and where they were disrupted no spacial orientation to aggregates of virus was evident. Although mitochondria were encountered in various stages of disintegration they did not appear to participate in viral development.

SUMMARY

Vaccinia and fowl pox viruses were visualized by the electron microscope in sections of infected chorioallantoic membrane of chicken embryos. The viruses were of similar structure and size, averaging $200 \times 300 \text{ m}\mu$ with con-

³ T. H. Flewett and G. Eaves reported observations on the structure of viruses of the pox group at the International Conference on Electron Microscopy in London, July 16-21, 1954. Using a technique similar to that described in this paper they demonstrated intracytoplasmic aggregates of viral particles in cells of chicken embryo chorioallantoic membranes infected with fowl pox virus. The aggregates could be interpreted as inclusion bodies and consisted mainly of particles whose internal structure resembled that of extracellular forms shown in Fig. 14. The tissues were examined 6 days after infection and a different strain of virus was used.

siderable individual variation. Intracytoplasmic viral particles contained a dense, nucleus-like body (nucleoid) separated from granular material (viroplasm) by a zone of lesser density. They were enclosed by a single membrane. Near the surface of the host cell and in the extracellular space the particles consisted of a central body of variable shape and density enclosed by a double membrane. The initial sites of development were confined to the cytoplasm of the host cell. Before release from the host cell the viral nucleoids appeared to enlarge and to occupy a central position within the particle, which became enclosed by a double limiting membrane. The brick-shaped forms found after removal of the embedding plastic from thick sections indicated that drying caused characteristic distortion of certain viral particles.

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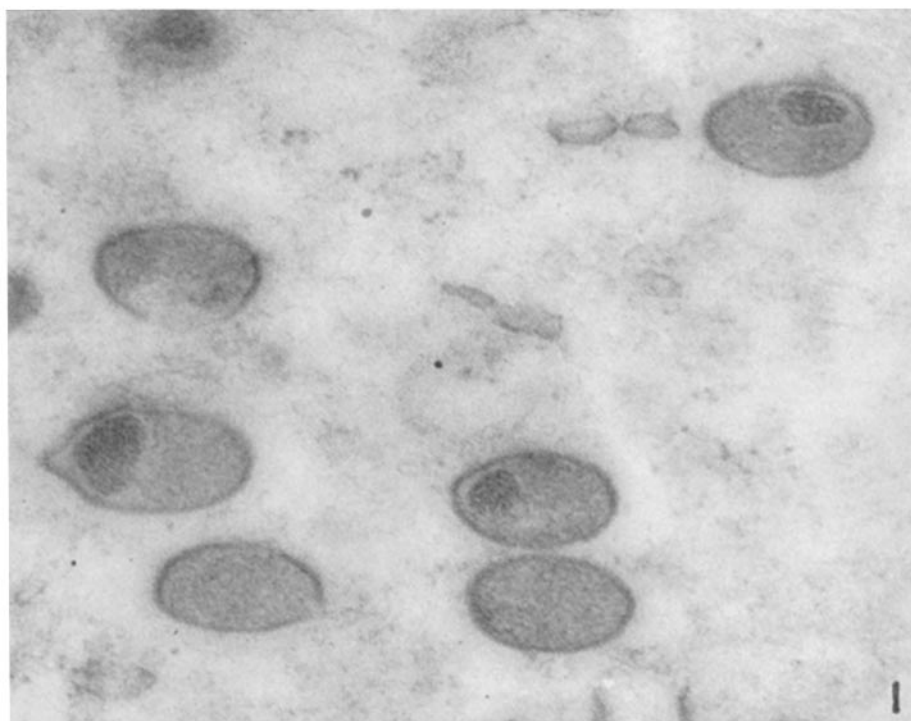
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EXPLANATION OF PLATES

PLATE 31

FIG. 1. Intracytoplasmic vaccinia virus composed of characteristic granular material (viroplasm) enclosed by a single, limiting membrane. Three particles possess an eccentric nucleus-like body (nucleoid). One particle appears to have been sectioned through the zone of decreased density which separates the nucleoid and the viroplasmic components. $\times 74,000$.

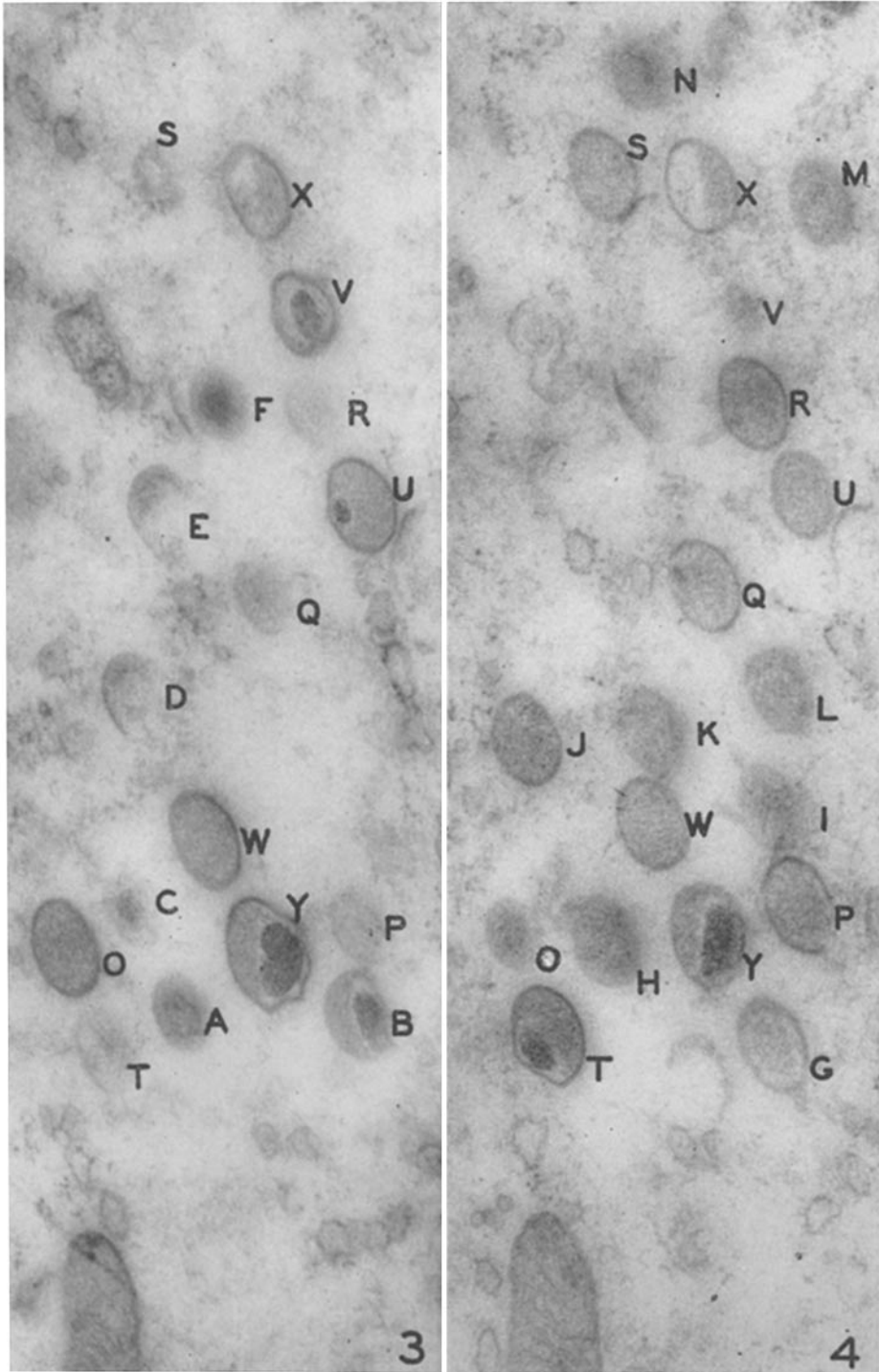
FIG. 2. Two viral particles which show a finely granular structure. The limiting membrane of the upper particle is indistinct, suggesting that it does not lie perpendicular to the plane of section. $\times 160,000$.



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

PLATE 32

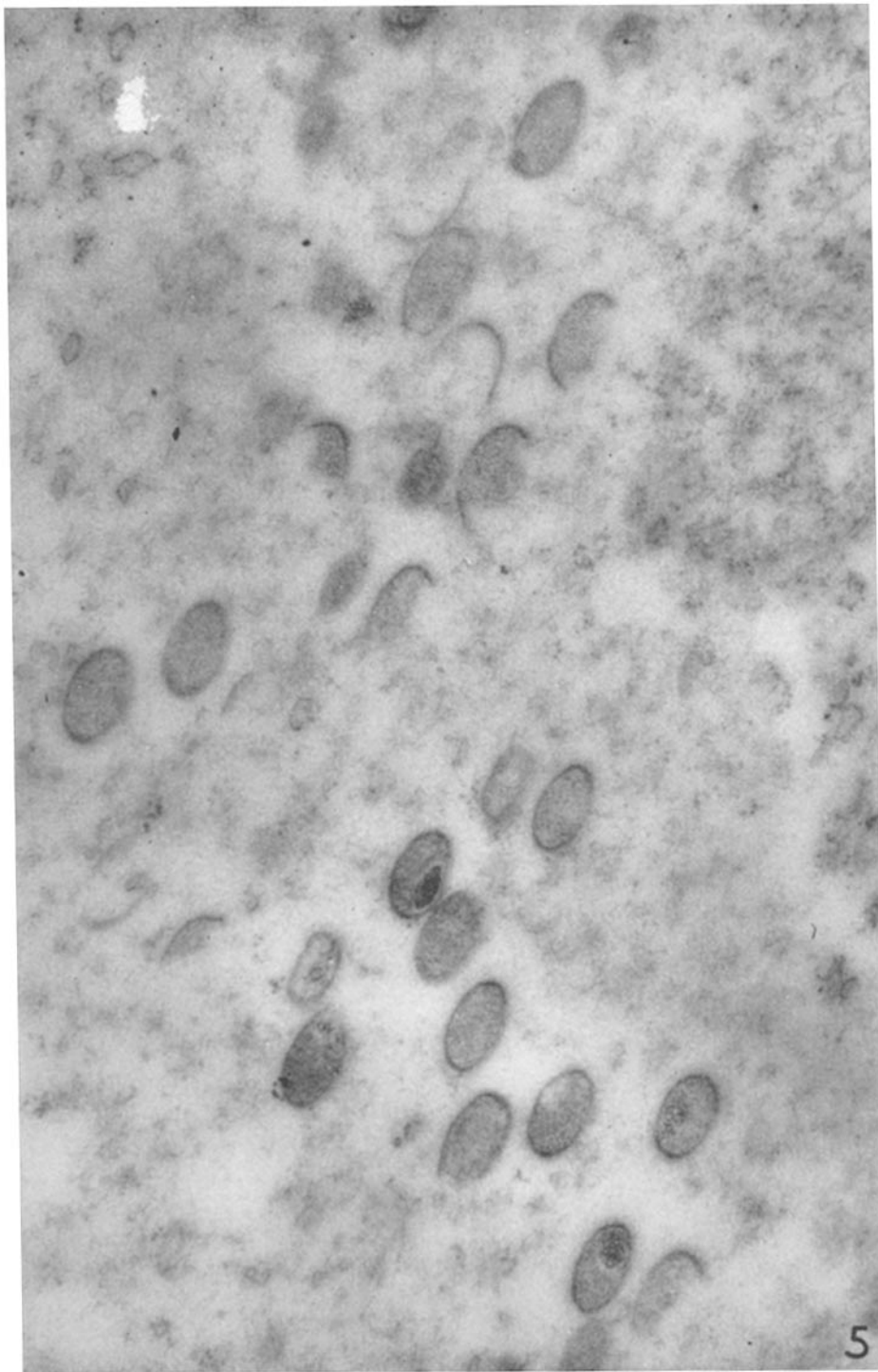
FIGS. 3 and 4. Consecutive serial sections illustrating variations in appearance of the virus produced by the level of transection. Particles *A* to *F* are transected in Fig. 3, but lie outside the plane of section in Fig. 4. The reverse is the case for particles *G* to *N*. Particles *O* to *S* appear smaller and less distinct at one level of section, but are recognizable as virus in the other, indicating that a peripheral section causes loss of density and superimposition of structures. Particles *T*, *U*, *V* contain nucleoids in one section but not in the other. Only the viroplasm of the particles *W* and *X* has been sectioned. The nucleoid in particle *Y* has been transected twice. $\times 46,500$.



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

PLATE 33

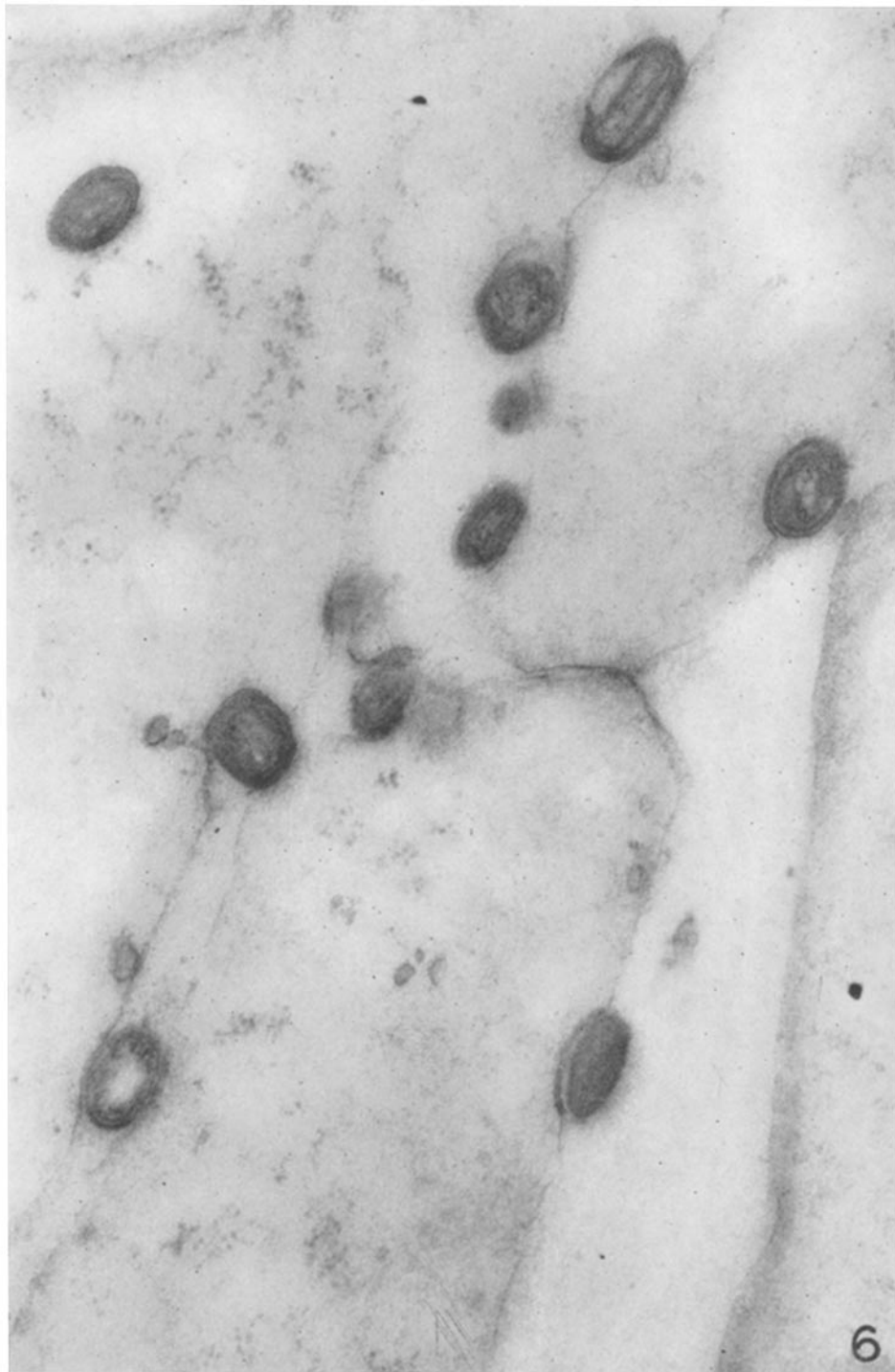
FIG. 5. Incomplete membranes, believed to represent developmental forms, near viral particles containing nucleoids. $\times 54,000$.



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

PLATE 34

FIG. 6. Viral particles lying in the extracellular space between four cells. They are composed of an inner body which may be disk-shaped, and a double limiting membrane. Several particles near the center of the illustration have been sectioned eccentrically. One particle near the upper left corner is within the cytoplasm of the host cell. $\times 60,000$.

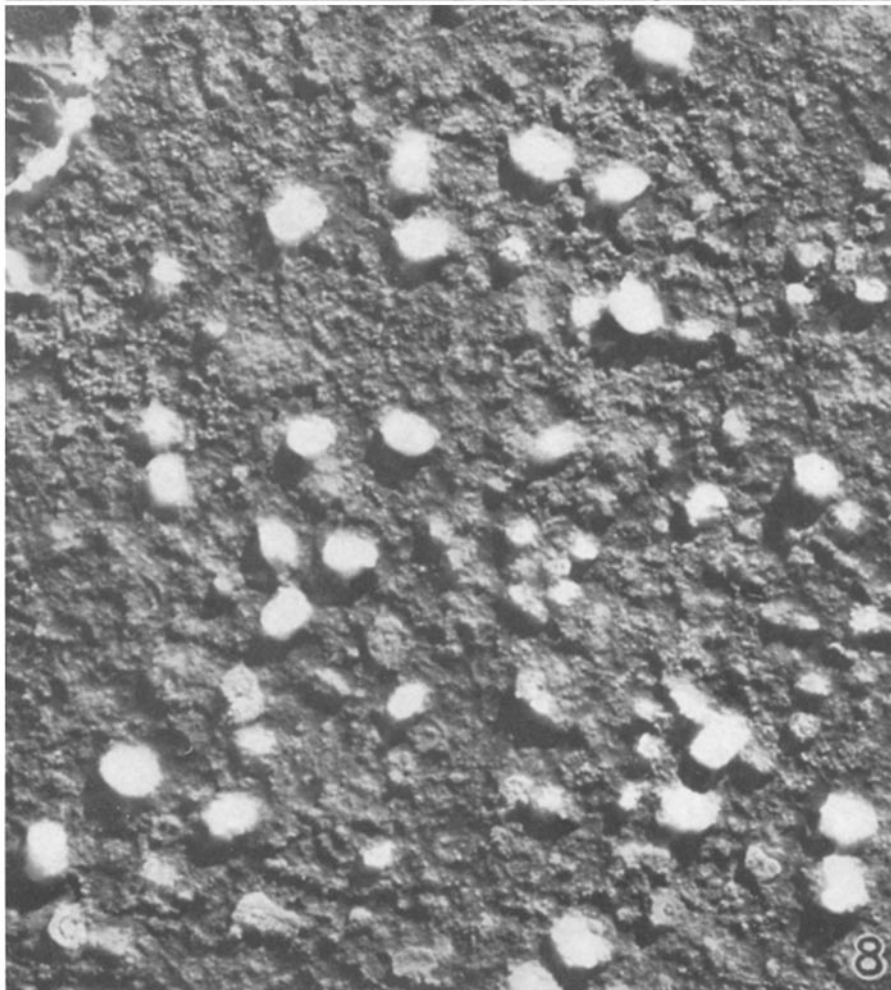
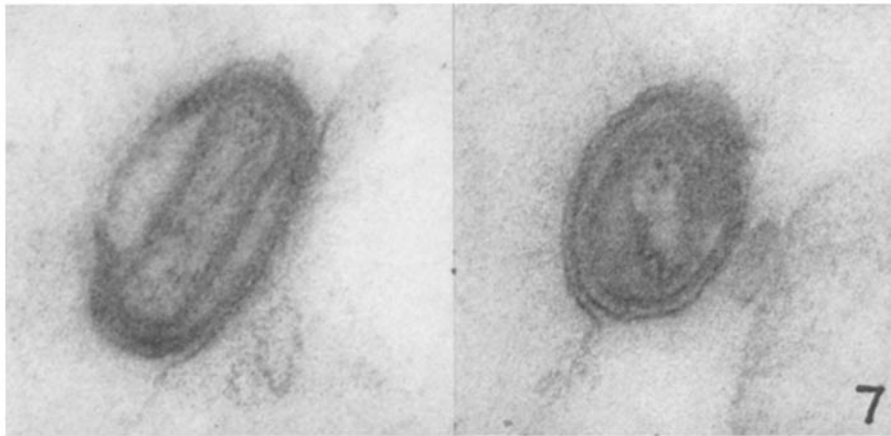


(Morgan *et al.*: Structure and development of viruses. II)

PLATE 35

FIG. 7. Parts of the preceding figure at higher magnification. Bordering the left side of particle at the right, the indented membrane of an adjacent cell exhibits marked increase in density to the electron beam. The internal structure of the particle at the left can be more clearly seen. $\times 131,000$.

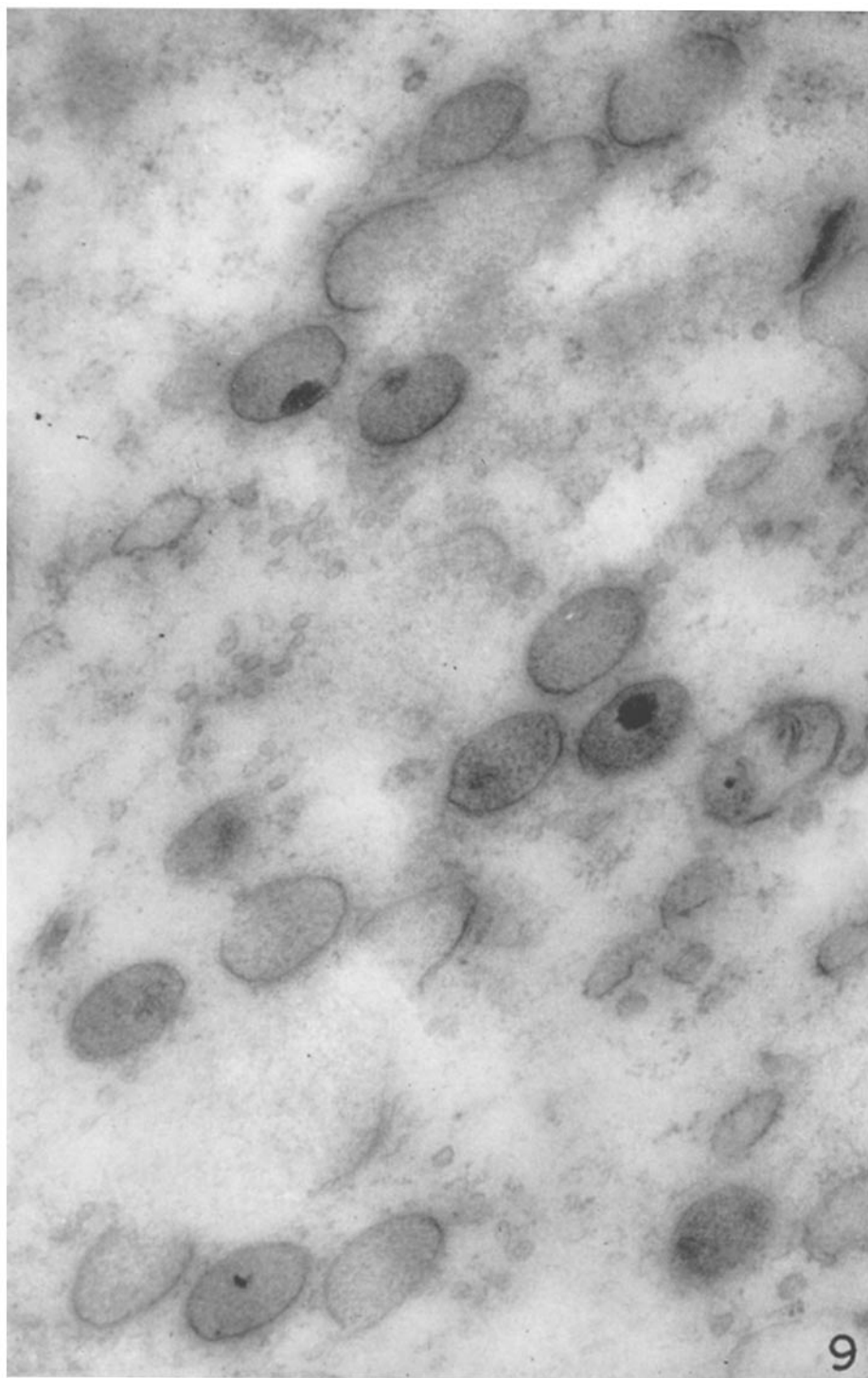
FIG. 8. A thick section through the cytoplasm of a cell containing vaccinia virus. The methacrylate was dissolved out by amyl acetate and the section was dried before shadowing. The presence of brick-shaped particles resembling those observed in ultracentrifuged preparations suggests that drying of the virus causes characteristic distortion. This figure is printed as a negative. $\times 25,000$.



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

PLATE 36

FIG. 9. Fowl pox virus in the cytoplasm of a host cell. Its structure resembles that of vaccinia virus. Incomplete membranes border aggregates of granular material which have replaced the characteristic components of cellular cytoplasm. Near the right border of the figure lies a mitochondrion which is nearly the same size as the virus but can be identified by the internal structure. $\times 56,500$.



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

PLATE 37

FIG. 10. The cytoplasm of a cell containing a large aggregate of granular material. Adjacent viral membranes are incomplete. Particles somewhat removed from this focus possess characteristic nucleoids, viroplasm, and limiting membranes. Several mitochondria are present near the lower border of the cell. Collagen fibers lie in the extracellular space adjacent to the upper border. $\times 38,000$.

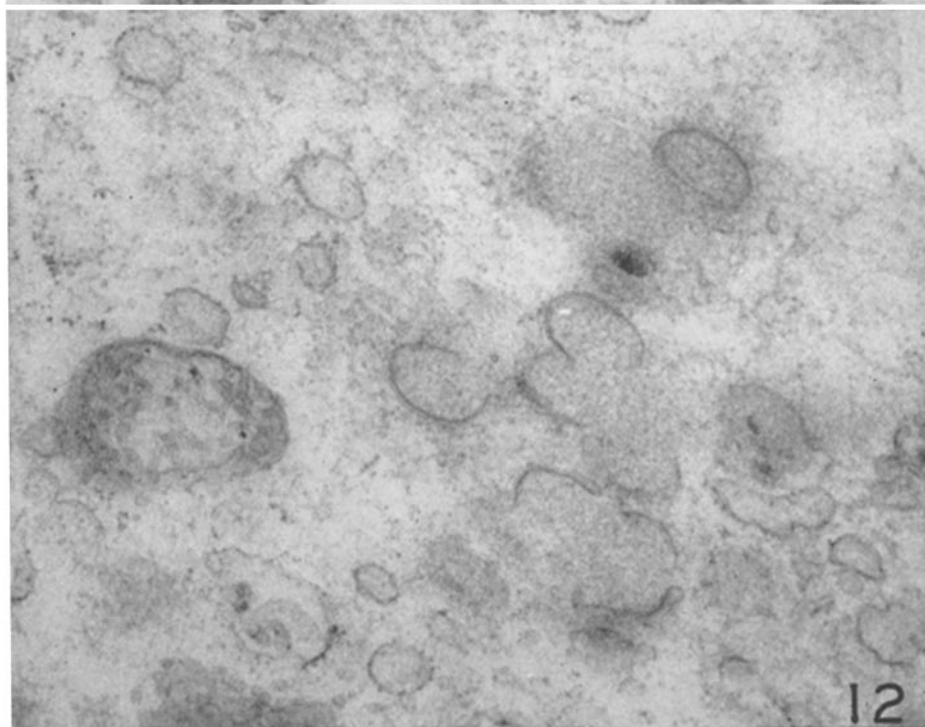
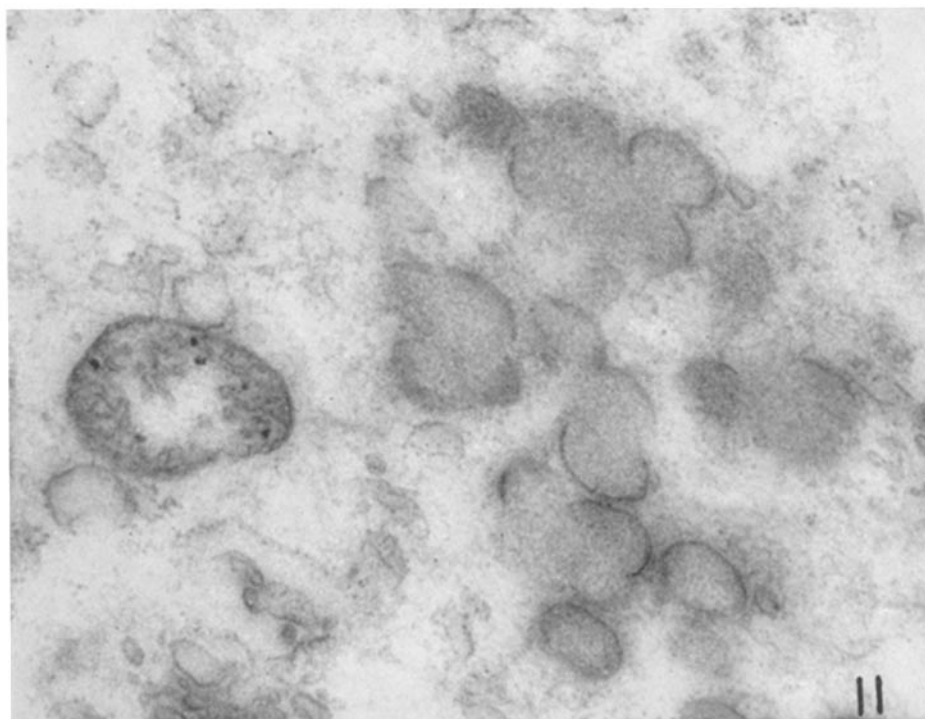


(Morgan *et al.*: Structure and development of viruses. II)

PLATE 38

FIG. 11. Multiple foci of granular material bordered by incomplete membranes. A mitochondrion lies near the left margin of the illustration. $\times 43,700$.

FIG. 12. The same field sectioned at a slightly different level. A dense structure which resembles a nucleoid lies within one aggregate of granular material. $\times 43,700$

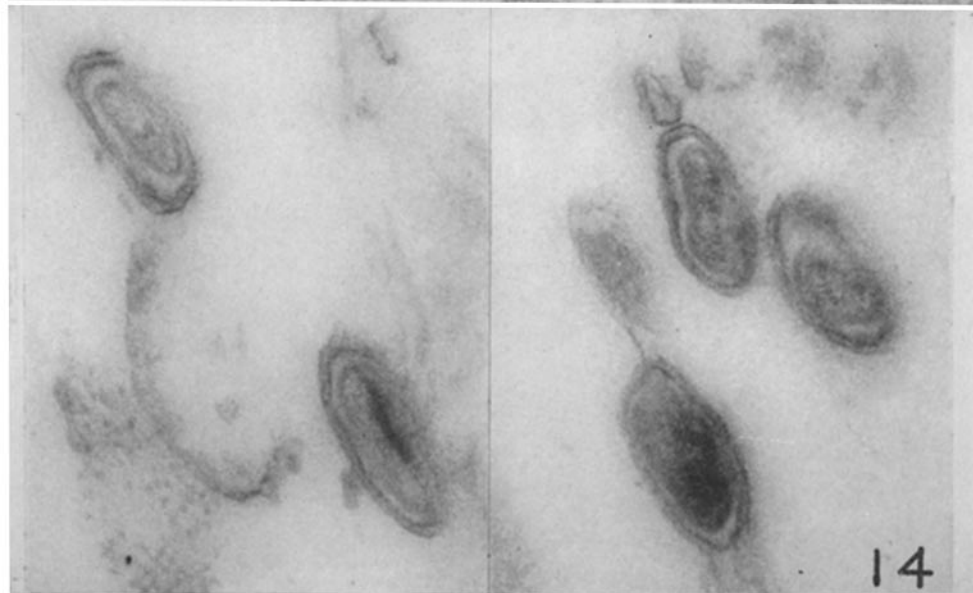
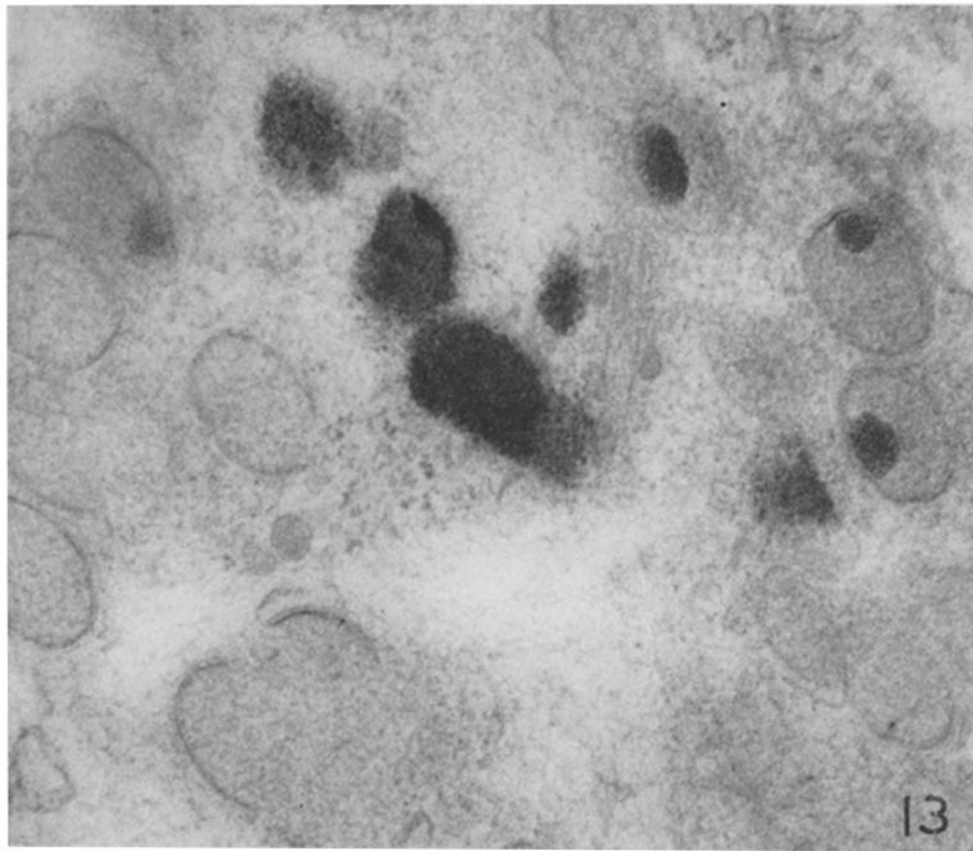


(Morgan *et al.*: Structure and development of viruses. II)

PLATE 39

FIG. 13. Multiple foci showing granular material and incomplete membranes. In addition, aggregates of a dense material similar to that constituting the nucleoids are present. $\times 63,600$.

FIG. 14. Extracellular forms of fowl pox virus. $\times 81,000$.



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