INTRACELLULAR FORMS OF POX VIRUSES AS SHOWN BY THE ELECTRON MICROSCOPE (VACCINIA, ECTROMELIA, MOLLUSCUM CONTAGIOSUM)*

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Plates 13 to 25

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Intracellular forms of viruses of the pox group are particularly well suited to study by the electron microscope because the relatively large size and characteristic morphology of the virus particles (15, 27) allow them to be recognized within infected cells cut in thin sections.

Morgan and Wyckoff's study of fowl pox was the first clear demonstration of the practicability of thin sectioning for study of animal viruses within host cells (19). Vaccinia-infected chorioallantois has been investigated by Bang (5) who suggested a relationship between mitochondria and virus, and by Wyckoff (31) who pointed out certain structures of low electron density which might have a role in virus development. Using similar material, we have called attention to the perinuclear position of early vaccinia virus development and to a matrix enveloping the earliest forms (13, 14). Molluscum contagiosum virus has been identified in thin sections of the human lesion in two laboratories (4, 9). In particular, Banfield *et al.* (4) presented evidence to suggest that the virus particles in such lesions were formed by segmentation and condensation from material comprising the septa which separated nests of mature virus. An impression might be gained from the above electron microscopic observations that pox inclusion bodies are merely aggregates of virus particles, similar to bacterial colonies, whose definition is hidden at times by an electron-dense matrix.

The present study was carried out to obtain further information on the development of pox viruses in cells having inclusion bodies as well as in cells in which they are absent. With the exception of molluscum contagiosum, studies of the pox group to date have been carried out using infected chorioallantoic membrane as a source of tissue. Beveridge and Burnet (7) have pointed out that it is rare in this tissue to find classical inclusions of vaccinia, and for this

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reason we have also studied infected rabbit cornea where inclusion bodies are plentiful. Inasmuch as true inclusion bodies can be seen in the chorioallantois infected with ectromelia (mouse pox), such material was also included. In addition, for purposes of comparison, a few observations were made of human skin infected with molluscum contagiosum, using the same methods of fixation and preparing thin sections as with the experimentally produced pox lesion.

Materials and Methods

Viruses[1].—The Nelson strain of vaccinia in its 16th egg passage was used for egg inoculation and a dermal strain passed 72 times in chick embryo tissue culture was used for the rabbit cornea experiments. Ectromelia virus was employed as the 6th egg passage, after isolation by Trentin from a spontaneous outbreak in mice (28, 29). Tissue infected with ectromelia virus was brought into our laboratory only after fixation. Molluscum contagiosum was received as biopsy material.

Eggs.—Eggs from a commercial hatchery were incubated at 35°C. for 15 days before inoculation in order to have well formed membranes.

Inoculation.—Chorioallantoic membrane inoculation was carried out according to the methods of Beveridge and Burnet (7). Inocula consisted of infected membranes ground with 1 ml. of saline per membrane, dilutions being planned to yield the type of lesion desired. For vaccinia, it has been found that confluent lesions, in which almost every cell is infected, yielded readily available infected cells under the microscope; consequently, undiluted material was inoculated. Because of a different tissue response, discrete pocks of ectromelia-infected tissue were found to be more workable, and such lesions were excised from membranes of a titration series regardless of the dilution of starting material.

Rabbit corneas were inoculated with vaccinia by placing a drop of tissue culture fluid on a freshly scarified surface and rubbing gently with the eye closed.

Harvest and Fixation.—Egg material was harvested at 72 hours and corneas were harvested at 96 hours. Whole egg membranes were removed and small pieces 3 to 4 mm. on a side were rapidly excised and placed in Palade's buffered osmium (21). 2 per cent osmic acid and veronal buffer were prepared separately and mixed in equal quantities immediately before using so that the final mixture was a 1 per cent solution of OsO_4 buffered at pH 7.3–7.4.

Cornea material was obtained by excision with a sharp scalpel from a rabbit immediately after it was sacrificed. The tissue was fixed as above, washed, and placed in 70 per cent alcohol before the lesion was cut from the tissue. The molloscum lesion was treated in similar fashion.

The period of fixation was 4 hours, followed by an hour of washing, and serial dehydration in 70 per cent, 95 per cent, and absolute alcohol each for hourly intervals. The tissue was imbedded according to the method of Newman, Borysko, and Swerdlow (20). It was placed in a 1:9 mixture of methyl:butyl methacrylate which was polymerized at 60°C. overnight with 2,4-dichlorobenzoyl peroxide as catalyst. This procedure leaves the tissue imbedded in hard, clear plastic.

Cutting.—Sections were cut on a thermal expansion microtome designed by Dr. Keith R. Porter using the glass knife of Latta and Hartmann (17). Ribbons were collected on 10 per

[1] We are indebted to the following investigators for supplying us with these materials: Dr. Bernard A. Briody and Dr. Francisco Duran-Reynals for the vaccinia agents; Dr. John J. Trentin for the ectromelia-infected eggs and histological slides; and Dr. Jack J. Albom for the molluscum contagiosum lesion.

cent acetone. Slightly wrinkled sections were often flattened by flotation on water at 60°C. Section thickness was evaluated by the light interference method of Porter (23) only red or gold sections being used.[2] Sections were mounted on collodion-covered grids and then usually viewed directly without removal of the imbedding medium. In a few special cases, the methacrylate was removed with toluene and the section shadowed with palladium before examination. An RCA type EMU electron microscope was used.

Criteria for Recognition of Virus Particles.—Identification of mature[3] virus particles was fundamental to this study because they are the end result of the developmental process and the key to its elaboration. The following characteristics are common to vaccinia, ectromelia and molluscum contagiosum and were useful for identification: (*a*) ***density***, mature virus particles are always the most dense structures in a tissue section causing them to be conspicuous; (*b*) ***size and shape***, mature particles are always round to oval, and uniform in appearance. The size of the particles was difficult to determine in sections because the particles were not always cut at their major or minor axis. Purified virus has been given values of about 210 by 260 mμ and the virus particles which we have seen in thin sections are of this order of magnitude. The uniformity of size, shape, and density of the virus particles enable one to readily differentiate them from mitochondrial fragments which vary in all three of the above properties; (*c*) ***numbers and occurrence***, virus particles are found only in infected tissue and never in normal controls. They are present in large numbers both intra- and extracellularly and, in the case of vaccinia, 72 hour lesions contained more of the particles than 48 hour lesions.

RESULTS

Normal Cells.—In eggs, the ectodermal layer only was infected with vaccinia virus, and all the observations recorded here pertain to that layer of tissue. Normal ectodermal cells (Fig. 1) were close packed with little intercellular space except at the surface where some drying effects were present. The cells were roughly hexagonal, although they varied greatly in outline, and the nuclei were usually oval. Nuclear and cell membranes were sharply defined and the double nuclear membranes and endoplasmic reticulum in the cytoplasm of sectioned cells described by Palade (21) appeared often. Nucleoli were small and varied in number and shape.

Close attention was given to the morphology of normal mitochondria because they were present in the cytoplasm and bore a similarity in cross-section to virus particles. Unlike virus, they were randomly distributed and varied considerably in size, shape, and electron density. Round fragments predominated but fragments of various lengths were common. Long filaments and branching mitochondria were rare. Some of the round fragments had diameters of 200 to 300 mμ, placing them in the size range of the virus particles but ir-

[2] We are indebted to Dr. Keith R. Porter, The Rockefeller Institute for Medical Research, for permitting us to have a copy of his microtome made, and for instructing us in his light interference method, as yet unpublished.

[3] The term "mature" virus has been adopted in order to differentiate complete and, presumably, infectious virus particles from the structures to be designated "developmental bodies." In addition, it is felt that the older term, elementary bodies, is ambiguous in view of the findings reported in the present paper.

regularity of size, shape, and density of the mitochondria contrasted sharply with the uniformity of the virus particles. Furthermore, mitochondria in infected cells were usually greatly swollen and no difficulty was encountered in differentiating them from virus.

Mitochondrial swelling was seen in some cells of normal tissue (Fig. 2). Swelling of fragments of various lengths produced bizarre forms and the phenomenon was considered to be associated with unhealthy cells which were perhaps becoming dehydrated. Normal membranes had been "dropped" as for inoculation and carried for 3 days, as were the inoculated eggs, thus enhancing the probability of non-specific degeneration in the outer layer of cells.

It was often difficult to orient small bits of a membrane in the methacrylate block and epithelial cells of the entoderm resembled, at times, those of the ectoderm. Certain guides were available, however, and there was seldom any doubt as to the nature of a given field under observation. The outermost layer of entodermal cells always had prominent microvilli (6) and the same cells contained rounded osmiophilic granules in great numbers. These granules, probably lipid, were replaced by a spongy network of vacuoles in infected membranes. The effect was non-specific because no virus was present in the entoderm, and it was seen in membranes infected with other viral agents.

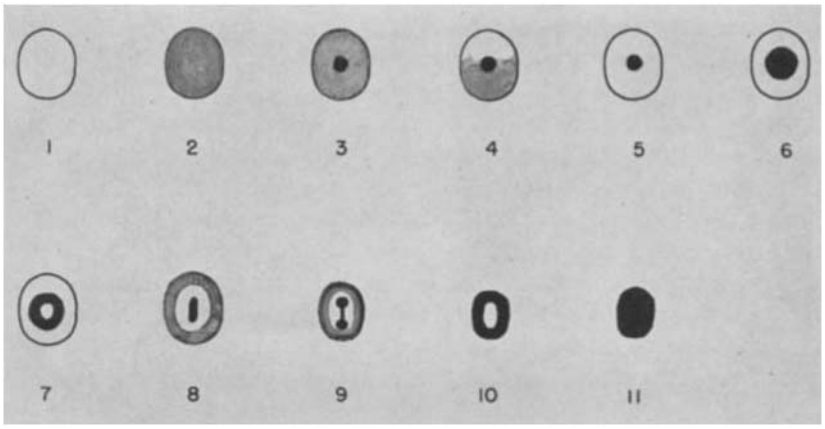
Cells of normal rabbit cornea presented a slightly different appearance (Figs. 10 and 11). In the deeper layers, cells were close packed and nuclei were osmiophilic. Mitochondria were thread-like and located close to the nucleus (Fig. 10). At the surface of the cornea, however, the cells were flatter, the intercellular spaces larger, and bridges between cells were common. The nuclei of such cells had lost their osmiophilia and the mitochondria were more widely distributed in the cytoplasm. Pale nuclei and scattered mitochondria have been assumed to be indicative of dead or senescent cells and all observations in infected tissue were confined to the lower layers of cells for that reason.

Vaccinia-Infected Cells of the Chorioallantois.—After 72 hours, vaccinia lesions of the chorioallantoic membrane presented a picture of complete ectodermal involvement; every cell observed was infected. Except for hyperplasia there was little tissue response. The basement membrane was apparently not broken and no virus was seen in the mesoderm or entoderm. The ectoderm contained much more virus than at 48 hours (14) and individual cells were more heavily infected. It was, nevertheless, possible to find cells in all stages of involvement.

A typical 72 hour infected cell (Figs. 4 and 5) appeared as follows: The nucleus was often distorted and the nucleolus enlarged. In the cytoplasm, mature virus particles could be recognized as electron-dense bodies of uniform elipsoidal shape with minor axes ranging around 160 mμ and major axes often twice that of the minor axis. They were present in great numbers, particularly at the periphery of a heavily infected cell. Near the nucleus a matrix area of

greater density than the rest of the cytoplasm was present within which were located what were taken to be "immature" or developmental forms of the virus. Mitochondria were reduced in number and those still present were swollen to many times their normal size.

The viral forms were round to oval in outline with diameters of 280 to 300 mμ. They were always bounded by a thin membrane, and their internal structure was variable. In areas where such forms were few in number (Fig. 3) the



TEXT-FIG. 1. Schematic diagram of various forms found in infected cells and apparently related to the development of pox viruses. It is assumed that these figures represent particles which have themselves been sliced, with the exception of No. *11*.

1, empty membrane or hollow sphere; *2*, membrane surrounding a homogenous material of low electron density; *3*, membrane, low density material, and a very dense central granule; *4*, similar to number *3* except only partially filled with the homogeneous material; *5* and *6*, membranes surrounding granules (centrally or excentrically located) of different sizes; *7*, a "hollow" central granule; *8* and *9*, forms seen particularly with ectromelia in the chorioallantois and with vaccinia in the cornea, resemble mature virus but contain bars or dumbell-shaped interior structures; *10*, thick walled virus in section; *11*, the most common form of mature virus.

majority of them appeared as "empty" circles, representing cross-sections of hollow spheres. At other times the circles were filled with a homogeneous material of low electron density (Figs. 4 and 5). In some, a dense granule was visible within and in others the granule was present in sizes ranging from the almost invisible up to that of mature virus. In many, the low density material only partially filled the structure, or was absent, giving the impression that the granule increased in size at the expense of the less dense material.

Thus, in the perinuclear areas the following viral forms were seen and they are represented diagrammatically in Text-fig. 1: *1*, membranes alone with apparently hollow interiors; *2*, membranes enclosing a homogeneous material of

low electron density; *3*, the same as *2* but with a dense central granule; *4*, membranes containing the granule but lacking some of the low density material; *5* and *6*, membranes containing the granule alone in various sizes; *7*, membranes containing a doughnut-shaped segment of a large granule; *i.e.*, a granule which had been sliced by the knife. These forms were concentrated in the matrix area of increased electron density, but in advanced stages of cellular infection they were also visible scattered among the mature particles. Such matrix areas of greater density were less obvious or absent in cells filled with mature virus. Mature virus, either intra- or extracellular, was always uniform and dense. In very thin sections, where the virus particles had been sliced, the interior was often transparent as though the particles were hollow (Fig. 5).

Ectromelia-Infected Cells of the Chorioallantois.—Ectromelia virus grew slower in eggs than did vaccinia and at the end of 72 hours the lesions produced by ectromelia macroscopically resembled the 48 hour vaccinia lesion. A profound difference in tissue response was observed, however, under both the light and electron microscopes. In ectromelia lesions the ectodermal basement membrane was completely destroyed and no sharp demarcation between ectoderm and mesoderm could be observed. The entoderm was intact, however, and vacuolated as described above. In addition to the destruction of ectoderm, which accompanied its proliferation elsewhere, there was an infiltration of leucocytes in great numbers. In the electron microscope the granules of these cells were irregular in size and shape and very dense. The nuclei always displayed an osmiophilic band around the edge and no virus was seen within them. Virus was seen in the mesoderm as far as the basement membrane of the entoderm.

The intracellular development of ectromelia was also strikingly different from that of vaccinia. In contrast to the situation in vaccinia, the optical microscope revealed many eosinophilic inclusion bodies of varying size in the cytoplasm of ectodermal cells. Under the electron microscope (Figs. 6 and 7) they appeared as large, dense, amorphous masses, usually round, but occasionally oval or bean-shaped. Although there was a sharp line of demarcation between inclusion body and cytoplasm, no limiting membrane was observed. Large inclusion bodies 5 to 6 mμ were almost as big as the nucleus and small ones, about 1 μ in diameter, approached the size of swollen mitochondria but the first stages of the inclusions could not be determined. The majority of cells contained only one inclusion but it was not uncommon to see two in one cell.

Small numbers of virus particles were usually associated with each inclusion and they were located around the edge of the latter in a clear zone (Figs. 7 and 9). The developmental bodies similar to those described for vaccinia were seen (Fig. 8) and one additional feature was noticed. Occasionally, virus particles the size and shape of mature virus, instead of being hollow, exhibited a bar or dumbell-shaped structure in the center (Fig. 9).

In addition to the clear halo around the inclusion bodies, the inclusion it-

self at times had some internal structure which looked to us like plaques and which gave the inclusion a moth-eaten appearance (Fig. 7).

Vaccinia-Infected Cells of the Cornea.—Vaccinial lesions of the rabbit cornea as disclosed by the optical microscope were thoroughly described by Tyzzer (30) in 1904 and corneal tissue has long been considered the tissue in which Guarnieri inclusion bodies could be best demonstrated.

In our corneal material, the macroscopic lesion produced by vaccinia 4 days after infection was a small crater 2 to 3 mm. in diameter. Microscopically, it was determined that only a narrow band at the perimeter contained inclusion bodies. The center of the crater was devoid of epithelium and the cells around it appeared to be normal. The basement membrane was intact and few signs of infiltration were noted, although an occasional leucocyte could be tentatively identified. (Fig. 13).

With the electron microscope a dual cellular response to the vaccinia virus was observed. Some cells contained dense, amorphous masses which resembled the inclusion bodies of ectromelia in every respect (Figs. 12, 13, 15, 17 to 19). In other cells, however, no inclusion bodies could be found and virus forms were seen as described above for vaccinia-infected chorioallantois within typical matrix areas (Figs. 12 to 14, 16).

In infected corneal cells containing such matrix areas, the latter were close to the nucleus and often in contact with it (Fig. 12) causing an indentation of the nuclear membrane. In such areas, rich in the apparently early forms of the virus, mature virus was formed without the production of true inclusion bodies. It was possible to detect the limits of the dense matrix area even in sections from which the imbedding medium had been removed (Fig. 16).

The true inclusion bodies (Fig. 15, 17) were much more dense to electrons than were the matrix areas. They were of varying size with some as large as 4 μ in diameter. The edges were more distinct than those of the matrix areas and even of ectromelia inclusions. At times (Fig. 15), it seemed as if a limiting membrane were present, and the interiors were not uniform in appearance. No indication of the origin of inclusion bodies could be found with certainty. Occasionally within inclusions, structures with diameters of 0.5 to 0.75 μ could be found, these being larger than the virus forms but not too large to be swollen fragments of mitochondria. In addition, there was an apparent decrease in the number of mitochondria in infected cells but no satisfactory method of quantitation has been found to ascertain the significance of the phenomenon.

On the other hand, it was possible to recognize stages in the change from inclusion body to new virus and to present a working hypothesis of the probable series of events. Pitting, to give the moth-eaten appearance described above for ectromelia, was seen (Fig. 15) and, in other instances, such pitting had advanced to a point where the dense material remained only as isolated islands (Figs. 17 to 19). Virus developmental bodies were clustered in the spaces

between the islands in large numbers giving the impression that the breaking up of the inclusion was related to their production, (Fig. 18). In more advanced stages the islands were smaller and mature virus replaced the developmental bodies (Fig. 19). The virus particles at this stage contained internal structures (bars and dumbells) as does the ectromelia virus surrounding an inclusion body (Fig. 9). They are considered to be transitional between virus developmental bodies and the mature virus (Figs. 5 and 6).

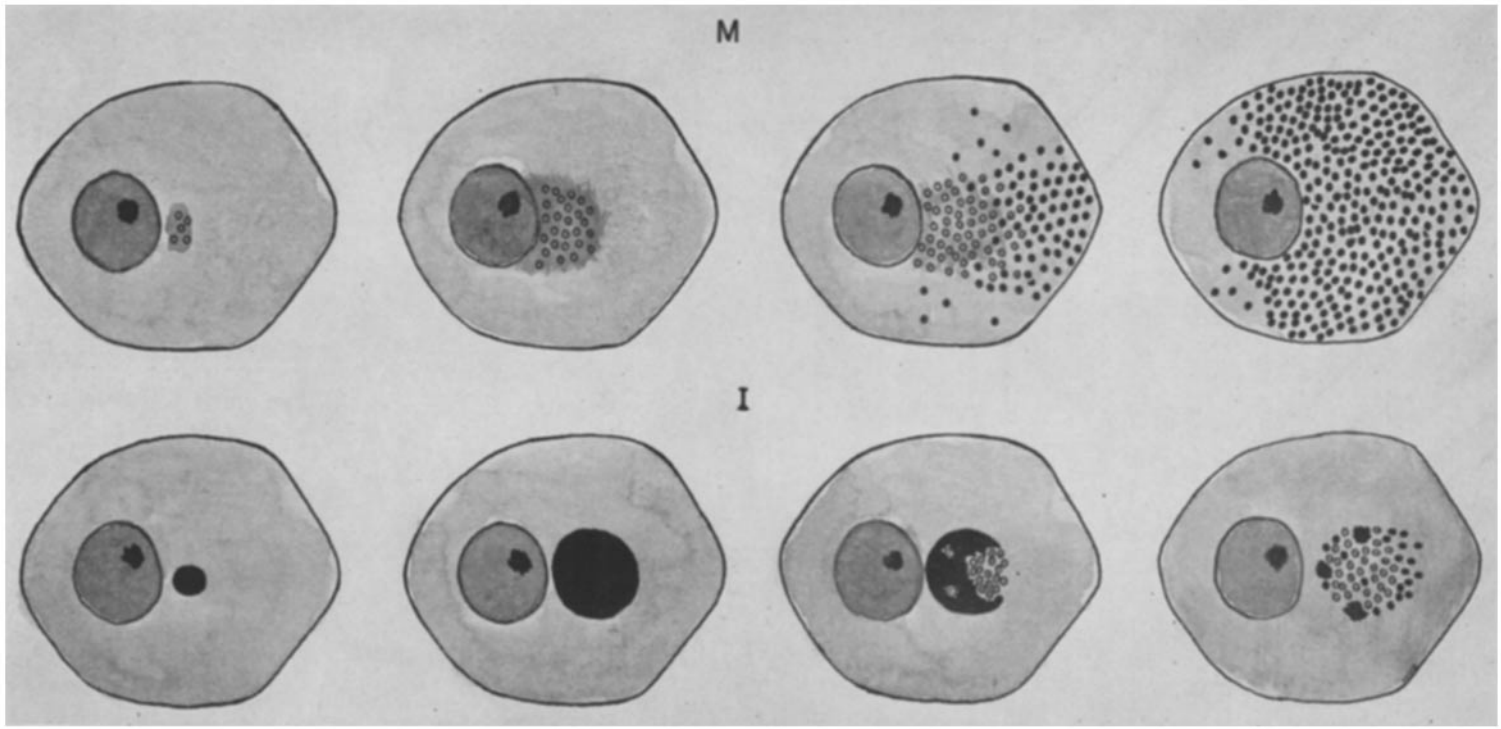
In the basal layers, virus particles were seen ringing some of the cells and virtually filling the intercellular spaces (Fig. 20). The cells in these regions exhibited little or no involvement and it has been assumed that the virus was liberated from infected cells located in the upper layers.

Molluscum Contagiosum.—The structure of the molluscum inclusion has recently been studied by Rake and Blank (24), Banfield *et al.* (4), and by Blank (9) and was reexamined here only for the purpose of comparison with vaccinia and ectromelia. As was indicated in the micrographs of Banfield *et al.* (4), developmental bodies were found in the cytoplasmic septa which separate the pockets of virus particles. When the plastic imbedding medium was not removed from sections, the developmental bodies resembled those described for the other two viruses. (Figs. 21 to 23).

DISCUSSION

A scheme of intracellular virus development based on morphological evidence alone is subject to uncertainties in assigning a place to the various observed stages in the chronology of events. In this study, only evidence based on repeated observations was credited, and single or anomalous findings were not considered. Absolute quantitation of the frequency of a given observation has not been possible because each tissue studied exhibited a wide range in respect to the degree of involvement of different cells and, in addition, the preparation of usable thin sections has not been sufficiently reproducible for this purpose.

The data outlined have stimulated us to propose the following interpretation as a working hypothesis. The accompanying diagram (Text-fig. 2) is a schematic illustration of the proposed mode of development for vaccinia which can proceed in one of two ways, either with or without the production of inclusion bodies. The upper line of the diagram, labelled *M*, illustrates the type of host-parasite interaction seen in the absence of inclusion bodies. The first sign of virus development of the *M* type is the formation of an electron-dense matrix in the cytoplasm near the nucleus. Within the matrix "empty" circles or "hollow" spheres can be seen. As the matrix increases in size, the structures within it, which we have called "developmental forms" of the virus, increase in number and complexity. As the numbers of developmental forms increase so do the numbers of finished or "mature" virus particles. The change from developmental forms to mature virus is accompanied by a disappearance of the matrix, the end result being a cell filled with mature virus only.



TEXT-FIG. 2. Diagrams representing the two observed patterns of vaccinia virus development, involving matrices or inclusions. Our interpretation of these observations follow: Upper row (*M*): A matrix is formed near the nucleus and developmental forms appear within it. At this stage most of them are of the "hollow sphere" type. As the matrix becomes larger the developmental bodies increase in number and the forms shown in Text-fig. 1 (*1* to *7*) can be seen. As the matrix begins to thin out, mature virus can be seen at its edges. When the infection has progressed to the extent that the cell is filled with mature virus, no matrix or developmental bodies can be seen. Bottom row (*I*): Dense homogeneous inclusion bodies appear and increase in size. Eventually they appear to be digested away revealing developmental bodies and, finally, mature virus.

When inclusion bodies are formed, as in the type I development, the whole process is obscured by a very dense homogeneous mass but the developmental bodies can be seen as the inclusion body disappears. However, the underlying processes of virus maturation appear to be similar to the above.

Unlike mature virus, developmental bodies are not randomly distributed in the cell. They occur in clusters or nests within matrix areas or inclusion bodies and occasionally scattered throughout the cytoplasm of heavily infected cells. They are found only in infected cells and not in the controls and there is an intimate association between developmental bodies and mature virus. They are uniformly round to oval and slightly larger than mature virus particles, ranging in size from 290 to 300 mμ. They are less dense to electrons than the mature virus and exhibit a more complex morphology. That they are unique structures peculiar to infected cells is clear, but the evidence for assuming them to be precursors of the virus particles is more subtle. For purposes of argument one might assume that they are the result of virus degradation instead. If this were the case, one would expect to find a large number of them in cells filled with virus because more of the particles would be older; by the same token, they should be visible extracellularly. None are seen in either circumstance. In the newly infected cell, developmental bodies are always located near the nucleus in the matrix area (Fig. 3). In cells in which there are numerous developmental bodies, there is also an increase in mature virus (Fig. 4) and the developmental bodies are still found in the matrix area. When cells are filled with mature virus, the matrix area is not present and only a few developmental bodies can be seen. These arguments pertain to the development of vaccinia in the egg (and in some corneal cells), but similar observations on ectromelia and molluscum lend support to the concept that the developmental bodies are early forms of the virus.

No matter where they are found, the developmental bodies display the same characteristics, even though they have been studied more intensively in vaccinia-infected chorioallantoic membranes than in the other host-virus systems investigated. When developmental bodies are few in number the "empty" circle or hollow sphere type is most common and this is assumed to be the earliest observable form. Levinthal and Fisher (18) have studied the structural development of a bacterial virus with the electron microscope and the events described by them seem to have many features in common with those described here. They were able to follow phage development in respect to time and showed that "empty spheres" were the first structures to appear. These spheres, which looked like doughnuts in their preparations, increased in number followed by an increase in numbers of mature phage particles. As the amount of mature phage increased the early forms gradually disappeared. This time-study strongly supports the views that the doughnuts are, indeed, precursors of the phage. Anderson *et al.* (3) showed that the doughnut shaped structures were ghost-

like empty spheres. Schlesinger and Werner's observations (33) also bear on this problem. Forms seen by them during a study of incomplete influenza virus were about twice the size of mature virus and gave the appearance of "doughnut shaped–ghosts."

The next form in the development of the pox viruses is thought to be the type which is filled with homogenous material of low electron density. When a dense, internal granule is present, it is often accompanied by a disappearance of the homogeneous material. Granules of different sizes can be seen and, in our working hypothesis, they grow at the expense of the homogeneous material. The largest granules are approximately as large as mature virus but the transition from developmental bodies to mature virus is not clear. At times large granules are themselves "empty" when sectioned, giving the whole developmental body the appearance of two concentric circles. On the other hand, the very young virus at the edges of ectromelia inclusions, within vaccinia inclusions, and occasionally in molluscum contagiosum–infected cells, definitely has internal structure in the form of bars or dumbells. This type of interior has not been seen when the mature virus at the periphery of cells or outside of cells is sectioned. In such cases the virus particles again appear empty displaying dense, thick walls. Banfield *et al.* (4) have reported similar findings of "empty" virus particles with molluscum contagiosum virus in formalin-fixed thin sections. Dawson and McFarlane (10) examined isolated vaccinia virus particles after having treated them with pepsin and found that they contained a dense "nucleus" and a thin membrane surrounding each particle. The substance between the outer limiting membrane of the virus and the "nucleus" was missing giving a picture of the particle which was exactly the reverse of that seen in the present work for both osmium and formalin (14) fixed mature virus. It would seem that the material removed by the pepsin constitutes the thick wall of our particles. The center "nucleus" portion is absent and may have been extracted by the fixative procedure, but we have no satisfactory explanation. We have never seen the outer limiting membrane on a mature virus particle but it could be tightly adsorbed to the somatic portion and not distinguishable. Peters and Nasemann (22) have confirmed Dawson and McFarlane's (10) work with vaccinia virus particles removed directly from a lesion and treated with pepsin. Their pepsin-treated vaccinia particles resemble members of the psittacosis-lymphogranuloma venereum group of viruses.

The stages of development described are, obviously, only part of a complete cycle. No information is at hand regarding the entrance of virus into the cell or the events leading to the formation of the matrix area or the inclusion body. Virus particles touching and even indenting cell walls were seen, but recently infected cells cannot be recognized until enough particles or developmental bodies are present to differentiate them from mitochondrial fragments. In

fact, little can be said of the extracellular fate of the virus. Particles have been observed crowding the intercellular spaces in areas where the cells are uninfected, or only slightly involved. (Fig. 20). One may suppose them to have come from neighboring cells, which have released virus in large quantities, and infiltrated into the new areas through the intercellular spaces.

Apparently the three pox viruses studied undergo a complex life cycle involving developmental bodies at one stage. These bodies seem to be obligatory parts of the cycle and independent of the presence or absence of inclusion bodies. Inclusion body production, on the other hand, is a response depending on a little understood host-parasite relationship. One virus, vaccinia, produces them regularly in the cornea but not in the egg, and one tissue, the chorioallantois of the chick embryo, possesses them frequently with ectromelia but not with vaccinia. The underlying process of virus maturation through a series of changes in the developmental bodies appears to be the same, however, and both types of development can be seen in a single section of corneal epithelium.

In spite of the fact that virus formation looks the same in matrices and inclusion bodies, there is marked difference between them for which we have no explanation, unless the difference in growth rate of the virus in a given host is a factor. Vaccinia produces no inclusions in the chorioallantois but the slower growing ectromelia does. In the cornea, where vaccinia grows more slowly than in the egg, inclusions are produced some of the time. There is no way to distinguish the difference between viral matrices and inclusions with the electron microscope except that the latter have a greater density to electrons. It has not been possible to determine the origin of either matrix or inclusion. Both the nucleus and the mitochondria have been considered but neither can be definitely implicated. Matrices are often in contact with the nucleus and always near it. Nuclear distortion and nucleolar enlargement are common findings. Anderson and Wilbur (2) have shown that heparin will release a nuclear gel without disrupting the nuclear membrane and it is conceivable that other agents could do the same. The present finding of a decreasing amount of matrix or inclusion material as the amount of virus increased correlates well with the studies of Bland and Robinow (8). They followed the development of vaccinia in cultures of rabbit cornea and reported that small inclusions were strongly Feulgen-positive. As the inclusions grew the affinity for the dye decreased and elementary bodies were only weakly positive or negative. Their work allows us to infer that the matrix observed in the electron microscope may be richer in desoxypentose nucleic acid than the virus which is being formed within it. On the other hand, molluscum contagiosum virus apparently does not form inclusion bodies or matrices of the type shown for vaccinia or ectromelia, and here the mature virus particles themselves are Feulgen-positive. Rake and Blank (24) have demonstrated a progressive increase in the intensity of the reaction

indicating that differences in chemical composition exist between members of the pox group, if molluscum contagiosum can be rightly included in this group.

Bang (5) suggested that there is a relationship between mitochondria and vaccinia virus and Ackermann and Kurtz (1) have found herpes virus associated with the mitochondrial fraction of cells. However, Schwerdt and Pardee (26) found the bulk of Lansing poliomyelitis virus in the submicroscopic (microsomal) fraction of infected cotton rat tissue. In the tissues observed in the present study there seems to be a reduction in the number of mitochondria in infected cells, and developmental bodies often occur in clusters reminiscent of mitochondria, but they have never been observed within a mitochondrion. The reduction of mitochondrial numbers at a time when inclusion bodies appear would lead one to suspect that they might be interrelated. The mitochondria might have migrated to the area near the nucleus and there yielded their materials (enzymes) for the synthesis of the matrix and inclusion material in which the virus particles develop. There is no proof that this occurs but inclusion bodies do, at times, contain structures that bear a strong resemblance to mitochondria in the swollen state. Attempts at correlating mitochondrial numbers with specific changes are difficult because there is thought to be a normal decrease in old cells (32), but future methods may overcome this difficulty.

The virus of rabbit myxomatosis has recently been considered to belong to the pox group (12) and electron microscopic studies of infected tissues have been carried out by Epstein, Reissig, and DeRobertis (11). They observed dense particles from 50 to 100 mμ with some as large as 900 mμ but the majority were within a range of 150 to 350 mμ. Such a range of particle sizes has not been observed with other pox viruses. They were frequently surrounded by a clear halo but a limiting membrane was not described, and they feel that the halo might be a reaction phenomenon of the cytoplasm itself (25). Many smaller, less dense particles were seen, often in clumps suggesting a growth process by condensation to form inclusions. Their specimens were fixed in either 10 per cent buffered formalin or 2 per cent buffered osmium tetroxide but the imbedding medium was always removed, a procedure which causes the production of artifacts as pointed out by Hillier and Gettner (16), and Palade (21).

SUMMARY

The intracellular development of three pox viruses has been studied with the electron microscope using thin sections of infected tissue.

Cells infected with vaccinia, ectromelia, and molluscum contagiosum viruses all form developmental bodies preliminary to the production of mature virus. Developmental bodies, believed to be virus precursors, are round to oval,

slightly larger than mature virus particles, less dense to electrons, and have a more varied morphology.

It is suggested as a working hypothesis that the process of maturation of a virus particle takes place as follows. In the earliest form the developmental bodies appear as hollow spheres, imbedded in a very dense cytoplasmic mass constituting an inclusion body, or in a less dense matrix near the nucleus in cells without typical inclusion bodies. The spheres become filled with a homogeneous material of low electron density. A small, dense granule appears in each developmental body and grows in size at the expense of the low density material. Following growth of the granule, particles are found with the dimensions of mature virus and having complex internal structure resembling bars or dumbells. Mature virus is ovoid and very dense to electrons. An "empty" interior may be found within its thick walls.

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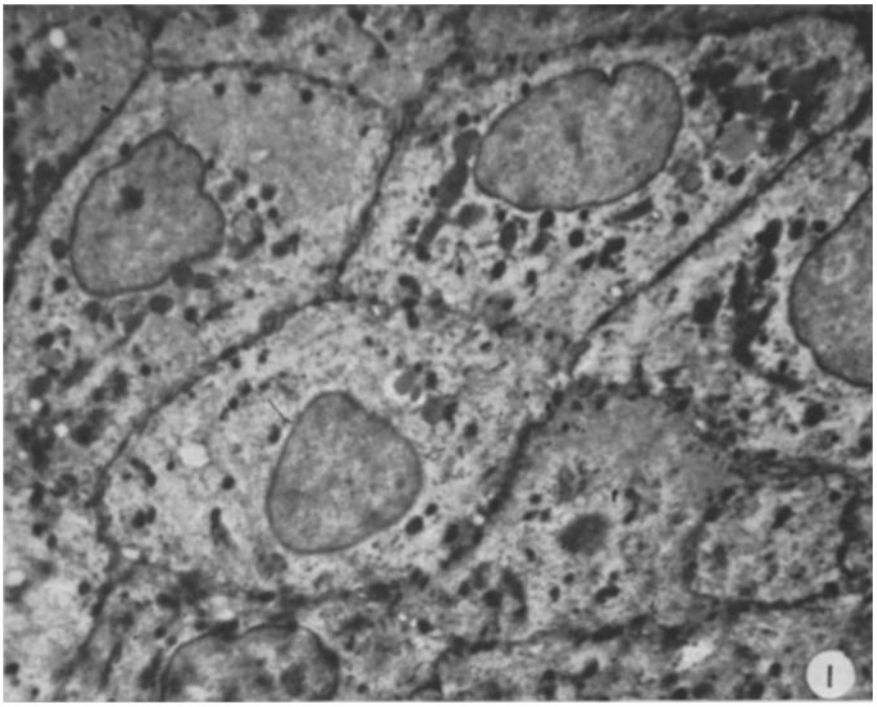
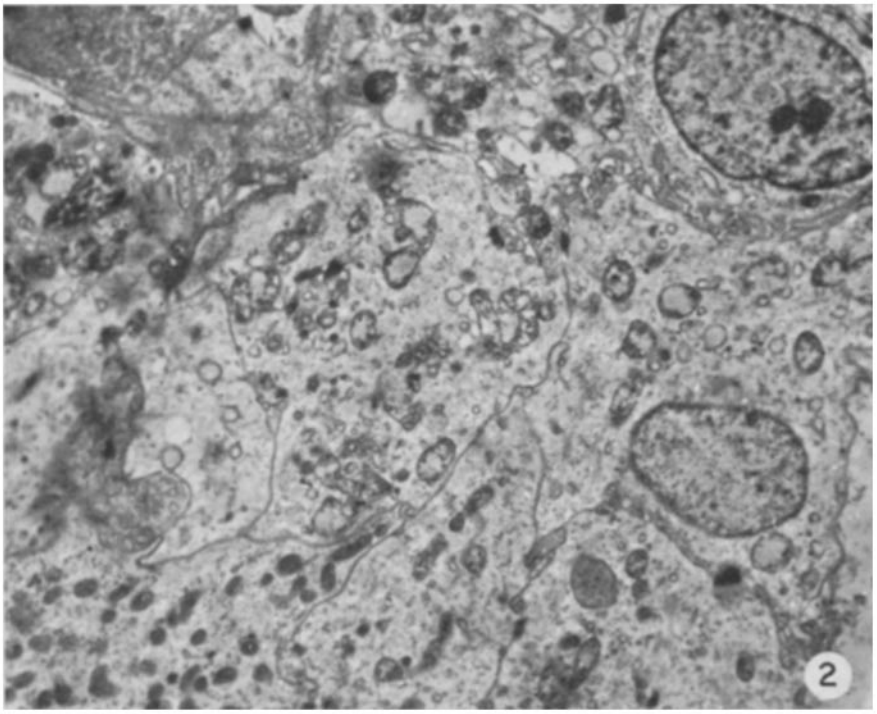
EXPLANATION OF PLATES

PLATE 13

FIG. 1. Cells from a normal chorioallantoic membrane. They are close packed and the nuclei are only slightly distorted. Fragmented and swollen mitochondria can be seen in the cytoplasm; note irregularity of their size, shape, and density. × 5000.

FIG. 2. Cells from a normal membrane which appear to be degenerating. Most of the mitochondria are greatly swollen but small particles and spheres are present. Their size overlaps that of virus particles but there is no regularity to their morphology. × 6,500.





(Gaylord and Melnick: Intracellular forms of pox viruses)

PLATE 14

FIG. 3. Nucleus and matrix of chorioallantoic cell in early stages of vaccinia infection. Developmental bodies in matrix are few in number and look like "empty" membranes. Some mature virus is present in the cytoplasm and the nucleus is not distorted. × 9,500.

FIG. 4. Ectodermal cell in more advanced stage of vaccinia infection. Nucleus is distorted and the nucleolus enlarged. Matrix is larger than that shown in Fig. 3 and contains many developmental bodies in various stages of maturation. Much more mature virus is present and there are fewer mitochondria than in normal cells. × 8,500.

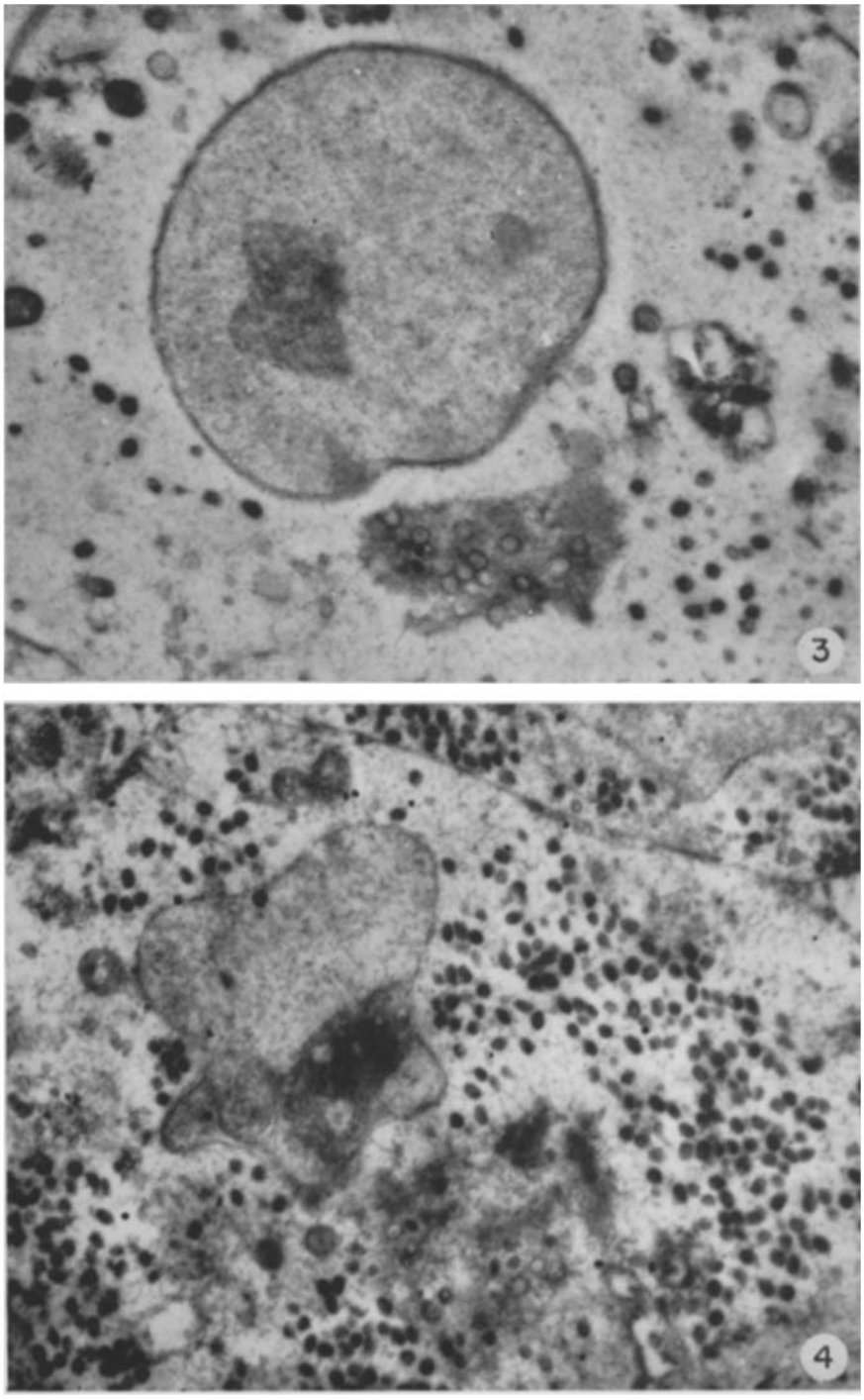


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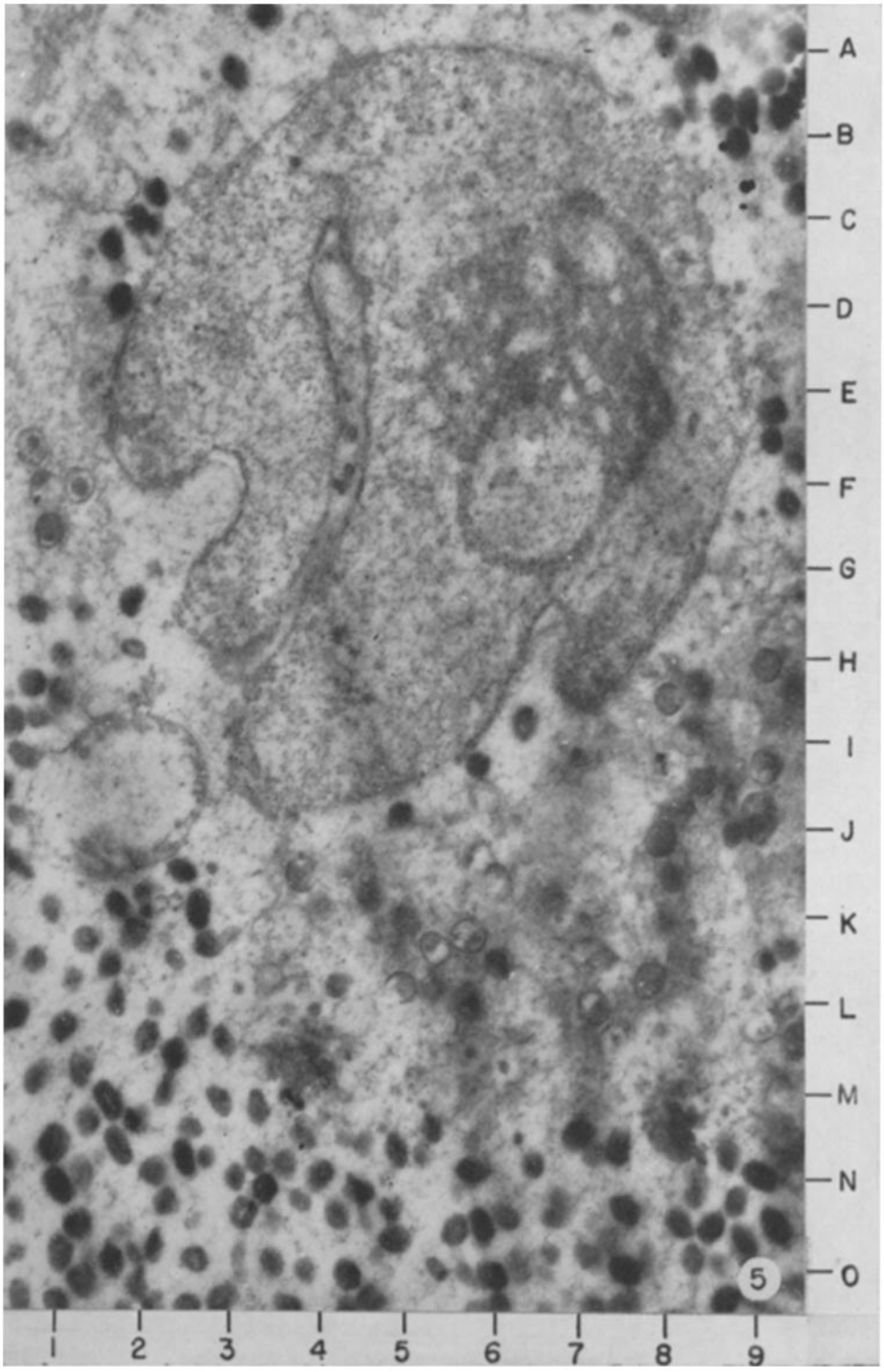
PLATE 15

FIG. 5. Enlarged view of an ectodermal cell perinuclear area. Matrix region (J3 to M9) contains developmental bodies in various stages of maturation. At H9 is a developmental body filled with a homogeneous material of low electron density. The group at F1 shows the granule in different sizes. In the developmental body with the largest granule the low density material has disappeared. A "hollow" mature virus can be seen at N3, and at M2 there are two mature particles having axial ratios of almost 2:1. One of them is also "hollow." The large circle at I2 is a swollen mitochondrion. Small black particles at B9 are precipitated osmium. × 20,000.

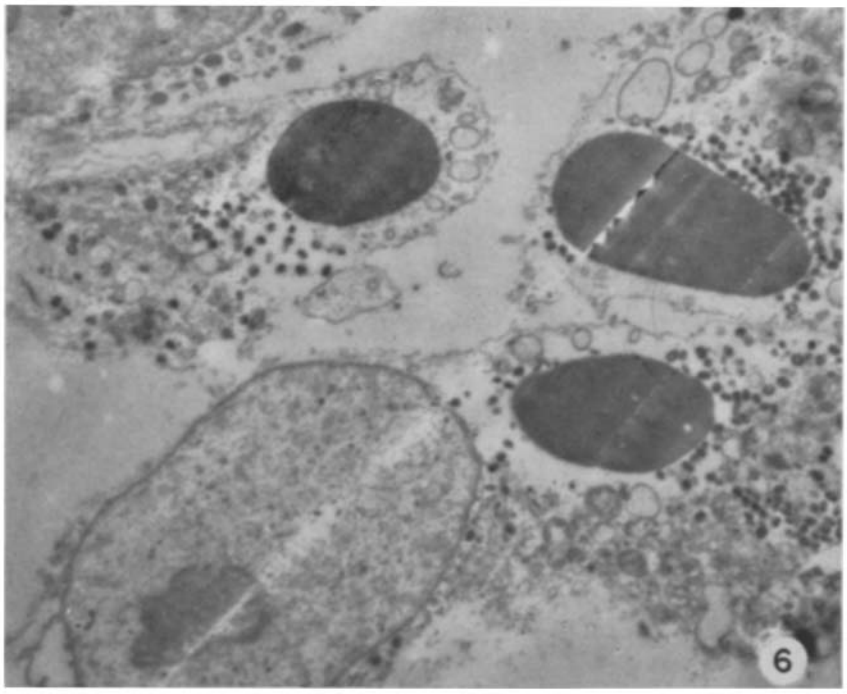


(Gaylord and Melnick: Intracellular forms of pox viruses)

PLATE 16

FIG. 6. Chorioallantoic cells containing inclusion bodies of ectromelia (Marchal bodies) and mature virus, some of which show empty interiors like vaccinia. Circles of different sizes are mitochondria. A nucleus is present at the lower left. × 6,500.

FIG. 7. Ectromelia inclusion body ringed with mature virus. Plaque-like areas in the inclusion are few in number. A few developmental bodies and transitional stages of mature virus are visible. A nucleus is present at the lower left. × 9,000.



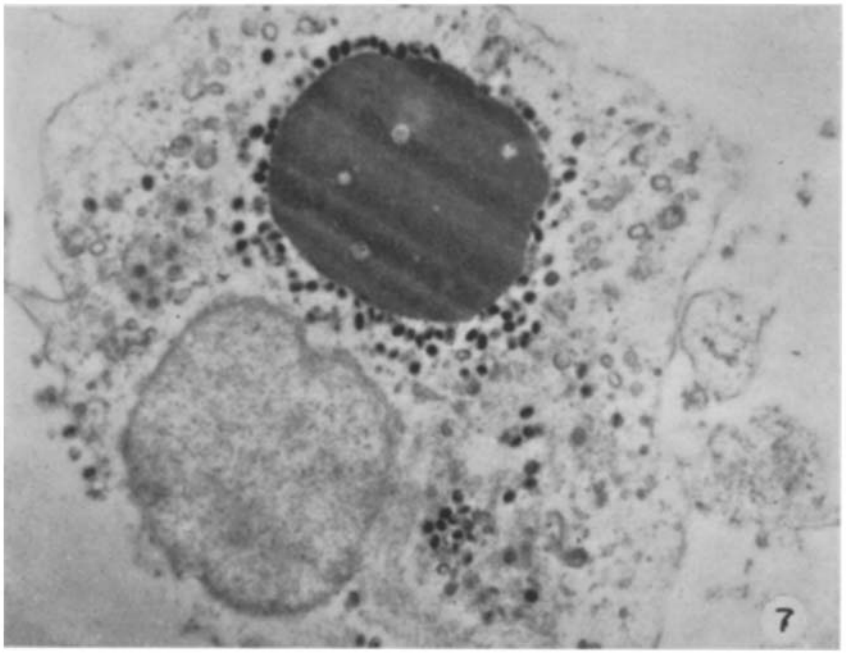


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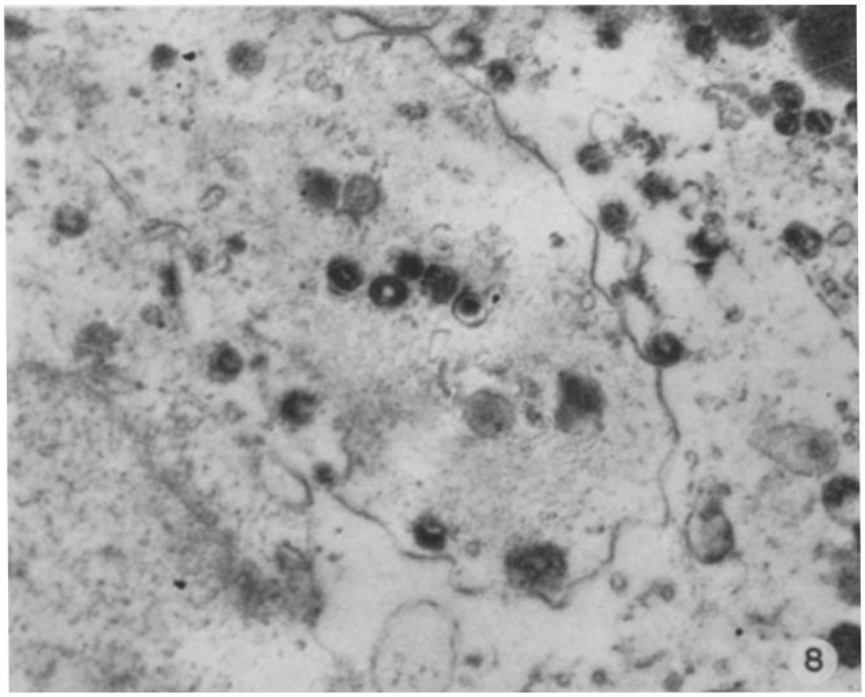
PLATE 17

FIG. 8. Developmental bodies of ectromelia virus. They appear similar to those of vaccinia. × 19,000.

FIG. 9. Ectromelia inclusion surrounded by particles resembling mature virus more than developmental bodies. The interiors instead of being hollow show bars, dumbells, and double circles. Lines at right are endoplastic reticulum. Part of a nucleus is at left and a mitochondrion can be seen between the nucleus and the inclusion. Grey patch at 4 o'clock is probably part of inclusion material. × 16,000.



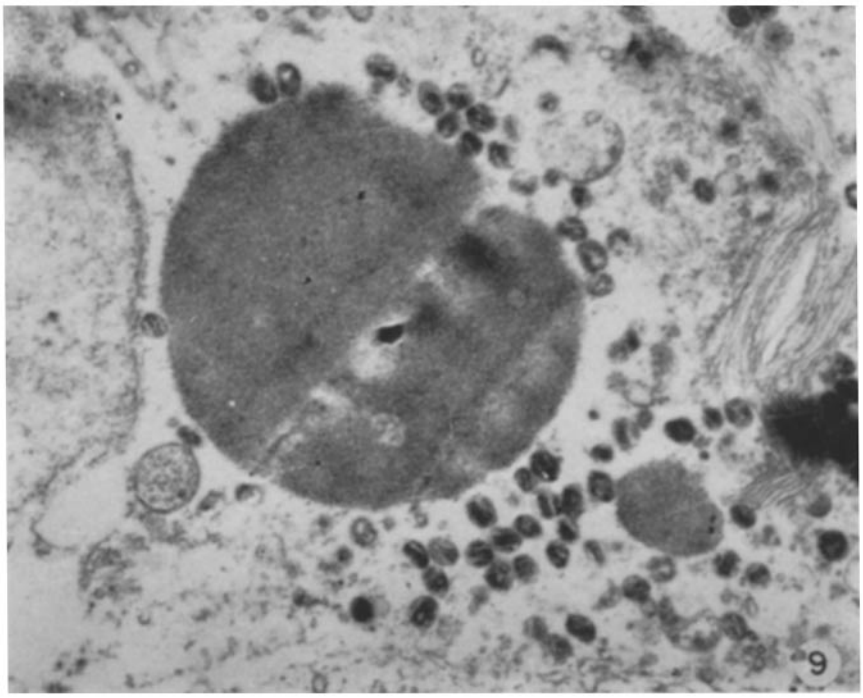


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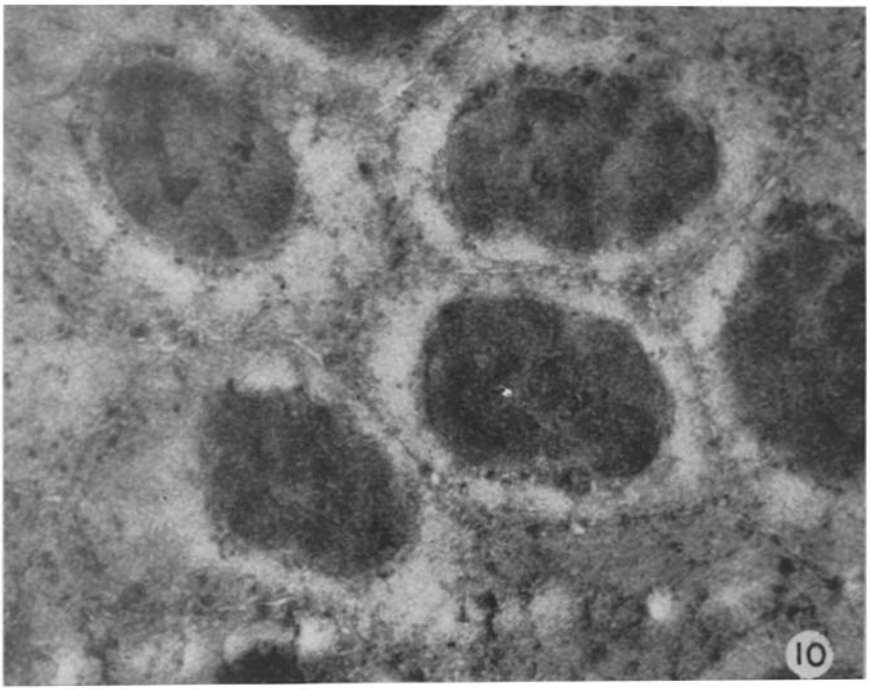
PLATE 18

FIG. 10. Epithelial cells from a normal rabbit cornea. Tiny thread-like mitochondria are clustered around the dense nuclei. × 6,500.

FIG. 11. "Normal" cells from an infected cornea, but away from the infected area. Some degeneration may have started because mitochondria are present in clumps. × 5,000.



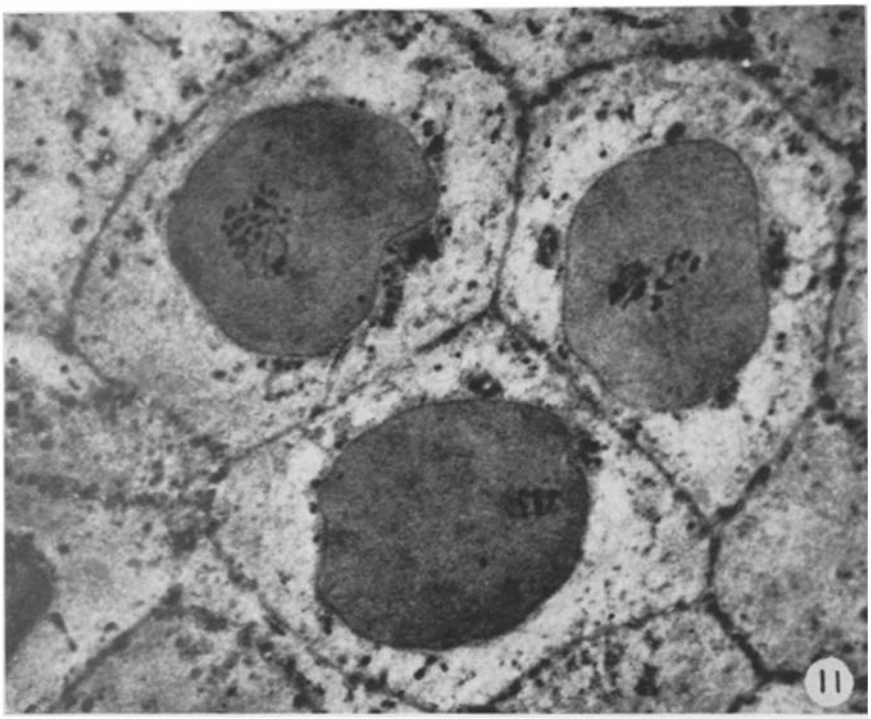


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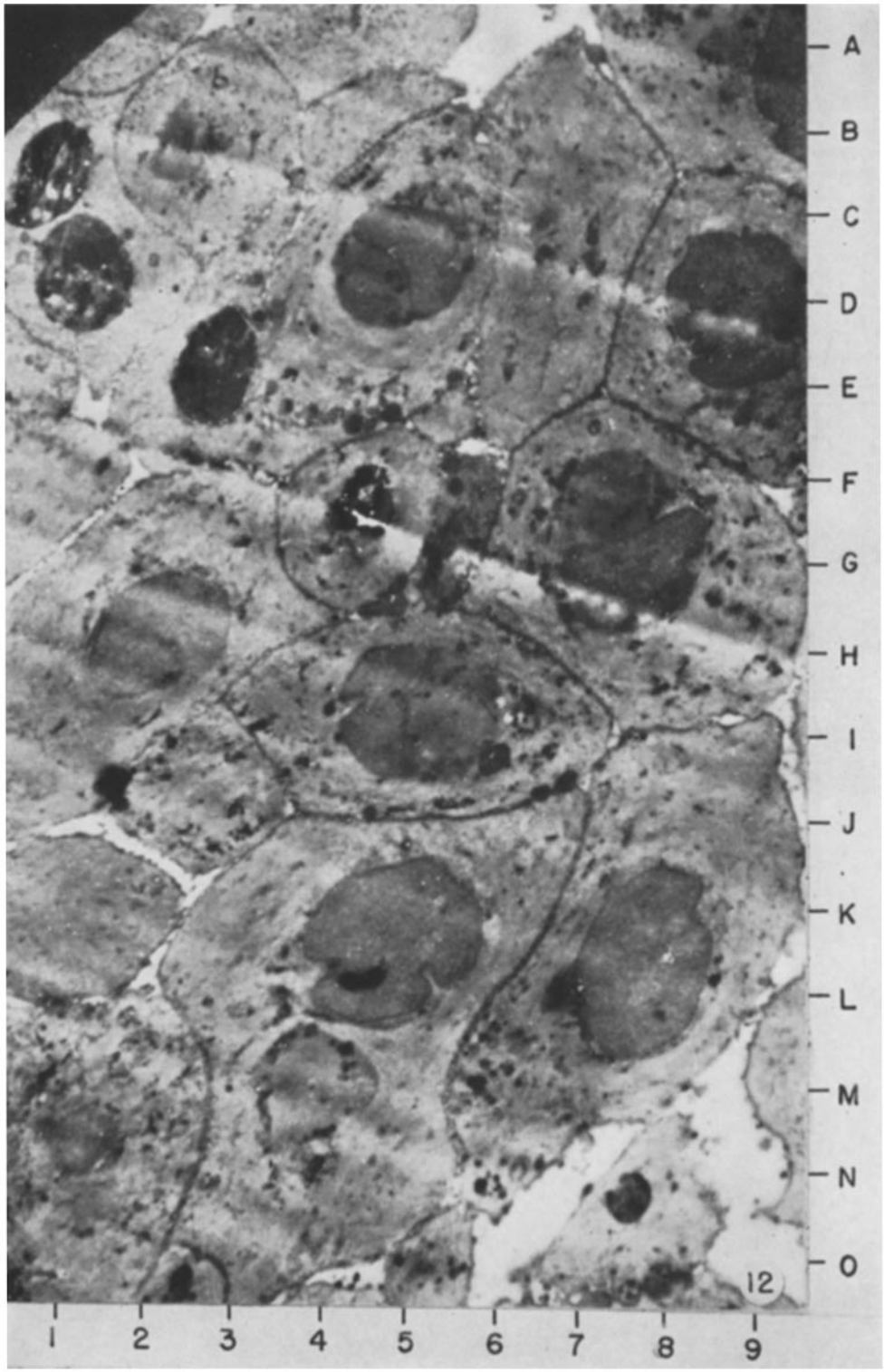
PLATE 19

FIG. 12. Vaccinia-infected cornea. Inclusion bodies (Guarnieri bodies) are present at B1, C1, and E2. Matrices indenting the nucleus are present at C4, F7, and I5. Matrix away from the nucleus can be seen at M4. Beginning matrix is present at L7. Very little mature virus is present and this field represents an early stage of infection. Some extracellular virus can be seen at O4 and N9. × 4,500.



(Gaylord and Melnick: Intracellular forms of pox viruses)

PLATE 20

FIG. 13. Vaccinia-infected cornea at a more advanced stage than shown in Fig. 12. There is an increased amount of dense, mature virus present. Inclusion bodies can be seen at F1, F8, G9, and I8. Cells at F6 and J8 are probably leucocytes with dense granules. An advanced matrix can be seen at I5 indenting nucleus. A matrix is present at M6 and there is more extracellular virus in this region than in Fig. 12. × 4,000.

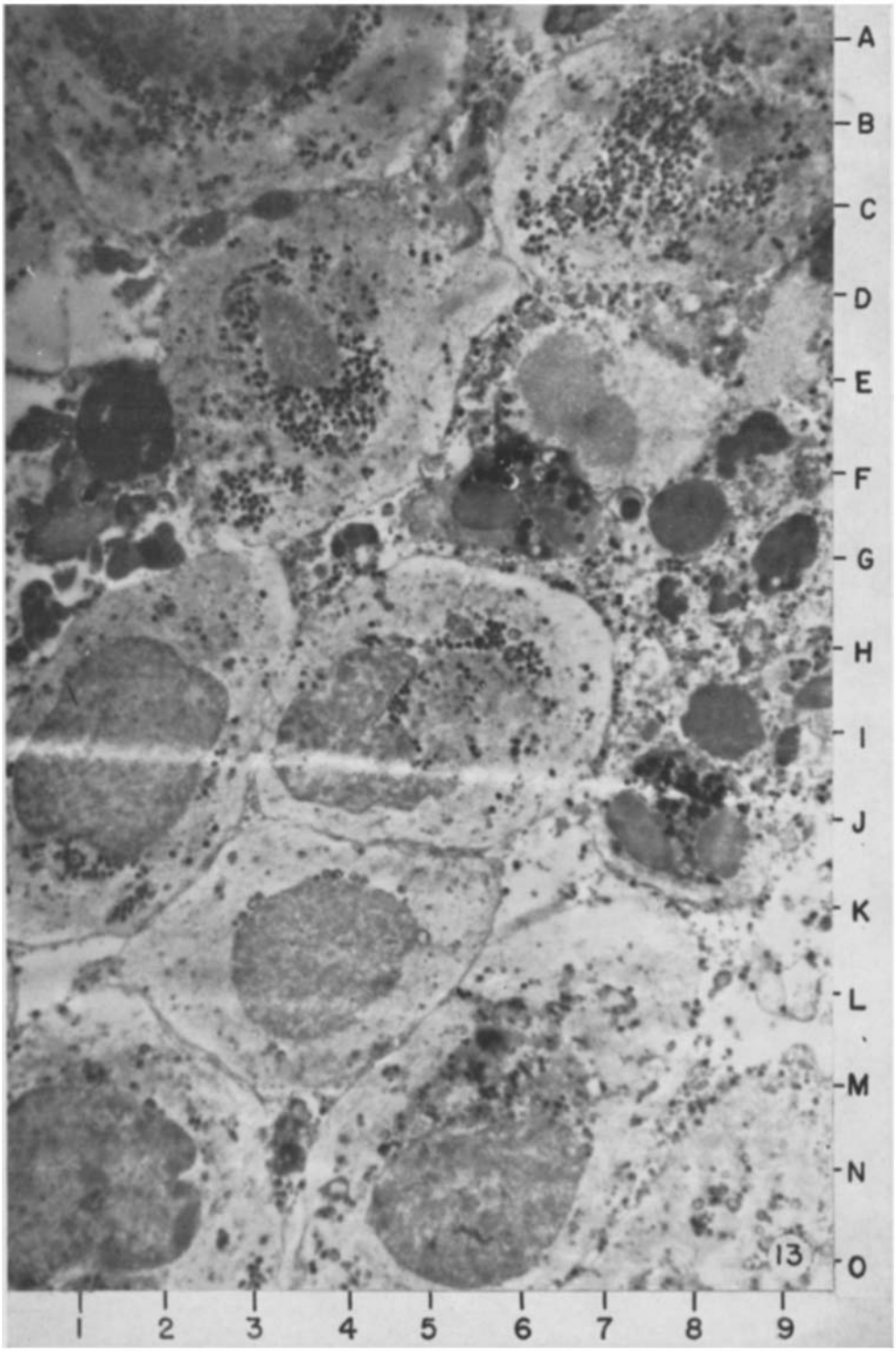


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PLATE 21

FIG. 14. Matrix area of a corneal cell infected with vaccinia for comparison with matrices found in the chorioallantois (Figs. 3 to 5). Developmental bodies are present within the area of increased density. Note extracellular virus in upper right. × 16,000.

FIG. 15. Three inclusion bodies of vaccinia-infected cornea for comparison with ectromelia-infected chorioallantois (Figs. 6, 7, 9). × 5,000.

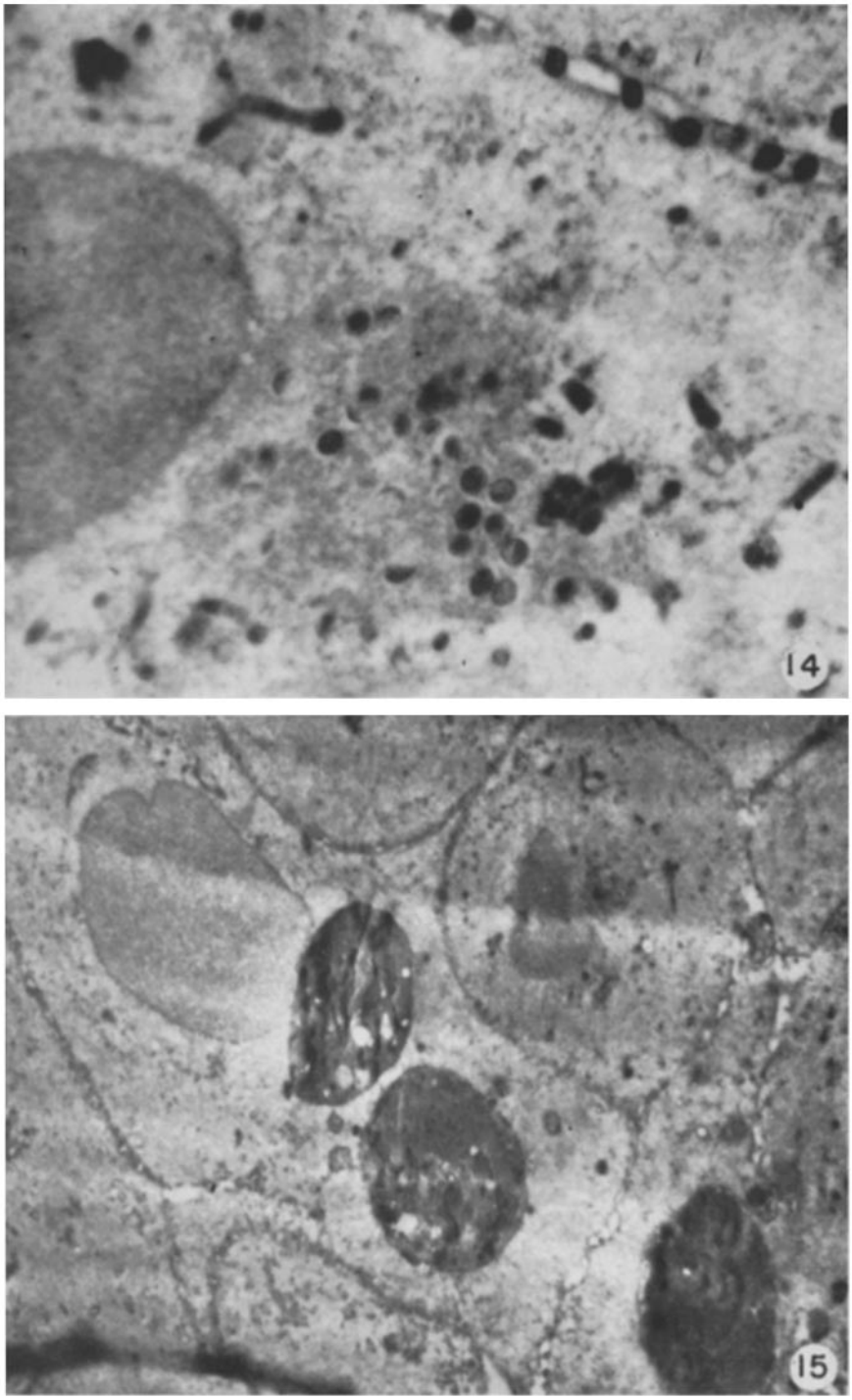

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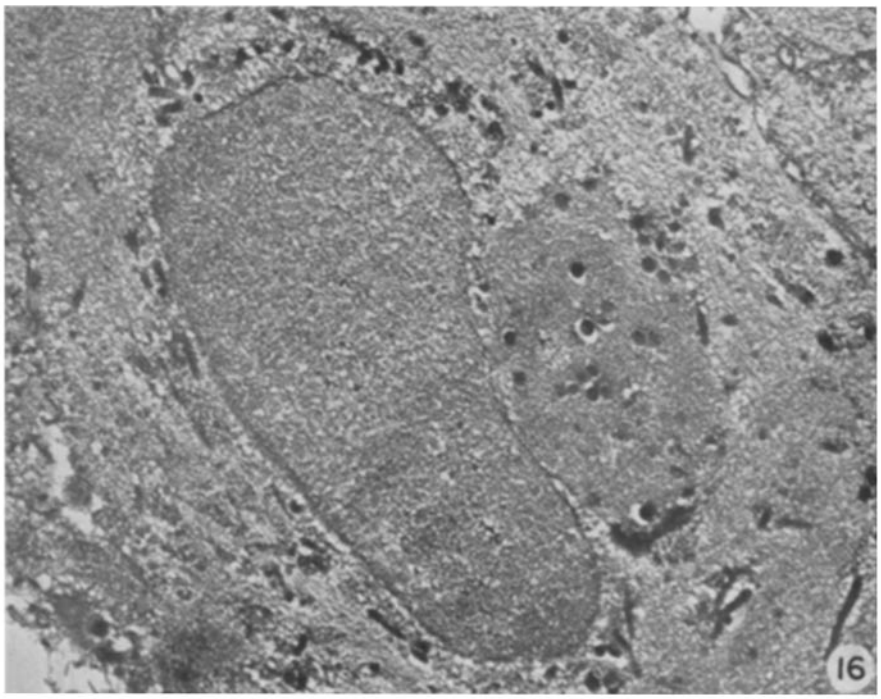
PLATE 22

FIG. 16. Vaccinia-infected cornea. Structure of matrix in which developmental forms of virus are found, virus compared to surrounding cytoplasm when methacrylate is removed. The section was shadowed with palladium before examination in the electron microscope. Nucleus is at left. × 8,000.

FIG. 17. Vaccinia-infected cornea. Four inclusion bodies with varying structures in a single cell. Nucleus is at upper right. Methacrylate removed, palladium shadowed. × 6,000.



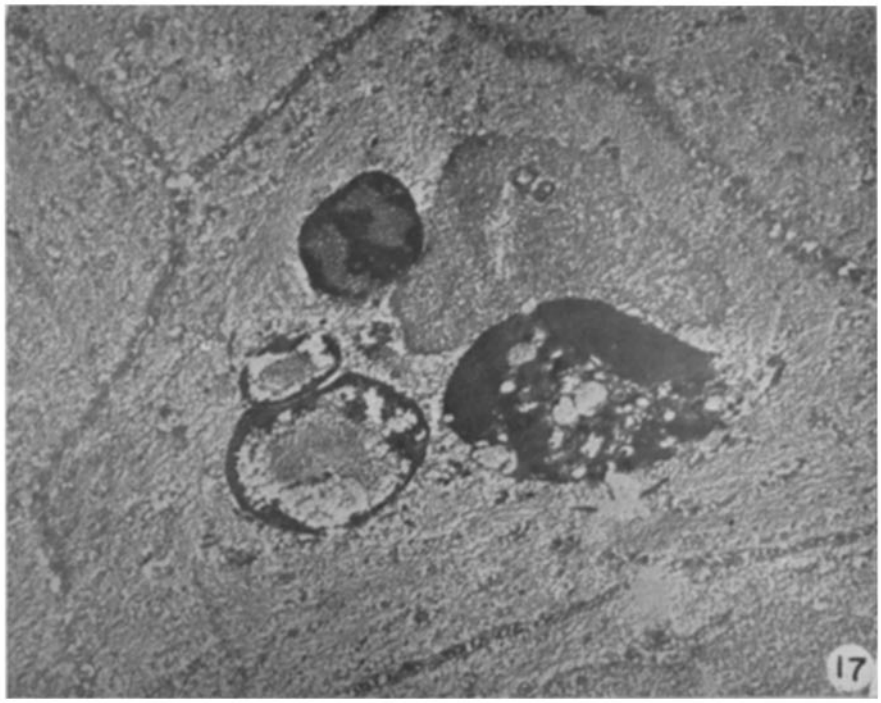


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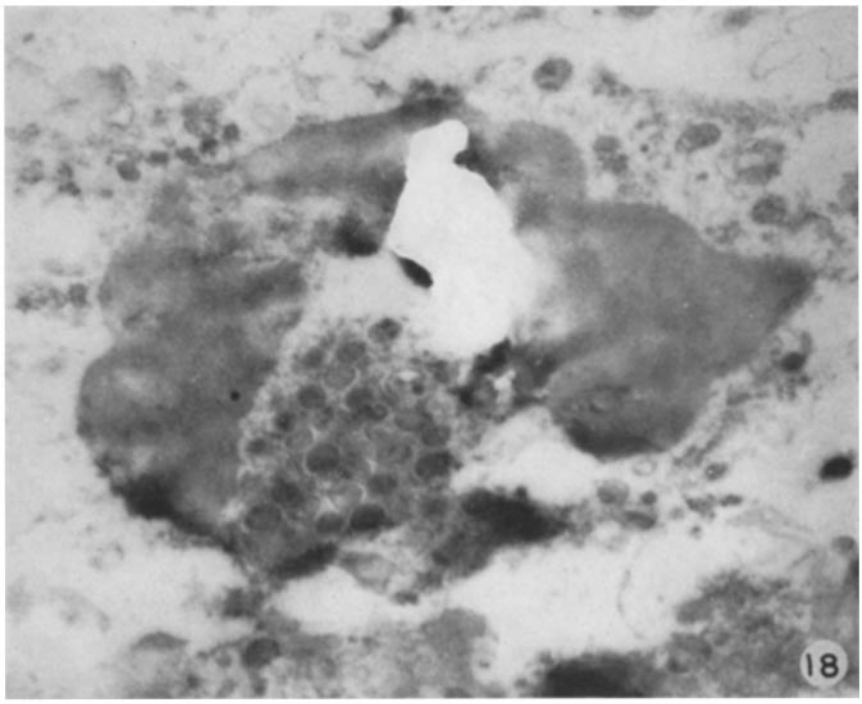
PLATE 23

FIG. 18. Inclusion body of vaccinia in cornea. An area of the inclusion apparently is being transformed into virus developmental bodies which are seen in various stages of maturation. × 19,000.

FIG. 19. A similar inclusion body at a more advanced stage of transformation and disintegration. Virus particles between islands of inclusion material show internal structure in the shape of bars and dumbells. (Compare with ectromelia, Fig. 9). × 17,000.



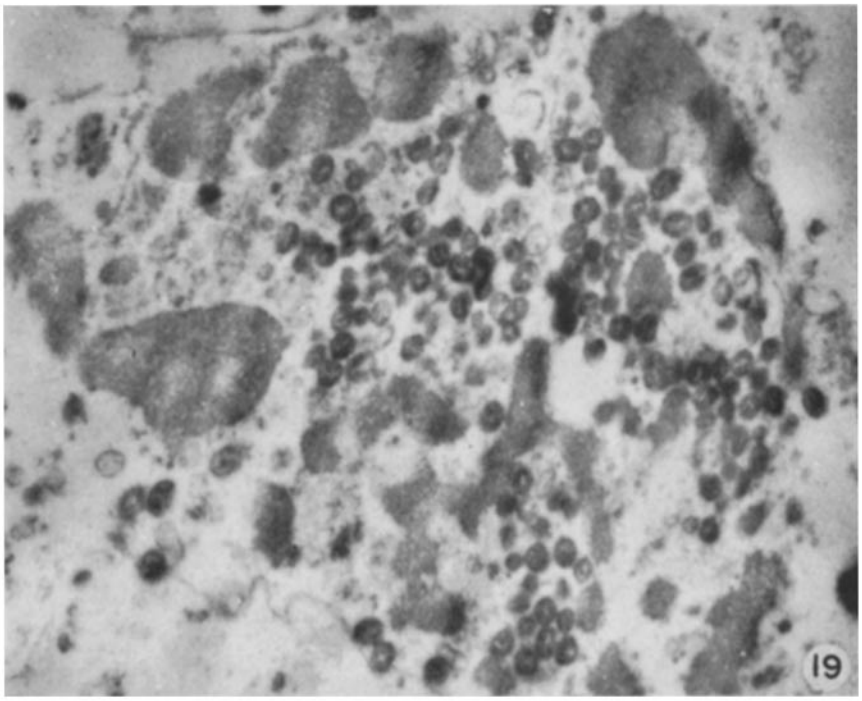


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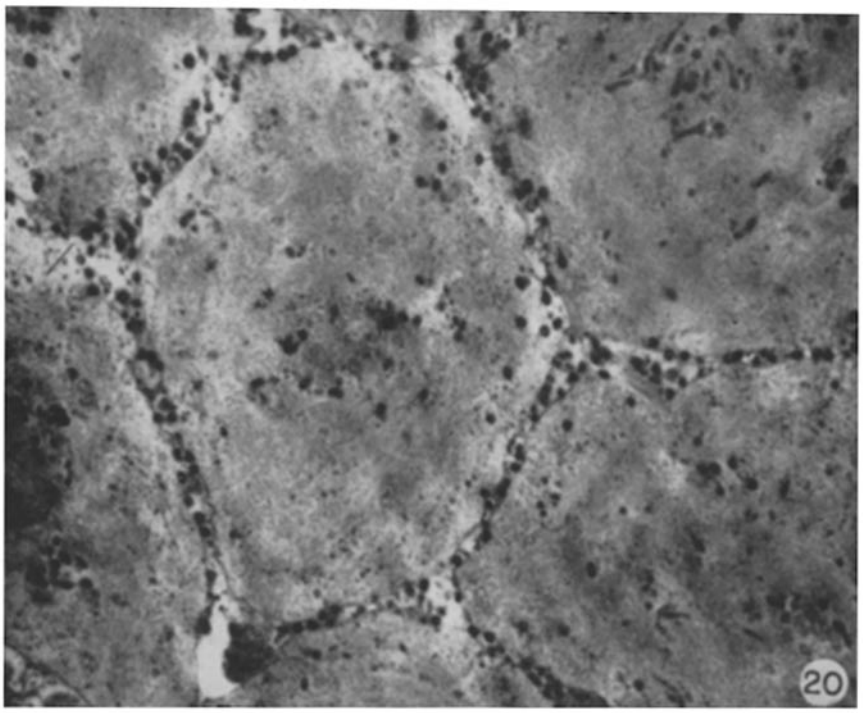
PLATE 24

FIG. 20. Corneal cells surrounded by extracellular vaccinia virus. The cells in this area are only slightly involved in infection and the virus must have come from neighboring areas. × 6,000.

FIG. 21. Molluscum contagiosum infected cell (Henderson-Paterson body). Note strands of cytoplasmic material separating pockets of mature virus. Nucleus is pushed to one side (lower left of cell) and the nucleolus is very dense. × 8,000.



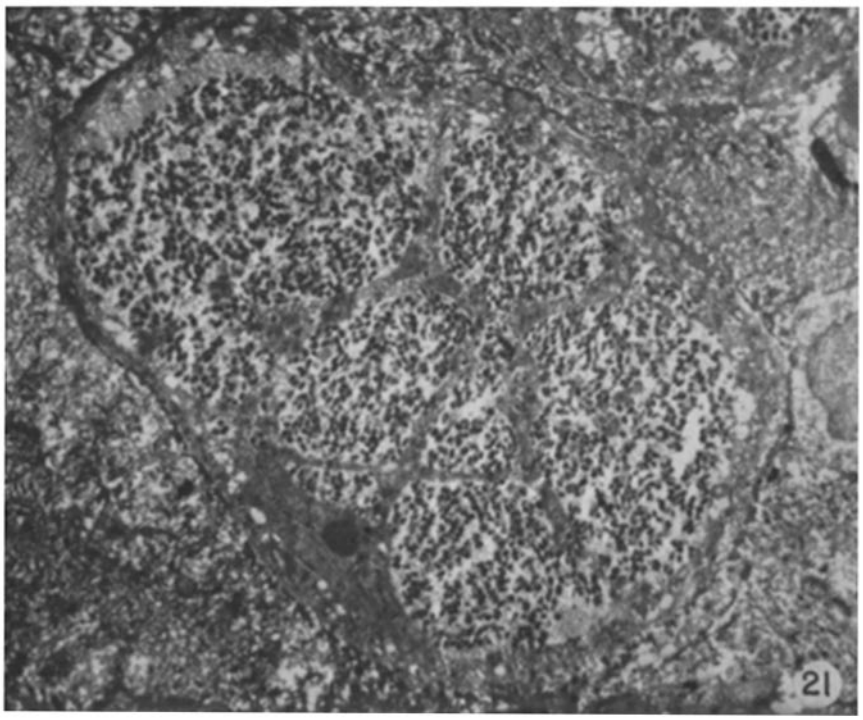


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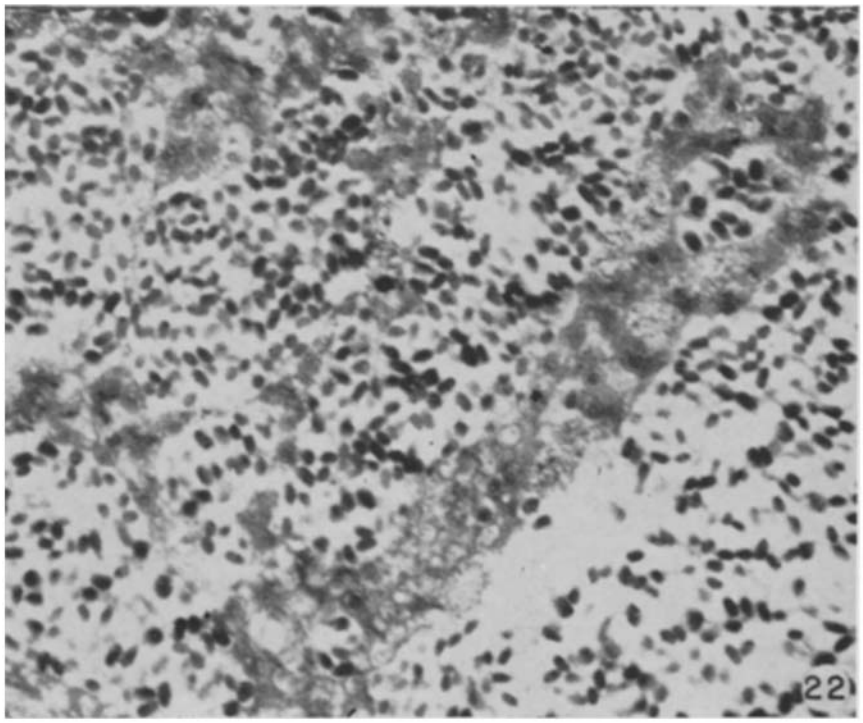
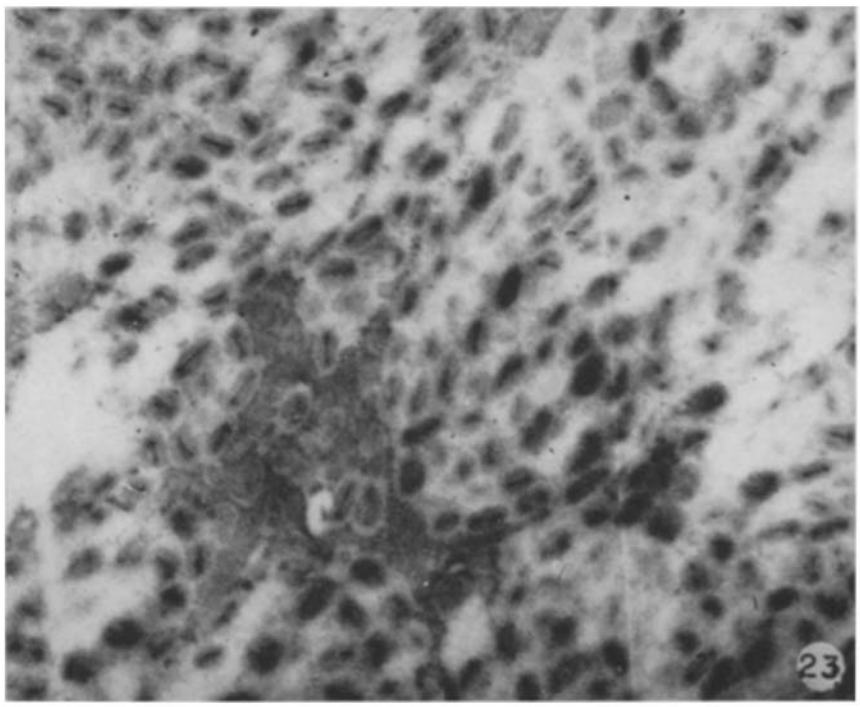
PLATE 25

FIG. 22. Virus developmental area in molluscum contagiosum infected cell. Developmental bodies resembling those of vaccinia are present imbedded in the remaining strands of material which serve to separate the nests of mature virus particles. × 13,500.

FIG. 23. Molluscum contagiosum infected cell. All stages of developmental bodies can be seen from the virus precursors clustered in remnant of cytoplasm to the viruses having dumbell shaped interiors and to those which are uniformly dense to electrons. × 26,000.





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