CYTOPATHOLOGIC CHANGES IN FOWLPOX (TURKEY ORIGIN) INCLUSION BODY FORMATION

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The initiation of pox virus replication is characterized by the formation of focal areas of dense particulate material (viroplasm) in the cytoplasm, which is usually associated with clusters of ribosomes.[1-8] Foci of viroplasm undergo sequestration by incomplete membranes which close to form "immature" viral particles, many of which contain dense nucleoids. Recent studies indicate that viroplasm is involved with the synthesis of viral DNA and structural protein. Following inoculation of cell cultures with vaccinia virus whose DNA had been labeled with thymidine-^{3}H, labeled material was detected in foci of viroplasm.[1] The inhibitory influence of actinomycin D on ribosomal clusters in areas of viroplasm and of fluorodeoxyuridine on nucleoid development has been reported for vaccinia virus in cell cultures.[2]

The process whereby immature viral forms develop to mature particles associated with inclusion bodies has not been adequately studied *in vivo*. The role of lamellar structures, fine fibrils, tubule forms and inclusion body membranes, all of which are characteristic of pox virus infection,[3-8] have not been demonstrated in the process of viral replication.

The fowlpox inclusion body has been used since the early days of the light microscope as a model to study the intracellular process of pox virus replication.[9-11] Recent electron microscopic studies have suggested that the inclusion body arises directly from foci of viroplasm.[12] Other investigators have proposed that viroplasmic areas and inclusion bodies represent two distinct models of viral replication.[13]

Additional morphologic studies are required to resolve the uncertainties concerning the role of the inclusion body and other cytoplasmic structures in the process of viral particle formation and maturation. To this end the following report is presented.

MATERIAL AND METHODS

The virus (supplied by Dr. James Norman, National Animal Disease Laboratory, Ames, Iowa) was originally isolated from skin lesions occurring on the heads of young turkey poults (supplied by Dr. L. A. Page, National Animal Disease Laboratory). It had been passed several times in embryonating hens' eggs and twice in primary swine kidney cells. The virus was inoculated onto the chorioallantoic membrane

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(CAM) of 10-day-old chicken embryos. Infected membranes were harvested 8 days later. A 10 per cent membrane suspension was prepared by grinding infected membranes in a TenBroeck grinder with saline. This suspension was centrifuged for clarification (20 minutes at 800 × g), for bacterial clearance (20 minutes at 2,000 × g), and for virus sedimentation (60 minutes at 60,000 × g). The resulting pellet was re-suspended with 10 drops of distilled water and portions were taken for negative staining. The remainder was diluted with saline to 20 cc and was used as the stock virus inoculum. It produced CAM lesions when diluted to 10^{-4} but not at 10^{-5}.

Stock virus was inoculated onto the dropped CAM of twelve 10-day-old chick embryos obtained from commercial inbred lines and also inoculated intradermally into the scalp of three 3-week-old chickens. The CAM lesions were harvested at 4 (early) and 8 (late) days post-inoculation. Dermal lesions from infected chickens were removed surgically when the papule stage was reached. All tissues were fixed in 2.5 per cent glutaraldehyde, postfixed in osmium tetroxide, dehydrated in ethanol and embedded in Epon. Ultrathin sections were stained with lead hydroxide [14] and examined with a Philips model 200 electron microscope.

In addition to fowlpox viral particles, viral particles resembling Rous sarcoma virus were noted. The CAM inoculation was thus repeated using chicken embryos from flocks allegedly free of Rous sarcoma agent as well as other avian pathogens (SPAFAS, Inc., Norwich, Conn.).

Results

The development of inclusion bodies in the epithelium of chick skin and in the CAM appeared to be identical.

In most cells of the early lesions and in basal cells of advanced lesions, intracellular evidence of viral infection consisted of early viral forms and precursor material (Fig. 1). Viroplasm was, in most instances, surrounded by incomplete membranes which appeared to be closing together to form immature viral forms. These immature forms, subsequently referred to as viroplasmic particles, consisted of dense viroplasm surrounded by a double membrane and were nearly always located near areas of viroplasm. They often contained an electron-dense nucleoid. The formation of viroplasm was not associated with any special cytoplasmic organelle although it was frequently seen adjacent to early inclusion body forms.

Long filaments with a characteristic periodicity were seen throughout cells in the early lesions. Glycogen and viroplasmic particles were often associated with these and occasionally the filaments appeared to be contributing an outer coat to the viroplasmic particle. In rare instances viral forms in varying stages of completion were present in progression along the filaments (Fig. 2). An intermediate viral form was characterized by an inner core similar to the early viroplasmic particles but surrounded by an additional coat.

The most mature viral forms seen free in the cytoplasm were also felt to be intermediate stages (incomplete virions). They were similar to the complete virion with the exception of the absence of the external

coat. The core and inner membranes were constricted centrally giving a dumbbell-shaped appearance when they were seen in longitudinal or cross section. Lateral bodies appeared to have been formed due to thickening of the outer coat on the broad face of the virion. A small, round electron-dense body was located in the outermost coat of these viral particles. Incomplete virions were seen scattered throughout the cytoplasm, often attached to a membrane structure. They were most often indented into inclusion body forms but were also seen associated with mitochondria and, in macrophages and adnexal cells, with endoplasmic reticulum (Fig. 3).

Degeneration of mitochondria was common in all infected cells. Mitochondria were enlarged, the cristae were disorganized and granular material was present on the interior. Although some fixation artifacts were undoubtedly present due to glutaraldehyde, most changes were believed to be antemortem. Many degenerated mitochondria had an incomplete virion attached to their surfaces.

Hypertrophy of the Golgi apparatus with the formation of many vesicles was common. Numerous aggregates of lipid-containing vacuoles which appeared to be surrounded by a limiting membrane (Fig. 4) were commonly associated with the Golgi apparatus. Clear areas were present in some of these vacuoles. Incomplete virions which had apparently formed in adjacent portions of the cytoplasm were attached to the surface of these vacuoles (Fig. 5). Attachment occurred only along the broad face of the incomplete virion, not on the narrow side or end. In some cases, they were indented into the membrane of the vesicle (subsequently called the inclusion body vacuole).

Inclusion body vacuoles were seen in various forms throughout most infected cells. Small vacuoles had one or more incomplete virions attached to them. Larger vesicles contained various numbers of rodlets. The rodlets were long slender dense structures and were seen in all large inclusion body vesicles.

Small, long tubular protrusions were seen on a few small clear vesicles which were present in the vicinity of the Golgi apparatus. These were occasionally associated with groups of dense rodlets. The rodlets were seen in aggregates in the cytoplasm (Fig. 6), massed in large membrane bound aggregates (Fig. 7), collected in the interior of the early inclusion vacuoles (Fig. 8), at the surface of the inclusion vacuoles, and, on rare occasions, astride the inclusion body vesicle membrane (one portion inside, the other out).

The inclusion bodies of cells in advanced stages of viral replication contained masses of electron-dense rodlets and scattered virions (Fig. 9). Central vacuolar areas containing the uniformly granular homoge-

neous substance were gradually obliterated by masses of rodlets and virions. Hypertrophy of the Golgi apparatus and mitochondrial degeneration were advanced (Fig. 10).

Electron-dense rodlets were more abundant in the smaller inclusion bodies. On occasion they filled the inclusion which contained only scattered virions. Some contained round or oval protrusions at their ends (Figs. 11 to 13). Some of these protrusions appeared to have become detached and were scattered throughout the interior of the inclusion body. They varied in size from 40 to 250 mμ and were similar in appearance to the viroplasmic particles seen free in the cytoplasm. Electron-dense rodlets were attached to the outer coat of virions in the interior of the inclusion body. They appeared to be forming the most external coat. In cross section they could be seen located around the virion as a "string of pearls". In longitudinal sections the rodlets appeared to be draped around the virion.

The mature virion which occurred inside the inclusion body contained two additional coats over those of the incomplete virion (outside the inclusion body). The innermost of these was thin and appeared to be obtained by indentation during passage through the inclusion body membrane. The outer coat, thicker and more electron-dense, appeared to be formed from rodlets on the interior of the inclusion body. An ovoid electron-dense body was present on the interior of the mature virion.

Discussion

The initial development of fowlpox virus, as with most pox viruses, involved the formation of viroplasm which was sequestered by membranes to form viroplasmic particles. The viroplasmic particles evolved by maturation and condensation into the incomplete virion which occurred free in the cytoplasm. Whether the limiting membrane of the early viroplasmic particle became the core membrane or the outer membrane of the incomplete virion could not be determined. The latter is believed to be true for vaccinia.[15]

The origin of the nucleoid of the viroplasmic particles is not apparent. Studies on ectromelia have shown the nucleoid to be composed of linearly-orientated electron-dense filaments and this material has been seen entering (or being extruded from) the viroplasmic particle.[6] Nucleoid-like structures free in viroplasmic material have been observed in vaccinia-infected cells.[15] Serial sections of vaccinia viroplasmic particles have indicated that nucleoids are always present [16]—the eccentric location in the particle readily explains its absence in cross section. The DNA component of the virus is probably located in the nucleoid.

The gray appearance of lipid in the early inclusion body vacuoles suggested a relatively high degree of saturation of fatty acids as op-

posed to the strong osmiophilia of lipid droplets containing large amounts of unsaturated fatty acids. The lipid vacuoles appeared to be membrane bound. This is not in agreement with current views on lipid droplets wherein the so-called "false limiting membrane" is believed to be due to a more intense reduction of osmium at the lipid-ground substance interface.[17] The close association of mitochondria with these lipid-containing inclusion body vacuoles may be related to the enzyme and energy requirement for the conversion of neutral triglycerides to the dense lipid structures which form the external coat of the virus. This close association of mitochondria with lipid is common in structures with high energy requirements; e.g., muscle.[17]

The requisites for initiation of viral replication in the inclusion body vacuole are not known. For the immature virion to become complete, it requires an outer membrane and an outer dense coat. The propensity of the virion for a membrane system might well bring about its attachment to the inclusion body vacuole (or aberrantly, to any other available membrane).

Whether the fine tubular protrusions seen on some Golgi vesicles were, in fact, related directly to the formation of dense rodlets could not be established. It was possible that the rodlets arose *de novo* at the inclusion body membrane upon stimulation by the entry of the incomplete virion. Rodlets, whatever their origin, could be pluripotential structures. They provided an external coating for incomplete virions. In addition, they also appeared to be the source of round bodies which resembled the earliest viroplasmic particles produced in the cytoplasm. One could not be certain, however, that the process of virion production was completed from viroplasmic particle to virion within the confines of the inclusion body.

Incomplete virions formed in the cytoplasm were coated in the process of migration through the inclusion body membrane. Within the inclusion body, rodlets were probably arranged over the surface of the virion thus forming the external coat. Whether rodlet material represented the lipoprotein complex responsible for the hemagglutinating property of the virus remains to be proven. The size of the rodlet in cross section was very similar to that of the 65-mμ particles shown to be associated with the hemagglutinating antigen of vaccinia virus.[18] The latter antigen has been shown to be nonessential for infectivity and this property can be disassociated from that of agglutination by centrifugation.[19]

The significance of the small dense body is not known.[20] It was always seen on or near the lateral bodies of the virion. Whether these structures were directly associated with the viral genome remains to be demonstrated.

The function of the inclusion body is probably to provide an efficient

mechanism of virus production; i.e., to localize the production of coat precursor material into a structure to which the incomplete virion has ready access. It is apparent that this mechanism of viral maturation does not occur in all poxvirus infections; nor is it certain that fowlpox virus inclusion body formation is utilized as efficiently in cell culture systems as in chicken skin and CAM.

One cannot reconstruct a dynamic process from static electron micrographs. Correlating the morphologic evidence with more finite chemical studies on vaccinia virus, however, the following sequence probably occurs after entry of infective fowlpox virus into susceptible epithelial cells: (1) viroplasm is produced and is sequestered by membranes to form viroplasmic particles, (2) viroplasmic particles undergo maturation by condensation and acquisition of an additional outer membrane to incomplete virions, (3) incomplete virions move to lipid-containing vacuoles, attach and migrate through the vacuolar membrane thereby obtaining a membrane coat, and (4) rodlets produced within the inclusion body provide the external lipid coat of the virus.

Summary

The cytopathologic features of chicken chorioallantoic membrane and chicken skin infected with fowlpox virus (turkey origin) have been investigated by electron microscopy. Sequestration of viroplasm to form viroplasmic particles free in the cytoplasm and the occurrence of more complete viral forms within the inclusion body agree with previous studies.

Stages of development of inclusion bodies were seen from small lipid vacuoles to large bodies packed with viral forms and electron-dense rodlets. Incomplete virions contained a viral core surrounded by an inner coat of 2 membranes and an outer coat containing 2 lateral expansions and an electron-dense body. Incomplete virions appeared to move to the inclusion body membrane, attach and migrate through it. In the process, this membrane became a second outer coat. The mature virion was complete when a third outer coat was added by an overlay process of rodlets onto the incomplete virion.

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[*Illustrations follow*]

Legends for Figures

FIG. 1. Two epithelial cells contain early fowlpox viral forms. Several areas of viroplasm are being sequestered by incomplete membranes. Adjacent to these are viroplasmic particles, some containing nucleoids (arrows). Filaments are present in the cytoplasm. × 7,000.

FIG. 2. Five viral forms are arranged in increasing degrees of completeness along a banded filament. The center particle may be in the process of forming an outer coat from the filament. The incomplete virion in the upper left is the most mature viral form seen free in the cytoplasm. × 56,000.

FIG. 3. Development of virus in an adnexal cell of the dermis (desmosomes are absent). No inclusion body vacuoles are evident. Incomplete virions appear to be migrating through membranes of the endoplasmic reticulum (arrow). × 12,000.

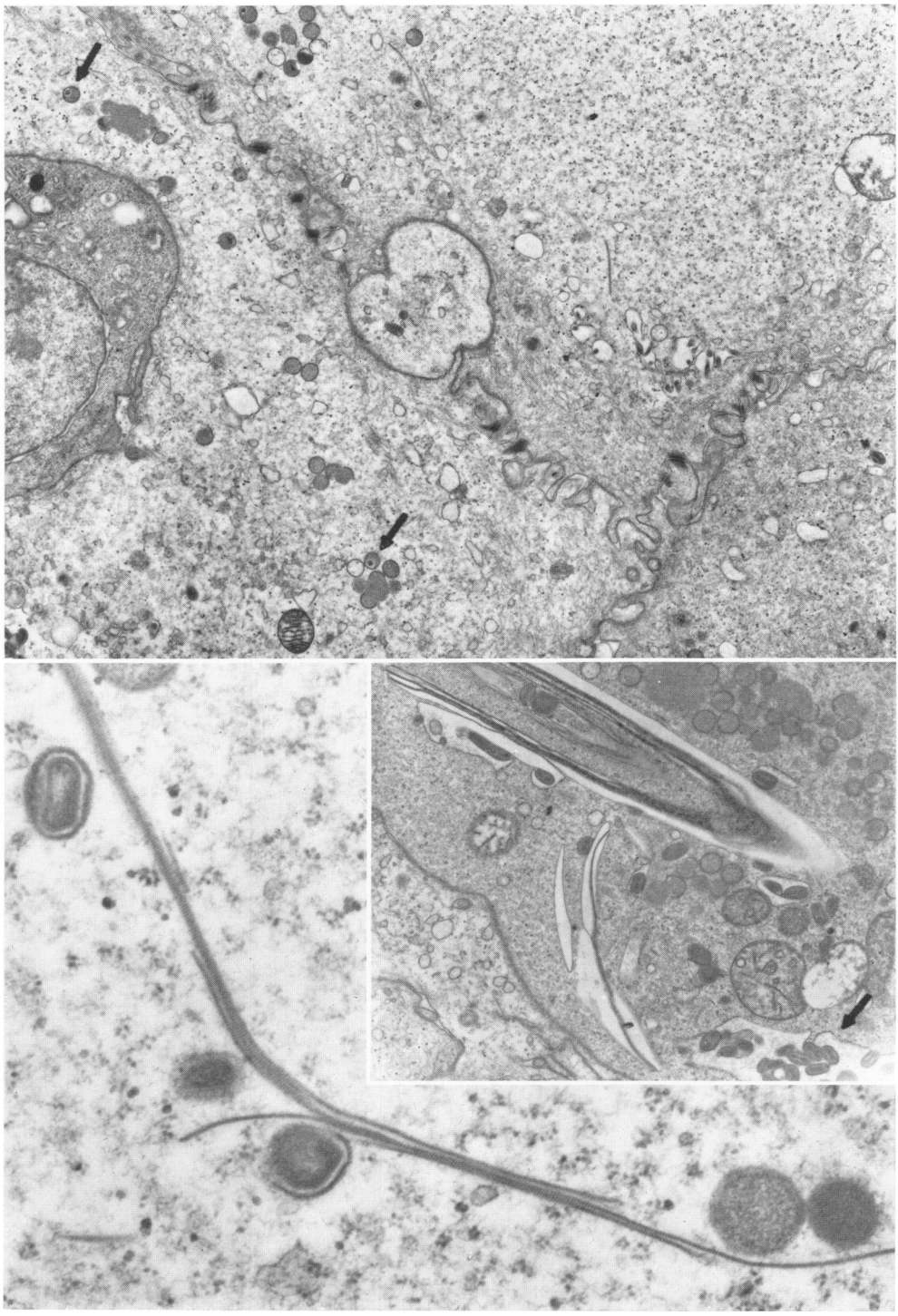

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FIG. 4. Early lipid-containing inclusion body vacuoles are surrounded by incomplete virions. Small lipid vacuoles have viral forms attached to their surfaces (arrow). Degenerated mitochondria are shown. × 14,000.

FIG. 5. A group of electron-dense rodlets lie free in the cytoplasm. One is attached to a vesicle (arrow). An incomplete virion is attached to a mitochondrion. × 17,500.

FIG. 6. Inclusion body vacuoles are surrounded by incomplete virions. × 23,000.

FIG. 7. Membrane bound aggregates of dense rodlets appear in a basal cell of an advanced lesion (small arrow). These are closely associated with developing viral forms (large arrow). × 9,000.

FIG. 8. An early inclusion body vesicle contains numerous electron-dense rodlets. If such structures are serially sectioned, one or more immature virions can be seen indented into the inclusion body membrane. × 13,000.

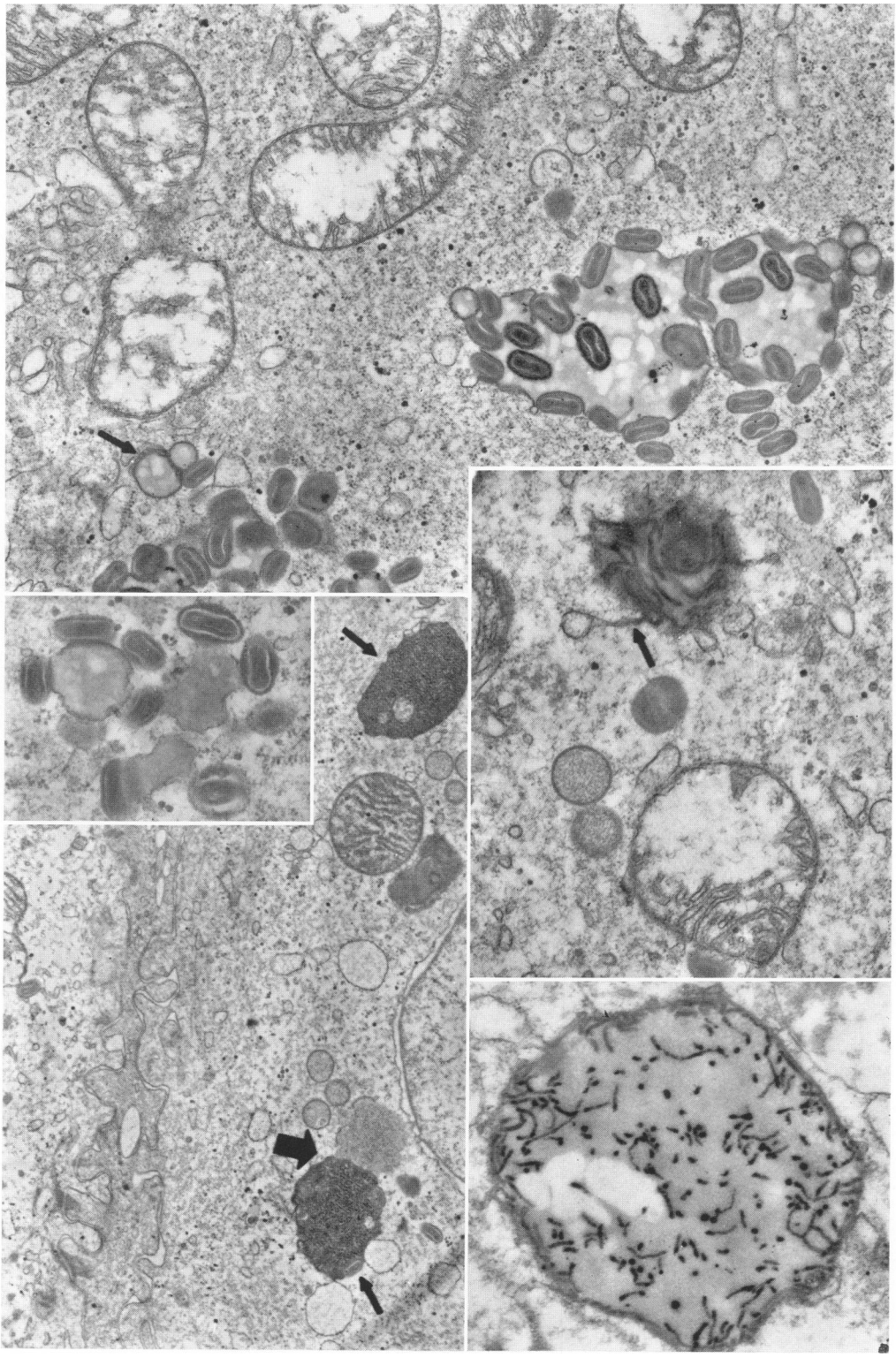

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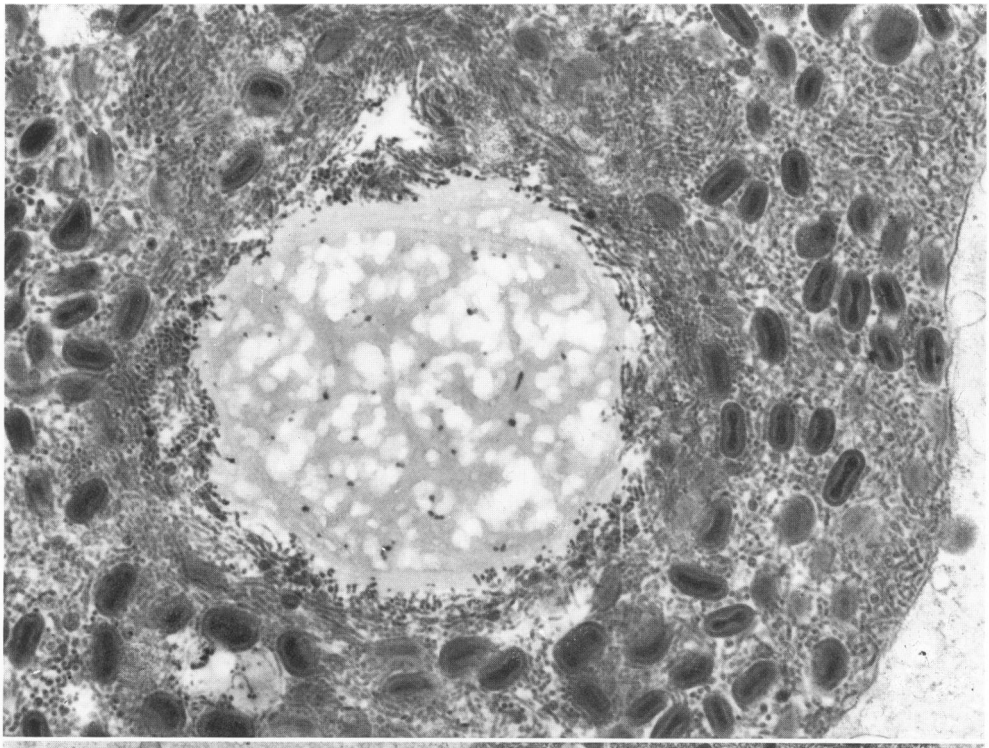
FIG. 9. A large inclusion body contains many rodlets. Some of these exhibit enlarged end bodies. Viral forms in various stages of completion are scattered throughout. The large central vacuolar space is typical of intermediate-sized inclusion bodies (5 to 10 μ). × 17,500.

FIG. 10. A cell contains multiple large (7 to 15 μ) inclusion bodies. Hypertrophy of the Golgi apparatus and mitochondrial degeneration are evident in the central area. Numerous fine tubules are interspersed between the small vesicles (arrow). × 7,500.



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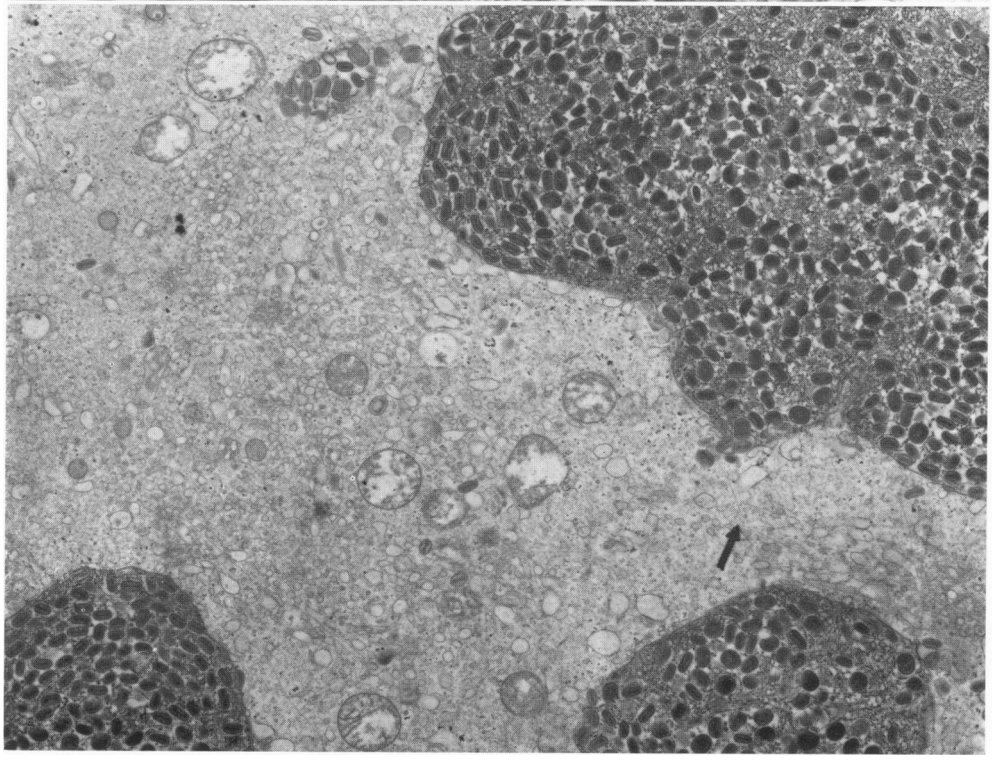

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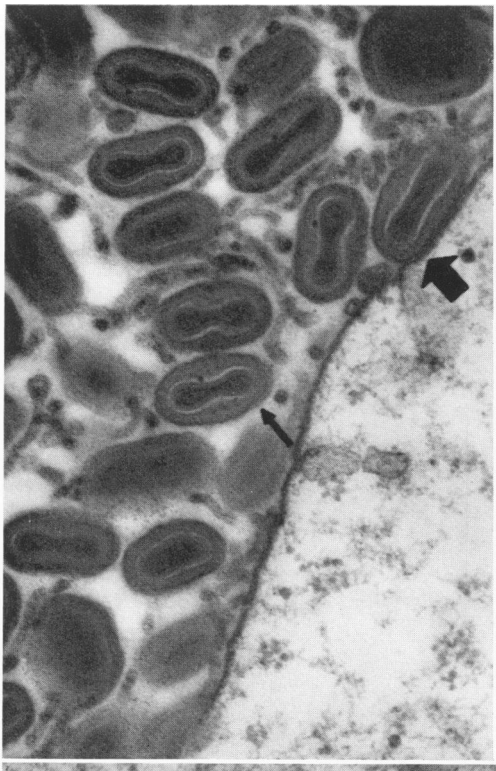
FIG. 11. An enlargement of Figure 10. The fowlpox virions have been sectioned longitudinally (large arrow) and crosswise (small arrow) and in various tangential cuts between these. × 68,000.

FIG. 12. A fowlpox virion is surrounded by electron-dense rodlets, one of which is enlarged in the midsection (arrow). A small round electron-dense body is shown in the virion. The virion is composed of a core, core membrane, two inner membranes, an intermediate membrane (which is enlarged over the broad face of the virion), and an outer membrane and a dense outer coat. × 110,000.

FIG. 13. An inclusion body membrane lies adjacent to Golgi vesicles. One cytoplasmic vesicle appears to be forming a long tubule (large arrow). Electron-dense rodlets protrude through the inclusion body membrane (small arrow) in several places. Note the varied appearance of the rodlets within the inclusion body. Many of these have club-shaped ends. Others are attached to round structures which resemble early viroplasmic particles seen in the cytoplasm. × 49,500.



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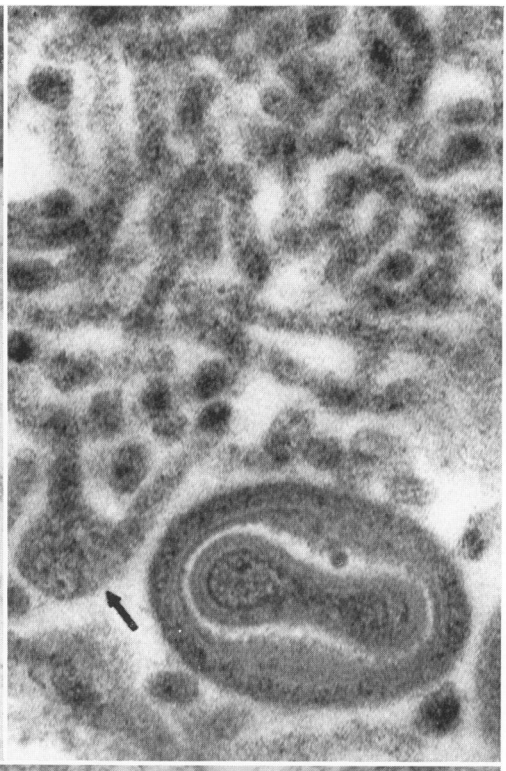

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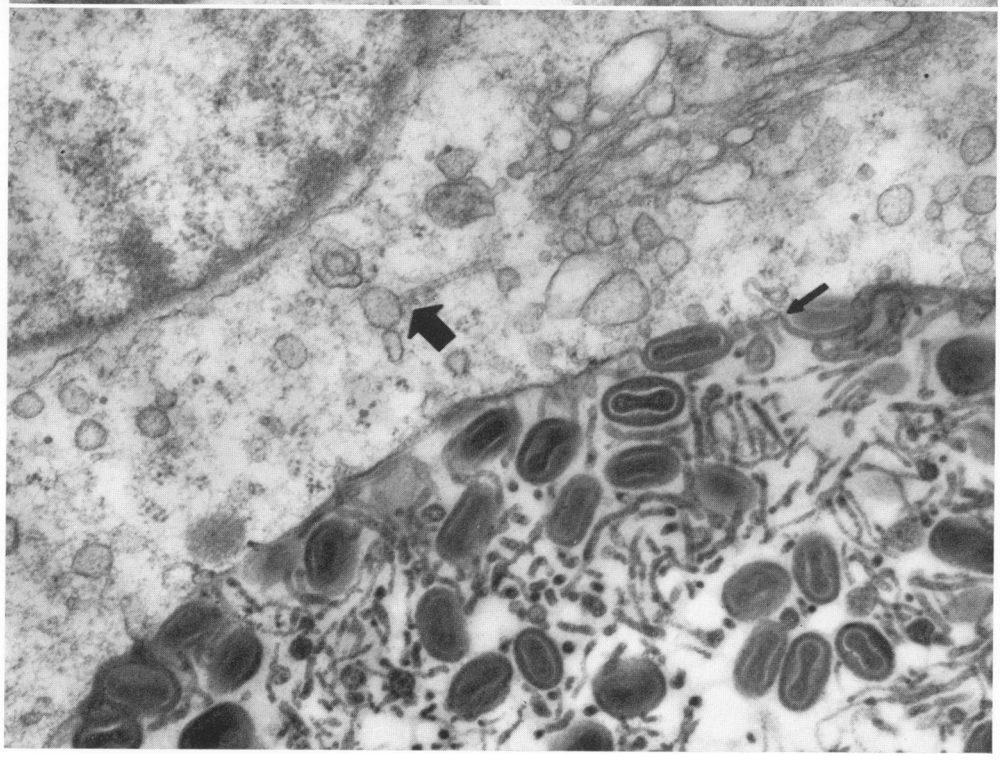

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