THE DEVELOPMENT OF VACCINIA VIRUS IN EARLE'S L STRAIN CELLS AS EXAMINED BY ELECTRON MICROSCOPY

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ABSTRACT

A favorable system which is amenable to frequent and reproducible sampling, consisting of suspension cultures of strain L cells and vaccinia virus, was employed to study the animal virus–mammalian host cell relationship. The three principal aspects investigated concerned the adsorption and penetration of vaccinia into the host, the relationship between the sequence of virus development and the production of infectious particles, and the changes in the fine structure of the host cells. Experiments in which a very high multiplicity of infection was used revealed that vaccinia is phagocytized by L cells in less than 1 hour after being added to the culture, without any apparent loss of its outer limiting membranes. Regions of dense fibrous material, thought to be foci of presumptive virus multiplication, appear in the cytoplasm 2 hours after infection. A correlation between electron microscope studies and formation of infectious particles shows that although immature forms of the virus appear 4 hours after infection, infectious particles are produced 6 hours after infection of the culture, at the time when mature forms of vaccinia appear for the first time in thinly sectioned cells. Spread of the infection is gradual until eventually, after 24 hours, virus is being elaborated throughout the cytoplasm. Addition of vaccinia to monolayer cultures induced fusion of L cells and rapid formation of multinucleate giant forms. In both suspension and stationary cultures infected cells elaborate a variety of membranous structures not present in normal L cells. These take the form of tube-like lamellar and vesicular formations, or appear as complex reticular networks or as multi-laminar membranes within degenerating mitochondria.

INTRODUCTION

The combined techniques of thin sectioning and electron microscopy have proved very successful in providing information about viral development in host cells. Such morphological studies become even more informative when correlated closely with the time sequence of multiplication of infectious units. To make such a correlation we have employed a virus-cell system which offers many advantages for studies with the electron microscope. This system permits the frequent collection of uniform samples, allows an accurate enumeration of both virus and cells for estimation of virus/cell ratios, is amenable to accurate assays for infectious particles by the plaque method, and uses a virus which multiplies to high titres so that high virus-to-cell ratios can be reached, thereby ensuring an almost simultaneous infection of nearly all of the cells in a culture. Strain L cells, which can be grown readily in suspension cultures, support the rapid multiplication of vaccinia and,

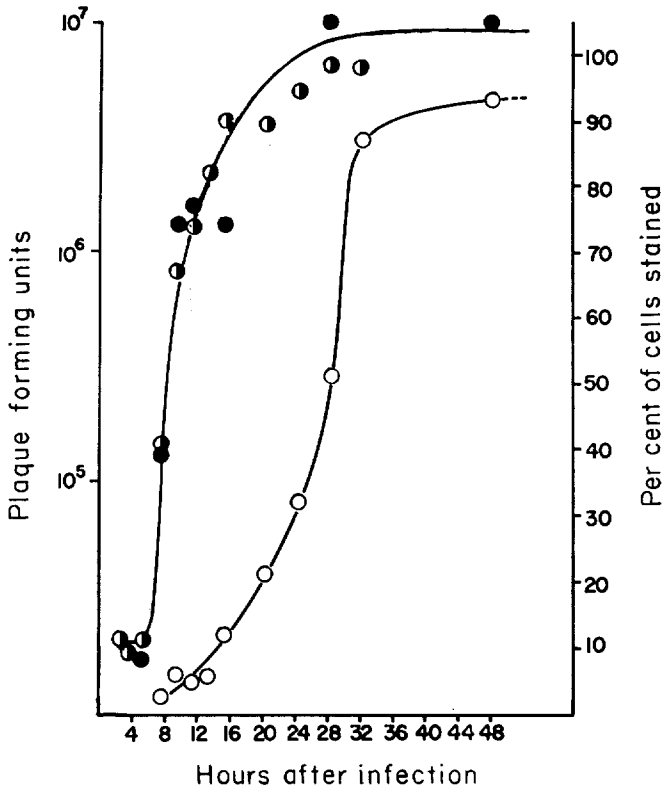

FIGURE 1

Growth curve of vaccinia and changes in L cell permeability due to infection with the virus. The dots and half filled circles represent two separate virus assays on samples from a representative experiment. The open circles represent the percentage of cells in the culture permeable to erythrocin.

therefore, fulfill the requirements of the host in this system. Vaccinia virus, a member of the pox group, was chosen because it is large and possesses a characteristic, readily identifiable internal morphology, features which have made these viruses favorite material for studies with the electron microscope. Thus the pox viruses were among the first to be visualized intracellularly (39), and in which immature developmental stages have been recognized (2, 11, 14, 19, 23).

The present report describes investigations on three different aspects of the development of vaccinia virus in strain L cells. First, the early stages of the infectious process were studied in order to visualize the steps in the early association between the virus and its host. Secondly, the stages of vaccinia virus maturation, assembly and release, as visualized in thin sections, have been correlated in time with the formation of infectious particles. Finally, cells have been examined at various times after infection for any changes in their fine structure.

MATERIALS AND METHODS

Cells

For most of the experiments, the cells used were L-60, a line of cells derived from a clone known as AMK 2-2 (28). From chromosomal and transplantation studies it seems quite certain that both the L-60 and AMK 2-2 cell lines originated from Earle's L strain (31). The cells were grown in suspension in roller tubes as previously described (34).

Medium

Cells were propagated in medium CMRL 1066 (13) containing 5 to 10 per cent of a horse serum selected for non-toxicity. Virus stocks were prepared in the same medium. Overlays for plaque assays

consisted of medium CMRL 1066 containing 2 per cent horse or calf serum and 1 per cent Bacto agar.

Preparation of Virus

The vaccinia virus stock was obtained from Dr. T. Hanafusa. This virus may be propagated readily in L cells cultured either in suspension or on glass. Lysates containing virus were prepared as follows. Individual suspension cultures of L cells were infected with vaccinia and 36 to 48 hours later, when nearly all of the cells in the culture showed cytopathic effects, the cultures were pooled and subjected to 4 to 6 cycles of freezing and thawing to release cell-associated virus. Generally these lysates contained $1–2 \times 10^7$ plaque-forming units (PFU's)/ml.

High titre stocks of virus were prepared from pooled lysates by partial purification using a series of differential centrifugation steps similar to those employed by Weil *et al.* (38), for the purification of the virus of encephalomyocarditis (EMC) from infected mouse brains. The large cellular debris was removed by low-speed centrifugation in the cold. The bulk of the virus in the clarified culture medium was then spun into a pellet by further centrifugation, in the cold, at 15,000 *g* for 30 minutes. The virus in these pellets, resuspended into fresh nutrient medium, was used in some of the studies employing a high multiplicity of infection. However, for determinations of particle counts the cellular proteinaceous material, normally present in these high speed pellets, was partially digested with 0.125 per cent trypsin in an isotonic saline solution (PBS of Dulbecco and Vogt, 8). Following the digestion, the virus was again spun into pellets at 15,000 *g* for 30 minutes and finally dispersed in PBS.

Plaque Assay for Virus

A plaque assay method developed in our laboratory (35) was used. L-60 cells in the logarithmic phase of growth in suspension cultures were centrifuged and resuspended at a concentration of 4×10^6 cells per ml in medium CMRL 1066 (13), containing 2 per cent foetal calf serum. One ml of this cell suspension was placed in a 60 mm Petri dish and 4 ml of medium were added. Under these conditions the cells adhere to the glass and form a complete monolayer in 1 to 2 hours. Virus concentrations were determined on these dishes using a modification of Dulbecco and Vogt's (8) plaque method. One-tenth ml of virus was added to the layer of cells and ½ hour allowed for adsorption. The overlay was then added in two portions. The first consisted of 2½ ml of 1 per cent Bacto agar containing medium CMRL 1066 and 2 per cent horse or calf serum. After the agar had solidified, 2½ ml of the same medium but without agar was added to the plate. The plates were then incubated at 37°C in an incubator flushed continuously with humidified air containing 5 per cent CO_2. Plaques developed in 2 to 3 days and were counted after staining with neutral red.

Assay of Particle Concentration

To determine what proportion of the vaccinia particles in our preparations were infectious, the partially purified virus suspension was mixed with a suspension of 340 mμ polystyrene latex spheres of a known concentration. A large droplet of this mixture was placed on a formvar membrane, dialysed, mounted on a grid, dried and shadowed, as described by Pinteric (24). The number of physical units estimated by this counting procedure in the electron microscope was compared with the number of infectious units as measured by plaque assay. This ratio in our preparations was 4:1 and is similar to the optimum ratios for vaccinia found by Overman and Sharp (21), who titrated vaccinia from calf lymph on chorioallantoic membranes, and by Dumbell *et al.* (9), who titrated cowpox in rabbit skin.

Methods Used for Infecting Cells and Sampling

For experiments in which both virus multiplication was studied and electron microscope observations were made, between 5×10^7 and 10^8 cells were suspended in 10 ml of pooled lysates containing $1–2 \times 10^7$ PFU's of vaccinia per ml and this suspension was shaken for 1 hour at 37°C. Any unadsorbed virus was removed by three cycles of washing with nutrient medium and centrifugation. Thereupon, the cells were suspended in about 500 ml of fresh nutrient medium, and were incubated at 37°C in suspension culture tubes. Samples were taken at regular intervals for a period of 48 hours. One portion of each sample was frozen at −80°C. This fraction was later thawed and then frozen and thawed 5 times and titrated for infectivity. Another fraction was used for the determination of cell viability by staining with erythrocin (30), and a third portion was centrifuged at low speed for several minutes and the pellet of cells obtained was prepared for electron microscopy.

In order to examine the early stages of infection more carefully, very high multiplicities of infection were used and samples were taken for electron microscopy only, for 3 hours after adding the virus. To achieve the high multiplicity of infection, about 10^7 cells were added to 5 ml of a concentrated virus preparation containing 10^9 PFU's in nutrient medium. Thus approximately 100 infectious units or 400 physical particles of vaccinia per cell were present. This suspension was shaken at 37°C and three consecutive samples were taken from it for

electron microscopy at hourly intervals after infection.

Multinucleate Cells

Multinucleate cells were obtained from infected stationary cultures of L cells as follows. About 10^5 to 10^6 PFU's of vaccinia were added to monolayer cultures of L cells in petri dishes. About 48 hours later these cultures, which contained a large proportion of multinucleate giant cells, were harvested for examination by optical and electron microscopy.

Electron Microscopy

Pellets of cells, from the control and infected cultures, obtained by centrifugation at 800 to 1,200 RPM for several minutes, were fixed in a 1 per cent solution of osmium tetroxide buffered at pH 7.4 (22), dehydrated, and embedded by standard procedures used for thin sectioning and electron microscopy. For improvement of contrast of the thinly sectioned material, the sections, mounted on copper grids, were stained by floatation on a solution of lead hydroxide, using the procedure of Watson (37).

RESULTS AND DISCUSSION

I. Virus Multiplication and Cell Permeability

Before dealing with the electron microscopic observations, the course of vaccinia virus multiplication in L cells growing in suspension will be considered. A representative growth curve for vaccinia virus is shown in Fig. 1. As indicated in Materials and Methods, the multiplicity of infection in this experiment was high enough to ensure that all, or nearly all, the cells were infected with at least one virus particle. It may be seen that following a latent period of less than 6 hours the virus titre rose appreciably until about 12 to 16 hours after infection and, thereafter, the rate of virus formation decreased and leveled off after about 28 hours. When the infected culture was examined at various times for loss of permeability regulation (as measured by staining with erythrocin), it was found that changes in permeability lagged behind the curve for virus formation by about 10 to 20 hours. With vaccinia, therefore, it seems that formation of infectious virus affects permeability only very gradually.

II. Development of Vaccinia Virus as Examined by Electron Microscopy

Electron microscopic observations were made on cultures where all, or nearly all, the cells were infected with at least one virus particle. However, 2 different types of cultures were examined. For investigations in which virus development was followed for a long period (48 hours), the input multiplicity of infection was about 3 to 5 infectious virus particles/cell. Infectious particle assays were also done on these cultures (see above). Where the early stages of infection were followed in detail, a

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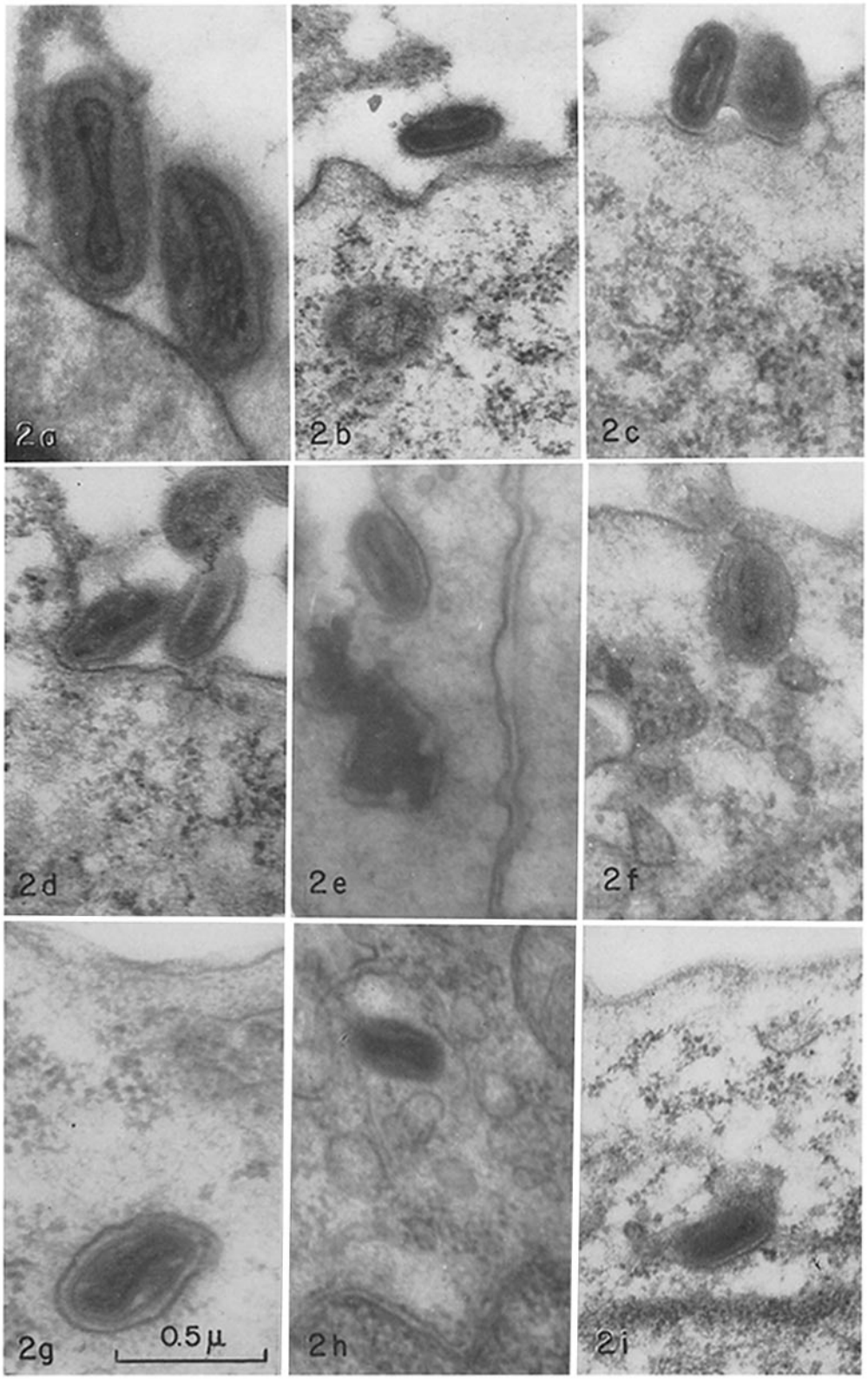
Key to Markings on Electronmicrographs

N, nucleus
m, mitochondria
f, lipid droplets
v, vaccinia virus
Va, vacuole or vesicle

FIGURES 2*a* to *i*

Nine micrographs selected to illustrate stages in the adsorption and penetration of vaccinia into the cytoplasm of L cells. All these examples demonstrate cells sampled 1 hour after infection with a high multiplicity of virus. In each example the cell membrane is uppermost and the center of the cell is below the lower margin of the micrograph. The adsorbed virus particles are attached to the membrane with either their long or short axes perpendicular to it, as seen in *a* to *b*. Stages in the engulfment or phagocytosis of vaccinia at the membrane are illustrated in micrographs *c* to *f*. In *e* some dense cytoplasmic debris is also being engulfed. The particle in *g* lies entirely within a vacuole which presumably formed at the surface by invagination. Another particle, in *h*, lies free in the cytoplasm. The upper portion of the virus shown in *i* appears to have been disrupted and the integrity of its membranes has been lost.

Magnification, 48,000 except Fig. *a*, magnification 120,000.


2a
2b
2c
2d
2e
2f
2g
0.5 μ
2h
2i

much higher virus-cell ratio was used, *i.e.* about 100 infectious particles/cell. The electron microscopic observations on the latter cultures will be described first.

(*a*) *Early Steps in the Infectious Process Studied by Electron Microscopy Using a High Multiplicity of Infection:* Under conditions of high multiplicity of infection, all or nearly all of the cells adsorb a large number of vaccinia virus particles very soon after the virus is added to the culture, and the chance of observing an infected cell during the early periods after infection is considerably enhanced. In fact, virus was observed in about one out of ten cell sections sampled 1 hour after its addition. In these cultures we have followed the process of adsorption of virus to the cell membrane, the mechanism of penetration through the membrane, and the early fate of the infecting particle in the cell.

The successive events which are thought to occur during the 1st hour after adding vaccinia to the cultures are illustrated in the nine micrographs of Fig. 2, *a* to *i*. Adsorption to the membrane (Fig. 2, *a* to *c*) occurs with both the short and long axes of the virus oriented perpendicularly to the membrane, thus indicating that adsorption sites may be present at all points on the surface of the brick-like particles. This observation is in contrast to that made by Higashi *et al.* (14), who found a preferential adsorption when the long axis of ectromelia and variola (viruses of the pox group) was oriented perpendicularly to the membrane of the host cell.

Examination of a large number of cells indicated that vaccinia passed into the cytoplasm by phagocytosis (Fig. 2, *c* to *f*) where the virus is observed in vesicles (Fig. 2, *g*) presumably found at the cell surface. However, particles not enclosed in vesicles were also observed in the cytoplasm at early times after infection (Fig. 2, *h*), suggesting that a direct penetration through the membrane may also occur.

One hour after infection, a large fraction of the cells contained virus in the cytoplasm. Of special significance is the observation (Fig. 2, *g*) that during penetration the virus does not lose any of its outer membranes, for intracellular particles observed at early times after infection appear indistinguishable from those which were observed to be adsorbed to the outer cell membrane.

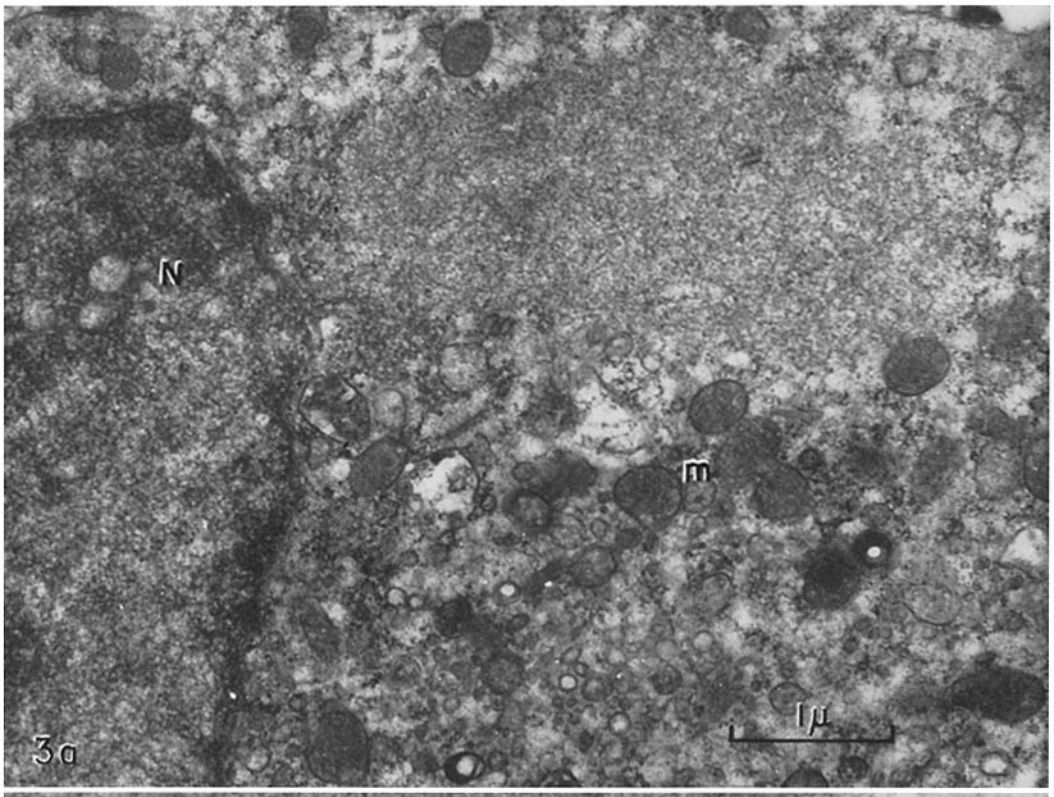
At later times after infection, complete virus particles became less and less evident within the cytoplasm: there were few particles observed in association with L cells in the sample taken at 2 hours, and very few at 3 hours after virus was added. However, areas of dense material which were not present in cells sampled at 1 hour were quite common in those examined 2 hours after infection, and these areas were more numerous in cells sampled 3 hours after infection. Examples illustrating the appearance of such foci of dense material are shown in Fig. 3 *a*, *b*. The dense material resembles very closely the material found in the nucleus and is composed of randomly oriented short lengths of fine threads or fibrils ranging in width from 25 to 80 A and some small granules of the same diameter as the threads. In one cell, sampled at 3 hours after virus was added, three such areas were observed. This suggests that in any one cell exposed to infection by a high virus multiplicity these foci may be quite numerous.

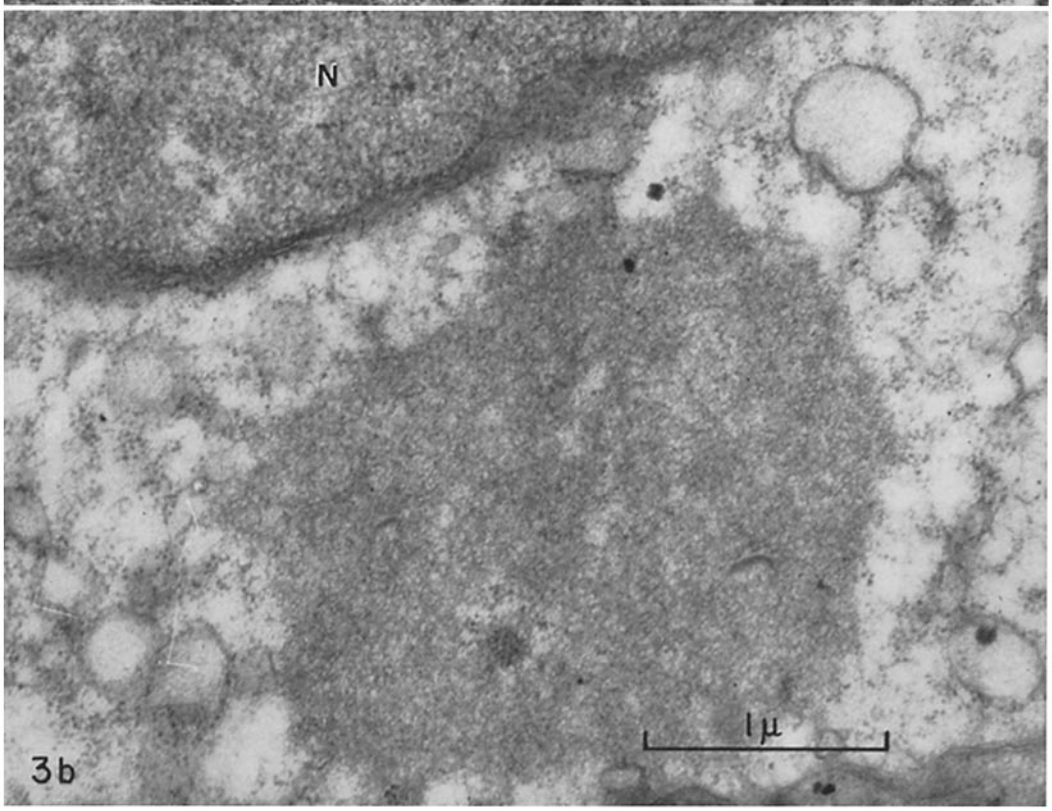
Another type of fibrillar structure, ranging in width from 40 to 80 A, was observed in the cytoplasm of L cells 2 hours after adding virus to the culture and became prominent in cells sampled 3 hours after infection. These fibrils were arranged either in loose, haphazardly oriented formations (Fig. 4 *a*), or were grouped into tight bundles in which the individual units were oriented parallel

FIGURE 3

a. Portion of the nucleus and cytoplasm of a cell sampled 3 hours after infection. In the upper part, next to the nucleus, is an area of dense material in the form of short lengths of granules and randomly oriented fine threads. The disposition of the mitochondria and other cytoplasmic elements suggests that they have been displaced by the dense, fibrillar material. × 22,500.

b. Another example showing a large area of dense fibrillar material in the cytoplasm. × 33,500.


N
m
1μ
3a


N
1μ
3b

with one another over considerable distances, as shown in Fig. 4 *b*. Such fibrillar formations have been described in a variety of animal cells following viral infection, including HeLa cells infected by *Herpes simplex* (Morgan *et al.*, 20), and chorioallantoic membrane by ectromelia, reported recently by Siegel (33). Although these filamentous structures are observed relatively frequently in L cells after vaccinia is added, they are not formed specifically as a result of infection since, upon careful examination, they were also observed occasionally in cells sampled from uninfected cultures. They have also been observed by us in Krebs 2 ascites cells and in HeLa cells (6). The significance of this cellular component is at present obscure. Perhaps these fibrils are formed within L cells in response to injurious stimuli.

(*b*) *Later Stages in the Development of Vaccinia Virus as Studied by Electron Microscopy:* To examine the later stages in the development of vaccinia virus, samples were taken for 48 hours from the culture infected with a lower multiplicity of infection (3 to 5 infectious particles/cell). From these electron micrographs, the sequence of steps in the elaboration of vaccinia virus in L cells has been determined. These include the formation of the limiting membranes of the virus, the condensation into an immature particle, the formation of the nucleoid, the transition into mature form, and finally the stage of virus release. All of these stages have been observed sequentially in the infected culture. However, because of asynchrony in virus development, samples taken later than 6 hours after infection contained, in different cells, examples of many stages of virus maturation. For convenience, the illustrations which show the sequence of virus development were chosen only for the clarity with which they illustrate a particular stage. The time during the latent period at which the various developmental forms were observed will be indicated, however.

The first stages of virus "condensation" were observed in the cytoplasm of cells sampled 4 hours after infection. Foci of developing virus, such as the one illustrated in Figs. 6 *a* and *b*, were observed to contain spherical particles with complete membranes and eccentric nucleoids, as well as clumps of viroplasm partially enclosed by incomplete membranes.

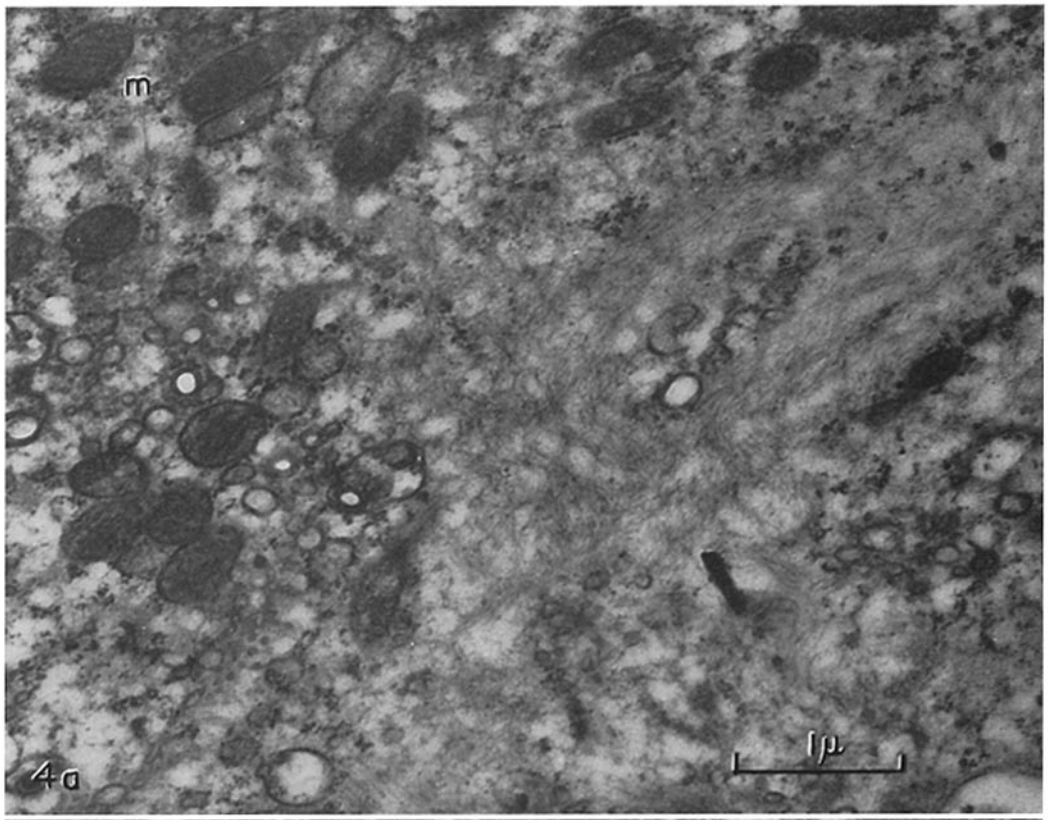
Condensation into presumptive virus particles first occurred in the dense fibrillar material (Figs. 3 *a* and *b*) described above, and seen in cells at 2 to 3 hours after infection. The dense material aggregated into compact clumps of dense filaments within and around which the limiting membranes of the virus were formed (Fig. 5 *a*). In the example shown at a higher magnification (Fig. 5 *b*), it may be seen that the membranes "condensed" both within the dense material and at the periphery of it. In other examples (Fig. 6 *c*), it appeared that at some points several, perhaps as many as four, membranes condensed in parallel with one another around the presumptive virus fibrous material or viroplasm. These multiple membranes, each approximately 30 A in width, were so closely apposed that they appeared often as a single wide membrane having an indistinct outline. Pictured in three dimensions, the membranes must first have been formed as caps at one edge of the clumps of viroplasm and these caps must gradually have grown to form a spherical surface, which eventually completely surrounded the viroplasm to form an immature particle.

Infectious vaccinia virus contains a dumbbell-shaped core of very dense material. The presumptive material for this core was first observed at 3 to 4 hours after infection and appeared as a nucleoid in immature virus. Formation of the nucleoids commenced by condensation of dense material at small foci among the fibrous elements of the viroplasm. There was some suggestion that the process of nucleoid formation could precede or follow that of membrane elaboration (compare

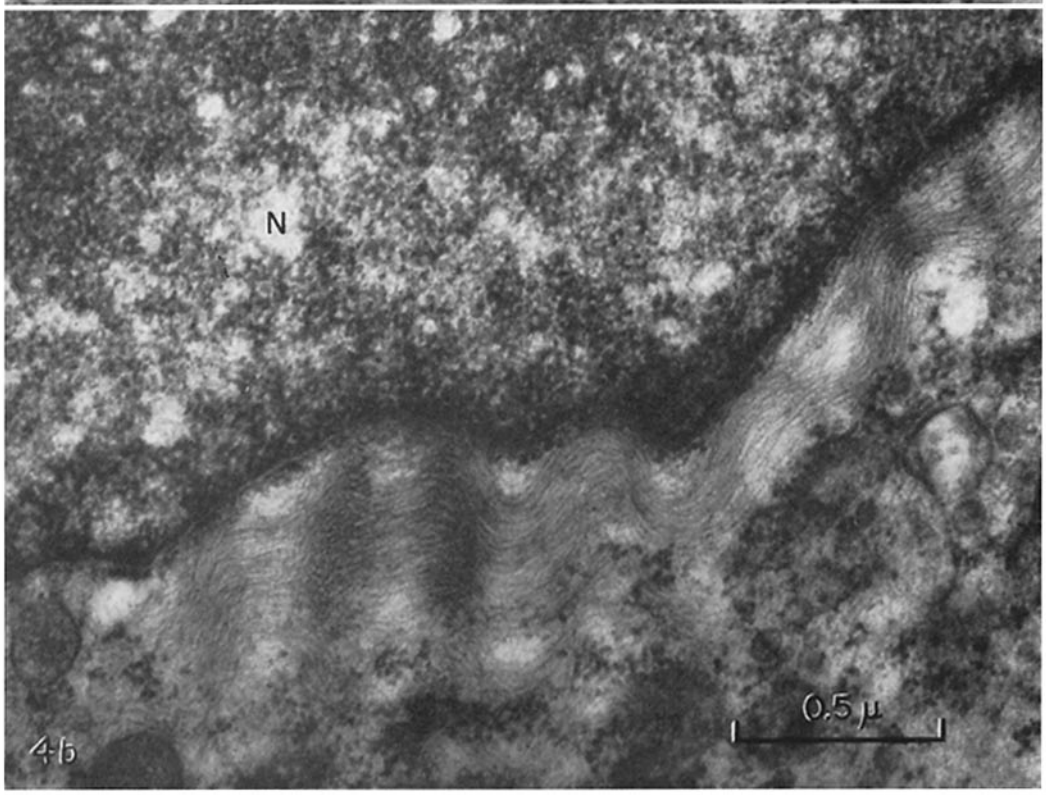
FIGURE 4

a, *b*. Portions of two cells sampled 3 hours after infection. Note the bundles of randomly oriented fibers occupying the center of the micrograph and a group of mitochondria on the left. × 22,000.

b. A portion of the nucleus and cytoplasm of another cell. Bundles of fine fibrils, arranged parallel to each other over considerable distances, are prominent in the paranuclear region. × 56,000.



4a



4b

the group of immature particles illustrated in Fig. 6 *a* with that shown in Fig. 6 *b*), suggesting that the two structures of the virus particle were formed independently. However, the alternative explanation that our observations were the result of sectioning artifacts is not ruled out. Although the entire space within an immature particle, with the exception of a zone or halo of less dense amorphous material, was filled by material of a filamentous or fibrous nature, that of the nucleoid was composed of coarser fibers or threads, generally 50 to 100 A in width (Fig. 6 *d*).

At later times during the latent period an intermediate stage between the spherical immature virus and the mature forms was observed. This is illustrated in Fig. 7 *a*, in which are present several particles, each having an oval or rectangular outline; these possess an elongated nucleoid at their center, indicating that the nucleoid increased in volume during maturation of vaccinia and the fibrous nature of the material within it became obliterated. Concomitantly the halo zone became wider and the central (viroplasm) portion of the previous stage now occupied a peripheral region.

The last or mature stage of vaccinia is illustrated in Fig. 7 *b*, in which a large group of particles is present. A single mature vaccinia, transected diagonally, in which some detail of internal structure is evident, is illustrated at a higher magnification in Fig. 7 *c*. A dumbbell-shaped core of very dense material, 30 to 35 mμ, occupies the center of the particle and is surrounded by a region of lower density about 25 mμ wide. Two other zones of intermediate density occupy the region between the less dense layer and the outer membranes on either side of the particle. The outer membranes are not resolved in this figure because the section was relatively thick. For a more comprehensive description of the morphology of mature vaccinia the reader is referred to a review article on this subject by Peters (23).

Mature particles of vaccinia were observed for the first time 6 hours after infection at the time when virus titre had commenced to rise (Fig. 1). At a later stage, 8 to 10 hours after infection of the culture, the observed areas of virus formation had enlarged and contained small groups of mature particles lying in close proximity to groups of particles in their formative stages. Very frequently such areas of developing virus were encompassed by numerous mitochondria having a disposition which may have resulted either from mechanical displacement at the time of the establishment in the cytoplasm of zones of fibrous material (see Fig. 3 *a*), or from the migration of mitochondria to the sites of virus multiplication. There is, however, no evidence from the material examined in the present investigation that in L cells vaccinia may be formed from the mitochondria, as suggested in previous studies on other pox-virus infections by Croissant *et al.* (4), and by Peters (23).

A rapid increase in the size and number of foci of virus development and in the number of mature virus particles present in the cytoplasm was observed in L cells sampled between 10 and 24 hours after infection, a result consistent with the rapid rise in virus titer recorded during this time interval (Fig. 1). Twenty-four hours after infection, when the titer had almost reached a maximum, vaccinia particles were evident in virtually all regions of the cytoplasm as large groups of mature and immature particles (Fig. 8).

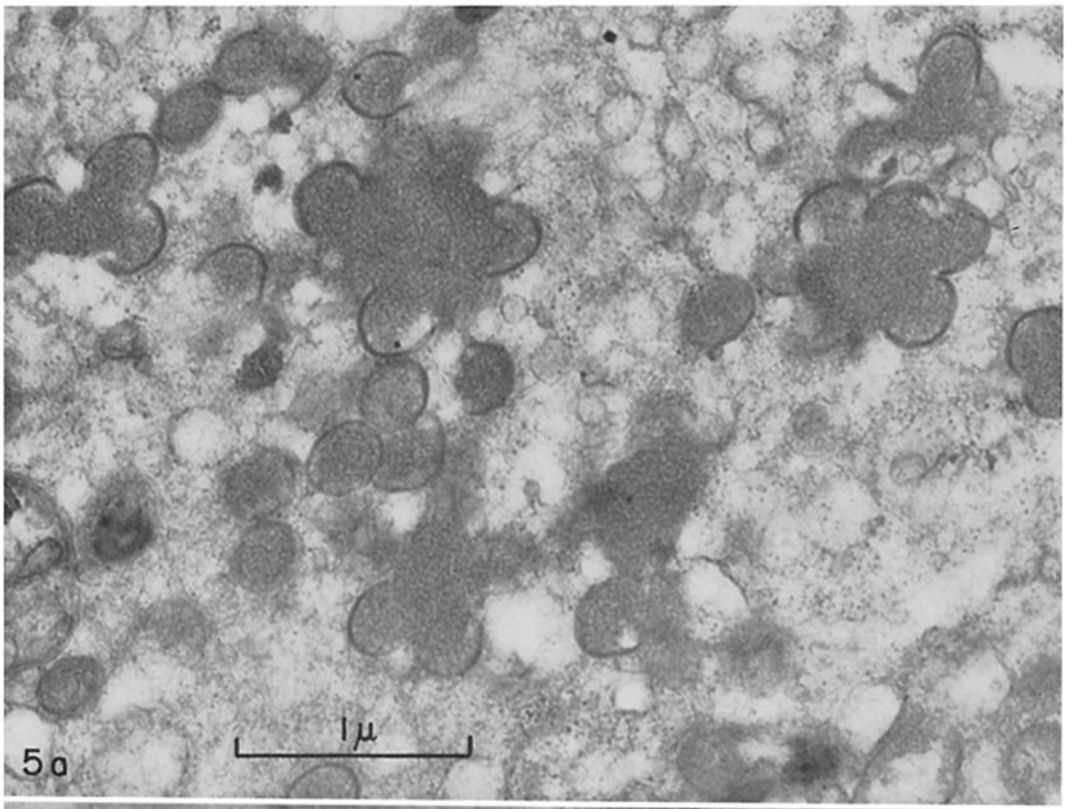
FIGURES 5 to 7

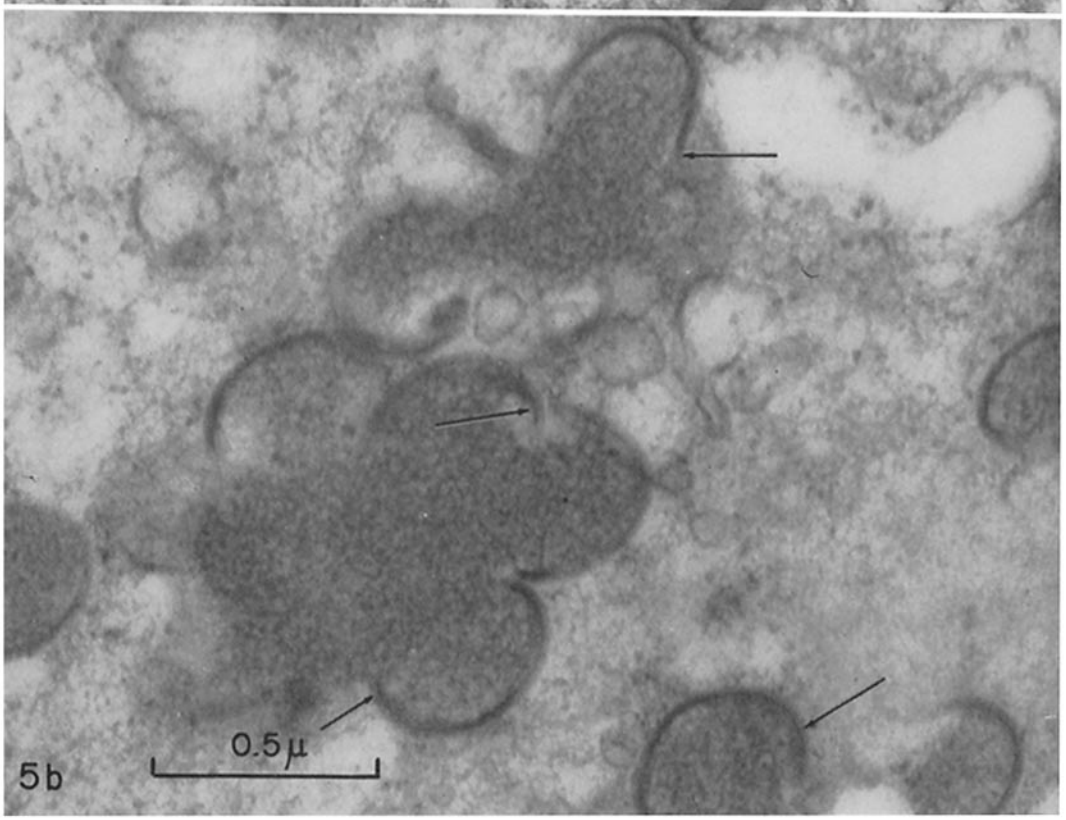
Micrographs of selected areas to illustrate stages in the assembly and maturation of vaccinia in L cells.

FIGURE 5

a. Dense viral membranes are shown forming within and around clumps of dense fibrillar material. In several particles the membranes have completely enclosed the presumptive virus material, or viroplasm. × 32,000.

b. One of the groups of particles in the course of development, illustrated in *a*, is shown here at a higher magnification. Arrows indicate sites where the elaboration of the dense limiting membranes has occurred within the foci of fibrillar material. × 61,000.


1μ
5a


0.5μ
5b

(*c*) *Emergence of the Virus:* From 10 hours after infection onward, during the most rapid rise in titer (Fig. 1), there were mature particles present in close association with microvilli at the cell surfaces (Fig. 9 *a*, *c*, *d*) and also near the membranes of intracytoplasmic vesicles (Fig. 9 *b*). Release of vaccinia from the cytoplasm may thus occur either directly through the microvilli or at the cell membrane through channels by which the vesicles may communicate with the cell exterior. Conclusive evidence substantiating either of these possible mechanisms of release was, however, not obtained.

(*d*) *Summary of sequence of steps in the multiplication of vaccinia virus in L cells:* A diagrammatic representation of the sequence of events during viral adsorption, penetration into the cytoplasm, multiplication and maturation is illustrated in Fig. 11. Until, however, more conclusive evidence is obtained about the fate of vaccinia following its penetration into the cytoplasm of the host cell, stages 3 and 4 in this diagram must be considered as only hypothetical.

III. Effects of Vaccinia Virus Multiplication on the Host Cell as Examined by Electron Microscopy

(*a*) *Vesicular and Tube-Like Formations in the Cytoplasm of L Cells:* Late in the infection, about 24 hours after the addition of vaccinia to the cultures, L cells were found to contain in their cytoplasm groups of smooth walled vesicles and very long "tubes." An example showing a whorl-like arrangement of the vesicles is shown in Fig. 12 *b*, and a tube, traversing the cytoplasm from the nucleus to the periphery of the cell and open at both ends, is illustrated in Fig. 11 *a*. Both longitudinal and transverse sections of tubes were observed in some cells, suggesting that either a very long tube had curved along its course through the cytoplasm or that more than one tube may be present in a single cell. The diameter of these tubes is variable and their actual length cannot be determined from thin sections, but some were more than 10 μ in length. A careful examination of tubes which had been sectioned tangentially near their open end revealed that the walls were composed of lamellae and very flat vesicles, arranged parallel to one another (Figs. 11 *b*, 12 *a*). In cross-sections (Fig. 12 *b*) the walls were also found to consist of concentrically arranged lamellae and very flat vesicles which had their walls apposed so closely that the lumen was barely visible. The material within the tubes appeared to be the same as that in the surrounding cytoplasm, which indicated that the centre of the tubes was in direct contact with the cytoplasm. A three-dimensional interpretation of the structure of a tube is shown in Fig. 13. In this diagram connections between vesicles have not been indicated, although they undoubtedly exist. The morphology of the walls suggests that lamellae, which may have formed first, split at some points to form the walls of vesicles. Such a mechanism of vesicle formation is suggested by bulbous swellings at the tips of lamellae (Fig. 12 *a*, arrow), by the morphology of the flat vesicles observed in cross-sections of tubes (Fig. 12 *b*), and by the aspect of rows of vesicles which continue when the lamellae stop at the ends of tubes, as shown in Fig. 12 *b*. The interchangeability between the appearance of a tube and the concentrically arranged and more loosely grouped flat vesicles (such a group is shown in Fig. 12 *c*) can be imagined if the latter were packed into a smaller volume, causing the walls of the vesicles to become apposed and arranged in concentric patterns.

(*b*) *Formation and Fine Structure of Multinucleate Cells in Stationary Cultures:* Multinucleate giant cells formed rapidly after infection of monolayer

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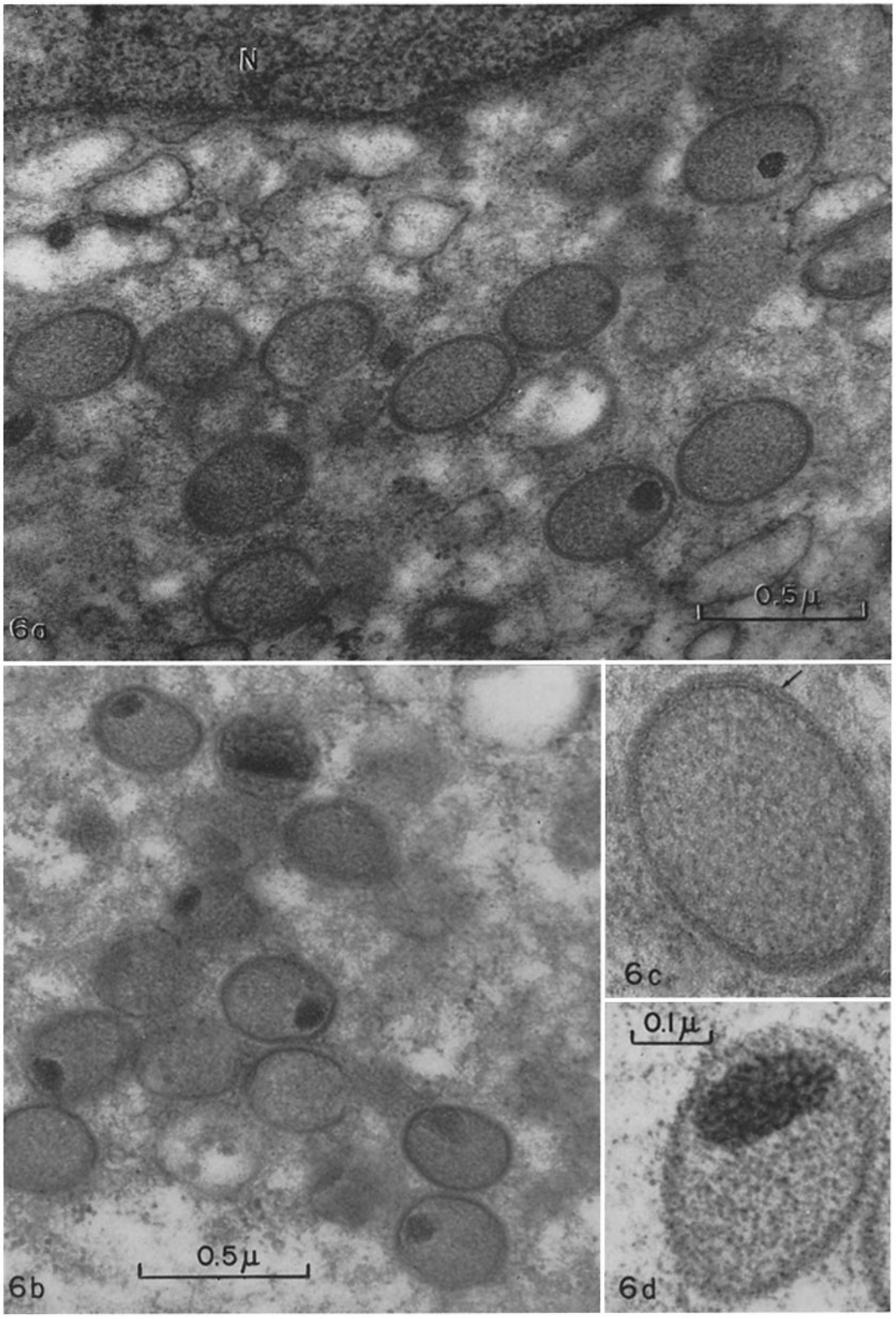
FIGURE 6

a. A group of immature particles, most with complete limiting membranes. In only two of these is a dense, eccentric nucleoid clearly distinguishable. × 54,000.

b. Eccentric, dense nucleoids are present in a large proportion of the particles present in this area. Note the "halo" of material of low density surrounding the nucleoids. × 54,000.

c, *d*. Two examples of immature virus. In *c*, the arrow points to a region where multiple membranes 30 A in width are resolved. In *d*, note the very dense, fibrous elements within the region of the nucleoid. × 130,000.


N
0.5μ
6a
0.5μ
6b
6c
0.1μ
6d

cultures of L cells with vaccinia. As early as 1 day after infection large cells were evident, and after 2 days many multinucleate cells, some measuring nearly 100 μ, were evident. When monolayers are infected with virus at high dilution (several hundred PFU's or less per Petri dish), plaques were formed within 2 days which had an appearance such as that shown in Fig. 14 *a*. The centre of the plaque was almost entirely devoid of cells, whereas the periphery was lined by a ring of multinucleate cells. Each large multinucleate cell consisted of a very dense, granular centre and a peripheral zone of nuclei, which may number several hundred in some cells. The appearance of a living multinucleated cell in the phase-contrast microscope is shown in Fig. 14 *b*.

The morphology of thinly sectioned multinucleate cells examined in the electron microscope corresponded closely to that expected from their appearance in the phase-contrast microscope (compare Figs. 14 *b* and *c*). The numerous nuclei occupied practically the entire cell periphery, and the cytoplasm contained very dense elements, including large numbers of mature vaccinia particles. In the example shown in Fig. 14 *c* there are two areas of membranes arranged into a reticulum. Such areas are characteristic of these multinucleate cells.

Present also in the central zone of the cytoplasm were oval or rounded structures, each several microns in diameter, bounded by either a single or double membrane. Dense granules, small vesicles, and larger regions of low density encompassed either partially or completely by wide dense bands were also observed within the membrane-bounded bodies. These rounded intracytoplasmic bodies, a number of which are present in the area shown in Fig. 16 *a*, are almost certainly degenerating mitochondria: in several examples intermediate stages were encountered, in which one portion of these organelles had clearly recognizable double outer membranes and cristae, as in mitochondria from cells in control culture, and the remainder had the appearance of the structures described above. An examination of the wide dense bands at higher magnifications showed them to have a complex morphology. They are formed of concentric layers of fine dense membranes or lamellae, each 20 A wide and separated from the next membrane by a zone of lesser density about 30 A wide (Fig. 16 *b*). In some instances as many as 14 multilaminar layers were counted within a single dense band. Disposition of the membrane bands within these mitochondrial structures indicates that they form as thickenings of the inner mitochondrial membranes, which normally are, however, wider than a single fine lamellar membrane.

GENERAL DISCUSSION AND CONCLUSIONS

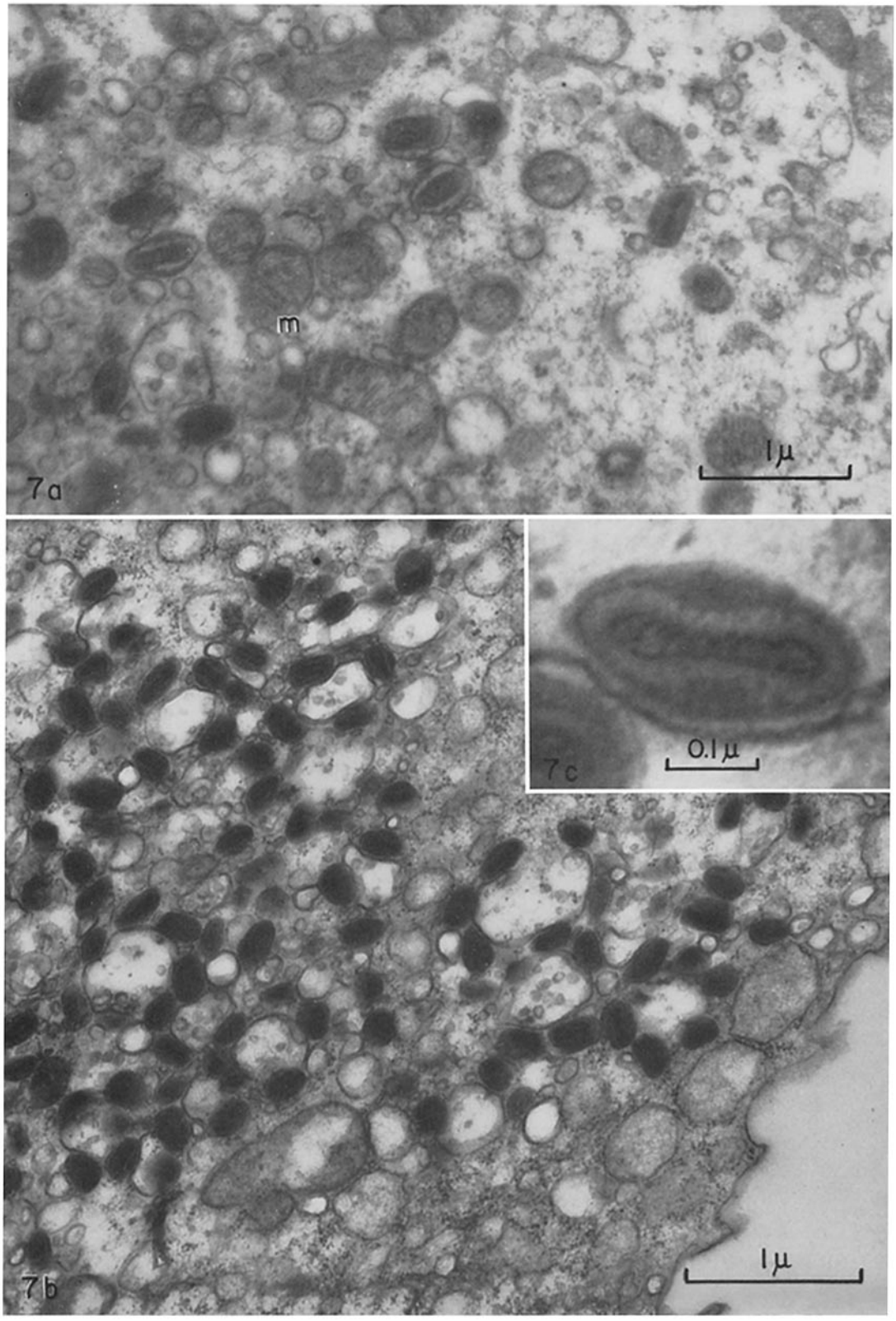
Evidence obtained from experiments using high virus multiplicities suggests that infection results from the adsorption and phagocytosis of vaccinia by L cells, because 2 to 3 hours after being added to the culture the cell-associated virus disappears whereas foci of dense fibrous material, such as those in which developing virus is observed later, appear in the cytoplasm. Since comparable

FIGURE 7

a. An intermediate late stage in maturation of vaccinia. The nucleoids, which have elongated, occupy a large volume in the center of the particles and are surrounded by a zone of material having a low density. The particles have assumed an oval or rectangular outline, in contrast with the spherical one of the less mature forms. One mature particle, at the right side of the micrograph, is distinguishable by its great density, rectangular outline and dumbbell-shaped core. × 24,000.

b. A large group of mature vaccinia. The particles are smaller and denser than in their formative stages. The characteristic dumbbell core can be distinguished in particles sectioned along the appropriate plane. × 28,000.

c. A single mature particle shown at a high magnification to illustrate detail of the internal structure. The dense, dumbbell-shaped core is surrounded by a zone of material of lower density. Two bodies of equal size and intermediate density occupy the region between the less dense layer and the outer membrane. A dense membrane, not associated with this particle, traverses the micrograph just below it. × 150,000.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investigations on the early stages of infection with other animal viruses have not as yet been reported, it is not known whether phagocytosis is a general mechanism for entry of animal viruses into host cells.

The fate of the virus 1 hour after infection is especially interesting. At this time, in a large majority of infected cells, vaccinia virus lies freely in the cytoplasm of the cell, and does not seem to have lost any of its limiting membranes. This means that no separation of the nucleic acid and protein moieties of the virus occurs on infection. This behaviour is to be contrasted with that of the bacterial viruses where such a separation does occur.

In several examples in different cells, sampled about 1 hour after infection, disrupted particles of vaccinia virus were observed in which one side of the particle had intact membranes, whereas the other side of the virus had a fuzzy outline with no distinguishable membranes, and denser viral material had spread out beyond the limits of the outline of the particle (Fig. 2, *i*). It seems probable that in these particles the various components of vaccinia virus are being separated and that this represents the first step in development of progeny virus. Our observations do not, of course, indicate how the disruption of the infecting particles takes place, but one possibility is that a proteolytic enzyme(s) is induced by the presence of foreign virus protein which, in turn, causes hydrolysis of the virus coat.

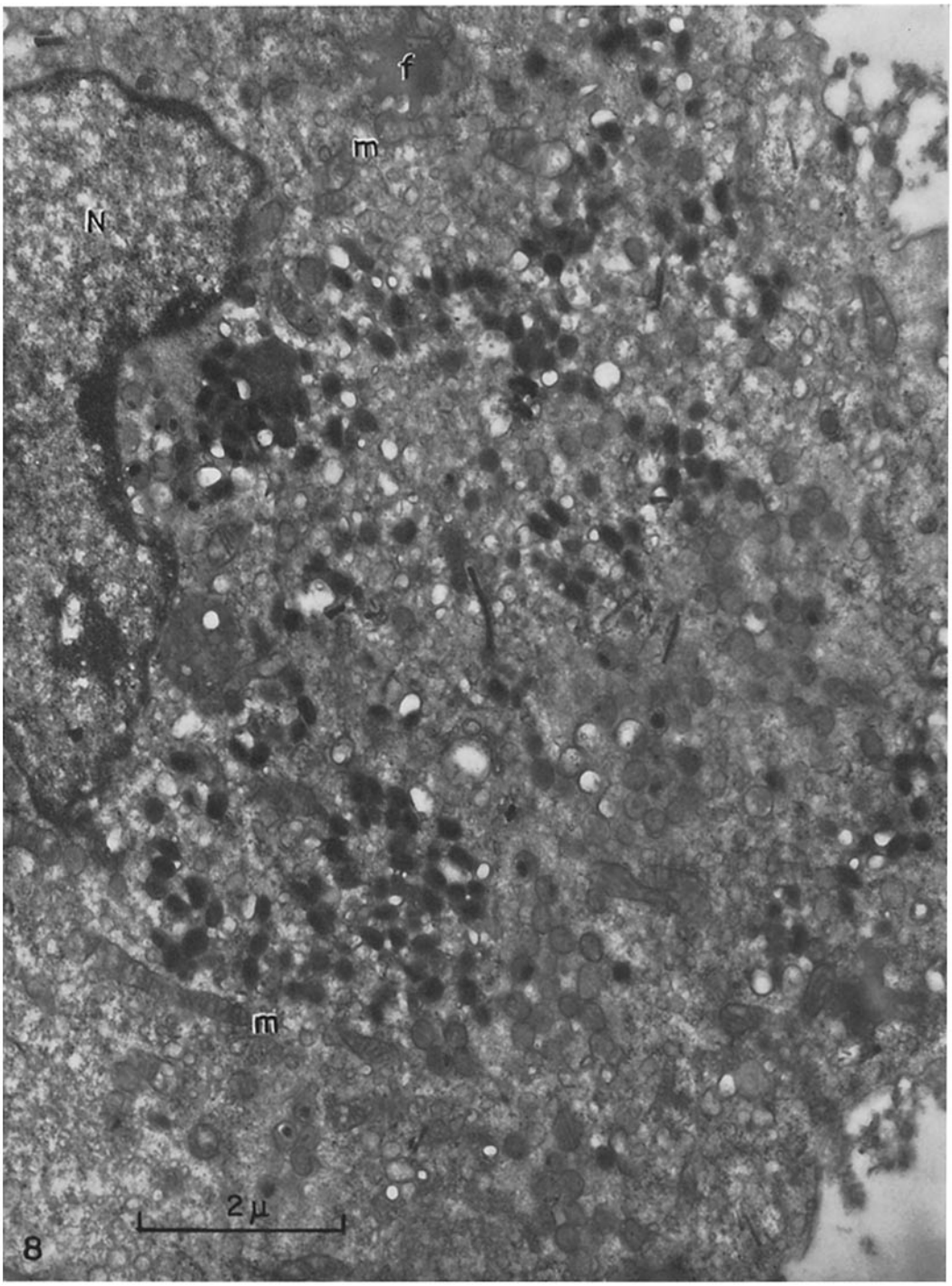
The discrete zones of randomly oriented threads or fine fibrils which appeared 2 hours after infection of L cells with vaccinia (Fig. 3 *a*), and which became more numerous 3 hours after infection, are almost certainly foci of potential viral multiplication because they were found only in infected cells, and at times when infecting particles had almost completely disappeared. Furthermore, in cells sampled at a later stage in viral multiplication early developmental stages of virus formation were observed in similar such areas. The presence of areas of "reticulogranular" material, similar in morphology to that described here, has been observed by Higashi *et al.* (14, 15) in HeLa cells 4 hours after infection with vaccinia. It is also of interest in this connection that the appearance of these foci coincides with the reported time of commencement of viral DNA synthesis (18, 3). Thus the discrete areas of fibrous material which develop in infected L cells at 2 to 3 hours after infection with high virus multiplicities are probably sites of viroplasm within which virus development occurs. The disposition of the mitochondria and other cytoplasmic elements near these foci suggests that as the fibrous material is being elaborated it displaces the normal cytoplasmic components. Thus, although a close spatial relationship between presumptive areas of virus formation and the mitochondria may develop fortuitously, nevertheless the mitochondria may be, as already suggested by Dourmashkin and Bernhard (7), who studied the development of molluscum contagiosum in skin, closely involved in the elaboration of the virus.

Our observations on the developmental stages of vaccinia multiplying in strain L cells are, in general, the same as those made previously by other workers (2, 7, 11, 19, 23) who studied the development of this and other pox viruses in other host cells. Certain details in the developmental process, however, are worthy of emphasis. Thus the appearance of zones of dense fibrous material in the cytoplasm of infected L cells, within which the early stages of immature vaccinia can be observed in later samples (see Figs. 3 *a* and *b*), has not previously been related to the sequence of vaccinia adsorption and penetration into the cytoplasm.

If, as indicated by the work of Peters (23), the DNA of vaccinia is situated within the core of the virus particle, which is formed from the nucleoid of the immature particle, it appears possible that the fibrillar material formed at the foci of presumptive virus elaboration, prior to the appearance of the nucleoids, is protein in nature. The observed presence of both nucleoids and viral membranes in cells sampled at 4 hours after infection (see Fig. 5 *a* and *b*) might have been

FIGURE 8

Portion of the nucleus and cytoplasm of a cell in a sample taken 24 hours after infection of the culture. The virus, present in some foci in its immature stages and in others as dense mature particles, is distributed throughout most of the cytoplasm. × 15,000.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anticipated from the work of Cairns (3), who, by the combined techniques of autoradiography and fluorescein-coupled antibody, found that viral DNA and surface protein synthesis commenced simultaneously, 3.5 hours after infection of KB cells with vaccinia.

The correlation between the time when the virus titre commenced to rise and the time when newly formed mature virus was first observed in the cytoplasm indicates that vaccinia multiplying in L cells becomes infectious only when its maturation is completed. In contrast to our observations, Peters (23) found a rise in titre 10 hours after infection of HeLa cells with vaccinia but did not observe mature particles until 15 hours after infection, and thus suggested that the immature form of the virus might also be infectious.

The events which bring about a spread of vaccinia infection in the cytoplasm of L cells remain obscure. It is possible that synthesis of viral materials spreads in ever widening areas around each infectious focus. Evidence for this has been presented by Cairns (3), who found that when several foci were established in a single host cell and viral material commenced to be synthesized, these foci spread with time and eventually merged into one another.

The appearance of vaccinia near the cell surface, often within microvilli, at 10 hours following infection of the culture is 2 to 4 hours later than the time when mature particles first become evident in the cytoplasm. However, virus multiplication continues, spreading to the hitherto unaffected regions of the cytoplasm. This contrasts with the progress of infection by the small RNA-containing animal viruses, some of which are released in a burst, followed soon by cell lysis (17, 30).

The manner in which cells of normal size fuse to form multinucleate cells is suggested by the example illustrated in Fig. 15 *a*, in which one of the smaller cells, occupying the lower right side of the micrograph, is attached along the entire surface of contact to a multinucleated cell by complex interdigitations of the membranes forming a reticulum. Presumably a breakdown of the membranes occurs at some stage of the fusion process so that a direct contact between the cytoplasm of the two cells undergoing fusion is established. Perhaps, when integration between these cells occurs the reticular formations become incorporated into the cytoplasm of the multinucleate cells (see the example in Fig. 15 *b*). Quite frequently mature vaccinia particles become "trapped" within the membranes of the reticulum (Fig. 15 *c*) and are thus isolated from the cytoplasm proper. We have no evidence to suggest that these membranes contribute to any external viral membranes.

The presence of several hundred nuclei in some giant multinucleate cells, within 48 hours after infection of the culture, suggests very strongly that the virus predisposes these cells to agglomeration and eventual fusion and that, therefore, they are not formed as a result of a process of repeated nuclear division, which would have to occur with an impossible rapidity. It is such fusion of very many L cells of normal size which brings about the formation of holes in the monolayer cultures, or plaques. Vaccinia, however, is only one of several viruses which produce cytopathic changes relatively slowly and stimulate multinucleate cell formation. Among those which have been observed by optical microscopy to stimulate formation of such cells are the viruses of *Herpes* (29), measles (29), mumps (27), and ectromelia (1).

FIGURES 9 *a* to *d*

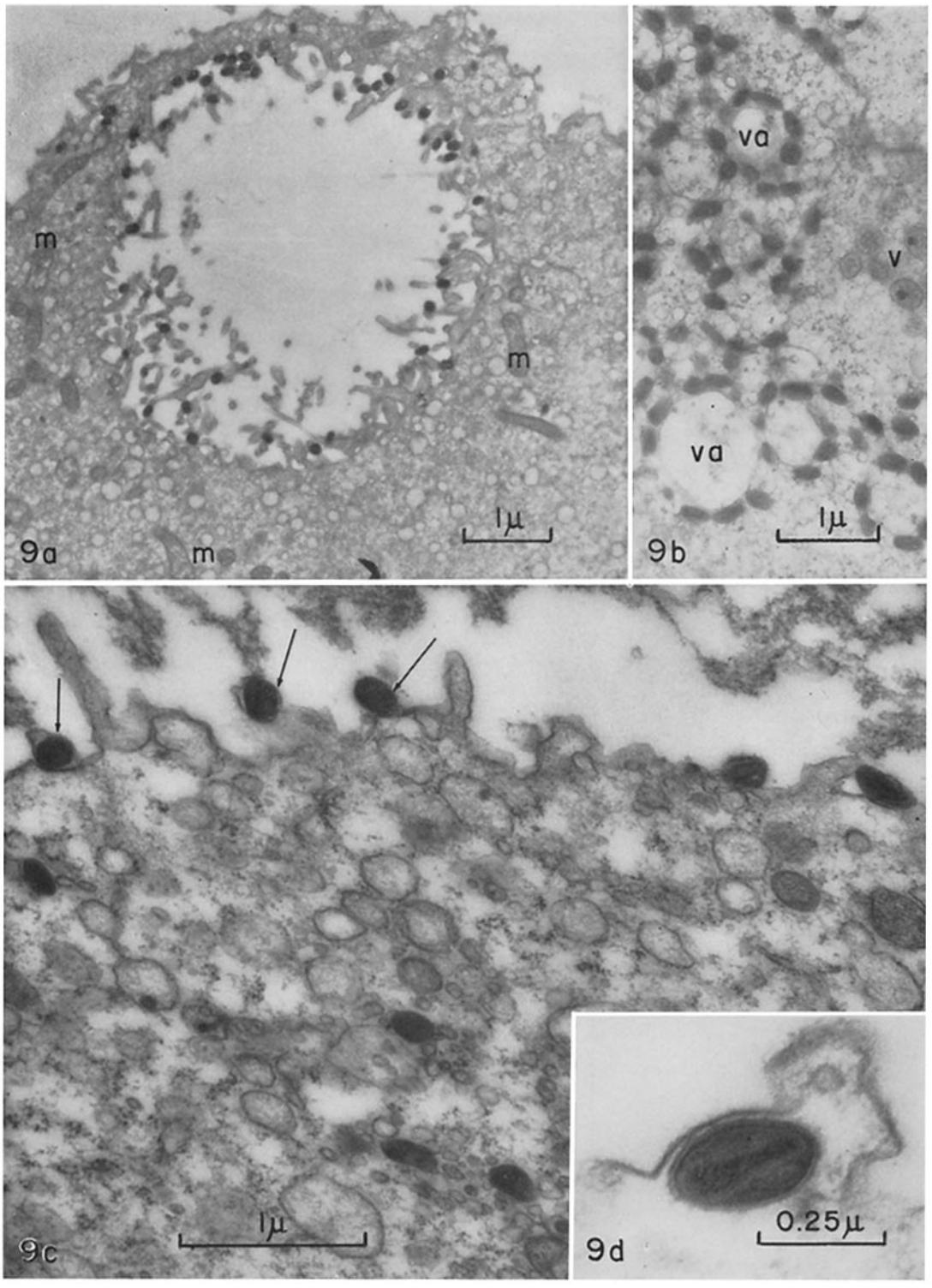
Stages in the process of virus release.

a. Section through either a large vacuole or an invagination near the cell surface, showing the projection into the lumen of numerous microvilli. The dense, rectangular particles associated with the microvilli are mature vaccinia. × 13,500.

b. "Rings" of mature virus close to the membranes of several cytoplasmic vacuoles. Note that most of the particles have their long axes parallel to the membranes. × 16,500.

c. Portion of the outer cell membrane from which project several microvilli. Arrows indicate microvilli enclosing vaccinia particles. × 28,500.

d. A single virus particle at the base of a microvillus. × 83,000.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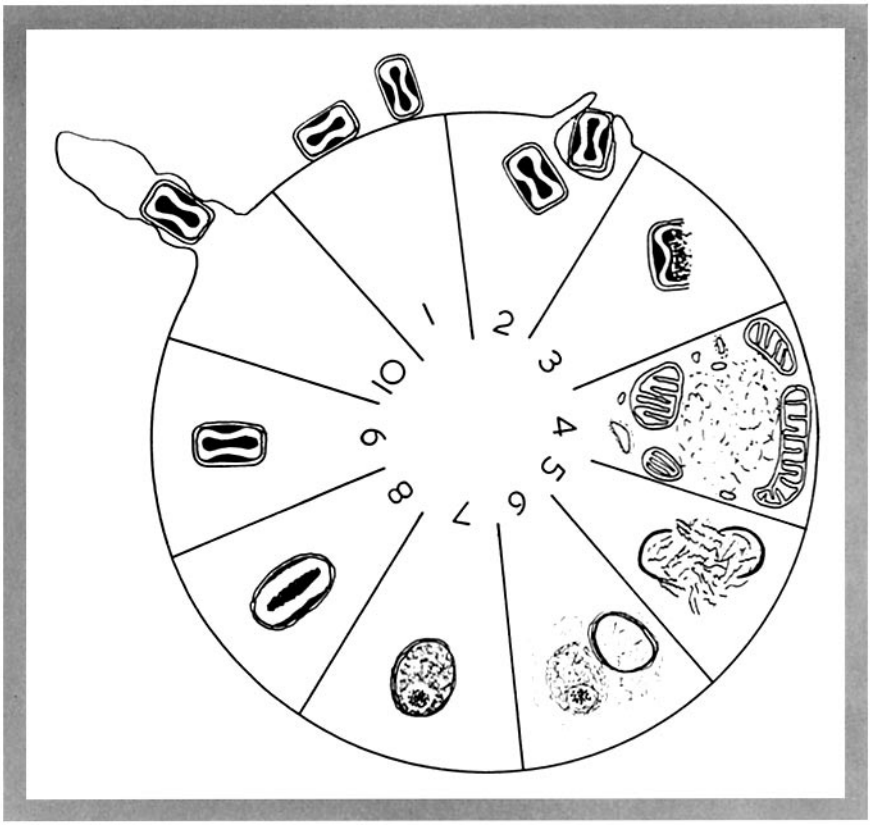


FIGURE 10

The sequence of development of vaccinia virus, followed by its assembly and release from L strain cells. The time scale commencing with the addition of vaccinia and the first appearance of a particular stage is as follows: *1* to *3*, 1 hour; *4*, 2 hours; *5* to *7*, 4 hours; *8* to *9*, 6 hours; *10*, 10 hours.

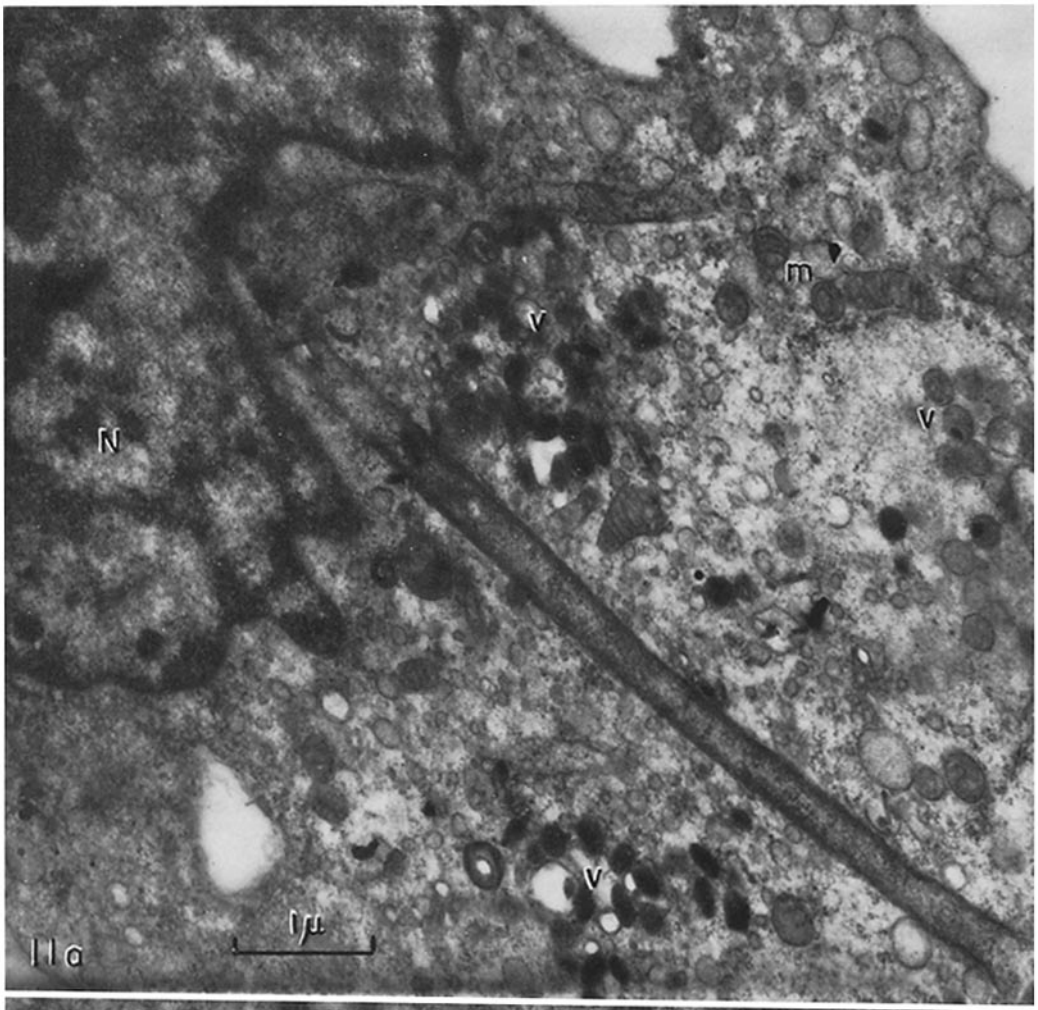
FIGURES 11 and 12

The morphology of tube-like structures and smooth walled vesicular components of the cytoplasm of infected cells twenty-four hours after addition of virus to the culture.

FIGURE 11

a. Portion of the nucleus and cytoplasm of an infected cell. A tube open at both ends is shown traversing the cytoplasm diagonally. Within the upper portion of the tube there is a single mature virus. Two groups of mature vaccinia are located directly above and below the tube, and an area of developing particles is present in the upper right side of the micrograph. × 18,000.

b. Peripheral areas of a cell showing the terminal portion of a tube (at left of centre) which has been sectioned obliquely to its long axis. Parallel lamellae or very flat vesicles appear to communicate with the other flat vesicles (arrows) traversing this region of the cytoplasm. × 24,000.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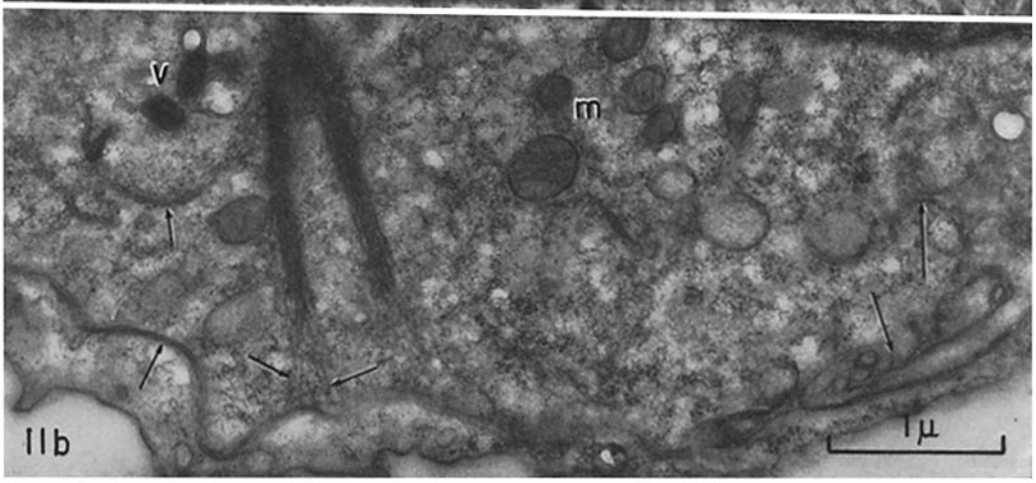
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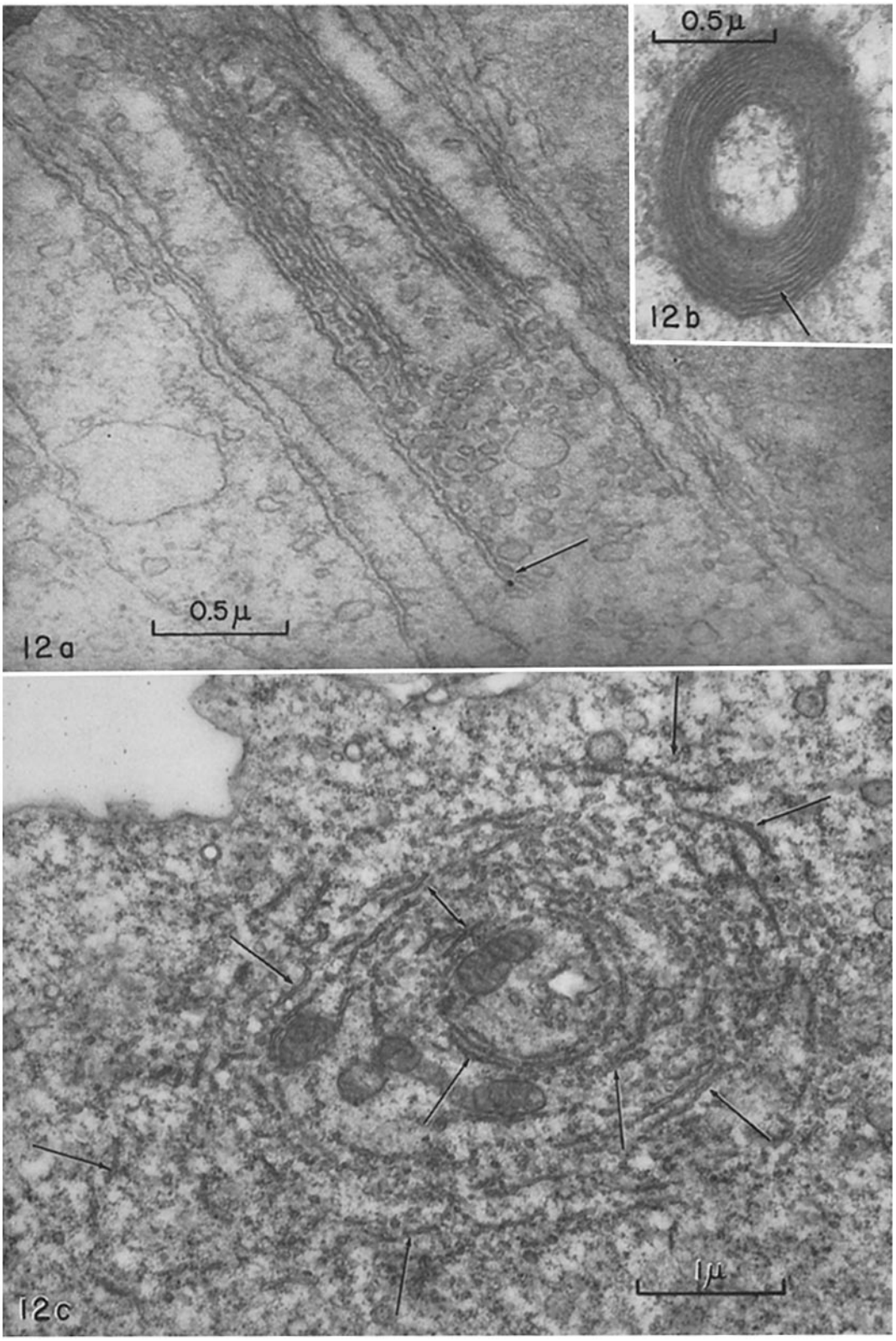
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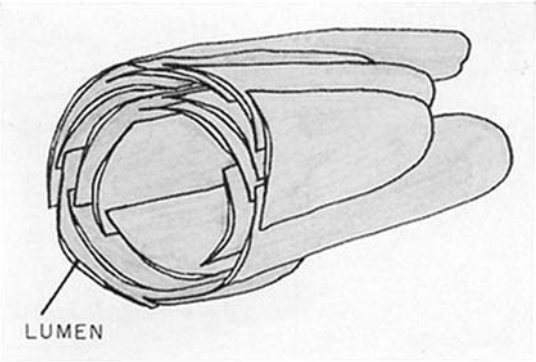

FIGURE 13

A three-dimensional diagrammatic interpretation of a portion of a "tube" found in the cytoplasm of infected L cells.

Vaccinia virus infection leads to very striking changes in the structure of L cells as evidenced by the formation in their cytoplasm of vesicular and membraneous structures not found in uninfected L cells. Several aspects of this response are (*a*) the appearance of tube-like structures and concentric groupings of the endoplasmic reticulum in cells from suspension cultures sampled one day after infection, (*b*) the presence in stationary cultures of complex reticular formations at surfaces where contact between cells, and their eventual fusion, occurs, and (*c*) the formation of multilaminar complexes within cytoplasmic organelles, believed to be degenerating mitochondria. There is some reason to believe that at least the last group of membrane complexes is phospholipid in nature, since it corresponds very closely in both dimensions and appearance to the myelin figures produced by Stoeckenius (36), using model systems of phospholipid-extracts suspended in water as starting material for electron microscopy.

All of these membranous formations are an example of a cellular response in a variety of animal cells which is elicited not only by viruses but also by toxic agents. Thus after viral infections, membranes arranged into double lamellae were observed by Morgan *et al.* (20), in HeLa cells infected with *Herpes* virus; parallel perinuclear and intranuclear lamellae were observed by Gregg and Morgan (12) in adenovirus-infected cells; and "crystalloids," morphologically similar to the "tubes" observed in the present investigation, appeared in Shope fibroma-infected cells (2). The transformation of practically the entire cytoplasm into very numerous small vesicles following infection with EMC virus (5) probably also corresponds to this type of cellular reaction.

Among the non-viral agents which bring about the elaboration of similar membranous structures are colloidal silica (25), particles of India ink (16), carcinogenic dyes (10, 26), and prolonged exposure of lung cells to carbon dioxide (32). Perhaps future investigations, by elucidating either one common specific biochemical response, or a number of them, of the cell to this variety of agents, will elucidate the significance of this stimulus to membrane formation.

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FIGURE 12

a. Terminal portion of a tube, the walls of which are composed of lamellae and flat vesicles lying parallel to one another. The arrow indicates a region where a lamella terminates in a vesicle. × 43,000.

b. Transverse section through a tube having walls of concentrically arranged lamellae and flat vesicles. One of the flat vesicles, the walls of which are so closely apposed that the lumen is barely distinguishable, is indicated by the arrow. Compare micrographs *A* and *B* with the diagram, Fig. 13. × 40,000.

c. Region of the cytoplasm of another cell showing a group of flat, smooth walled vesicles (indicated by arrows) arranged concentrically. × 24,000.

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The micrographs in Figures 14 and 15 illustrate cells sampled from stationary cultures 48 hours after infection.

FIGURE 14

a. Very low-power photomicrograph of a portion of a monolayer culture in a Petri dish, following staining with neutral red. The centre of the field is occupied by the area of a plaque which has at its periphery a ring of multinucleate cells, indicated by arrows. × 50.

b. Phase-contrast micrograph of a living multinucleate cell. Note the dense granular material in the centre and the numerous nuclei at the periphery of the cell. × 500.

c. An electron micrograph of a giant cell, showing the peripheral grouping of the nuclei and the dense, central cytoplasm. A reticulum of membranes in two regions of the cytoplasm is indicated by arrows. Note the numerous particles of vaccinia scattered throughout the cytoplasm, including the areas of the reticulum. × 4,200.

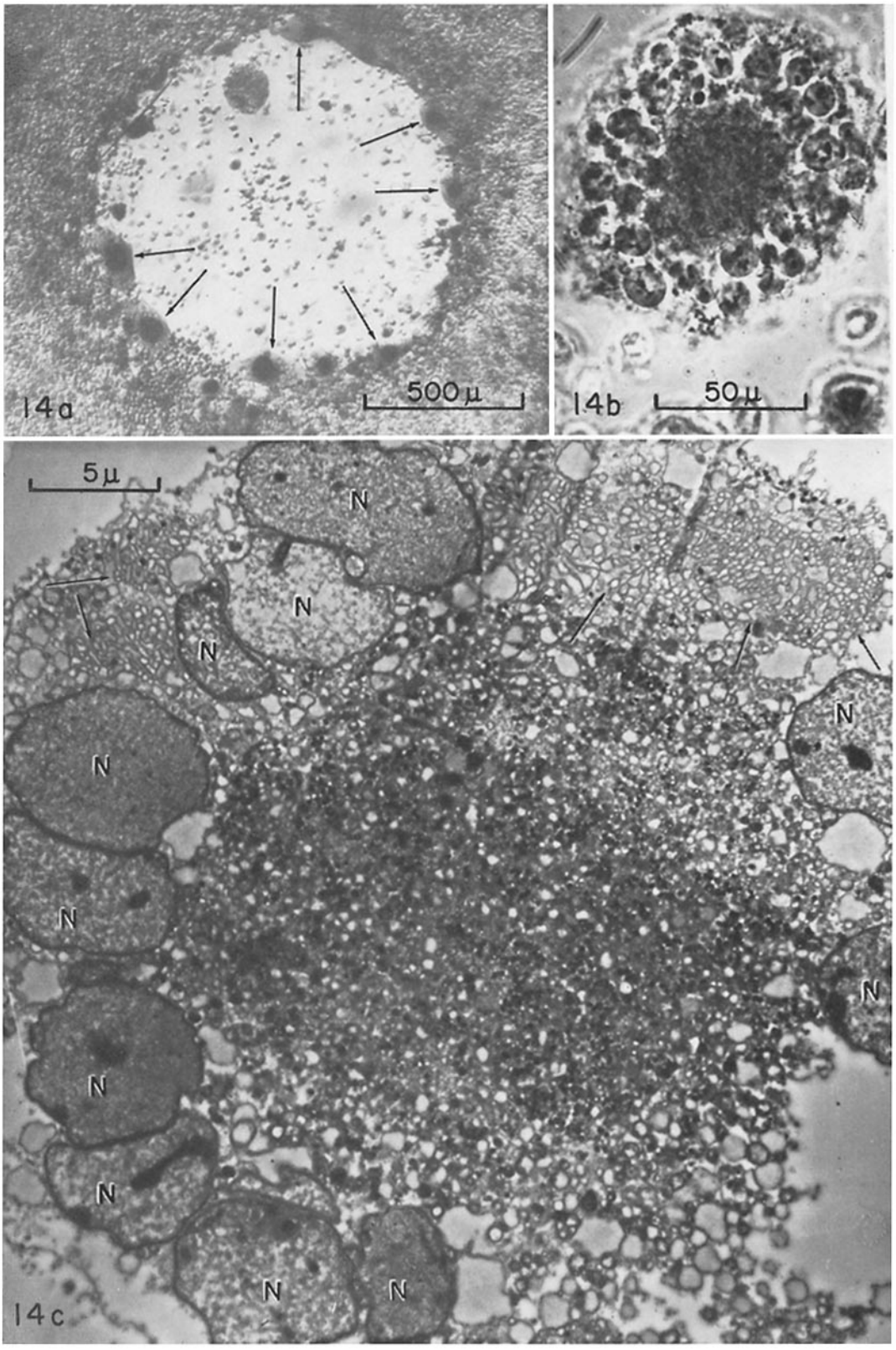
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FIGURE 15

a. An electron micrograph showing a region where a cell of normal size has become attached to a multinucleate cell. A reticulum of membranes which has formed along the entire surface of attachment is indicated by arrows. × 12,500.

b. A small area selected to illustrate the nature of the reticular complex of membranes in a multinucleate cell. × 10,000.

c. A group of mature vaccinia enclosed within membranes of a reticular complex. × 25,000.

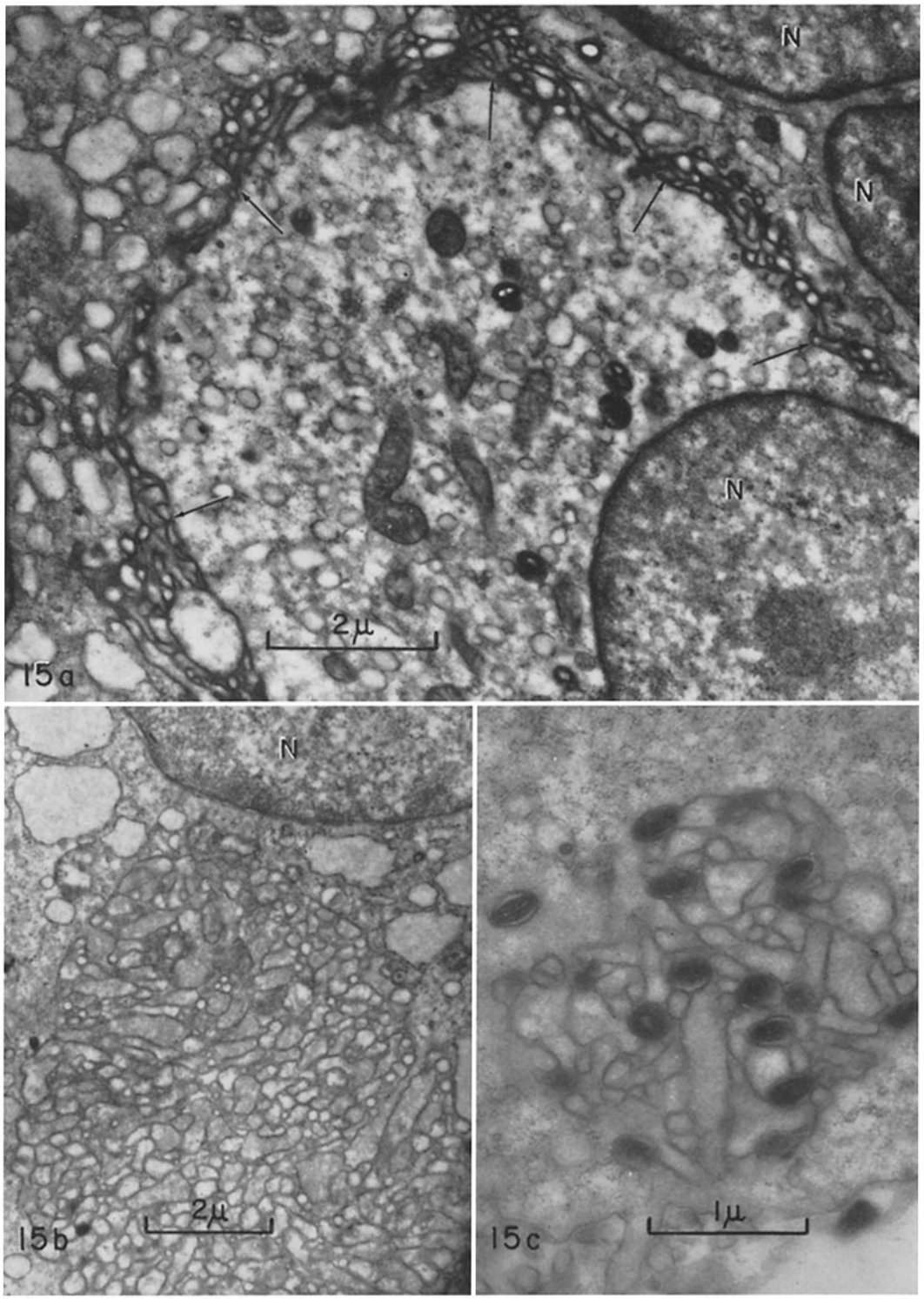
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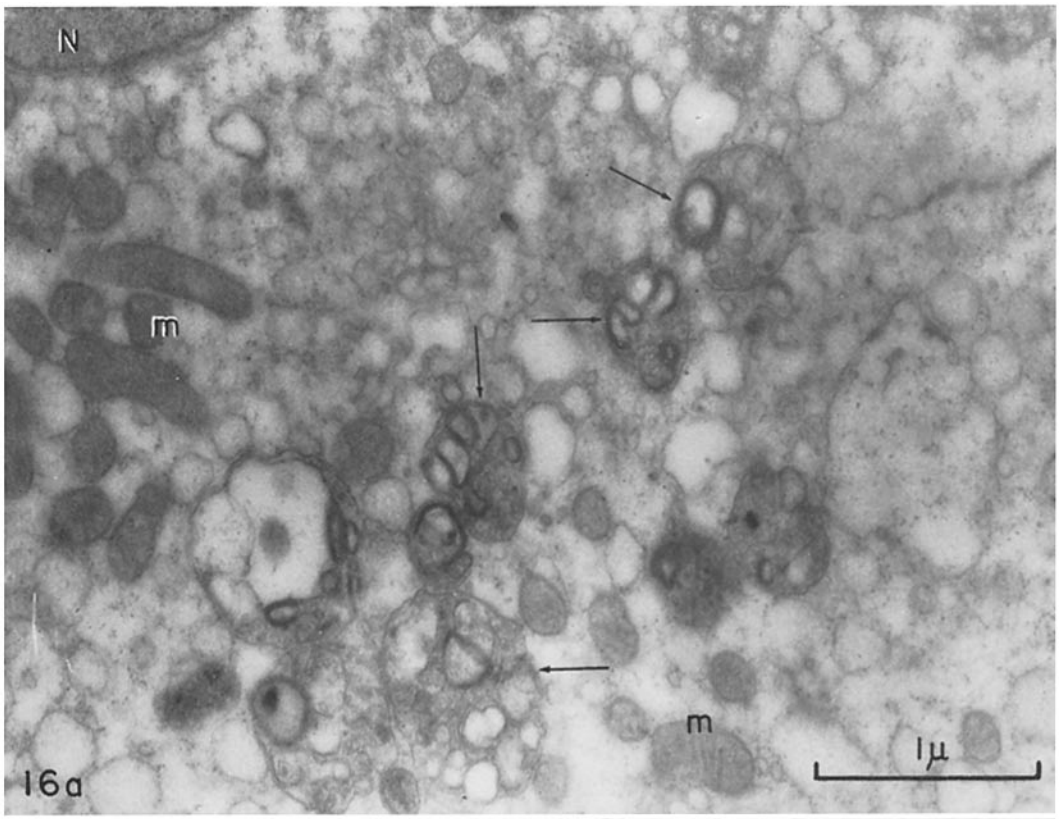
FIGURE 16

a. An area of the cytoplasm of a multinucleate cell showing groups of mitochondria of abnormal (arrows) and normal morphology. In place of the cristae, which are no longer recognizable in these swollen degenerate mitochondria, there are present dense granules and vacuoles, the latter being surrounded either partially or completely by wide dense bands. × 29,000.

b. Fine structure detail of a single wide band within what is, most probably, a degenerate mitochondrion. Note the pattern of repeating lamellae 20 A wide separated by material of lesser density approximately 30 A in width. × 190,000.



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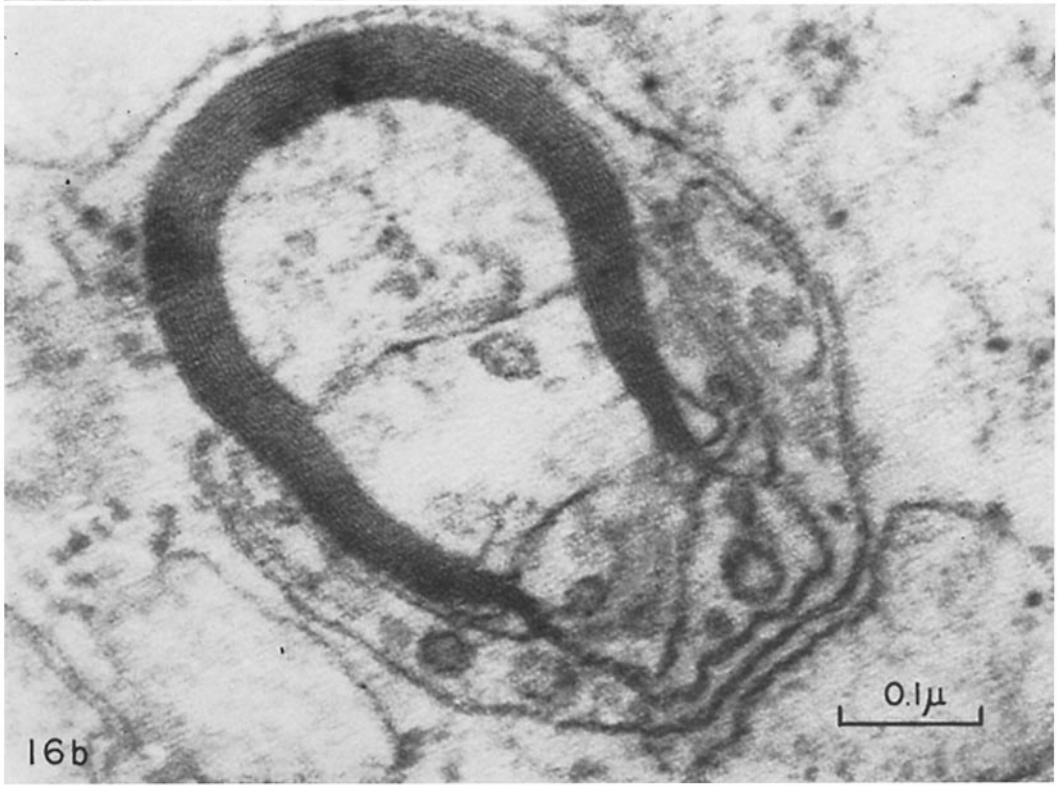

16b