THE UPTAKE AND DEVELOPMENT OF VACCINIA VIRUS IN STRAIN L CELLS FOLLOWED WITH LABELED VIRAL DEOXYRIBONUCLEIC ACID

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ABSTRACT

Vaccinia virus which had its DNA labeled with thymidine-H^3 was purified and used as inoculum for L cells growing in suspension. Samples taken over an 8-hour period after infection were studied by light and electron microscopic autoradiography. Within 20 minutes of its being taken up at the cell membrane in phagocytic vesicles, the outer coat of vaccinia becomes disrupted and the virus core containing the labeled DNA passes into the cytoplasmic matrix. Within 1 hour after inoculation the labeled material passes out of the cores into zones of viroplasm, where cores or remnants of cores are gathered and the label becomes more concentrated by 3 hours after inoculation. Most of the label is conserved in the viroplasm areas during the remainder of the experiment. However, 6 hours after inoculation a very small proportion of progeny virus in the cytoplasm, morphologically distinct from the cores of the inoculum, has associated with it labeled material, perhaps derived from the DNA of the inoculum.

INTRODUCTION

Both the disposition and the role of viral nucleic acid have been satisfactorily elucidated in studies of bacterial infection by T bacteriophages. A process by which viral DNA is injected into the host bacterium was originally postulated by Hershey and Chase (27), on the basis of radiochemical evidence, and later supported by direct electron microscopic observations (2, 23). Identification of the viral DNA within the host became possible after the discovery of an unusual base, 5-hydroxymethylcytosine, in the DNA of the *T*-even bacteriophages (48). The successful initiation of infection with extracted and purified DNA or RNA of animal viruses has finally provided clear evidence that in animal systems also the nucleic acid is the infectious component (11, 18).

However, a "syringe-like" mechanism, evident in the T bacteriophages, is not found in animal viruses and thus far no unusual bases have been reported in the nucleic acids of these viruses that could provide a clear distinction between the host and viral molecular species. Accordingly, the site of release of viral nucleic acid is unknown and its subsequent fate cannot be followed easily. To obviate the difficulty of distinguishing virus and host cell material, animal viruses have been tagged by radioisotopes and the fate of the labeled material ascertained chemically during the virus-host cell interaction (30, 46). As a result of such experiments with myxoviruses, it was suggested that initial release of the infecting component of influenza virus may occur at the cell surface (30). More recently Joklik, studying isotopically labeled pox viruses, concluded that degradation of virus particles begins immediately following adsorption and that the initial attack on viral protein and phospholipid occurs close to the cell surface (31). Electron microscopic evidence obtained on thin sections indicates that both *in vitro* (13, 16, 17) and *in vivo* (49) animal viruses are engulfed in

vesicles formed at the cell surface in a process termed by Fazekas "viropexis" (22). Only after this phagocytic event is there evidence of breakdown of the virus particles (17).

To integrate the evidence thus far obtained by radiochemical means with that coming from morphological observations, an attempt was made to follow a viral nucleic acid within the host cell by electron microscopic autoradiography. By this means, it might be possible to discover the way in which the infectious nucleic acid gains access to the site of viral multiplication and to establish its subsequent fate in the infected cell. For the fulfillment of these objectives, advantage was taken of recently developed methods (9, 10) which permit the localization, within 0.1 to 0.2 μ, of a suitable tagged macromolecule present in a thinly sectioned cell. Vaccinia, one of the largest of animal viruses, was chosen as the infective agent because the localization of the nucleic acid within the viral particles is well within the resolution attainable by this technique.

It was anticipated that release of nucleic acid from the inoculum particles and its fate inside the host cell could be followed chronologically in synchronously infected cells by means of frequent sampling. The addition of suitably high virus multiplicities to suspension cultures could bring about almost simultaneously infection of nearly all of the cells in a culture and at the same time provide material in which there was a good chance to observe cell-associated virus particles in the electron microscope. Thus it might be possible to follow the fate of the labeled DNA in relation to development of vaccinia virus.

MATERIALS AND METHODS

The strain of cells and of virus used and the techniques for their propagation have been described previously (13, 16). Either CMRL 1066 (26) or Eagle's MEM nutrient medium (21) supplemented with foetal calf serum was used throughout.

Preparation of H^3-Thymidine Labeled Virus

Monolayer cultures of L cells were infected with lysates of vaccinia virus at a multiplicity of 1 to 2 plaque-forming units (PFU) per cell. The virus, dispensed in aliquots of 1.0 ml per each 12 oz. bottle, was allowed to adsorb for 1 hour. To each culture were then added 15 ml of medium MEM, containing 5 per cent foetal calf serum and methyl-H^3-thymidine (5.0 C/mM, New England Nuclear Corp.) at a final activity of 20 μc/ml. Incubation at 37°C was continued for 36 to 48 hours, until the characteristic cytopathic effect was evident throughout each monolayer.

In the present studies the release of cell-associated virus from pooled lysates was effected by a single cycle of freezing and thawing followed by treatment in a Raytheon 10 KC sonic oscillator for 10 to 20 minutes. Concentrated suspensions of partially purified virus were obtained using several cycles of differential centrifugation, as described previously (16). Additional steps were employed for the removal of DNA not contained within virus particles. To this intent, the pellets obtained after treatment with trypsin were washed once, resuspended in 10 ml of medium MEM and recovered by centrifuging at 15,000 *g* for 30 minutes, and then dispersed and incubated at 37°C for 1 hour in 2.0 ml of phosphate-buffered saline (PBS, 19), containing 50 μg/ml desoxyribonuclease (crystalline, Worthington). A final sedimentation of the virus at 15,000 *g* for 30 minutes was followed by resuspension in medium MEM.

Test for the Specificity and Degree of Labeling of the Viral DNA

To test whether all of the non-viral DNA was being removed, a mixture of "hot" cells and "cold" virus lysate was subjected to a purification procedure identical with that used for labeled vaccinia. About 100 ml of virus lysate, derived from 5×10^6 cells, were mixed with 5×10^6 cells of a control culture grown for 12 hours in the presence of 20 μc/ml of H^3-thymidine. Large concentrations of label could be demonstrated specifically in the nuclei of "hot" cells by means of light and electron microscopic autoradiography (see below). Aliquots of partially purified virus (20 λ) were counted in a gas flow Geiger counter prior to and following the enzymatic digestion steps. Trypsin digestion followed by digestion with DNAse reduced the counts from several hundred CPM to zero. Absence of non-viral H^3-DNA was also demonstrated by infecting L cells in suspension with the virus purified from a "hot" cell-"cold" virus mixture and examining sections of cells sampled at 1 and 6 hours postinfection, by light microscopic autoradiography. Cell-associated grains in excess of the number expected from the background alone were absent.

If all of the thymine present in the DNA of the vaccinia virus grown as given above were derived from H^3-thymidine, it can be calculated, using published values for the content of vaccinia DNA (28), that on the average 7 disintegrations should occur per particle per day. The actual label present was estimated by counting an aliquot of purified "hot" virus and correcting for the efficiency of the counter.

The final result indicated that labeling of thymine in viral DNA was only 10 to 20 per cent complete.

Method for Cell Infection and Sampling

Concentrated cell suspensions (about 10^7 cells/ml) were mixed with the virus, at a final concentration of approximately 8 PFU per cell, as described previously. Throughout the 1st hour after inoculation, cells were sampled, suspended in warm nutrient medium, and pelleted at 800 *g*. Before being fixed or frozen they were washed three times by resuspension and centrifugation to remove unadsorbed virus. The time elapsing between sampling and fixation was about 5 minutes. One hour after inoculation the remainder of the cells was also washed and diluted to a concentration of 2×10^5/ml in warm nutrient medium. Incubation was then continued to allow virus development to proceed.

Plaque Assay for Virus

The assay used was similar to that described by Gomatos *et al.* (25). After a period of 1 hour, to permit virus to adsorb to the monolayers, each Petri dish culture received 10 ml of overlay consisting of equal volumes of a twice concentrated nutrient medium with 10 per cent foetal calf serum and a 1.8 per cent solution of Bacto-Difco agar. The plates were incubated for 3 days at 37°C, with a gas phase of 5 per cent CO_2 in humidified air. The cells were subsequently fixed for 10 minutes, through the agar, with 5 ml of a 30 per cent formalin solution, and stained for 10 minutes with Ehrlich's haematoxylin after removal of the agar. Excess stain was washed away with tap water and the preparations were allowed to dry at room temperature. Plaques, visible as unstained areas in the monolayer, could be counted easily.

Electron Microscopy

NEGATIVE STAINING: Vaccinia virus particles from suspensions were examined and counted, using negative contrast procedures, as previously described (5, 13).

PREPARATIONS FOR THIN SECTIONING: Pellets of cells, fixed and dehydrated by conventional techniques, were embedded in either epoxy-resin mixtures (35) or methacrylate (4 parts butyl:1 part methyl). The contrast of thinly sectioned cells was increased by lead staining according to the procedure of Karnovsky, method B (32).

Autoradiography

Three separate experiments were conducted with labeled virus. To randomize the observations, data for each time point in any single experiment were obtained from sections cut from 2 or 3 blocks at two or more well separated levels. To minimize differences which could result from a variability in the photographic emulsion, especially those due to differences in emulsion thickness, each batch of sections, mounted either on glass slides or specimen grids and representing cells sampled at various time-points throughout the experiment, was coated with the same freshly prepared batch of emulsion. For light microscopy the methods of Caro (8) were employed on 0.5 μ sections, whereas for electron microscopy more recent techniques, using Ilford L-4 emulsion and chemical development, were employed (9, 10). Although contrast of the thinly sectioned cells was sometimes enhanced by staining with uranyl acetate without removal of the photographic emulsion, most commonly removal of gelatin and simultaneous staining was achieved by application of alkaline solutions of lead salts, as suggested by Revel and Hay (45).

TABLE I

Disappearance of Infectious Particles from the Suspension Medium of Cultured L Cells

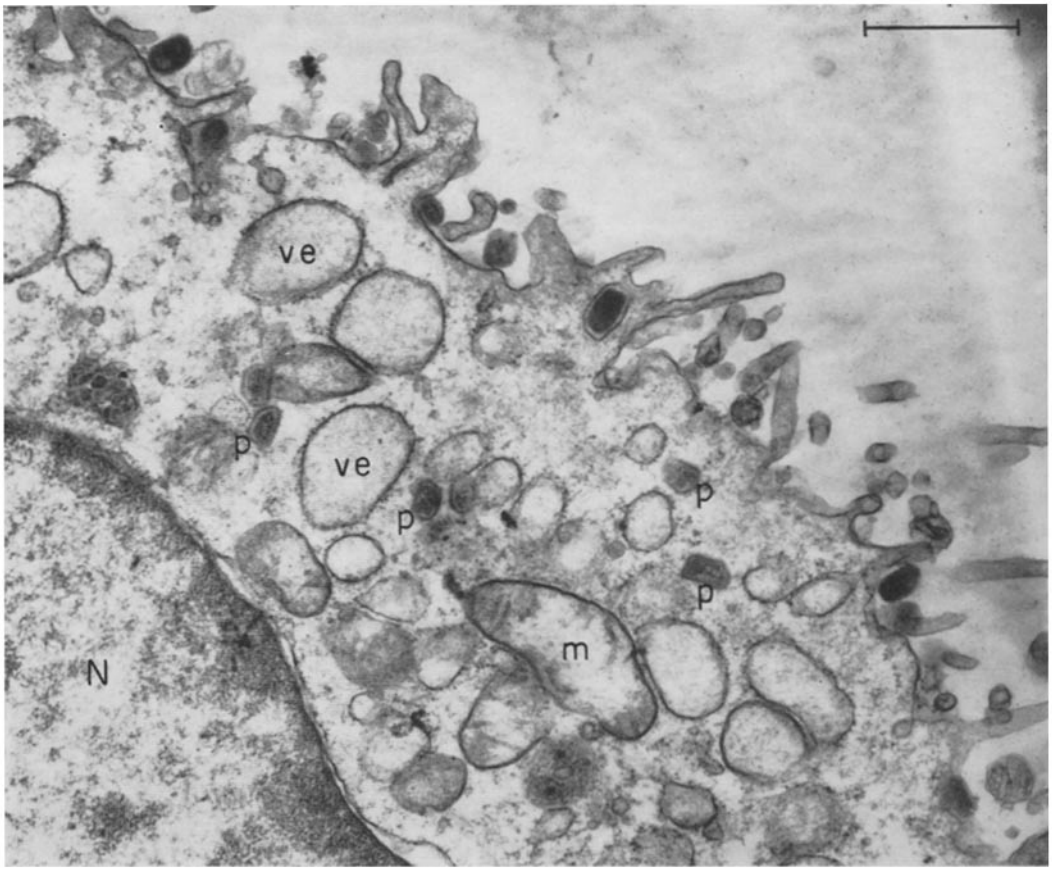
Time following inoculation	PFU/ml	Per cent virus removed
min.		
0	1.0×10^8	0
10	4.0×10^7	60
20	3.2×10^7	68
30	2.5×10^7	75
60	1.8×10^7	82
120	1.6×10^7	84

The data come from a representative experiment in which 1.2×10^7 cells/ml suspended in nutrient medium were mixed with a concentrated virus suspension containing 3.9×10^8 PFU/ml to give a concentration of 8 PFU/cell of vaccinia in the mixture. Aliquots, removed at intervals, were centrifuged at 1,500 RPM for 2 minutes and the amount of virus remaining in the supernate was determined by plaque assay.

RESULTS

Uptake of Infectious Particles

To study the rate at which infectious particles become cell-associated after the inoculation of suspension cultures, the disappearance of vaccinia from the suspending medium was followed by plaque assay. The results (Table I) revealed that about one-half of the virus had disappeared, and presumably became cell-associated, in 10 minutes;



Key to Figures

Au, autoradiogram; days of exposure of the emulsion are given in parentheses.
E, epoxy resin embedding.
M, methacrylate embedding.
m, mitochondria.
N, nucleus.
p, central biconcave plate or virus core.
ve, vesicle.
vp, clump of viroplasm or "factory" area.

FIGURE 1

This figure illustrates portion of the nucleus and cytoplasm of a cell sampled 20 minutes after inoculation. Particles of vaccinia are present 1) on the outer cell membrane, 2) partly or 3) completely enclosed by the membrane, and 4) lying "free" in the cytoplasm. Note that the "free" particles, actually virus cores, are less dense than particles close to the surface. *E* (see Key), × 20,000.

two-thirds in 20 minutes; and almost all of it within 1 hour after inoculation. Furthermore, the decrease in titer, recorded in 20 minutes, was 85 per cent of that observed at 1 hour.

Ratio of Physical Virus Particles to Infectious Units

As shown previously for this system (16) and for similar vaccinia-cultured cell systems (20, 31), the ratio of virus particles to infectious units is generally low. In the current experiments several assays of two suspensions of purified labeled virus, prepared independently from lysates obtained at different times, gave an average ratio of 12:1 (3.9×10^9 virus particles/3.2×10^8 PFU/ml).

Early Association of Cells and Virus

By the use of thin sections, cells and associated vaccinia particles were counted directly on the image screen in the electron microscope and

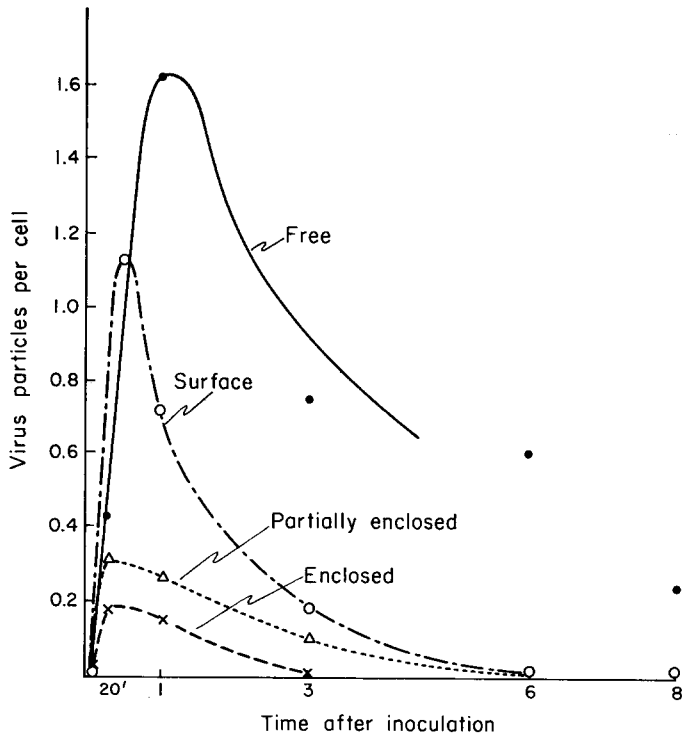


FIGURE 2

The concentration of virus particles at various extra- and intracellular sites as a function of time. Average numbers of virus profiles per cell profile are given on the ordinate.

divided into four categories according to their location: 1) on the outer surface; 2) partly enclosed by the outer cell membrane; 3) completely enclosed in phagocytic vesicles; 4) "free" in the cytoplasmic matrix. Actually the "free" particles are only viral cores. These four stages are evident in the example illustrated in Fig. 1. Fig. 2 summarizes the results obtained by counting about 200 cell-profiles for each time-point and shows that the maximum accumulation of virus occurred at the cell surface 20 minutes after inoculation, whereas the greatest concentration of "free" particles was evident in the cytoplasmic matrix 1 hour after inoculation and remained relatively high throughout the remainder of the experiment. An explanation for this last observation will be provided below. Concentrations of virus particles, either partially or fully enclosed in cell membrane pockets, were relatively low even during the 1st hour.

From (a)—the multiplicity of virus added, (b)—the ratio of virus particles to infectious units, and (c)—the rate at which infectious virus became cell-associated, it can be estimated that 20 minutes after inoculation one cell had become associated with an average of 65 particles and that 1 hour after inoculation one cell would have been complexed with an average of 80 particles.

Another independent estimate[1] of the number of cell-associated virus particles was obtained from direct counts (Fig. 2) of virus present with cell profiles. It yielded averages of 76 particles per cell at 20 minutes and 98 particles per cell

[1] Since an L cell has an average diameter of 10 μ it is expected to yield 100 to 200 sections (500 to 1,000 A thick), and since each vaccinia particle measures 2,500 to 3,000 A it is expectedly present in 2.8 to 5.5 sections. Using the formula $\frac{sc \times np}{sp}$, where sc is the number of sections through one cell, np the average particle count per section, and sp the number of sections through one virus particle, it is possible to calculate the average number of virus particles per cell. For 500 A thick sections, sc = 200, and sp = 5.5; for 1,000 A sections, sc = 100, and sp = 2.8. np was 2.1 at 20 minutes and 2.7 at 1 hour.

at 1 hour after inoculation. These values agree closely with those predicted from the infectivity experiments summarized in Table I. Accordingly, it is concluded that infectious particles became cell-associated at the same rate as all the virus particles present in the system.

Autoradiography and Light Microscopy

To obtain quantitative information about the degree of conservation and the gross localization of the viral DNA of the inoculum throughout the early stages of virus reproduction, L cells previously inoculated with "hot" purified vaccinia were sampled at specified intervals, processed for autoradiography, and finally examined by light microscopy. The counts of autoradiographic grains over sectioned cells are summarized in Table II and an example showing the distribution of silver grains over a group of cells is shown in Figs. 3 *A* and *B*. It is apparent from the average counts presented in column (3) of Table II that within the sensitivity of the method used the concentration of labeled DNA remained constant in the cytoplasm throughout the experiment. Good correspondence between grains actually observed over the nucleus and grains expected over an equivalent area due to background (compare columns (4) and (5) of Table II) indicates that viral DNA did not penetrate into the nucleus during the experimental period of 8 hours.

To test whether the label registered by autoradiography remained restricted to DNA throughout the experiment, samples of infected cells, similar to those used for autoradiography, were taken at 1 and 6 hours after inoculation, prepared by a modified Schmidt and Thannhauser extraction, as described by Siminovitch *et al.* (47), and subsequently counted in a gas-flow Geiger counter.[2] Comparison of the two time-points indicated that, calculated on the basis of the amount of label per cell, virtually all of the label was still present in the 6-hour sample, although a 30 to 40 per cent decrease in the cell number, observed in the later sample, might have resulted from increased fragility of infected cells caused by the longer incubation in the 6-hour sample.

TABLE II

Autoradiographic Grain Counts per Cell in Sections of L Cells Inoculated with H^3-Thymidine-Labeled Vaccinia Virus

Time after inoculation (1)	Total grains in cytoplasm (2)	Actual grains in cytoplasm with standard deviation (3)	Grains over the nucleus (4)	Background grains over nucleus (expected) (5)
20 minutes	2.93	1.91 ± 0.08	0.29	0.29
1 hour	2.98	2.12 ± 0.37	0.41	0.28
3 hours	3.58	2.81 ± 0.46	0.48	0.24
6 hours	3.81	2.74 ± 0.52	0.41	0.31
8 hours	3.14	2.43 ± 0.28	0.44	0.28

For each time-point in this representative experiment grains were counted, in a phase-contrast microscope, over 1,000 to 2,000 cell profiles. Three lots of sections 0.5 μ thick, cut from 2 blocks, were covered on three separate occasions by three batches of Ilford K-5 emulsion. Exposure time was 9 days. The grain count over the nucleus and cytoplasm, resulting from background alone, was ascertained by first obtaining measurements of the average area of the nucleus and cytoplasm from enlarged prints of about 500 sectioned cells. In these sections the nucleus occupied about one-quarter of the cell area. The grains in the cytoplasm (column (3)) are total grains less the background.

[2] Briefly, pellets of cells which had been treated with cold trichloroacetic acid (TCA) were extracted with cold and then boiling 95 per cent ethanol. The ethanol was evaporated and the residue treated with 0.3 N potassium hydroxide, at 37°C for 20 hours. The digest was neutralized with 1.5 N perchloric acid; further addition of a 5 per cent solution of TCA resulted in the formation of a precipitate. After washing in TCA the precipitate was treated with 0.15 ml of hydroxide of hyamine 10-X (Rohm and Hass) and the mixture was diluted with 15 ml of phosphor fluid in preparation for counting.

Morphology of Mature Virus

In general, thin sections have provided less information about virus structure than negative contrast preparations. To interpret successfully any possible changes resulting from interaction of vaccinia with L cells, it was considered desirable to compare the fine structure of vaccinia as revealed by these two techniques. The brief description of mature virus particles used as the inoculum is being given at this point as an aid to the interpretation of subsequent observations on the penetration and multiplication of vaccinia.

As was shown previously, the surface of vaccinia examined by the negative contrast method is covered by surface ridges having the appearance of rodlets or tubules, each about 70 A wide and up to 1,000 A in length (Fig. 4). Some virus particles which had first been centrifuged at 15,000 *g* for 30 minutes in a saline-sucrose continuous gradient (25 to 40 per cent sucrose) and then negatively stained were found to be partially disrupted. In such particles the internal double-concave plate or inner core and the two lateral masses, previously described in the classical work of Peters (43), became clearly visible (Figs. 5 to 8). The surface of this plate is covered by fine, short projections resembling the spikes present on influenza virus, each approximately 50 A wide and 200 A long. Separation of the inner core from the outer membrane of the virus, on which are located the surface ridges, may have resulted from an osmotic shock following the rapid transfer of the virus from the hypertonic sucrose medium to phosphotungstate medium of lower tonicity.

The appearance of thinly sectioned vaccinia virus, which had been maintained in isotonic solutions prior to fixation, is shown in Figs. 9

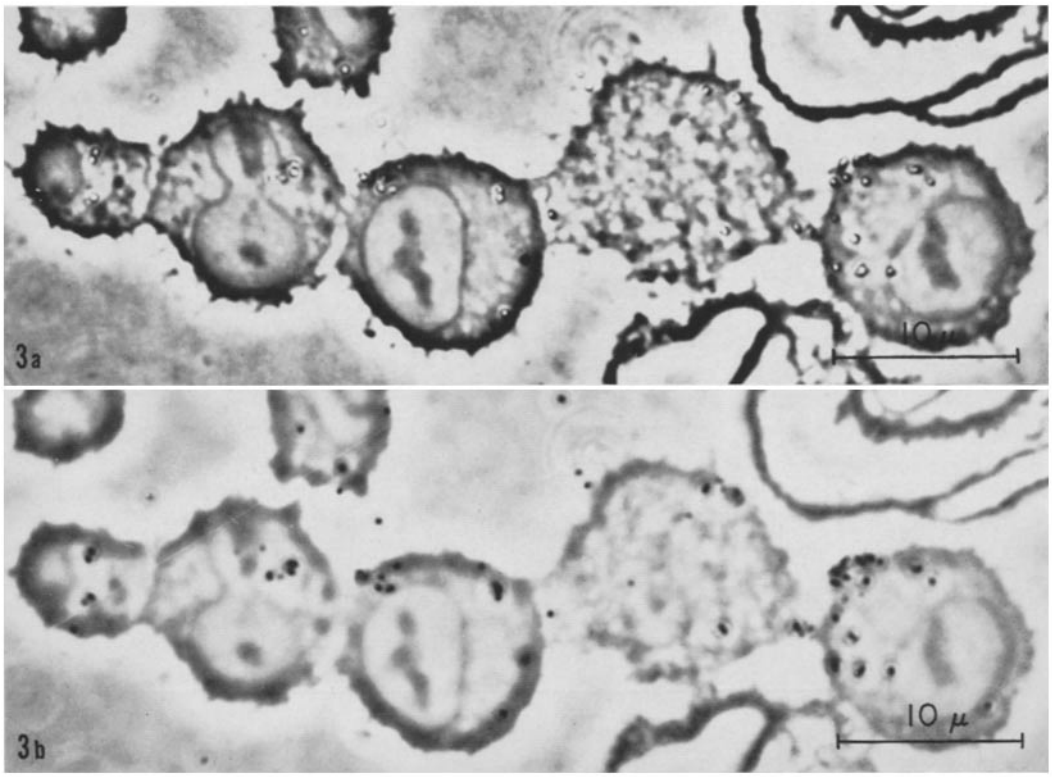


FIGURE 3 *A* AND *B*

A selected section, 0.5 μ thick, of a group of cells from a pellet prepared 1 hour after inoculation with labeled virus. Note the absence of grains in the nuclei and an unusually high number over the cell on the far right. *A*, focused at the level of the cells; *B*, focused on the emulsion. Phase contrast, *Au* (9), *M*, × 2,500.

and 10. As in the whole mount, it is possible, in the narrow aspect, to distinguish the two lateral dense masses filling the space between the outer membrane and the biconcave plate or core. The rectangular shape of the latter can also be discerned when the virus is sectioned parallel to its broad face. The morphology of vaccinia prepared by these two quite different techniques appears to be the same.

Stages in the Uptake and Development of Vaccinia Virus

A plausible sequence of events in the uptake and development of vaccinia can be reconstructed from the appearances encountered in samples fixed at given time-points during the 8-hour period of the experiment. The relationship between time elapsed after inoculation and frequency of occurrence of stages of virus penetration and development, as observed in cell profiles by electron microscopy, is summarized in Fig. 11. After an initial decline in the proportion of cells in which the early stages of virus penetration were evident, there was a rapid increase, by 3 hours, of cells containing one or more areas of viroplasm. The "free" particles, which are actually the cores or central plates of vaccinia, remained at a high concentration even at the later stages. Immature and mature forms of vaccinia, illustrated in Figs. 28 to 38, became evident between the 3rd and 6th hours of the experiment.

The results already obtained by light microscopic autoradiography indicated the possibility of using, in a similar manner, a DNA-labeled viral inoculum for autoradiography and electron microscopy, to obtain a more precise localization of vaccinia as penetration and replication were proceeding. To obtain adequate contrast, the sections used for autoradiography were cut from cell samples embedded in methacrylate. Since embedding in methacrylate resulted in a certain amount of structural damage, however, fine morphological details will be illustrated by micrographs of unlabeled vaccinia virus in equivalent stages of development taken from material embedded in epoxy resin.

In cells sampled 20 minutes after inoculation, intact labeled virus was observed on the outer cell membrane (Figs. 1, 13, 14) and enclosed in vesicles (Figs. 1, 12). Other particles appeared to be undergoing disintegration, whereby the core had become free of the enclosing outer viral membrane and had separated from the two lateral dense elements, as illustrated in Figs. 15 and 16. The cores were also found at this time lying free within the cytoplasmic matrix. These structures, within which was contained the labeled material, had lost their hour-glass shape in their narrow

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FIGURE 4

A whole vaccinia particle from purified suspension negatively stained. The surface is covered by ridges which may be long rodlets or tubules. × 300,000.

FIGURES 5 AND 6

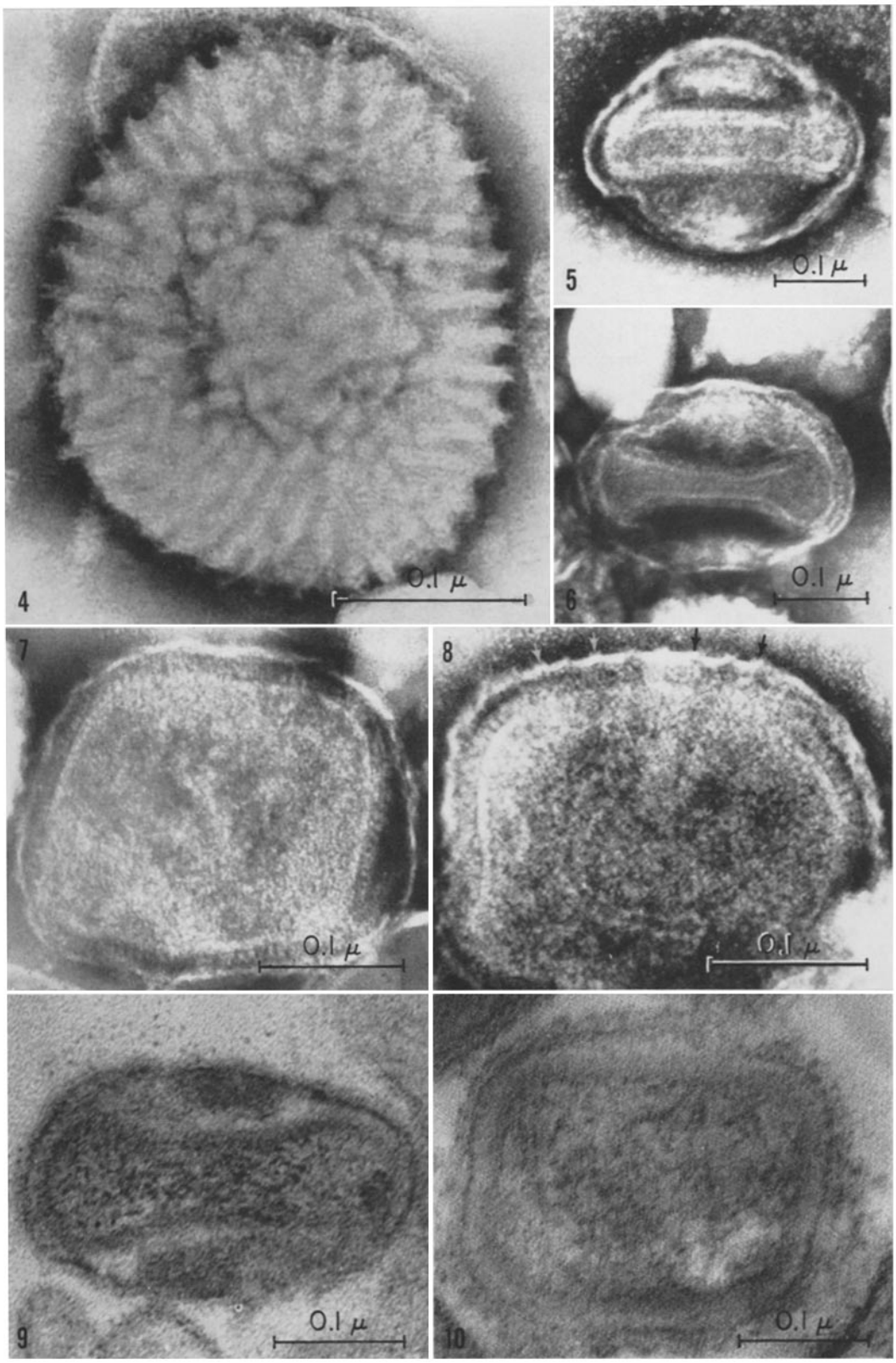
Two negatively stained whole mounts showing the narrow aspect of vaccinia taken from a suspension centrifuged in a sucrose gradient. Short projections cover the central biconcave plate. The ridges, normally present on the outer membrane, are not evident here. × 140,000.

FIGURES 7 AND 8

Two examples from a similar preparation, showing the broad, rectangular side of the virus. Short projections emanating from the coat of the core are visible. Arrows indicate protrusions on the outer coat which may be related to the surface ridges of undamaged virus particles. × 220,000 and × 250,000.

FIGURES 9 AND 10

These figures illustrate the narrow and broad aspect of vaccinia as observed in thin sections. There is a close similarity between these images and those seen in whole mounts after negative staining. *E*, × 200,000.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aspect and had become rectangular in outline, as shown in Figs. 1, 17 to 20. Otherwise, they were intact and consisted of a dense filamentous network at their center and were enclosed by a membrane plus a layer of fuzzy material. Although these cores, included in the category of "free" virus remained in the form of cores which were found free in the cytoplasmic matrix, although some of these rectangular structures were partially disrupted, as illustrated in Figs. 21, 22. Frequently at this time clumps of dense filamentous material could be observed adjacent to the virus cores and

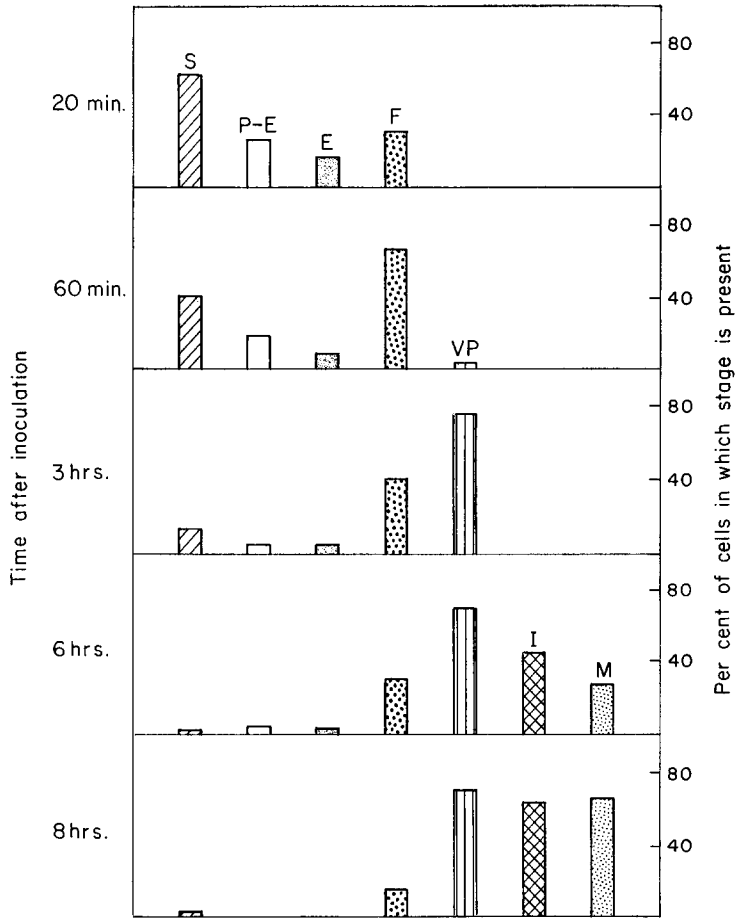


FIGURE 11

Frequency of various stages of virus penetration and development as a function of time elapsed after inoculation. Stages represented in the bar diagram were obtained on about 200 cell profiles for each time-point. *S*, surface vaccinia, *P–E*: partially enclosed; *E*, in vesicles; *F*, "free" virus; *VP*, viroplasm areas; *I*, immature virus; *M*, mature vaccinia.

virus in Figs. 2 and 11, remained evident in the cytoplasm throughout the experiment, they subsequently lost some of their dense content and with it the label, as shown below. Upon this loss, which occurred between 1 and 3 hours after inoculation, these structures became only "shells" of cores and will be so termed.

After 1-hour exposure, most of the inoculum "shells." The diameter of the threads inside these clumps was greater than that of threads within virus cores, suggesting that upon their separation from the core a dense material had been deposited on or near the threads. Frequently these clumps had overlying autoradiographic grains (Figs. 23 to 25). Sometimes when several cores or "shells" were gathered at a single locus, the dense material

occupied an area as large as 1 μ^2. Such relatively large areas of viroplasm were counted as single "factories" (Fig. 11).

Three hours after inoculation, the dense filamentous material of the "factories" was observed in about 80 per cent of the cell profiles examined, and frequently 3 or 4 foci of viroplasm were present in the cytoplasm of a single profile (Fig. 26). Some of the larger "factories" occupied areas almost 5 μ^2. The appearance of typical viroplasm or "factory" areas, each consisting of dense filamentous elements 30 to 50 A wide and of indeterminate length, is shown in Figs. 26 and 27. The material of the viroplasm generally resembled that observed in interphase nuclei and in chromosomes of L cells (12). Surrounding each "factory" there were numerous cores and/or "shells" often having a content of lower density than those found in the 20-minute samples (Figs. 25 to 27). Silver grains were superimposed infrequently on these "shells" but appeared preferentially concentrated over the "factories" themselves (Figs. 25 to 27).

At 6 hours postinoculation, both immature and mature forms of virus were evident in the cytoplasm. Invariably the immature particles were to be found embedded in the matrix of "factory" areas and occasionally were close to autoradiographic grains (Figs. 28, 32). The membranous coats of immature virus encompassed, either partially or completely, clumps of the filamentous viroplasmic matrix. When these coats were examined at a higher magnification it was possible to resolve in each one a "unit" membrane, 70 A wide, plus an outer 100 A layer of material of intermediate density (Figs. 29, 30). Occasionally, some degree of organization was apparent among the dense filaments, 15 to 40 A wide, which constitute the nucleoid (Fig. 31).

Maturing or mature forms of vaccinia, when first observed at 6 hours postinoculation, were invariably found either at the periphery of the zones of viroplasm or at a distance of several microns from them, but never within the viroplasm. These particles had two characteristic features, as illustrated in Figs. 33 to 36. First, they had become reorganized internally and possessed a core or central plate compressed in its narrow aspect by two dense lateral masses. Secondly, near the outer surface of the particles were present vesicles of the endoplasmic reticulum which sometimes appeared to have fused into a flat sac and enveloped the entire virus, as illustrated in Fig. 35. Since progeny virus, having a core and two lateral masses within an outer coat, could be distinguished readily from the inoculum particles in the cytoplasm of infected cells, it was possible to recognize label in the newly formed particles.

In fact, autoradiographic grains were encountered five times over the profiles of new mature or maturing particles out of several thousand such particles examined in the 6- and 8-hour samples (see Figs. 37, 38 for examples), whereas in the inoculum virus, evident at the 20-minute time point, from 5 to 40 per cent of the virus profiles were labeled.

The reconstructed sequence of vaccinia uptake and replication in L cells is summarized diagrammatically in Fig. 39.

DISCUSSION

Experiments using infectivity assays in parallel with light and electron microscopic autoradiography have confirmed and extended previous information about the uptake of vaccinia and have made possible the location of viral DNA throughout the cycle of virus multiplication.

Uptake of Vaccinia in Relation to Infection

These observations are consistent with the view that infection begins with phagocytosis of the virus, as shown by an association of label with intact virus particles, which are found initially only in pockets and vesicles formed at the cell membrane. Other viral components carrying the DNA label, such as cores and dense filamentous threads, were not detected at the cell membrane. Since the ratio of infectious units to virus particles is 1 to 12 and, therefore, quite low, it is considered likely that a penetration mechanism other than phagocytosis would have been detected among the large number of early stages which were recorded. The evidence presented here, which shows that adsorption of infectious virus occurs at the same rate as that of virus particles from the population in general, is also in favor of this view. Furthermore, pretreatment of L cells with fluoride, an inhibitor of phagocytosis, does not prevent attachment of vaccinia particles but does stop their engulfment at the cell membrane. Such exposed particles remain susceptible to neutralization by specific antivaccinia serum, which reduces the titer, whereas in the controls, where virus

engulfment occurs, antiserum becomes relatively ineffective (15). This evidence is in line with our previous observations and those of others, indicating that "viropexis" is a general phenomenon in animal virus infections (13, 14, 16, 17, 49) and quite unlike the injection process found with the tailed bacteriophages. Elucidation of the structure of mature vaccinia proved useful for the interpretation of electron micrographs showing virus penetration.

A close correspondence between images observed after negative staining and thin sectioning substantiates the general structure of vaccinia as elucidated by the work of Peters (43). The existence of the short, regularly spaced projections emanating from the coat of the core has not been reported heretofore, although similar structures have been found previously on the surfaces of myxoviruses (29), and some tumor viruses (40). In the present context, the morphological distinction which it is possible to make between the outer covering and the "shell" or coat-covering of the core of vaccinia might indicate the presence of two kinds of proteins or lipoproteins, only the outer one of which is susceptible to degradation in the environment of the phagocytic vesicle.

The dense filamentous component, 15 to 40 A wide, which is evident in the nucleoids of immature forms and within the core of mature vaccinia, could be DNA or a DNA-protein complex, as indicated by the presence of label in the core and by the enzymatic digestion experiments of Peters (44). These findings are not in accord with the idea that the surface ridges or tubular elements of vaccinia represent the viral nucleoprotein (42).

Contrary to our previous work suggesting that the entire virus particle is transferred to the cytoplasm (16), the present observations, carried out on more satisfactory material, clearly indicate that only the core containing the DNA gains access to the cytoplasmic matrix. The outer membrane and surface ridges or tubules as well as the two lateral bodies apparently remain behind, at the site of the initial disintegration. Thus a disruption of the virus and release of an inner structure containing the infectious component, postulated for influenza virus previously (17), apparently occur in the case of vaccinia.

The chemical composition of the virus surface and the complexity of the viral architecture could be factors controlling the events within a phagocytic vesicle. Should a surface protein or a lipoprotein be resistant to hydrolysis, the virus could escape intact from the vesicle and become transferred to the deeper regions of the host cell, as appears to be the case with Adenovirus 7 (13). The situation with vaccinia could be explained by assuming that the outer virus coat is susceptible to digestion, whereas the inner one is not. In the case of the simple RNA-containing viruses, release of the infectious molecule, after disintegration of the protein shell, could be the disruptive mechanism, as suggested for polio (39) and foot-

FIGURE 12

Thin section through the peripheral cytoplasm of a cell sampled 20 minutes after inoculation. A particle contacting the surface and another in a vesicle are evident. *E*, × 120,000.

FIGURES 13 AND 14

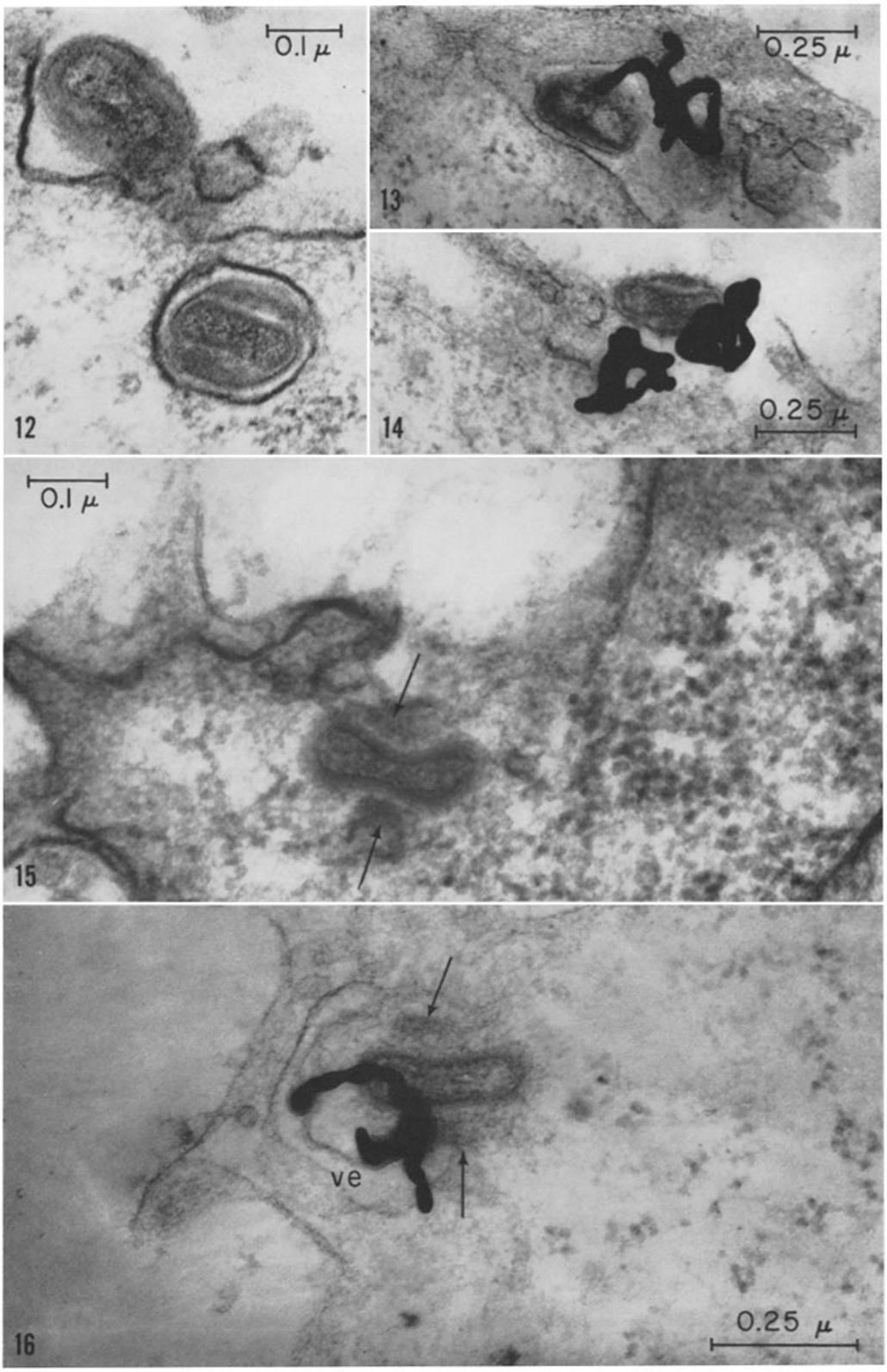
Two examples of vaccinia particles and associated grains, on the outer membrane of cells sampled 20 minutes after inoculation. *Au* (43), *M*, × 60,000.

FIGURE 15

Peripheral cytoplasm of a cell sampled 20 minutes after inoculation, showing a virus particle which may be undergoing partial disruption. Note that the outer viral coat is missing and the two lateral dense masses, indicated by arrows, appear to be separated from the biconcave core. *M*, × 110,000.

FIGURE 16

An example like the one in Fig. 15 above, but showing also an associated silver grain. A flat vesicle may have been originally the phagocytic vesicle. *Au* (66), *M*, × 90,000.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12
0.25 μ
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ve
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and-mouth disease virus (6). The morphological evidence thus far obtained can now be used to design experiments for the study of the enzymes associated within virus-enclosing vesicles and their interactions with the components of viral coats.

Inoculum-Virus DNA and Vaccinia Replication

These autoradiographic studies have also made possible the localization of the DNA coming from inoculum virus throughout the cycle of virus replication. By following the label, it was demonstrated that the morphological sequence of virus development, which heretofore had been reconstructed from a chronological series of samples (3, 24, 41), is, in fact, the sequence related directly to the synthesis of vaccinia particles. Association of the DNA first with the core and subsequently with the viroplasm indicates that separation of the labeled molecule from the cores occurs 1 to 3 hours after inoculation. This is in accordance with observations of Joklik (31), showing that inoculum DNA of vaccinia becomes accessible to degradation by the nuclease commencing at 1 to 2 hours after adsorption. Release of the DNA could occur either after a profound disruption of the dense coat of the core or after development of a fine break in this coat sufficient only to allow the DNA to pass out. Both mechanisms are compatible with the present morphological evidence which shows broken down as well as apparently intact "shells" free of viroplasm. The mechanism by which a specific uncoating enzyme, postulated by Joklik (31), may act in such a release process has yet to be elucidated.

A dense fibrous material, occasionally observed as small clumps in cells 1 hour after inoculation, is, no doubt, connected with the larger, more numerous "factories" which are evident at 3 hours after infection. Even at 1 hour after infection, however, some matrices of viroplasm are quite extensive and have at their periphery several "shells." At 3 hours, greater numbers of "shells" are present close to the "factories." At this time both DNA and viral protein are being synthesized in these "factories" (7, 37), but so far there is no evidence that such synthesis starts as early as 1 hour after inoculation. Perhaps the fibrous material lodged in the cytoplasm at the earlier time is only the inoculum DNA or DNA-protein which has fused into a single mass after becoming separated from several cores.

Whereas 5 to 40 per cent of the inoculum

FIGURES 17 TO 20

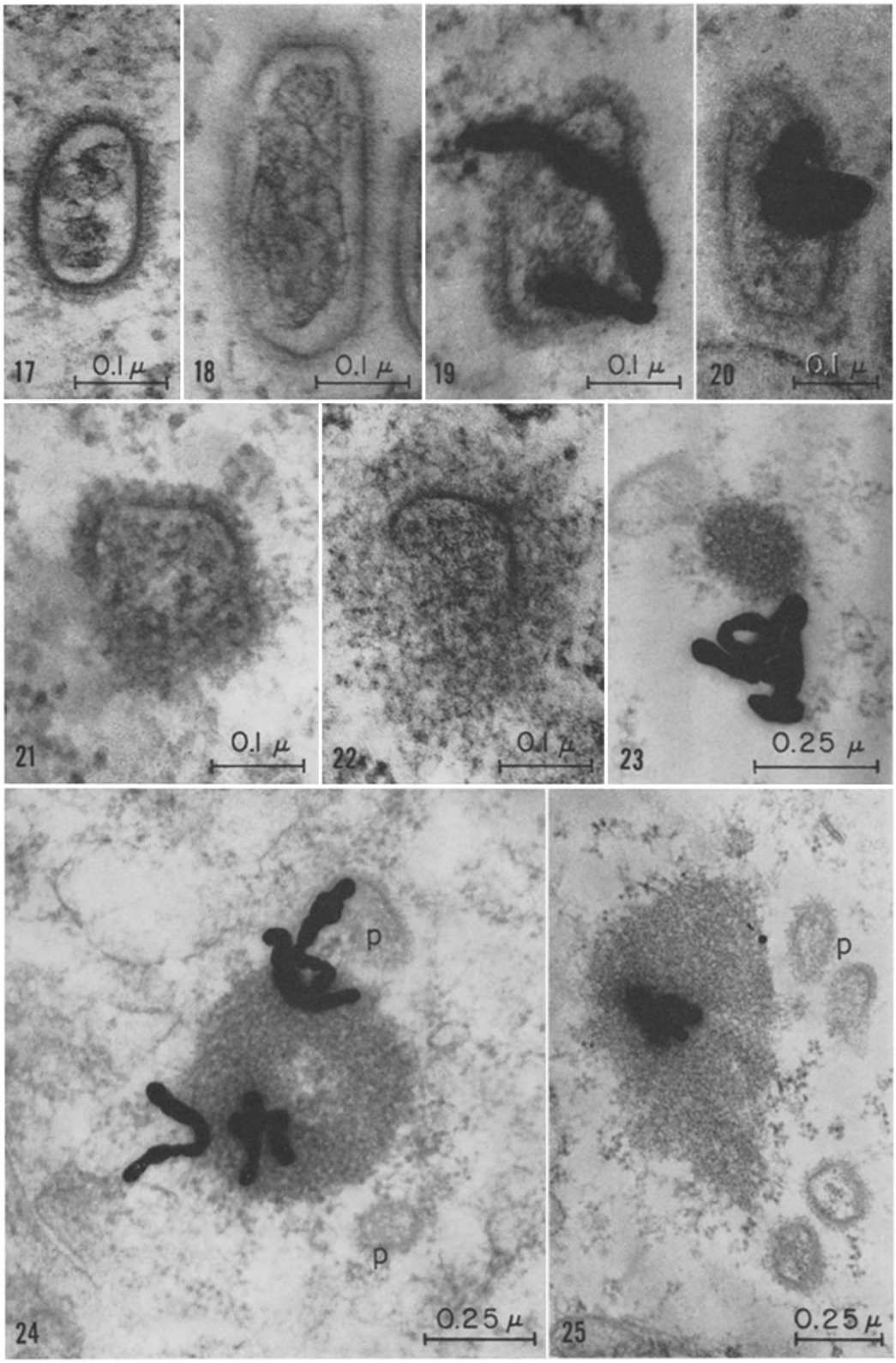
These figures illustrate four examples of the central plates or virus cores observed lying free in the cytoplasm of thinly sectioned cells sampled at 20 minutes and 1 hour after infection. Note the dense filaments inside the core in Fig. 18. Label is associated with cores shown in Figs. 19 and 20.
Figs. 17 and 18: *E*, × 140,000.
Figs. 19 and 20: *Au* (66), *M*, × 140,000.

FIGURES 21 TO 23

Three examples from thinly sectioned cells preserved 1 hour after inoculation. Breakdown of the coats of the cores is evident in Figs. 21 and 22. In Fig. 23, a "shell" or a profile of a core appears next to the clump of dense fibrous material and an associated grain.
Figure 21: *M*, × 140,000.
Figure 22: *E*, × 140,000.
Figure 23: *Au* (43), *M*, × 140,000.

FIGURES 24 AND 25

Two examples showing clumps of material in the cytoplasm of cells sampled 1 hour after inoculation. The label is within the clumps, whereas the "shells", present at the periphery, have a light content and no grains over them.
Figure 24: *Au* (66), *M*, × 68,000.
Figure 25: × 50,000.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19
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21
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22
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23
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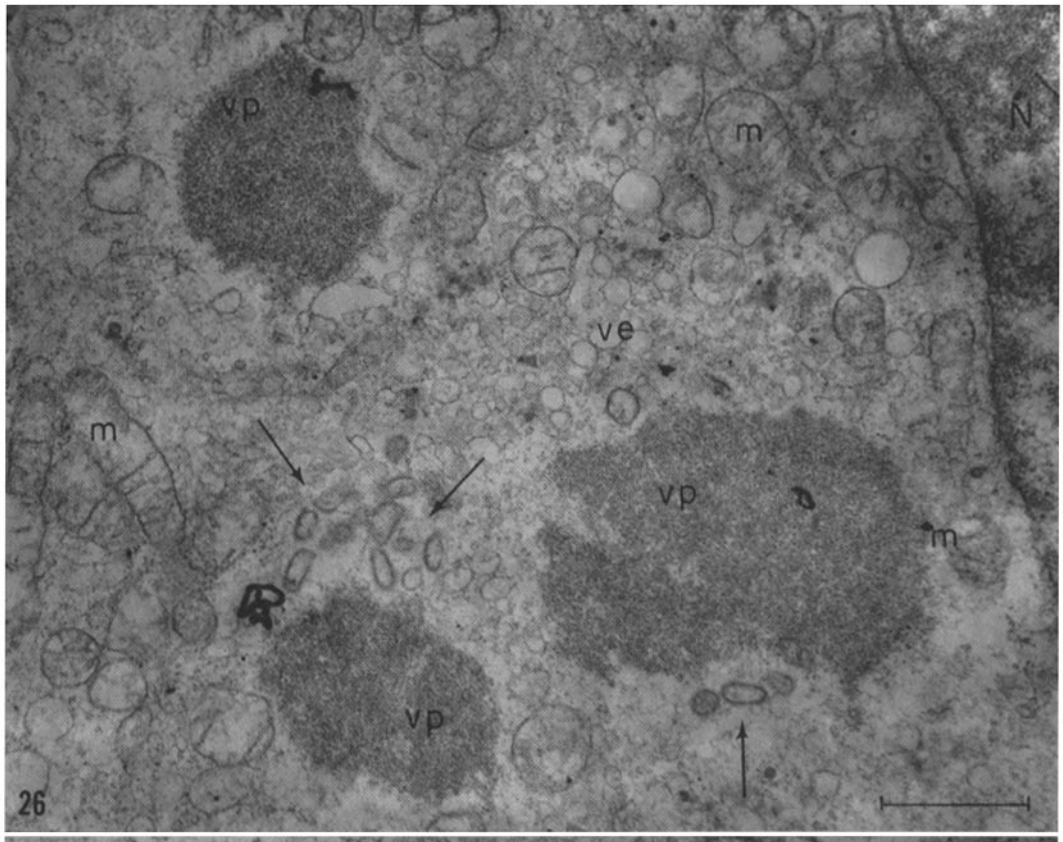
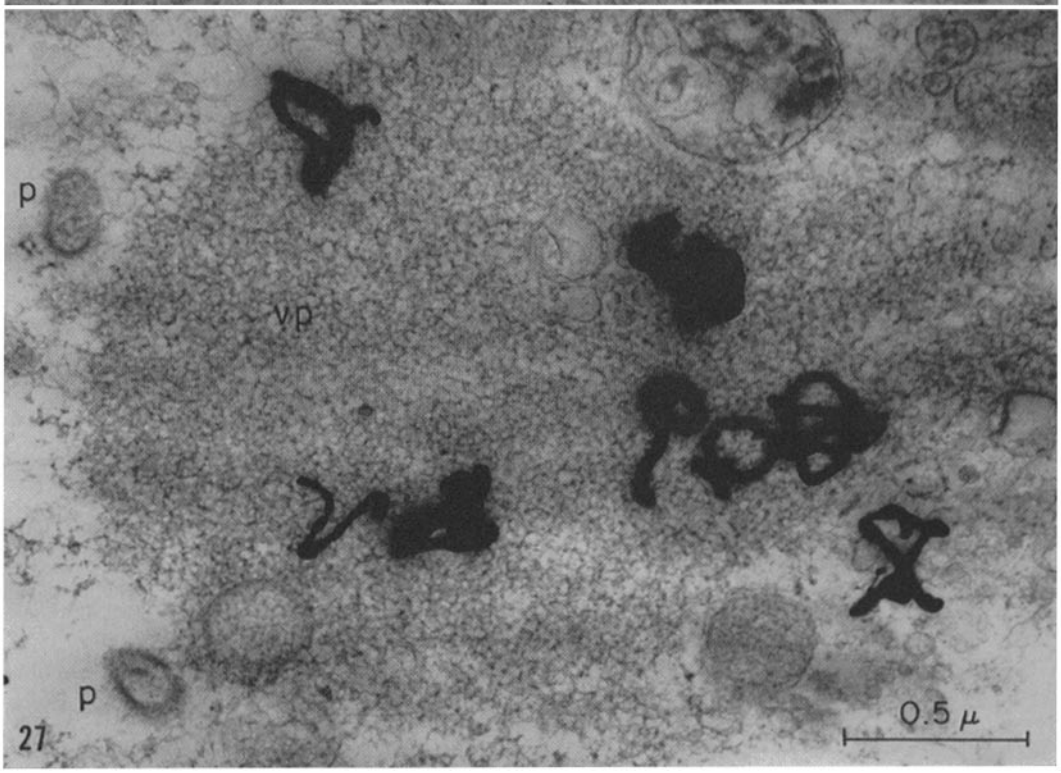
p
24
0.25 μ
p
25
0.25 μ


vp
m
N
ve
m
vp
m
vp
26


p
vp
p
27
0.5 μ

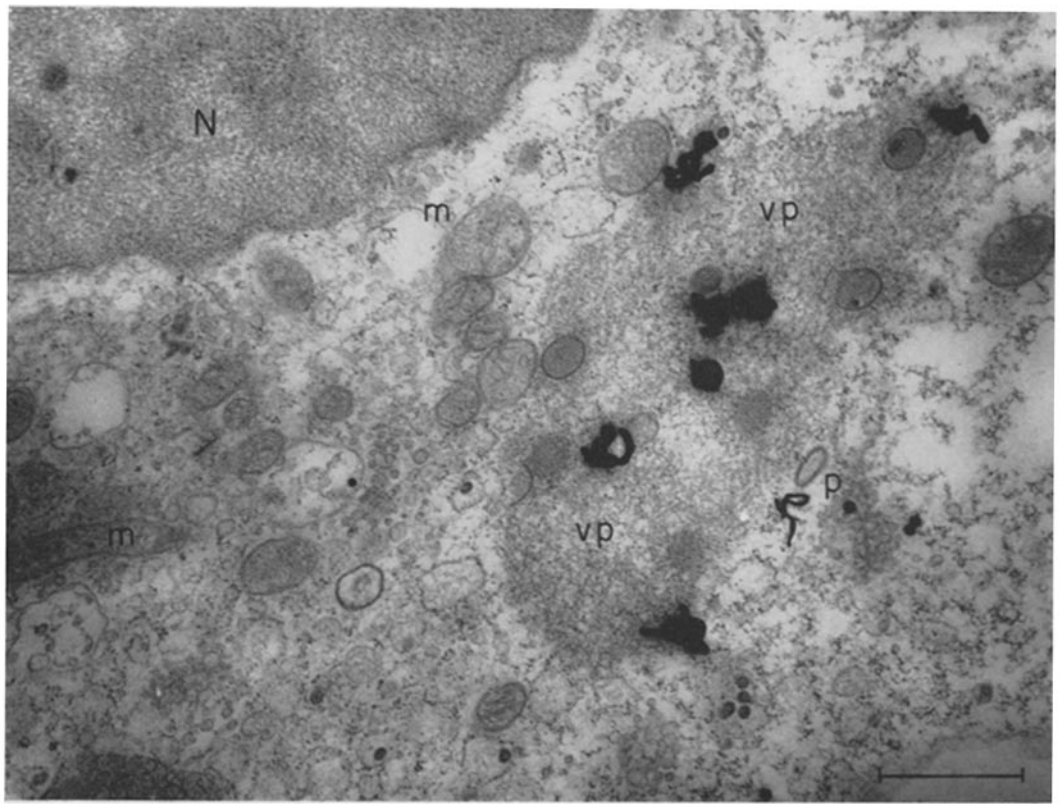


FIGURE 28

An example showing the distribution of label within "factories" in the cytoplasm of cells sampled 6 hours following inoculation. Immature forms of vaccinia are present in the viroplasm. *Au* (66), *M*, × 20,000.

particles, observed in thin sections, had associated silver grains, only a fraction of 1 per cent of the maturing or mature progeny virus was labeled. Unfortunately, adequate quantitative estimates of the kind obtained with Pneumococcus-transforming DNA (34) have not been made on the transfer of the parental genome to the progeny of vaccinia. However, in view of the apparent stability of inoculum DNA (31 and this paper) and the rapid accumulation of relatively large amounts of viral DNA in the cytoplasm several hours after infection (36), it seems unlikely that the label found in newly formed vaccinia particles represents merely reincorporation from a pool assumedly derived

FIGURE 26

Portion of the nucleus and cytoplasm of a cell 3 hours after inoculation. Label is associated with the three foci of viroplasm. Arrows point towards inoculum-virus "shells," present at the periphery of the clumps. *Au* (66), *M*, × 20,000.

FIGURE 27

An example similar to those shown above but at a greater magnification. Note that the viroplasm consists of fine coiled threads, each less than 50 A in width. Two "shells" are evident on the left of the "factory". *Au* (66), *M*, × 50,000.

FIGURES 29 TO 36

Stages in the formation of vaccinia reconstructed from samples taken at 4 to 6 hours postinoculation.

FIGURE 29

Incompletely formed surface components consisting of a unit membrane, indicated by arrows, and a layer of fairly dense material on the outside. *E*, × 125,000.

FIGURE 30

An immature particle completely enclosed in a coating consisting of a membrane (arrows) plus some outer dense material. An eccentric nucleoid is also visible. *E*, × 220,000.

FIGURE 31

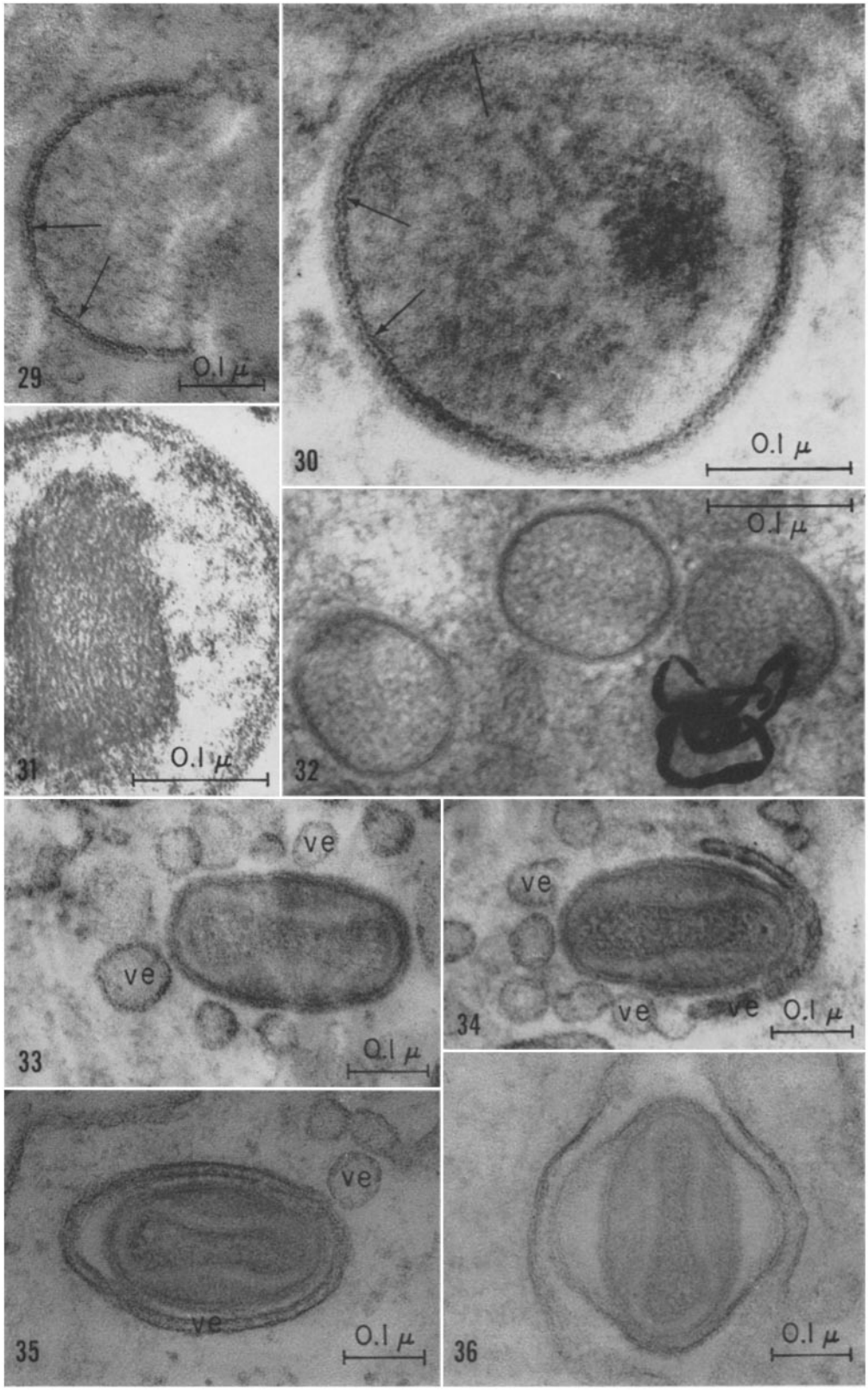
Portion of an immature particle with an elongated, central nucleoid. The fine, dense threads, each 15 to 40 A in width, appear to be oriented parallel to the long axis of the nucleoid. Double fixation with 1 per cent glutaraldehyde followed by 1 per cent OsO_4. Sections stained 1 hour with aqueous 1 per cent uranyl acetate. *E*, × 215,000.

FIGURE 32

Three immature particles, one of which is partly obscured by a silver grain. *Au* (30), *M*, × 85,000.

FIGURES 33 TO 36

Four examples of maturing or mature progeny virus. In the first three, vesicles of the endoplasmic reticulum surround the virus partially or completely. The particle in Fig. 36 is lodged between two cells. *E*, × 120,000.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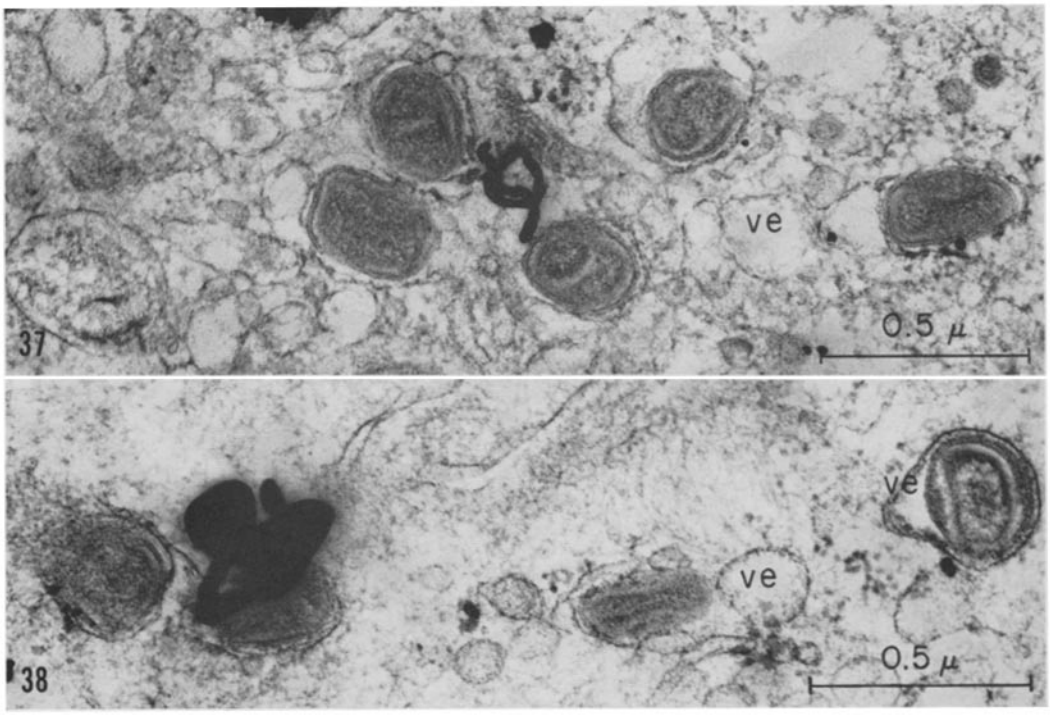
from a breakdown of the parental molecule but may represent either large pieces or entire strands of the inoculum DNA. It might be possible to obtain further information on this point by following quantitatively the transfer of label from the inoculum to progeny, using purified virus from cells 6 hours after inoculation and at intervals thereafter.

As has been observed previously by both light (4, 7) and electron microscopy (16), several centers of virus synthesis may occur, scattered in the cytoplasm of a single cell. Since each infecting particle can induce its own center of synthesis, as shown experimentally by Cairns (7), the observed presence of several "factories" in the cytoplasm of some L cells sampled from cultures inoculated with 8 PFU per cell was to be expected.

It is not yet clear whether the "initiator" particle postulated by Cairns (7) is, in the present system, one of the twelve able to produce a plaque on monolayers of L cells, or whether several particles cooperate to initiate a vaccinia "factory." It has been shown, however, that when purified vaccinia particles are first irradiated and then purposely clumped, a direct relationship exists between the number of particles in an average clump and the extent of multiplicity reactivation which occurs (1). This suggests that cooperation of several particles may be required to initiate a replicating center in the cytoplasm of L cells. In either case, the site of virus multiplication is evidently a focal point for an accumulation of viral cores. Within the sensitivity of the techniques used, all of the viral DNA remains in the cytoplasm up to 6 to 8 hours following inoculation, and none, apparently, enters the host-cell nucleus. Thus each cytoplasmic "factory" can be thought of as a kind of miniature nucleus in which resides the control of the synthetic capacity for the production of virus. Such a concept is consistent with the observation that damage to the host-cell genome by mitomycin C or by porfiromycin, which bring



FIGURES 37 AND 38

Two groups of maturing or mature vaccinia virus particles, 6 hours after infection. One silver grain is lying between two particles in Fig. 37, and directly above another particle in Fig. 38. *Au* (66), *M*, × 60,000.

about an irreversible inhibition of DNA synthesis of HeLa cells, does not prevent vaccinia multiplication (38). Further, in a mutant sub-line of strain L cells normally lacking thymidine kinase this enzyme can be synthesized after infection with vaccinia, and a cytoplasmic synthesis of vaccinia DNA can occur when nuclear-DNA synthesis is absent or very low (33). Future biochemical investigations may show whether, among the initial steps in vaccinia reproduction, viral nucleic acid polymerases are induced and act within these cytoplasmic "factories," resulting in an orderly synthesis and assembly of viral components.

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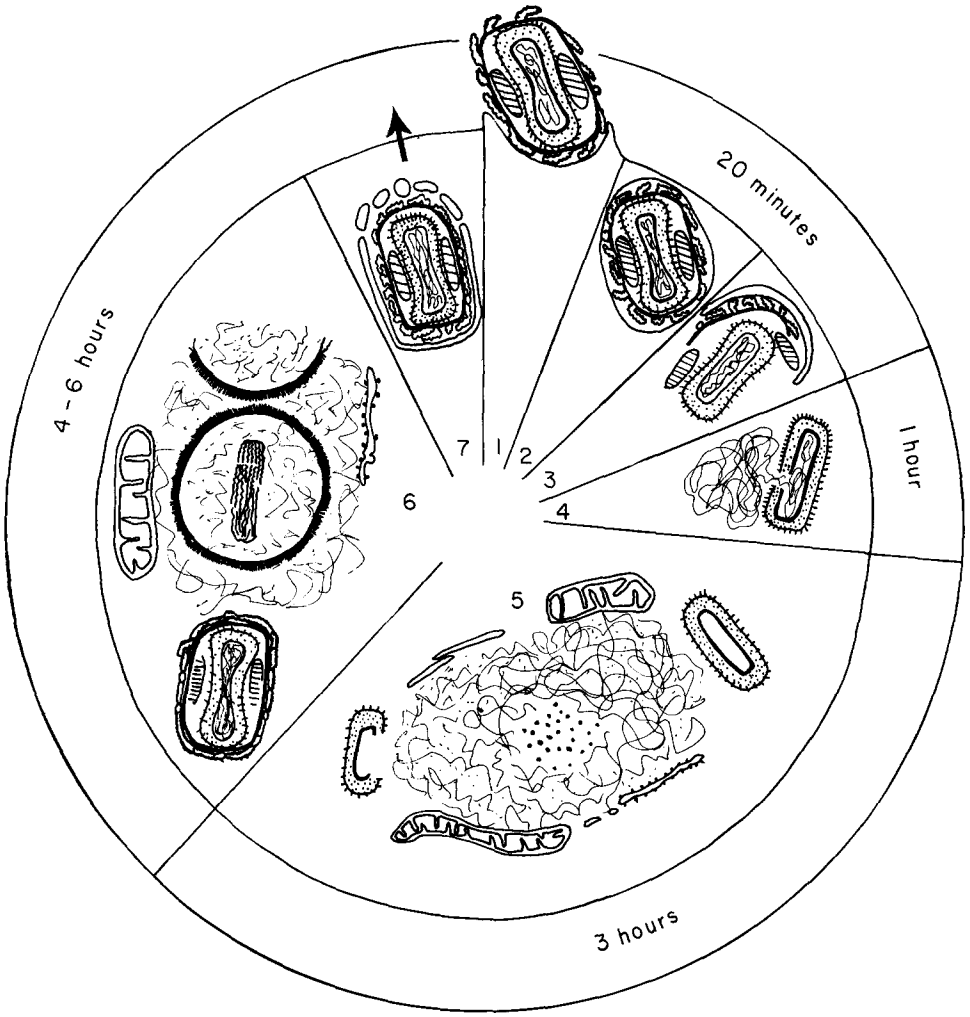

FIGURE 39

Diagram illustrating the sequence of vaccinia uptake and replication in L cells.

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