A COMPARISON OF THE CHANGES IN FINE STRUCTURE OF L CELLS DURING SINGLE CYCLES OF VIRAL MULTIPLICATION, FOLLOWING THEIR INFECTION WITH THE VIRUSES OF MENGO AND ENCEPHALOMYOCARDITIS

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ABSTRACT

The virus of encephalomyocarditis (EMC), examined by the negative-contrast method, is indistinguishable from the serologically related Mengovirus. The particles are 270 to 280 A in diameter. The surface of EMC is composed of an undetermined number of subunits. Frequent sampling of infected cells was carried out throughout one-step cycles of viral multiplication to observe cytopathic changes occurring in L cells infected by these two related RNA viruses. EMC and Mengovirus, which multiply at equal rates, in most respects elicit similar alterations in cell fine structure. Rearrangement and changes in nuclear material accompanied by formation of small vesicles in the centrosphere region commence at 4 to 6 hours after infection. Thereafter a progressive degeneration of the nucleus and vesiculation of the cytoplasm are observed up to 18 to 20 hours. Increased numbers of small dense granules, indistinguishable from ribonucleoprotein particles, appear in the cytoplasm between 8 and 14 hours after infection. L cells infected with Mengovirus become permeable to Erythrocin more slowly than those infected with EMC. Only in the case of Mengovirus infection are large aggregates of dense material first observed in the cytoplasm at 8 hours, followed by the appearance of crystals probably composed of Mengovirus particles, at 12 hours. Differences in the rates of cell permeability after infection with EMC and Mengovirus are discussed in relation to formation of virus crystals and plaque-type mutants.

INTRODUCTION

Cytopathic changes which occur in host cells during single cycles of virus multiplication have been examined by both light (25) and electron microscopy (22). Comparisons of such changes, resulting from the infection of a particular host by several related viruses within a group, have also been undertaken (2, 28), and more recently a report was published comparing, by light microscopy, changes in cell morphology which accompany the infectious cycle of two serologically related virus mutants (18). To date, however, comparisons of changes in the fine structure of cells infected by closely related viruses, during a single cycle of virus reproduction, have not been reported.

The present study was prompted by some

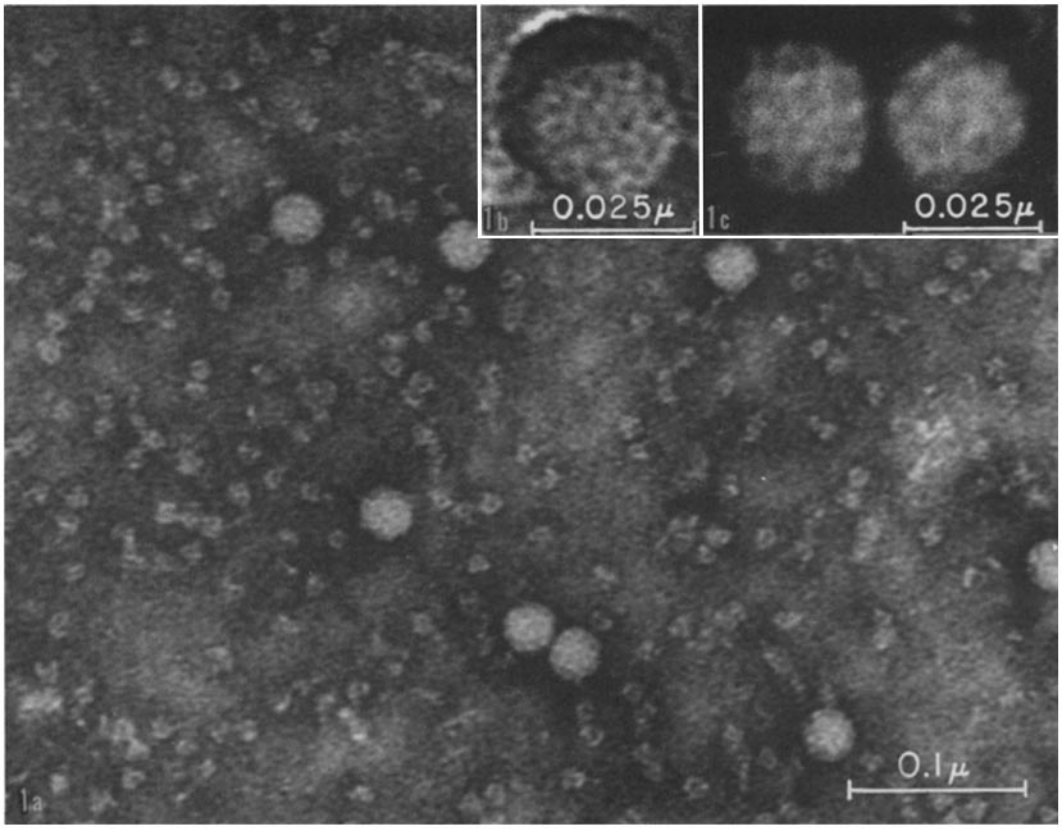


FIGURE 1

a. Negatively stained EMC. Note the smaller component, perhaps viral material, scattered among the virus particles. × 230,000.

b, c. Insert shows three virus particles at a higher magnification. *b*, × 880,000; *c*, × 700,000.

preliminary observations which indicated differences in cytopathic effects in L cells following their infection with either Mengovirus or the virus of encephalomyocarditis (EMC). Although examination of cells infected with EMC by either light or electron microscopy failed to detect the presence of virus aggregates (8), masses of viral material were observed by light microscopy in Mengovirus-infected cells (15). The fine structure of these aggregates will be described in this report.

The two infecting agents employed are small RNA viruses belonging to the Columbia SK group. They are serologically related and both multiply to high titers in a common host, strain L mouse fibroblasts (4, 8, 14, 17), cultured in suspension. This provided us with a suitable system for carrying out quantitative and morphological comparisons on virus multiplication and changes in the fine structure of the host.

MATERIALS AND METHODS

Source of Cells and Virus

The cells used were subline L-60, derived from clone AMK_{2-2} (35), which had been derived from Earle's L-strain fibroblasts (38). These cells were grown in suspension, using roller tubes (39).

The virus of encephalomyocarditis (EMC) was obtained from Dr. T. S. Work (17) and Mengovirus was obtained as a mouse brain suspension from Dr. M. Theiler and subsequently passaged in L-929 cells, as described previously (14). Both viruses multiply to high titers in L cells.

Nutrient Media

The nutrient medium used for suspension cultures was CMRL 1066 (20), supplemented with 10 per cent non-toxic horse serum or fetal calf serum (Colorado). Overlays for plaque assays contained either synthetic medium 1066 or Eagle's MEM

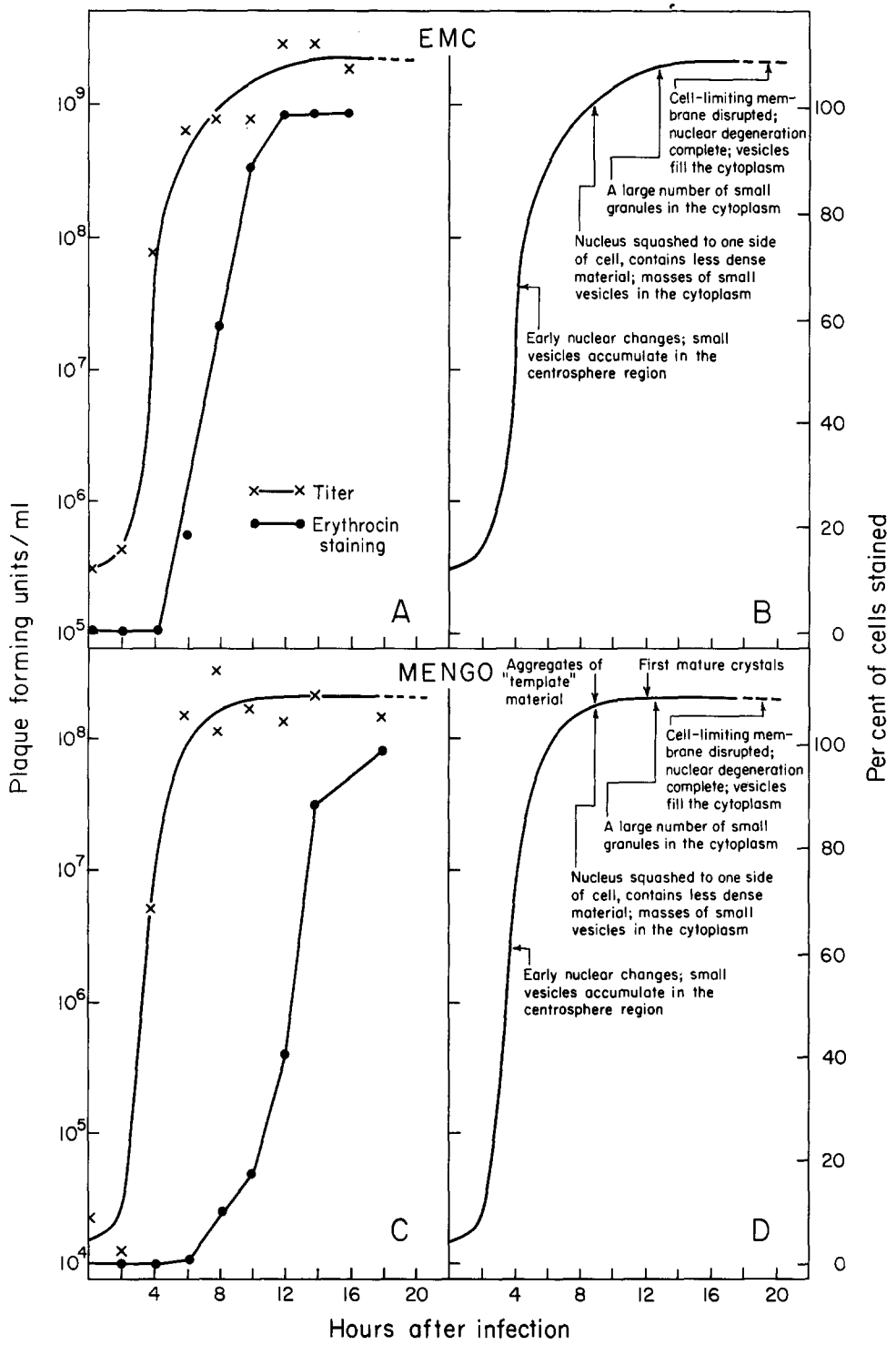


FIGURE 2

A, C. Permeability of L cells to Erythrocin during one-step cycles of multiplication of EMC and Mengovirus.

B, D. Changes in fine structure at various times during virus replication.

plus 5 per cent horse serum and 0.9 per cent Bacto Agar.

Preparation of Virus

Lysates containing virus were prepared by infecting suspension cultures with virus multiplicities greater than was sufficient to infect each cell in the population. When nearly all of the cells in the culture showed cytopathic effects, 18 to 24 hours after infection, the cultures were frozen and thawed at least three times to release all of the cell-associated virus. Generally, these lysates contained between 10^8 to 10^9 plaque-forming units/ml.

Virus was partially purified from pooled lysates, by the use of a series of differential centrifugation steps similar to those employed by Weil *et al.* (41) for the purification of EMC from infected mouse brains. Large cellular debris was removed by low speed centrifugation in the cold. The bulk of the virus in the clarified medium was spun into pellets by centrifugation in the cold at 104,000 *g* for 1 hour. The pellets were resuspended in 0.125 per cent trypsin dissolved in an isotonic saline solution (PBS of Dulbecco and Vogt (11)), for the digestion of the proteinaceous cellular debris, after which the virus was again spun at 104,000 *g* for 1 hour and was finally dispersed into either PBS or a 0.25 M ammonium acetate solution at neutral pH.

Plaque Assays

Concentrations of infectious particles were determined by methods described previously for Mengo (14), and for EMC (17), which are based on the technique of Dulbecco and Vogt (11).

Method for Infecting Cells and Sampling

About 1×10^8 to 2×10^8 cells, concentrated into a pellet by low speed (1,000 RPM for 5 min) centrifugation, were resuspended in 5 ml of nutrient medium containing about ten infectious units per cell and this suspension was agitated continuously for 1 hour, at 37°C, to facilitate adsorption. Unadsorbed virus was removed by three washes with nutrient medium. Thereafter the infected cells were suspended in about 500 ml of CMRL 1066 plus serum and were incubated at 37°C in suspension cultures. Samples for assays of virus and morphological examination were removed at regular intervals during the 18- to 20-hour period of the experiment, as described previously (10).

Cytochemical Techniques

Uptake of the dye Erythrocin by infected cells, ascertained as before (10), was assumed to indicate an irreversible loss of permeability regulation leading to cell death (37). Monolayers of cells, grown on coverslips, were infected in the manner described previously (15). Following their fixation by freeze-substitution, infected cells were examined for the presence of protein and viral nucleic acid using the fluorescent antibody technique and acridine orange staining, in conjunction with enzymatic digestion procedures, also described previously (15).

Labeling of RNA in infected cells was carried out by the addition of 1 μc/ml of uridine-H^3 (New England Nuclear Corp., Boston, Mass.) to the nutrient medium of suspension cultures of infected cells. 0.33 mM of unlabeled thymidine (1000-fold molar excess) was also added to inhibit DNA labeling (42). After 12 hours' infection, cells were fixed and embedded by the method described below. Sections 1 μ thick, cut from these samples, were mounted on glass slides and coated with stripping film photographic emulsion (Kodak AR-10). Standard autoradiographic procedures, as previously described (16), were used.

Electron Microscopy

Suspensions of partially purified virus were examined by the negative staining technique of Brenner and Horne (3).

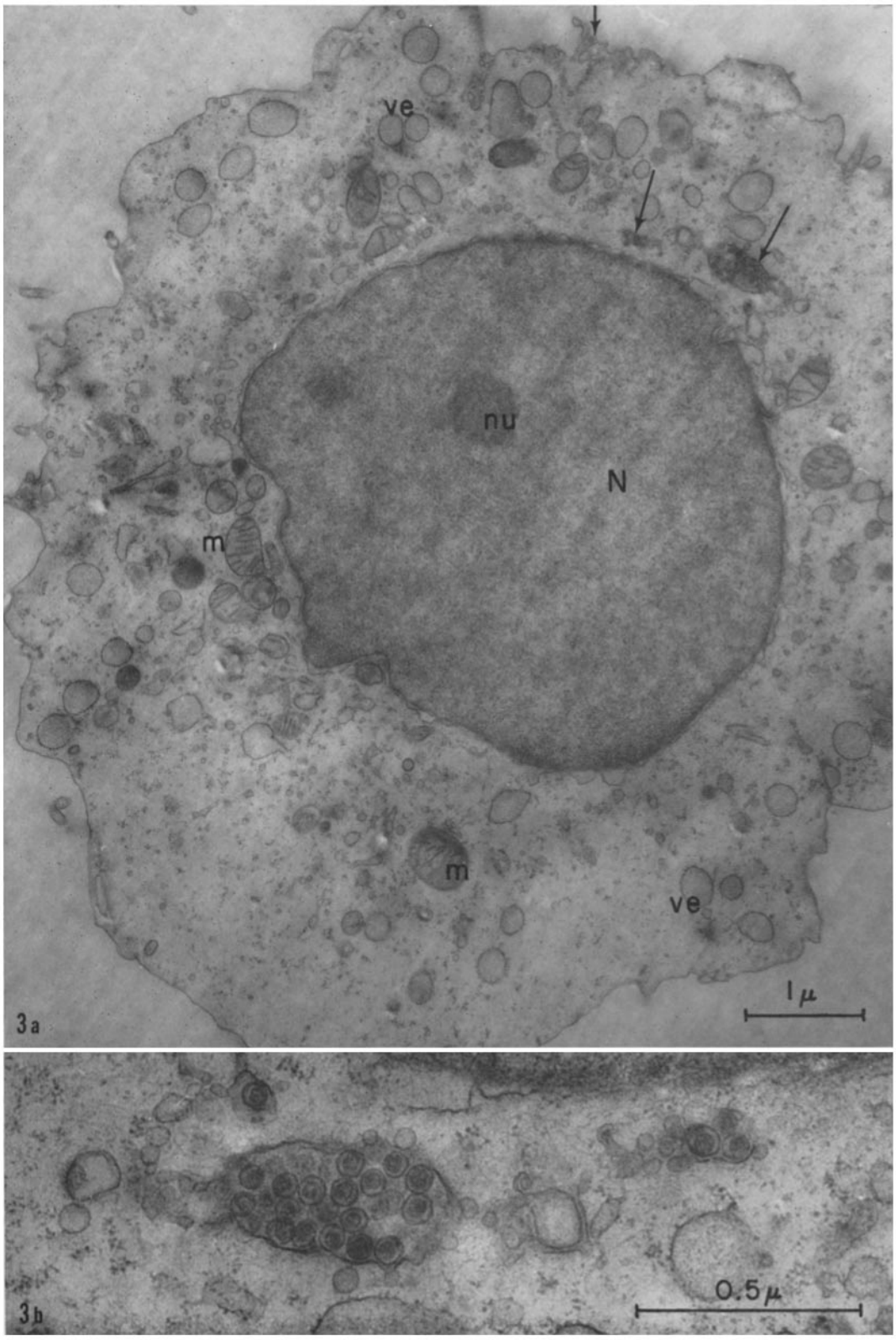
Cells were prepared for thin sectioning by being

Key to Abbreviations

N, nucleus
nu, nucleolus
m, mitochondria
VL, virus-like particles
ve, vesicles
(K), stained by Karnovsky's formula B
(M), stained by Millonig's formula

FIGURE 3

a. A cell from a non-infected suspension culture. Apart from the components usually found in cells of this type, note the presence also of VL particles, indicated by arrows. × 18,000 (M).
b. Portion of the same cell at a higher magnification to show groups of VL particles. × 70,000.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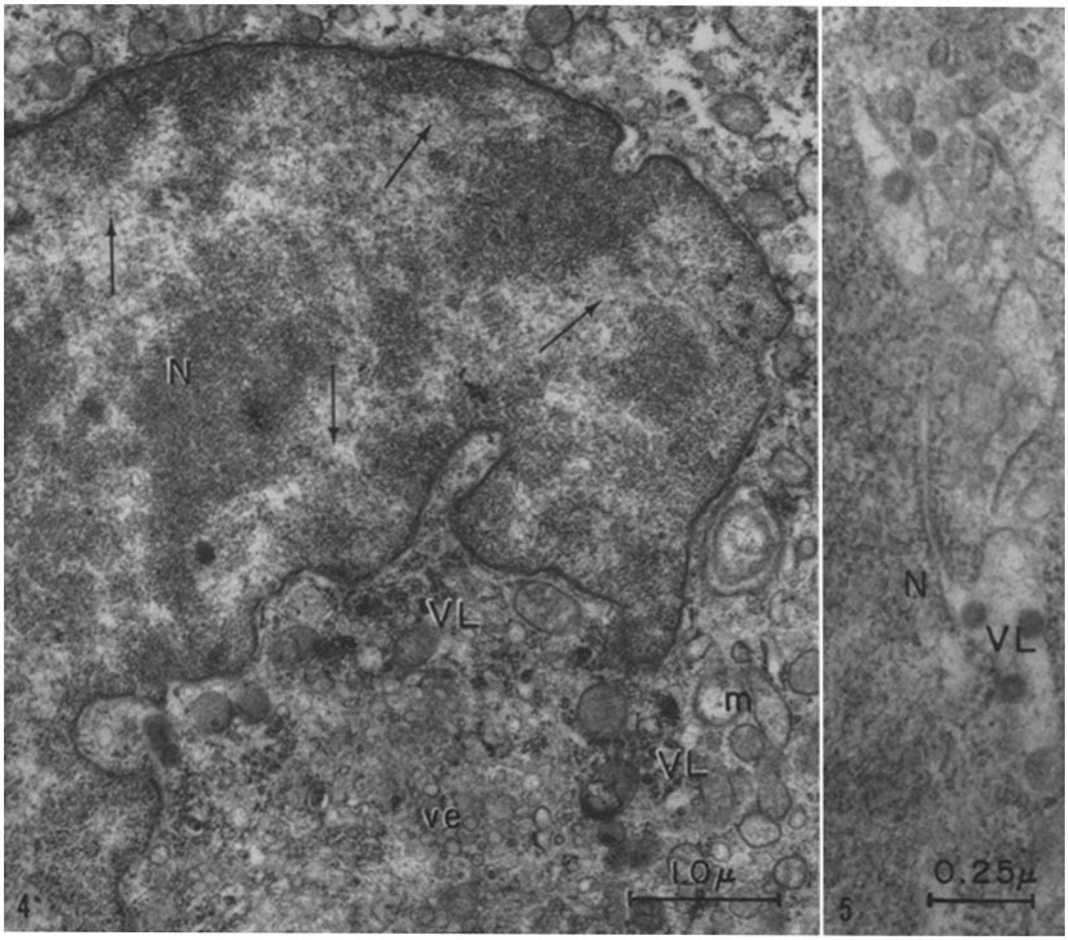


FIGURE 4

Portion of the nucleus and cytoplasm of a cell sampled 6 hours after infection with Mengovirus. Regions devoid of dense material are indicated in the nucleoplasm by arrows. The nucleus is indented at a region above a mass of cytoplasmic vesicles, which have formed in the centrosphere region. × 20,000 (M).

FIGURE 5

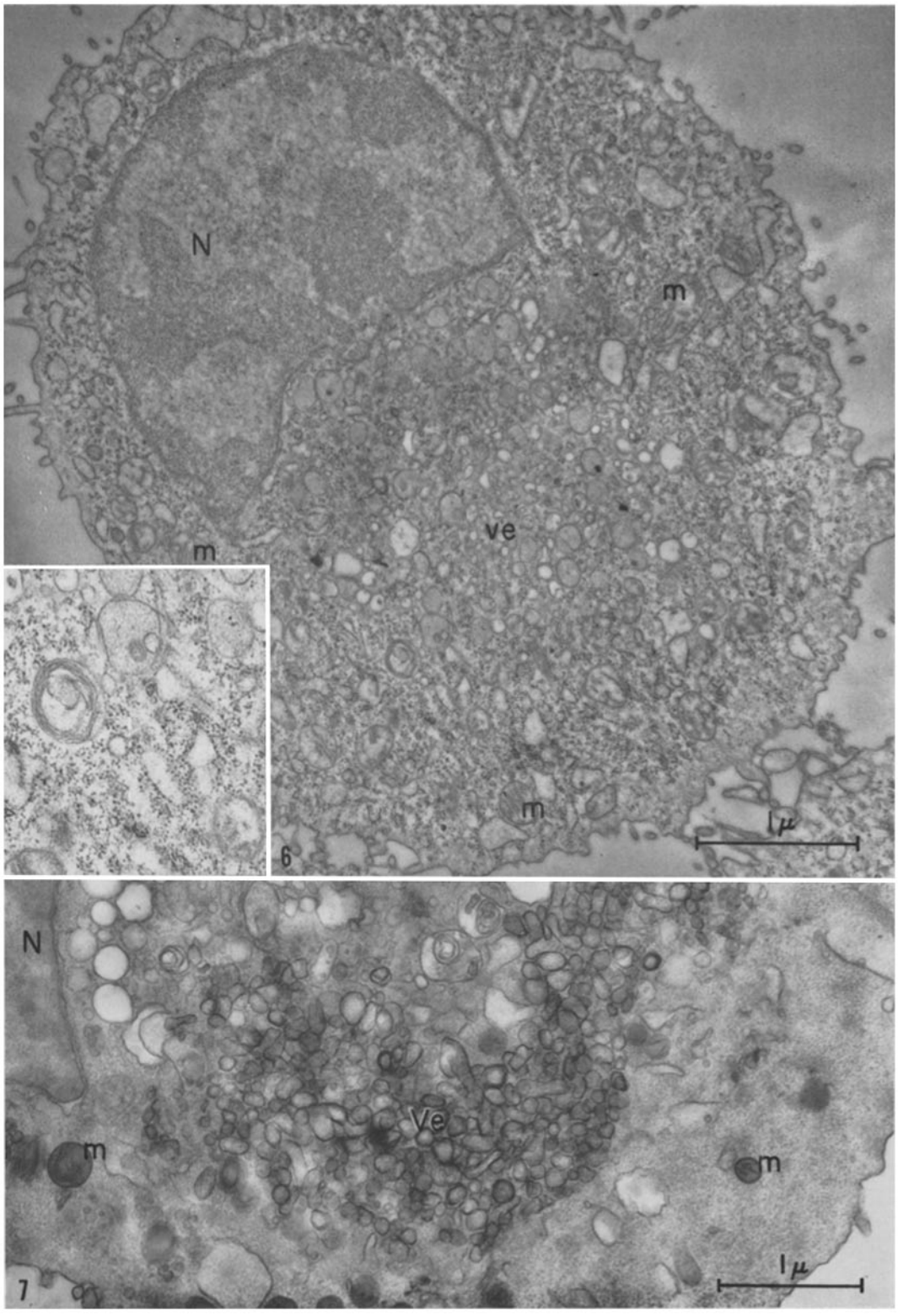
A small area showing the periphery of a nucleus and adjacent cytoplasm of a cell sampled 6 hours after infection with Mengovirus. Within the swollen sacs of the nuclear envelope are virus-like particles of type A, indigenous to these cells. × 55,000 (M).

FIGURE 6

A cell sampled 8 hours after infection with EMC. × 25,000 (M). Insert: enlargement of a portion of the cytoplasm to show the numerous dense granules. × 65,000.

FIGURE 7

Portion of the nucleus and cytoplasm of a cell sampled 8 hours after infection with Mengovirus. × 22,000 (M).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pelleted, fixed, and dehydrated by means of the conventional techniques (31). For embedding, either methacrylate (4 parts butyl:1 part methyl) or epoxy-resin mixtures, as described by Luft (24), were used and contrast of thinly sectioned cells was increased by staining with a solution of lead salts according to the procedure of Millonig (27) or of Karnovsky, method B (23).

RESULTS

A. *Physical Characteristics of the Virus*

EMC particles obtained from L-60 cells have a diameter of 270 to 280 A as measured in negative-contrast preparations, corresponding closely to the size of EMC virus which had been isolated from other host cells (13, 41). EMC examined after such staining possessed an undetermined number of dense and light spots (Figs. 1 *a*, *c*). At a higher resolution (Fig. 1 *b*), the surface appeared to consist of a number of closely packed components, each possessing a central hole 10 to 20 A across, surrounded by a less dense ridge 20 to 25 A wide. The resolution attained, however, was still too low to resolve any possible regularity in packing of the viral surface units. In similar preparations, Mengovirus was indistinguishable in size and appearance from EMC.

In the purified preparations of EMC suspended in ammonium acetate, elements smaller than the virus were also present, scattered throughout (Fig. 1 *a*). Some of this material was in the form of structures similar in appearance to the viral surface units; the remainder appeared as short rodlets, granules, and fine threads, frequently coiled into loops. The latter forms could also have been viral components which had become partially obscured by the phosphotungstate. Very frequently, partially disaggregated virus particles were also observed, in which one portion was intact whilst the remainder consisted of a number of loosely aggregated elements similar in appearance to those found scattered among the virus. This small particulate material, which resembles in size and morphology the structures seen on the virus particles, may be of viral origin.

B. *Virus Multiplication and Cell Permeability*

One-step growth curves for EMC and Mengovirus, as well as the time course of changes in cell permeability resulting from infection, are shown in the results of two typical experiments (Fig. 2). From these data it is clear that most of the infectious viruses had been synthesized by 8 to 10 hours after the completion of adsorption. The similarity in rates of multiplication of these two viruses was not, however, paralleled by the rates at which cell permeability to Erythrocin increased. In the infectious cycle with EMC, L cells became permeable to Erythrocin about 3 hours after virus had been formed, whereas when Mengovirus was the infecting agent the increase in cell permeability lagged behind viral multiplication by 8 to 10 hours. Thus, although most of the infectious Mengovirus is made as fast as EMC, changes in cell permeability show up much more slowly with Mengovirus.

C. *Changes in the Fine Structure of Cells Associated with Virus Multiplication*

i) Controls

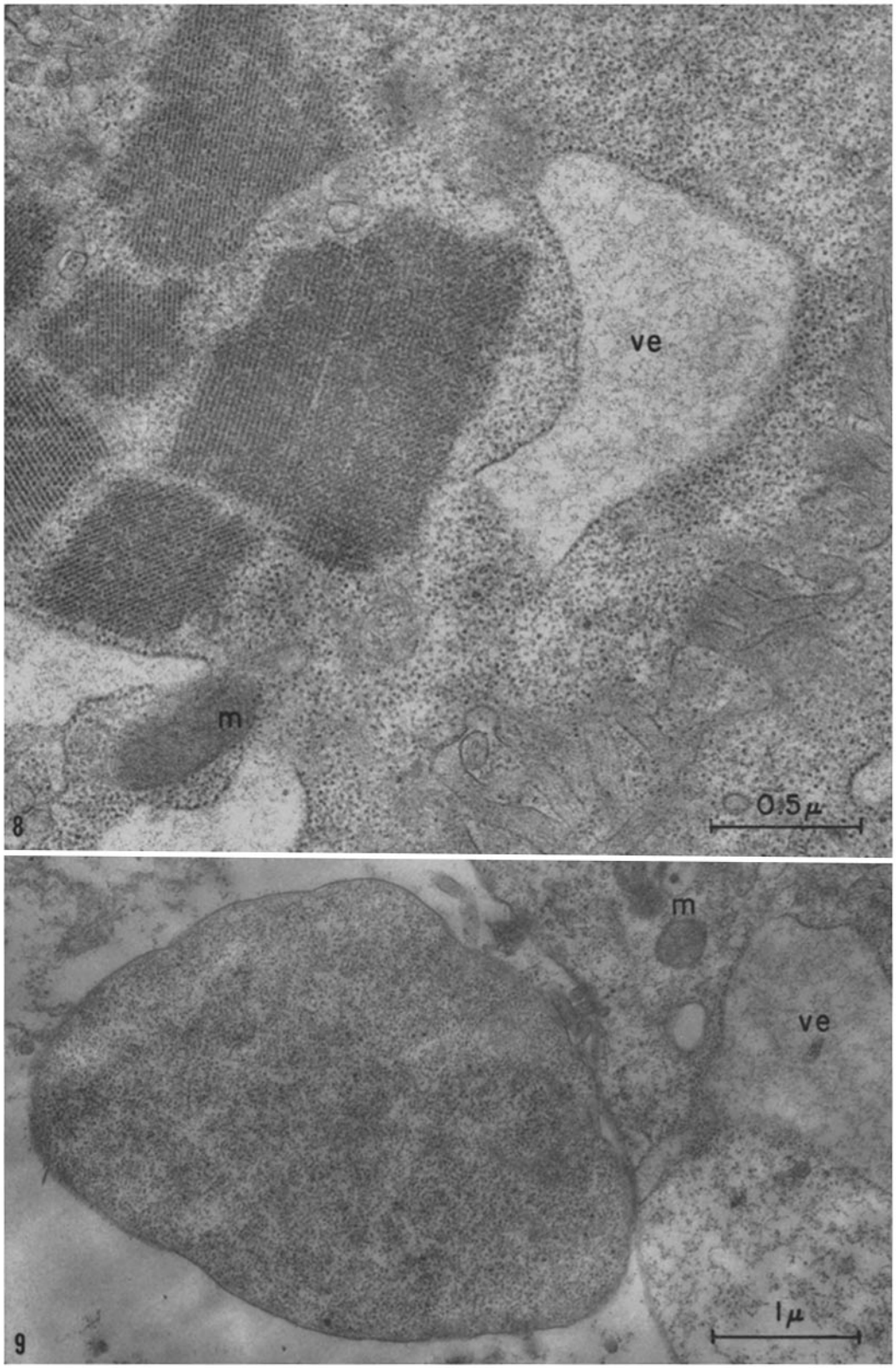
Uninfected L-60 cells, examined as controls, showed components which are usually present in cultured cells of the fibroblastic type. An example of one such cell is shown in Fig. 3. Distributed randomly throughout the nucleoplasm of the spherical, centrally placed nucleus is a dense filamentous and granular component, ranging in width from 50 to 200 A. This material aggregates during mitosis into the chromosomes and is

FIGURE 8

Regions at the outer periphery of two cells sampled 12 hours after infection with Mengovirus. The interdigitating microvilli indicate the outer membrane of each cell. In addition to the crystals present in the cytoplasm of the cell on the left, note the masses of small dense granules in the cytoplasm of both cells. × 47,000 (M).

FIGURE 9

Portion of a cell sampled 12 hours after infection with Mengovirus. The cytoplasmic mass or bleb protruding beyond the remainder of the cell contains masses of small, dense granules. × 22,000 (M).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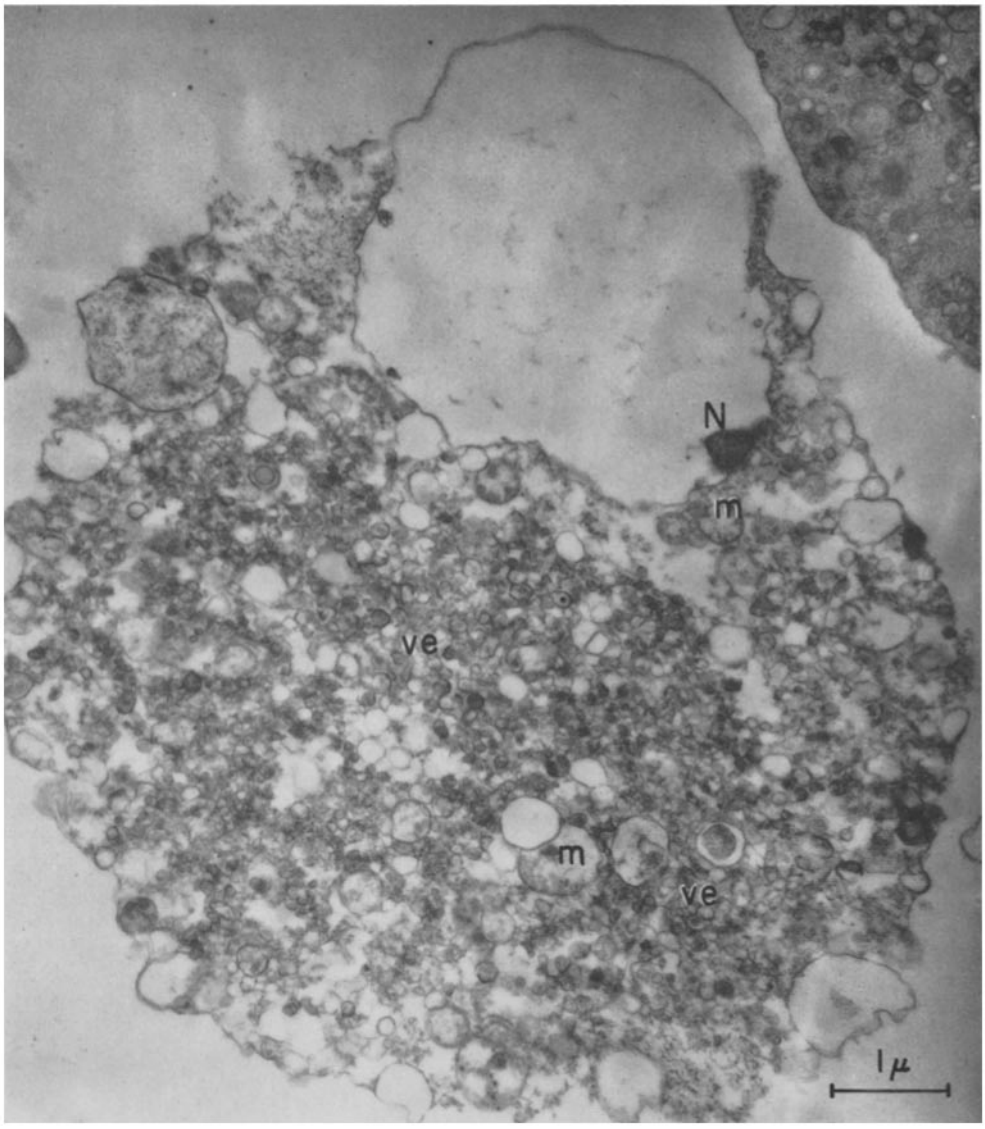


FIGURE 10

A cell from a sample taken 10 hours after infection with EMC. Only a small clump of chromatin and a large vesicle remain at the site probably occupied formerly by the nucleus. Some of the small vesicles which occupy the cytoplasm are filled with dense material. × 15,000 (M).

Figures 11 to 16 show cytoplasmic aggregates and crystals in Mengovirus-infected cells.

probably the chromatin of the nucleus (6). Also found within the nucleus are dense, tightly packed aggregates of granules and short filaments which make up the material of the nucleoli. The nuclear envelopes consist of flat cisternae of the endoplasmic reticulum.

In the cytoplasm, in addition to the rough and smooth walled elements of the endoplasmic reticulum (Fig. 3 *b*), free ribonucleoprotein particles and mitochondria, there are present virus-like (VL) particles of two types (9): Type A particles, having a center of low density, measure 600 to 800 A in diameter and were present within small cytoplasmic vesicles in most cells; Type C

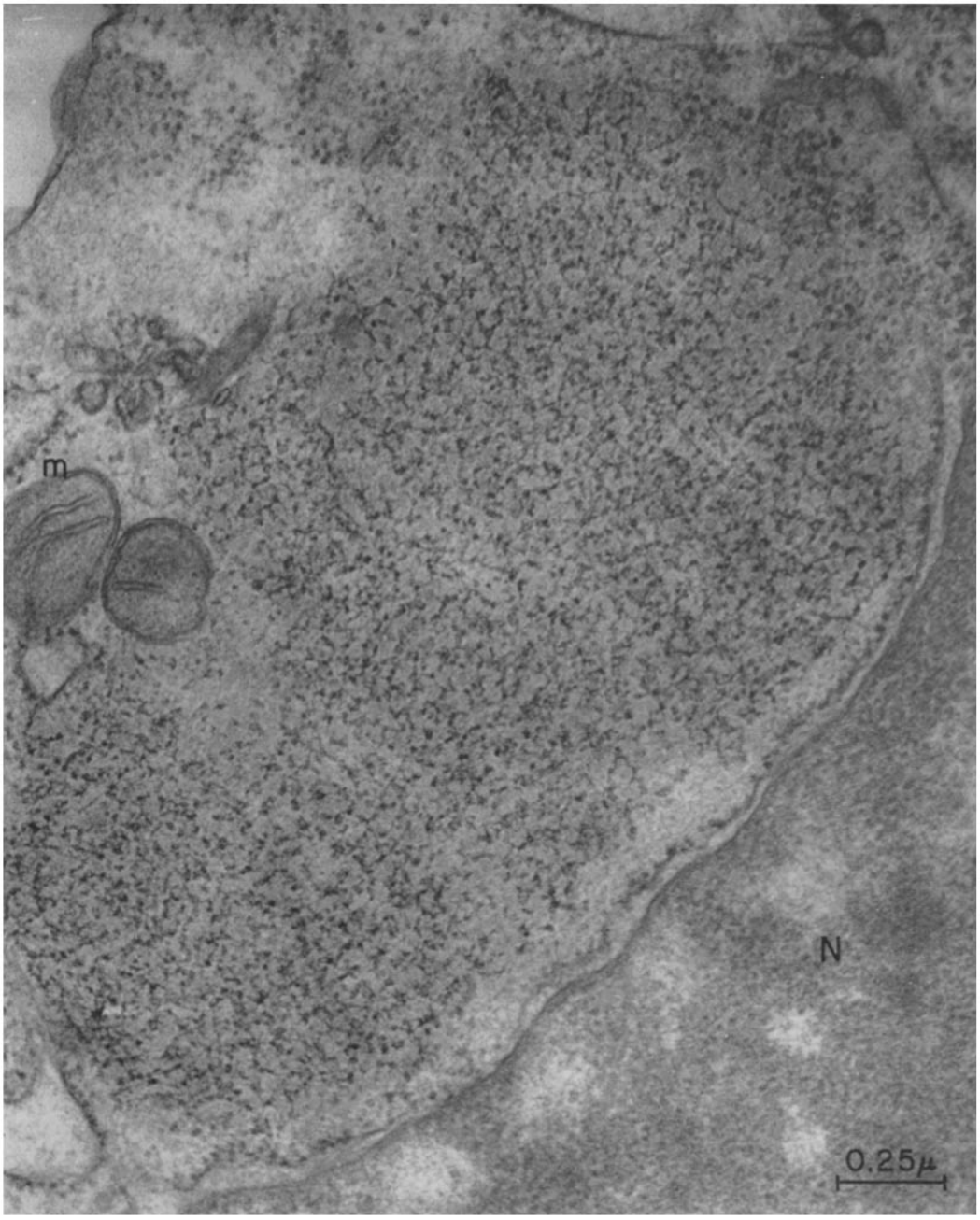


FIGURE 11

Portion of the nucleus and cytoplasm of a cell 10 hours after infection. Note the large inclusion of dense, randomly oriented filamentous material in the cytoplasm. × 60,000 (K).

particles, which possess a dense nucleoid (Fig. 3 *b*) and measure 600 to 1,100 A in diameter, were seen both within cytoplasmic vesicles and on the outer cell membrane in 10 to 20 per cent of the cells.

The Golgi or centrosphere region, not evident in Fig. 3, is of moderate size.

ii) Cells Infected by Mengovirus

Although changes in cell structure resulting from viral infection appear to be closely synchronized when viewed by light microscopy, alteration in the fine structure of cells shows a greater variability at any given time. When a particular alteration in cell structure was being related to a given time-point during the infectious cycle, such a change was first observed in a large fraction of cells in a population.

Since, in many respects, the fine structure of L cells was changed in a similar manner by infection with either EMC or Mengovirus, in most cases an example is used from only one of the systems to illustrate a particular feature.

1. THE NUCLEOLUS: Nucleoli, readily distinguishable from the surrounding chromatin by their density, were present in most cells up to 14 hours after infection and could even be distinguished in some cells 18 hours after. However, commencing 4 to 6 hours after infection these organelles became progressively more dense and generally smaller, so that when 10 to 12 hours had elapsed from the time of infection it became difficult to distinguish individual threads and granules within the mass of the nucleolus.

2. THE NUCLEUS: One of the characteristic features of the infection, a progressive nuclear degeneration, first became evident 4 hours after infection, when small patches devoid of chromatin material appeared at various points in the nucleoplasm, but most frequently at the periphery of the nucleoli. These patches became progressively larger (Figs. 4, 6) until by 16 to 18 hours after infection practically all of the dense material had disappeared, except for small marginal clumps. In many cells sampled 18 to 20 hours after infection only a large vacuole remained present, most probably at the site previously occupied by the nucleus (Fig. 10). The shape of the nucleus also underwent marked changes. There was, first of all, an indentation (Fig. 4), which was followed later by a very pronounced flattening and displacement of the nucleus to one side of the cell (Fig. 6). Extreme squashing was evident late in the infection, just prior to cell disintegration.

3. CYTOPLASMIC ORGANELLES: With respect to both their size and morphology, the *mitochondria* were unchanged in most cells up to 12 hours after infection (Figs. 7, 11, 12, 16). Moderate swelling was observed in other cells (Fig. 6). Late in the infectious cycle, pronounced mitochondrial enlargement was evident in most cases, at a time when the integrity of the outer cell membrane had been disrupted (Fig. 10). Nevertheless, even at this stage of virtually complete cellular disintegration, the double outer membranes and cristae remained clearly recognizable.

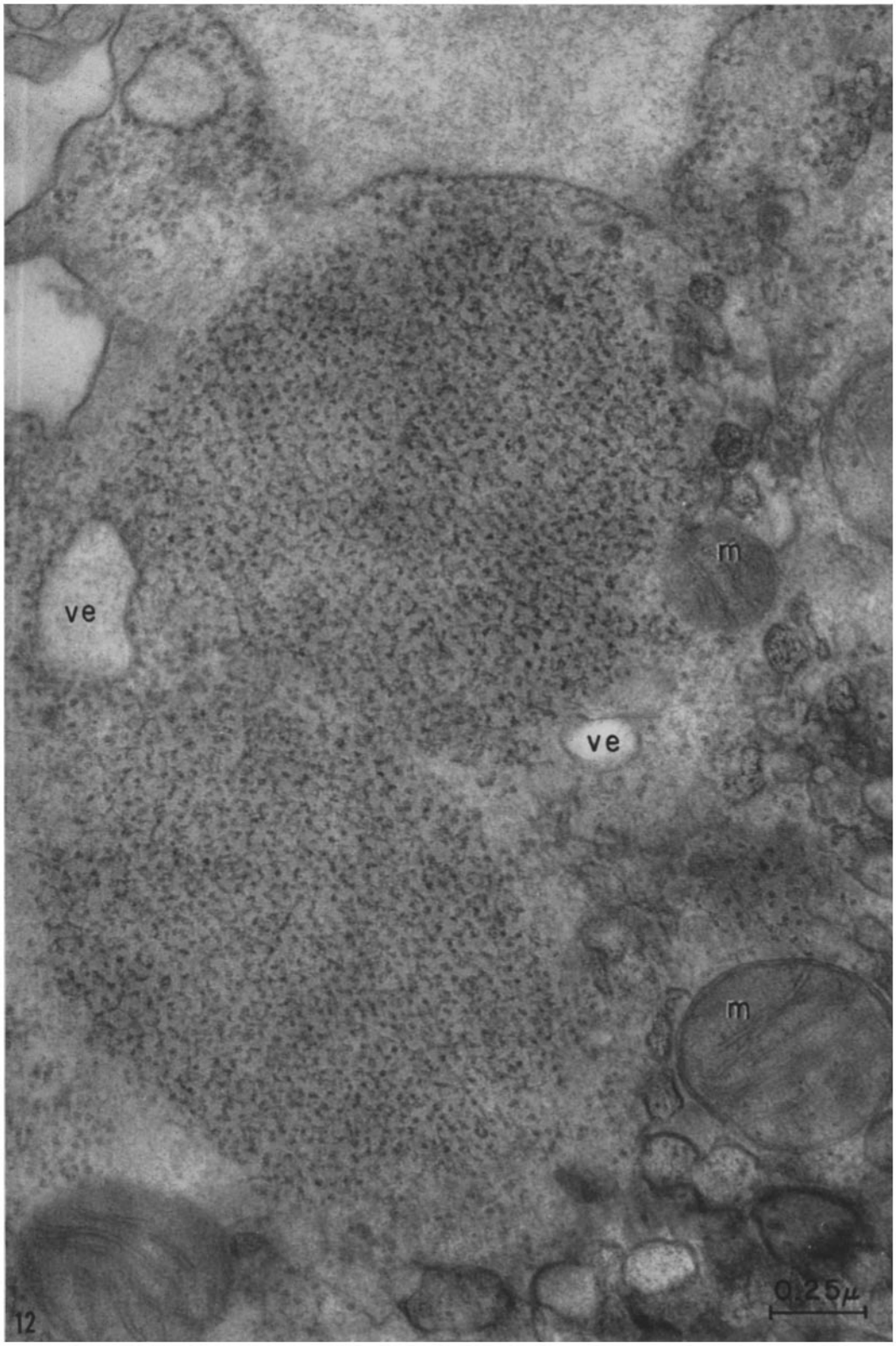
Another distinctive response to infection by either virus was shown by the *endoplasmic reticulum*, *i.e.*, there was a formation, in the centrosphere region of the cytoplasm, of an increased number of small, smooth walled vesicles, each 500 to 2,000 A in diameter. This proliferation of vesicles became evident 4 hours after infection, at the time when virus multiplication was rapid (see Fig. 2). The number of vesicles increased and they came to occupy ever more space in the cytoplasm (Figs. 6, 7), until by 18 to 20 hours practically the entire cytoplasm was a mass of vesicles (Fig. 10), many of which had become filled with a dense material. The larger, terminal vesicles at the cell periphery measured approximately 0.5 to 1.0 μ across.

The flat sacs of the nuclear envelopes became dilated in some cells 6 to 8 hours after infection, and in the majority of cells by 12 hours (Fig. 16). Within these swollen sacs and projecting into them from the limiting membrane were numerous type A(9)VL particles (Fig. 5). Increased numbers of such VL particles were also observed within vesicles throughout the cytoplasm, at 6 to 8 hours after infection.

A similarity in size and density between ribonucleoprotein granules and any scattered virus which may have been present in the infected cells made it difficult to distinguish between the two types of *granules*, when observed in thinly sectioned

FIGURE 12

In this example, also sampled at 10 hours, a paracrystalline organization of dense material within the inclusion is discernible. × 60,000 (K).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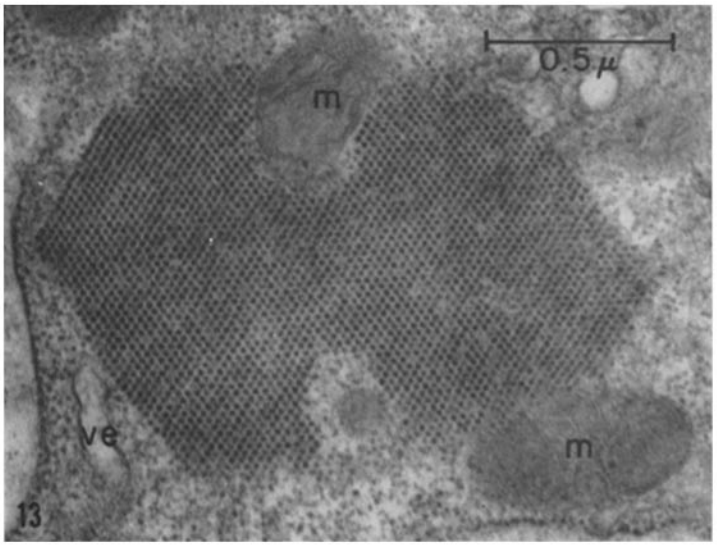
cells, and no such distinction will be attempted here.

In cells sampled 4 to 6 hours after infection there was an increased amount of the *granular component*, especially in the cytoplasm peripheral to the nucleus (Fig. 4). Later, at 8 to 10 hours after infection, small granules were found scattered throughout the cytoplasm of the majority of cells in much larger numbers than in uninfected cells (Fig. 6). When advanced degeneration had occurred, at subsequent times, cell-associated granules became very few in number (Fig. 10). Examples of masses of these small granules are shown in Figs. 6, 8, and 16. Blebs or large processes, projecting beyond the main body of a small proportion of cells sampled 12 to 16 hours following infection, also contained masses of small granules, as shown in Fig. 9.

About 8 hours after infection with Mengovirus, large aggregates of dense material were found in the cytoplasm of L cells. Such *cytoplasmic inclusions* were, however, absent from cells infected with EMC virus.

The aggregates of dense material, such as those shown in the examples, Figs. 11 and 12, consisted of masses of granules and "kinky" filaments which frequently appeared either to be branched or to cross over one another within the thickness of the section. The filaments in some aggregates were distributed haphazardly (Fig. 11), sometimes surrounding the larger cytoplasmic organelles. In other aggregates, some of which were examined in serial sections, the dense threads were in a *paracrystalline* arrangement within regularly spaced parallel rows, although many points of disturbances in the lattice were evident, with filaments crossing from one plane to another (Fig. 12). The threads and granules were measured on prints enlarged three times from the original negatives to a final magnification of 130,000 diameters. In



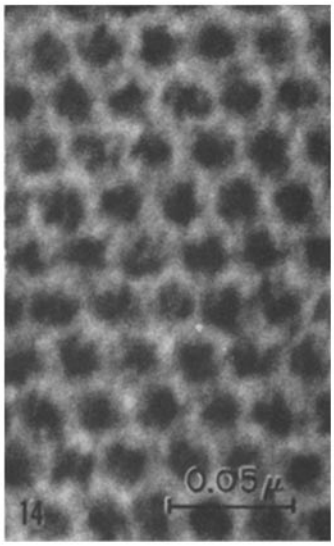


FIGURE 13

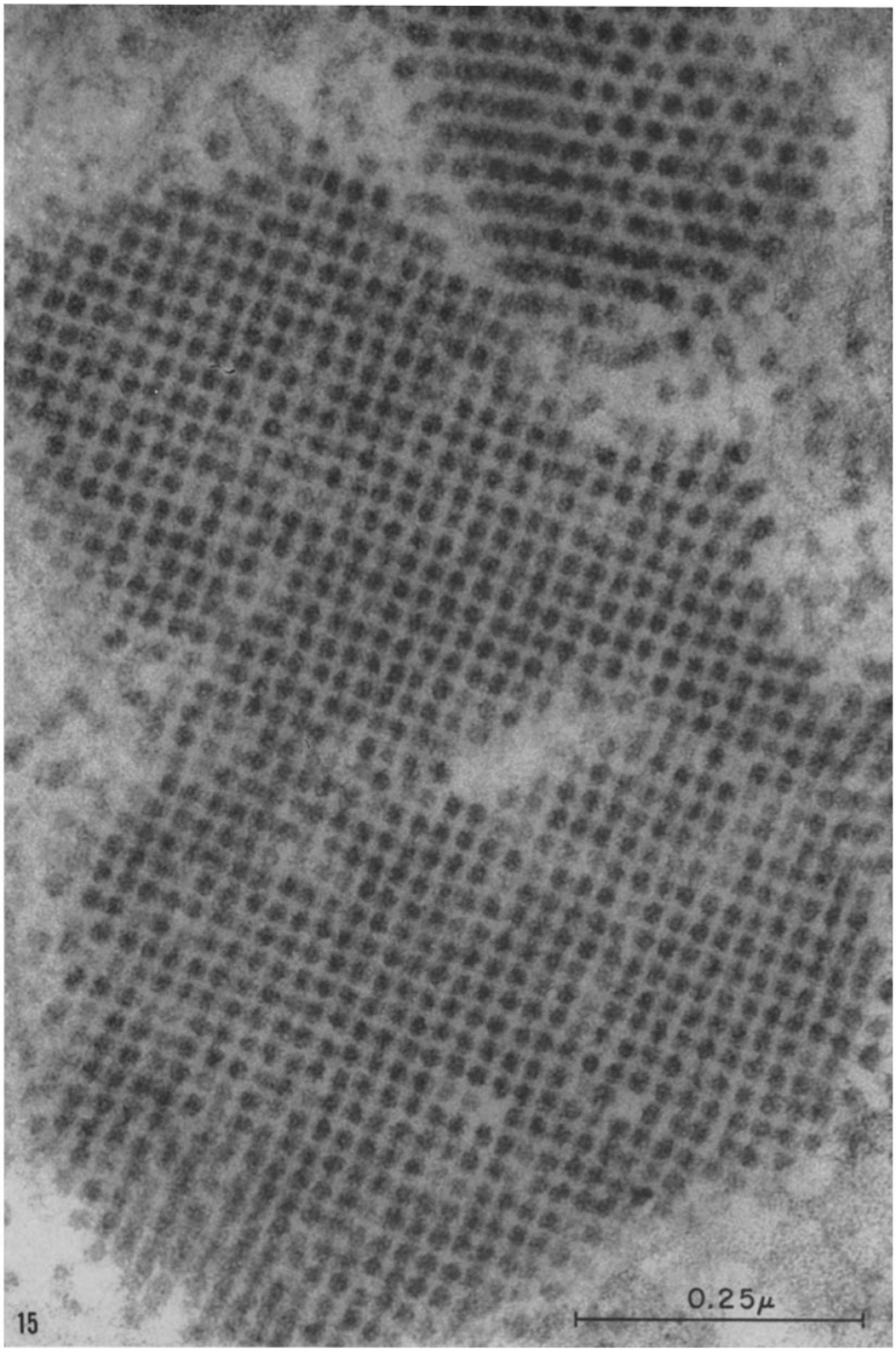
Portion of the cytoplasm of a cell 12 hours after infection. Particles, most probably virus, are tightly packed within the crystal. × 48,000 (M).

FIGURE 14

Example from another crystal, shown at a higher magnification. The hexagonal packing is quite clear. × 300,000 (M).

FIGURE 15

A large crystal in which cubic packing is evident. × 180,000 (K).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material stained according to Karnovsky's formula B (23), the width of the "kinky" filaments ranged from less than 50 to about 80 A and the diameter of the granules from 70 to 100 A. After "staining" by Millonig's procedure (24) these elements appeared to be smaller.

From 12 hours after infection until about 16 hours, approximately half the aggregates were in the form of dense spherical particles (Fig. 13). The average center-to-center spacing, measured on well formed crystals from twenty different cells, was about 250 A, which is some 10 per cent less than the size of virus particles from suspension preparations and may represent shrinkage resulting from preparative procedures. In two well formed crystals out of nearly 60 examined, the center-to-center spacing was much narrower, ranging from 160 to 200 A. Both square nets (Figs. 15, 16) and hexagonal packing (Figs. 13, 14) were encountered.

Occasionally crystals were found in which a different packing arrangement was apparent. In these the space between the rows of particles was nearly twice that of the particle diameter. When examined in serial sections such crystals were found to be composed of a series of plates of particles, since the rows were in close register from section to section, as shown by a series of four consecutive sections which had been superimposed on one another photographically (Fig. 17, *a* to *d*, and composite *e*). It is unlikely that such an unusual spacing had resulted from a sectioning artifact if it is assumed that at least two layers of particles were embedded in each section, the thickness of which is more than 500 A.

As was the case with the dense aggregates found in the earlier samples, the crystals had frequently formed around and between mitochondria and vesicles (Figs. 13, 16). In some cells, sampled at 12 to 14 hours postinfection, extensive cytoplasmic inclusions were found, in which some of the material was in the form of crystalline masses of particles, whereas the remainder consisted of filamentous aggregates, as described above.

4. CHARACTERIZATION OF THE CRYSTALS: L cells were grown on glass coverslips and then exposed to Mengovirus. After 12 hours, cells were fixed and stained with acridine orange or fluorescent antibody. The results are shown in Figs. 18 to 21. Of 1,000 cells examined after staining with fluorescent antibody, 20 per cent contained brightly fluorescing inclusions. Among 1,000 cells treated with RNAse and subsequently stained with acridine orange, 39 per cent showed the presence of either one or several brightly fluorescing orange-red foci. The percentages of cells containing fluorescing centers are certainly higher than the percentage of cells in which crystals were found by electron microscopy, as was to be expected from a comparison between whole mounts and thin sections. Also, many of the fluorescing inclusions are probably cytoplasmic blebs in which virus is not in a crystalline aggregate.

Infected cells were also exposed to tritiated uridine for the entire 12-hour period of the infection. Cells were fixed, dehydrated, and embedded in epoxy-resin prior to being sectioned and subjected to autoradiography. The label in some cells had been incorporated into well defined cytoplasmic inclusions (Figs. 22, 23). These highly labeled aggregates most probably correspond to the crystals found by electron microscopy.

iii) Cells Infected with EMC Virus

With two obvious exceptions, cytopathic changes resulting from infection with EMC virus were identical with those found in L cells infected with Mengovirus and occurred at correspondingly similar times during the infectious cycle (Fig. 2 *B*, *D*). The two differences were an absence of blebs or cytoplasmic projections and an absence of cytoplasmic aggregates of dense material, including crystalline arrays, from cells supporting EMC replication.

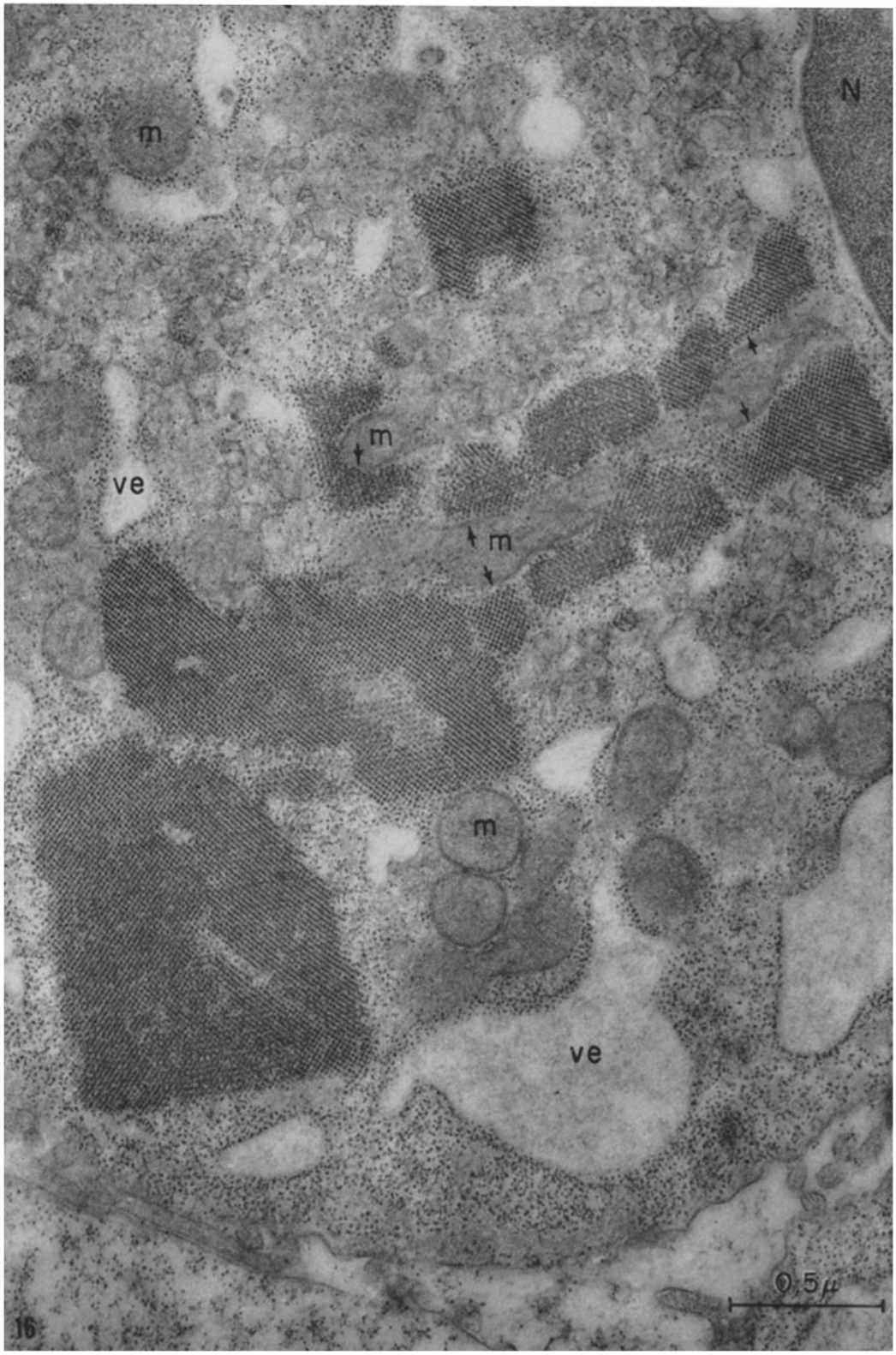
DISCUSSION

A. *Changes in Fine Structure*

In general, the cytopathic effects resulting from infection by Mengovirus and EMC were similar. Such changes in cell fine structure have, in fact, already been reported in a variety of animal cells infected by various small RNA viruses, including

FIGURE 16

Portions of two cells sampled 12 hours after infection. Much of the cytoplasm of the upper cell is occupied by crystalline material. Some of it surrounds the mitochondria at points indicated by arrows. In these crystals the center-to-center spacing ranges from 160 to 200 A. × 45,000 (M).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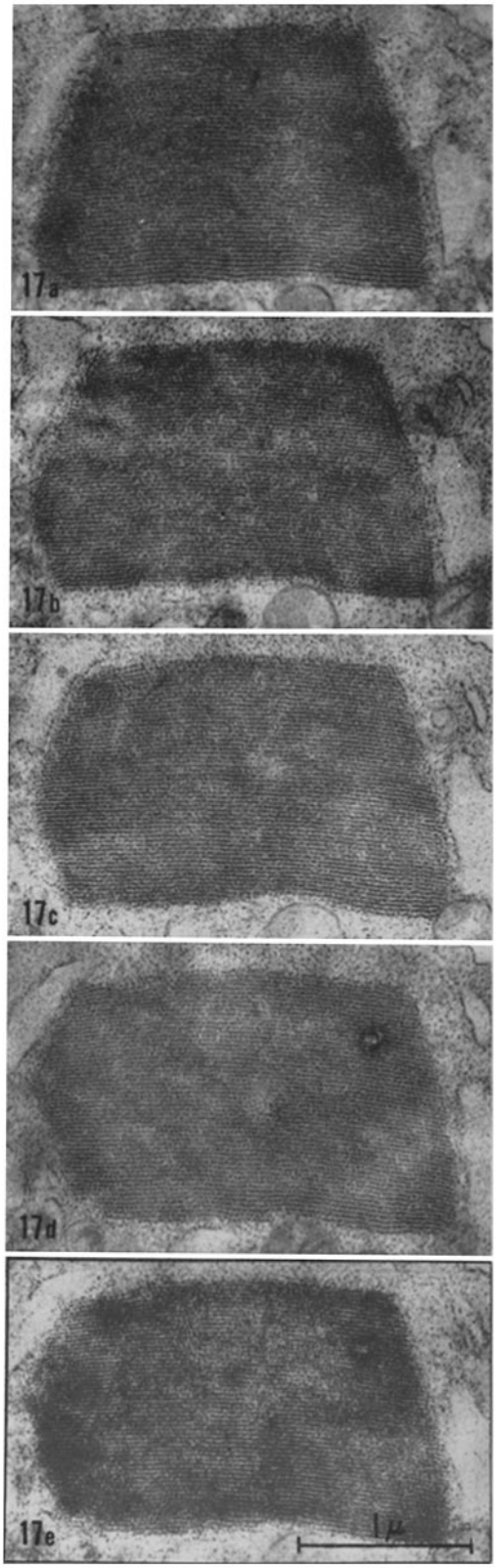

poliovirus (22) and ECHO 9 (34). One of the changes commonly found is a progressive clumping of the chromatin material at the nuclear periphery and a decrease of this material at the center. This change was first detected in the present study at 4 to 6 hours after infection, at the time when virus multiplication was at its fastest. The significance of this observation is not understood at present since there was no visual evidence of viral reproduction within the nucleus. Biochemical data suggest, on the contrary, that these viruses are not dependent upon synthesis of host cell deoxyribonucleic or ribonucleic acid, which are of nuclear origin, for Actinomycin D and Mitomycin C, inhibitors of all nucleic acid synthesis in the host cell, fail to affect production of Mengovirus (32, 33). Perhaps also related to the changes in nuclear morphology is the observation that the DNA-dependent RNA polymerase of L cells infected with Mengovirus is markedly inhibited (1), as is the incorporation of RNA precursors into the nucleus (16, 26).

Another feature commonly found during infection by the small RNA viruses, and observed here, is a squashing or flattening of the nucleus and its displacement towards one side of the cell. No doubt this occurs when large numbers of small vesicles are formed, first in the centrosphere region and later throughout the entire cytoplasm. Thus the degree to which the nuclear shape and position are altered is directly related to the quantity of the cytoplasmic vesicular component.

Apart from elaborating small vesicles, cells respond to viral infection by producing a variety of membranous structures. Application of chemicals and other injurious agents similarly elicits such a response, as already discussed in a previous report (10). In the case of infection with poliovirus, vesicle formation in the cytoplasm might be equated with increased synthesis of phospholipids (5). Increased formation of VL particles in the cytoplasm of infected L cells might also occur as a

FIGURE 17

a to *d*. Consecutive sections through a crystal. Note that the spacing between the dense rows is greater than the diameter of the individual particles.

e. A composite of the above serial sections shows that the rows are in register through all four sections. × 24,000 (M).

result of some such metabolic stimulus, for these particles are elaborated at membrane surfaces (9).

Until the very last stages of the infectious cycle, when cells become freely permeable to Erythrocin, mitochondrial structure is not affected appreciably. These organelles are frequently encompassed by Mengovirus (?) material, as observed in cells sampled 8 hours after infection and later. There is, however, no evidence that mitochondria are here involved specifically in virus reproduction for, in fact, the multiplication of EMC is the same under aerobic or anaerobic conditions (7).

As was anticipated from previous work using light microscopy (15), large accumulations of RNAse-resistant viral material were again recognized in blebs protruding from cells infected with Mengovirus. It was, however, not possible to distinguish the virus from ribonucleoprotein particles (RNP) in the electron micrographs so that it is doubtful which of the granules observed in large numbers at the cell periphery and within protrusions or cytoplasmic blebs are virus and which are RNP.

Although demonstration of the viral nature of cytoplasmic inclusions was more satisfactory when observed 12 hours after infection with Mengovirus, information is lacking about the chemical composition of the filamentous and granular material present in aggregates at earlier times (8 to 10 hours postinfection). Presence of dense, non-crystalline inclusions, termed "template sites," has also been observed by Rifkind *et al.* in ECHO 9 virus-infected cells (34). The temporal sequence of template formation and the first appearance of crystalline masses of particles in cells infected with Mengovirus suggest that the dense spherical particles could be formed from pools of dense material which concentrates in the cytoplasm. A paracrystalline organization which is evident within some of the aggregates at 8 to 10 hours after infection might indicate that at some stage of their development the spherical particles, which are later found to be packed within crystals, have already acquired their spherical shape at 8 to 10 hours but are not yet susceptible to the preservation procedures employed and, therefore, unfold into the "kinky" filaments and granular elements. Or, as seems more likely, this dense component represents a morphological stage in the progression towards larger, spherical particles. If the latter explanation is the correct one and the crystals are aggregates of virus, then virus condensation may proceed within a crystalline lattice, a process different from aggregation of preformed mature virus into crystals, as has been suggested by some workers.

Both the cubic face-centered and the close hexagonal types of packing, which were observed in Mengovirus crystals in the present study, have also been found within intracytoplasmic crystals after infection by some of the other small RNA viruses, including poliovirus (40), Coxsackie (29), and ECHO 19 (30).

In the majority of crystals the size of the particles, as measured by the center-to-center spacing, is compatible with their being Mengovirus. In some exceptional cases, however, where the spacing was smaller, the identity of the dense particles remains obscure.

The formation of large numbers of particles approximating the size of Mengovirus between 10 and 12 hours after infection did not cause the infectious titer to rise although such an increase might have been anticipated. A maximum estimate, based on the percentage of cells containing crystals, each of which is assumed to contain 2×10^5 to 1×10^6 particles, indicates that the number of virus particles in the lysate should double in the period between 10 and 12 hours after infection. Absence of an increase in the infectivity of the lysates between 8 and 12 hours could be the result of a combination of factors such as balance of infectious virus formation by inactivation due to some unknown variable.

B. *Comparison of EMC and Mengovirus Infections*

The one important morphological difference resulting from the infection by these two viruses was the formation of large virus aggregates in L cells infected with Mengovirus, whereas none were found with EMC. Part of the explanation for this difference might be sought in the differences in rates at which L cells lose control of their permeability to metabolites. During the multiplication of EMC in Krebs 2 ascites cells (36) and of a related Columbia SK virus in L cells (19), viral RNA is formed first and soon thereafter becomes incorporated into infectious particles. From the present studies it is clear that changes in cell permeability commence much earlier after EMC infection than after Mengovirus infection, so that cells which have harbored EMC lyse some 6 to 8 hours ahead of those in which Mengovirus repli-

cates. Such a delay in the latter case may make it possible for cells to continue the process of coating virus RNA with protein within the large pools or templates resulting in crystal formation. We have no evidence that either Mengovirus or EMC is formed within membrane-bounded inclusions, as has been postulated for poliovirus (21).

It is possible that such differences in rates at which cell permeability is changed may be one of the genetic characteristics which determines the small differences in cytopathology observed on monolayer cultures. It has been shown that release of new EMC virus in suspensions of Krebs 2 cells is correlated in time with a wave of "stainability" which passes through the culture (37). Perhaps the genetic differences distinguishing some of the plaque-type mutants of EMC and Mengovirus which have been isolated (17, 12) are related to processes governing the rates at which cells become permeable and virus is released.

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FIGURE 18

Acridine orange-stained cells infected with Mengovirus for 12 hours. Crystalline concentrations of RNA are evident in several cells. × 440.

FIGURE 19

A preparation similar to the one above except that the cells were treated with ribonuclease prior to staining. A large crystal stands out clearly in one of the cells. Small foci of red fluorescent material within the nucleus in some cells come, most probably, from the overlying cytoplasm and are not nucleolar in origin.

FIGURE 20

Portion of a culture of cells infected with Mengovirus for 12 hours, stained with fluorescein-labeled antibody to the virus by the indirect method. The numerous brightly fluorescing foci at the periphery of cells are most probably cytoplasmic blebs. × 440.

FIGURE 21

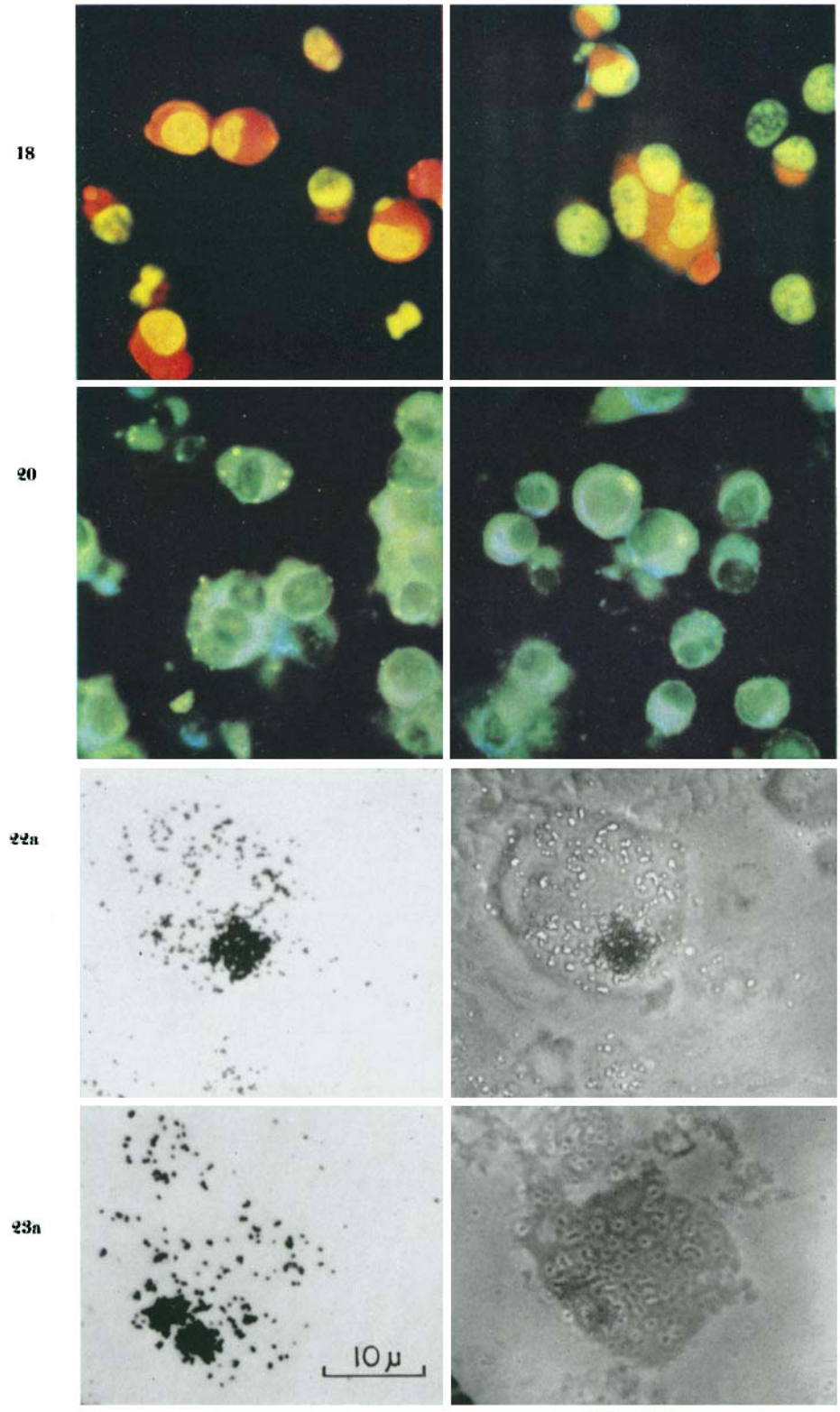
Another example from a preparation such as that shown in Fig. 20. The brightly fluorescing areas in the cytoplasm of two adjacent cells are probably virus crystals. × 440.

FIGURES 22a and 23a

Two examples showing autoradiographs of cells infected with Mengovirus for 12 hours and exposed to H^3-uridine during the entire period of infection. Note the dense concentration of grains, conforming to the shape of a crystal, in Fig. 22 *a*. × 1,400.

FIGURES 22b AND 23b

Phase contrast micrographs of the two cells carrying label demonstrated on the left. × 1,400.


18
19
20
21
22a
22b
23a
23b
10 μ

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