CYTOMEGALOVIRUS INFECTION

VIRAL ULTRASTRUCTURE WITH PARTICULAR REFERENCE TO THE RELATIONSHIP OF LYSOSOMES TO CYTOPLASMIC INCLUSIONS

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Morphologically the human cytomegalovirus (HCMV) and the mouse cytomegalovirus (MCMV) are virtually indistinguishable. In both infections viral particles are produced in characteristic nuclear inclusions. After release into the cytoplasm these particles frequently become embedded in electron dense material which has many characteristics of lysosomes. This may represent a reaction by the infected cell against virus particles produced in its own nucleus and as such could be pathogenetically important in limiting or terminating infection.

The chief purpose of the present investigation was to compare the electron micro-histochemical features of mouse cytomegalovirus particles in the liver with that observed in the salivary gland and to attempt to correlate these findings with the quantity of virus found in those organs. In addition the development of human cytomegalovirus in cell cultures of human fibroblasts has been compared with that of mouse cytomegalovirus in various tissues.

MATERIAL AND METHODS

Mouse Cytomegalovirus

The preparation of the virus, the animals employed, the methods of inoculation and of virus assay have been described previously.[1,4] Two types of experiment were performed. In the first of these 0.2 ml homogenate containing 10^5 tissue culture doses (TCD_{50}) of virus was inoculated intraperitoneally into weanling Swiss Webster mice. This resulted in a cumulative mortality of 60 per cent by the sixth day. Three mice were killed on each of the fourth, fifth and seventh days. Their livers and spleens were examined by light and electron microscopy and the quantity of virus in these organs determined. In the second type of experiment 0.2 ml of homogenate containing a smaller dose of virus was inoculated. This resulted in a cumulative mor-

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tality of approximately 20 per cent. Two animals were killed on each of the fifth, tenth, 16th, 22nd and 28th days. Their livers and salivary glands were studied by light and electron microscopy and their virus titers determined.

Human Cytomegalovirus

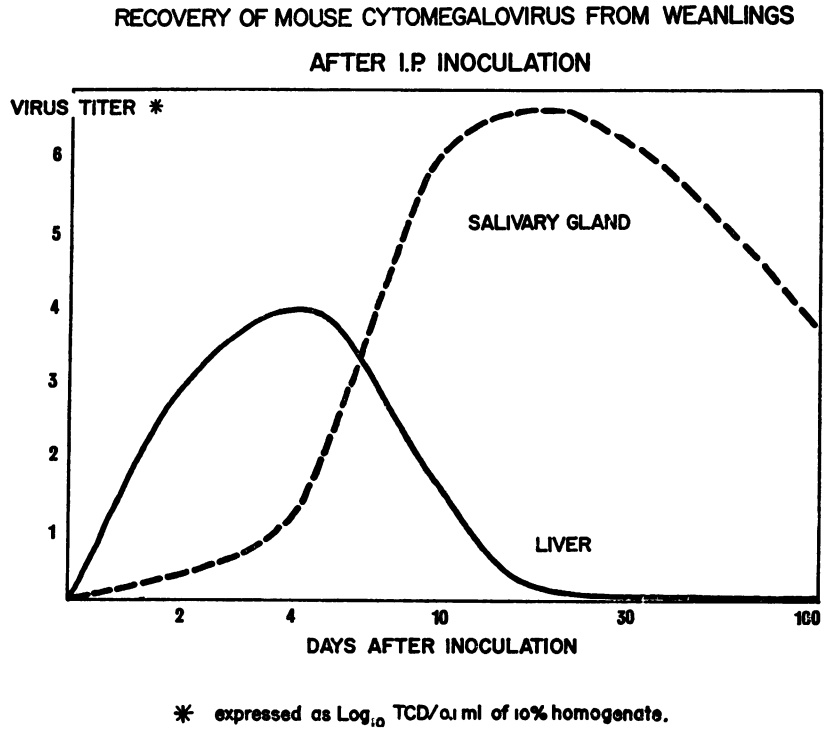
The Ad strain of virus was grown in MAF, WI26 or WI38 strains of diploid human fibroblasts grown in monolayers.

Histologic Techniques

In addition to hematoxylin and eosin staining and the Feulgen reaction paraffin sections of the liver and salivary gland were studied using the periodic acid-Schiff (PAS) reaction and a simultaneous coupling azo dye method for acid phosphatase activity employing naphthol AS-TR[5].

Electron Microscopy

Tissue and tissue cultures were fixed in phosphate buffered 1 per cent osmium tetroxide, embedded in Araldite and cut with a Porter Blum II microtome.[1,2] Thin sections for electron microscopy were stained with uranyl acetate or lead citrate.[6] In addition thin sections of the salivary gland and liver from infected animals as well as human fibroblasts were examined electron microscopically using the periodic acid silver methenamine (PASM) reaction.[7] This method results in the precipitation of finely granular silver salt by many substances which are PAS-positive to light microscopy. The same tissues were also investigated electron micro-histochemically for acid phosphatase activity.[8,9]



RESULTS

Replication of Infective Mouse Cytomegalovirus in Various Tissues

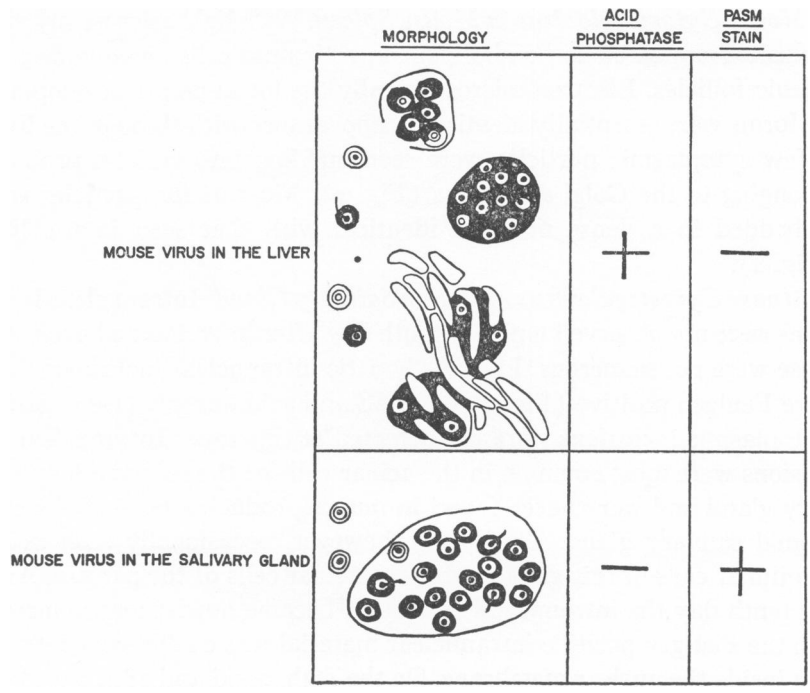
Text-figure 1 is a diagrammatic representation of the mean of many experiments in which the virus titers were measured in the liver and salivary gland at various times after infection. The quantity of infectious virus in the liver reached a maximum on approximately the fifth day after inoculation and then declined steeply. In the salivary gland the virus titer rose more slowly but reached a maximum higher than the liver on approximately the 20th day. Measurable titers of virus could be regularly recovered from the salivary glands for 100 days and less regularly for 1 year.

Virus Development

Text-figure 2 is a diagrammatic representation of the electron microscopic features and histochemical reactions of the mouse cytomegalovirus in the various tissues which were examined.

Mouse Cytomegalovirus in Mouse Liver. Intranuclear type A^{10} inclusions were conspicuous by light microscopy from the third to the sixth

MORPHOLOGY AND HISTOCHEMICAL REACTIONS OF CYTOPLASMIC CYTOMEGALOVIRUS PARTICLES



days. These inclusions were Feulgen positive but PAS and acid phosphatase negative. They were accompanied by considerable hepatic necrosis and inflammation. Cells with intranuclear inclusions frequently also contained cytoplasmic inclusions which were Feulgen and acid phosphatase positive (Fig. 1) but PAS negative. In the later stages of the infection viral inclusions were hardly ever seen and focal necrosis became much less conspicuous.

The intranuclear development of this virus was virtually identical with that of the human cytomegalovirus in fibroblasts.[2] The dense material which surrounded the great majority of cytoplasmic virus particles appeared to be produced close to the Golgi zones, probably by the endoplasmic reticulum. Cytoplasmic viral inclusions seemed to be formed by coalescence of single particles embedded in dense material (Fig. 3). Acid phosphatase activity was localized in this dense material (Fig. 4) but its PASM reaction was negative. In addition lattice-like crystals of irregular shape and measuring up to 1 μ in diameter were occasionally seen in nuclei but more frequently just outside the nuclear membrane which often appeared ruptured in the vicinity (Fig. 5). The crystals exhibited a 75° dot and line pattern with a distance of approximately 650 Å between lines (Fig. 6). Crystals could be demonstrated only after staining with uranyl acetate or uranyl acetate and lead citrate but not with lead citrate alone. They were acid phosphatase negative.

Mouse Cytomegalovirus in Mouse Spleen. With light microscopy viral inclusions appeared to be abundant in reticulum cells surrounding the splenic follicles. Electron microscopically the intranuclear developmental forms were essentially identical in appearance with those in the liver. A few cytoplasmic particles were seen budding into vesicles probably belonging to the Golgi apparatus (Fig. 7). Most of the particles were embedded in a dense material identical with that seen in the liver (Fig. 8).

Mouse Cytomegalovirus in Mouse Salivary Gland. Intranuclear inclusions were not observed until the tenth day after infection and even then these were not numerous. Like the hepatic intranuclear inclusions these were Feulgen positive (Fig. 2) but PAS and acid phosphatase negative. Cytoplasmic inclusions were not detected at this time. Intranuclear inclusions were most common in the acinar cells of the submaxillary salivary gland and were never found in mucus-producing cells of the sublingual salivary gland. They were, however, occasionally seen in the demilunar cells of this gland and in the acinar cells of the parotid. After the tenth day the intranuclear inclusions became much more numerous and the Feulgen positive intranuclear material was chiefly concentrated just inside the nuclear membrane. On the 16th, 22nd and 28th days both

nuclear and cytoplasmic inclusions were conspicuous, particularly in the acinar cells of the submaxillary gland. The cytoplasmic inclusions were PAS and Feulgen positive (Fig. 2) but exhibited no detectable acid phosphatase activity. Occasional necrotic cells were also seen and the entire submaxillary gland contained a diffuse interstitial chronic inflammatory reaction.

By electron microscopy the intranuclear inclusions resembled those of the liver and spleen. Many infected nuclei contained conspicuous round areas composed of fine granules scattered through the zones of virus formation (Fig. 9). The virus particle consisted of a pleomorphic core which stained strongly with uranyl acetate (Fig. 10), surrounded by a single membrane demonstrated more clearly by lead citrate (Fig. 11) or by uranyl acetate followed by lead citrate. The particles were interspersed with strands of fibrils which were much better developed in this tissue than in the other organs studied (Figs. 10 and 11). In the later stages of the infection these fibrils frequently occupied the greater part of the central zones of the nucleus and displaced the virus particles to the inner side of the nuclear membrane. Higher magnification suggested that the fibrils were similar in structure and staining reaction to the membranes surrounding viral cores and in some places the fibrils appeared to fuse with these membranes (Fig. 11). No periodicity could be detected in the fibrils which were approximately 100 Å wide and on cross section appeared somewhat rounded. In the center of the fibrils there was an unstained zone which sometimes also could be seen in longitudinal sections (Fig. 11).

The cytoplasmic inclusions observed by light microscopy when viewed with the electron microscope consisted of groups of virus particles frequently surrounded by a single membrane apparently derived from the Golgi apparatus or endoplasmic reticulum (Fig. 12). Occasionally these vesicles contained only a single particle but usually the number was much higher, up to a hundred or more. The persistence of cytoplasmic particles in these vesicles up to the 28th day was striking and quite unlike the rapid decrease in the number of particles in the liver. Immediately after release from the nucleus cytoplasmic particles consisted of a nucleus surrounded by two envelopes (Fig. 13). In the vast majority of particles, however, dense material obscured the space between the outer and inner envelopes (Fig. 13). Unlike the cytoplasmic particles in the liver and spleen, individual virus particles remained separate from one another and virus containing inclusions composed of dense material like those in the liver and spleen were not observed. Although this dense material was scanty it gave a positive reaction to the PASM stain (Fig. 14) and was therefore almost certainly responsible for the PAS positivity of the

cytoplasmic inclusions encountered with conventional microscopy. The virus containing vesicles in acinar cells frequently lay quite close to the acinar lumens and occasionally their membranes had ruptured. Virus particles were often seen in the lumens of acini and ducts.

Human Cytomegalovirus in Cultured Human Fibroblasts. The features of intranuclear virus particles have been previously described[2] and closely resembled those of the mouse cytomegalovirus. Cytoplasmic particles were almost always embedded in dense material and several particles frequently coalesced to form a cytoplasmic inclusion (Fig. 15). With electron micro-histochemistry the dense material surrounding individual virus particles and forming the matrix of the larger cytoplasmic inclusions was acid phosphatase positive (Fig. 16) and gave a positive PASM reaction (Fig. 17).

Discussion

Intranuclear Virus Development

Intranuclear virus particles generally consisted of spherical or rod shaped cores surrounded by single membranes (Figs. 3, 7 and 9) but occasionally only naked cores were found. This was particularly striking in human fibroblasts infected with the human virus.[2] Particles were generally embedded in a skein of finely granular material resembling chromatin.[2,3,11] This skein was particularly well-developed in the mouse salivary gland where it consisted of short filaments rather than granules. In addition virus infected nuclei, particularly in the salivary gland and spleen, contained striking, roughly spherical, dense areas composed of fine granules (Fig. 9). These dense areas were frequently situated in close proximity to developing virus particles. Similar structures have been considered to be "virus factories" by previous investigators.[12,13] Similar changes have also been observed in the nuclei of cells infected by adenovirus, type 12, and in this system they did not appear to be related directly to the production of virus particles.[14]

In the later stages of the infection, particularly in the mouse salivary gland, striking fibrils developed in the center of the nucleus (Figs. 10 and 11). Their staining affinity for lead citrate and their structure suggested that they were related to the protein coat of the virus. This was supported by the relatively decreased Feulgen staining in the central areas of the nucleus where fibrils were situated predominantly (Fig. 2). This finding, as well as the relative excess of viral cores in the early stages of the infection suggested a certain asynchrony in the manufacture of the two components of the mature cytomegalovirus particles.

Intranuclear and cytoplasmic crystals composed of subunits smaller than mature viral particles were observed in the liver of mice infected

with cytomegalovirus (Figs. 5 and 6). Such crystals have been observed in hepatic parenchymal cells infected by the murine cytomegalovirus.[1] Similar crystals were illustrated by David [15] in a hepatic biopsy specimen from a patient with cholestasis. The relationship of these structures to cytomegalovirus infection therefore must remain problematic.

Cytoplasmic Particles

Only virus particles in the vicinity of the nucleus exhibited two distinct envelopes surrounding the viral cores (Fig. 13). In all the tissues which we examined amorphous dense material soon obliterated the space between the two capsules (Figs. 3, 8, 13 and 15). In the liver, spleen and in fibroblasts a variable amount of this material also surrounded individual particles and frequently formed larger inclusions in which the particles were embedded (Figs. 3, 8 and 15).

Cytoplasmic bodies of this type have, thus far, been illustrated in only a few members of the Herpes virus group such that causing bovine malignant catarrhal fever [16] and in the Lucké renal adenocarcinoma.[17]

The structure of these inclusions suggested that they represented a form of lysosome.[1] The demonstration of increased acid phosphatase activity in infected fibroblasts [2,3] supported this idea. The present investigation has localized the acid phosphatase activity electron micro-histochemically in the dense material surrounding virus particles in both the mouse liver and in human fibroblasts (Figs. 4 and 17). Of the lysosomal features studied in this investigation, acid phosphatase activity is the most characteristic. The absence of demonstrable enzyme activity from particles in the salivary gland correlated with the scantiness of the dense material found in this tissue. A positive PAS reaction has frequently been demonstrated in lysosomes.[18] There was good correlation between this staining reaction and acid phosphatase activity in infected human fibroblasts (Figs. 17 and 18). There was no correlation in infected murine hepatic parenchymal cells which were PAS-negative, however, in spite of the presence of many acid phosphatase positive lysosomes. Correlation was also lacking in the salivary gland which contained strongly PAS-positive inclusions but lacked acid phosphatase activity. The possession of a membrane is another well-recognized feature of lysosomes. This is not invariable, however, particularly in recently developed lysosomes.[19] The absence of a membrane around many cytoplasmic viral inclusions, therefore, does not obviate the need to consider them lysosomes.

The origin of the dense material comprising the lysosomes remains controversial.[19] Our general impression is that the dense material is formed outside the Golgi complex, probably by the endoplasmic reticu-

lum. Very soon, however, it accumulates in smooth membraned vesicles which probably are part of the Golgi apparatus. Unlike MacGavran and Smith [3] we found that lysosomes seemed to increase in number and size before the release of virus particles into the cytoplasm so that the presence of cytomegalovirus in the nucleus appeared to stimulate the formation of lysosomes.

Persistence of Viral Titers in Relation to the Structure of Cytoplasmic Particles

A possible explanation for the shorter period of virus replication in the liver (in contrast to the salivary gland) appears to be related to the fact that the liver is capable of responding to mouse cytomegalovirus infection by producing an interferon. Investigations carried out concurrently with those reported here, however, indicate that this virus does not stimulate interferon production *in vitro* or *in vivo* and is relatively insensitive to interferons.[20] The structure of the cytoplasmic virus particles in the mouse liver had led to the suggestion that the viral nucleoids were destroyed by enzymes in the surrounding dense lysosomal material.[1] In this investigation acid phosphatase activity was demonstrated in the hepatic cytoplasmic viral inclusions but not in the cytoplasmic particles in the salivary gland which were surrounded by little amorphous material. The relative lack of lysosomal activity in the salivary gland, therefore, may be related to the remarkable persistence of virus particles and infectious virus in this organ (Text-fig. 1).

Differential Tissue Susceptibility

The variation in susceptibility of various tissues to cytomegalovirus is fascinating but still remains unexplained. In the mouse liver virus particles were chiefly seen in parenchymal cells. Kupffer cells were frequently damaged but only relatively few contained virus particles.[1] In the mouse spleen reticulum cells were affected and follicular lymphocytes were spared. In the mouse salivary gland our observations have confirmed those of previous investigators [21,22] who found that the characteristic inclusions were localized chiefly in the acinar cells of the submaxillary gland.

Summary

The development of the mouse cytomegalovirus in the salivary gland and liver have been compared by electron microscopy and histochemistry. In the early intranuclear stages of infection more viral cores appeared to be produced than protein coat material, while in the later stages this was reversed.

Cytoplasmic virus particles persisted almost unchanged in the submaxillary salivary gland for at least 28 days. Viral titers showed relatively little decrease up to 100 days. In hepatic parenchymal cells, however, cytoplasmic virus particles soon became surrounded by dense material with lysosomal characteristics. Virus particles were rarely seen after the fifth day and viral titers dropped steeply at about the same time. It is suggested that the persistence of the mouse cytomegalovirus in the salivary gland may be related to the relatively low lysosomal activity in this organ.

The cytoplasmic particles of human cytomegalovirus in human fibroblasts resemble those produced by mouse cytomegalovirus in the liver rather than those in the salivary gland.

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LEGENDS FOR FIGURES

FIG. 1. Mouse liver. Fourth day after infection with mouse cytomegalovirus. A characteristic acid phosphatase positive cytoplasmic inclusion is shown (arrow). In the nucleus there is an indistinct acid phosphatase negative inclusion. × 600.

FIG. 2. Mouse salivary gland, 16th day after infection. Two large cells contain characteristic intranuclear and cytoplasmic inclusions. Intensely staining zones appear just inside nuclear membrane (arrow). Feulgen stain. × 600.

FIG. 3. Mouse liver, fourth day after infection. A typical cytoplasmic inclusion (arrow) appears above the nucleus which also contains a few virus particles. × 22,200.

FIG. 4. Mouse liver, fourth day after infection. The same animal shown in Figure 3. A cytoplasmic inclusion gives a positive acid phosphatase reaction. A few distinct particles surrounded by the reaction product can be discerned (arrow). × 22,200.

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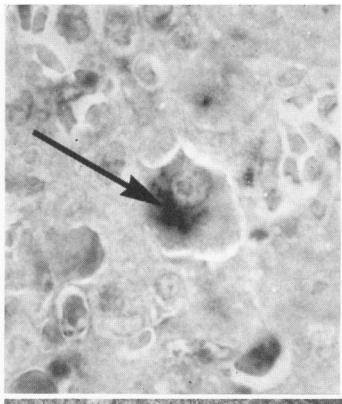

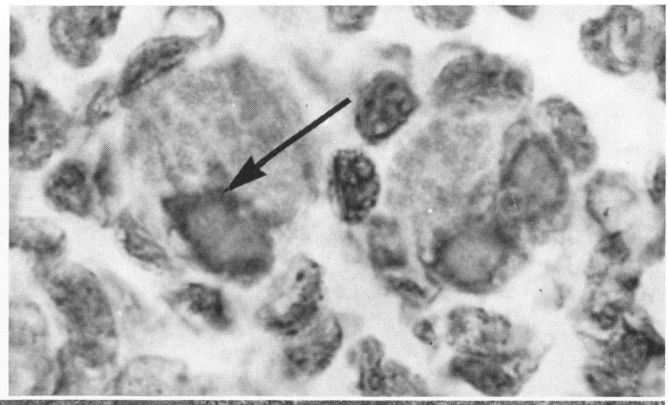

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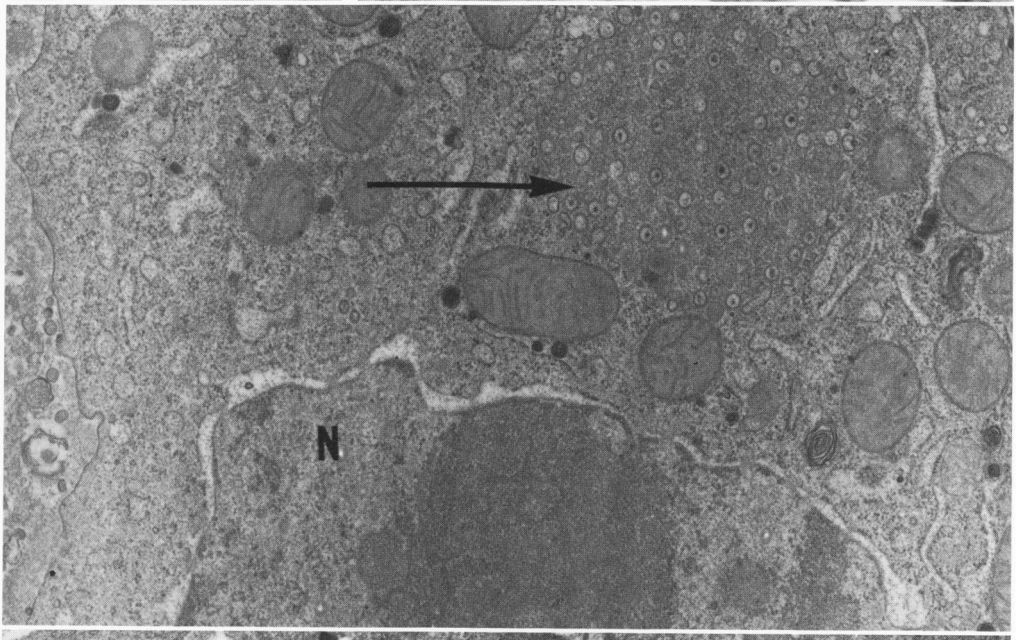

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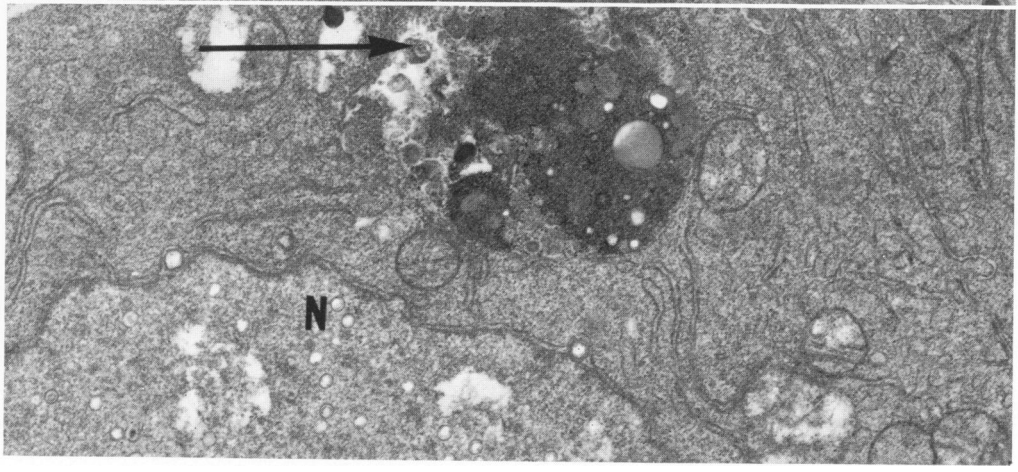

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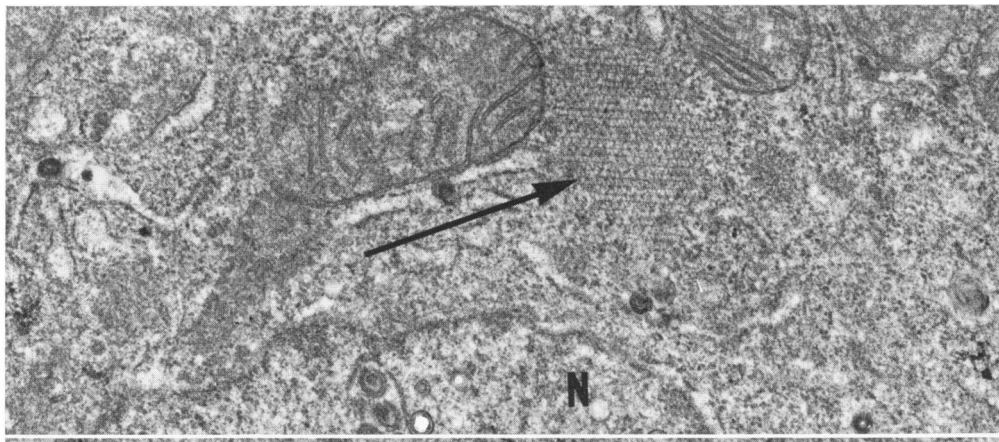

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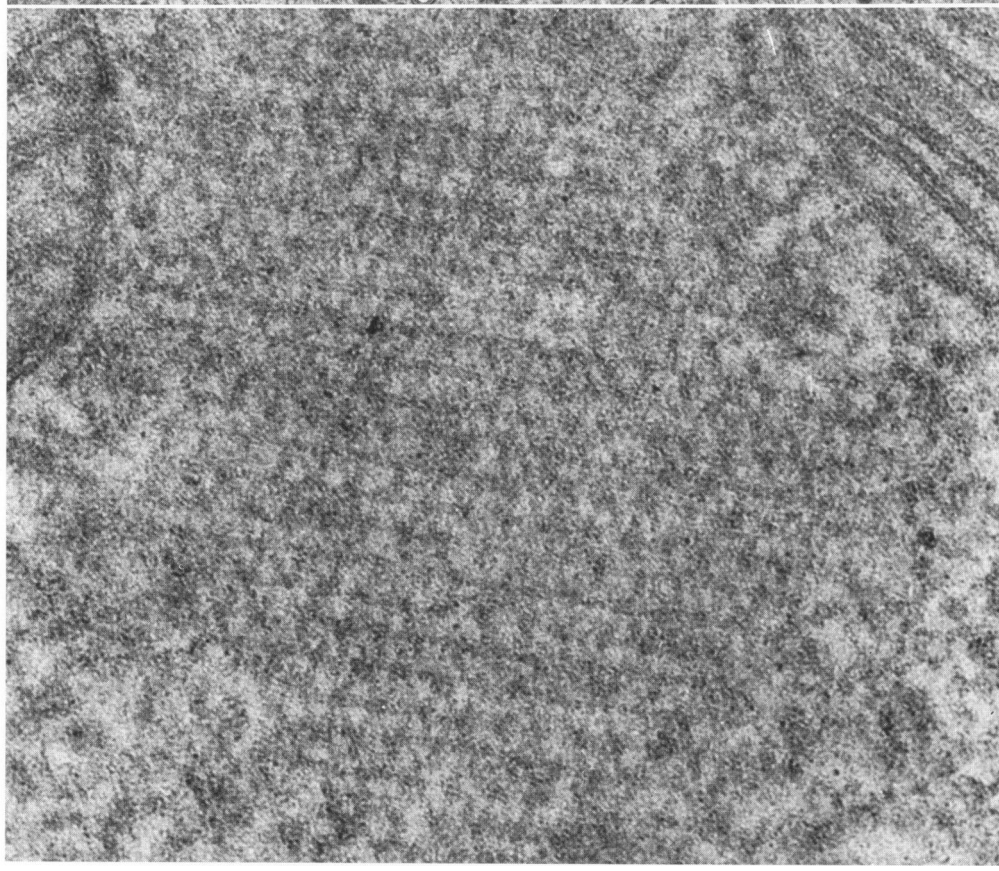
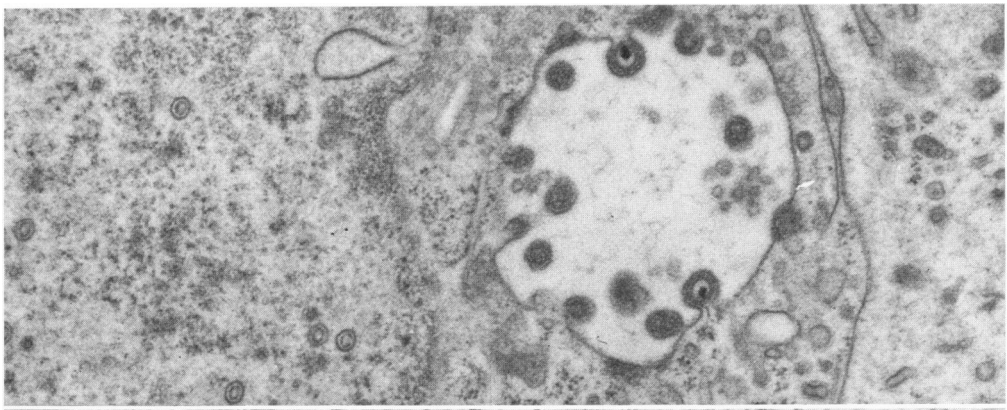


FIG. 5. Mouse liver, fourth day. A crystal (arrow) lies just above the nucleus which contains virus particles. Uranyl acetate stain. × 22,200.

FIG. 6. The same crystal shown in Figure 5. Uranyl acetate stain. × 134,000.



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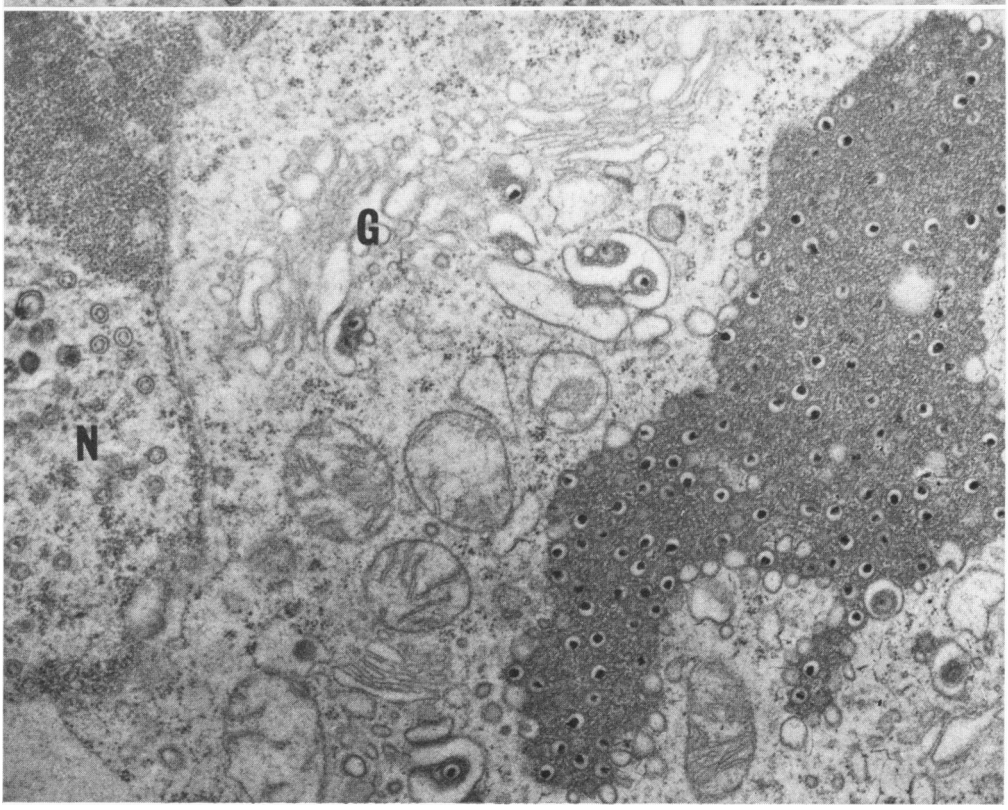

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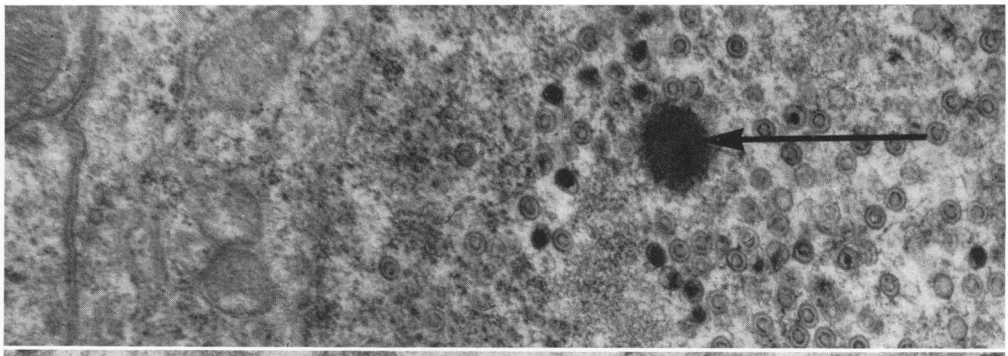
FIG. 7. Spleen, fifth day. Cytoplasmic particles are budding through a membrane (? Golgi). Uranyl acetate stain. × 21,000.

FIG. 8. The same specimen as that shown in Figure 7. Particles are seen in the nucleus (N), are budding into Golgi vesicles (G) and in a cytoplasmic inclusion. Uranyl acetate stain. × 26,000.

FIG. 9. Salivary gland, 16th day after infection. Intranuclear virus particles surround a dense spherical area composed of fine granules (arrow). Uranyl acetate stain. × 33,600.

FIG. 10. Salivary gland, same specimen as shown in Figure 9. Intranuclear virus particles exhibit strongly staining cores. Hollow appearing fibrils are conspicuous. Uranyl acetate stain. × 100,500.

FIG. 11. The same specimen as shown in Figure 10 after staining with uranyl acetate and lead citrate. The membranes of the virus particles and the fibrils are both more conspicuous. In cross section the fibrils appear hollow. × 100,500.



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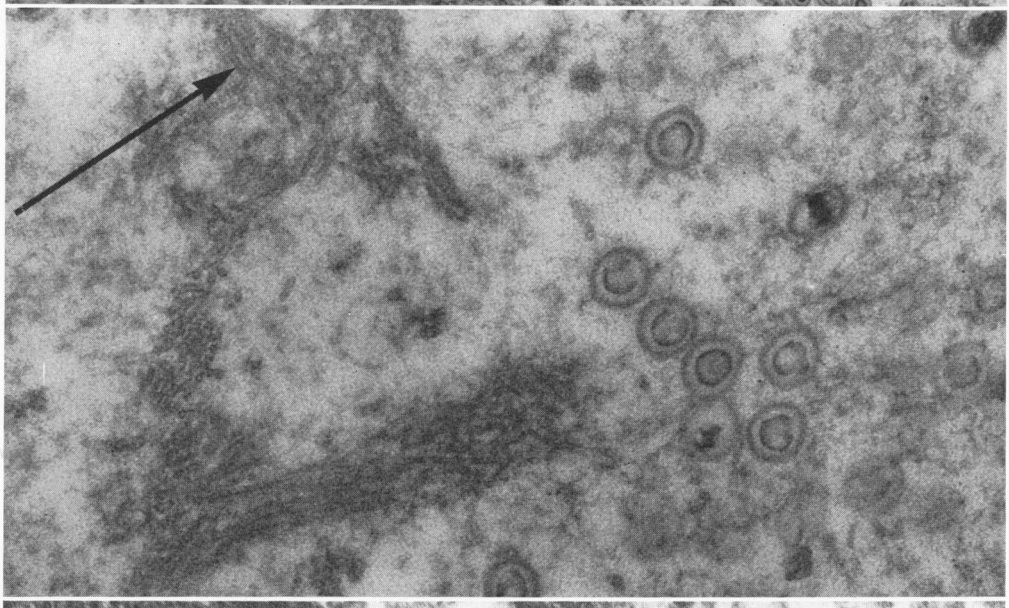

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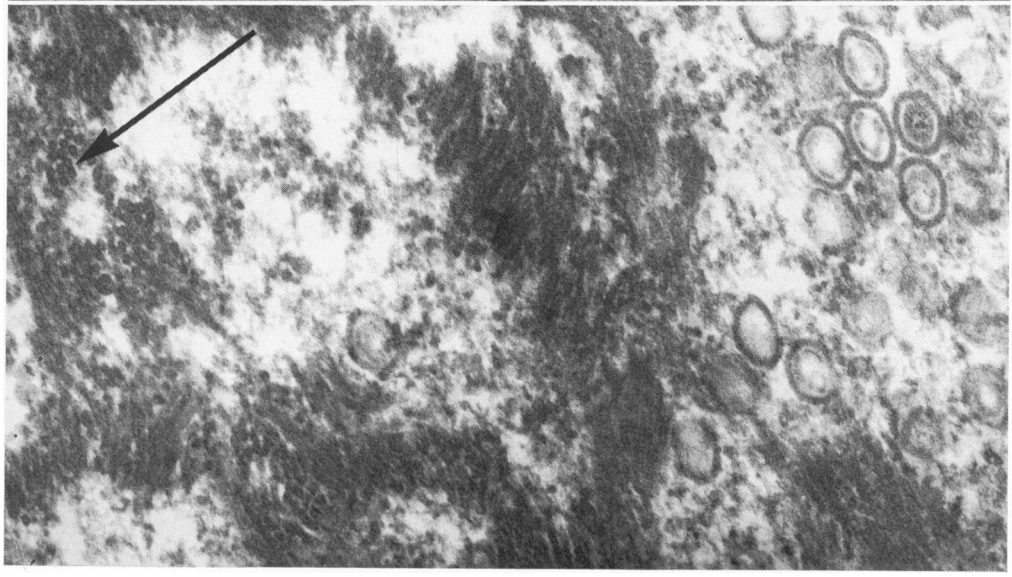

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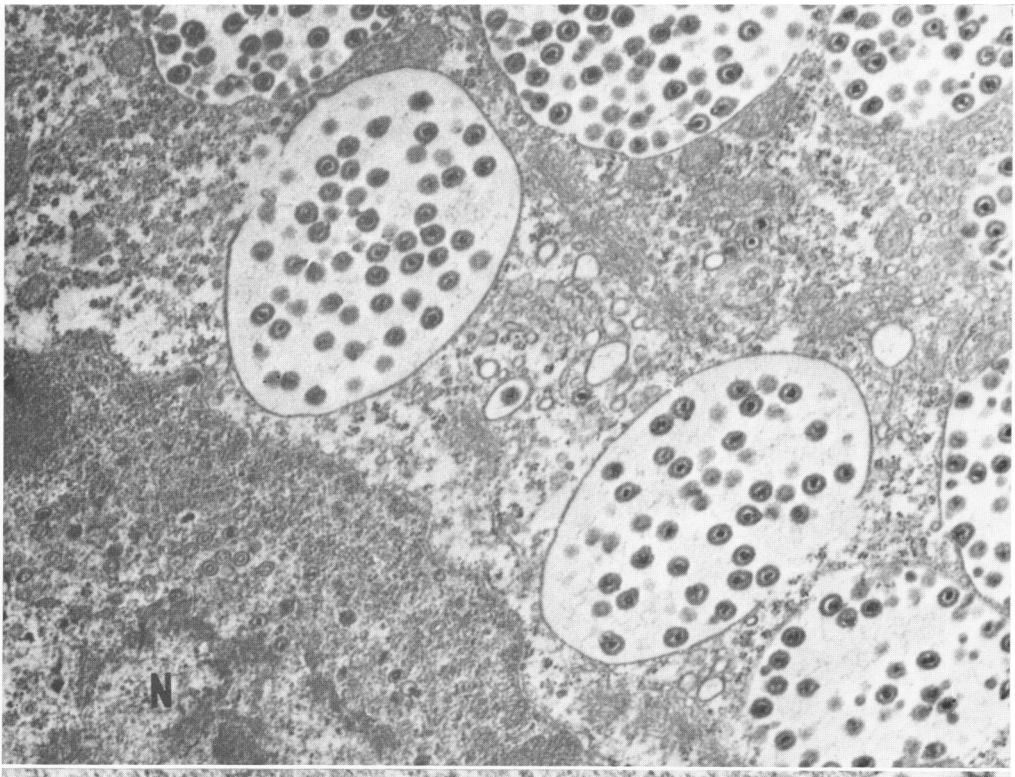
FIG. 12. Salivary gland, 16th day after infection. Particles appear in the nucleus and in cytoplasmic vesicles. The nucleus also contains some fibrils. Uranyl acetate stain. × 16,300.

FIG. 13. The same specimen as shown in Figure 12. Two particles, just emerged from the nucleus, have distinct double membranes. This cannot be distinguished in more mature particles in vesicles. Uranyl acetate stain. × 37,700.

FIG. 14. Same specimen as shown in Figures 12 and 13. The PASM reaction in the membranes of the cytoplasmic virus particles is strongly positive. × 32,000.



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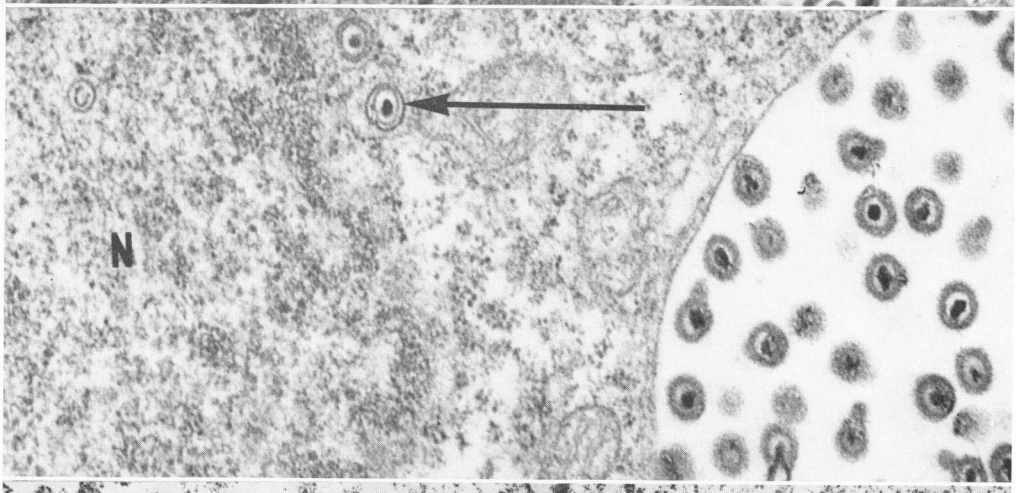

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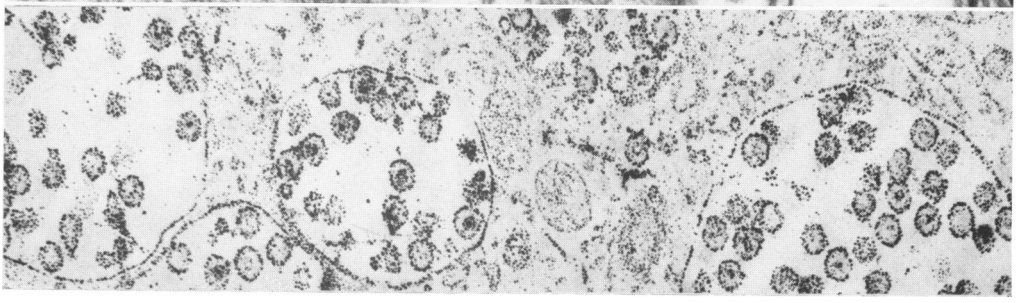

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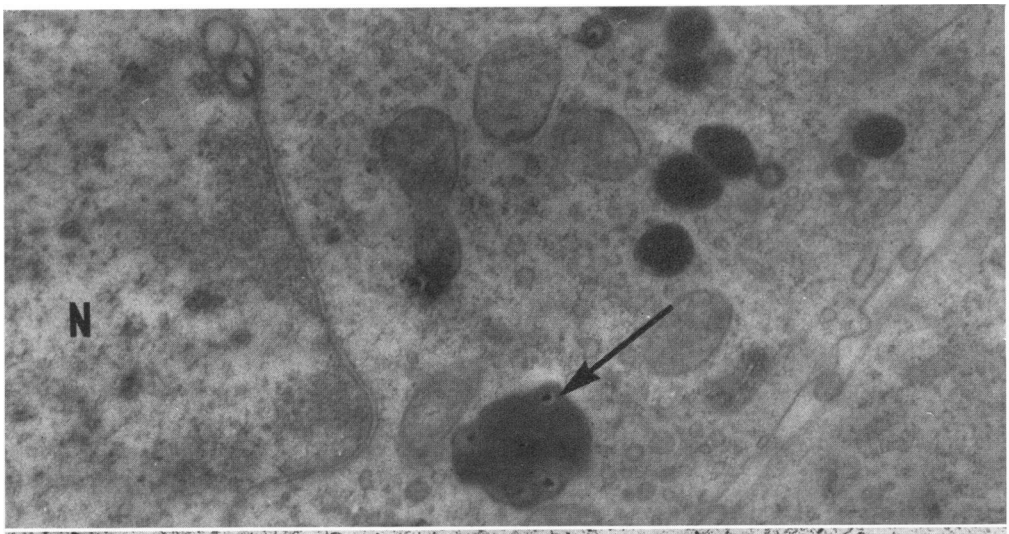
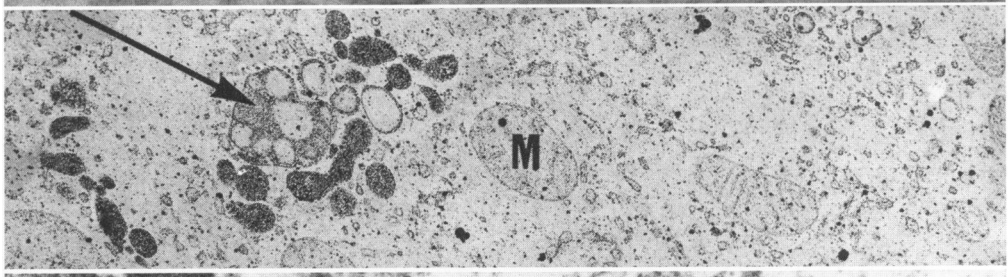
FIG. 15. Human fibroblast, fifth day after infection with human cytomegalovirus. A cytoplasmic inclusion containing virus particles (arrow) lies close to the nucleus. Uranyl acetate stain. × 21,000.

FIG. 16. Same specimen as shown in Figure 15. A cytoplasmic inclusion (arrow) is PASM positive. A mitochondrion (M) gives a negative reaction. × 21,000.

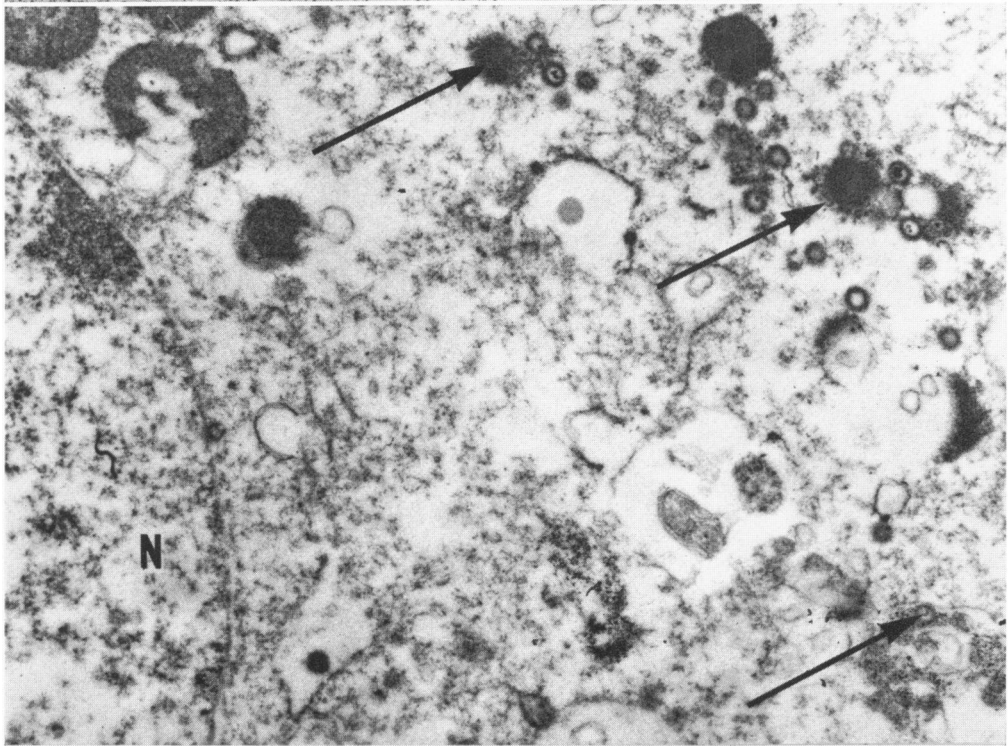
FIG. 17. Human fibroblast, fifth day after infection. Acid phosphatase positive cytoplasmic inclusions (arrows) appear in the cytoplasm. Uranyl acetate stain. × 21,000.



15



16



17