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Characteristic Ultrastructural Changes in Organs of Newborn Mice Infected with EMC Virus[1] (38249)

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The encephalomyocarditis (EMC) virus is highly infectious to newborn mice. When newborn mice are inoculated intraperitoneally with EMC virus culture fluid ($10^{-6}TCID_{50}$), as many as half will die within 24 hr and mortality can be as much as 100% by 72 hr. Tissues collected for electron microscopy from EMC virus infected mice showed cytopathic changes frequently associated with the presence of viral particles arranged into crystalline patterns. The presence of such crystals indicates tissue invasion with multiplication of the virus. We have found crystals only in mice inoculated with virus and never in control animals.

Experiments in our and other laboratories have shown the EMC virus to be highly cardiotropic and to damage readily the myocardium (1–3), cardiac valves (4), endocardium (4), aorta (5), kidney (6), pancreas (7, 8) and liver (9) of mice and other mammals. The cytopathic changes were similar to cytopathology produced by infection with various picornaviruses in tissue culture cells (10, 11) and in experimental animals (12, 13). We have studied and compared the cytopathic changes produced in various tissues of our infected animals, and it is the purpose of this report to review these changes which appear to be characteristic of picornaviral infection, at least of infections with EMC virus.

Methods and Materials. A total of 56 newborn mice of a random HaM/ICR strain was used. The mice were inoculated intraperitoneally with 0.05–0.10 ml EMC virus culture fluid (1) with a titer of $10^{-6}TCID_{50}$ and killed 24 hr later. Tissues collected for electron microscopy included myocardium, cardiac valves, kidney, pancreas, liver and aorta. The tissues were excised rapidly and minced into 1 mm^3 blocks in a pool of cold fixative. Fixation was begun in 5% glutaraldehyde in phosphate buffer for 2 hr, followed by a buffer wash and postfixation in cold 1% osmium tetroxide in phosphate buffer for 1.5 hr. All tissues were dehydrated in a series of ascending concentrations of methanol. Embedment was in epoxy resin. Thin sections for electron microscopy were stained with aqueous uranyl acetate and lead citrate and examined with a Siemens Elmiskop I electron microscope.

Results. General. Cellular damage was seen most frequently in the myocardium, pancreas and liver and less often in the valvular tissue. The lesions observed electron microscopically were usually focal, involving only one cell, but involvement of two or more cells in a lesion was common. Many lesions occurred in all tissues. Damage to individual cells ranged from mild to severe. The most remarkable and consistent cytopathic change produced by the EMC virus was a readily recognizable intracellular proliferation of membrane components, characterized by vesiculation and vacuolization with formation of membrane-vesicle complexes (MVC) (Fig. 1). Often the

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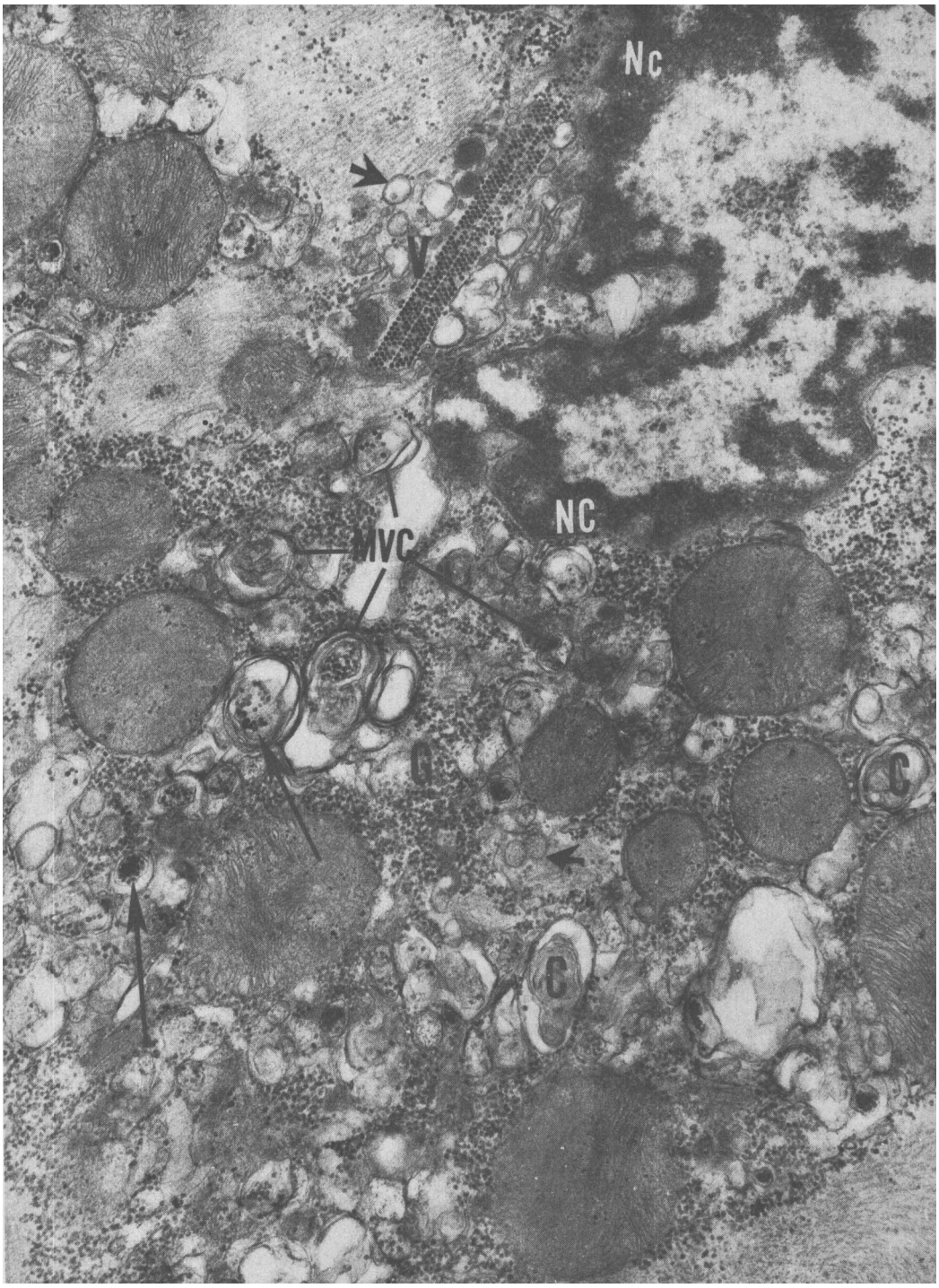

FIG. 1. Perinuclear necrotic area in a myocyte of a newborn mouse inoculated with EMC virus culture fluid. Nuclear chromatin (NC) is marginated. The lesion consists of membrane-vesicle complexes (MVC) which contain cytoplasmic (C) or granular material, small vesicles, and vacuoles (short arrows), and an elongate viral crystal (V). Myofibrils are absent in this cytonecrotic zone. Some of the granules within the membrane-vesicle complexes are similar to glycogen (G), whereas others are of greater electron density (long arrows). Magnification ×30,000.

MVC contained particles of cytoplasmic material or granular material of several types (Fig. 1). Crystalline aggregates of viral particles were frequently observed in close proximity to the lesions (Fig. 1), whether or not the lesions were of mild or severe degenerative morphology. The viral crystals were also observed in the interstitium and extracellular spaces. Table I summarizes the incidence of observed EMC viral crystals in the relatively small portions of tissues studied electron microscopically. Margination and/or pyknosis of nuclear chromatin was found in all tissues studied. Dilatation of the rough endoplasmic reticulum (RER) was common to all tissues, being most noticeable in fibroblasts of the valvular stroma and aortic adventitia. There was some evidence of inflammatory infiltration of macrophages and polymorphonuclear neutrophils, but neutrophilic involvement in the inflammatory response was more pronounced in the liver than in other organs.

Myocardium. Focal necrosis of the myocardium usually involved single myocytes. The cytopathic lesions were identified by the formation of MVC (Fig. 1). Myofibrils were absent from necrotic areas, but the myofibrils adjacent to these areas were often well organized (Figs. 1, 2). The necrosis was generally perinuclear. Viral crystals were often associated with the necrosis (Figs. 1, 2). Many of the MVC contained fragments of cytoplasmic material. We have also observed granular material either scattered randomly in necrotic areas or contained within membrane-bound vesicles. The granular materials were generally (a) similar in size and electron density to glycogen (Fig. 1), (b) similar in size to glycogen but of greater electron density (Fig. 1), or (c) larger in size than glycogen and of greater electron density (Fig. 2). This third type of granule was larger (300–340 Å) than viral particles contained within crystalline aggregates (220–240 Å).

Heart valves. Necrotic cells were not numerous in the valve leaflets. The lesions were focal, usually limited to single cells. The damaged cells were fibroblasts which were most frequently observed near the attachment site of the valve leaflets to the atrioventricular ring (Fig. 3). The RER of the fibroblasts was dilated and the cytoplasm contained a number of MVC (Fig. 3). Viral crystals were observed within the necrotic cytoplasm (Fig. 3).

Kidney. The cytopathic changes in the kidney included significant necrosis of glomerular mesangial cells, tubular epithelial cells and interstitial cells (6). Mesangial cells were occasionally ruptured, with the formation of numerous vacuoles. Morphologic degeneration was most pronounced in tubular epithelial cells. Numerous MVC in association with viral crystals were found, particularly in the tubular interstitium (6). The RER was often dilated.

Pancreas. Both acinar cells and beta cells of the pancreas showed necrotic changes consisting of intracellular vesiculation and vacuolization with formation of MVC. The damage was often marked. The RER was dilated, the mitochondria were swollen, and some of the zymogen granules were dis-

TABLE I. Incidence of Observed Intracellular Crystalline Aggregates of EMC Viral Particles in Various Tissues from Newborn Mice Inoculated with EMC Virus Fluid.

	Myocardium	Heart valve	Kidney	Pancreas	Liver	Aorta
Number of tissues observed	39	9	13	8	31	10
Number of tissues with observed viral crystals	31	5	13	8	7	8
% with viral crystals	80	56	100	100	23	80

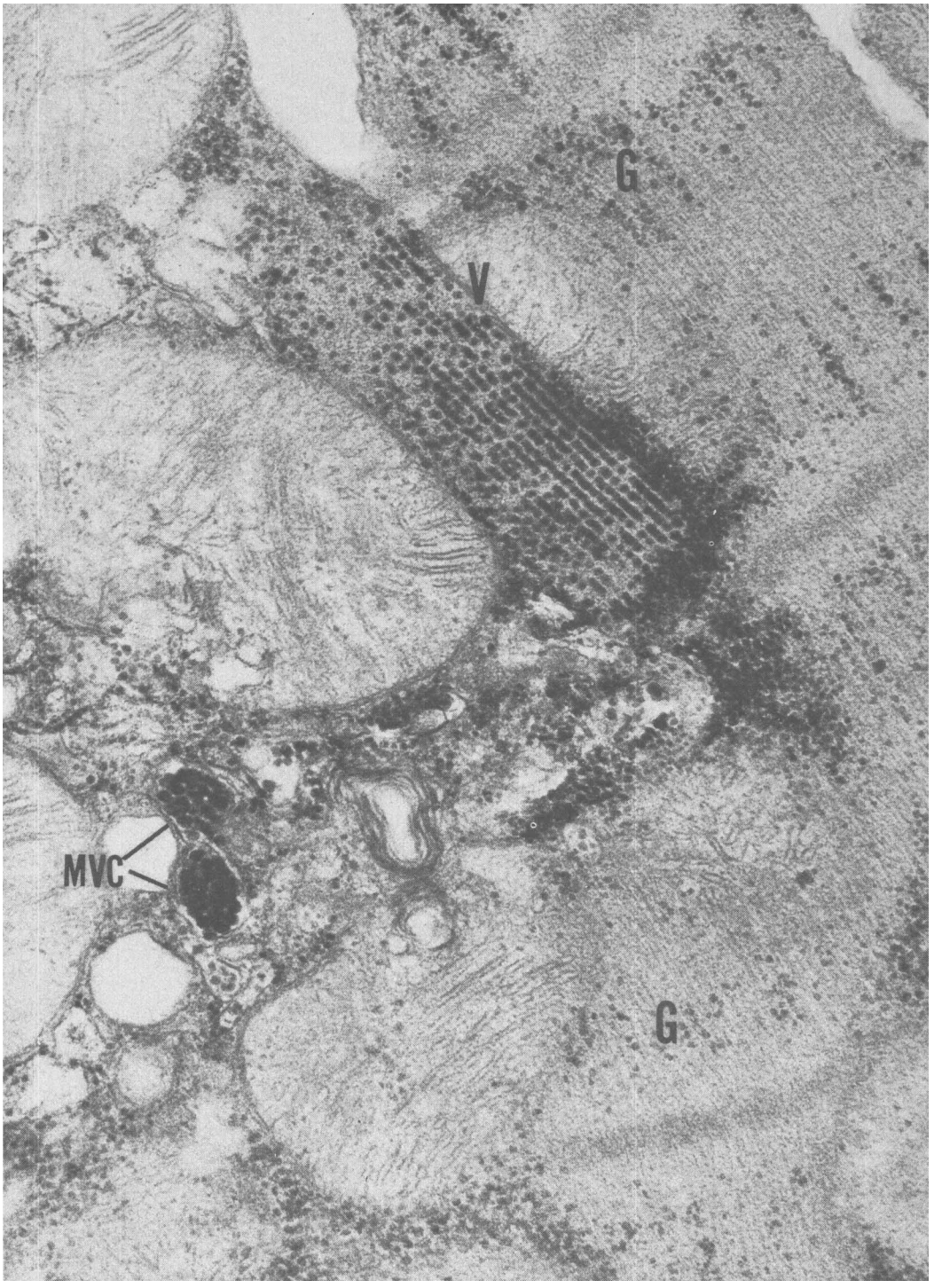

FIG. 2. A portion of a myocyte of a newborn mouse that was inoculated with EMC virus culture fluid and killed 24 hr later showing a small necrotic zone in which there are two membrane-vesicle complexes (MVC) containing granules that are larger and of greater electron density than glycogen (G). These vesicles are in close spiatal relationship to a viral crystal (V). Magnification ×64,000.

FIG. 3. Section from the region at which the valve leaflet inserts into the atrioventricular ring of the heart of a mouse that was inoculated with EMC virus fluid. Fibroblasts (F) within the stroma are separated by a matrix of collagen fibrils (C). In the lower right area of the illustration is a portion of a diseased myocyte (MY). A large part of the cytoplasmic extension of the fibroblast in the center shows necrosis in which membrane-vesicle complexes and mitochondria (M) are evident. A small viral crystal (V) is located within the necrotic area. Magnification ×15,000.

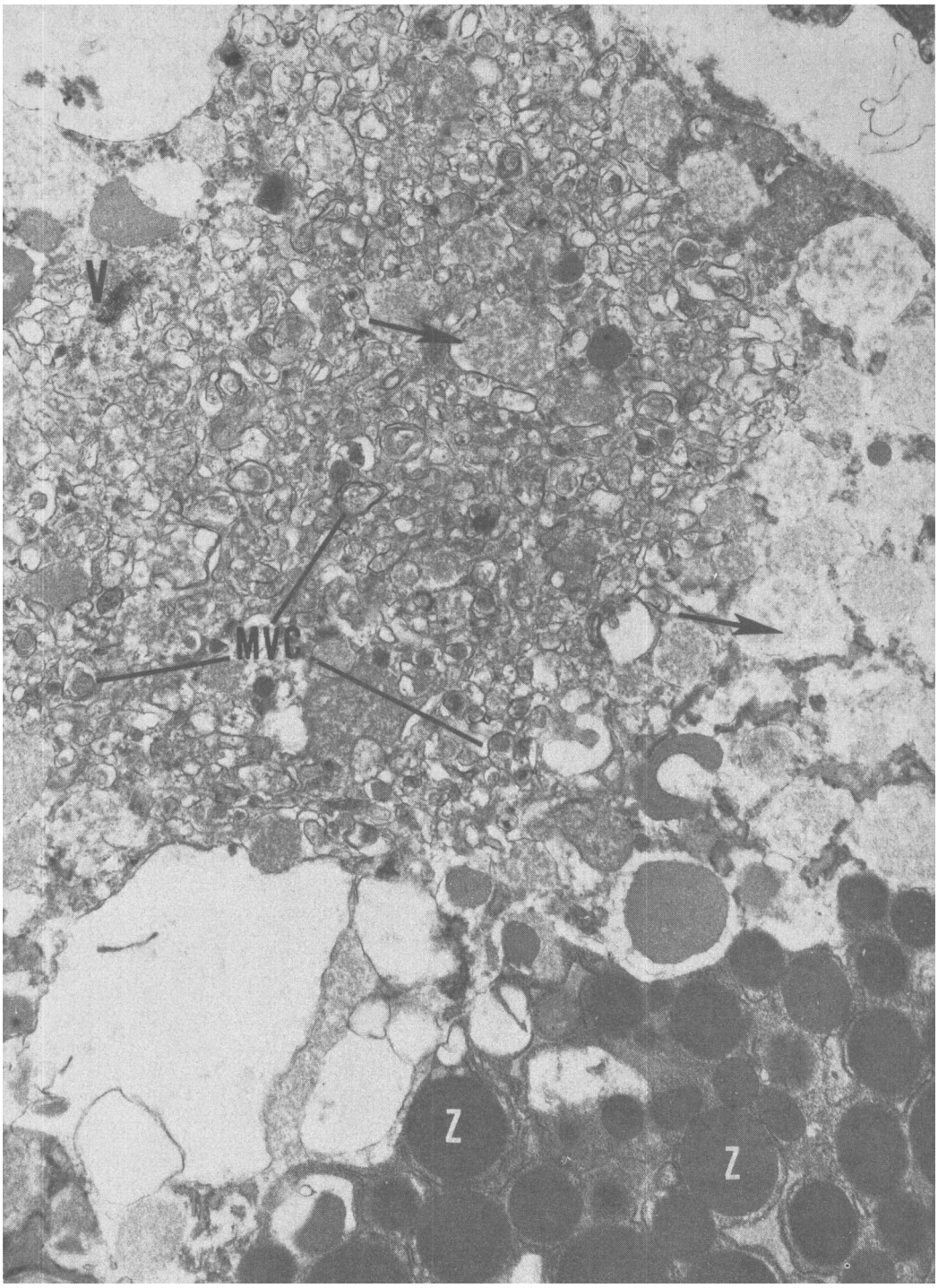

FIG. 4. An acinar cell from the pancreas of a newborn mouse that was inoculated with EMC virus culture fluid. Necrosis of this cell is extensive, with a large area showing severe degeneration. The cytonecrosis consists of disrupted cytoplasm in which numerous membrane-vesicle complexes (MVC) and degenerated zymogen granules (arrows) are located. A small viral crystal (V) is situated within the cytonecrotic area. Unaffected zymogen granules (Z) in more normal cytoplasm appear in the lower right portion of the illustration. Magnification ×14,000.

solved (Fig. 4). Typical EMC viral crystals (Fig. 4) were found in both acinar and beta cells.

Liver. As with the other tissues, damage to the liver was focal, ranging from moderate to marked in affected parenchymal and Kupffer cells (9). Usually a single cell was affected, but areas with several necrotic cells were not uncommon. In several animals, the sinusoids and, less frequently, the spaces of Disse, were congested with cellular debris.

The proliferation of membrane materials with vesicular lamellarform structures was clearly observed in the liver. Viral crystals were found in association with slight or marked degeneration. Phagocytic polymorphonuclear neutrophils were occasionally observed within degenerative areas (Fig. 5).

Aorta. Crystalline aggregates of EMC viral particles were found in adventitial fibroblasts and medial smooth muscle cells in association with vesiculation and MVC (Fig. 6), as noted in the lesions of other tissues reported here. Fibroblasts were often markedly altered, with the nuclear chromatin marginated, the RER dilated, and the cytoplasm containing abundant vesicles. In the smooth muscle cells (Fig. 6) the necrotic areas were perinuclear and similar in appearance to those in fibroblasts.

Discussion. Intracellular necrosis associated with viral crystalline arrays has been observed electron microscopically in the myocardium, cardiac valves, kidney, pancreas, liver, and aorta of newborn mice which were injected intraperitoneally with EMC virus culture fluid and killed 24 hr later. By comparing the cytopathic lesions produced in these various tissues, we noted that the intracellular necrosis was similar in all these tissues and was indicative of infection by the EMC virus. This intracellular necrosis ("cytonecrosis") was found most frequently and was most widespread in the myocardium, pancreas and liver and was least often observed in the valvular tissues and aorta; but, it must be remembered constantly that the electron microscopic examination is limited to such extremely small areas of tissue that involved areas and cells are readily missed. Cells of mesenchymal or mesodermal origin seemed particularly susceptible to infection by EMC virus. In all infected cells, regardless of origin or specialization in function, a similar characteristic cytopathology ("virocytonecrosis") associated with EMC viral crystals was observed. We have observed this type of focal lesion in all of our previous studies with viruses and have been impressed all along with its characteristic features. Similarly altered cellular morphology has been observed by other investigators in tissue culture cells (10, 11) and experimentally mediated *in vivo* systems (13) infected with other picornaviruses. But, we are not aware of any other single injurious agent which will induce identical cytonecrosis, i.e., formation of membrane-vesicle complexes, dilatation of RER, margination and/or pyknosis of nuclear chromatin and inflammatory infiltration, as observed here in a variety of tissue types.

The characteristic viral induced necrosis which we have chosen to designate as "virocytonecrosis" is well illustrated by Fig. 1. It is generally of perinuclear localization, consisting of proliferation of cytomembranous components with the formation of membrane-vesicle complexes (MVC), margination of nuclear chromatin, and dilatation of RER. Fragments of cytoplasmic material of different degrees of degeneration and disintegration are found in all of these complexes of vacuoles and vesicles. Inflammatory infiltration with macrophages and polymorphonuclear neutrophils, the latter being most pronounced in the liver, was a common observation in the tissues studied. This is regarded as indicative of acute infection.

We have also observed granular materials of unknown nature within the membrane-vesicle complexes of the cytonecrotic lesions, especially in the myocardium. The granular materials are generally (a) similar in size and electron density to glycogen (Fig. 1), (b) similar in size but of greater electron density than glycogen (Fig. 1), or (c) larger in size and of greater electron density than glycogen (Fig. 2). It is suggested that the latter two types of granules may represent different and heretofore un-

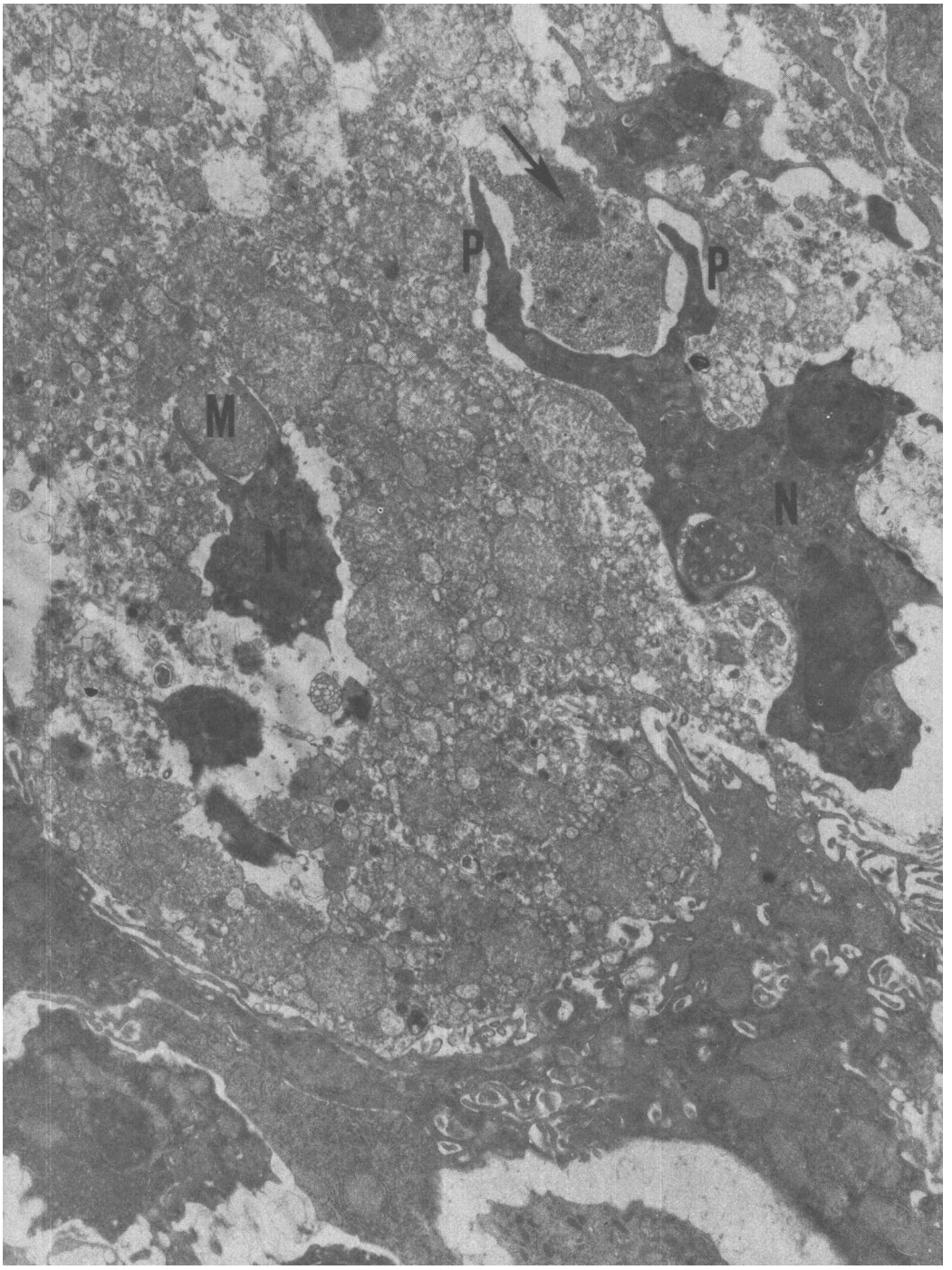

FIG. 5. Portion of degenerative hepatocyte from liver of a newborn mouse infected with EMC virus and sacrificed 24 hr later. Within the cytoplasm are profiles of neutrophils (N) engaged in active phagocytosis of cytoplasmic material. One phagocyte is engulfing a mitochondrion (M) while another has extended pseudopods (P) around homogenous granular debris (arrows). Magnification ×9,000.

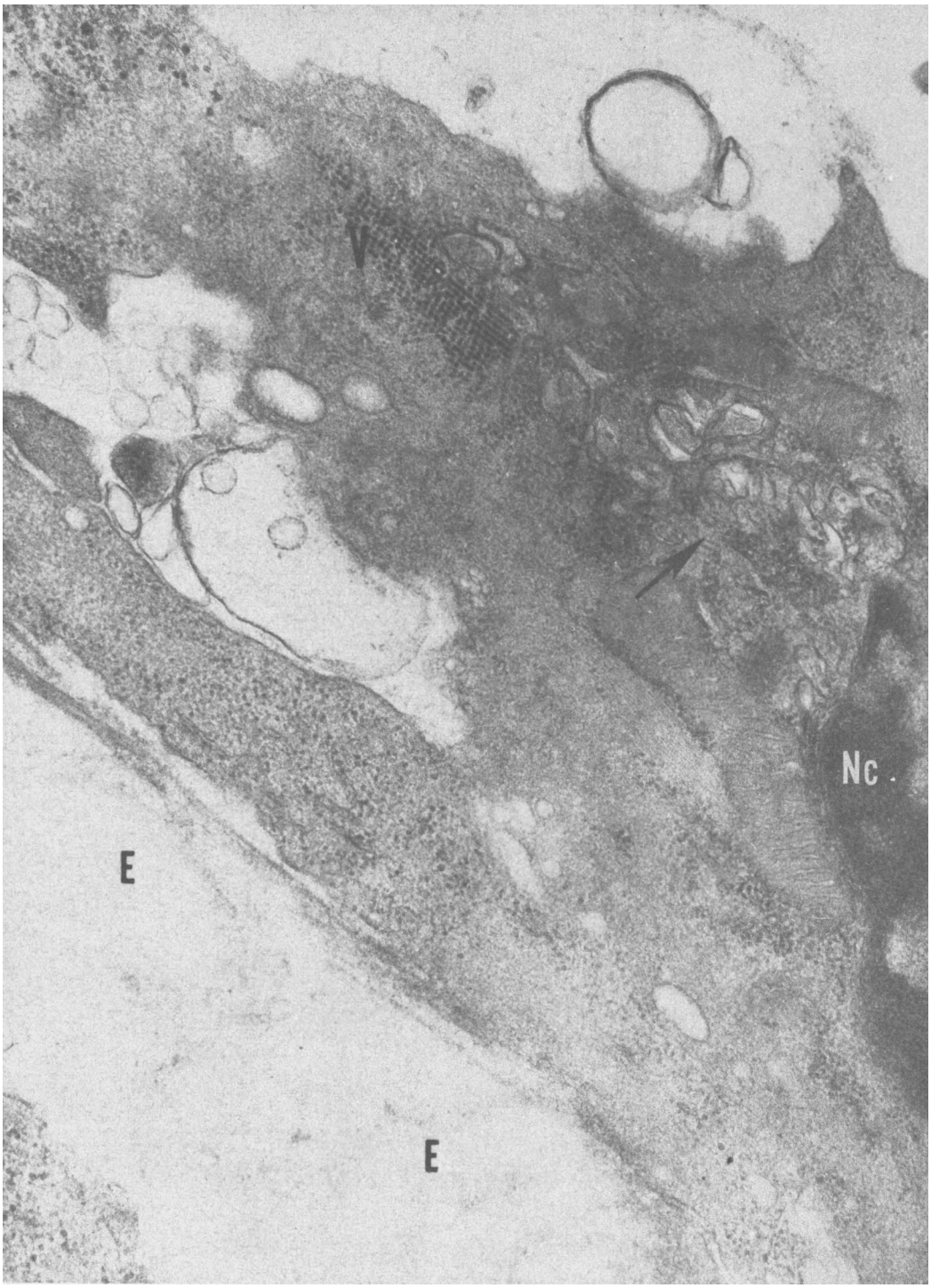

FIG. 6. Part of the medial smooth muscle cell from the aorta of a mouse that was inoculated with EMC virus fluid. The cell is bordered on one side by elastic fibers (E). Within the perinuclear cytoplasm is a small area of cytonecrosis (arrow). A viral crystal (V) is situated near the necrotic zone. The nuclear chromatin (Nc) is marginated. Most of the remaining cytoplasm appears unaffected. Magnification ×39,000.

recognized forms of active viral material during the replicative stages which are distinct from crystalline aggregates of viral material. This suggestion is supported by several factors: (1) these granules resemble neither ribosomal nor glycogenous materials; (2) the larger size and greater stainability with uranium and lead salts infer properties common to viral material rather than to glycogen or ribonucleoprotein; (3) these granular forms have been seen only in the infected animals and not in controls (1); and (4) the granules are frequently associated with characteristic virocytonecrosis. Just as we can expect different stages of infection in individual cells at the end of any infective period of 24 hr, we might also expect the viral material to appear in different forms during the infectivity cycle. It has been shown previously that viral or viral-like particles can be enclosed within cytoplasmic vesicles (14, 15). Further studies, using other than the morphologic approach as reported here, of the granular materials within the membrane-vesicle complexes are needed to clarify their role and importance in the necrotic process produced by these viral infections.

Proliferation of membranous components (10, 12) and increased synthesis (16) of cytomembrane material, as well as formation of membrane-vesicle complexes, are important and consistent observations in picornavirus infection. It has been suggested that these changes represent a host cell-virus interaction (12) characteristic of picornavirus infection (13, 14). All the tissues reported here exhibited similar cytopathology which was consistent with previous observations in our laboratory with other picornavirus infections.

The association of viral crystalline aggregates in close proximity to clearly discernable cytonecrosis supports the contention that direct cellular infection by EMC virus has occurred. Furthermore, neither viral crystals nor cytonecrosis was found in tissues of the control animals. The data in Table I show that viral crystals were observed in approximately 65% of the tissues studied. This high incidence cannot be considered coincidental. It offers strong support for the contention that direct infection by EMC virus has occurred in the animals studied. Furthermore, the presence of viral crystals cannot be ruled out in the other (35%) tissues studied when one realizes how little of the tissue is subjected to electron microscopic examination.

These observations again demonstrate the widespread damage to many important organs of mice produced by viruses. Although the relationship of these studies to infection with the picornaviruses in man, with the production of acute cellular damage and old residual scars in all the structures of the heart, aorta, kidney, pancreas, liver, etc., can only be interestingly conjectured, it deserves consideration.

Summary. A specific ultrastructural cytopathologic response to EMC viral infection was observed in various organs of newborn mice infected with EMC virus. This cytopathology is characterized by intracellular proliferation of membrane material with formation of membrane-vesicle complexes, margination of nuclear chromatin, dilatation of rough endoplasmic reticulum, accumulation of cytoplasmic and unidentifiable granular material within membrane-bound cytoplasmic vesicles, and inflammatory infiltration. That the vesicle-bound granules may represent forms of viral materials during the replicative stage is suggested. A high incidence of viral crystals in association with this cytopathology was demonstrated. This host cell-virus interaction is similar to cytopathic changes observed in infections with other picornaviruses. We have designated this intracellular necrosis as "cytonecrosis" and when it is produced by viruses as "virocytonecrosis."

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