FUCHSINOPHILE GRANULES IN THE TISSUES OF MICE INFECTED WITH THE CONNECTICUT-5 STRAIN OF COXSACKIE VIRUS*

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PLATES 11 AND 12

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The Connecticut-5 strain of Coxsackie virus calls forth a variety of lesions in the tissues of suckling mice (1–3). There have been described hepatitis, pancreatitis, myositis, myocarditis, adipositis, and encephalomyelitis. In fully grown mice, the pancreas is selectively affected (4).

In studying these lesions in sections stained with a modified Masson method, our attention was caught by the presence of minute fuchsinophile granules, usually cytoplasmic, in the degenerating cells. Similar bodies could not be found in the tissues of control uninoculated mice, nor in mice injected with material from uninfected animals. Therefore it would seem of interest to describe their appearance and distribution in some detail, and to discuss their possible significance.

Methods

Suckling mice were inoculated intraperitoneally or intracerebrally with various dilutions of infected carcass suspensions. After opening the body cavities, the entire animal was fixed for several days in Bouin's solution, and thin transections at various levels embedded in paraffin for sectioning. 4 μ sections were stained as follows:—

1. Bullard's hematoxylin—3 to 5 minutes.
2. Wash in tap water.
3. 5 per cent acid alcohol followed by dilute ammonia water.
4. Wash in distilled water.
5. Acid fuchsin, 1 per cent in 1 per cent acetic acid—5 to 10 minutes.
6. Wash in distilled water.
7. Phosphomolybdic acid, 1 per cent—1 minute.
8. Wash in distilled water.
9. Lichtgrün, 2 per cent in 1 per cent acetic acid—30 minutes.
10. Wash in distilled water.
11. Phosphomolybdic acid, 1 per cent—1 minute.
12. Distilled water, alcohols, xylol.

FINDINGS

Appearance of the Granules.—

The fuchsinophile granules, hereafter designated as F granules, are for the most part spherical in shape, and of fairly uniform size. The diameter, as es-

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timated from photomicrographs taken at a magnification of 1700, is of the order of 550 to 600 mμ. They stain intensely red with acid fuchsin, save when they are embedded in material which is densely colored with the bluish green counterstain, in which case they assume a lavender tinge. Each granule is surrounded by a narrow, pale halo. This is not limited by an outer membrane. No fission or budding forms have been observed.

Distribution of Granules in Various Organs or Tissues.—

In general, the F granules are found in the cytoplasm of degenerating parenchymal cells, wherever lesions are produced by the Conn.-5 strain of Coxsackie virus. Thus they have been found in myocardium, liver, pancreas, fetal adipose tissue, brain, spinal cord, and skeletal muscle. They are absent from tissues not affected by the virus—salivary glands, thyroid, adrenals, spleen, lymphnodes, bone marrow, ovary, and testis. In a few preparations, they have been present in great numbers within the epithelial cells of the small intestine, and probably also in the lumen. They are regularly found in the lumina of the renal tubules, and less abundantly within the epithelial cells of the convoluted tubules.

A detailed description of their distribution in the affected tissues follows.

Liver.—As described in a previous publication (1), the Conn.-5 virus produces in 0 to 2 day mice severe hepatitis, characterized by necrosis of many liver cells, and the appearance of large, oval, phospholipid-containing cells in the sinusoids. The incidence of hepatitis in mice infected on the day of birth, or on the following day is about 90 per cent; inoculation on the 2nd day produces hepatitis in less than 10 per cent; on and after the 3rd day, the liver becomes completely resistant.

Minute fuchsinophile granules in livers showing hepatitis are present in the cytoplasm of many of the liver cells in great numbers (Fig. 1 A). They are especially distinct in large parenchymal cells with pale cytoplasm and shrunken pycnotic nucleus, in which the granules are often somewhat large, less sharp in outline, and less vividly stained than are the granules in the less severely affected cells, in which they are very minute, sharply defined, and intensely stained with the acid fuchsin.

In the oval, phospholipid-containing cells (1), granules are present at a stage when these cells are not too densely stained (Fig. 1 B). But in the majority of these cells,—which take on an opaque, deep red, or greenish red color, and become very dense and refractile,—granules can no longer be discerned.

In general, the number of granule-containing liver cells parallels the severity of the hepatitis, and the concentration of virus used for inoculation. In two 3 day old mice, inoculated intracerebrally with 0.01 cc. of 10^{-3} suspension of infected carcass, no hepatitis was produced. There were no fuchsinophile granules in the cells of the liver parenchyma, but clumps of brightly stained granules were present in many of the Kupffer cells, and occasionally free granules were present in the sinusoids. In mice surviving for 7 days granules were found only in the Kupffer cells.

No granules were found in the epithelium of bile ducts, nor in any of the many immature blood cells in the sinusoids. The cytoplasm of the megakaryocytes, which are numerous at this stage, takes a pale greenish blue stain, and is completely free of granules.

Pancreas.—Massive necrosis of the acinal epithelium is produced in 100 per cent of mice

inoculated intraperitoneally with the Conn.-5 strain of Coxsackie virus during the first 2 days of life (1). The islands of Langerhans and the pancreatic ducts are spared.

With the acid fuchsin–Lichtgrün stain, the zymogen granules are brilliantly red, and in the normal pancreas at this age, the epithelial cells of practically every acinus contain large numbers of these zymogen granules. In the necrotizing cells of the infected pancreas, the zymogen granules are dispersed, and many cells are completely devoid of them. However, one does find in the cytoplasm of the degenerating acinal cells, often in all, myriads of minute granular bodies, much smaller than zymogen granules, and readily distinguished by their staining a reddish lavender, in contrast to the brilliant red of the zymogen. In appearance and size, they are identical with the granules described in the liver cells (Fig. 2). F granules have also been demonstrated in the acinal cells of the pancreas of adult mice infected with the Conn.-5 strain.

Myocardium.—When lesions are present, which is infrequently the case with the Conn.-5 strain, granules are found in the necrotic muscle fibers and free in the adjacent interstitial tissue, but not elsewhere (Fig. 3).

Skeletal Muscle.—In mice with well marked myositis, there are great numbers of fuchsinophile granules within the empty sheathes of the fragmented necrotic fibers—less abundant and distinct in the hyaline coagulated clumps—and in the protoplasmic strands of the sarcolemma cells (Fig. 4). Small clusters of granules have been seen in the cytoplasm of occasional muscle cells of normal fibers, but in general, unaffected muscle tissue is free from granules.

Adipose Tissue.—Necrosis of the fetal fat lobules, with subsequent calcification, is one of the most characteristic lesions produced by this group of agents.

In the infected mice, fuchsinophile granules may be found in enormous numbers in the embryonic fat cells, even before the development of lesions. They are especially numerous in the periphery of the lobules where the necrotizing lesions are most intense. In the older calcifying lesions, the granules are obscured by the dense, bluish staining calcific deposit, but towards the center of the lobules, individual fat cells of large size, with degenerating pycnotic nucleus, are seen to be stuffed with granules. The calcified material is at first fairly particulate and only later fuses into large masses. It is possible, though this is difficult to establish with certainty, that the calcium is deposited on or about the fuchsinophile granules.

Preparations prepared by Gomori's alkaline phosphatase method, and subsequently counterstained with acid fuchsin–Lichtgrün, show the granules with particular distinctness. They are not blackened. In control preparations, the fat cells show affinity for the fuchsin, but the stain surrounds the individual fat vacuoles, and appears as a network, not as discrete granules.

Brain and Spinal Cord.—Granules are found in and about the lesions in both brain and cord. The characteristic cells with swollen, hydropic nuclei (degenerating astrocytes?) frequently contain profuse aggregates of fuchsinophile granules in their cytoplasm, but the granules are also thinly scattered through the neighboring ground substance (Fig. 5). No granules are found within cells that can be definitely identified as ganglion cells. In the lesions, these often become shrunken and sclerotic, under which circumstances, they stain diffusely and intensely with the acid fuchsin.

The unaffected portions of the brain and cord are completely free.

Kidney.—No lesions of the kidney have previously been noted (1). With the modified Masson stain employed in this study, however, many of the convoluted tubules contain large hyaline epithelial droplets, which tend to take a greenish blue color, and are probably indicative of protein resorption.

F granules may be found in the cytoplasm of the epithelial cells of the proximal convoluted tubules. Usually they lie beneath the brush border (Fig. 6). They are never present in great number, as in hepatic and pancreatic cells, and in many preparations, no intra-epithelial granules were found. Within the lumina, F granules are easily demonstrable in the form of

compact aggregates, which seem to be embedded in a shreddy coagulum. Very rarely, clusters of granules may be found in the capsular space of a glomerulus. We have not been able to show clearly that the granules are discharged from the epithelial cells through the brush border, and the route through which they gain entrance into the tubules remains obscure.

Whether the granules within the lumina of the renal tubules are identical with the cytoplasmic particles may perhaps be questioned. Against the idea that they are merely a nonspecific protein precipitate is the fact, clearly brought out in the photograph, that each particle is surrounded by a characteristic halo. Furthermore, in sections stained with Heidenhain's iron hematoxylin, the granules are stained dark grey or black, in contrast to the unstained shreddy protein coagulum in which they are embedded.

The granules are easily demonstrated in the centrifuged sediment of urine from infected mice, but not in the urine of normal mice of the same age.

Intestine.—The occurrence of F granules in the epithelial cells of the small intestine is of interest, because of the known presence of virus in the feces. When found, they are situated in the supranuclear portion of the cytoplasm, beneath the cuticular membrane. There are myriads of granules, sharply stained and of uniform size. Many desquamated cells, in various stages of disintegration, are filled with the granules, and with the breaking up of the cells, they appear to be set free within the lumen of the gut.

Granules in abundance also accumulate between the epithelial cells and the underlying stroma. They appear somewhat coarser, less deeply stained and less uniform in size than the intracellular granules, and it is hard to be certain that they are not precipitated protein in edema fluid.

The time after inoculation at which F granules first appear in the tissues corresponds fairly well with the time of appearance of the lesions. This is shown in the following table:—

TABLE I

Tissue	Heart	Liver		Pancreas	Kidney	Fat	Muscle	CNS
		P	*K*					
Days after inoculation when F granules appear	3–6	3–4	7	2–4	3–9	7–9	3–7	7–9

P—parenchymal cells; *K*—Kupffer cells.

The F granules are thus seen to appear early in the heart, liver parenchyma cells, and pancreas; later in embryonic fat lobules, brain, and spinal cord.

F Granules in Lesions Caused by Other Strains of Coxsackie Virus.—Although only a limited number of preparations have been studied, one can state with certainty that similar granules are demonstrable in lesions caused by the Powers, DeMole, Ohio R, and High Point strains of Coxsackie virus. The High Point strain, in our experience, produces lesions restricted to the skeletal muscles, and it is only in this tissue that we have been able to find F granules.

DISCUSSION

The foregoing observations show that the lesions produced by the Conn.-5 strain of C virus are characterized by the appearance of minute fuchsinophilic

granules in the degenerating cells of various tissues. Their interpretation offers some difficulties. The size of the virus particles of the Dalldorf type A of Coxsackie virus has been estimated by Quigley (5) on the basis of filtration through Elford membranes and by ultracentrifugation, as about 6 to 9 mμ. Melnick (2) gives 15 to 23 mμ, as determined with gradocol membranes, as the size of Conn.-5 virus. Recently Melnick, Rhian, Warren, and Breeze (6) have published detailed studies, indicating a comparable size for the Conn.-5, Ohio 1, High Point, and Texas strains of 15 to 23 mμ according to filtration and approximately 24 to 32 mμ from sedimentation data. Particles of these dimensions would obviously not be visible in stained microscopic preparations. This leaves for consideration various possible interpretations of which the following will be dealt with:—

1. The granules are non-specific, and are merely indicative of a peculiar type of cell degeneration.

2. The bodies represent forms of a pleuropneumonia-like microorganism, which has been introduced as a contaminant of the injected material, or which was previously latent in the affected tissues.

3. The stained particles are either aggregates of elementary bodies or stages in a life cycle during which the virus particles have assumed larger dimensions.

4. The particles represent elementary bodies magnified by the adsorption of stainable surrounding cytoplasmic material.

As regards the first possibility, certain considerations speak strongly against it. F granules can be found in the cytoplasm of cells showing no other evidence of degeneration, *e.g.*, myocytes or Kupffer cells in the liver. Moreover, although the granules are primarily intracellular, they are sometimes found free in the intracellular spaces, or in the lumina of renal tubules.

Another point against the view that the F granules are non-specific signs of cell degeneration, is their uniform size, and the invariable presence of a surrounding halo. Furthermore, the F granules are found in a variety of cell types —liver cells, pancreatic acinar cells, myocardial and skeletal muscle fibers, fetal fat cells, and astrocytes. Even when free from cells, as in the lumina of the renal tubules, they conserve the same morphology. It seems to us unlikely that such definite structures, occurring in such diverse cellular elements, should represent merely a banal type of cytoplasmic degeneration.

The suggestion has been made that the F granules may be forms of pleuropneumonia-like microorganisms, introduced as a contaminant of the injected material, or occurring as latent parasites of normal mouse tissue, but roused to activity and multiplication at the site of the lesions. The available evidence, we believe, is against either possibility

1. F granules have been found after inoculation of material from widely different sources and localities—human feces, throat washings, and sewage, obtained in Connecticut, Ohio, Massachusetts, and North Carolina. It would

seem unlikely that the same contaminant should be present in material from such widely different sources.

2. 10 units of streptomycin were added as routine to each cc. of suspension used for inoculation, and in a recent experiment, 20 units of streptomycin were given to 0 day mice with the virus inoculum, and again on the 1st and 2nd says after injection. All the mice succumbed with typical lesions, in which F granules could be demonstrated. Streptomycin has been found to inhibit the growth of different strains of pleuropneumonia-like microorganisms in concentration of 1.0 to 0.1 units per cc. (7) when added to culture media. Three doses of 20 units, administered to newborn mice averaging about 1 gm. in weight should have been adequate to inhibit the growth of these microorganisms.

3. Through the kindness of Dr. Dienes, attempts were made by him to cultivate pleuropneumonia-like microorganisms from the tissues of 3 day old mice, infected with suspensions containing Conn.-5 virus. They were unsuccessful.

4. The suspensions were still infective after filtration through gradocol membranes of pore size 50 mμ and 60 mμ.[1] This should hold back pleuropenumonia-like microorganisms, filtrable forms of which are estimated tρ be from 125 to 150 mμ. The F granules present in the lesions of the filtrate-infected mice could not have derived from pleuropneumonia microorganisms present in the unfiltered material. Against the third possibility, that the stained particles represent aggregates of elementary bodies, is the general uniformity in size. Were they aggregates, one might expect to find greater variability. As to their being stages in a life cycle, such as has been described for the psittacosis-lymphogranuloma group, this would be mere speculation.

Positive evidence that the elementary bodies have adsorbed cytoplasmic constituents, and have thus been made larger and demonstrable in stained preparations, is not at present available. There are experiments in the literature indicating that alkaline phosphatase may be incorporated in, or adsorbed upon, the elementary bodies of vaccine virus (8, 9). Smadel (10) points out that biologically active substances such as various enzymes may be found in appreciable quantities in preparations of elementary bodies. It has been shown that the particles have the capacity to adsorb these enzymes upon their surface, and to hold them tenaciously through repeated washings.

Of the various interpretations discussed, this then would seem to us the most reasonable—namely that the stained particle represents an elementary body surrounded by an envelope of altered cytoplasmic material having an affinity for the fuchsin.

The halo which invariably surrounds the individual particle can be interpreted in one of two ways. It may represent a digestion zone in the contiguous cytoplasm; or it may be some sort of capsular material. In favor of the latter possibility is the observation that the halo is seen about free forms within the

[1] We are greatly indebted to Mr. Harold Amos for carrying out these filtrations.

renal tubules. Moreover, though pale in contrast with the granule itself, the material does take a pale stain, and therefore is probably not a mere zone of liquefaction.

Further investigation will be necessary before one can associate the F granules with virus activity. There is, however, some indirect evidence in support of such an association. Having found the F granules in great numbers within the renal tubules, it was an obvious experiment to test the infectivity of the urine. This has been done and a detailed report of the experiments has been reported elsewhere (11). It can be said here that the urine of infected suckling mice has been found to contain the virus in high concentration. This does not prove that the activity of the virus is bound to the F granules within the renal tubules, but is in accord with such a possibility.

SUMMARY

Minute cytoplasmic fuchsinophilic granules, characteristically surrounded by a halo, have been demonstrated in the lesions produced in suckling mice by the Connecticut-5 strain of Coxsackie virus. Their possible significance is discussed.

The author wishes to express his indebtedness to Miss Sheila Richardson for technical assistance and to Mr. Frank White for taking the photographs. He is particularly grateful to Dr. Dienes for his attempts to cultivate pleuropneumonia-like microorganisms from infected mice, and to Mr. Harold Amos for preparing gradocol filtrates.

BIBLIOGRAPHY

1. Pappenheimer, A. M., Daniels, J. B., Cheever, F. S., and Weller, T. H., *J. Exp. Med.*, 1950, **92**, 169.
2. Melnick, J. R., *Bull. New York Acad. Med.*, 1950, **26**, 342.
3. Melnick, J. R., and Godman, G. C., *J. Exp. Med.*, 1951, **93**, 247.
4. Pappenheimer, A. M., Kunz, L. J., and Richardson, S., *J. Exp. Med.*, 1951, **94**, 45.
5. Quigley, J. J., *Proc. Soc. Exp. Biol. and Med.*, 1949, **72**, 434.
6. Melnick, J. R., Rhian, N., Warren, J., and Breeze, G. S., Jr., *J. Immunol.*, 1951, **67**, 151.
7. Leberman, P. R., Smith, P. F., and Morton, H. E., *J. Urol.*, 1950, **64**, 167.
8. MacFarlane, M. G., and Salamon, M. R., *Brit. J. Exp. Path.*, 1938, **19**, 184.
9. MacFarlane, M. G., and Doby, D. E., *Brit. J. Exp. Path.*, 1940, **21**, 219.
10. Smadel, J. E., *in* Virus and Rickettsial Infections of Man, (T. M. Rivers, editor), Philadelphia, J. B. Lippincott Company, 1948, 329.
11. Pappenheimer, A. M., Kunz, L. J., and Richardson, S., *Proc. Soc. Exp. Biol. and Med.*, 1951, **77**, 405.

EXPLANATION OF PLATES

All photographs taken at 1525 magnification from 4 μ paraffin sections stained with modified Masson trichrome.

PLATE 11

FIG. 1. Mouse 3809. Inoculated intraperitoneally on day of birth with 0.02 cc. 10^{-1} bone carcass suspension containing Conn.-5 strain of Coxsackie virus.

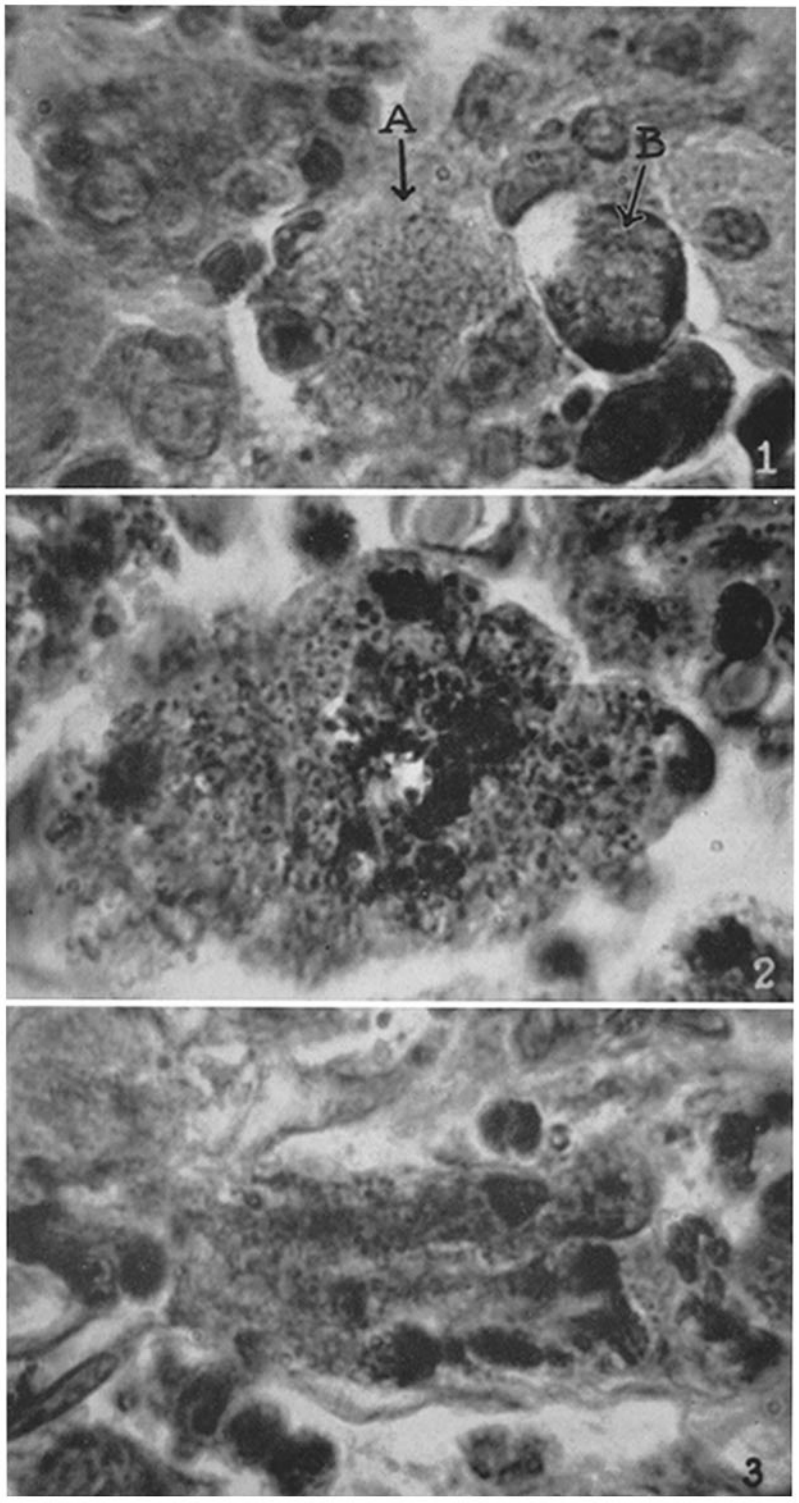
The liver is the seat of diffuse hepatitis. Several parenchymal liver cells are shown filled with F granules, each surrounded by pale halo. A large oval phospholipid-containing cell lying free within a sinusoid is also filled with granules.

FIG. 2. Mouse 4267. Inoculated intraperitoneally on day of birth with 10^{-1} carcass suspension of Conn.-5 virus. Sacrificed on 3rd day.

Pancreas is the seat of diffuse necrosis. The degenerating acinar cells shown in the photographs are stuffed with F granules. Towards the lumen larger zymogen granules are indistinctly shown.

FIG. 3. Mouse 4577. Inoculated intraperitoneally with 10^{-2} Conn.-5 suspension on day of birth. Sacrificed on 4th day.

Myocardium.—There are several focal areas of necrosis in left ventricles. Photograph shows necrotic muscle fibers filled with F granules.



(Pappenheimer: Fuchsinophile granules and Coxsackie virus)

PLATE 12

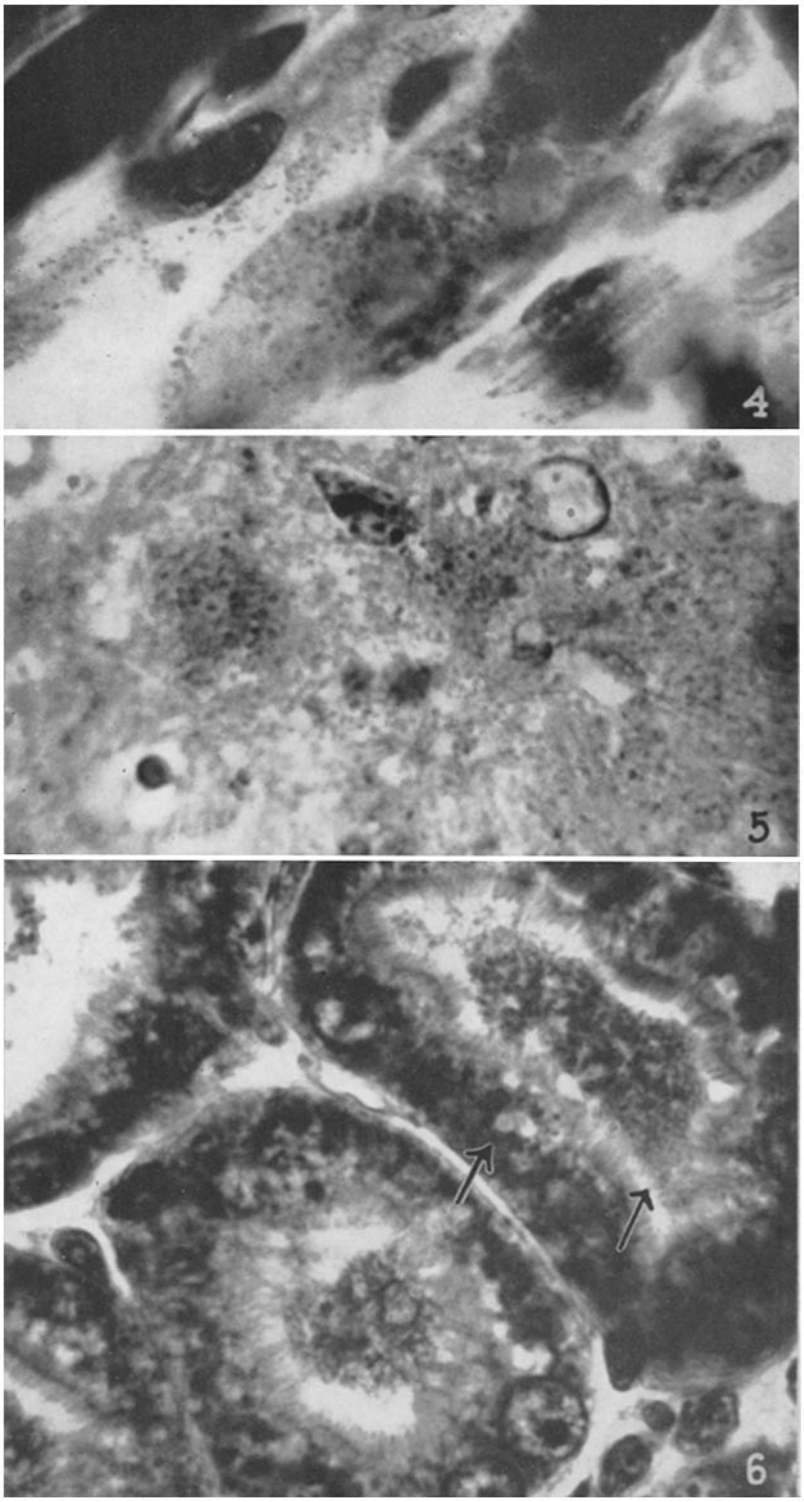
FIG. 4. Mouse 4577. Skeletal muscle fibers have undergone coagulative necrosis. F granules are shown embedded in the coagulated material, and very abundantly within the empty and collapsed sarcolemma sheaths.

FIG. 5. Mouse 4108. Inoculated with 2×10^{-5} dilution of Conn.-5 virus on the 4th day after birth. Sacrificed 6 days later, at which time the animal was paralyzed and tremulous. Histologic examination showed extensive myositis, encephalomyelitis, and necrosis of fat pads with calcification.

Spinal Cord.—Many typical F granules are found in the vicinity of hydropic degenerating astrocyte (?) nuclei; no granules are present in the nerve cells.

FIG. 6. Mouse 3623. Inoculated intraperitoneally on day of birth with 10^{-1} suspension of Conn.-5 virus. Sacrificed on 3rd day.

Kidney.—Many convoluted tubules contain compact aggregates of intensely stained F granules. The halo about each particle is distinctly shown. Similar bodies are seen in sparse number within the cytoplasm of the renal epithelial cells.



(Pappenheimer: Fuchsinophile granules and Coxsackie virus)