STUDIES BY ELECTRON MICROSCOPY OF THIN SECTIONS OF INFECTIOUS MYXOMATOSIS IN RABBITS*

By B. EPSTEIN, D.V.M., MAGDALENA REISSIG, M.D., and E. De ROBERTIS, M.D.

(From Departamento de Ultraestructura Celular, Instituto de Investigación de Ciencias Biológicas, Montevideo, and Instituto de Biología Animal, Ministerio de Ganadería y Agricultura, Pando, Uruguay)

Plates 9 and 10

(Received for publication, June 20, 1952)

The study with the electron microscope of normal and pathological tissues at a submicroscopic level has been made possible through recent improvements in the technique of thin sectioning. From this point of view the changes produced by viruses and particularly those involving a new growth of tumoral nature are of considerable interest.

The infectious myxomatosis of rabbits is one of these pathological processes having both inflammatory and neoformative characteristics. It is caused by a virus agent and its outstanding feature is the production of tumors, mainly subcutaneous and also at the site of inoculation. Since first described by Sanarelli (1898) in Uruguay (1), epizootics of the disease have been recorded in Argentina, Brazil, Uruguay, and also in California. 4 or 5 days after being injected by any route, rabbits develop the typical symptoms of the disease. The first sign to appear is blepharoconjunctivitis. It is soon followed by small subcutaneous tumors scattered throughout the body and swelling at the base of the ears and at all the mucocutaneous junctions. The general symptoms of the disease include gradual anorexia, cachexia and, in the last stages, fever. The spleen and lymph nodes, and according to some authors also the testes and bronchii (2, 3), show pathological changes even though true tumors are not found within them (for literature see Levaditi *et al.* (4)).

From a histological point of view, inflammatory changes with infiltration by polymorphonuclear cells, mainly eosinophiles, occur in the first stages. There is also edema and swelling of the connective spaces which fill up with mucoid material. Later on the proliferative changes are outstanding and the typical hypertrophied stellate cells make their appearance. Several workers have described inclusion bodies in the cytoplasm of these cells (5–10). Aragao described bodies within the nucleus (11), although he denied their presence later (12). Also large basophilic bodies and small acidophilic inclusions have been described by Rivers (6, 13), in epithelial cells, and confirmed later by others (9, 10).

The virus agent seems to proliferate intensely within the tissue, since an emulsion of it is infective in a 1:100,000 dilution (14).

* Work supported by a grant from The Rockefeller Foundation.

There are but few studies on the morphology of the myxoma virus. It belongs to the so called pox group viruses that include other large viruses such as vaccina, smallpox, chicken pox, herpes, molluscum contagiosum, and so forth. Because of its filtration properties its diameter has been estimated at 175 mμ (15). Electron microscope studies of isolated elementary bodies give dimensions of 225 × 225 × 290 mμ (16). They have also been seen with the optical microscope in eye-washing material, and the diameter calculated to be about 310 to 360 mμ (17).

These facts and the lack of further morphological data on the virus-host-cell relationship in infectious myxomatosis made its study with the electron microscope a very promising one. This work has been carried out in thin sections of tumor tissue after experimental inoculation with material containing myxoma virus.

Methods

The strain of myxoma virus employed was originally isolated from natural infections found in Uruguay (Department of Colonia). This strain is the so called C.P.M. from the Instituto de Biología Animal.[1] At present it has been transferred 43 times and it is maintained in glycerine-buffer at low temperature.

For this experiment, myxomatous tumor tissue was ground in a mortar with sterile sand, diluted 1:10 in physiological saline solution, and then centrifuged at 2,500 R.P.M. for 5 minutes. Two rabbits (C_1 and C_2) were inoculated subcutaneously with 5 ml. of the supernatant. Both of them showed all the typical symptoms of the disease and presented tumors at the site of inoculation. They died on the 9th day.

For the second transfer, two other rabbits (C_3 and C_4) were inoculated with red blood cells from C_1 and C_2 obtained by heart puncture on the 8th day. The blood was received in heparin and the erythrocytes were washed three times in saline in a centrifuge at 2,000 R.P.M. Finally they were injected in amounts corresponding to 2 ml. of whole blood. Rabbits C_5 and C_6 were inoculated with tumor material from C_3 and C_4 and died on the 6th day.

Pieces of the tumors from the different animals were obtained at the final stage of the disease just before or after death. They were cut into fragments of 1 mm. and fixed for 15 to 30 minutes in a 10 per cent formalin solution in veronal-acetate buffer at pH 7.4 and in 2 per cent osmic acid in veronal-acetate buffer at the same pH.

The tissues were sectioned and studied histologically with the current techniques. The material for examination with the electron microscope was embedded according to the technique described by Newman, Borysko, and Swerdlow (18). The tissues were washed thoroughly, dehydrated in the usual series of alcohols, and passed through three changes of a monomer composed of 9 parts of butylmethacrylate and 1 part of methylmethacrylate. They were finally embedded in a similar mixture previously polymerized with 1 per cent benzoyl peroxide to a viscous consistency. The final polymerization was carried out at 55°. This treatment avoids the distortion and destruction frequently observed when the monomer undergoes direct polymerization.

Sections were made with glass knives in a special thermal expansion microtome devised at the Rockefeller Institute.[2] After removal of the plastic with acetone and amyl acetate, the sections were covered with a parlodion film and the final grids were made.

[1] Thanks are given to B. Szifres from the Laboratorio de Biología Animal Ministerio de Ganadería del Uruguay, who has kindly provided us with the myxoma virus strain for these experiments.

[2] Thanks are given to Dr. K. R. Porter for his contribution to the construction of a microtome of this type in this Department.

In most cases the tissue sections were directly observed with an R.C.A. EMU-2 C electron microscope provided with the wide field pole piece described by Hillier (19). In a few instances the sections were shadowed with palladium at an angle of 11°. Electron micrographs were obtained at magnifications ranging between 2,000 and 4,000.

RESULTS

The sections examined were obtained from tumors fixed between the 6th and 9th days of evolution. Cytological observation with the optical microscope permitted the demonstration of few small inclusion bodies within the cytoplasm of the myxomatous cells, as has previously been reported by several authors (5–10). In thin sections observed under the electron microscope, some round cells having an oval, frequently indented nucleus can be observed (Fig. 2), but the typical stellate myxomatous cells are predominant (Fig. 1). In most cases only segments of these cells are seen. Intercellular spaces are rather large. To some extent they seem due to retraction artifacts, but in most cases we find, within these spaces and surrounding the cells, an increase of collagenous fibrils of about 50 to 60 mμ in diameter, showing typical banding and frequently dissociated into thinner filaments. Alterations of the collagen intercellular pattern by edema and infiltration with amorphous material are apparent in most electron micrographs, but they will not be described further.

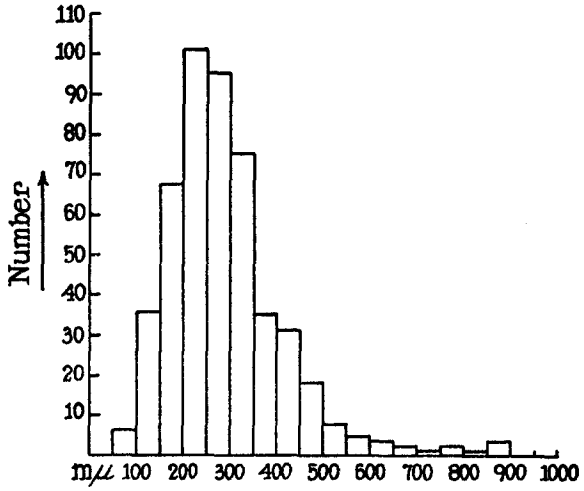
Both in the round and in the stellate cells, the most conspicuous change is the presence of dense bodies of varying size which sometimes fill the entire cytoplasm leaving the nucleus apparently intact.

The typical myxomatous cell of Fig. 1 shows an apparent thickening of the nuclear membrane. This thickening seems to be due to the deposit of an amorphous material of high electron density on the nuclear membrane. The same nuclear changes are seen in Figs. 2 and 3. All over the cytoplasm of the cell of Fig. 1, from the perinuclear zone to the farthest expansion, a particulate material of varying size and electron density can be seen. The number, size, and distribution of the particles vary from cell to cell as can be observed if Figs. 1, 2, 3, and 4 are compared.

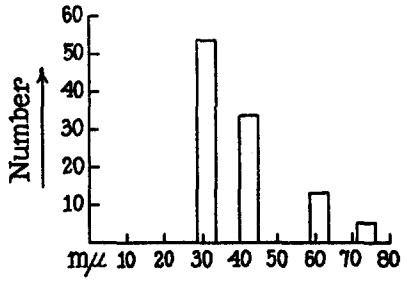
The largest bodies are very dense, mostly oval shaped, but sometimes rather rectangular with flattened sides. Their size and characteristics correspond to the described elementary bodies of the myxoma virus. It seems of interest to remark the fact that there is a continuous range of sizes between those large dense virus bodies and other much smaller particles. Their electron density is also variable. In the cytoplasm of the cell of Fig. 2 (upper center) round patches are seen, having a greater density than the remaining cytoplasm. These patches seem to become more and more distinct through an increase in electron density. The cell of Fig. 4 shows a wide variety of particles disseminated throughout the cytoplasm. Many elementary bodies are homogeneous and appear to be surrounded by a halo of less dense material having a fine granular structure. The less dense bodies show an internal constitution. They are formed by the tight clumping of small, dense particles. Sometimes this granular

structure is observed throughout the whole body; in others only the edge appears scalloped, corresponding to the projection of granules (see Figs. 5 and 6).

Measurements of the diameter of 488 typical dense bodies show a continuous range of sizes from 50 or 100 mμ to 650 mμ. In a few cases inclusions of 700 to



TEXT-FIG. 1. Distribution curve of measurements of diameter (in millimicrons) of 488 cytoplasmic inclusion bodies found in ultrathin sections of myxomatous tumors.



TEXT-FIG. 2. Distribution curve of measurements of diameter (in millimicrons) of 106 smaller particles contained within the cytoplasmic inclusion bodies.

900 mμ were found. This range of variation seems too large to be exclusively due to subtangential sections passing through the virus particles.

The distribution curve of Text-fig. 1 also shows that the highest frequency of sizes is found between the range of 150 and 350 mμ (representing 69.5 per cent of the total). Taking 300 mμ as the limit for the resolution of the optical microscope, one sees that at least 57.5 per cent of these elementary bodies are submicroscopic and cannot be observed by optical means in tissue sections.

As was mentioned above, in certain cases smaller particles can be shown within the elementary bodies. Measurements in 106 of these particles (Text-fig. 2) show that most of them (92.5 per cent) are of the order of 30 to 45 mμ. Particles of a similar size and electron density are found in great number throughout the cytoplasm, either isolated or associated in small groups, but they were not included in the measurements.

DISCUSSION

The relationship between virus and host cell has been intensely studied in recent years with the electron microscope. These studies cover a variety of animal, plant, and bacterial viruses. One of their main objects is the determination of the mechanism of virus reproduction and multiplication within the host cell. Results have been particularly interesting for the large size viruses of the pox group.

Morgan and Wyckoff (20) working with the fowl pox virus, arrived at the conclusion that "the virus particles seem to arise through differentiation and condensation of the cellular cytoplasm rather than by any process of fission." But they did not eliminate the possibility that the latter process may also take place.

In a recent study on the development of virus particles in lesions of molluscum contagiosum it has been suggested (21) that the virus may arise from material composing the matrix of the intracellular septa of the epithelial cells "by a process of segmentation into a provirus phase followed by condensation to form the mature virus." The authors base this conclusion mainly on the fact that all gradations in size and density among the different particles have been found.

On the other hand Blank (22), also in molluscum contagiosum, shows the presence of a "subvirus" particle approximately one fourth the diameter of the virus itself. He mentions that "the close association of these smaller particles with fully formed virus and the lumpy irregular surface of the virus suggest that it may be formed by an aggregation of smaller particles."

Results reported here show the presence of a wide variety of particles differing both in size and electron density, within the cells infected with the myxoma virus. The largest ones have the size and morphological characteristics of the mature infective virus. Below this range there is a series of smaller, probably non-infective particles. Some of the inclusions, of lower electron density, frequently show a complex constitution. They are formed by the clumping of smaller units present in the cytoplasm, perhaps by a progressive process of growth and condensation of materials.

Since it is known that the myxoma virus can develop and grow in the chorioallantoic membrane of the chick embryo (23), experiments are under way to study the virus-cell relationship in this material. This technique may provide a clearer insight into the mechanism of virus formation.

SUMMARY

Rabbits were inoculated with the C.P.M. strain of myxoma virus and the resulting subcutaneous tumors were fixed, embedded, and sectioned for observation with the electron microscope.

Both round cells and the typical stellate myxomatous cells were observed in addition to changes in the collagen pattern at the intercellular spaces. The cytoplasm of the cells showed a great number of bodies of varying size and density, the largest of them having the size and other characteristics of the elementary bodies of the virus. Some of the bodies showed an internal structure, being formed by the tight clumping of small dense particles.

Distribution curves of the diameter of the elementary bodies and of the smaller internal particles are presented.

The morphological problems involved in the virus-host cell relationship are discussed in the case of the myxoma virus.

BIBLIOGRAPHY

1. Sanarelli, G., *Centr. Bakt., 1., Abt.*, 1898, **23,** 865.
2. Ahlstrom, C. G., *Acta path. scand.*, 1940, **17,** 377.
3. Stewart, F. W., *Am. J. Cancer*, 1931, **15,** 2013.
4. Levaditi, C., Lepine, P., and Verge, J., Les ultravirus des maladies animaux, Paris, Librairie Maloine, 1943.
5. Splendore, A., *Centr. Bakt., 1. Abt., Orig.*, 1909, **48,** 300.
6. Rivers, T. M., *Proc. Soc. Exp. Biol. and Med.*, 1927, **24,** 435.
7. Lipschutz, B., *Wien. klin. Woch.*, 1927, **40,** 1101.
8. Findlay, G. M., *Brit. J. Exp. Path.*, 1929, **10,** 214.
9. Lewis, M. R., and Gardner, R. E., *Am. J. Path.*, 1932, **8,** 583.
10. Hyde, R. R., and Gardner, R. E., *Am. J. Hyg.*, 1933, **17,** 446.
11. Aragao, H. De B., *Brazil-méd.*, 1911, **25,** 471.
12. Aragao, H. De B., *Mem. Inst. Oswaldo Cruz*, 1927, **20,** 225.
13. Rivers, T. M., *J. Exp. Med.*, 1930, **51,** 965.
14. Hobbs, G. R., *Am. J. Hyg.*, 1928, **8,** 800.
15. Stanley, W. M., Science in Progress, 1939, cited by Levaditi (4).
16. Von Borries, B., Ruska, E., and Ruska, H., *Klin. Woch.*, 1938, **17,** 921.
17. Van Rooyen, C. E., and Rhodes, A. J., *Centr. Bakt., 1. Abt., Orig.*, 1937, **140,** 117.
18. Newman, S. B., Borysko, E., and Swerdlow, M., *J. Research Nat. Bureau Standards*, 1949, **43,** 2020.
19. Hillier, J., *J. Appl. Physics* 1951, **22,** 135.
20. Morgan, C., and Wyckoff, R. W. G., *J. Immunol.*, 1950, **65,** 285.
21. Melnick, J. L., Bunting, H., Banfield, W. G., Strauss, M. J., and Gaylord, W. H., *Ann. New York Acad. Sc.*, 1952, **54,** 1214.
22. Blank, H., *Ann. New York Acad. Sc.*, 1952, **54,** 1226.
23. Haagen, E., and Du Dscheng-Hsing, *Zentr. Bakt., 1 Abt., Orig.*, 1938, **143,** 23.

PLATES

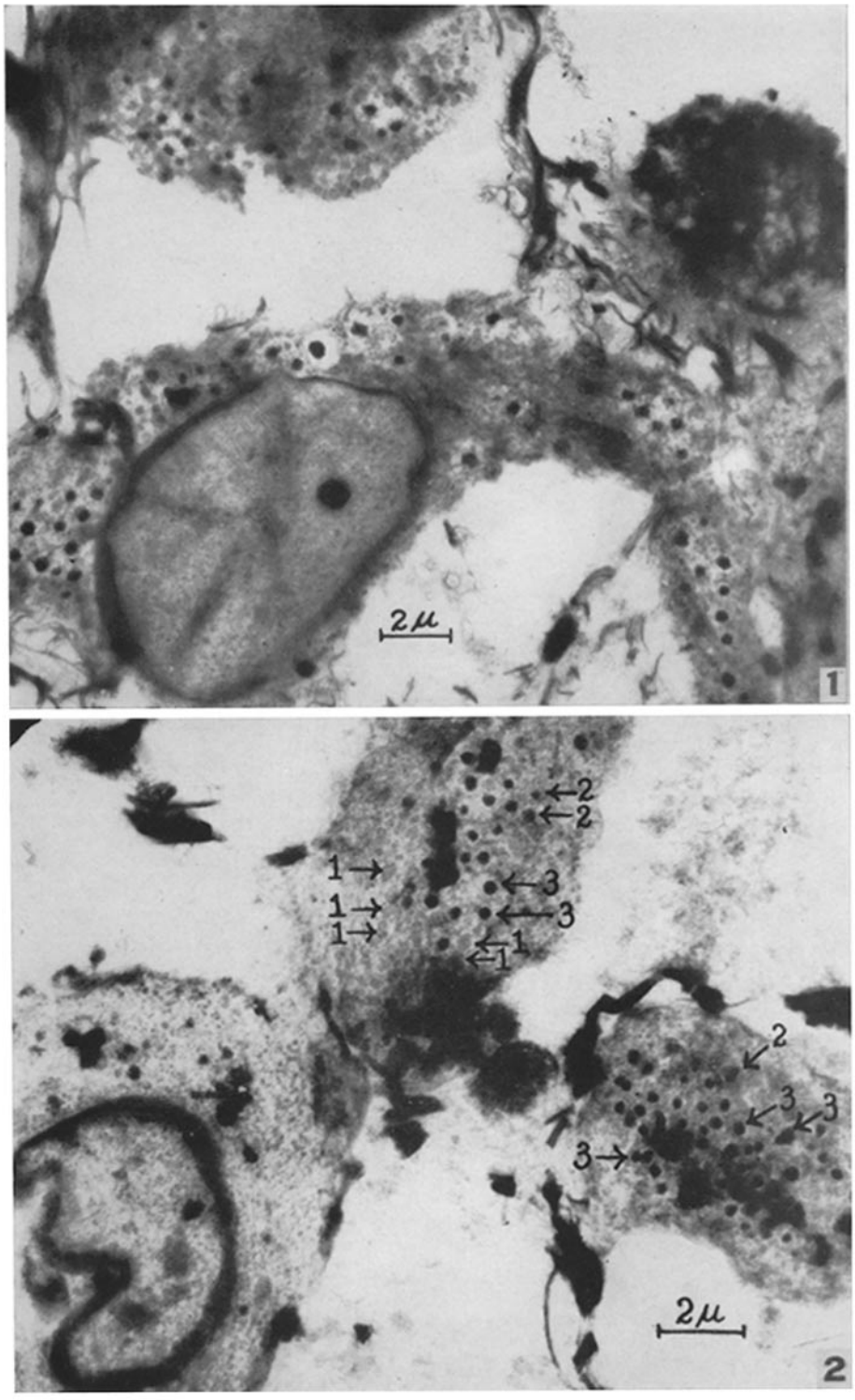
EXPLANATION OF PLATES

All figures are from ultrathin sections of myxomatous tumors. Fixation in 2 per cent osmic acid in veronal-acetate buffer at a pH of 7.4. The specimens of Figs. 4, 5, and 6 were shadowed with palladium at an angle of 11°.

PLATE 9

FIG. 1. Electron micrograph of an ultrathin section of a myxomatous tumor. Particles of varying size and density are seen in the cytoplasm of the stellate cell. At upper left a round cell, containing a few dense particles and numerous bodies of lower electron density. × 4,860.

FIG. 2. At lower left a round cell having an indented nucleus. Its nuclear membrane is covered by an amorphous and dense material. At the right and at upper left, parts of cells containing particles of varying density. The numbers 1, 2, 3 indicate different degrees of electron density. × 6,390.



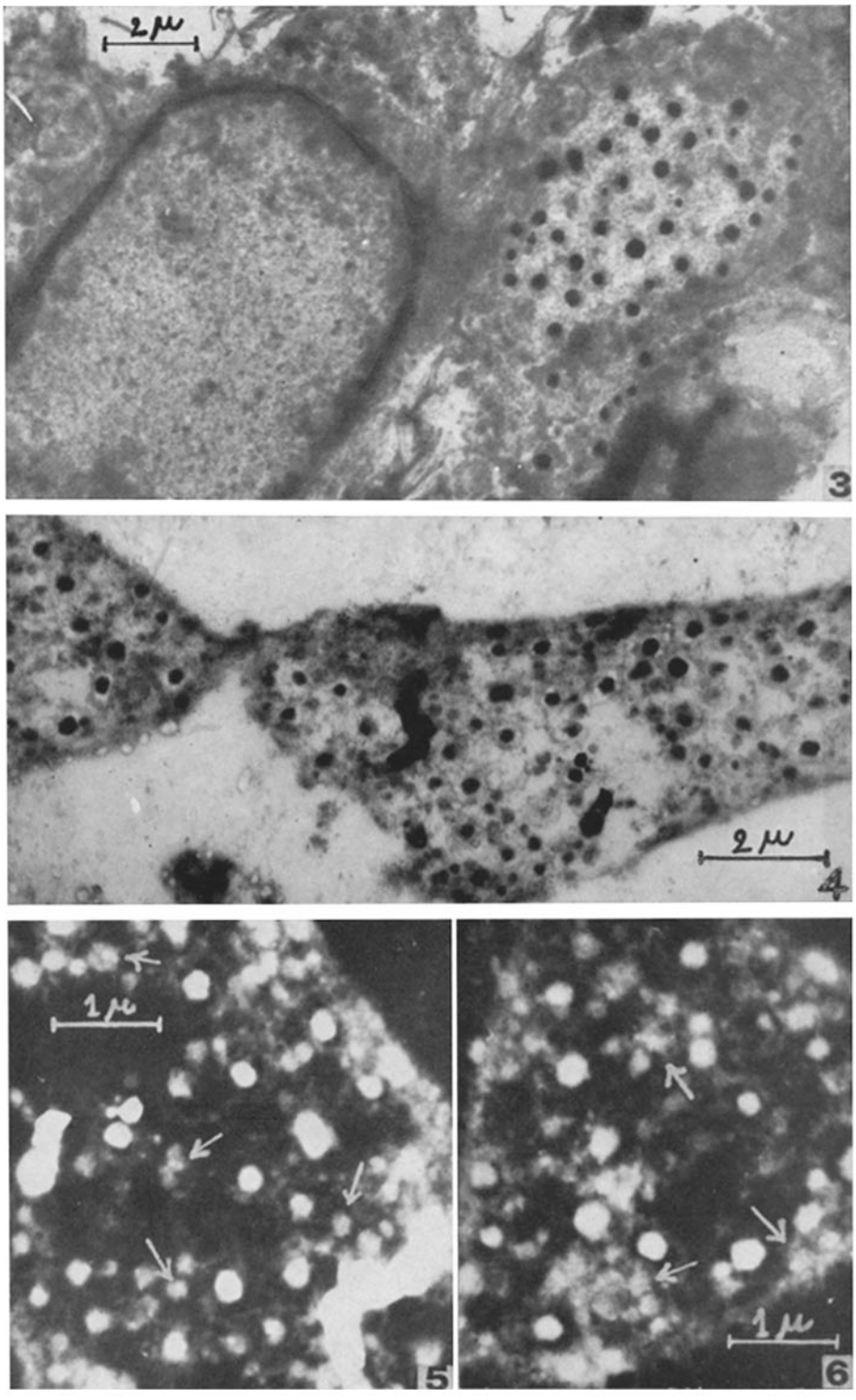
(Epstein *et al.*: Electron microscope study of infectious myxomatosis)

PLATE 10

FIG. 3. At the right, numerous particles are seen within the cytoplasm. × 6,480.

FIG. 4. Expansion of a stellate cell filled with particles of different sizes. Many of them are surrounded by a clear halo. The less dense ones show an internal constitution. × 8,680.

FIGS. 5 and 6. Enlarged negative print of Fig. 4. Homogeneous elementary bodies can be seen, together with less dense particles formed by the clumping of small granules. Arrows indicate particles in which internal constitution is best defined. × 15,450.



(Epstein *et al.*: Electron microscope study of infectious myxomatosis)