

ELECTRON MICROSCOPE STUDIES OF RABIES VIRUS IN MOUSE BRAIN

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ABSTRACT

The cells of brains of 2- and 3-day old mice infected with street rabies virus were examined in the electron microscope. It was observed that characteristic rod-like or elongated particles were found within a "matrix" in the cytoplasm of nerve cells and of astrocytes. These rod-like particles can be separated into two types, on the basis of slightly different morphological features. One particle is 110 to 120 $m\mu$ wide and has double-membraned coats; the other is 120 to 130 $m\mu$ wide and is covered by a single limiting membrane. The former is closely associated with the endoplasmic reticulum. The biological relationship between the two types is unknown, but both types of particles are considered to be street rabies viruses because of their structural features. It is believed that segmentation and branching of elongated particles may play a role in virus multiplication. Negri bodies appear as dense round bodies containing various coarse structures but no virus particles.

INTRODUCTION

Since the discovery of the filterable nature of rabies virus by Pasteur (1), there have been many studies on the epidemiology, immunology, and pathology of rabies virus. The biological properties of the virus, however, have remained poorly understood. One reason for this has been the lack, until recently, of a satisfactory tissue culture system for the virus. Successful cultivation of fixed rabies virus in hamster kidney cell cultures has now been attained (2, 3).

In a preliminary report (4), we described rod-like particles within nerve cells of mice infected with street rabies virus. Because of the regular size, form, and characteristic appearance of these particles, we considered them to be the virus.

The present paper expands upon the previous report and includes studies of the Negri body which is pathognomonic of rabies. A major difficulty in the study of Negri bodies has been the limited

number of them present even around Ammon's horn of the hippocampus, which makes it hard to find them in an electron microscope field. We have used a strain of virus which occasionally produces great number of Negri bodies around Ammon's horn in young mice.

MATERIALS AND METHODS

Two strains of street rabies viruses were used. The first has been described (4); the second, isolated from a naturally infected spotted skunk found in California, was obtained from Dr. Harald N. Johnson.

C₃H mice, 2 or 3 days old, were inoculated intracerebrally with about 10^8 LD₅₀'s of virus. The mice showed typical paralysis 10 to 12 days after inoculation. At this time, small pieces of Ammon's horn of the hippocampus were immediately fixed in 2 per cent buffered osmium tetroxide containing 5.4 mg CaCl₂/ml, refrigerated for 2 hours, dehydrated in graded alcohols over 2 hours, and embedded in Epon

812 or Araldite A. Thin sections were cut with glass knives on a Porter-Blum microtome and stained with uranyl acetate or lead hydroxide (5). All preparations were observed in a Siemens electron microscope.

Thick sections of resin-embedded and of paraffin-embedded brains were stained, respectively, with McNamara's modification of Giemsa (6) and with hematoxylin and eosin.

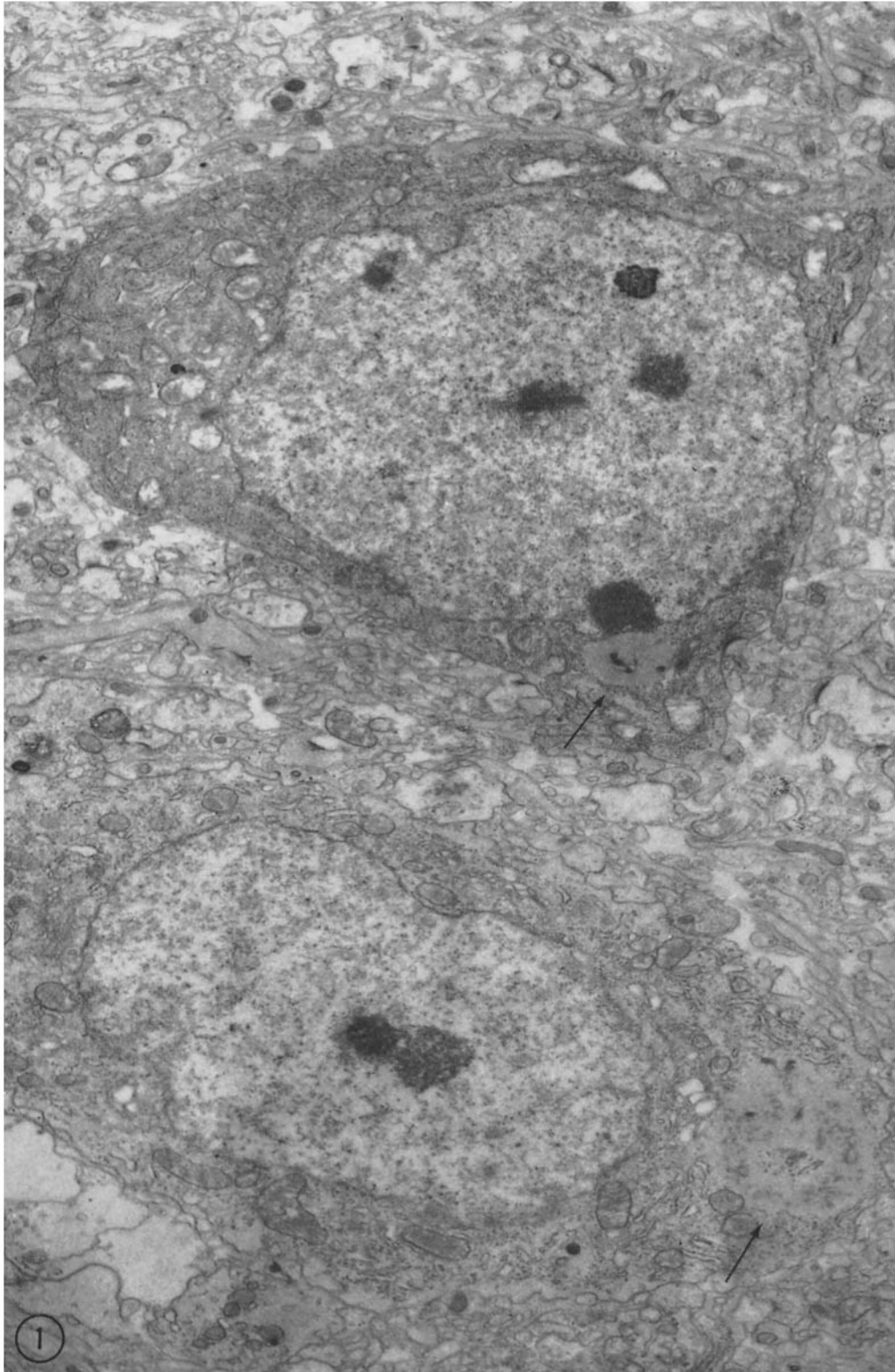
RESULTS

Fig. 1 illustrates two typical nerve cells, both with small foci of infection (see arrows). The nuclei are relatively large and oval, with little chromatin near the nuclear membrane and a few prominent nucleoli. The cytoplasm contains a well developed Golgi complex, many mitochondria, rough-surfaced endoplasmic reticulum of irregular disposition, and numerous ribosomes, most of which are free in the ground substance. The cytoplasm of the upper cell is dense, due to the large number of free ribosomes; these are fewer in the lower cell, and there is a homogeneous clear area around the peripheral zone of the cytoplasm. At the lower right of the upper cell and at the center right of the lower cell, respectively, unusual masses have replaced the cytoplasmic components. In other sections these masses are located in the peripheral zone of the cytoplasm and are of variable sizes and irregular shapes. They are homogeneous but contain a few dark particles. In the lower cell, around the mass are some vacuoles of varying sizes which seem to correspond to cytoplasmic vesicles. Since infection with both strains of virus produced masses having a similar fine structure, the following description applies to both, except where specific differences are pointed out.

Figs. 2 and 3 show the masses at higher magnification. The homogeneous ground substance of the mass, hereafter referred to as "matrix," is composed of relatively electron-opaque filamentous aggregates. As shown in Fig. 2, small vesicles, some clear and others poorly defined, are infrequently scattered within it. These presumably are cytoplasmic components. In Fig. 3, elongated rods of uniform width are seen cut at various planes within the matrix. As previously mentioned (4), two slightly different types of particles are recognizable. One is 120 to 130 μ in width and has a single limiting membrane and

a light interior. The other one is 110 to 120 μ in width and has a moderately dense peripheral space bounded by parallel double membranes. The interior of the rods exhibits a ground substance similar to that of the matrix. A round inner structure is found within an obliquely sectioned rod (upper central portion of Fig. 4); some inner structures of various shapes are also seen within the rods in Figs. 6 and 10. Occasionally the swollen rough-surfaced endoplasmic reticulum has rod-like structures within it, and though the structural detail is not clear, the rods seem to have uniform features. The rods that appear most clearly are shown in Figs. 5, 8, and 9. To the left in Fig. 5, completely surrounded by matrix, are two structures contained within a portion of swollen endoplasmic reticulum. Ribosomes are attached at some areas. These two structures show some similarity, especially as regards double membranes, to the rods which are embedded in the matrix. The interior of the rod at the right lower corner of Fig. 5 opens directly into the ground substance of the matrix. Fig. 6 shows one single- and two double-membraned particles (arrows) containing core-like structures. However, such core structures, which vary in shape and are of relatively low density, appear infrequently and seem to be the exception. Some rods, especially when viewed in cross-section, have an apparent third membrane closely apposed to the inner layer of the double membrane, as shown in Fig. 7. In Fig. 8, all the rods, although cut in various planes, clearly belong to the dense and double-membraned type of particle. In this section, in which there is a relatively high population of rods, the matrix is scanty. As indicated by arrows, the swollen endoplasmic reticulum contains a few rods near the matrix. These rods when seen in cross-section are entirely surrounded by the membranes of the endoplasmic reticulum. Their width, 90 to 100 μ , corresponds to that of the inner membrane of the double-membraned rod. Fig. 9 shows a clump of double-membraned rods with an extraordinary pattern. They are closely packed within the lumen of the endoplasmic reticulum which has a few attached ribosomes. No trace of matrix is visible. Several of the particles, seen in longitudinal section, are continuous at one end with the endoplasmic reticulum,

FIGURE 1 Two nerve cells. The cytoplasm of each cell contains a homogeneous "matrix" area (arrows) which has some dense particles. $\times 18,000$.



which sometimes extends into the interior of the particle. These particles are also approximately 90 to 100 $m\mu$ in width. Extremely long rods such as the one shown in Fig. 4 are rare.

A typical matrix containing several double-membraned rods is seen in Fig. 11. Here several rods cut in longitudinal section are clearly seen to branch, or join, and there is a round translucent "hole" at the point of branching or junction. The longest rod exhibits a well defined membranous structure, as well as a discontinuity (see arrow) that suggests the possibility of segmentation as one means of multiplication.

Fig. 12 shows a portion of a nerve fiber with matrix containing both double-membraned rods and in addition a few curved double-membraned structures. The latter are similar to the rods, with respect to their electron opacity and the distance between their parallel membranes, but they have ampulla-like enlargements, as indicated by arrows, that seem to be continuous with their limiting membrane. This type of structure is very prominent in the brain of a mouse inoculated with the California strain of virus which produced a great number of Negri bodies.

The double membranes seen in Fig. 13, at high magnification, run in different directions, and the ends of some of them are continuous with membranes of vesicles, which appear to belong to the endoplasmic reticulum. No well defined, uniform particle is visible.

In Fig. 14 some irregular zones of increased density are apparent in the matrix. Again these are associated with double-membraned structures, some of which occur in parallel at a width of about 110 $m\mu$, thus having much the same character as the uniform rods, and some of which are irregular. Evidence of a relationship between these structures and the endoplasmic reticulum is manifest in Fig. 15 where many curved double membranes are seen in the peripheral areas of the matrix.

Fig. 16, a photomicrograph of Ammon's horn, shows many Negri bodies within and near nerve cells. They are eosinophilic, but many of them contain strongly basophilic granules in their sub-

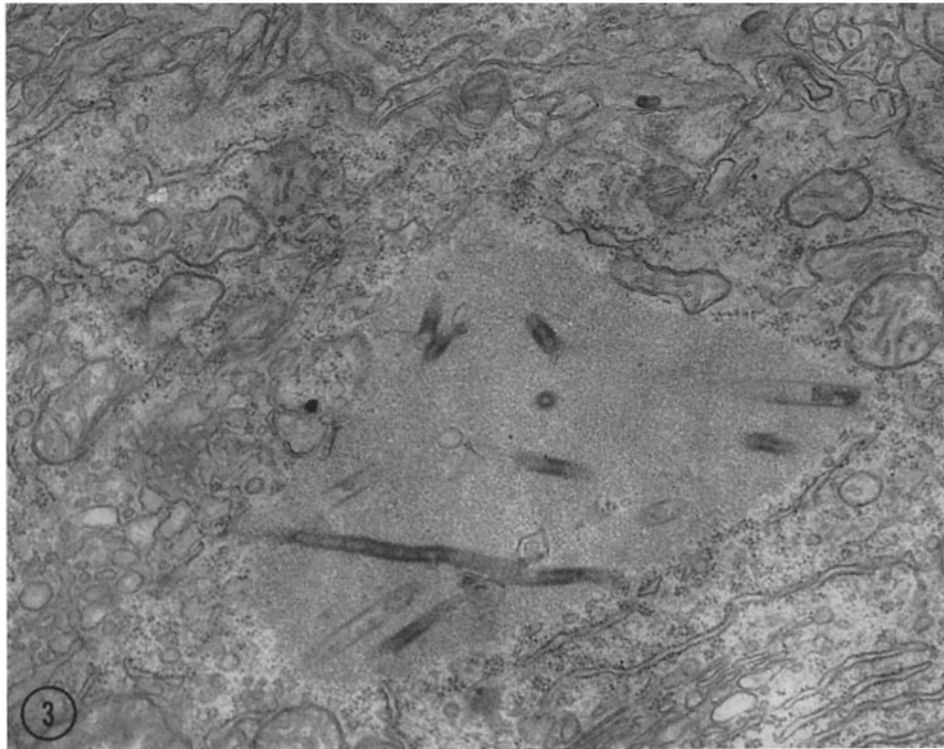
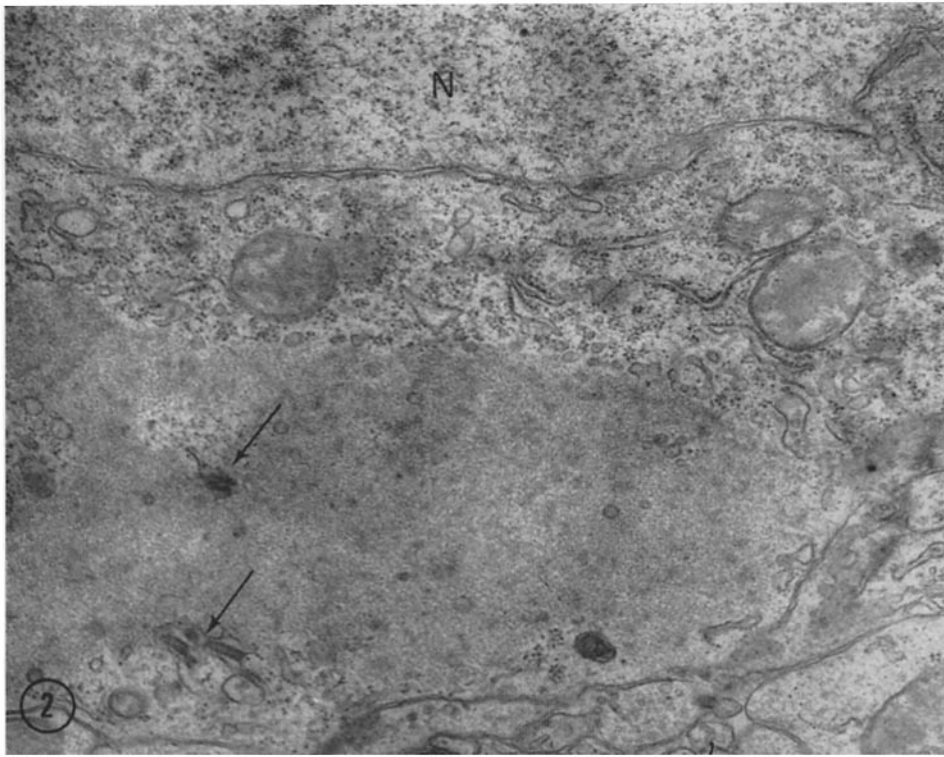
stance, especially in the large inclusion bodies. These granules are of irregular shape and are centrally or eccentrically located in the inclusion bodies. From this same brain, electron microscope sections have been obtained and are shown in Figs. 17 to 20. In Fig. 17, irregular membranous particles similar to those of Fig. 15 are present throughout the cytoplasm. The cell shown in Fig. 17 has a sharply outlined round body, located close to one matrix, that is covered by a limiting membrane and filled with dense fine granules and coarse substance. Despite the existence of a well developed Golgi complex and an abundance of endoplasmic reticulum, the classification of this cell is problematic because of the strange nature of the nucleus. In Fig. 18, a typical myelin-like structure close to the matrix is shown at the juxtannuclear portion of a nerve cell in which two oval shaped, electron-opaque inclusion bodies are visible. The body at the right contains many vacuoles, some of which are surrounded by dense granules, and although such small granules are not clearly detectable in the left one, other morphological resemblances are apparent: both have a homogeneous ground substance enclosed by thin double membranes and both contain many dense aggregates.

Figs. 19 and 20 demonstrate oval inclusion bodies with similar morphological features. They appear near mitochondria and other cellular components.

Two different kinds of cell are shown in Fig. 21. The upper portion of the figure shows ribosomes and rough-surfaced endoplasmic reticulum typical of a nerve cell, whereas the lower portion shows a rather opaque cytoplasmic ground substance and very few ribosomes; at the peripheral zone of the cytoplasm near the cell membrane some prominent sheaves of fine filaments identify this lower cell as an astrocyte. The left two-thirds of Fig. 22 shows portions of a cell with the same morphological features, especially the sheaves of fine filaments at the upper center portion. The cytoplasm of both these astrocytes contains typical virus matrices and shows rods cut in various planes.

FIGURE 2 The lower left portion of the cytoplasm is replaced by a matrix which contains many small vesicles, some indistinct and some clear. Arrows show rod-like particles in longitudinal sections. N, nucleus. $\times 23,000$.

FIGURE 3 At the center, a matrix contains many rods cut in various planes. $\times 23,000$.



DISCUSSION

Elongated rods, or virus particles, of two general types have been described in this paper. One of them is 110 to 120 $m\mu$ wide and has a double outer limiting membrane enclosing a relatively dense zone, as well as a fine, third internal membrane. The outer membrane of this particle is frequently connected with the surrounding endoplasmic reticulum. The other particle has a slightly greater width, 120 to 130 $m\mu$, a single limiting membrane, and a less dense interior. The latter particle has no demonstrable connection with the endoplasmic reticulum. Despite these differences, certain similarities exist between them: both particles appear within a characteristic matrix in the cytoplasm, and are frequently intermingled; both are rod shaped; and in both of them the ends are open to the ground substance of the matrix. They may, therefore, belong to a common category, but nothing to suggest a transitional stage has yet been observed.

A few studies on the street or fixed rabies virus particle have been published. A recent preliminary report, by Almeida *et al.* (7), which is of interest is that on the electron microscopic appearance of particles obtained from hamster kidney cultures infected with rabies virus which titered to $10^{-4.5}$. These authors describe the particles as being similar to the myxoviruses, but much more data will be needed before a final decision can be made.

Three groups of workers have reported on the size of this virus as estimated by the graded colloidion membrane method. Galloway *et al.* (8) and Yaoi *et al.* (9) estimate the size of the fixed virus to be between 100 to 150 $m\mu$; on the other hand, Levaditi *et al.* (10) report 140 to 210 $m\mu$ for fixed virus and 160 to 240 $m\mu$ for street virus. As suggested by Lépine and Sautter (11), it is possible that street and fixed viruses are of the same size and that the reported differences are due to vary-

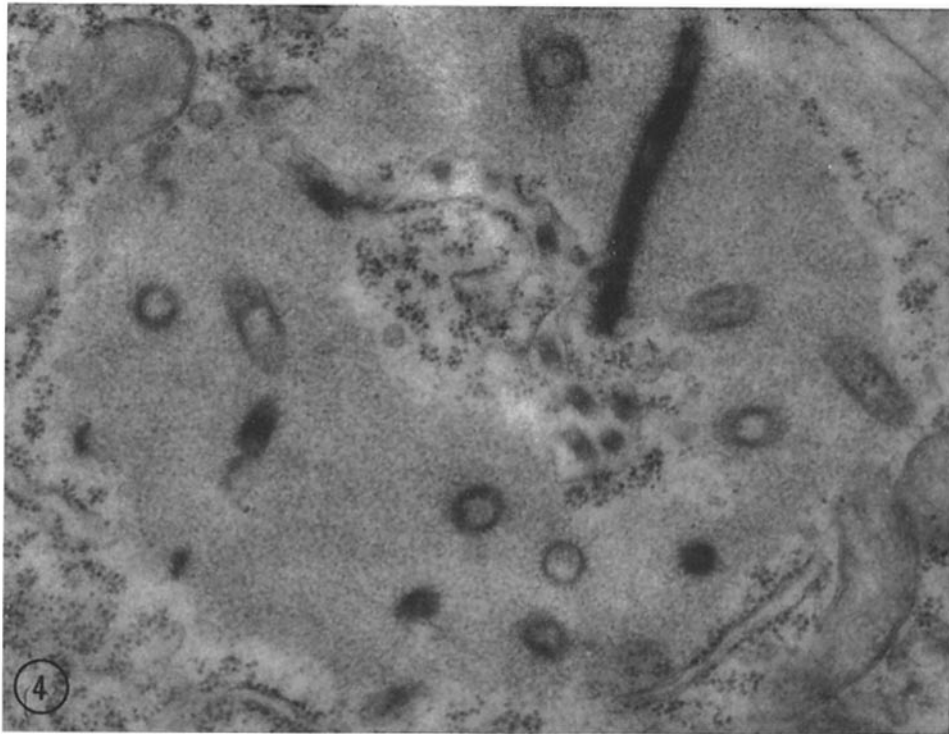
ing titers of the filtrates used for inoculation of the animals. Although we have no idea what the estimated size of the rod-like particle would be if it was measured by the filtration method, there is agreement between the above reported estimates and the width demonstrated in the electron microscope.

Other evidence in favor of the concept that the elongated particle is rabies virus is the constant association of a matrix with the particles. Many investigators (12-17) have indicated that some viruses, especially the large ones, produce special areas in which progeny viruses grow. These areas have been studied in detail by electron microscopy. The shape, electron opacity, and fine ground substance of the matrices produced by the pox viruses are the same as those of the matrices present in the rabies-infected cells. We have not found characteristic particles or matrix in uninfected control animals. Purified virus material must be obtained of course in order to make a final identification of the elongated particle.

The electron micrographs have shown that the outer membrane of the double-membraned particles is often connected with the membrane of the endoplasmic reticulum and that it presents the picture of a rod-like projection into the lumen. Such projections of cellular or intracytoplasmic membranes are well known as the mechanism of release of viruses such as influenza (18), Newcastle, mumps (19), western equine encephalomyelitis (20), vesicular stomatitis (21), visna (22), and many oncogenic viruses (23-29), even though there are certain differences among them. Some of the cylindrical forms of the Moloney agent (29) are very similar in appearance to the rabies virus particles seen in Fig. 9 (arrows). The mode of release of the presumed rabies virus, however, differs from that of the above mentioned agents in several ways: (a) the intracytoplasmic membranes with which the rabies virus particles are associated are

FIGURE 4 The ground substance of the matrix is composed of filamentous aggregates. The rod-like particles within the matrix can be classified as dense double membraned particles and single membraned ones with slightly larger width. The part central to the plane of section exhibits endoplasmic reticulum with attached ribosomes. Some dense rod-like particles are found within its lumen. $\times 37,000$.

FIGURE 5 A part of the matrix. Two double membraned rods, one of which (right lower) shows both ends opened to the surrounding matrix substance. An irregularly-shaped internal structure is visible within a single membraned particle at the right upper. Two dense particles are enclosed in an enlarged vesicle of the endoplasmic reticulum. $\times 116,000$.



mostly of the rough type, suggesting an origin in the endoplasmic reticulum; (b) the virus particles appear to be embedded in the matrix, and extrusion-like projections have not been found at the surface of the cell; (c) lastly, the particles within the lumen of the reticulum are smaller than those within the matrix.

As shown in Figs. 8 and 9, all particles within the lumen generally have a length of around 200 to 300 $m\mu$, which is much shorter than the long rods in the matrix. This suggests the possibility that these particles have been formed by the segmentation of long rods. In fact, a definite break in the continuity of one long particle has been photographed (4). The same kind of discontinuity is illustrated in Fig. 11. A similar feature has been demonstrated by negative staining of the long form of polyoma virus (30). Electron micrographs of budding formation have been obtained from meningopneumonitis virus (18, 31). In our studies, a formation that suggests a branching of the long rod-like particles also appears, as shown in Fig. 11. This evidence would seem to indicate, therefore, that some kind of segmentation of the elongated particle may play an important role in virus multiplication.

In Figs. 8 and 9, many virus particles are shown within the cytoplasm, but the matrix occupies only a relatively small area. Such an inverse relationship between the number of particles and the size of the area of matrix has been recognized in the case of ectromelia virus growth (13). In this case, studies of sequential stages of infection led to the interpretation that the matrix may be a source of virus material, and that it is gradually incorporated into the progeny viruses, which brings about a decrease in volume and density of the matrix. This interpretation is strongly suggested also by the present study, but it remains a working hypothesis, since a chronological study is not feasible on the mouse brain.

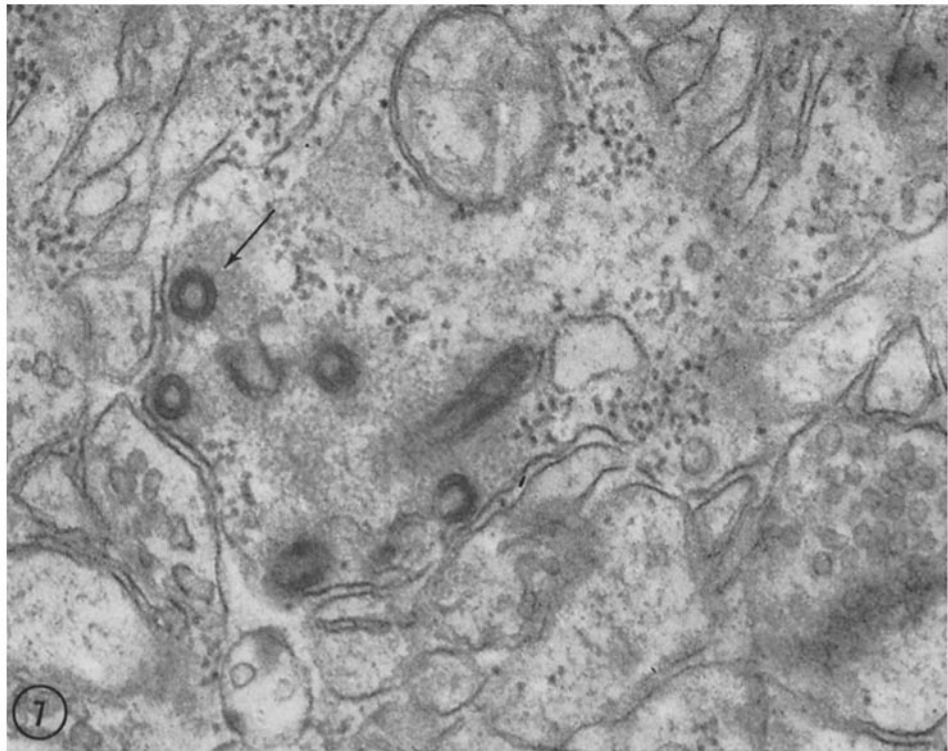
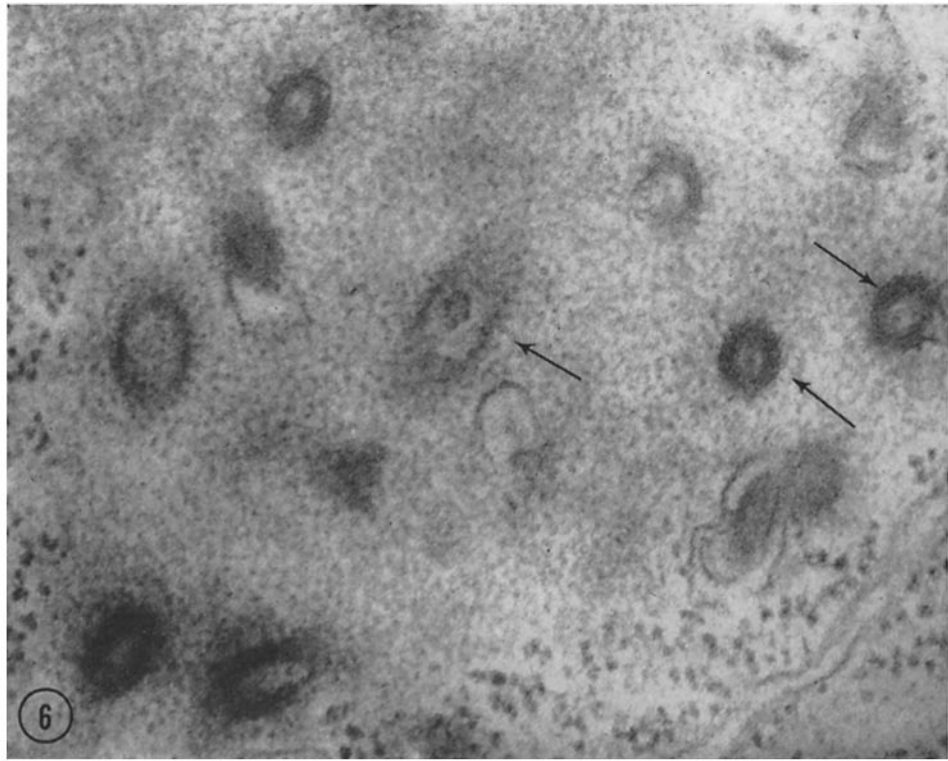
Since Negri (32) described the inclusion bodies characteristic of the street rabies virus infection, there have been a number of attempts to clarify the nature of the Negri body and its relationship to

rabies virus. Various morphological approaches, however, have not led to a satisfactory agreement. For instance, Hottle *et al.* (33) published the first reliable electron micrographs of Negri bodies but did not find the virus particles. With fluorescent antibody, Goldwasser and Kissling (34) found that the Negri body stained, which suggested its viral origin. When we first recognized the matrix area containing elongated particles in the nerve cell, the question of its relation to the Negri body was immediately raised. It is well known that the capacity of rabies virus to form inclusion bodies is usually lost after serial passage through experimental animals. This phenomenon is unique among many virus diseases, and the population of the Negri bodies is variable and not always high (35). The two strains of virus used in this study did not originally produce large numbers of Negri bodies, but, after intracerebral passage of the viruses in young mice, Negri bodies appeared around Ammon's horn in the hippocampus area of an occasional mouse. The matrix is not identical with the Negri body, since it appears constantly in infected brains, whether or not Negri bodies are present. In the electron micrographs, the dense and oval or round (presumed Negri) bodies look very much like the Negri bodies seen in the light microscope. They are not present in either control or infected brains which do not have Negri bodies. The bodies show only slight evidence of internal structure, they are commonly oval or round in shape, covered by a smooth limiting membrane, found near the matrix, and have a comparatively dense interior. They are similar to the Negri bodies shown by Hottle *et al.*, except that the latter have no outer covering membrane. It is quite possible that the preliminary paraffin embedding used in the study by Hottle *et al.* disturbed the morphology of the cellular structures and that the difference is between the bodies actually is negligible.

The appearance of two kinds of intracellular mass in rabies is reminiscent of pox virus infections. For instance, Marchal bodies which appear at the terminal stage of ectromelia virus infection are very

FIGURE 6 A part of the cytoplasm mostly replaced by matrix. Core-like structures can be found in three particles (arrows). $\times 65,500$.

FIGURE 7 A cluster of rod-like particles contained within the nerve fiber. A particle in cross-section (arrow) clearly shows the third inner membrane. $\times 62,500$.



similar to Negri bodies, especially under optical microscopy. Under the electron microscope, however, the Marchal bodies have a homogeneous ground substance, while Negri bodies contain some indistinct structures, one of which seems to be a degenerative cellular component. From some optical microscope studies of Negri bodies, it has been postulated that they contain characteristic basophilic granules, called "inner bodies," which give a weak positive reaction to basophilic dyes. Such basophilic granules have been found frequently within large extracellular Negri bodies in our materials, suggesting that this cytoplasmic in-

clusion body is derived from degenerative cellular constituents.

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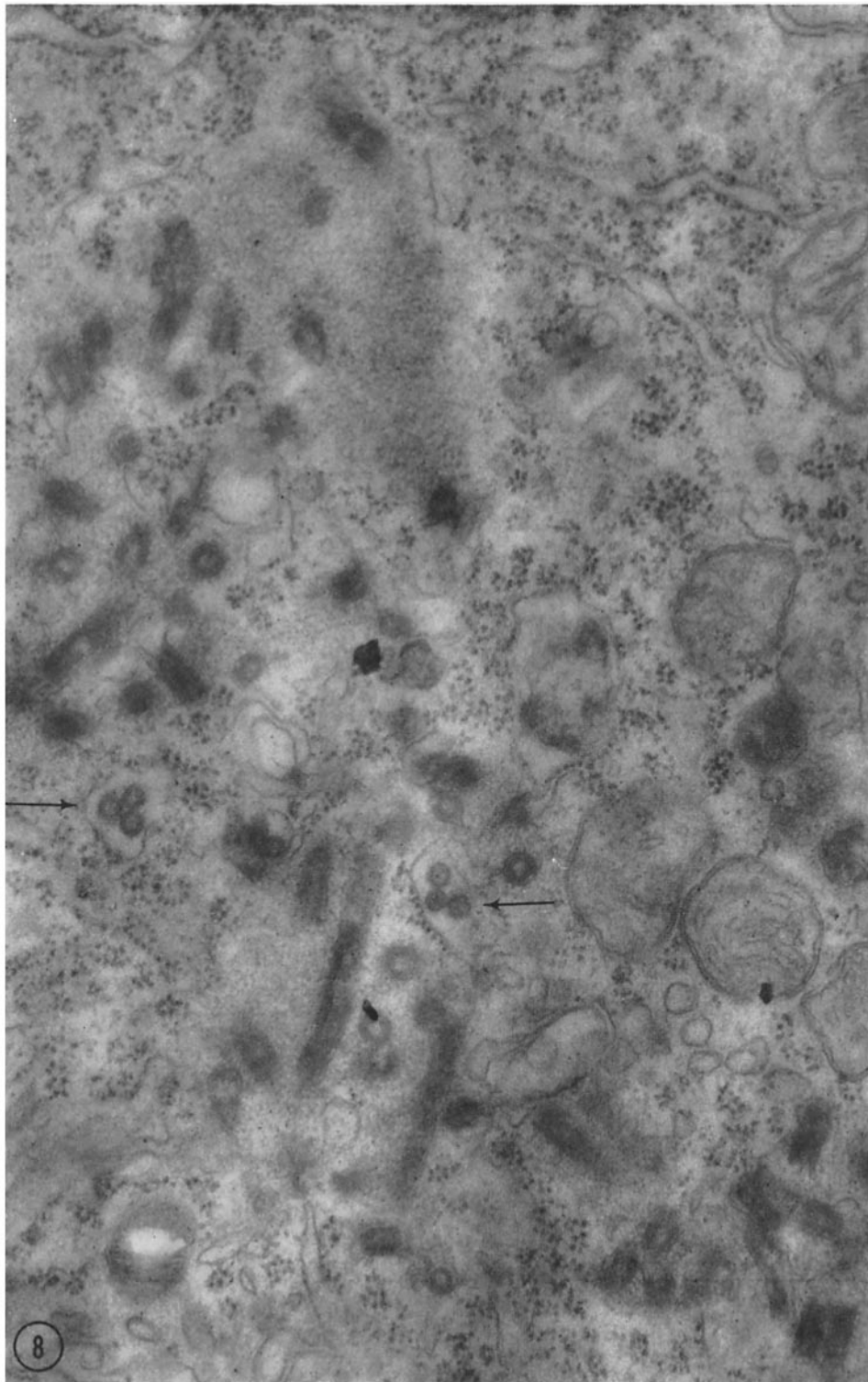
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FIGURE 8 Many double membraned particles are seen to be distributed within the cytoplasm, from the upper left to the lower right corners. The matrix occupies a relatively small area in the cytoplasm. Two groups of three particles (arrows), which are completely surrounded by membranes of the endoplasmic reticulum, have a width of 90 m μ . \times 43,000.



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FIGURE 9 A clump of double-membraned rods are closely packed within the lumen of the endoplasmic reticulum to which a few ribosomes are attached. The outer membranes of certain particles (arrows) are connected to the membranes of the endoplasmic reticulum. $\times 60,500$.

FIGURE 10 A part of a nerve fiber. Near the cell membrane is a matrix containing two single-membraned particles. Irregularly-shaped inner structures are visible within them. $\times 54,000$.

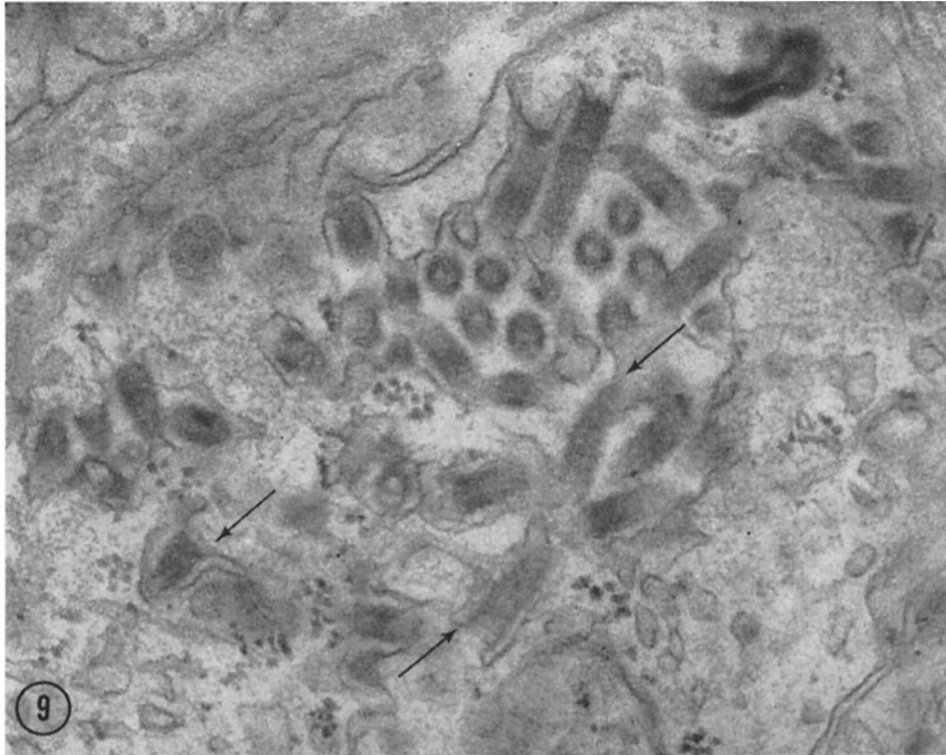


FIGURE 11 Matrices containing only rod-like particles with double membranes. One of the formations shows branching or joining of rods. A discontinuity of structure is visible (arrow). $\times 49,500$.

FIGURE 12 A part of a nerve fiber. It contains a few curved double-membraned structures, some of which are connected with the outer membranes of rods. $\times 44,000$.



FIGURE 13 Irregularly-arranged double-membraned structures within the matrix. The electron opacity of the space between a pair of membranes is relatively high. M, mitochondrion. $\times 103,000$.

FIGURE 14 The lower two-thirds of the figure is occupied by the matrix within which are some electron-opaque zones. Coincident with these are double-membraned structures. $\times 45,000$.

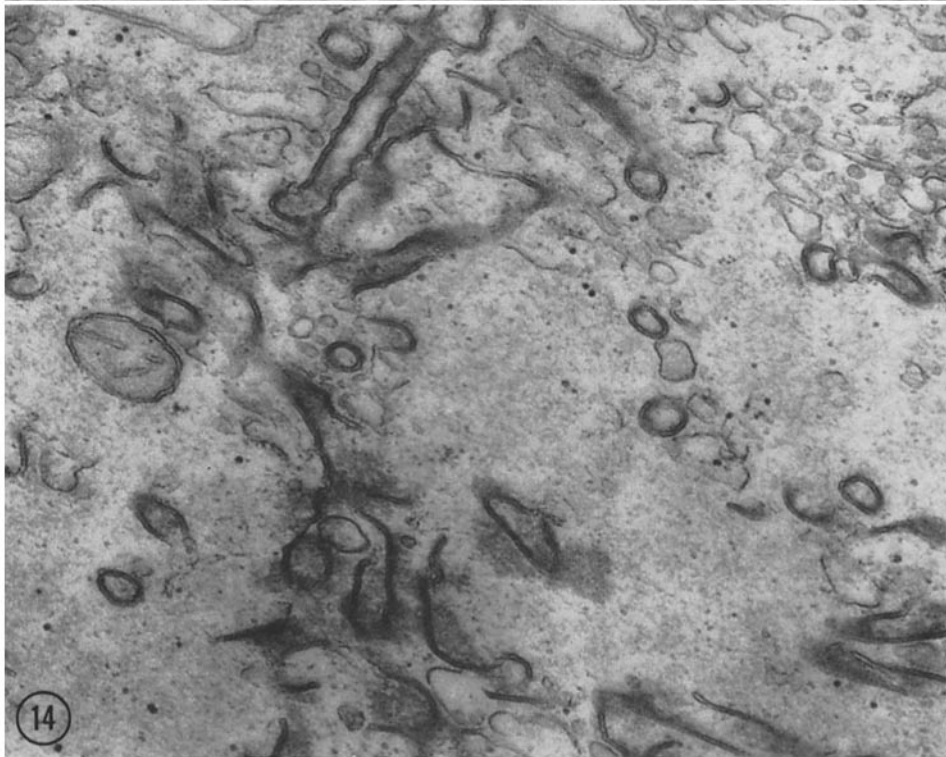


FIGURE 15 Numerous curved double-membraned structures which are similar to those shown in the preceding picture. Some of them are clearly connected with membranes of the endoplasmic reticulum. $\times 40,000$.

FIGURE 16 Photomicrograph of Ammon's horn in the hippocampus. Most of the large Negri bodies have dense internal parts which are strongly basophilic (arrows). $\times 1,000$.

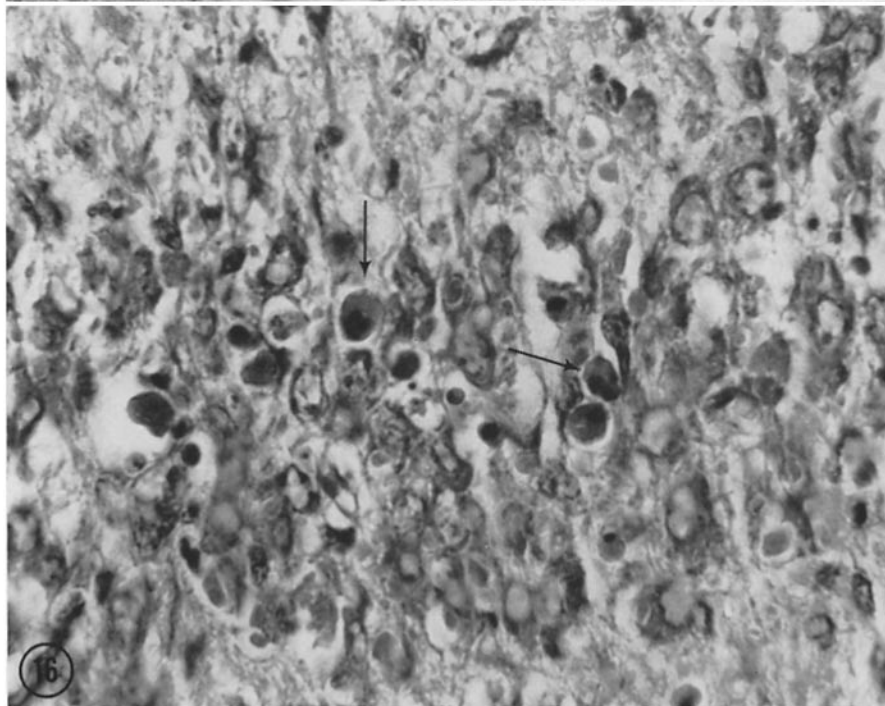
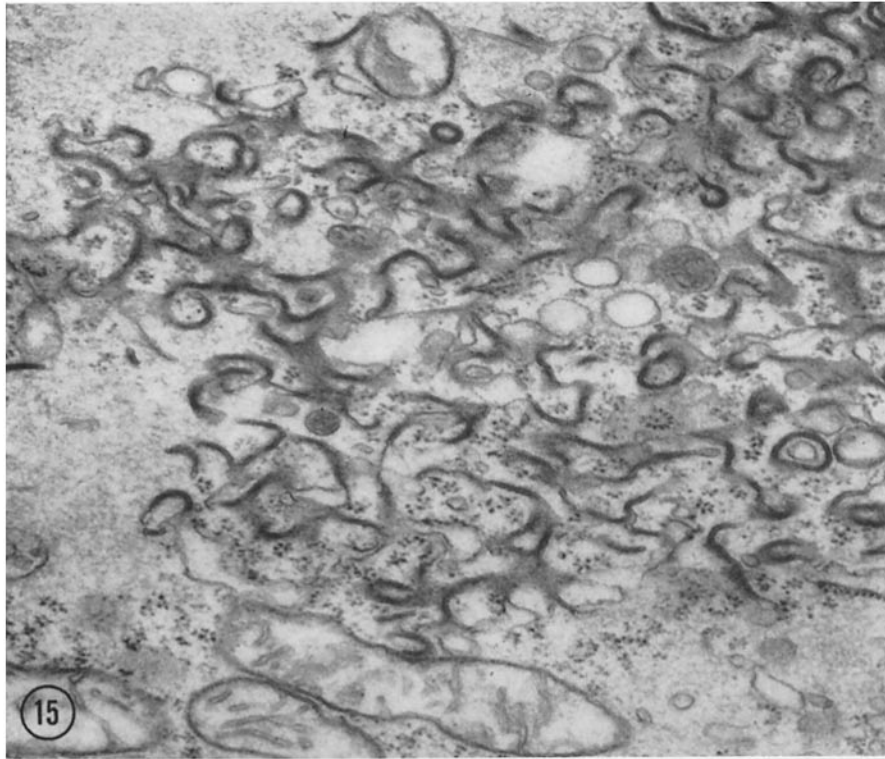


FIGURE 17 Two sharply outlined dense bodies are found at the upper part of the cytoplasm. A typical matrix is also present at either side of the nucleus of the same cell. \times 19,000.

FIGURE 18 Myelin-like structures located close to the matrix (*m*). \times 40,000.

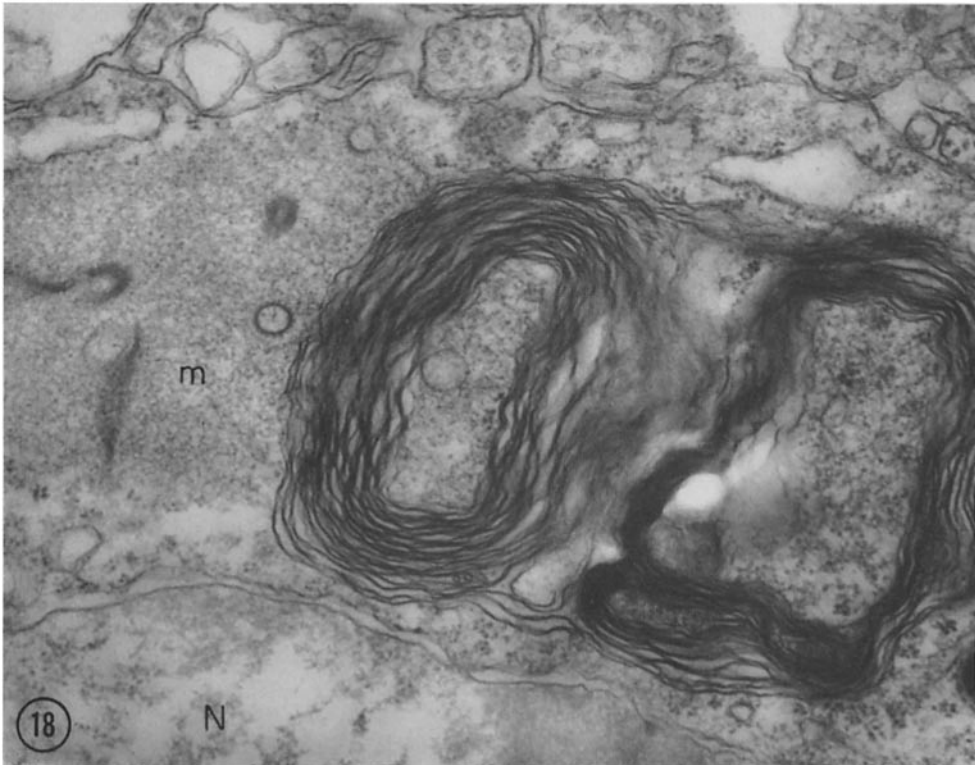
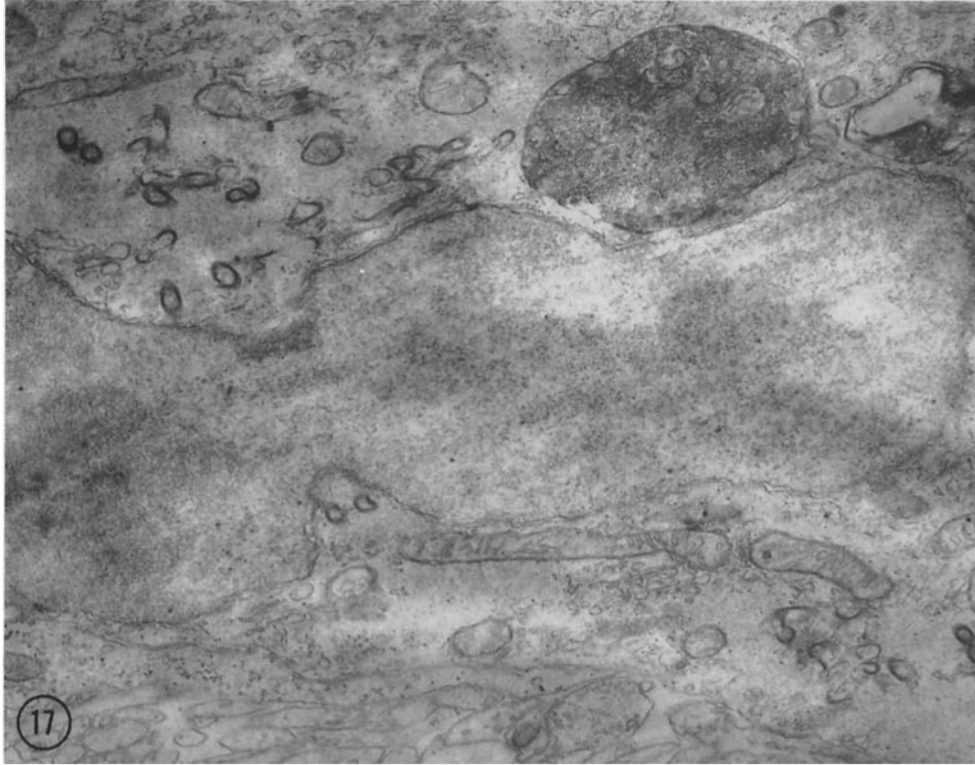


FIGURE 19 A portion of the cytoplasm of a nerve cell. Two oval inclusion bodies show irregular internal structures and are entirely enclosed by membranes. $\times 14,000$.

FIGURE 20 An oval body showing features very similar to those of the bodies in Fig. 19. $\times 20,500$.

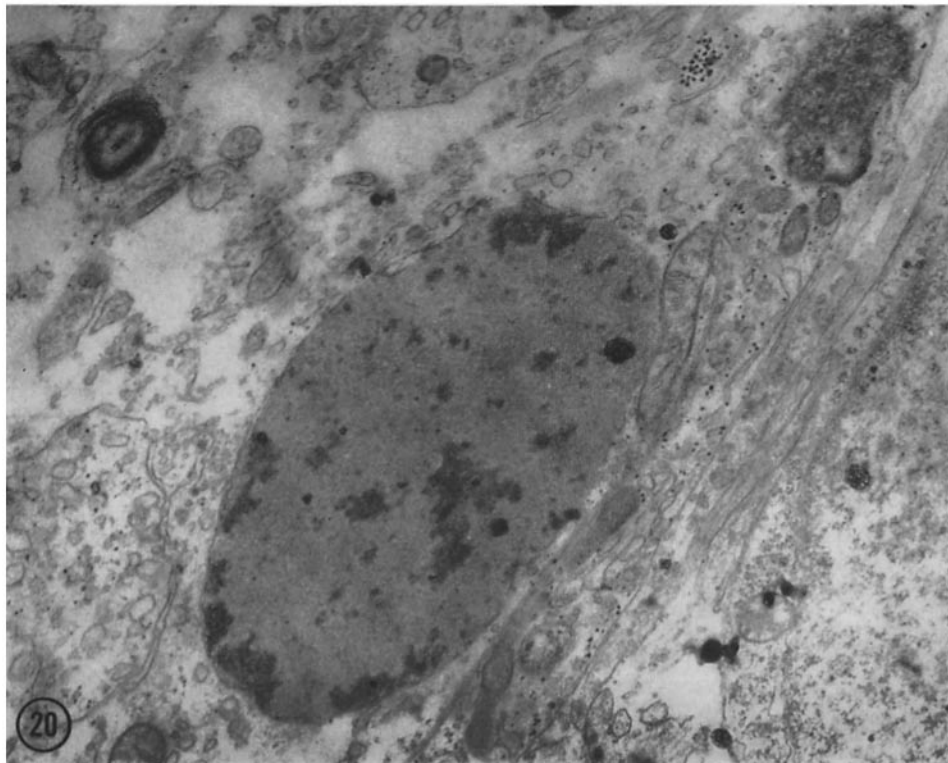
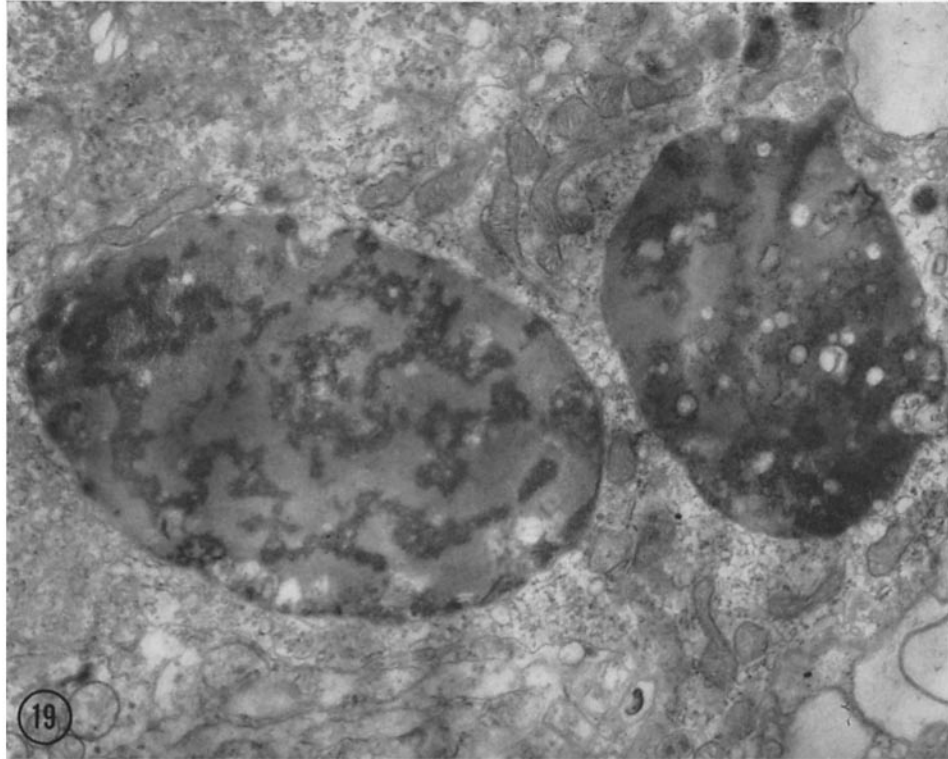


FIGURE 21 The upper third shows part of a nerve cell. The lower two-thirds is considered to show part of an astrocyte. In the cytoplasm of the astrocyte a typical matrix area containing two double-membraned particles is visible. $\times 35,000$.

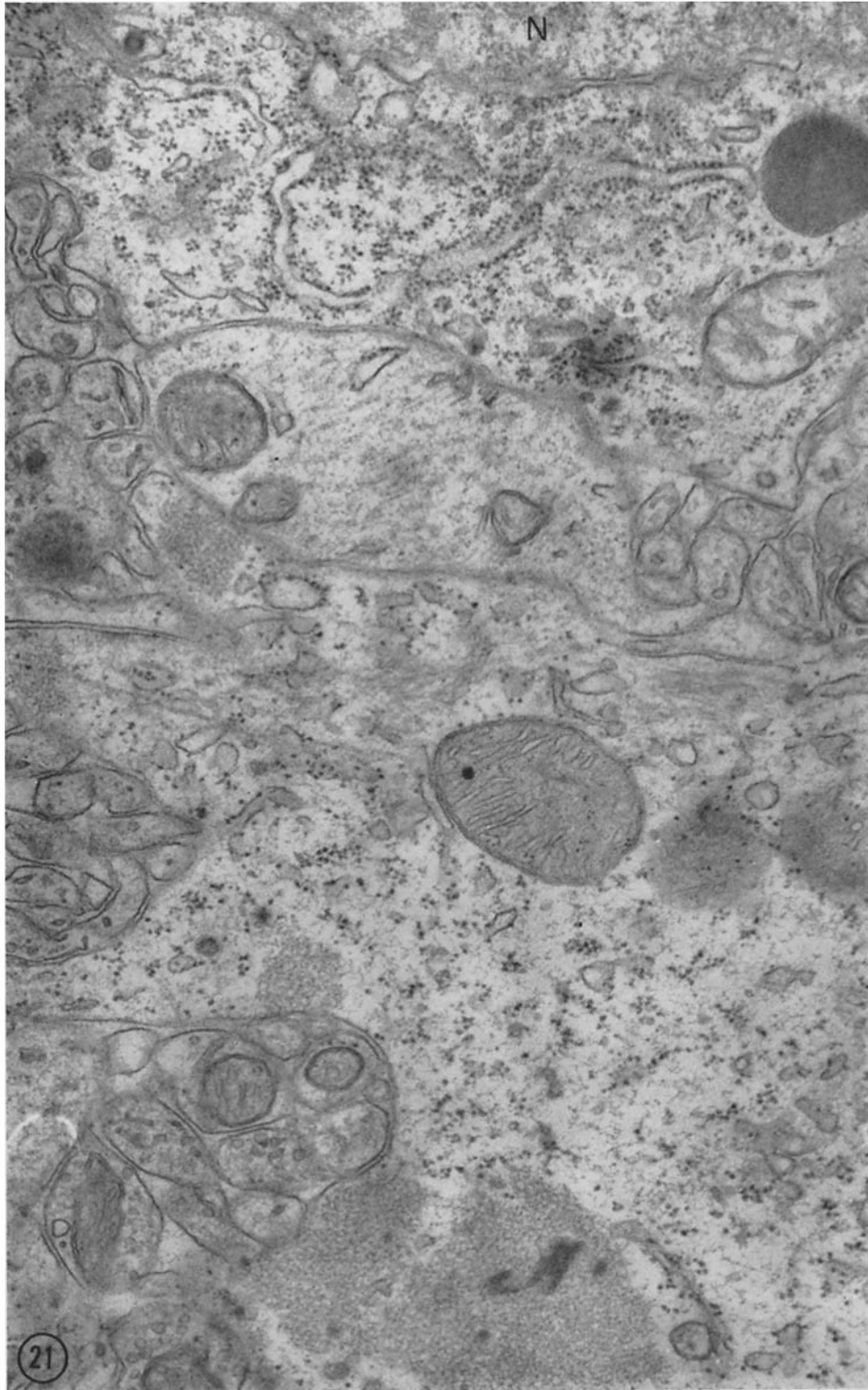


FIGURE 22 The left two-thirds is made up of the cytoplasm of an astrocyte. Sheaves of fine filaments are prominent at the upper center part. A typical matrix can be seen at the right lower corner. $\times 20,000$.

