AN ELECTRON MICROSCOPE STUDY OF NERVES INFECTED WITH HUMAN POLIOMYELITIS VIRUS*

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Plates 26 to 28

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The view that the poliomyelitis virus is transmitted centrally by way of nerve axons is supported by substantial experimental evidence (1–6). Particularly pertinent are the experiments in which infection was secured by exposure of the end of a sectioned nerve to a suspension of active virus (6–7). In all such experiments the criterion of infection is either paralysis or the occurrence of lesions in the affected nuclei of the central nervous system.

The relative rapidity of the central transport of the virus (2.4 mm. per hour according to Bodian and Howe) suggests that the microstructure of the axon may be important in the colloidal phenomena concerned with this process. It was therefore considered profitable to study infected nerves with the electron microscope. Thus far electron microscopy has been applied to the study of nerve structure chiefly by means of the fragmentation technique. In fragmented preparations of various types of vertebrate and invertebrate nerves the present authors observed fibrous structures which characteristically have dense edges (8). Because of this appearance the fibrils were tentatively called "neurotubules." Such structures had not previously been observed in other tissues and reasons were given for supposing that they may be axonic in origin.

Although the fragmentation technique, as applied to whole nerves, makes impossible the localization of structures to particular regions of the nerve fiber and also involves severe sampling difficulties, it appeared to be the only method applicable to the problem at the present time. In the experiments described in the present paper attention was focused upon the neurotubules. Some of the observations have been briefly reported previously (9).

Methods

The infections were made by Professor Harold K. Faber and Miss Rosalie J. Silverberg, of the Department of Pediatrics, Stanford University School of Medicine. After dissection

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and fixation the nerves were sent to this laboratory for examination. The authors are most grateful for this generous collaboration.

Under nembutal anesthesia, sciatic nerves of *rhesus* monkeys were transected and the ends of the proximal stumps dipped for 10 to 15 minutes in a 20 per cent suspension of CAM strain of human poliomyelitis virus isolated by the Stanford investigators. This strain proved to be relatively mild, having low invasiveness. After periods varying from 3 to 72 hours the infected nerves were dissected and fixed in 10 per cent formalin in mammalian Ringer solution at 4°C. The contralateral uninfected nerves were kept as a control. Other animals, similarly infected, were allowed to survive as controls to prove the virulence and transmissiblity of the virus. In most cases the inoculation was insufficient to produce paralysis, though the diagnosis of subclinical infection was confirmed by histological analysis of the spinal cord which showed lesions in the lumbar region which were most intense on the infected side.

Recently, similar preparations were made using the Wis.'45[1] strain of human poliomyelitis virus. This strain is highly virulent and invasive, producing paralysis in most of the control animals. A 50 per cent suspension was used, as compared with the 20 per cent suspension for the CAM strain. Also the dipping technique was somewhat better developed than that used with the CAM strain.

Controls were also made by dipping sectioned nerve ends in suspensions of normal spinal cord containing no virus.

The formalin-fixed nerves were cut into serial segments each about 10 mm long. The easily detached connective tissue was removed under the wide-field microscope and the segments were cut in 10 μ transverse sections with the freezing microtome. The sections were then fragmented by placing the suspension in contact with the nickel rod of a 9 kc. magnetostriction oscillator for 3 to 5 minutes. The sonic treatment was adjusted so as to avoid unnecessarily great disintegration of the material. After centrifugation for 2 to 3 minutes in a clinical centrifuge at about 1,000 R. P. M., preparations for electron microscope examination were made from the supernate without staining. The preparations were washed by treatment with water on the grids or by immersing the grids in distilled water. This was necessary in order to achieve sufficient resolution of the structure of the neurotubules.

To obtain uniformity of results all nerve segments were weighed (after removal of connective tissue) and diluted with a standard amount of distilled water—about 100 times the weight of nerve. The amount of fragmentation necessary varied from sample to sample, the criterion being the liberation of an abundance of fibrils in the supernate. To secure reasonable sampling of the material in each nerve segment some 20 to 30 grids of each supernate were prepared and scanned in the electron microscope. Electron micrographs were made of those regions judged to be significant. Obviously such a procedure involves subjective factors which are unavoidable but which were minimized in importance by exhaustive visual study of each specimen.

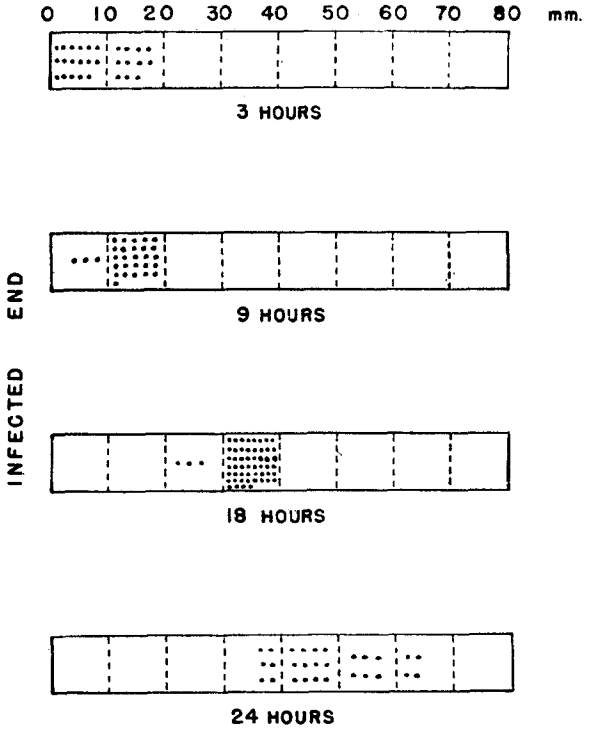
Most of the observations were made with an RCA Type EMU electron microscope equipped with a biased gun, though some of the observations, particularly of the Wis.'45 strain material, were made with an RCA Type EMB electron microscope.

RESULTS

In properly cleaned preparations the neurotubules from uninfected control nerves have relatively clear axial regions (Fig. 2) or show a faint periodic cross banding. This is true also of the great majority of neurotubules from infected

[1]This strain was sent to Professor Faber by Dr. Paul F. Clark.

nerves. However, a relatively small number of neurotubules show characteristic dense particles usually within the borders of the neurotubules. This is generally an all-or-none phenomenon. If particles are present at all on a particular fibril they are usually present in fair abundance throughout the extent visible on the grid.



TEXT-FIG. 1. Diagrammatic representation of the relative density of population of particles found along nerves infected with CAM strain of poliomyelitis virus.

Nerves were cut into serial segments, each 10 mm. long. Times of fixation after infection were 3, 9, 18, and 24 hours. Spots represent numbers of neurotubules containing dense particles found in each segment.

The dense particles are usually aligned in an irregular pattern within the dense edges of the neurotubules (Fig. 1), though in some cases the particles occur in the axis of the neurotubule and are disposed in a very regular pattern, possibly related to the periodic structure of the tubule (Fig. 5).

The particles vary considerably in size, shape, and density (Figs. 3, 4, 6, and 7). The width in the chief axis varies between about 150 and 500 Å. Measurement of 100 particles showed that about 70 per cent of the particle diameters lay between 250 and 350 Å with a mean of 330 Å. They may appear as dense

spots, rods, or ovoids having clear centers and dense edges. Such clear centers might well be due to action of the electron beam from the biased gun.

In most cases of material from nerves infected with CAM strain virus, of which micrographs were obtained, the dense particles are situated within the confines of the dense edges of the neurotubles (Fig. 1). In some cases the particles occur at fibrils edges and in the field about the fibrils. This is especially true in end segments of nerves fixed after only 3 hours' infection which are somewhat traumatized by the transection.

The position along the nerve at which the dense particles were found seems to be related to the length of time after infection at which the nerves were removed and fixed. The relative abundance of the particles in the serial segments is illustrated in Text-Fig. 1 which is based on a single series of experiments. Particles were found as far as 65 mm. from the nerve end 24 hours after infection. In two cases no particles were found, within the 65 mm. of nerve available, when fixed 48 hours after infection.

The general picture observed in material from nerves infected with virus of the Wis. '45 strain is similar to that of the CAM-infected nerves except that there was considerably more dense material of quite variable size and shape (Fig. 8); also the particles are found on and about the neurotubules rather than within their edges as seemed to be the case with the CAM-infected nerves. In two nerves[2] fixed 24 hours after infection the dense particles, though particularly abundant, were distributed along the length of the nerve in a manner somewhat similar to that of the CAM-infected nerves as illustrated in Fig. 8. Thus, although the relationship of the dense material to the neurotubules is much less obvious in the nerves infected with the Wis. '45 strain, the distribution of the dense particles along the nerve bears a relation to the time after infection similar to that found for the CAM-infected nerve.

DISCUSSION

The physical nature and the location of the neurotubules in the nerve fibres have an important bearing on the interpretation of the present observations on infected nerves. However, recent experiments indicate that these problems are more complex than was at first assumed. It seems best to consider the present observations without reference to the supposed axonal location of the neurotubules.

[2]In these particular cases the sectioned nerves were dipped in 25 per cent virus suspension in M/15 KCl solution. This variation was employed to test the possibility of aiding the migration of the virus by depolarization of the cut nerve end with KCl. The number of dense particles observed in these nerves appeared to be greater than in other nerves. However, more experiments would have to be performed to determine whether the effect is due to the presence of the KCl.

The evidence suggests that the dense particles described above are associated in some way with the process of infection of the nerves with the poliomyelitis virus. It is true that a certain amount of amorphous and particulate material may be observed even in preparations of normal nerves, depending upon the procedure used, and that the relative numbers of neurotubules showing dense particles in preparations of infected nerves, particularly with the CAM strain, are extremely small compared to the number of neurotubules which are devoid of particles. However, when particles are found in neurotubules the picture is qualitatively quite different from that observed in neurotubules from normal nerves (Fig. 2). Particularly important is the fact that the spatial distribution of the particulate material along the nerves varies with the time after inoculation in a manner which indicates that the morphological changes are associated with the central movement of the infection (if Bodian and Howe's figure of 2.4 mm. per hour be assumed to be approximately correct).

Negative results were obtained with nerves sectioned and dipped in a suspension of spinal cord containing no virus. Hence, the phenomenon is probably not due to trauma from the sectioning or to materials in the emulsion other than virus.

Assuming that the dense particles are associated in some manner with the infective process, two possibilities suggest themselves regarding their origin: (1) they may result from inflammatory or other reaction of the nerve fibers to the passage of the virus; (2) they may represent the virus particles themselves. These possibilities may now be examined.

The relative abundance of dense particles, in preparations from the Wis. '45 strain infections, occurring both upon the neurotubules and in the surrounding fields of the electron micrographs might suggest that the material may result from alteration of the nerve substance in the area of infection. The structure of the neurotubules themselves does not appear to be demonstrably altered under these conditions. However, this hypothesis encounters the difficulty that the neurotubules from regions distal to the wave front of the infection, *e.g.* over which the infection has already passed, may show little departure from normal structure.

Identification of the particles with the virus itself would seem possible as far as their size is concerned. Physical chemical studies indicate that the virus diameter is probably less than 500 Å; ultrafiltration experiments suggest a size range of 80 to 170 Å (10). However, there is no satisfactory standard of comparison from electron microscope data upon which to base such an identification. Electron microscope studies have been made of purified preparations of animal and human poliomyelitis virus including the SK strain (11), the MV strain (12), the Lansing strain (13), and the MM virus (14). The general conclusion is that the virus particles probably have diameters ranging from

100 to 350 Å. Unfortunately, even in relatively highly purified virus preparations, there remains a considerable contamination of macromolecules and particulates derived from the nerve tissue from which the virus was obtained. Until the virus is crystallized or otherwise quantitatively separated from contaminating nerve material it will probably be extremely difficult to demonstrate conclusively the morphology of the virus particles with the electron microscope.

It might be pointed out that, to account for the spatial distribution within the nerve (Text-fig. 1), the supposition that the particles are directly related to, or actually are, the virus particles themselves requires the assumption that the virus is transported centrally, largely independent of an actual multiplication.

In view of the difficulties of interpretation, it is perhaps best for the present not to speculate beyond the fact that the morphological changes described appear to be directly associated with the advancing virus infection. Identification of the dense particles, which are the chief feature of the present observations, must await more definitive evidence.

SUMMARY

Sciatic nerves of *rhesus* monkeys infected with CAM and Wis. '45 strains of human poliomyelitis virus were fixed in formalin, sectioned, fragmented, and examined in the electron microscope.

Most of the neurotubules of nerves infected with the CAM strain have normal appearance but a very small number show the presence of dense particles irregularly aligned within the edges of the neurotubules. The diameters of the particles range between 160 and 500 Å, the mean being 330 Å. The particles were found in regions along the nerve which varied with the time after infection, indicating a central movement of the morphological alteration of the order of 2 mm. per hour.

Relatively abundant dense particulate material was found in nerves infected with Wis. '45 strain virus and the particles were chiefly attached to the edges of the neurotubules and in the adjacent areas of the field.

The dense particles appear to be associated with the virus infection but no further characterization is possible at this time.

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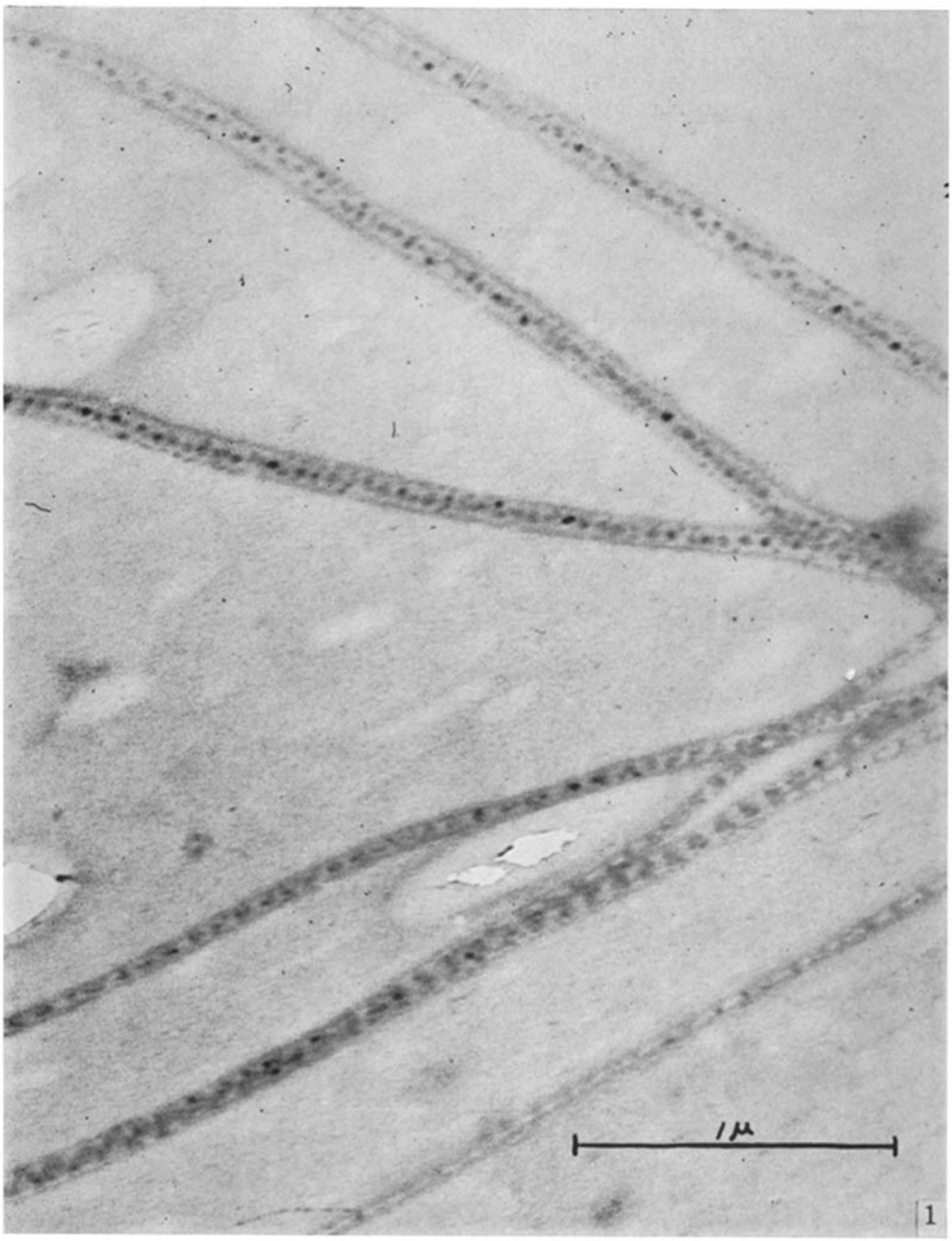
EXPLANATION OF PLATES

PLATE 26

FIG. 1. Neurotubules of a *rhesus* monkey infected with human poliomyelitis virus, CAM strain. 18 hours after inoculation. × 45,000.



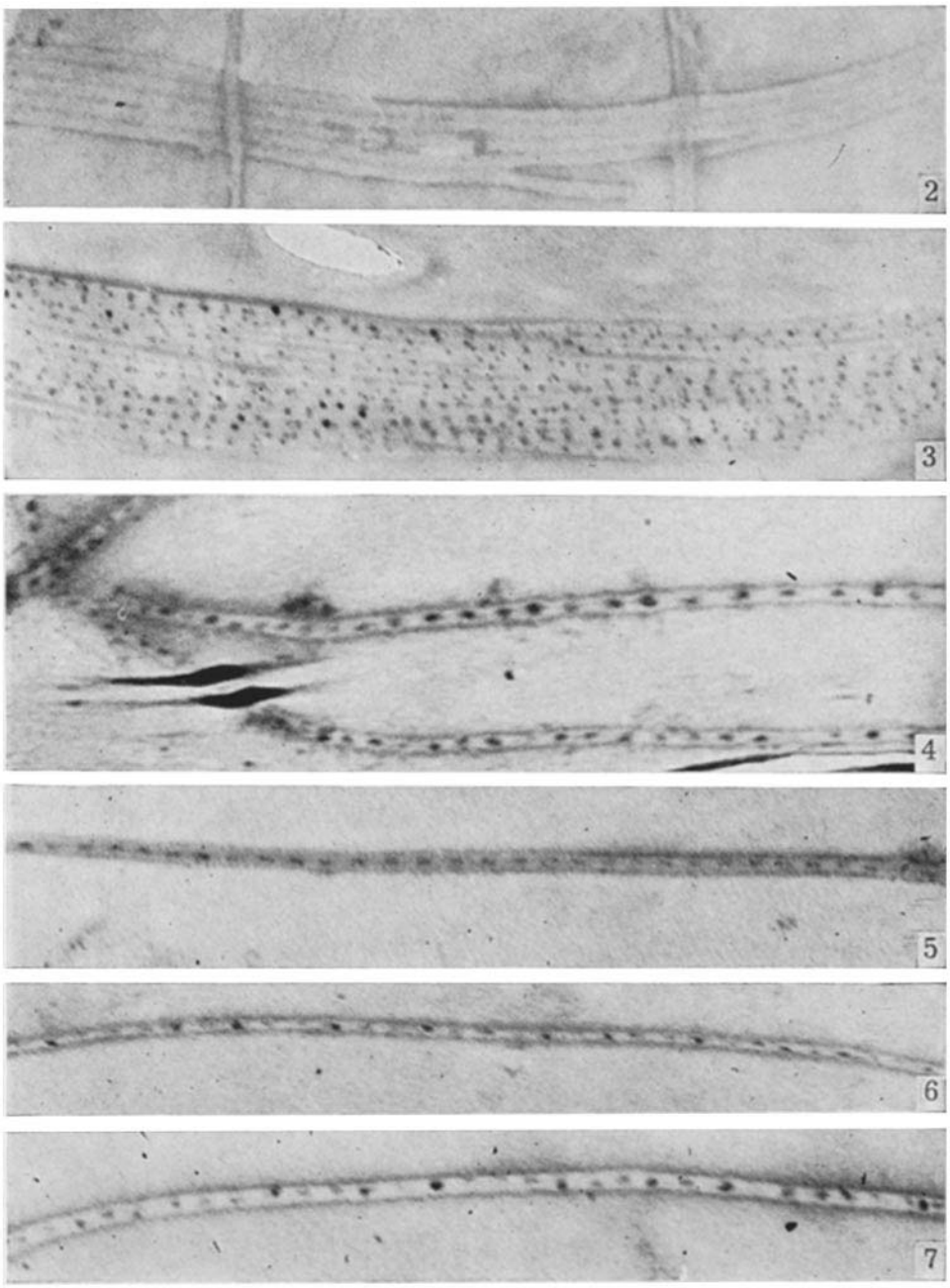
(De Robertis and Schmitt: Nerves infected with human poliomyelitis virus)

PLATE 27

FIG. 2. Bundle of neurotubules from a normal sciatic nerve of *rhesus* monkey. × 34,000.

FIG. 3. Bundle of neurotubules of a nerve infected with human poliomyelitis virus, CAM strain, 18 hours after inoculation. × 34,000.

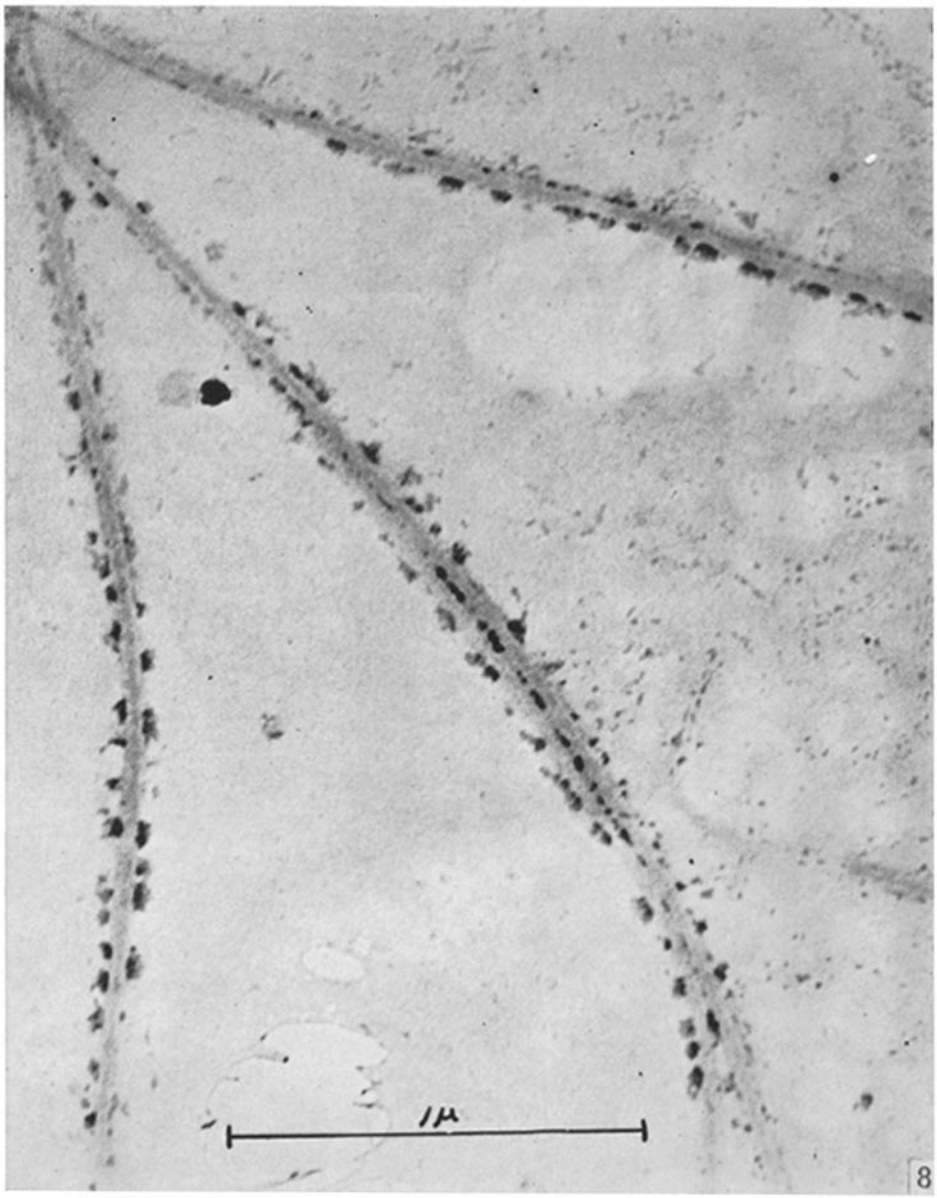
FIGS. 4, 5, 6, 7. Neurotubules of a nerve infected with human poliomyelitis virus, CAM strain, 24 hours after inoculation. × 45,000.



(De Robertis and Schmitt: Nerves infected with human poliomyelitis virus)

PLATE 28

FIG. 8. Neurotubules of a *rhesus* monkey sciatic nerve infected with human poliomyelitis virus (Wis. '45 strain). × 55,000.



(De Robertis and Schmitt: Nerves infected with human poliomyelitis virus)