The Early Changes in the Axoplasm during Wallerian Degeneration

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ABSTRACT

Axoplasmic changes were studied in the saphenous nerve of the albino rat during the early stages of Wallerian degeneration. The axons were examined at 0, 24, and 48 hours after the surgical transection of the nerve. The material was fixed in 2 per cent OsO_4 in phosphate buffer (pH 7.2-7.5) with sucrose (added to a final osmolar concentration of ~0.37 M).

The earliest changes were seen in the endoplasmic reticulum which became fragmented into rows of small vesicles. Then, between 24 and 48 hours, the neurofilaments underwent complete disintegration and the axoplasm became filled with finely granular material which later formed irregular clumps surrounded by a structureless matrix, probably fluid *in vivo*.

The fragmentation of the neurofilaments was accompanied by pronounced swelling of the mitochondria.

INTRODUCTION

The phenomena accompanying the degeneration of nerve fibers after sectioning have been studied with a great variety of techniques. (For reviews of these studies see references 1-3). The anatomical events involved in degeneration are relatively well known at the resolution level of the light microscope, but these same studies have been rather unrewarding in so far as the early phases of the degenerative process are concerned. The only alterations found before the fragmentation of the myelin are in the staining properties of the axons, and these changes are difficult to correlate with any of the accompanying biochemical or physiological changes. It seemed important therefore to study the degenerating nerve with the electron microscope with the intent of characterizing the early changes in the axoplasm which accompany the cessation of conduction of stimuli along the

nerve fiber. It was thought that a correlation of anatomical and physiological alterations in this period might yield important clues to the functional significance of the different axoplasmic constituents.

As an initial step in this line of research, the saphenous nerve of the rat was studied under the electron microscope at successive intervals during the degeneration process. The present report is concerned with changes in the axoplasm from the normal state until its morphological disintegration, which under the experimental conditions used, was found to occur at about 48 hours after the severance of the nerve.

Material and Methods

Twelve young albino rats of both sexes were used. With the animals under ether anesthesia and while observing aseptic precautions, the saphenous nerve was exposed and sectioned with a clean scissors stroke. The animals were divided into two groups and studied 24 or 48 hours after the operation. The fixation was performed by exposing the peripheral stumps of the severed nerves and flooding them with the fixative. This was allowed to act for 2 to 4 minutes *in situ*. A small segment of nerve, about 5 mm. in length was then excised at about 20 mm. below the level of the

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surgical lesion, divided into several small fragments and left in fixative at 0°C. for 2 hours.

Several mixtures of reagents were used as fixatives. The best results were obtained with OsO_4 in phosphate buffer, pH 7.2–7.5, and sucrose. The OsO_4 was used at a final concentration of 2 per cent in keeping with the observation of Palay and Palade (4), that concentrated OsO_4 was more effective in the fixation of nervous tissue. The final concentration of phosphate buffer was 0.17 to 0.25 M. Sucrose was added to the fixative according to Caulfield's suggestion (5) to attain a final osmolar concentration of ∼0.37 M.

Dehydration was accomplished with ethanol in graded concentrations, and acetone. Impregnation was achieved by two passages through n-butyl methacrylate monomer. To ensure a proper embedding, it was found very important to leave the tissue in monomer plus catalyst overnight. Polymerization was carried through at 60°C. By this procedure the preservation of the myelin sheaths was much improved over that observed after other procedures.

The blocks were cut with a Porter-Blum cantilever microtome in 0.05 to 0.3 μ sections. The thin sections were mounted on carbon-coated grids and examined under the electron microscope, while the thick ones were mounted on slides and, after removal of the plastic, were examined under phase contrast optics.

A modified RCA microscope (EMU II c), an Emiskop I, and an Elmiskop II Siemens microscope were used. Micrographs were obtained at 1500 to 20,000 diameters and enlarged photographically. The results reported are based on the study of many hundreds of normal and degenerating fibers.

RESULTS

A. Normal Axoplasm: The normal axoplasm consists of a homogenous matrix of very low electron density in which are embedded several distinct morphological entities.

1. Neurofilaments.—(Fig. 1) They appear as dense thread-like structures of indefinite length and with diameters ranging from 100 to 200 A. In high power micrographs, they seem to exhibit in places an axial periodicity of ∼200 A, but it cannot be ascertained whether this structural detail is real or only apparent (Fig. 2). The neurofilaments form a loose network with meshes predominantly oriented along the longitudinal axis of the axon; sometimes they are grouped over some distance into parallel arrays. Similar filaments have been described by various authors both in tissue sections and in axonic material prepared by different procedures (6–8, 10, 11, 15).

2. Endoplasmic Reticulum.—(Figs. 1 and 3) All micrographs of axons showed profiles of elongated vesicles, 250 to 500 A units in diameter, oriented with their long axes parallel to the major axis of the fiber. The shape of these vesicles is somewhat variable. Some of them are cylindrical while others appear as rows of fused spherical units. Occasionally a very small number of dense particles 100 A in diameter can be seen adhering to their walls. The vesicles are often surrounded by condensations of neurofilaments.

3. Mitochondria.—The axonal mitochondria are in general extremely elongated, slender structures, 0.15 to 0.25 μ in diameter (Figs. 1 and 5).

They are bounded by a well defined double membrane, and often show typical cristae. These assume sometimes the aspect described by Palade (9) in nerve cells, being arranged parallel to the longitudinal axis of the mitochondrion. This aspect is, however, infrequent, and it may be said that mitochondria in the myelinated axon show a rather small number of cristae when compared with images published of dendrites and synaptic endings (13). This observation may be of interest in view of the correlation suggested by Palade (9) between the number of cristae and the concentration of oxidative enzymes in various cell types.

4. Particulate Components.—Two different kinds of particles can be seen: (a) Small (100 A or less) dense particles which are seen lying free in the axoplasmic matrix or along the neurofilaments, or adhering to the vesicles of the endoplasmic reticulum; and (b) Large particles (160 to 300 A) of moderate density, which lie scattered throughout the axoplasm, often in contact with the neurofilaments (Fig. 4).

B. Axoplasmic Changes during Wallerian Degeneration:

1. Time-Course and Over-All Picture of the Degenerative Process.—The onset and time course of the degenerative change vary to a certain extent from one experiment to another. In some cases, nerves sectioned for 24 hours show almost no differences from the normal controls. Even when fibers within the same nerve are considered, it is evident that in some of them the degenerative process has advanced much further than in others within the same period of time.

Whatever the rate at which the axoplasmic changes in the individual fibers progress, they become completed at about 48 hours in the material used in these experiments. At this time, all axons show a complete disruption of their internal

structure (Figs. 7 to 9) with a disappearance of the neurofilaments and a clumping of the axoplasmic material as will be described below. Some of the fibers become extremely swollen (compare Figs. 10 and 11), the myelin sheaths acquire complex infoldings (Fig. 8), and the cytoplasm of the Schwann cells shows many signs of increased activity with the development of large numbers of mitochondria and a considerable amount of granular and agranular reticulum.

2. Axoplasmic Matrix and Endoplasmic Reticulum.—Very early in the degenerative process the density of the matrix is seen to increase, especially near the profiles of the endoplasmic reticulum and among the strands of neurofilaments.

The earliest changes in the formed elements of the axoplasm appear in the endoplasmic reticulum. It is exceptional to find normal endoplasmic reticulum profiles after 24 hours of degeneration. The tubular elements have become fragmented into rows of small vesicles. The aspect shown in Fig. 6 is seldom seen, but it is believed that these rows become disoriented very soon with the consequent scattering of the vesicles which will then appear as isolated units in the sections.

3. Neurofilaments.—Their alterations appear somewhat later than those described under paragraph 2, and it is likely that once they start in a given fiber they proceed very rapidly to completion. Intermediary stages of degeneration are seldom seen, most fibers appearing either normal or completely altered. What are interpreted as the earliest changes appear as a loss of the preferred orientation of the filaments. The predominantly longitudinal disposition is absent and the parallel arrays of filaments disappear. The axoplasm shows a reticulated pattern which differs somewhat from the normal one. Together with these changes, there is a considerable diminution in the number of filaments, which consequently appear more sparsely distributed. Some micrographs show a very marked reduction in length of the filaments. It is of course difficult to assess the worth of this latter observation in view of the great length of the filaments as compared with the thickness of the sections. It may be that this apparent fragmentation of the filaments is only another expression of the loss of their preferred longitudinal orientation.

As noted above within 24 to 48 hours after the severance of the nerve, the neurofilaments seem to undergo complete disintegration. In their place a finely granular material of marked density is found (Figs. 7 and 9). This material is at first homogeneously distributed throughout the axoplasm showing in rare occasions a reticulated pattern (Fig. 9), but later becomes segregated into irregular clumps often adhering to the axonal membrane (Figs. 7, 8, 11). The rest of the fiber is now occupied by a material of very low density, without any visible structure and presumably fluid *in vivo*. This latter conclusion is suggested by the behavior of the myelin sheath, which shows in places deep infoldings (Fig. 8 (c), (d), (e)) that reduce the lumen of the fiber to a cleft-like cavity and even obliterate it completely. In other places still, the fibers become extremely swollen and their shape strongly suggests that they contain fluid under pressure. Fig. 11 illustrates the enormous increase in diameter which may be attained by degenerating fibers after the destruction of the neurofilaments.

4. Granular Material.—Thick particles of the 160 to 300 A class become more abundant in the first stages of degeneration. Later on, it becomes impossible to distinguish them from the irregular axoplasmic clumps.

5. Mitochondria.—They remain relatively unaltered until the changes affecting the neurofilaments are clearly evident. At this time, they become swollen and filled with a material of low density (Figs. 7, 9). A statistical analysis was made of mitochondrial size at different stages of the degenerative process. For this purpose, the smaller diameter was measured in all mitochondrial profiles present in a number of micrographs chosen at random. The results of this study are illustrated in Text-fig. 1 which compares the size distribution of: (a) mitochondria of normal nerve fibers (108 profiles with a mean of 177 mμ and a standard error of the mean of ±4.3 mμ); (b) mitochondria in degenerating fibers before neurofilament alterations have become apparent (100 profiles with a mean of 170 mμ and a standard error of the mean of $\pm7, 8$ mμ); and (c) mitochondria in degenerating fibers after destruction of the neurofilaments (80 profiles with a mean of 288 mμ and a standard error of the mean of ±14.5 mμ). The latter group is thus found to be significantly different from the normal (a difference between the means of 111 mμ with a standard error of ±15 mμ); and from the degenerating fibers with normal neurofilaments (a difference between the means of 118 mμ with a standard error of ±16 mμ).
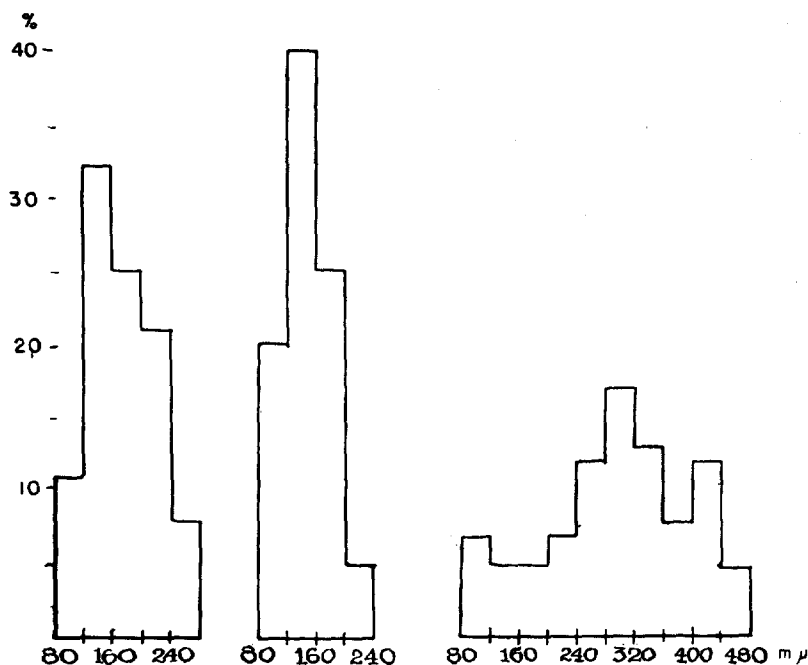
TEXT-FIG. 1. Size distribution of mitochondrial profiles in normal and degenerating nerve fibers.

From left to right: Mitochondria in normal fibers (108 profiles); in degenerating fibers with unaltered neurofilaments (100 profiles); and in degenerating fibers after the destruction of the neurofilaments (80 profiles); ordinates, per cent count of mitochondrial profiles, abscissae, smaller diameter of profiles in mμ.

In consequence it can be said that the swelling of the mitochondria takes place either at the same time as, or later than, the destruction of the neurofilaments.

DISCUSSION

The most striking alterations observed are those in the neurofilaments. Our results show that between 24 and 48 hours all neurofilaments disappear. There is some evidence that this phenomenon takes place as a progressive fragmentation of the filaments into shorter and shorter rods (Fig. 9), but from the present study not much can be said about the nature of this destructive process. Maxfield (10) showed that as the pH and ionic strength of a suspension of neurofilaments were raised, the filaments "depolymerized" into globular molecules of about 70,000 molecular weight. It is not possible to say whether any such process takes place in the degenerating nerve; but the readiness with which the neurofilaments become "depolymerized" *in vitro* suggests that they might show considerable lability in the altered medium of the degenerating axons. The observations reported seem compatible with the idea that after the destruction of the neurofilaments, the axoplasm assumes a fluid state, possibly by a gel-sol transformation. This change in physical state seems to be the immediate cause of the fragmentation of the nerve fiber. The hypothesis that this fragmentation is primarily due to a failure in the internal pressure of the axon brought about by an interruption of the axoplasmic flow from the cell body (16) does not seem to be tenable.

The experiments reported afford an interesting opportunity to test the hypothesis that neurofilaments are concerned with the conduction of nerve impulses (15). During Wallerian degeneration there is clearly a time at which the neurofilaments are completely destroyed and the possibility of testing such nerve fibers for conduction is obvious. Experiments along this line are now in progress. The extreme lability of the neurofilaments in the degenerative process seems to rule out the possibilities that they are either relatively inert structural components or "artifacts caused by longitudinal tension forces present in the axis cylinder" (11).

It is not possible for the moment to give a definite interpretation of the mitochondrial changes,

which as shown above, appear at the same time as or after the destruction of the neurofilaments. It cannot be said, however, that these events are causally related. Similar changes in mitochondria have been reported for instance by Rouiller and Gansler (12) in liver cells during starvation and by Okada and Peachey (14) in isolated mitochondria after gamma irradiation. The swelling can be therefore a non-specific manifestation of mitochondrial damage but it could also be due to an altered fluid balance in the inside of the nerve fibers with the mitochondria acting as osmometers. The tendency of the fibers to swell during degeneration might be taken as supporting the latter possibility. The earliest of all cytoplasmic changes in the axon are those of the endoplasmic reticulum. There is no clue as to the mechanism of these changes, but there can be no doubt that these vesicular elements are the axoplasmic structures most sensitive to the severance of the connections with the cell body.

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EXPLANATION OF PLATES

PLATE 261

FIG. 1. Normal axoplasm in a nerve fiber sectioned parallel to its longitudinal axis. Three mitochondrial profiles (*m*) and some tubular and vesicular elements of the endosplasmic reticulum (*er*) can be seen. The neurofilaments appear in places oriented in parallel arrays (arrows). × 45000.
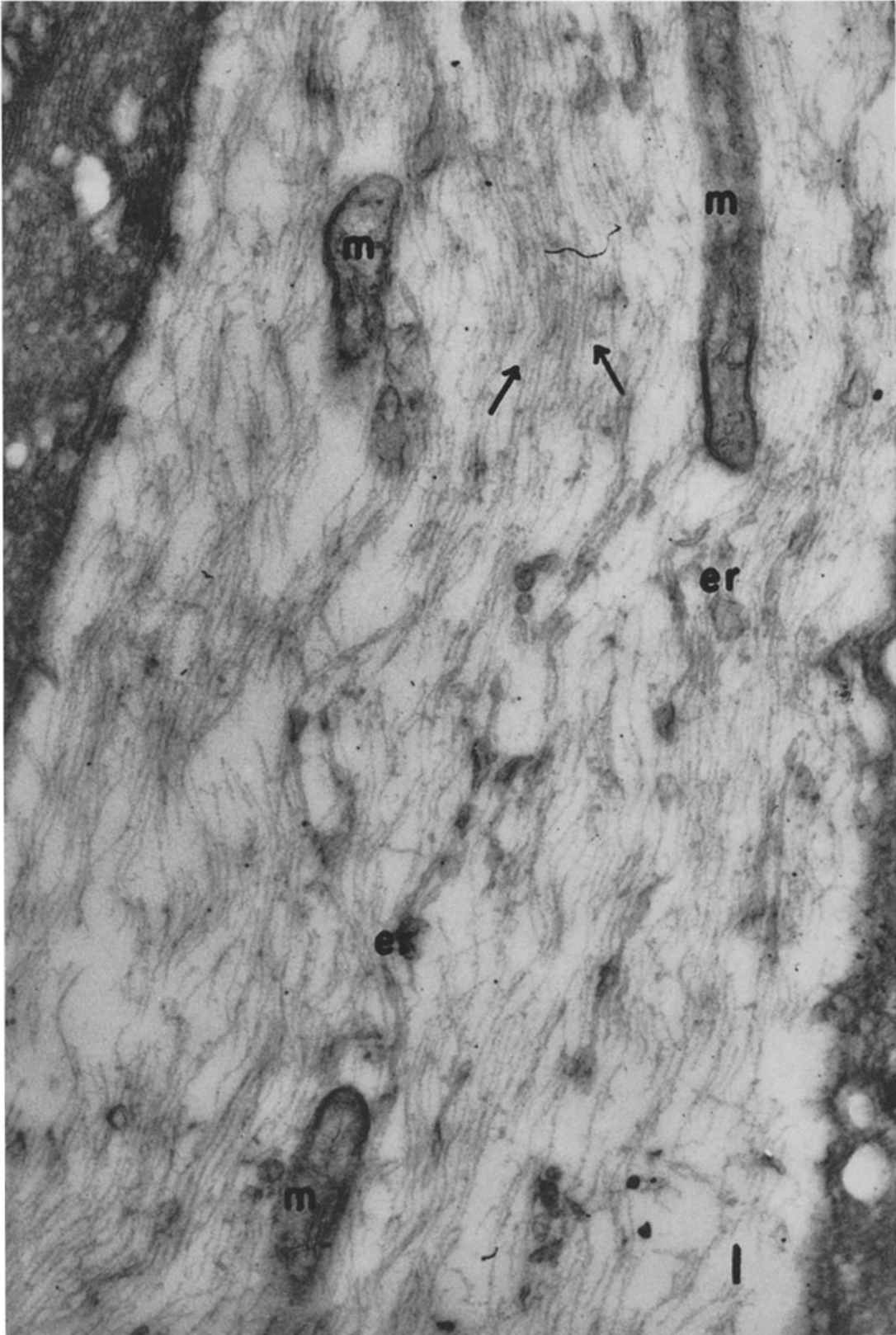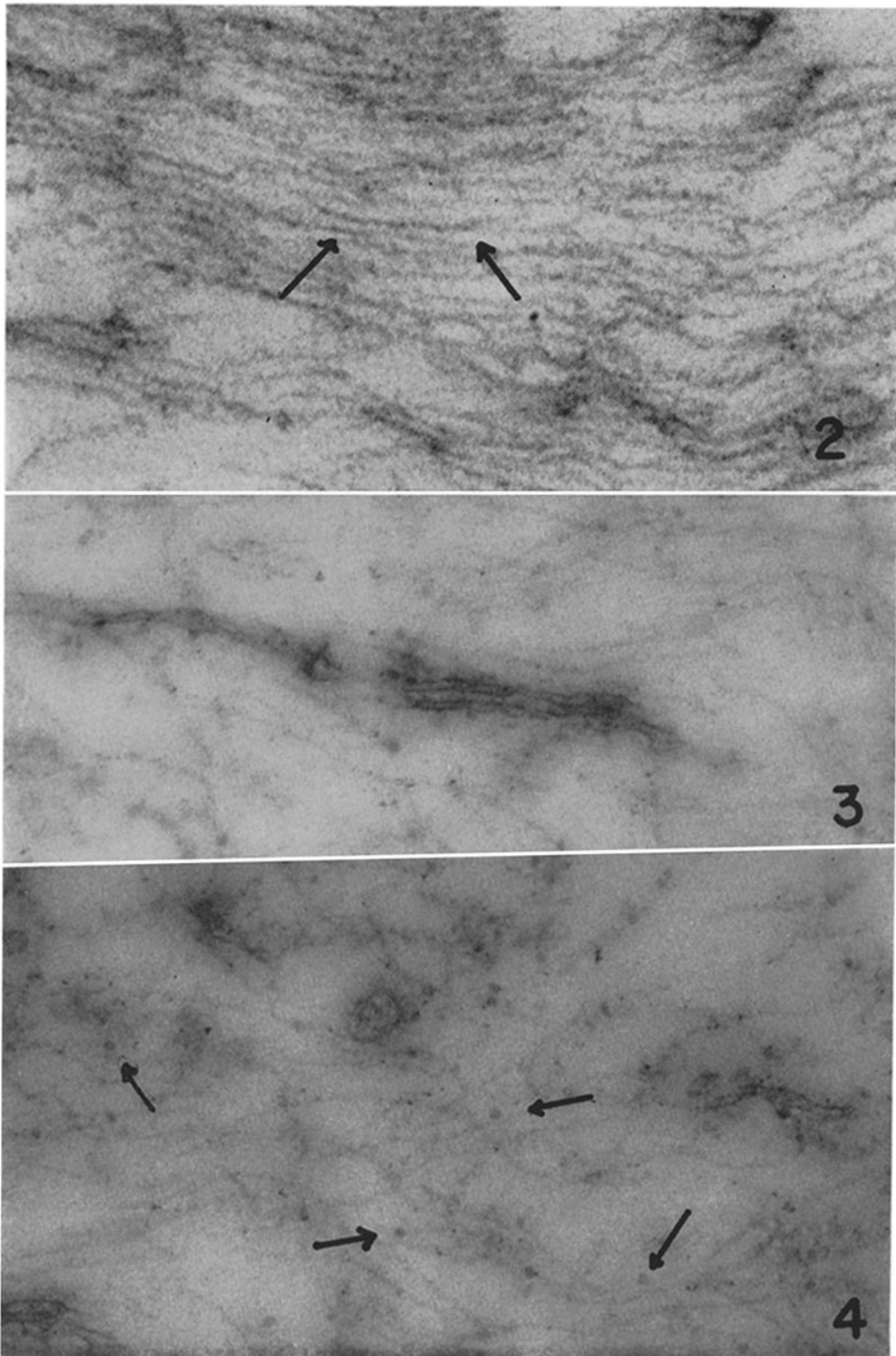
(Vial: Axoplasm changes and Wallerian degeneration)

FIG. 2. Neurofilaments in normal axoplasm. The arrows point to filaments in which a suggestion of periodic structure is shown. $\times$ 100,000.

FIG. 3. Endoplasmic reticulum in normal axoplasm, showing the usual tubular appearance of the profiles. $\times$ 84000.

FIG. 4. Normal axoplasm. The arrows point to moderately dense particles of uniform size ($\sim$200 A). Similar particles are consistently found in normal fibers. $\times$ 84000.
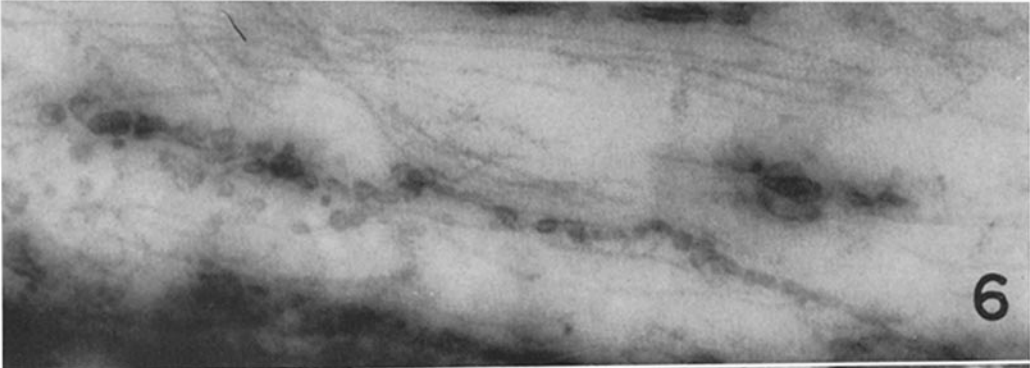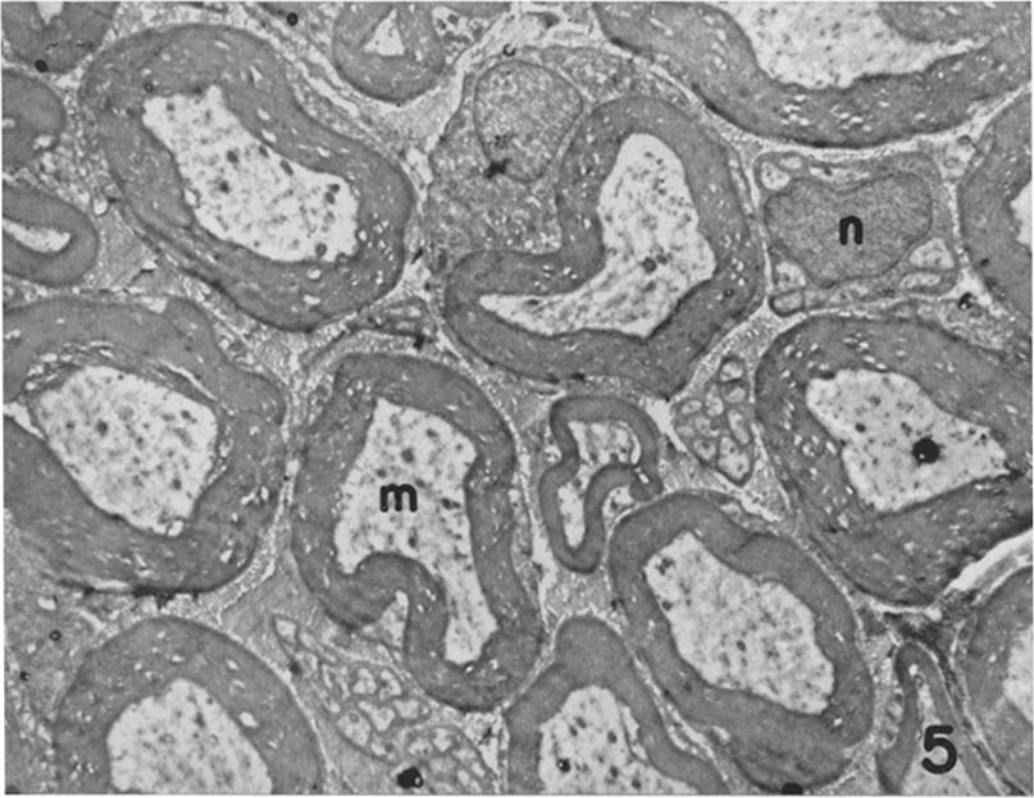
(Vial: Axoplasm changes and Wallerian degeneration)

FIG. 5. Cross-section of normal saphenous nerve. Myelinated (*m*) and non-myelinated fibers (*n*) can be seen. Myelinated fibers show an irregular outline and they are homogeneously filled with axoplasm showing the cross-sections of mitochondria. The space between the fibers is occupied by bundles of collagenous fibrils. × 4500.

FIG. 6. Nerve fiber 24 hours after severance, showing extensive vesicular fragmentation of the endoplasmic reticulum tubules in an otherwise normal fiber. × 57000.
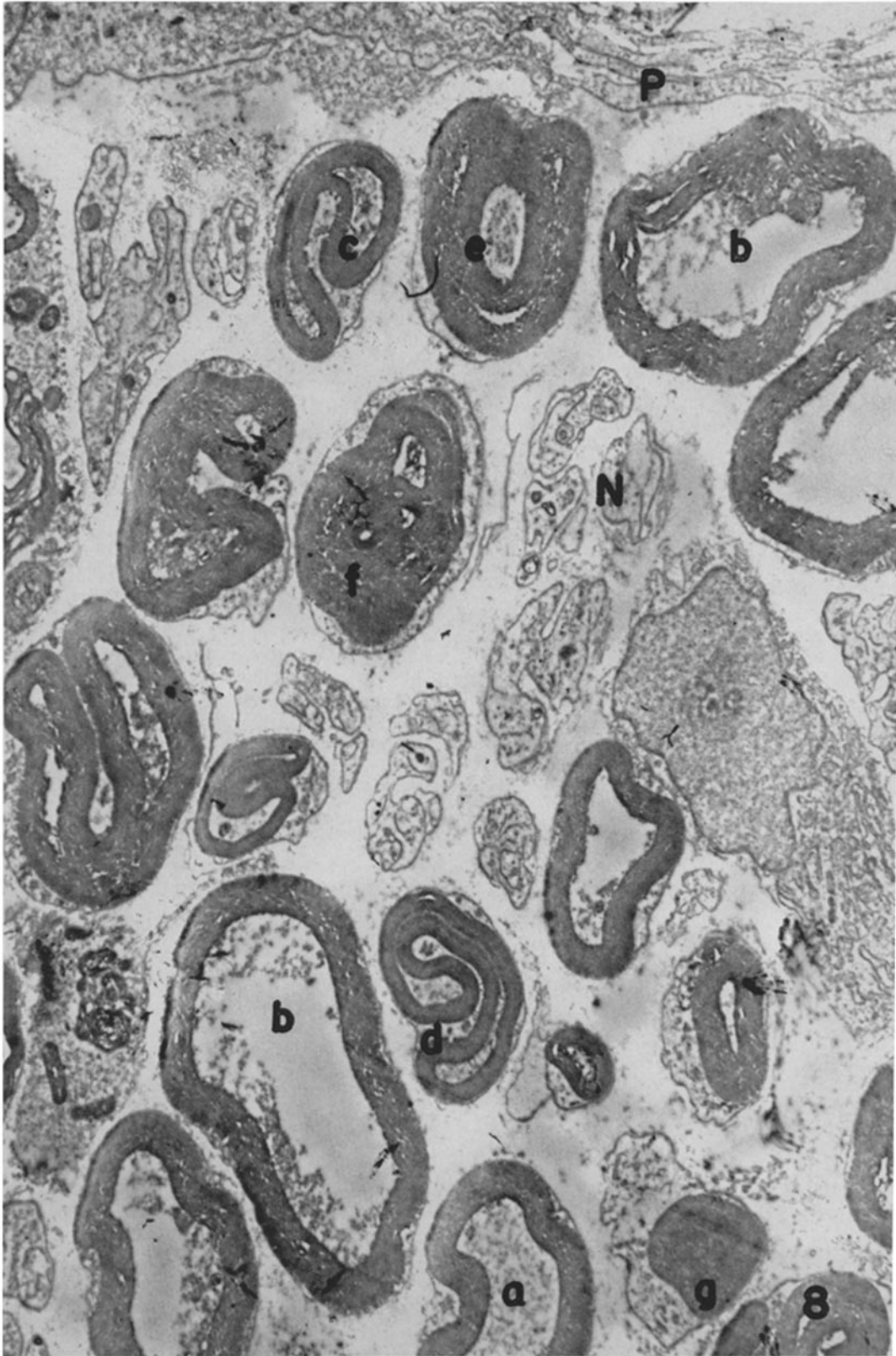
FIG. 7. Nerve fiber 48 hours after severance. Swollen, and possibly fragmented mitochondria are clumped together with remnants of axoplasm against the wall of the fiber. The rest of the fiber is occupied by a clear structureless material. × 16000.
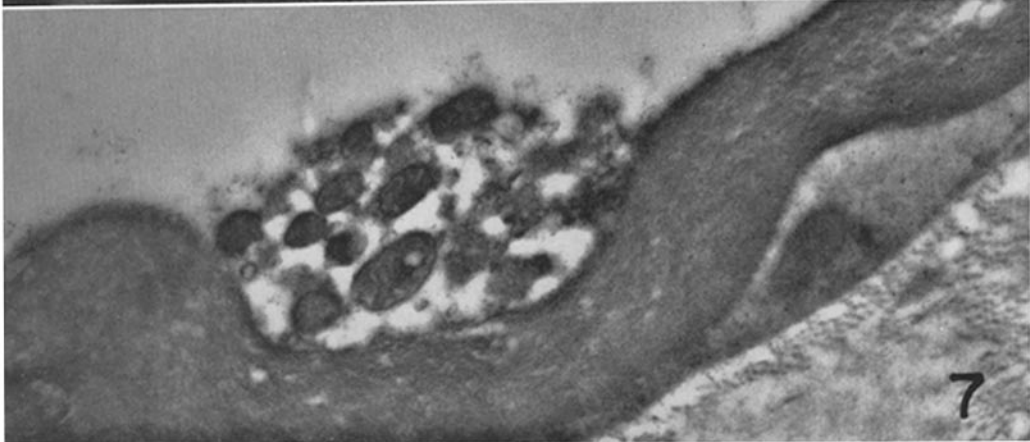
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FIG. 8. Cross-section of saphenous nerve, 48 hours after severance. *P*, perineurium. *N*, non-myelinated fibers. The neurofilaments have undergone complete destruction in all myelinated fibers. In one of them (*a*) the axoplasm rests are still filling homogenously the lumen of the fiber, while in others they have condensed into irregular clumps. Moderately swollen fibers are evident at (*b*). Collapsed fibers with extensive folding of the myelin are shown at (*c*) and (*d*). At (*e*) is shown another type of myelin infolding: in this case, the inner myelin ring surrounds invaginated Schwann cell protoplasm, while the axon is reduced to a virtual cavity. (*f*) and (*g*) show sections through fibers in which the myelin changes have completely occluded the axon. $\times$ 4500.

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FIG. 9. Nerve fiber 48 hours after severance. The neurofilaments are replaced by a fine, dense granular material arranged in places in a reticulated pattern. In some places, a partly disintegrated filamentous material is seen (arrows). Mitochondria (*m*) are swollen and possibly fragmented. × 44000.
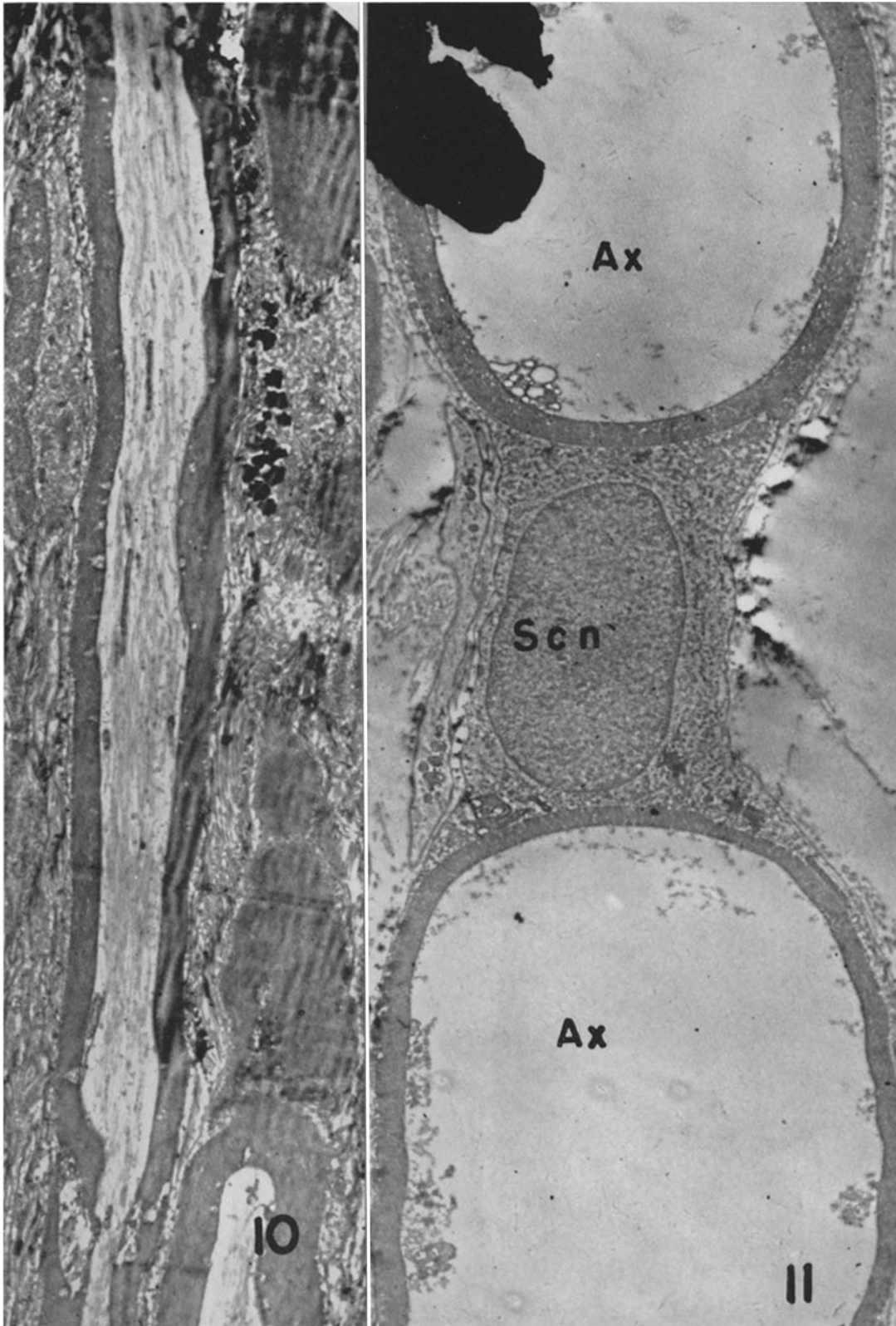
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PLATE 266

FIG. 10. Longitudinal section of a nerve fiber 24 hours after severance. The fiber shows an essentially normal aspect with preservation of the neurofilaments, and it should be compared with Fig. 11 × 4800.

FIG. 11. Longitudinal section of a nerve fiber 48 hours after severance. Neurofilaments are destroyed and the fiber appears practically empty. It has undergone an extreme degree of swelling and it is presumably fragmented into myelin ovoids. Between the two segments illustrated in this figure, the nucleus of the Schwann cell, can be seen. *Scn.* Schwann cell nucleus. *Ax*, axon. × 4800.

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