POLARIZATION AND ELECTRON MICROSCOPE STUDY OF FROG NERVE AXOPLASM*

BY WAYNE THORNBURG, PH.D., AND EDUARDO DE ROBERTIS,‡§ M.D.

(From the Department of Anatomy, University of Washington, Seattle)

PLATES 129 TO 132

(Received for publication, March 14, 1956)

INTRODUCTION

Recent work on the physicochemical changes in nerve which are associated with conduction have caused a renewed interest in the submicroscopic organization of axoplasm. Changes in light scattering (1), in viscosity (2), in opacity and volume (3), and in ultraviolet absorption (4) have been described. Although experimental techniques have not been sufficiently sensitive to relate the time course of any of these changes to a single propagated impulse, all these studies suggest that axoplasmic structures play an important role in nerve function (5).

In this paper findings will be presented on the ultrastructure of axoplasm as observed in electron micrographs of thin sectioned frog nerve and an attempt will be made to correlate measurements on the submicroscopic fibrillar component of fixed axon with the birefringence of fresh axon. The only quantitative work on the birefringence of axoplasm has been done by Bear *et al.* (6) on the giant axon of squid. An average value of $+1.5 \times 10^{-4}$ was obtained. On the basis of this and certain assumptions it was calculated that between 0.3 and 0.6 per cent of the total volume of the axon is occupied by axially oriented protein filaments. These authors point out, however, that the nature of the fibrillar component cannot be uniquely determined by birefringence measurements alone.

One of the difficulties encountered in making birefringence measurements on most nerve fibers lies in the fact that the relatively weak birefringence of the axoplasm is superimposed upon that due to the myelin sheath and/or connective tissue elements. This difficulty has been circumvented in the present study by dissecting away these obscuring elements. The method used was based upon the work of one of us (7) who was able to separate long pieces of fresh axon from myelinated nerves of toad and rat by an extrusion technique. Axoplasmic components of these nerves were studied with the phase and electron microscopes. By a similar technique suitable specimens have been obtained

* This work was supported in part by a grant from the Medical and Biological Research Fund of the State of Washington.

‡ Walker-Ames Professor of Anatomy, Fall Quarter, 1953.

§ Present address: Instituto de Investigación de Ciencias Biológicas, Montevideo, Uruguay.

from frog nerve for measurements with the polarizing microscope. The approximate agreement between the computed partial volume of an oriented submicroscopic component in fresh axoplasm and the partial volume occupied by the neuroprotofibrils in osmium tetroxide–fixed material indicates: (*a*) that the neuroprotofibrils are present in the living nerve, and (*b*) that the fibrillar component seen with the electron microscope is not a fixation artifact.

Experimental Methods

Optical Measurements.—Specimens of axon were prepared from frog sciatic nerve (*R. pipiens* and *R. catesbiana*) by teasing out short pieces of the nerve in a drop of Ringer's solution and then drawing small fiber bundles apart in an axial direction with fine forceps. Sheath elements tend to rupture at or near the nodes of Ranvier and to slide off the axon (see Figs. 1 and 2). Bare axons of several hundred microns in length were regularly obtained. Once the axon is separated from its sheath it tends to adhere to the coverslip. Thus fresh specimens can conveniently be mounted in Ringer's solution between two strain-free coverslips and examined with the polarizing microscope. All retardation measurements were made with a polarizing microscope designed and built by Dr. S. Inoué. A $\lambda/20$ compensator was adjusted to the position of best match between specimen and field (8). Duplicate readings were taken to the nearest minute in arc in two quadrants and average readings are considered to be accurate to ± 3 minutes. The compensation end-point proved to be particularly sensitive since slight variations in axon thickness produced a wave of contrast reversal which travelled along the axon cylinder as the end-point was approached. The thickness of the axon at the region selected for the end-point was measured with a calibrated fine-focusing adjustment. Values obtained are considered to be accurate to ± 0.5 micron. Axon widths were also recorded in order to obtain an average value for the ratio of the two diameters.

In order to determine what fraction of the total retardation was due to form birefringence, some of the same preparations were fixed in 10 per cent formaldehyde and dehydrated in alcohol, and the measurements were repeated in a graded series of imbibition fluids. Satisfactory values for form birefringence were obtained by locating the minima of the resulting curves. Attempts to determine the refractive index of the oriented component from these curves, however, tended to result in rather large errors. More accurate values were obtained by examining the specimens under the darkfield microscope and noting the refractive index of an imbibition fluid in which no light from the specimen area was scattered. A mismatch in refractive index of 0.0005 was readily detectable by this method. This method has been verified on preparations of formalin-fixed muscle in which values in the range of 1.57 have been obtained.

Specimens of axon were selected according to the following criteria: (*a*) straight region for a distance of at least 50 microns, so that its axis could be accurately determined, (*b*) proper association with a myelin sheath at one end to insure positive identification of the specimen as an axon, (*c*) an approximately uniform width and thickness as evidence of minimal mechanical damage from dissection, and (*d*) an optical field free of connective tissue or other interfering elements. When the myelin sheath was only partially separated (see Figs. 1 and 2) fine connective tissue filaments were often seen. When the sheath was completely separated (see Figs. 3 and 4) these filaments were removed and values for birefringence were more consistent and somewhat lower. The extent to which ultrastructure in the axon might be damaged by the dissection procedures was a matter of some concern. It was noted, however, that the length, diameter, and birefringence all tended to recover to their original values in axons that had been placed under tension for brief periods. Further, the birefringent component appeared

to be rather durable. The axon birefringence in nerves which had been removed from the animal and placed in cold Ringer's solution was retained for several days. The birefringence in freshly teased preparations decayed slowly over a period of several hours.

Electron Microscope Technique.—Frog nerves and sympathetic ganglia (*R. pipiens and R. catesbiana*) were fixed according to the method of Palade (9), embedded in methacrylate, and sectioned. Electron micrographs were taken at magnifications between 5000 and 7000 with an RCA 2C EMU microscope provided with a compensated objective lens, an objective

TABLE I

Polarizing Microscope Measurements on 32 Fresh Axons from Myelinated Frog Nerve

Thicknesses in microns; λ/20 compensator readings in minutes (difference between settings to match specimen with field in two quadrants = $2\beta_2$ in notation of Bear *et al.* (6)).

Thickness	λ/20 compensator reading	Thickness	λ/20 compensator reading
μ	*min*	*μ*	*min.*
5.1	59	7.5	101
6.0	69	9.6	72
7.2	83	11.0	118
5.1	55	5.0	115
6.1	65	3.2	70
5.5	62	6.0	117
5.0	58	8.0	121
6.0	102	5.8	123
11.5	151	4.0	115
7.7	153	7.0	63
14.2	218	6.0	135
5.1	104	8.5	153
6.0	171	8.2	120
7.6	114	10.2	120
6.2	85	9.6	83
5.2	58	8.0	150

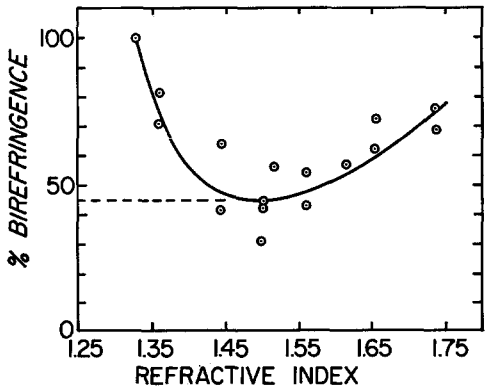
Average thickness..... 7.034 μ
Average compensator reading..... 105.7′
Average total birefringence..... 2.39×10^{-4}

aperture of about 25 microns, a condenser aperture of about 250 microns, and apertures in the projector lens.

OBSERVATIONS

Birefringence of Axons.—The average value of the total birefringence of 85 fresh myelinated axons from frog sciatic nerve was $+2.5 \times 10^{-4}$. The average thickness of these fibers was 6.4 microns (range 3.0 to 14.2 microns) and the average retardation was 1°41′ (range 58′ to 2°33′). The average value for the birefringence of 32 of the best specimens, selected according to the criteria listed above, was $+2.39 \times 10^{-4}$ (see Table I). No significant difference between the axons of *R. pipiens* and *R. catesbiana* was observed. The refractive index of components in axoplasm responsible for scattering light, according

to the darkfield method, was 1.523. All components of both nerve and connective tissue which had undergone fixation and dehydration disappeared simultaneously in a medium of this refractive index. Axons which had adhered to the coverslip proved to be somewhat flattened while in Ringer's solution, the average value of the ratio between width and thickness being 1.3. A representative curve of form birefringence is shown in Text-fig. 1. The scatter of experimental points was due, in part, to some variation of the thickness of these preparations as they were fixed, dehydrated, and passed through the various imbibition fluids. From this and similar curves it was deduced that



TEXT-FIG. 1. Form birefringence curve. Two sets of data on a single axon have been plotted to determine the percentage of form birefringence. Total birefringence in saline solution = $+4.4 \times 10^{-4}$.

about 55 per cent of the total birefringence of the fresh axon in Ringer's solution was positive form birefringence. This is interpreted as evidence for the presence of axially oriented filaments in the fresh axon. The remaining 45 per cent of positive intrinsic birefringence is believed to reside in the same structure.

Electron Microscope Observations on Myelinated and Unmyelinated Nerve Axons.—Some of the details of structure found in sections of myelinated and unmyelinated nerve fibers are illustrated in Figs. 5 to 8. The longitudinal sections show better the disposition of the axially oriented material of the axon. In addition to typical rod-shaped mitochondria several submicroscopic components of low density can be recognized within the amorphous axoplasmic matrix.

(*a*) *Fibrillar component:* Fibrils of approximately 90 A diameter and of an apparently indefinite length are seen to lie parallel to the axis of the fiber (Figs. 5, 6, and 8). These fibrils have smooth edges, show no periodic structure, and are similar to the ones found in the isolated axon after compression which have been referred to as neuroprotofibrils (7, 5). They probably also correspond to the neurofilaments found in invertebrate axons (10) and in nerve fibers

isolated from the central nervous system of mammals (11). The longitudinal disposition of these neuroprotofibrils is shown better in rather thick sections (Figs. 5 and 6) in which the internal structure of the mitochondria and of the myelin sheath is obscured. In thinner sections (Fig. 8) the lamellar disposition of the myelin sheath is clearly resolved but only few neuroprotofibrils and other components of the axoplasm lie in the plane of section. A qualitative comparison between myelinated and the unmyelinated nerve fibers indicates that, although they are similar in other respects, the concentration of neuroprotofibrils is higher in the latter (compare Figs. 5 and 6). The amount of endoplasmic reticulum and dense particles also appears to be greater in the unmyelinated nerve fibers.

TABLE II

Number of Neuroprotofibrils Counted in Electron Micrographs of Oblique Sections from Frog Nerve

Fiber	Corrected cross-sectional area	Total No.	No. per μ^2
	μ^2		
1	5.6	360	64
2	2.5	326	130
3	1.5	194	129
4	1.75	147	84
5	1.9	107	57
Average No. of neuroprotofibrils per μ^2			93

Counts were made of the number of neuroprotofibrils per square micron of cross-section in five selected electron micrographs of myelinated nerve (see Table II). More reliable counts could be made on oblique sections since in this case the neuroprotofibrils appeared as short lines. By measuring the major and minor axes, the elliptical areas were converted to equivalent cross-sectional areas. From these data an average value of 93 neuroprotofibrils per square micron was obtained.

(*b*) *Endoplasmic reticulum:* In the myelinated fibers and, even more conspicuously, in the unmyelinated fibers there are strands of canaliculi and vesicles about 300 to 400 A in diameter scattered along the axon cylinder (Fig. 6). These structures are similar to those which have been described in a variety of cells and referred to as endoplasmic reticulum (12).

(*c*) *Dense particulate component:* Many dense particles of spherical shape, 70 to 150 A in diameter, are scattered along the axon. These structures have no evident connection with other submicroscopic structures. They are similar to the dense particles found in other tissues by Palade (13) and seem to be particularly concentrated in the nerve cells (14, 15).

DISCUSSION

From simple geometrical considerations the partial volume of the neuroprotofibrils may be calculated from the electron micrographs. Thus:

$$d_1 = \frac{N\pi r^2}{1\mu^2}$$

in which d_1 is partial volume, N is the number per square micron, and r is the radius in microns. Length need not appear in this equation since both the axons and the fibrils are considered to be of indefinite length. Substituting the values obtained above for myelinated nerves:

$$d_1 = \frac{93\pi(0.0045\mu)^2}{1\mu^2} = 0.0059$$

or approximately 0.6 per cent.

The following statement of the Wiener equation for rod birefringence is based upon the fact that $(n_a + n_o)$ is approximately equal to $2n_1$. Since in this particular case, the error in the approximation occurs in the fourth significant figure we may say that:

$$n_a - n_0 = \frac{d_1 d_2 (n_1^2 - n_2^2)^2}{2n_1[(1 + d_1)n_2^2 + d_2 n_1^2]}$$

in which

d_1 = partial volume of fibrils;
$d_2 = 1 - d_1$ = partial volume of surrounding medium;
n_1 = refractive index of fibrils = 1.523 (as determined by the darkfield technique);
n_2 = refractive index of surrounding medium = 1.346;[1]
$n_a - n_o$ = form birefringence = 1.4×10^{-4} (55 per cent of the total birefringence).

By substituting these values in the above equation and solving for d_1 we obtain:

d_1 = 0.0069 or approximately 0.7 per cent.

These two values for the partial volume of an oriented component in frog axoplasm are in somewhat better agreement than might be expected from the quality of the data. They are in approximate agreement with the value obtained by Bear *et al.* (6) for squid nerve axoplasm and, if the same number were used for the refractive index of the oriented component in the interpretation of both sets of birefringence measurements, this latter agreement would be still better. It should be emphasized that neither the polarization nor the electron

[1] In this case n_2 is the refractive index of the isotropic portion of axoplasm. Its value was calculated on the assumptions that: (*a*) proteins were the only substance in the isotropic portion, (*b*) frog axoplasm is 8 per cent protein by weight as indicated by Engstrom (16), and (*c*) the refractive index increment for each 1 per cent protein was 0.0018 as shown by Barer (17). While a more direct approach is desirable it may be seen that rather large errors in the calculation will not greatly affect the final result.

microscope method leads to an unambiguous characterization of the oriented fine structure in axoplasm nor to a particularly accurate value for its partial volume. Polarization microscope measurements of axon thickness and of per cent form birefringence are susceptible to error and the way in which the partial volume is distributed cannot be determined from birefringence measurements alone. The accuracy of electron microscope measurements on the number of neuroprotofibrils per unit cross-section and on their average diameter is limited by the resolving power of this instrument. Uncertainties also arise as to the fidelity with which submicroscopic structure in the living cell is preserved by osmium tetroxide fixation. Both of these methods indicate, however, that axially oriented filaments are present in frog nerve axoplasm. The approximate agreement between the values obtained for the partial volume of this oriented component is considered to be substantial evidence for the existence of the neuroprotofibril in the living nerve. It also implies that this particular structure is preserved by osmium tetroxide fixation without major geometrical alteration.

It is of interest that about 10 per cent of the total protein present in axoplasm is found to be in the form of oriented filaments. The refractive index of this component was somewhat lower than the value obtained by Bear *et al.* (6) for squid giant axon (1.57 to 1.60), and was also lower than the value previously reported by one of us (18) for frog axon (1.56). This latter discrepancy was due to inaccuracy in locating the minima of form curves. The assumption that the neuroprotofibrils are the only birefringent component of axoplasm cannot be supported at the present time.

The electron micrographs permit recognition of further complexities in the submicroscopic organization of axoplasm. The neuroprotofibrils, which had been seen earlier in compressed axon preparations (7), are readily observed in sectioned material. Some variation in their diameter which has been noted in preparations from guinea pig, toad, and frog is believed to be a species difference.

SUMMARY

1. The submicroscopic organization of nerve axons from *R. pipiens* and *R. catesbiana* has been studied by means of polarizing and electron microscopes.

2. In measurements on a series of 85 fresh myelinated axons from which the sheaths had been removed average values were obtained for the total birefringence, $+2.5 \times 10^{-4}$, the form birefringence, $+1.4 \times 10^{-4}$, and the refractive index of the oriented component, 1.523. The average partial volume occupied by axially oriented filaments was computed to be 0.69 per cent.

3. Electron micrographs of fixed myelinated axons demonstrate an average of 93 axially oriented neuroprotofibrils per square micron of cross-section. The neuroprotofibrils are approximately 90 A in diameter, of indefinite length, and occupy a computed partial volume of 0.59 per cent.

4. Mitochondria, neuroprotofibrils, endoplasmic reticulum, and dense

particles are seen in electron micrographs of both myelinated and unmyelinated nerve axons.

5. It is concluded that the neuroprotofibrils are present in the living nerve, that they play an important but undetermined role in nerve function, and that these structures are not an artifact of osmium tetroxide fixation.

BIBLIOGRAPHY

1. Tobias, J. M., *Cold Spring Harbor Symp. Quant. Biol.*, 1952, **17,** 15.
2. Flaig, J., *J. Neurophysiol.*, 1947, **10,** 211.
3. Hill, D. K., *J. Physiol.*, 1949, **108,** 278; 1950, **111,** 283, 304.
4. Lüthy, H., *Helv. Physiol. et Pharmacol. Acta*, 1948, **6,** C-28.
5. De Robertis, E., *Gaz. Mêd. Portuguesa*, 1954, **7,** 253.
6. Bear, R. S., Schmitt, F. O., and Young, J. Z., *Proc. Roy. Soc. London, Series B*, 1937, **123,** 505.
7. De Robertis, E., and Franchi, C. M., *J. Exp. Med.*, 1953, **98,** 269.
8. Bear, R. S., and Schmitt, F. O., *J. Opt. Soc. America*, 1936, **26,** 363.
9. Palade, G. E., *J. Exp. Med.*, 1952, **95,** 285.
10. Schmitt, F. O., *J. Exp. Zool.*, 1950, **113,** 499.
11. Fernández-Morán, H., *Exp. Cell Research*, 1952, **3,** 282.
12. Porter, K. R., *J. Exp. Med.*, 1953, **97,** 727.
13. Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 59.
14. De Robertis, E., *J. Histochem. and Cytochem.*, 1954, **2,** 341.
15. Palay, S. L., and Palade, G. E., *J. Biophysic. and Biochem. Cytol.*, 1955, **1,** 69.
16. Engstrom, A., *Exp. Cell Research*, 1950, **1,** 80.
17. Barer, R., *Nature*, 1953, **171,** 720.
18. Thornburg, W., *Anat. Rec.*, 1954, **118,** 362.

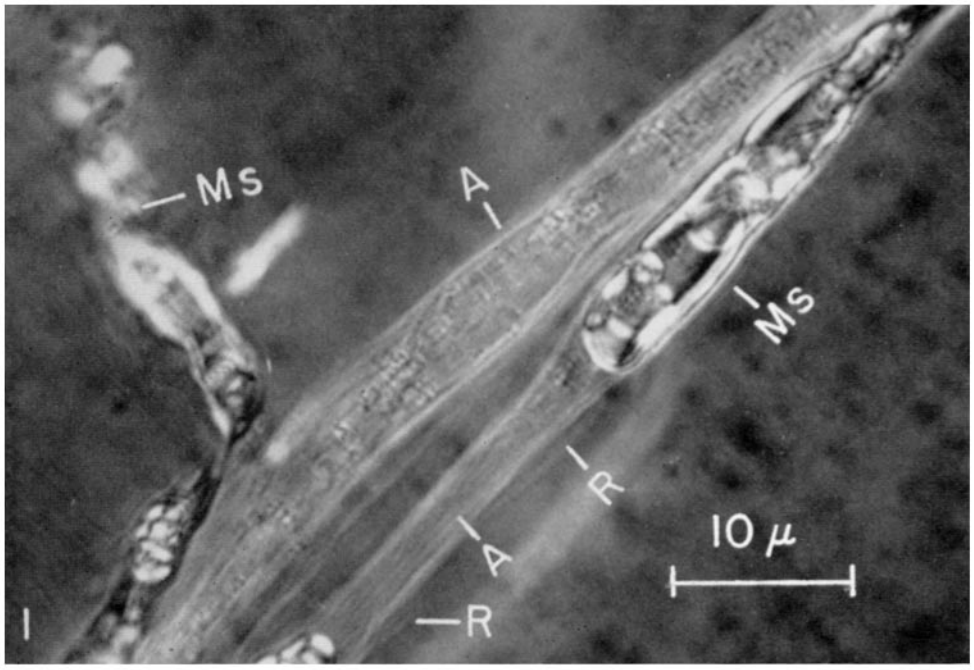
EXPLANATION OF PLATES

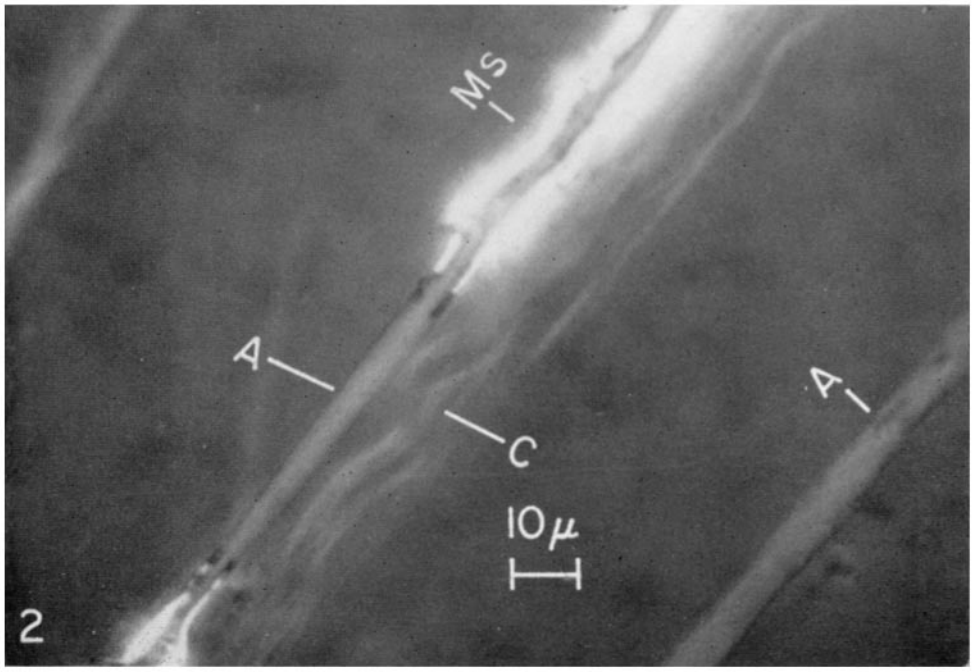
A, axon.
AM, axon membrane.
C, collagen.
ER, endoplasmic reticulum.
F, oriented filaments.
IS, intercellular space.
M, mitochondria.
MS, myelin sheath.
N, nucleus of Schwann cell.
NF, neuroprotofibrils.
P, dense particulate component.
R, remnants of nerve sheath.
SchC, Schwann cell.
V, vesicles of Schwann cell.

PLATE 129

FIG. 1. Photomicrograph taken during dissection of two fresh axons under polarizing microscope. Myelin sheaths have been partially removed. Sheath remnants lie parallel to axon at *R*. × 2300.

FIG. 2. At center, a length of fresh axon under tension during removal of myelin sheath segments; at lower right, a length of axon from which myelin sheath and interfering connective tissue have been removed. × 900.



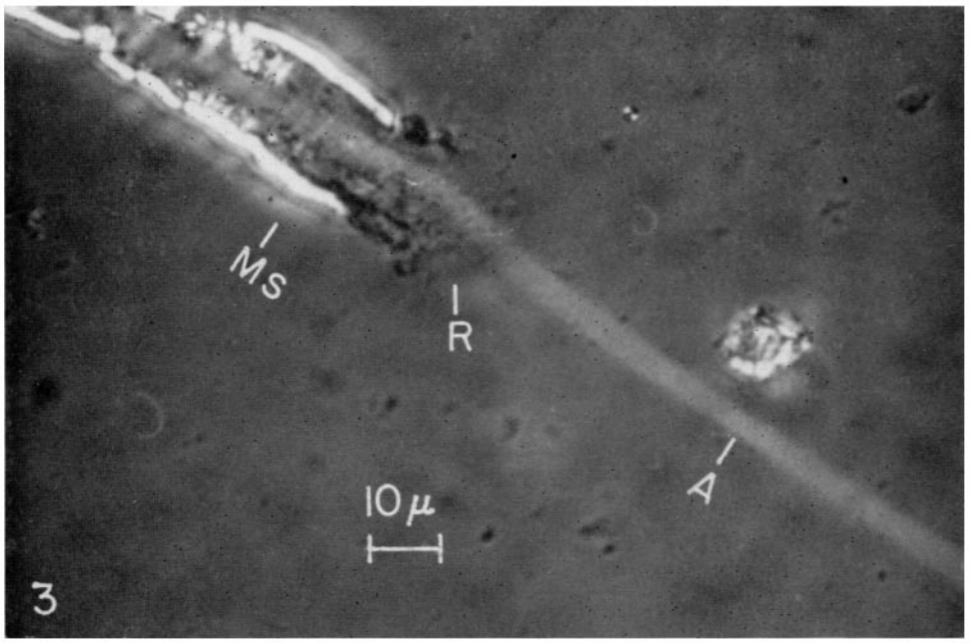


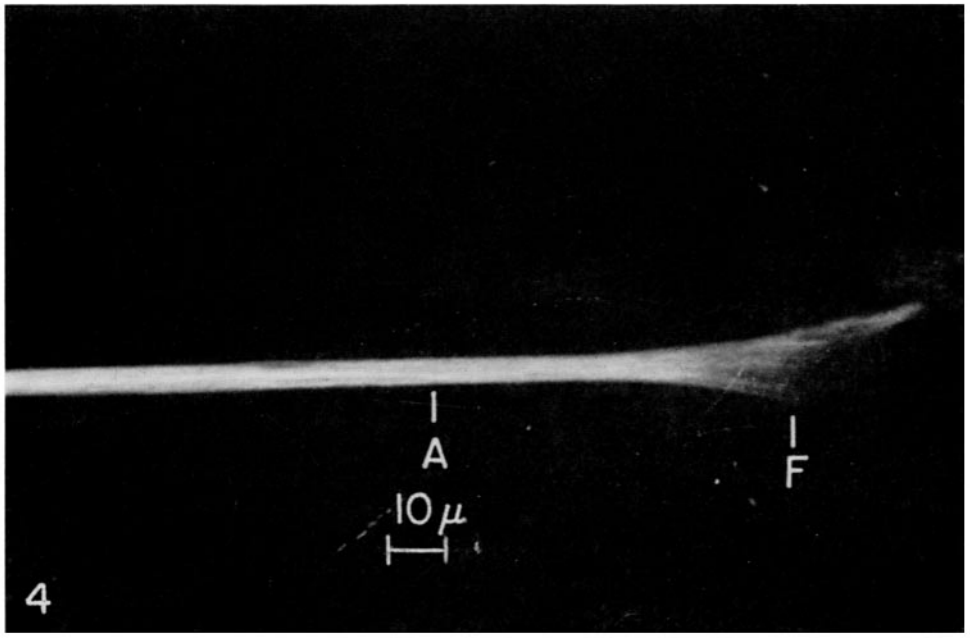
(Thornburg and De Robertis: Frog nerve axoplasm)

PLATE 130

FIG. 3. Photomicrograph of bare axon protruding from myelin sheath. Note relatively uniform diameter and absence of interfering connective tissue. Material at *R* is believed to be sheath remnants which have contracted toward the region of sheath rupture. × 1000.

FIG. 4. Photomicrograph of fresh axon 5.0 microns in diameter and high birefringence ($+6.5 \times 10^{-4}$). Note suggestion of frayed filament (*F*) at broken end. × 800.



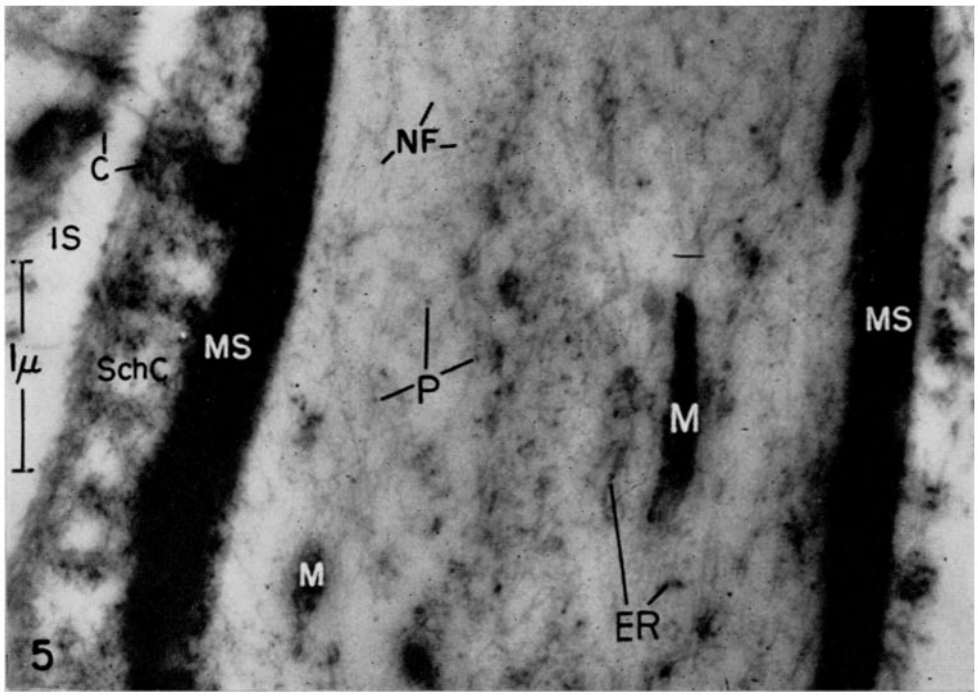


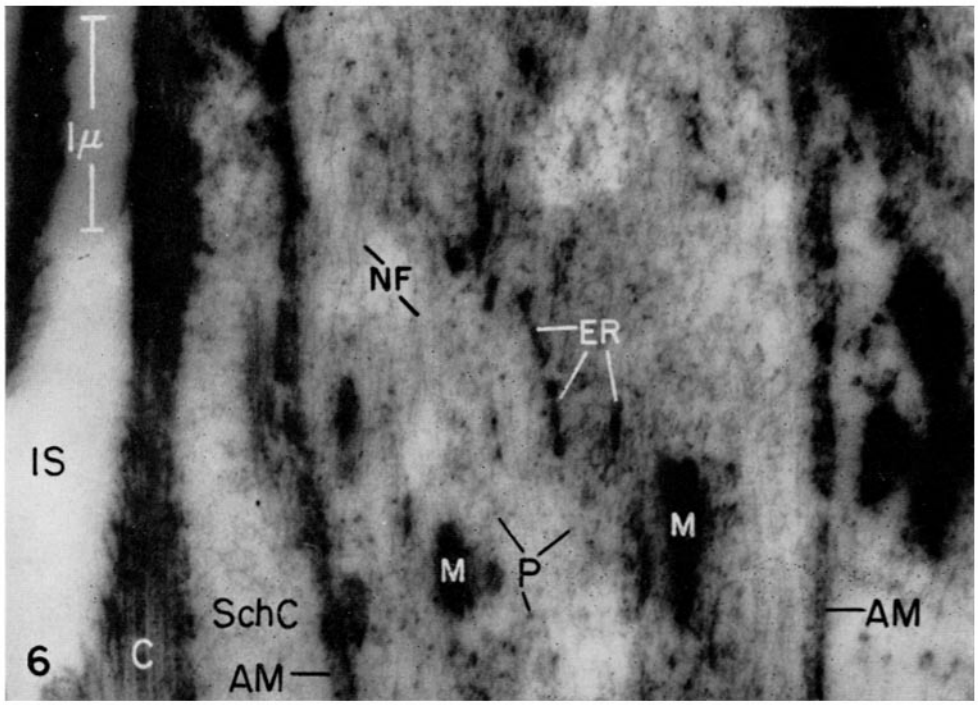
(Thornburg and De Robertis: Frog nerve axoplasm)

PLATE 131

FIG. 5. Electron micrograph (high contrast print) of a section of a myelinated nerve fiber of *R. catesbiana* showing the different components described in the text. × 28,000.

FIG. 6. Electron micrograph (high contrast print) of a section of unmyelinated nerve fiber of *R. catesbiana* (see description in the text). × 28,000.



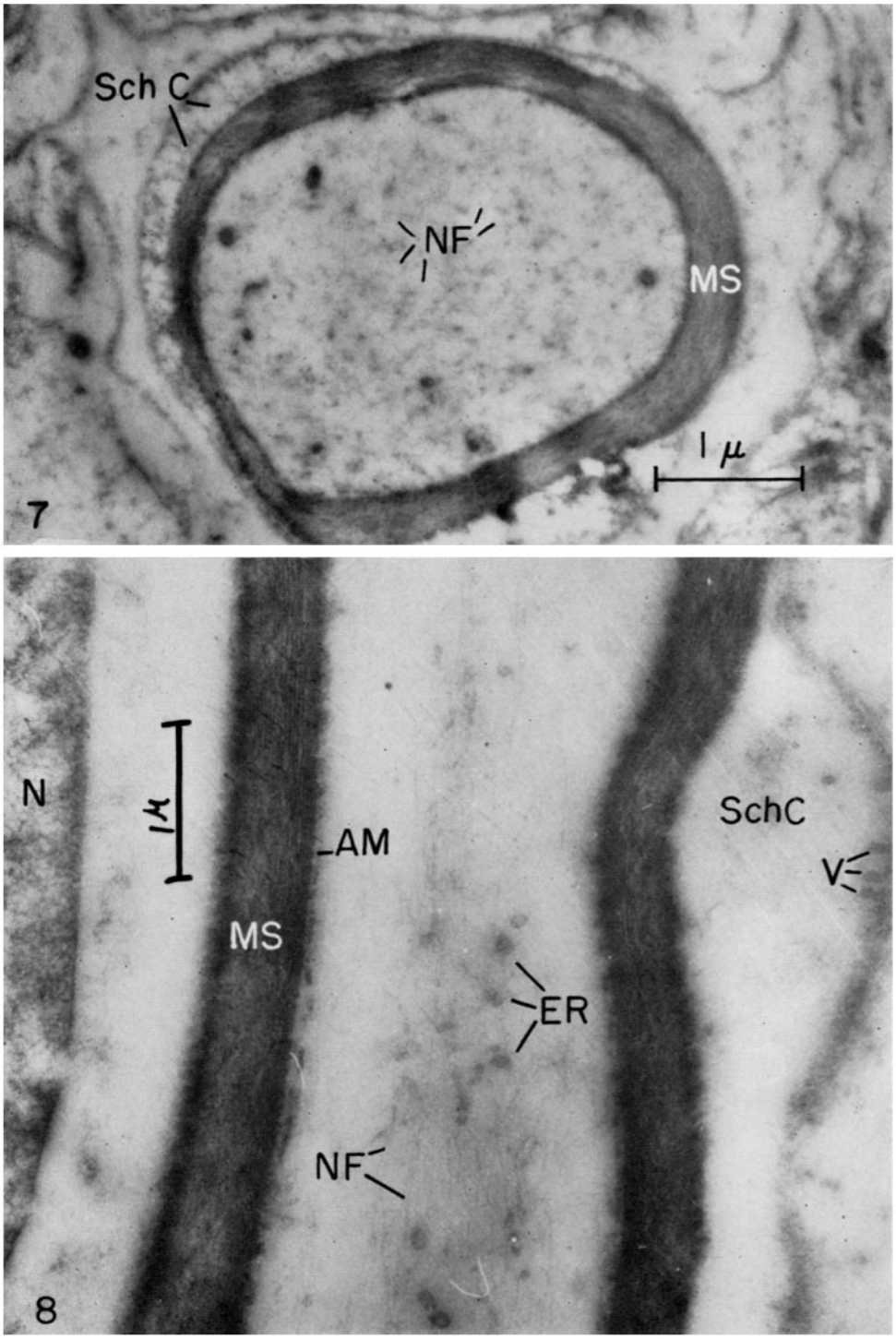


(Thornburg and De Robertis: Frog nerve axoplasm)

PLATE 132

FIG. 7. Electron micrograph of the cross-section of a myelinated nerve fiber of the bullfrog showing the lamellar structure of the myelin sheath and the surrounding Schwann cell. Within the axon most of the small dots and short rods correspond to the neuroprotofibrils. Measurements of the number of neuroprotofibrils can be better achieved in oblique sections. × 21,000.

FIG. 8. Electron micrograph of a longitudinal section of a myelinated nerve fiber. Since the section is much thinner than in Fig. 5 the lamellar structure of the myelin sheath is clearly visible. Only a few neuroprotofibrils and elements of the endoplasmic reticulum are seen within the axon. Note some of the vesicles of the Schwann cells. × 23,000.



(Thornburg and De Robertis: Frog nerve axoplasm)