THE POLARIZED LIGHT METHOD FOR THE STUDY OF MYELIN DEGENERATION AS COMPARED WITH THE MARCHI AND SUDAN III METHODS *

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Introduction

For many years the methods most commonly used for the demonstration of myelin degeneration in the peripheral and central nervous system have been the Weigert, the Marchi, the sudan III, and the scarlet red.

The use of the Marchi method has been criticized by numerous authors because of its capriciousness and because of the large amount of artefact frequently present which interferes considerably with correct diagnosis in cases of suspected early degenerative change. The Weigert, the sudan III and scarlet red methods, although apparently giving consistent results, have the disadvantage of not demonstrating the early phases of myelin change.

In connection with the study of vitamin B_1 deficiency in the rat, we have had occasion to examine the nervous tissues of approximately 500 animals. In these studies the variability of the Marchi method, and the unsuitability of the sudan III and scarlet red methods for the detection of early myelin change in the nerves of rats were thoroughly confirmed.

The results obtained by Sutton, Setterfield and co-workers [1-4] with polarized light in the study of early myelin changes in degenerating nerves indicated that it might be advantageously used in the study of nerves from vitamin B_1 deficient animals. As the method had not been generally used it seemed desirable to subject it to a critical study in which both normal and transected nerves would be examined by the polarized light method as well as by the conventional Marchi and sudan III methods. The results confirm and extend the findings of Sutton and his co-workers, and it is believed that the further observations made are fundamental and

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will be of value to anyone wishing to use the method in similar studies.

Experimental

The nerves for this study were obtained from normal rats 6 to 8 weeks of age and approximately 150 gm. in weight. They had been fed the complete ration used in the stock colony.[5] The right and left lumbosacral and sciatic nerve trunks from 142 rats were studied. Of these, 71 rats were used for studies of the normal nerve, 50 of which were studied by the polarized light method, 11 by the Marchi method, and 10 by the sudan III method. In the remaining 71 rats the right nerves were transected while the left nerves were left intact and studied as normal controls; in this group 28 were studied by the polarized light method, 23 by the Marchi, and 20 by the sudan III method.

For the study of progressive degenerative changes due to transection of the nerve, the animals were lightly anesthetized with ether, the sciatic trunk was cut as high up in the leg as possible, and about 1 mm. of the nerve was removed. When the desired length of time after transection had elapsed, these animals were killed by decapitation. The progress of degenerative change was studied at intervals of 24, 48, 72, 120 and 216 hours after transection. There was no evidence of infection during the interval between transection and killing. The animals used for studies of the normal nerve were either decapitated or killed with chloroform.

After the skin was removed from over the posterior extremities, the muscles were cut away to bare the lumbosacral and sciatic trunks of the right and left extremities so that rapid fixation would take place. As it was considered undesirable to remove the nerves from the legs before fixation, the entire pelvis and extremities were placed in the desired fixative.

The tissues used for the polarized light and sudan III methods were fixed in 10 per cent neutral formalin (Merck) for 48 hours, the fixative being changed at the end of 24 hours. The nerves were removed from the extremities at the end of 48 hours and stored in fresh 10 per cent neutral formalin until sectioned.

The tissues used for the Marchi method were fixed in Müller's fluid for 7 days, the fixative being changed daily for the first 3 days. The nerves were removed from the legs at the end of the 2nd day. After fixation the nerves were placed in Marchi's fluid for 7 days,

washed, dehydrated, and embedded in pyroxylin as rapidly as possible.*

Before the nerves were sectioned they were arbitrarily divided into three pieces: the "proximal" portion consisted of the lumbosacral trunk from its exit from the vertebral column to the head of the femur; the "medial" portion consisted of the sciatic trunk from the head of the femur to the knee joint; and the "distal" portion extended from this point to the heel. In transected nerves only the portions distal to the cut were studied. These corresponded to the "medial" and "distal" portions of the normal nerve.

For study by the polarized light method, frozen sections were made on a clinical microtome at 10, 20 and 35 microns. The sections were cut into distilled water and immediately mounted in glycerin or glycerin jelly.[6] Because of the clearing action of the mounting medium, visual observations were made about 30 minutes after mounting in order to secure as constant a picture as possible. With only a few differences, which will be discussed later in this paper, the technic of visual observation was essentially the same as that described by Sutton, Setterfield and Krauss.[1] As the mounts were not permanent, microphotographs were taken within 60 minutes after mounting and constituted the only permanent record of the section. The microphotographs were taken at 500 diameters at the point of greatest birefringence between crossed Nicols.

For study by the sudan III method, frozen sections were made at 20 microns,* stained, and mounted in glycerin jelly. The nerves prepared by the Marchi method were cut at 20 microns,† dehydrated, cleared in xylol and mounted in dammar.

Results

The Polarized Light Method

General Observations: During the early part of this study it was noted that when normal nerves were cut at 20 microns, numerous

* Comparison of frozen sections of unembedded nerves prepared by the Marchi method with those embedded in pyroxylin indicated that this rapid dehydration and embedding did not alter the resulting picture.

† Thinner sections (10 microns) were studied, but in this case the thickness of sections showed no apparent differences or advantages, and sections cut at 20 microns were used throughout this study.

fibers at the periphery and occasionally at the ends showed marked differences in structure from those in the body of the section. There was considerable darkening of the fibers as compared with the body of the section, and the proportion of the myelin sheath to the axis cylinder was greater. Nodes of Ranvier and incisures of Schmidt-Lantermann were also more frequent and more clearly seen. It was also observed that the same structure was exhibited by isolated fibers, the more distal portions of the nerve trunk, and occasional sections which were thinner than usual. It seemed apparent that the thickness of the section had some bearing on these differences, and a study of the effect of thickness on the details of structure was made.

In order to determine the effect of the thickness of the sections on the visibility of structural details, a series of sections 10, 20 and 35 microns in thickness was prepared. Figures 3 to 5 illustrate the results of this study in normal nerves, and Figures 6 to 8 show the effect of thickness in transected nerves. The structure of the fibers in sections cut at 10 microns (Fig. 1) more closely resembled the structure of isolated fibers (Fig. 1) than those in sections cut at 20 or 35 microns (Figs. 2 and 3). The sections cut at 10 microns constituted roughly a single layer of fibers, and the amount of birefringent material was reduced to a minimum. Visibility was thus increased and fibrillar and interfibrillar structures were more clearly depicted. The increase in birefringent material (myelin sheaths) in the thicker sections caused a haze, which almost completely obliterated any details of structure in the sections cut at 35 microns. It is interesting to note in this connection that in taking microphotographs of sections of different thickness, about one-fifth the exposure necessary for the sections cut at 10 microns was adequate for those cut at 35. Variation in the size of individual fibers within a given section could be clearly seen in the thinner sections (10 microns), whereas in the thicker ones (20 and 35 microns) the haziness often obliterated them altogether.

After considerable study of sections of different thickness, the sections cut at 10 microns were selected as offering the truer representation of the individual fibers and their relations to each other. This thickness was used throughout the remainder of the study.

Since it was desired to apply this method to the study of the peripheral nerves of vitamin B_1 deficient animals where autopsy

material was desired, a study was made to determine the effect of decapitation as compared with killing with chloroform. No apparent differences due to the method of killing were noted in any of the nerves studied.

In variation from the method of Sutton, Setterfield and Krauss,[1] some of the sections were mounted in glycerin jelly [6] rather than in glycerin. This semisolid medium allowed the sections to be safely photographed with the microscope in the horizontal position; it also had the advantage of making it possible to weight the coverglass, and as the material hardened the section remained flattened. A further advantage was that the nerve did not clear so rapidly as when mounted in glycerin. A comparison of sections mounted in both mediums showed that although the glycerin jelly gave a somewhat darker preparation, this did not interfere in any way with the study of the nerve.

The Normal Nerve and its Variations: In 10 micron sections the individual fibers of the nerve did not always follow the parallel course seen in preparations cut at 20 microns. However, the slight waviness seen at times would hardly be mistaken for the tortuosity present in degenerating fibers. Individual fibers were quite regular in size along their course except at the nodes of Ranvier, but differed in size as compared with other fibers in the same area. The fibers seen in the "medial" and "distal" portions of the nerve were usually smaller in diameter, and the proportion of the myelin sheath to the axis cylinder was usually less, although more variable, than in the "proximal" portion.

The myelin sheath was usually birefringent, brilliantly clear at the points of greatest birefringence, and rarely contained isotropic materials. All the materials in the sheath, however, did not become dark at the points of extinction; this gave the section a finely granular appearance. The nodes of Ranvier varied considerably in size and in structure, and were at times very granular. Part of the granulations remained light at the points of extinction. In the greater number of cases, however, the nodes showed club-shaped enlargements which were similar to those previously found in Busch preparations.[5] The incisures of Schmidt-Lantermann were not always present but were quite frequently seen. When visible, they were irregularly placed, their apices varied in direction, and they contained variable amounts of isotropic material. Although

they had been observed at all thicknesses, they were more frequently seen in the 10 micron (Fig. 1) than in the thicker sections (Figs. 2 and 3). The axis cylinders were isotropic and varied somewhat in size. Their borders were usually sharply defined, but at times were mildly roughened. Tortuosity of the axis cylinder was not observed in normal fibers. Interfibrillar connective tissue was isotropic, as were perineural fat deposits. The interrelationship of fibers could often be seen better by uncrossing the Nicol prisms and cutting down the light.

The Transected Nerve: The earliest period at which degenerative change was studied in these experiments was 24 hours after transection, at which time marked and consistent changes were observed. Although the severity of the degenerative change observed at any given interval after transection varied somewhat in different nerves, the progress of the degeneration after 24 hours was essentially as shown by Sutton, *et al.*,[1] and will not be repeated here. However, in the transected nerve as well as in the normal, thickness of the section was an important consideration. Although the edema consistently present in the transected nerves tended to separate the fibers to a variable extent and thus show more isolated fibers than in normal nerves, the thicker sections were hazy and details of structure were at times markedly obscured (Figs. 4–6).

In the earlier stages of the degenerative change localized areas of swelling and apparent fragmentation of the fibers were noted which did not become blackened at the points of extinction. Either this material was not birefringent, or else it was composed of numerous droplets of still normal myelin which did not allow rotation of the section to cause extinction because of its multiple refractive surfaces.

It was often of very great advantage when confronted with hazy undifferentiated threads of fibrillar tissue which seemed partially isotropic, or with a black space between the nerve fibers, to uncross the analyzer prism and cut down the light by lowering the substage condenser. By this means one could distinguish between a tangentially cut fiber which was discontinuous and showed its thready nature, and an isotropic portion of a fiber which usually showed the sheath elements and its continuity with the birefringent portions of the fiber. It could be determined in the same manner

whether a black structureless space between fibers was a split in the section or a darkened area composed of completely isotropic fibers (Figs. 16–19).

The above procedure was also of value in ascertaining whether the axis cylinder of a nerve fiber was swollen or whether there was periaxillar accumulation of isotropic material. In some cases fusiform areas of blackening were seen along the course of the nerve fibers. By uncrossing the Nicols one could often determine whether this represented an accumulation of degenerated myelin or a Schwann cell.

During the progress of the degenerative change many fibers were seen which assumed a "sausage-link" structure, apparently indicating fragmentation. However, when such fibers were viewed with the analyzer uncrossed numbers of them were found which were still contained within an intact sheath (Figs. 16 and 17).

The nerves of 8 animals (16 sciatics) were studied in the fresh state without previous fixation. Frozen sections were made 10 or 20 microns in thickness and mounted in glycerin. The sections were cut into distilled water but remained in it only a few seconds before being placed on a slide and mounted. Study of these nerves revealed a completely different picture from that observed in the fixed nerves (Figs. 13–15). The fibrillar structure was not clearly seen and the tissue reacted only minimally or not at all to rotation of the stage. Degenerated nerves showed more similarity to the fixed nerves than did the normal nerves. A comparison of sections cut at 10 and 20 microns showed that, as in the case of fixed nerves, the increase in myelin caused considerable distortion (Figs. 13 and 14).

The Marchi and the Sudan III Methods

Normal nerves prepared by the Marchi technic exhibited a marked variation in the type and amount of blackened material present. This variability was so great that correct diagnosis was at best exceedingly difficult and uncertain. Nerves transected for 96 hours were seen which showed very little staining with osmic acid, while normal nerves frequently contained copious amounts of blackened droplets. In the transected nerve the first stage at which degenerative change could be definitely ascertained was 72 hours after transection (Fig. 9). The amount of blackened

material indicative of degeneration present at any given interval varied more than was indicated in comparable preparations studied by the polarized light method. Segmentation and fragmentation could be seen clearly as the light was decreased by lowering the substage condenser in areas that showed no blackening with osmic acid. Sections prepared by the Marchi and polarized light methods at intervals of 72 and 120 hours after transection are compared in Figures 9–12. In contrast to the polarized light method only myelin changes are seen in the Marchi preparations.

The normal nerves prepared by the sudan III method showed no evidence of staining except for perineural fat deposits which were intensely dyed. The earliest definite staining of degenerated fibers in transected nerves was 120 hours after transection, although segmentation and other fibrillar changes could be seen clearly long before this time despite the lack of staining.

Discussion

The results obtained in studies of normal and degenerated nerves by the polarized light method show that it is a valuable supplement to the older methods for the demonstration of myelin change. Its chief disadvantages are the comparatively small amount of critical study to which it has been subjected and the necessity of taking routine microphotographs in order to obtain permanent records. As with all new technics, this method demands that careful and critical observations be made until one is thoroughly familiar with its intricacies. On the other hand, it has many advantages over the older methods. It is simple, rapid and consistent. Technical manipulations are reduced to a minimum, thus lessening the possibility of producing artefacts. Both axis cylinder and myelin sheath changes are shown in the same preparation. Moreover, myelin sheath changes after transection of nerves can be demonstrated much earlier by the polarized light method than by either the Marchi or sudan III methods.

The thickness of the section is very important in the polarized light method. It is believed that sections cut at 10 microns offer a truer representation of the structure of the individual fibers and their interrelations than do those cut at 20 or 35 microns. Since those cut at 10 microns represent roughly a single layer of fibers, the haziness due to the birefringent material is minimal and the

details are more clearly seen than in the thicker sections. The use of a microtome which is more accurate than the clinical microtome would be advantageous.

Although only a minimal amount of evidence is at hand, it indicates that the structures observed in normal and degenerated nerves by the polarized light method are to some extent the result of fixation in formalin. The myelin sheaths of fresh (unfixed) nerves exhibited only slightly the property of birefringence, although in two animals, whose sciatic nerves had been transected for 216 hours, the typical "Maltese cross" effect (Fig. 15) consistently seen in degenerated nerves fixed in formalin was visible but reacted only slightly to rotation of the stage. This effect of formalin, however, does not detract from the usefulness of the method.

Summary

1. A study has been made of the merits of the polarized light method as compared with the Marchi and sudan III methods in demonstrating myelin degeneration due to transection of the peripheral nerves of the rat.

2. The polarized light method was found to be rapid and accurate. The changes depicted were consistent and did not depend on numerous technical manipulations for their demonstration. Both myelin sheath and axis cylinder changes were visible in the same preparation.

3. As compared with the polarized light method, the Marchi method gave very inconsistent results. The sudan III method was consistent but failed to reveal the early changes following transection.

4. Marked and advanced changes were shown by the polarized light method in nerves which had been transected only 24 hours. The earliest degeneration shown by the Marchi method was 72 hours after transection and by the sudan III method 120 hours after transection.

5. The thickness of the section influenced the structural detail observed by the polarized light method. Sections 10 microns in thickness showed more detail than thicker sections.

6. It was found to be advantageous to uncross the analyzer prism for determining the continuity of fibers which appear seg-

mented, for distinguishing between edema of the axis cylinders and periaxillar accumulation of isotropic material, and for revealing the presence of isotropic fibers masked by the crossed Nicols.

7. Sections of fresh unfixed normal or degenerating nerves, when viewed by polarized light, presented an appearance considerably different from that of fixed nerves. This does not detract, however, from the usefulness or reliability of the method.

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DESCRIPTION OF PLATES

PLATE 42

All microphotographs were taken at a magnification of 500 diameters. Except for Figs. 9, 11, 17 and 19, all microphotographs were taken in polarized light at the point of greatest birefringence between crossed Nicols.

Wratten metallographic plates were used with a contrast developer (Eastman D–19) for photographs of preparations studied by the polarized light method. Wratten "M" plates were used for the photographs of the Marchi preparations.

FIG. 1. Normal nerve. Microphotograph of the "proximal" portion showing structural details as seen in a section cut at 10 microns. Note the fibrillar detail, the proportion of myelin sheath to axis cylinder, the presence of numerous incisures of Schmidt-Lantermann, and the nodes of Ranvier. Compare with Figs. 2 and 3. Polarized light method.

FIG. 2. Normal nerve. Microphotograph of the same portion of the same nerve shown in Fig. 1. Note the increase in haziness and loss of detail in sections cut at 20 microns. Polarized light method.

FIG. 3. Normal nerve. Microphotograph of the same portion of the same nerve as shown in Figs. 1 and 2. Note the almost complete loss of detail in sections cut at 35 microns. Compare the structure of the node of Ranvier in the middle right hand portion of the photograph with the nodes in Fig. 1. Polarized light method.

FIG. 4. Degenerated nerve. Microphotograph of a section of a nerve which had been transected for 48 hours. Edema, segmentation and increase in isotropic material in the individual fibers are clearly seen. Section cut at 10 microns. Compare with Fig. 1. Polarized light method.

FIG. 5. Degenerated nerve. Microphotograph of the same portion of the same nerve as shown in Fig. 4. Note the loss of detail and interference due to superimposed layers of fibers in sections cut at 20 microns. Polarized light method.

FIG. 6. Degenerated nerve. Microphotograph of the same portion of the same nerve shown in Figs. 4 and 5. Note increased interference and haziness in section cut at 35 microns. Polarized light method.

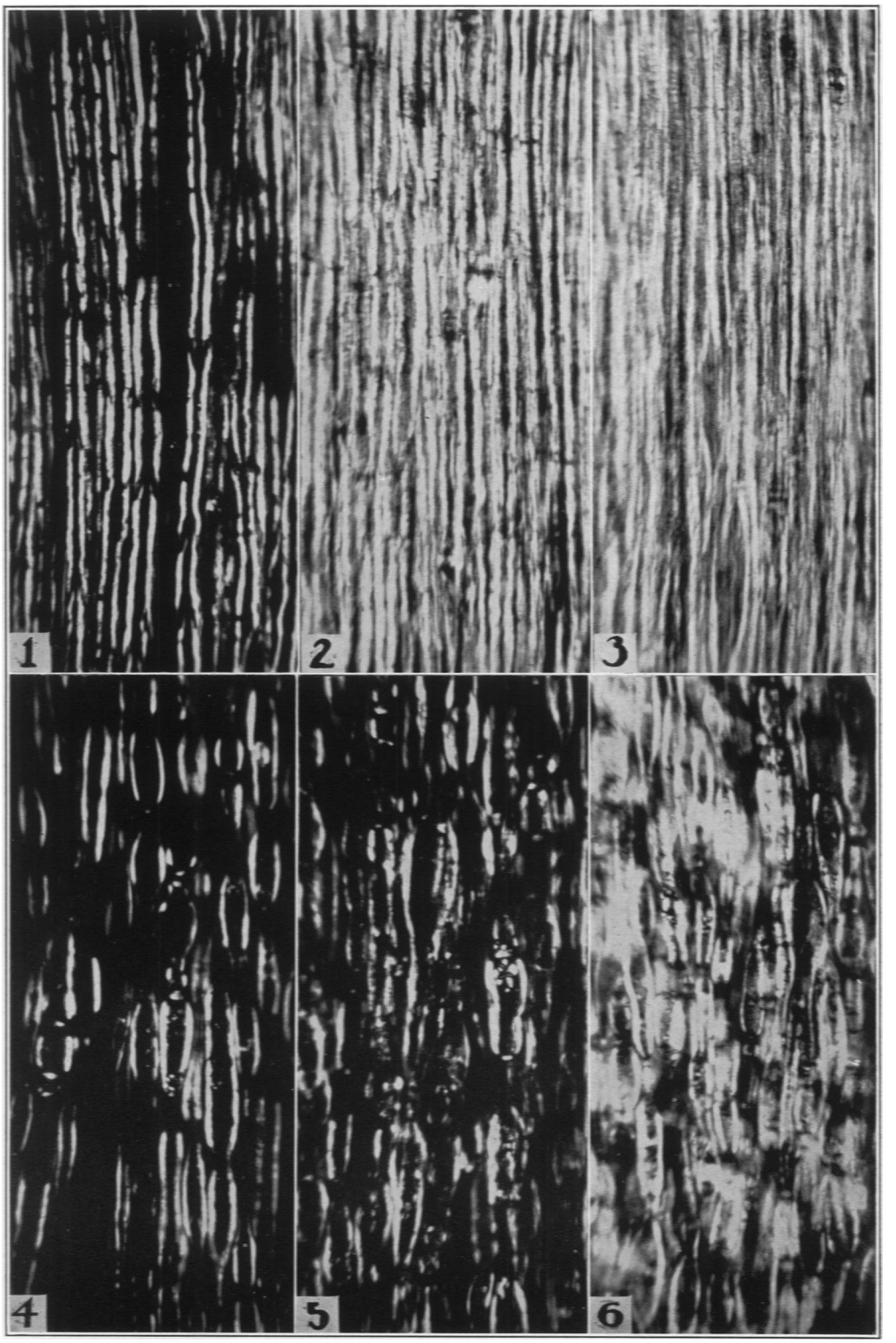

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PLATE 43

FIG. 7. Normal nerve. Microphotograph of the "medial" portion of the same nerve shown in Figs. 1, 2 and 3. Note the variations in size of the fibers and the proportion of myelin to axis cylinder. Compare with Figs. 1 and 8. Polarized light method. Section cut at 10 microns.

FIG. 8. Normal nerve. Microphotograph of "distal" portion of the same nerve shown in Figs. 1, 2, 3 and 4. Note the decrease in size of the fibers to the axis cylinders as compared with "proximal" and "medial" portions. Polarized light method. Section cut at 10 microns.

FIG. 9. Degenerated nerve. Microphotograph of a nerve which had been transected 72 hours showing the irregular black precipitation seen in Marchi preparations. Compare with Fig. 10. Marchi method. Section cut at 20 microns.

FIG. 10. Degenerated nerve. Microphotograph of a nerve that had been transected for 72 hours as shown by the polarized light method. Note the clear sheath and axis cylinder changes that are shown. Compare with Fig. 9. Polarized light method. Section cut at 10 microns.

FIG. 11. Degenerated nerve. Microphotograph of a nerve that had been transected 120 hours. Marchi method. Section cut at 20 microns.

FIG. 12. Degenerated nerve. Microphotograph of a nerve that had been transected 120 hours. Polarized light method. Section cut at 10 microns.

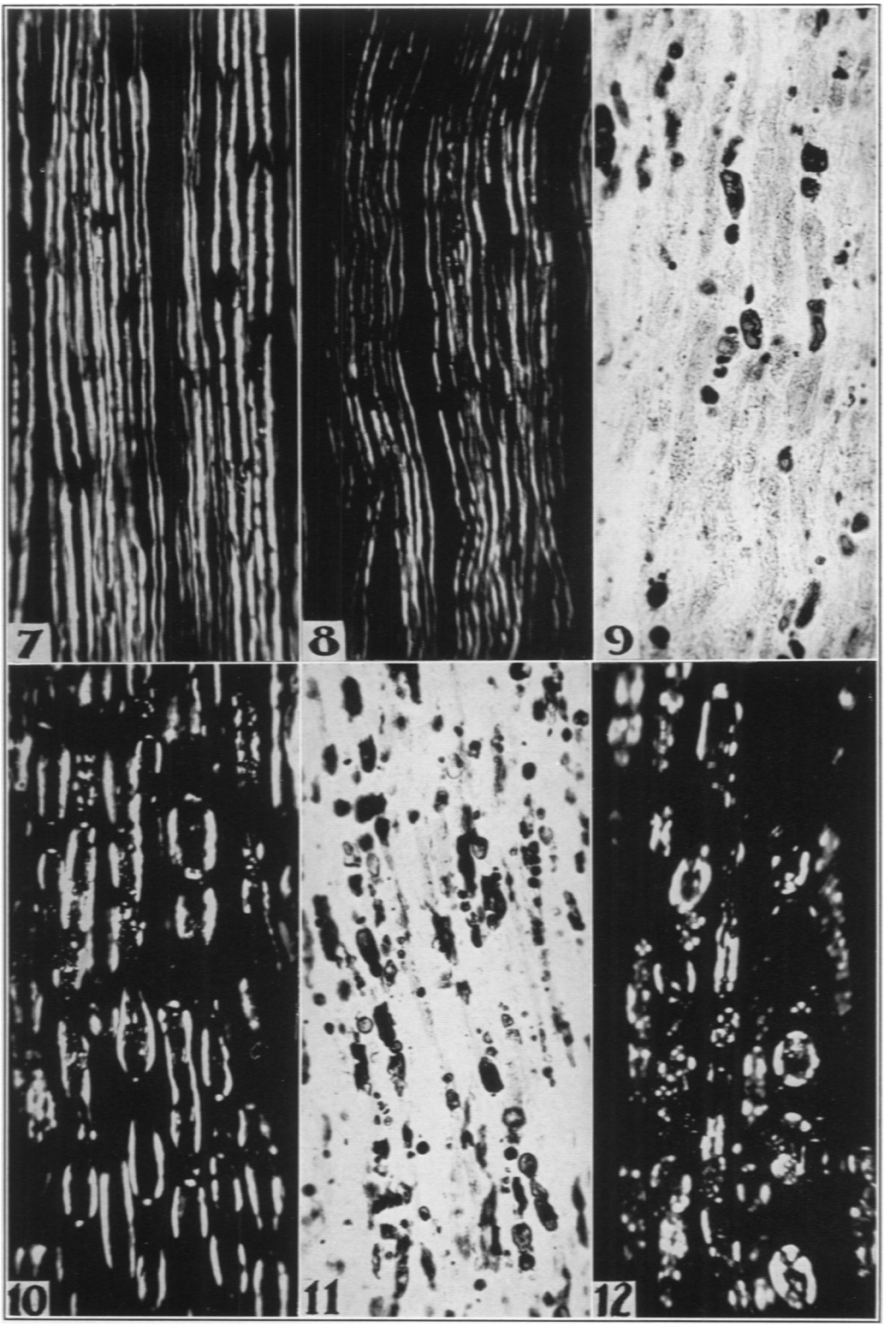

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PLATE 44

FIG. 13. Normal nerve. Microphotograph of a fresh (unfixed) nerve showing the lack of differentiation and structural detail as compared with the fixed nerve shown in Fig. 1. Such preparations react only minimally to rotation of the stage. Polarized light method. Section cut at 10 microns.

FIG. 14. Normal nerve. Microphotograph of a section cut at 20 microns of the same portion of the same nerve shown in Fig. 13. Note the increase in birefringent material as compared to Fig. 13 and the lack of typical fibrillar detail as shown by fixed nerves. Polarized light method.

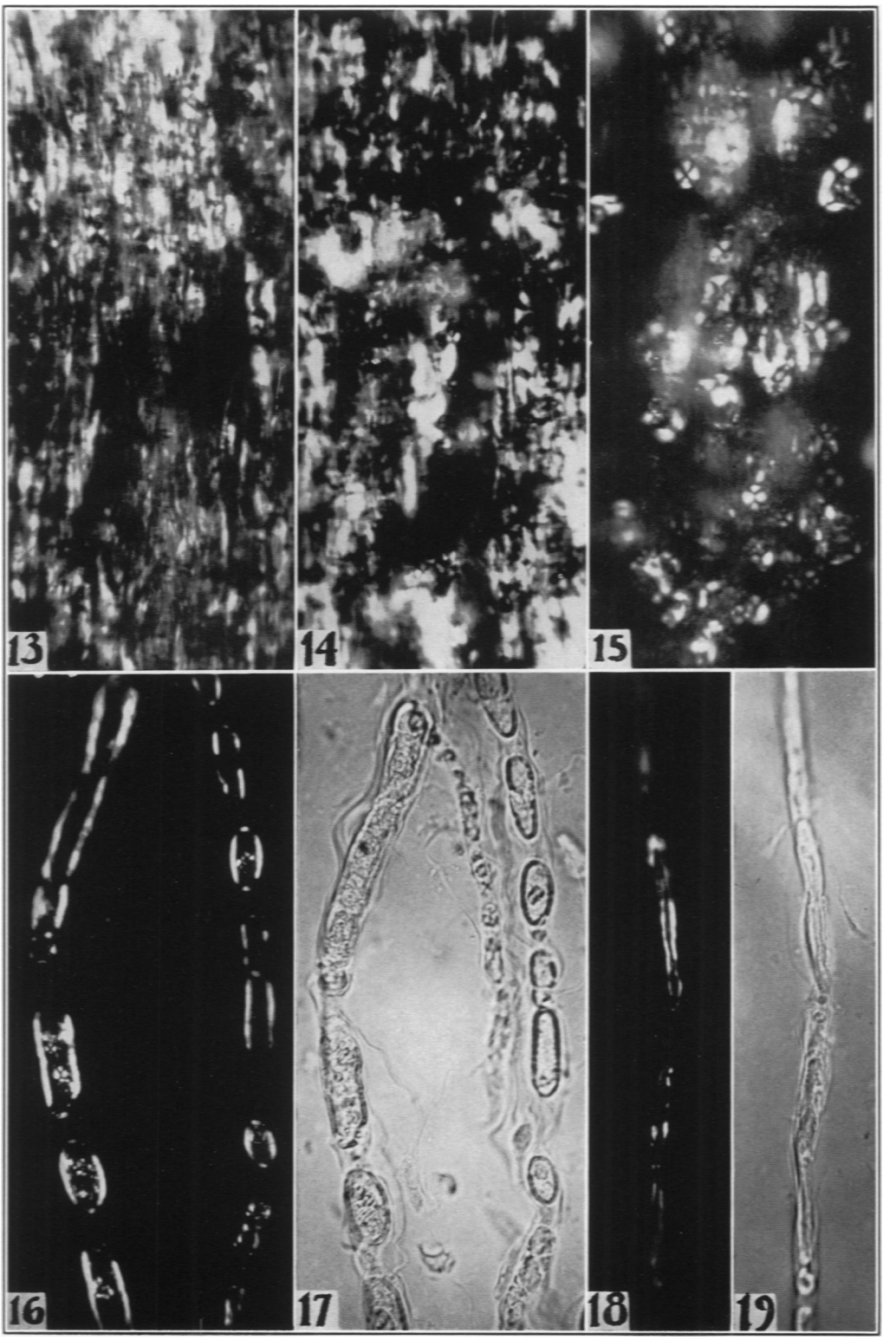
FIG. 15. Degenerated nerve. Microphotograph of a section of a fresh (unfixed) nerve that had been transected for 96 hours. The similarity between the fixed and unfixed nerve is greater than in the normal nerve. Such sections react slightly to rotation of the stage, but structural detail is much less clear and definite than in fixed nerves. Polarized light method. Section cut at 10 microns.

FIG. 16. Degenerated nerve. Microphotograph of isolated fibers from a nerve that had been transected 72 hours. Note the segmentation and the completely isotropic areas between the segments and between the two fibers shown. Compare with Fig. 17. Polarized light method. Section cut at 10 microns.

FIG. 17. Degenerated nerve. Microphotograph of the same portion of the same nerve shown in Fig. 16, showing the advantages of uncrossing the Nicol prisms to determine whether segmentation seen with crossed Nicols is indicative of fragmentation, and also to determine whether a blackened area is a split between fibers or an area of degenerated material that has become completely isotropic. Note the continuity of the sheaths of both fibers and the appearance under these circumstances of an isotropic fiber that had been "masked" by the crossed Nicols. The wavy material between the fibers represents interfibrillar connective tissue. Section cut at 10 microns.

FIG. 18. Degenerated nerve. Microphotograph of an isolated fiber from the same nerve shown in Figs. 16 and 17. Note the difference in size of the fibers in these sections. Compare with Fig. 19. Polarized light method. Section cut at 10 microns.

FIG. 19. Degenerated nerve. Microphotograph of the same portion of the same nerve shown in Fig. 18 with the Nicol prisms uncrossed. Note that the smaller size of the fiber is to a large extent the result of perifibrillar increase in isotropic material. Without examination with uncrossed Nicol prisms this fiber would have been considered merely a smaller or a less edematous fiber than those shown in Fig. 16. Section cut at 10 microns.



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