SILVER STAINING OF THE ENDONEURIAL FIBERS OF THE CEREBROSPINAL NERVES *

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The point of departure of any study of the connective tissue sheaths of the peripheral nerve must still be the work of Ranvier [1,2,3] and of Key and Retzius.[4,5] Review of recent literature shows scarcely a line added to the descriptions given by these masters fifty years ago. The endoneurium is described by the one as strands of fibrous connective tissue, by the other as a sheath, lying in immediate contact with the individual nerve fiber. Obviously, the endoneurium consists of cells as well as fibers: we shall consider the fibers only.

The fibers of the endoneurium are revealed best by the silver methods that have been devised for the study of collagen; in the writer's opinion, the best of them is his modification [6,7] of Hortega's technique described in a former number of the Journal. We shall proceed to apply this method to the endoneurial fibers of a cerebrospinal nerve.

THE DISTAL NERVE

As shown in Fig. 1, the endoneurial fibers of a distal nerve are arranged in two distinct patterns. There are longitudinal collagen fibers, and there is a delicate web around each nerve fiber.

The longitudinal fibers have been described by many authors. They constitute the *Fibrillenscheide* of Key and Retzius (1873,[4] page 354; 1876,[5] page 101), and the intrafascicular connective tissue of Ranvier (1875,[1] page 764; 1889,[2] page 585). Running between and over the nerve fibers, these longitudinal collagen fibers form a coarse network of meshes elongated in the direction of the nerve (Nageotte[8,9]).

The longitudinal fibers may be demonstrated fairly well by any good collagen stain but, like all fibers of the connective tissue group, they are brought out more effectively by silver. Stained with silver, they were described and illustrated by Ramón y Cajal (1909,[10] page 264; 1913,[11] page 76; 1928,[12] page 63).

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The web sheathing the individual nerve fiber is revealed only by silver and only by silver used in a particular way. It has been seen by few. Studnička,[13] Snessarew,[14] and Ranke [15] note in passing that their respective silver techniques reveal "a network in the Schwann sheath and in the capsules of the ganglion cells." However, Plenk,[16] (1927, page 380), of the Histological Institute of Vienna, was really the first to describe and illustrate this delicate network which dips in at Ranvier's nodes and forms a closely fitting sheath around each nerve fiber. In the summer of 1928, Herr Plenk had the kindness to show me his preparations and to look at mine. I believe that it is fair to say that we agreed that the technique described in this paper gives the clearer view of the web. Plenk's work is a mine of information about argyrophil webs in all parts of the body and there is a full bibliography, from which the references in this paragraph were taken.

Here and there, the longitudinal fibers give off branches to the web and, at points where the nerve fibers have been torn apart, delicate filaments may be seen to pass from one web to another; these observations were made first by Nageotte.[9] While we have dealt with them separately for the purpose of description, longitudinal fibers and web undoubtedly form a whole and are to be regarded as the ultimate distribution of fibrous connective tissue around the individual nerve fiber.

We have found the same construction of the endoneurium in man, and in all of the laboratory animals examined, cat, dog, rabbit, rat, monkey and guinea pig.

The Dorsal Root Ganglion

From the fibrous capsule of the ganglion, septa of fibrous connective tissue extend inward, forming sheaths around each nerve fiber and around each ganglion cell. All this is demonstrable easily by the usual collagen stains; but, at this point, silver takes up the tale and reveals around each ganglion cell a closely plaited web of argyrophil fibers, as shown in Fig. 2. Here and there on the web are coarse, parallel fibers, representing the longitudinal fibers of the endoneurium.

In these argyrophil webs, there seems to be only one orifice, the point of exit of the axis cylinder. Fortunate sections show the argyrophil fibers woven neatly and smoothly around this orifice and at this point the argyrophil web of the ganglion cell is continuous with the argyrophil web of the nerve fiber.

Over the ganglion cell, the web is stronger and bolder than on the distal nerve. We shall see it become still denser and more intricate as we follow the root upward to and into the cord.

Glomeruli

Immediately on leaving the ganglion cell, the axis cylinder describes a curious convolution known as the glomerulus. The argyrophil web clings closely to the nerve fiber and accompanies it in all of its twistings and turnings. In the glomerulus, heavy argyrophil fibers run around and around the nerve fiber, forming a tubular sheath which gives an impression of resistance and rigidity. The arrangement suggests the spiral wire reinforcement around a rubber garden hose. Counterstaining with azo carmin shows the winding axis cylinder inside of the argyrophil tube.

The Spinal Nerve Roots

Fig. 3 shows a sensory root entering the cord. In the roots, both the longitudinal fibers and the web are heavier and more prominent than in the distal nerve. Here the plexus arrangement of the endoneurium is particularly evident, heavy argyrophil fibers crossing the nerve bundle in all directions. As in the distal nerve, at points where the nerve fibers have been torn apart, delicate web filaments are seen to pass from one nerve fiber to another, (Nageotte[9]).

The Pia

Silver brings out in striking contrast the two layers of the medullary pia described by Key and Retzius [5] (1875, page 143). As the root joins the cord, it is seen clearly that endoneurium and perineurium are merely peripheral extensions of these two layers of the pia mater. The outer layer of the pia consists of concentric laminae of dense collagen; it continues out over the root as the laminated perineurium.

The inner layer, the intima piae of Key and Retzius, is a loosely woven network of collagen and reticulum fibers that splits into two layers to surround the roots. The outer layer of this intima piae extends out over the root just beneath the perineurium. In fact, it may be said that the peripheral nerve never succeeds wholly in getting outside of the pia mater. In cross-sections of such trunks as the sciatic and the tibial, these layers of the pia around the nerve bundle are recognized readily; even in the finest branches, the outer layer of the pia, the perineurium, continues as Henle's sheath.

The Pial Ring

The root slants upward and inward between the two layers of the intima piae and enters the cord through a hole in the inner layer. The margin of this hole is reinforced by a heavy ring of fibers from the intima piae. At the entrance of the relatively large sensory roots, the pial ring is strengthened further by fibrous partitions, dividing it into several smaller rings. Here and there, a small bundle of nerve fibers leaves the main bundle of the root and enters the cord through a small aperture of its own.

The pial ring merits our attention, for it explains the endoneurium. In favorable sections it can be seen plainly that the longitudinal fibers of the endoneurium spring from the pial ring. What appear to be holes in the inner layer of the intima piae are the points where its fibers stream out through the root around the individual nerve fibers to become the longitudinal fibers of the peripheral endoneurium.

The Intramedullary Endoneurium

As shown in Fig. 3, the endoneurium accompanies the nerve fibers of the root for a short distance into the cord; but there is a striking difference in its arrangement outside and inside of the pial ring. Outside, in the root, the strong "longitudinal" fibers run in all directions, binding the nerve fibers together into a bundle. Inside, where the nerve fibers are embedded in the substance of the cord, there seems to be no need of such collective support. Here the binding fibers are reduced to a few delicate filaments that can be traced from one nerve fiber to another. Inside of the cord, support is given rather to the individual nerve fiber by winding strong argyrophil fibers around and around it to form a tubular sheath, very like the tubular sheaths wound around the glomeruli in the ganglia. These winding argyrophil fibers can be traced to the pial ring. The pial ring, then, formed by the inner layer of the intima piae, supplies both kinds of fibers. Facing outward, the pial ring gives off the longitudinal fibers of the peripheral endoneurium; facing inward, it supplies tubular sheaths to the nerve fibers embedded in the cord.

These argyrophil tubes are seen especially well in thick sections, 20 to 25 microns. After penetrating the cord for a short distance, the bundle of tubes stops abruptly as if chopped off by a knife. The tip of each tube is conical, rounded off as neatly as if turned in a lathe. In many sections, a pale pink axis cylinder from the cord may be seen to enter each conical tip and run inside of the tube formed by the argyrophil web. Counterstaining with azo carmin brings out clearly the position of the axis cylinder inside of the argyrophil web. Motor and sensory roots present exactly the same structure.

The Pial Funnels

At the points where blood vessels from the pia enter the cord, Key and Retzius (1876,[5] page 5 and Table I) describe funnel-shaped extensions of the intima piae (*Piatrichter*) sunk into the cord, forming a loose sheath around the vessel. In silvered sections, these funnels are seen clearly outlined in black. As stated by these authors, here and there a root may enter the cord through such a pial funnel. Most of the roots penetrate the cord accompanied only by the argyrophil endoneurium and the membrane of Schwann.

The Membrane of Schwann

Ranvier (1875,[1] page 1073; 1882,[3] page 1069; 1889,[2] page 805) stated that the membrane of Schwann accompanies the root fibers for a short distance into the marginal glia of the cord and he gives an illustration that is strikingly like Fig. 3. According to Nageotte, this observation has been forgotten. Inside of the cord, the membrane of Schwann and the argyrophil endoneurial sheath have exactly the same distribution.

Technique

We shall give here only a résumé of the technique as modified for the peripheral nerves, referring the reader to the paper in a former number of the Journal (1929) where the methods and the formulas were described in detail.

For Distal Nerves:

1. Large nerves should be split into thin slices to ensure rapid penetration. Fix in Zenker from 3 to 5 hours, no longer. Wash in running water from 3 hours to overnight, as convenient.

2. Embed in paraffin.

3. Stick sections on the slide with Masson's gelatin glue; harden the gelatin in hot formol fumes overnight (*Am. J. Path.*, 1928, **4**, 206; *ibid.*, 1929, **5**, 245).

4. After removal of the paraffin, wash in running water for 5 minutes.

5. Mordant with the Mallory bleach:
   (*a*) 1 per cent tincture of iodin, 3 minutes; rinse in tap water.
   (*b*) 5 per cent hypo, 3 minutes; rinse in tap water.
   (*c*) $\frac{1}{4}$ per cent potassium permanganate, 5 minutes; rinse in tap water.
   (*d*) 5 per cent oxalic acid, 5 minutes; wash well in running water for 10 minutes.

6. Distilled water; change 3 times within 5 or 10 minutes.

7. Río-Hortega's lithium silver augmented to 10 per cent at 55 to 58° C. for 5 minutes.

8. Quick rinse by pouring distilled water over both sides of the slide.

9. Formol, 1 per cent in tap water, 3 minutes.

10. Rinse with distilled water.

11. Yellow gold chlorid, 1 to 500, at room temperature, 10 minutes.

12. Rinse with distilled water.

13. Oxalic acid, 5 per cent, 10 minutes.

14. Rinse with distilled water.

15. Hypo, 5 per cent, 10 minutes; change as often as it becomes turbid.

16. Wash well in running water to remove the hypo.

Counterstain as desired and mount in balsam. The best counterstains are the reds, such as erythrosin, 1 per cent, or azo carmin, $\frac{1}{2}$ per cent.

The silver solution and the gold solution may be used again and again. Filter the silver solution before use.

For Ganglia:

1. Fix in Bouin's fluid from 1 to 3 days; pass directly to absolute alcohol.
2. Embed in paraffin.

Subsequent steps as for distal nerves except that, in Step 4, Bouin sections should be washed in running water for 20 minutes to remove the picric acid thoroughly; and, in Step 7, the temperature and concentration of the silver bath should be lower. Ten per cent lithium silver at room temperature for 10 minutes or 2 to 3 per cent silver at 40 or 45° C. for 5 minutes give cleaner lines and better detail than the higher temperatures that are necessary for distal nerves.

As noted in the former paper, after Bouin fixation this technique differentiates ectodermic from mesodermic cells. Correspondingly, while all mesodermic cells are invisible, the ectodermic ganglion cells are silver-positive. To obliterate the ganglion cells and secure a pure picture of the collagen framework, as in Fig. 2, we have found several methods effective. The most reliable of them is to repeat the Mallory bleach. After the first Mallory bleach, leave the sections overnight in distilled water, changing it several times. The next day, repeat the bleach and continue from Step 6 as usual.

Ganglia fixed in Zenker not more than 3 hours and stained with silver at 40° C. or under, show good webs and colorless ganglion cells.

For Spinal Nerve Roots:

Fix in Bouin and treat as ganglia. The cord is likely to stain red or black, giving poor contrast with the black web on the roots. Here the double Mallory bleach is useless. A paler ground is secured by a quick rinse with weak ammonia water after the silver bath. The ammonia rinse should not be too long or too strong or the web on the root will be decolorized also. We add 5 drops of ammonia to 100 cc. of distilled water and pour it over the slide for exactly 5 seconds by the watch; then rinse quickly with distilled water and proceed from Step 8 as usual. If the ammonia is to be used, stain the sections at 40 or 45 ° C. Sections stained at room temperature decolorize too easily.

Zenker, Formol and Bouin Fixation:

Formol fixation may be rejected at once. In formol-fixed sections, the web and the finer details of the endoneurium remain invisible.

The universal dependence on formol fixation may be the chief reason why these details were not described long ago.

For distal nerves Zenker is the fixative of choice. For ganglia and roots, Zenker is one of the best fixatives but it is highly selective and somewhat erratic; it sensitizes the various components of the tissue in different degrees and demands some care in selecting the time in the fixative and the temperature and concentration of the silver bath. For ganglia and roots Zenker is a fixative for the expert; Bouin is the fixative for the routine worker, for with routine methods it gives uniform results.

Summary

The endoneurium consists of longitudinal fibers and a closely fitting argyrophil web. The distribution of the web is described together with the silver technique necessary for its demonstration.

This study was commenced and in great part completed in 1927 in the Laboratory of Neurocytology of the Presbyterian Hospital of New York City, at the instance of the Director, Wilder Penfield. The writer takes this occasion to thank both Professor Penfield and Professor William V. Cone for cordial assistance of every kind. That modern master of the histology of the peripheral nerves, Professor Nageotte, of the Collège de France, has been good enough to review and confirm these observations. The writer remains ever indebted to him for sound criticism and advice.

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DESCRIPTION OF PLATES

PLATE 93

FIG. 1. Sciatic nerve of cat. Paraffin section. The author's silver technique. The endoneurial fibers (longitudinal fibers and web). The axis cylinders, Schwann cells and myelin sheaths (neurokeratin) are invisible.

FIG. 2. Dorsal root ganglion of cat. Paraffin section. Author's silver technique. A pure picture of the framework of the ganglion; all else invisible. In the upper part of the figure, the web forms fibrous capsules over several (invisible) ganglion cells; over many of the cells it has been cut away.

Center of figure, portion of a glomerulus inside of a fibrous capsule; below this, an entire glomerulus and two fibrous capsules with tops cut off.

Lower part of figure, the endoneurium of the root fibers, showing the longitudinal fibers and the web.

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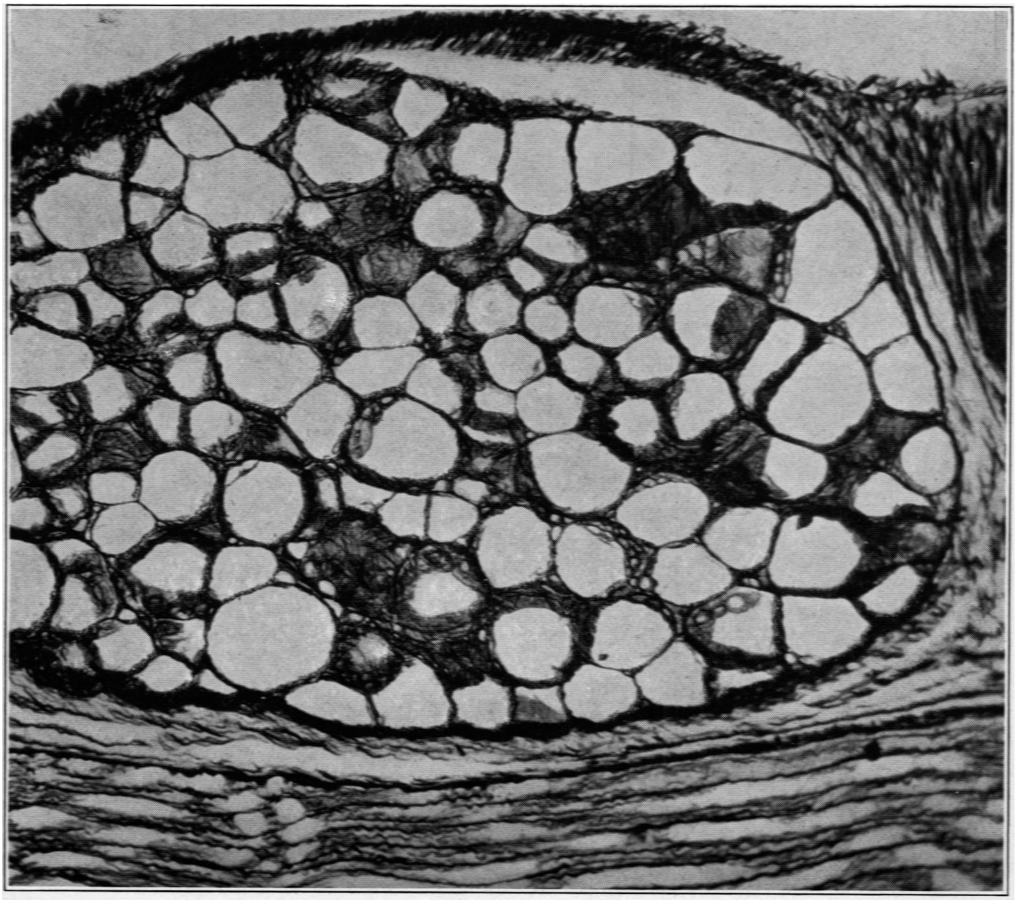

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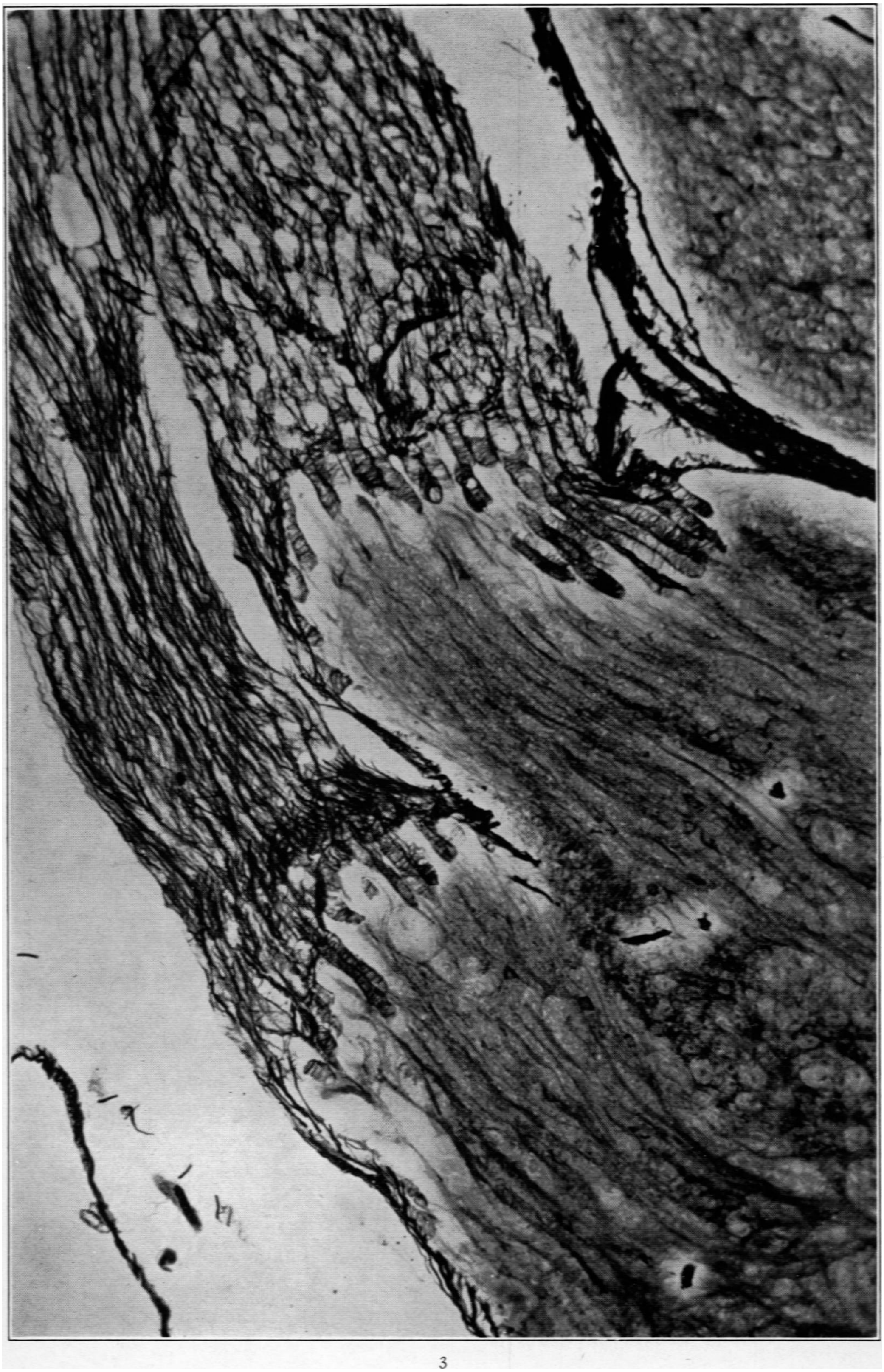
PLATE 94

FIG. 3. Cross-section of cord of cat; entrance of sensory root. Paraffin section. Author's silver technique.

Upper left, the endoneurium of the root with prominent longitudinal fibers and web. Center, the pial ring, forming two loops. The longitudinal fibers of the root are continuous with those of the pial ring.

Within the pial ring, the intramedullary endoneurium, accompanying the nerve fiber for a short distance into the cord. Here the longitudinal fibers are few and inconspicuous. The intramedullary endoneurium is seen to consist chiefly of spiral or circular fibers given off by the pial ring: it ends abruptly in a conical tip from which the axis cylinder emerges.



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Laidlaw Silver Staining of Endoneurial Fibers