Immunocytochemical Localization of P_0 Protein in Golgi Complex Membranes and Myelin of Developing Rat Schwann Cells

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ABSTRACT P_0 protein, the dominant protein in peripheral nervous system myelin, was studied immunocytochemically in both developing and mature Schwann cells. Trigeminal and sciatic nerves from newborn, 7-d, and adult rats were processed for transmission electron microscopy. Alternating 1-μm-thick Epon sections were stained with paraphenylenediamine (PD) or with P_0 antiserum according to the peroxidase-antiperoxidase method. To localize P_0 in Schwann cell cytoplasm and myelin membranes, the distribution of immunostaining observed in 1-μm sections was mapped on electron micrographs of identical areas found in adjacent thin sections. The first P_0 staining was observed around axons and/or in cytoplasm of Schwann cells that had established a 1:1 relationship with axons. In newborn nerves, staining of newly formed myelin sheaths was detected more readily with P_0 antiserum than with PD. Myelin sheaths with as few as three lamellae could be identified with the light microscope. Very thin sheaths often stained less intensely and part of their circumference frequently was unstained. Schmidt-Lanterman clefts found in more mature sheaths also were unstained. As myelination progressed, intensely stained myelin rings became much more numerous and, in adult nerves, all sheaths were intensely and uniformly stained. Particulate P_0 staining also was observed in juxtanuclear areas of Schwann cell cytoplasm. It was most prominent during development, then decreased, but still was detected in adult nerves. The cytoplasmic areas stained by P_0 antiserum were rich in Golgi complex membranes.

Peripheral nervous system (PNS) myelin is synthesized by Schwann cells, and one of its major constituents is P_0 protein. The P_0 protein is an integral membrane glycoprotein (30,000 mol wt) which comprises ~50% of the total myelin proteins in mammalian peripheral nerves (10). We studied its localization immunocytochemically in 20-μm-thick vibratome sections and found that P_0 antiserum stained all peripheral myelin sheaths intensely and uniformly (25). Similar results have been obtained using paraffin (26) and frozen (5) sections, but none of the methods used achieved cellular preservation adequate for a more detailed analysis of P_0 localization.

Recently, Baskin et al. (2, 3) described a method to immunostain osmium-fixed, epoxy embedded tissue according to the peroxidase-antiperoxidase (PAP [20]) method. We have used this postembedding immunocytochemical technique to detect the presence of P_0 in newly formed myelin sheaths and in perinuclear Schwann cell cytoplasm. We also show, by tracing the distribution of immunostaining on electron micrographs of adjacent thin sections, that P_0 is in cytoplasmic areas rich in Golgi complex membranes. A preliminary report of this work has already appeared (27).

MATERIALS AND METHODS

Tissue

Newborn, 7-d-old and adult Sprague-Dawley rats were fixed by intracardiac perfusion with an aldehyde solution containing 1.5% glutaraldehyde and 0.5% paraformaldehyde in 0.08 M phosphate buffer. Segments from the trigeminal and sciatic nerves were dissected from these animals and fixed for an additional hour. To obtain better fixation of adult rat sciatic nerves, additional animals were perfused through the abdominal aorta. The tissue was postfixed in 2% osmium

tetroxide, dehydrated in ethanol, and embedded in Epon. Human sural nerve biopsies were fixed in 3.6% glutaraldehyde and processed in the same way as the rat tissue. 1-μm-thick sections were cut with glass knives, mounted on glass slides, and encircled with a diamond scribe. 12 serial sections were cut from the newborn and 7-d trigeminal nerves. Even-numbered sections were stained immunocytochemically with P_0 antiserum; the others were stained with paraphenylenediamine (PD), a cytological reagent that stains myelin sheaths intensely (8). From the 7-d trigeminal nerve, additional 1-μm-thick sections were cut adjacent to thin sections with silver interference colors. The 1-μm sections were stained with P_0 antiserum. The thin sections were mounted on a Formvar-coated slot grid, stained with uranyl acetate and lead citrate, and examined with a Philips 400 electron microscope. 26 immunostained Schwann cells were photographed; the negative images were enlarged and projected onto electron micrographs of the same cells so that nuclear and cellular outlines corresponded. Areas of immunostaining then were traced on the electron micrographs.

Immunostaining Procedure

1-μm-thick Epon sections mounted on glass slides were placed in a 60–80°C oven for 24–48 h before immunostaining. Pretreatment of Epon sections before immunostaining was similar to that described by Baskin et al. (2). Epon was removed from the tissue by immersing the slides for 15 min in a solution of saturated sodium ethoxide/absolute ethanol (1/2 vol/vol). Before use, the saturated sodium ethoxide was aged until it was dark brown (~2 wk). The slides were rinsed three times in absolute ethanol and then placed in 0.2% hydrogen peroxide for five min. After this bleaching step, the slides were rinsed three times in 0.5 M tris buffer (pH 7.6) and stained with a 1:500 dilution of P_0 antiserum by the peroxidase-antiperoxidase method as previously described (11). All sections were examined microscopically with bright-field illumination.

P_0 antiserum was prepared in rabbits. The purity of the P_0 protein used in preparing the antiserum and the specificity of this serum for P_0 protein has been described (25). The specificity of P_0 immunostaining was tested by incubating 1-μm sections of peripheral nerves with preimmune serum or P_0 antiserum, which was absorbed with purified P_0 protein.

Comment

The most critical step in immunostaining Epon sections was the removal of Epon by sodium ethoxide. Incomplete removal of Epon decreased or prevented immunoreactivity of the primary antiserum with its antigen. Excessive sodium ethoxide treatment damaged or often completely removed the tissue from the slides. Before use, the saturated sodium ethoxide had to be aged until it attained a dark-brown color (2). The ability of this solution to remove Epon increased with prolonged storage. Various epoxy resin mixtures attain different degrees of polymerization. Thus, the potency of the sodium ethoxide and the degree of polymerization of the Epon changed the duration of treatment and/or concentration of sodium ethoxide necessary for complete Epon removal. We examined slides with a phase-contrast microscope following sodium ethoxide treatment to make sure that the plastic was removed and tissue remained undamaged.

Tissue fixed with a mixture of 1.5% glutaraldehyde and 0.5% paraformaldehyde, or with 3.6% glutaraldehyde and then postfixed in 2% osmium tetroxide, or else fixed in osmium tetroxide alone (results not shown), were intensely stained by P_0 antiserum. Other fixatives or aldehyde concentrations were not tested. To obtain intense P_0 immunostaining, sections had to be bleached with H_2O_2 after Epon removal. Potassium-ferrocyanide-reduced osmium tetroxide is often used as a postfixative for ultrastructural analysis of myelin membranes (13). This solution provides increased contrast in tissue sections, and the trilaminar structure of unit membranes is more readily appreciated. The use of ferrocyanide-reduced osmium tetroxide decreased the intensity of immuno-precipitate when 1-μm-thick Epon sections were stained by the procedure employed here.

RESULTS

In PD-stained sections of newborn rat trigeminal nerves, many Schwann cells had established a 1:1 relationship with axons (Fig. 1). Compact myelin sheaths, which were intensely stained by PD, surrounded some of these sheaths. The diameter of these axons (~1–3μm) and the thickness of their myelin sheaths were similar in light micrographs of PD-stained sections and in electron micrographs of serially-cut thin sections. PD also stained mitochondria in Schwann cell cytoplasm.

When adjacent 1-μm-thick sections of newborn trigeminal nerves were treated with P_0 antiserum, many more single axons were surrounded by detectable staining than in PD-treated sections (Figs. 1 and 2). Completely compact sheaths were thick, densely stained rings. They were thicker than the same sheaths in PD-stained sections, and comparison showed that axonal areas were reduced by this increase in thickness (Figs. 1 and 2). Thinner rings often were incomplete and sometimes stained less intensely. They were shaped like horseshoes, commas, or curved strips (Figs. 2 and 3).

Particulate P_0 staining also occurred in the cytoplasm of Schwann cells. The particles differed in size and distribution from mitochondria. Schwann cells that contained cytoplasmic P_0 immunostaining usually were sectioned through their nuclei and also had periaxonal staining. However, particulate cytoplasmic staining was present in occasional Schwann cells that did not contain periaxonal staining (Fig. 3). Still, these Schwann cells and all others immunostained by P_0 antiserum had isolated single axons. No staining was observed in Schwann cells that surrounded multiple axons or were dividing.

In comparable regions of 7-d trigeminal nerves, PD-stained sections contained many large compact myelin sheaths (Fig. 4). Mitochondria in axons and Schwann cell cytoplasm also were intensely stained.

In adjacent sections that were treated with P_0 antiserum, all myelin sheaths were intensely stained (Fig. 5). As in the newborn nerves, myelin sheaths were thicker than they were in the PD-stained sections. Comparisons of the same sheaths in light and electron micrographs showed that axonal areas were reduced by spread of PAP reaction product at the inner margins of myelin sheaths. Outer margins of myelin sheaths corresponded closely, as did the boundaries of Schwann cells and their nuclei. Epoxy resin removal by sodium ethoxide did not change myelin sheath thickness because thicker sheaths were not observed in sections treated with preimmune or P_0 absorbed serum. In these control sections, no staining was observed (Fig. 6).

Schwann cells in 7-d nerves contained particulate P_0 staining in perinuclear regions. The distribution of this cytoplasmic staining was traced on electron micrographs of the same cells in adjacent thin sections (Fig. 7). In all cells studied, P_0 staining was restricted to areas rich in Golgi complex membranes. This comparison also demonstrated that myelin sheaths with as few as three lamellae were stained by P_0 antiserum. Noncompacted portions of myelin sheaths were not stained by P_0 antiserum, or they stained with less intensity than compacted myelin lamellae.

In sections of adult nerves, P_0 antiserum stained all myelin sheaths intensely and uniformly (Fig. 8). There were gaps in myelin staining at Schmidt-Lanterman clefts; pockets of cytoplasm located paranodally or elsewhere along myelin segments also were unstained. There were no lightly stained sheaths in adult nerves. Schwann cells that had formed myelin sheaths in adult nerves still contained particulate P_0 staining in perinuclear regions. The intensity of this staining was less than that found in developing nerves.

To test the effect of prolonged storage of Epon-embedded nerves on the intensity and distribution of P_0 staining, we sectioned and stained blocks of human sural nerves that were embedded in Epon 19 yr ago (Fig. 9). The results were similar to those obtained with freshly processed rat tissue (Fig. 8).

DISCUSSION

Our results clearly demonstrate that the immunostaining technique described here is a very sensitive, highly specific method for detecting P_0 protein in Schwann cell cytoplasm and myelin

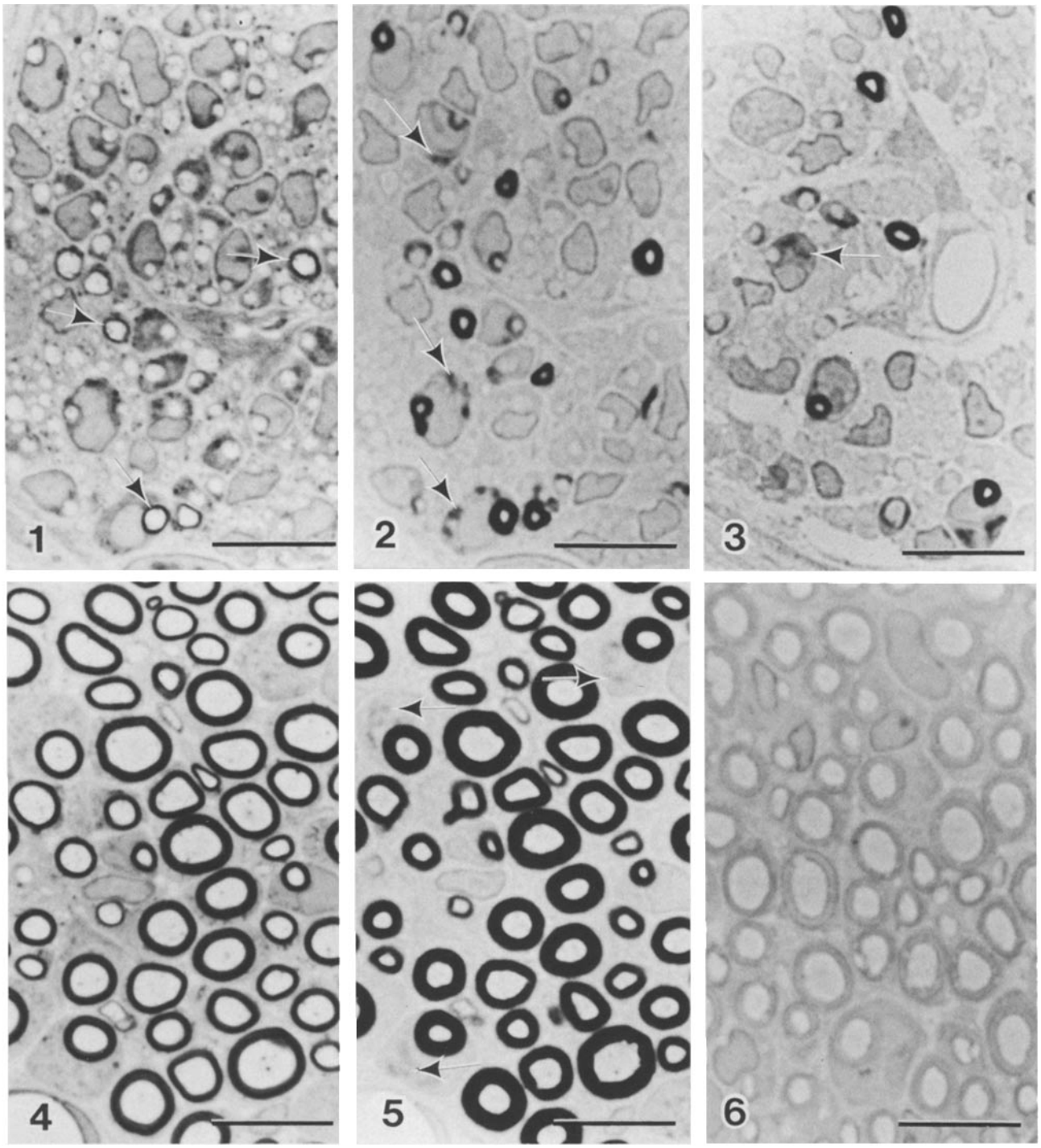

FIGURE 1 Transverse section of a newborn trigeminal nerve stained with paraphenylenediamine (PD). Numerous Schwann cells surround single axons and there are some thin compact myelin sheaths (arrows). Densely stained dotlike profiles in Schwann cell cytoplasm are mitochondria. Phase-contrast. Bar, 10 μm. × 2,000.

FIGURE 2 This section is adjacent to that shown in Fig. 1; it was stained with P_0 antiserum (1:500) and counterstained with hematoxylin. Many axons are surrounded by intense P_0 staining, whereas others are surrounded by less intense and often incomplete staining. Intense particulate staining is present in the cytoplasm of some Schwann cells (arrows). Bright-field. Bar, 10 μm. × 2,000.

FIGURE 3 Transverse section of a newborn trigeminal nerve stained identically to that shown in Fig. 2. P_0 staining is present around axons and in Schwann cell cytoplasm. One Schwann cell (arrow) contains particulate P_0 staining within its cytoplasm, but no staining is detectable around its axon. Bright-field. Bar, 10 μm. × 2,000.

FIGURE 4 Transverse section of a 7-d rat trigeminal nerve stained with PD. Myelin sheaths are intensely stained. There also is particulate staining of mitochondria in axons and Schwann cell cytoplasm. Phase-contrast. Bar, 10 μm. × 2,000.

FIGURE 5 This section, stained with P_0 antiserum (1:500) and counterstained with hematoxylin, is adjacent to that shown in Fig. 4. Myelin sheaths are intensely stained. There is particulate staining in Schwann cell cytoplasm (arrows), and its distribution differs from that shown in Fig. 4. Bright-field. Bar, 10 μm. × 2,000.

FIGURE 6 Control transverse section of a 7-d trigeminal nerve immunostained with preimmune serum (1:500). There is no staining of myelin sheaths or Schwann cells. Phase-contrast. Bar, 10 μm. × 2,000.

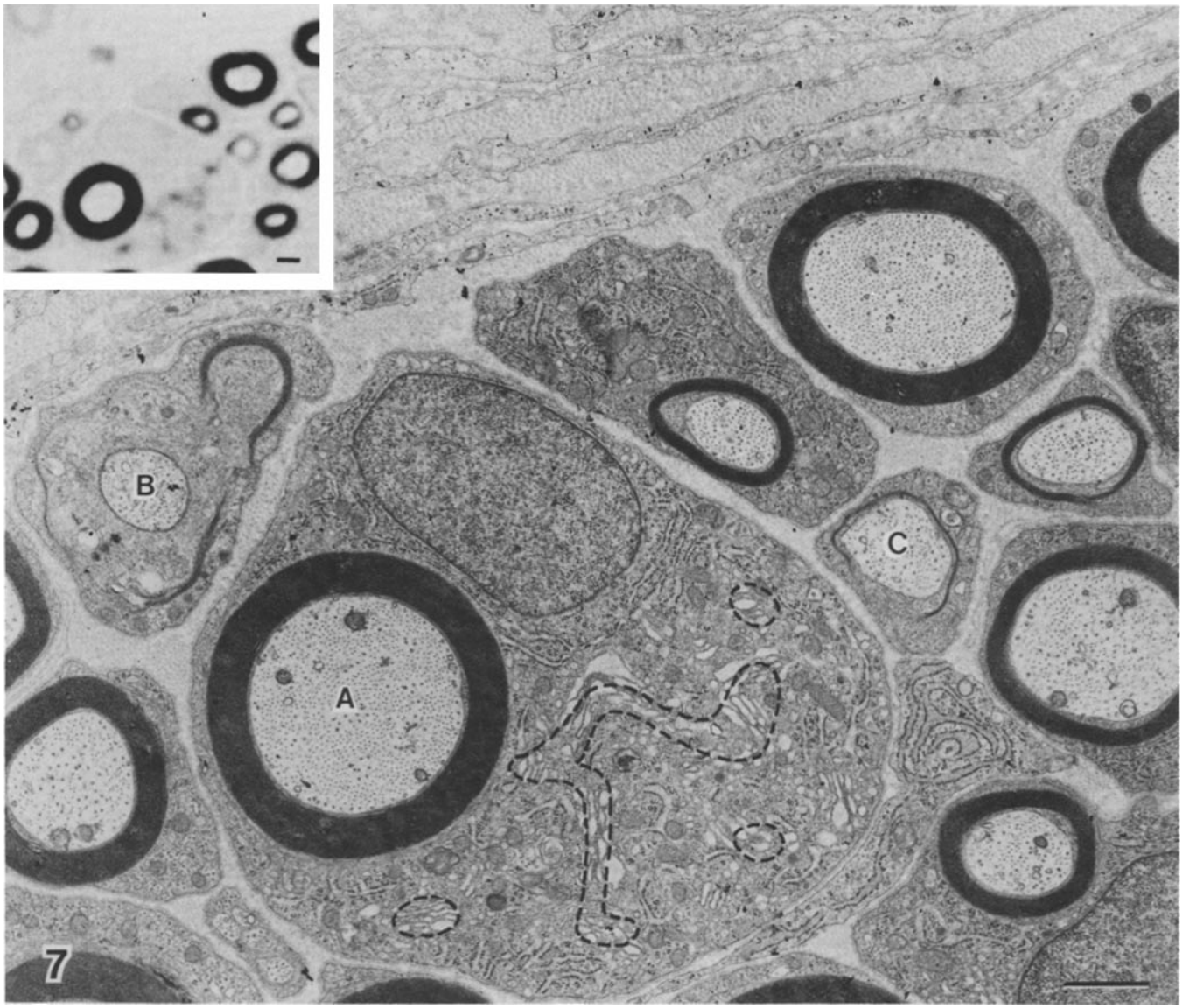

FIGURE 7 Electron micrograph (EM) of 7-d trigeminal nerve. The insert shows the same area in an adjacent section that was stained with P_0 antiserum and hematoxylin. Fiber *A* is surrounded by a thick myelin sheath which is intensely stained by P_0 antiserum in the 1-μm section. Particulate P_0 staining is present in Schwann cell cytoplasm. Its localization (outlined regions) in Golgi complex-rich areas was determined by tracing immunostained areas in a micrograph of the thick section (inset) onto an electron micrograph of the same cell in an adjacent thin section. Fiber *B* is sectioned through a paranodal region. Myelin lamellae are present and they are immunostained in the adjacent 1-μm-thick section. Fiber *C* is surrounded by a sheath with only three lamellae. The compact portion is stained by P_0 antiserum in the adjacent thick section. Bars, 1 μm. EM × 13,000. Inset × 3,500.

sheaths of developing and mature nerves. P_0 protein has been detected in newborn rat sciatic nerves with immunocytochemical methods (5, 27) and with a combination of polyacrylamide gel electrophoresis, immunochemical, and autoradiographic techniques (5), but not in conventionally stained polyacrylamide gels (30, 32). Similarly, myelin basic protein and myelin-associated glycoprotein can be detected immunocytochemically in the newborn rat central nervous system (CNS) (22, 23) but have not been detected by radioimmunoassay (7, 12) or polyacrylamide gel electrophoresis. Our immunocytochemical technique appears more sensitive than standard biochemical procedures for detecting myelin proteins, but it is limited by the inability to quantify the amount of protein present.

During peripheral nerve development, Schwann cell processes separate axons into bundles of fibers (29). As the Schwann cells proliferate, they subdivide the fiber bundles and most of them eventually surround a single axon. This 1:1 relationship between Schwann cells and axons precedes myelin formation, and the axon may provide a trigger for the initiation of myelination by the Schwann cell (1). Our data support this hypothesis, as P_0 staining occurred only in those Schwann cells which had established a 1:1 relationship with an axon. Most of these cells have immunoprecipitate around their axon, although occasional cells contain only particulate cytoplasmic staining. In the developing CNS, the occurrence of myelin proteins in oligodendrocytes before myelin formation (22) is more frequent than we have found in Schwann cells at comparable stages of PNS development. The distance between the site of synthesis of myelin proteins and the point of their initial insertion into myelin membranes is less in the PNS (28) than in the CNS (21) and may account for the lower percentage of Schwann cells containing only cytoplasmic staining.

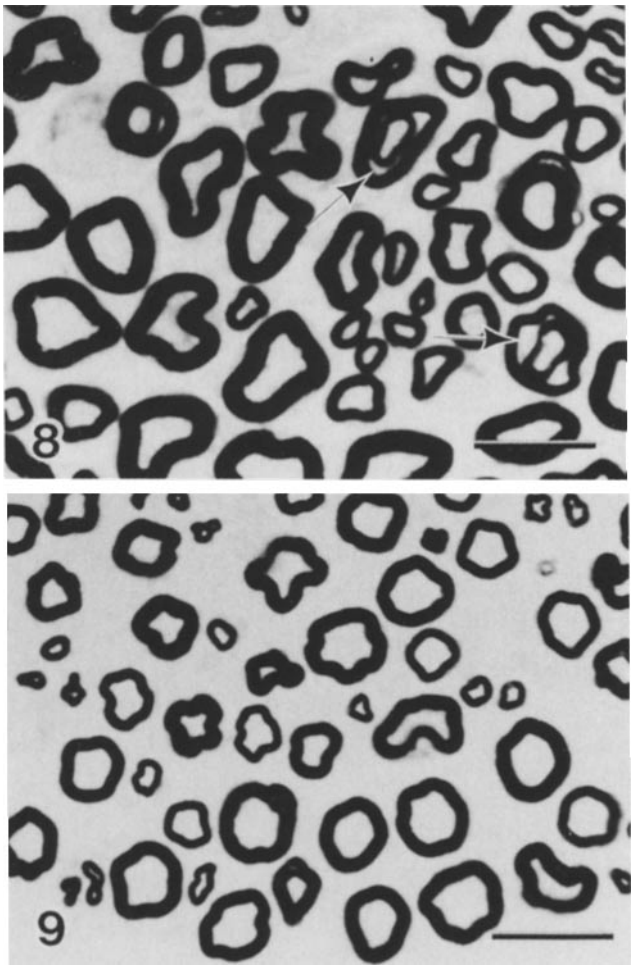

FIGURE 8 Transverse section, adult sciatic nerve, stained with P_0 antiserum (1:500). All myelin sheaths are intensely stained, and particulate P_0 staining is present in the cytoplasm of some Schwann cells. Schmidt-Lanterman incisures are unstained (arrows). Bright-field. Bar, 10 μm. × 1,600.

FIGURE 9 Transverse cross section of a human sural nerve biopsy stored in Epon for 19 yr before sectioning and staining with P_0 antiserum (1:500). The intensity and distribution of P_0 staining is similar to that found in freshly processed rat tissue. Bright-field. Bar, 10 μm. × 1,600.

Myelination includes mesaxon formation, spiral growth of the mesaxon, compaction of the multilamellar spiral, and growth of the compact sheath. It is not known whether P_0 protein is present in the Schwann cell plasma membrane or mesaxon during their initial wrapping around the axon. P_0 protein is the first myelin-specific protein detected in developing peripheral nerves (27), and there is a positive correlation between its accumulation and the degree of myelination (32). Ultrastructural localization of P_0 protein may determine when it is first inserted into the Schwann cell plasma membrane or mesaxon. At the light microscopic level, P_0 protein was detected in as few as three compact myelin lamellae. Variations in the shape and intensity of P_0 staining around axons in developing nerves demonstrated that compacted myelin lamellae stain more intensely than myelin membranes that are separated by cytoplasm. A dense continuous ring of P_0 staining surrounds axons that are ensheathed by at least 3–6 compact myelin lamellae. Schmidt-Lanterman incisures and isolated pockets of Schwann cell cytoplasm are unstained in these more mature myelin sheaths. The intensity of P_0 staining in Schwann cell cytoplasm increased during the time of active myelination and then decreased to still detectable levels in Schwann cells from adult nerves. In plastic sections of adult rat peripheral nerve, P_0 is the only myelin protein that has been detected within the cytoplasm of Schwann cells. Myelin basic protein, myelin-associated glycoprotein and P_2 protein were not detectable in adult Schwann cell cytoplasm.[1] Myelin basic proteins are present in much smaller amounts than P_0 protein in PNS myelin and are likely to be synthesized in free ribosomes which are evenly distributed throughout the cytoplasm (6). Results from autoradiographic and biochemical studies demonstrate that myelin proteins turn over in adult animals (4) and probably are present in Schwann cell cytoplasm. Basic proteins present in the cytoplasm of adult Schwann cells are probably at levels below the sensitivity of detection in 1-μm-thick sections stained by the PAP method.

When the thickness of myelin sheaths was compared in serial sections that were alternately stained with P_0 antiserum and PD, a selective spread of immunostaining reaction product occurred around the inner diameter of all immunostained myelin sheaths. The external diameter of a myelin sheath was identical in these P_0 and PD sections. This spread occurred over a constant distance; it was not affected by the diameter of the myelin sheath or axon and thus decreased the apparent axonal area more in small myelinated fibers than in large fibers. This spread of immunostaining reaction product also occurs when sections of peripheral nerve were stained with antisera directed against basic protein, P_2 protein, and myelin-associated glycoprotein.[1] Removal of plastic by sodium ethoxide and etching of the sections with H_2O_2 did not reduce axonal areas of unstained myelinated fibers. Unmyelinated axons had the same diameters in immunostained and PD sections. Morphometric analysis of immunostained fibers should be restricted to measurements of the external diameter of myelinated fibers due to this decrease in axonal area.

P_0 protein is an integral membrane glycoprotein whose carbohydrate moiety is located at the intraperiod lines of myelin (16, 17, 31). The intraperiod lines are continuous with the extracellular surface of Schwann cell plasma membranes. Studies on the biosynthesis of glycoproteins indicate that amino acids and core sugars are incorporated in the rough endoplasmic reticulum, whereas elongation of carbohydrate moieties occurs in the Golgi complex (19, 24). Completed plasma membrane glycoproteins are believed to be transported to the cell surface in vesicles that fuse with the plasmalemma (24). Glycoproteins are orientated in these vesicles so that their oligosaccharides will be exposed at the external surface of the cell. Assuming that P_0 protein is synthesized in the same way as other integral membrane glycoproteins, it is not surprising that it can be detected immunocytochemically in Golgi complex membranes of the Schwann cell. P_0 protein was not detected in membranes of the rough endoplasmic reticulum, although the majority of the molecule would be expected to be synthesized at that site. There are two possible explanations for this. First, the carbohydrates added in the Golgi complex may be the antigenic sites recognized by the P_0 antiserum. This seems unlikely, as only 6% of the total molecule is carbohydrate and P_0 antiserum does not cross react with other glycoproteins that contain sugar moieties similar to those of P_0 protein (25). A second, more plausible explanation is that P_0 protein is trans-

[1] B. D. Trapp. Unpublished results.

ferred more rapidly from the rough endoplasmic reticulum to Golgi complex membranes than from Golgi complex membranes to either the plasma membrane or myelin and that, therefore, it accumulates in the Golgi complex. This appears to be the case in the biosynthesis of protein G, an integral membrane glycoprotein of vesicular stomatitis virus (14, 15, 18).

The metabolism of peripheral nerve glycoproteins has been studied by autoradiography following the injection of tritiated fucose into peripheral nerves of adult mice and developing rats (9). The majority of tritiated fucose injected into peripheral nerves is incorporated into P_0 protein. Following a 1-h pulse, the label was confined to juxtanuclear Schwann cell cytoplasm in areas rich in Golgi complex membranes. With longer labeling periods, the number of grains associated with the Golgi complex membranes decreased, and there was a concomitant increase in silver grains over myelin. This study suggests that P_0 protein is fucosylated in the Golgi complex of the Schwann cell and is consistent with our results showing its presence in Golgi complex membranes.

Our results clearly demonstrate the usefulness of a sensitive immunocytochemical method for investigating the involvement of individual proteins in myelin formation and maintenance. The results with P_0 glycoprotein support the concept that the Golgi complex apparatus of Schwann cells plays a pivotal role in the biogenesis of the myelin sheath.

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REFERENCES

1. Aguayo, A. J., L. Charron, and G. M. Bray. 1976. Potential of Schwann cells from unmyelinated nerves to produce myelin: a quantitative ultrastructural and radiographic study. *J. Neurocytol.* 5:565–573.
2. Baskin, D. G., S. L. Erlandsen, and J. A. Parsons. 1979. Immunocytochemistry with osmium-fixed tissue. I. Light microscopic localization of growth hormone and prolactin with the unlabeled antibody-enzyme method. *J. Histochem. Cytochem.* 27:867–872.
3. Baskin, D. G., S. L. Erlandsen, and J. A. Parsons. 1979. Influence of hydrogen peroxide or alcoholic sodium hydroxide on the immunocytochemical detection of growth hormone and prolactin after osmium fixation. *J. Histochem. Cytochem.* 27:1290–1292.
4. Benjamins, J. A., and M. E. Smith. 1977. Metabolism of myelin. *In* Myelin. P. Morell, editor. Plenum Publishing, Corp., New York. 233–270.
5. Brockes, J. P., M. C. Raff, D. J. Nishiguchi, and J. Winter. 1980. Studies on cultured rat Schwann cells. III. Assays for peripheral myelin proteins. *J. Neurocytol.* 9:67–77.
6. Campagnoni, A. T., G. D. Carey, and Y.-T. Yu. 1980. In vitro synthesis of the myelin basic proteins: subcellular site of synthesis. *J. Neurochem.* 34:677–686.
7. Cohen, S. R., and M. Guarnieri. 1976. Immunochemical measurement of myelin basic protein in developing rat brain: an index of myelin synthesis. *Dev. Biol.* 49:294–299.
8. Estable-Puig, J. F., W. C. Bauer, and J. M. Blumberg. 1965. Technical note paraphenylenediamine staining of osmium-fixed, plastic-embedded tissue for light and phase microscopy. *J. Neuropathol. Exp. Neurol.* 24:531–535.
9. Gould, R. M. 1977. Incorporation of glycoproteins into peripheral nerve myelin. *J. Cell Biol.* 75:326–338.
10. Greenfield, S., S. Brostoff, E. H. Eylar, and P. Morell. 1973. Protein composition of myelin of the peripheral nervous system. *J. Neurochem.* 20:1207–1216.
11. Itoyama, Y., N. H. Sternberger, M. W. Kies, S. R. Cohen, E. P. Richardson, and H. deF. Webster. 1980. Immunocytochemical method to identify myelin basic protein in oligodendroglia and myelin sheaths of the human nervous system. *Ann. Neurol.* 7:157–166.
12. Johnson, D., R. H. Quarles, and R. O. Brady. 1980. A radioimmunoassay for the myelin associated glycoprotein. *Fed. Proc.* 39:1831.
13. Karnovsky, M. J. 1971. Use of ferrocyanide-reduced osmium tetroxide in electron microscopy. Eleventh Annual Meeting American Society for Cell Biology. 146 (abstr.).
14. Knipe, D. M., H. F. Lodish, and D. Baltimore. 1977. Localization of the two cellular forms of the vesicular stomatitis viral genome. *J. Virol.* 21:1221–1127.
15. Knipe, D. M., D. Baltimore, and H. F. Lodish. 1977. Separate pathways of maturation of the major structural proteins of vesicular stomatitis virus. *J. Virol.* 121:1128–1139.
16. Peterson, R. G., and D. C. Pease. 1972. Myelin embedded in polymerized glutaraldehyde-urea. *J. Ultrastruct. Res.* 41:115–132.
17. Peterson, R. G., and R. W. Gruener. 1978. Morphological localization of PNS myelin proteins. *Brain Res.* 152:17–29.
18. Rothman, J. E., H. Bursztyn-Pettegrew, and R. E. Fine. 1980. Transport of the membrane glycoprotein of vesicular stomatitis virus to the cell surface in two stages by clathrin-coated vesicles. *J. Cell Biol.* 86:162–171.
19. Schachter, H., and S. Roseman. 1980. Mammalian glycosyltransferases: their role in the synthesis and function of complex carbohydrates and glycolipids. *In* The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Publishing, Corp., New York. 85–160.
20. Sternberger, L. A., P. H. Hardy, J. J. Cuculus, and H. G. Meyer. 1970. The unlabeled antibody enzyme method of immunohistochemistry. Preparation and properties of soluble antigen-antibody complex (horseradish peroxidase-antihorseradish peroxidase) and its use in identification of spirochetes. *J. Histochem. Cytochem.* 18:315–333.
21. Sternberger, N. H., Y. Itoyama, M. W. Kies, and H. deF. Webster. 1978. Immunocytochemical method to identify basic protein in myelin forming oligodendrocytes of newborn rat CNS. *J. Neurocytol.* 7:251–263.
22. Sternberger, N. H., Y. Itoyama, M. W. Kies, and H. deF. Webster. 1978. Myelin basic protein demonstrated immunocytochemically in oligodendroglia prior to myelin sheath formation. *Proc. Natl. Acad. Sci. U. S. A.* 75:2521–2524.
23. Sternberger, N. H., R. H. Quarles, Y. Itoyama, and H. deF. Webster. 1979. Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rats. *Proc. Natl. Acad. Sci. U. S. A.* 76:1510–1514.
24. Struck, D. K., and W. J. Lennarz. 1980. The function of saccharide-lipids in synthesis of glycoproteins. *In* The Biochemistry of Glycoproteins and Proteoglycans. W. J. Lennarz, editor. Plenum Publishing, Corp., New York. 35–84.
25. Trapp, B. D., L. J. McIntyre, R. H. Quarles, N. H. Sternberger, and H. deF. Webster. 1979. Immunocytochemical localization of rat peripheral nervous system myelin proteins: P_2 protein is not a component of all peripheral nervous system myelin sheaths. *Proc. Natl. Acad. Sci. U. S. A.* 76:3552–3556.
26. Trapp, B. D., Y. Itoyama, L. J. McIntyre, and H. deF. Webster. 1979. Immunocytochemical localization of myelin proteins in human peripheral nerve. *J. Neuropathol. Exp. Neurol.* 38:323.
27. Trapp, B. D., N. H. Sternberger, R. H. Quarles, Y. Itoyama, and H. deF. Webster. 1980. Immunocytochemical localization of peripheral nervous system myelin proteins in epon sections. *J. Neuropathol. Exp. Neurol.* 39:392.
28. Webster, H. deF. 1971. The geometry of peripheral myelin sheaths during their formation and growth in rat sciatic nerves. *J. Cell Biol.* 48:348–367.
29. Webster, H. deF., J. R. Martin, and M. F. O'Connell. 1973. The relationships between interphase Schwann cells and axons before myelination: A quantitative electron microscopic study. *Dev. Biol.* 32:401–416.
30. Wiggins, R. C., and P. Morell. 1980. Phosphorylation and fucosylation of myelin protein in vitro by sciatic nerve from developing rats. *J. Neurochem.* 34:627–634.
31. Wood, J. G., and B. J. McLaughlin. 1975. The visualization of concanavalin A binding sites in the interperiod line of rat sciatic nerve myelin. *J. Neurochem.* 24:233–235.
32. Wood, J. G., and E. L. Engel. 1976. Peripheral nerve glycoproteins and myelin fine structure during development of rat sciatic nerve. *J. Neurocytol.* 5:605–615.