

# Immunocytochemical Localization of Basic Protein in Major Dense Line Regions of Central and Peripheral Myelin

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**ABSTRACT** To localize basic protein (BP) in the lamellar structure of central and peripheral myelin, we perfused newborn and 7–11-day rat pups with a phosphate-buffered fixative that contained 4% paraformaldehyde and 0.05 or 0.2% glutaraldehyde. Teased, longitudinally split or “brush” preparations of optic and trigeminal nerves were made by gently teasing apart groups of myelinated fibers with fine forceps or needles. Some of these preparations were immunostained without pretreatment in phosphate-buffered antiserum to BP according to the peroxidase-antiperoxidase method. Others were pretreated in ethanol before immunostaining. Then, all of them were dehydrated, embedded in Epon, and sectioned for electron microscopic study. In optic and trigeminal nerves that were not pretreated, myelin, glial cells, and their organelles were well preserved. BP immunostaining was present on cytoplasmic faces of oligodendroglial and Schwann cell membranes that formed mesaxons and loose myelin spirals. In compact central and peripheral myelin, reaction product was located in major dense line regions, and the myelin periodicity was the same as that observed in unstained control myelin that had been treated with preimmune serum. In ethanol-pretreated tissue, the myelin periodicity was reduced but dense line staining still was present. Our immunocytochemical demonstration of dense line localization of BP in both CNS and PNS myelin that was not disrupted or pretreated with solvents is important because of conflicting evidence in earlier immunostaining studies. Our results also support biochemical and histochemical evidence suggesting that BP exists *in vivo* as a membrane protein interacting with lipids on the cytoplasmic side of the bilayer in the spirally wrapped compact myelin membrane.

Basic protein (BP) is a major constituent of central nervous system (CNS) myelin (3, 9, 23, 40). It has a molecular weight of about 18,500, is antigenic, and is thought to have an important role in the formation and maintenance of myelin's compact lamellar structure (5, 9, 23, 48). Myelin isolated from the peripheral nervous system (PNS) contains smaller amounts of a basic protein called P<sub>1</sub> (7, 8, 15). Since P<sub>1</sub> and BP have almost the same molecular weight (7, 8, 15) and amino acid sequence (7), they probably are identical or very closely related proteins (4, 7, 8, 15, 49).

The localization of BP in the lamellar structure of myelin is an important issue for those interested in membrane assembly and maintenance. Investigators have used immunocytochemi-

cal methods and specific antisera to study this directly and both dense line (16, 39) and intraperiod region (27, 43) localizations have been described. But in all of these experiments, immunostaining was only observed in myelin pretreated with solvents like ethanol (27), in myelin lamellae broken by tissue processing procedures (16, 39), or in myelin disrupted by isolation for biochemical study (43).

Since different localizations were obtained and the methods used in the above studies broke up membranes or could have extracted some of their constituents, our goal was to create conditions that would preserve myelin fine structure and still allow specific immunostaining of compact lamellae. Here, we report that BP antibodies stain dense line regions of CNS and

PNS myelin that is not disrupted and has not been pretreated with solvents or detergents. A summary of our method and findings has been published (30).

## MATERIALS AND METHODS

### Tissue Preparation

Newborn and 7–11-d-old Osborne Mendell rat pups were perfused through the heart for 10 min with a solution containing 4% paraformaldehyde, glutaraldehyde (either 0.05 or 0.2%), and 0.1 M phosphate buffer. The pH of the fixative was 7.2 and it was warmed to 36°C just before use. The optic and trigeminal nerves were exposed and kept covered with 0.2 M phosphate buffer during the subsequent dissection. The meninges were gently peeled off the optic nerves *in situ*; a 1–2 mm segment was removed and split longitudinally by teasing with fine forceps and needles. After removing the perineurium from trigeminal nerves *in situ*, fine needles were used to isolate one fascicle and make “brush” preparations consisting of small bundles and individual fibers. These “brush” preparations and split optic nerve segments were immersed in the perfusion fixative for 2 h at 4°C, were rinsed twice for 15 min each in 0.1 M phosphate buffer (4°C) and rinsed twice more (15 min each, 4°C) in 0.08 M phosphate buffered saline (PBS), pH 7.2. Most specimens were not pretreated in detergents or solvents. Some were pretreated in ethanol at 4°C as follows: 5, 25, and 50% for 4 min each; 75% overnight; 50, 25, and 5% brief rinses; PBS, one rinse.

### Immunostaining

Optic and trigeminal nerve specimens were immunostained according to the peroxidase-antiperoxidase (PAP) method (42) as described for the demonstration of BP (44) with the following modifications. PBS (0.08 M) was used instead of Tris buffer in all rinses, all sera, and in the diaminobenzidine HCL (DAB) solution which was millipore filtered just before use. Intervals for each step were: (a) 3% normal rabbit serum for 1.5 h at 4°C; (b) 1:500 or 1:5,000 goat antiserum to rabbit BP for 1 h at room temperature (RT), then overnight at 4°C; (c) PBS rinses; two for 30 min each at 4°C and one for 30 min at RT; (d) rabbit antigiant IgG for 1 h at RT; (e) three PBS rinses; two for 30 min each at RT and one for 30 min at 4°C; (f) 1% normal rabbit serum for 30 min at 4°C; (g) goat peroxidase-antiperoxidase for 1 h at 4°C; (h) four rinses in PBS; three for 30 min each at 4°C and one for 30 min at RT; (i) 0.05% DAB + 0.01% H<sub>2</sub>O<sub>2</sub> in PBS (millipore filtered) for 5 min at RT; (j) four rinses in PBS; one for 30 min at RT

and three for 30 min each at 4°C; (k) 2% phosphate buffered OsO<sub>4</sub> for 5–7 min at 4°C. Then the specimens were dehydrated rapidly in ethanol, rinsed in propylene oxide, and embedded in Epon. Thin sections were mounted individually on Formvar-coated single-slot grids. To study the distribution of reaction product and the fine structure of the same glial cells and myelin sheaths, alternating serially cut thin sections were stained with uranyl acetate and lead citrate. The others were not stained before they were examined in the electron microscope.

Production and specificity of the goat antiserum to rabbit BP has been described (18). Briefly, high titer antiserum was produced by immunizing a young female goat with 5 mg of a rabbit BP and porcine thyroglobulin (TG) conjugate dissolved in saline and emulsified in complete Freund's adjuvant (CFA). The rabbit BP was purified according to the method of Deibler et al. (12) and formed a well-defined, single band on SDS gel electrophoresis. The molar ratio of BP to TG in the conjugate was 100:1.5. After boosting the animal with similar injections of BP-TG in incomplete Freund's adjuvant (IFA), blood was collected and tested for BP antibodies by radioimmunoassay (10). Binding capacity of this antiserum was 100 mg BP/ml.

As controls for staining specificity, optic and trigeminal nerve preparations were incubated in step two with either serum collected from the goat before immunization or absorbed antiserum in which the specific antibodies had been removed by precipitation with purified BP in amounts sufficient to eliminate all anti-BP reactivity detectable by radioimmunoassay.

To test for possible differences in immunoreactivity and staining distribution, we also used rabbit antibodies to rat central myelin large BP in a few experiments. These antibodies have been described (6) and were a gift of Dr. Dale McFarlin. Rabbits in his laboratory were immunized with multiple injections (in IFA) of an acid extract of spinal cord. Pooled sera were passed over an immunoabsorbant of Sepharose conjugated to purified rat central large basic protein and the purified antibodies were eluted with 0.1 M acetic acid. Control tissues in these experiments were incubated in step two with normal rabbit serum.

## RESULTS

When semithin sections of teased optic and trigeminal nerves that had not been pretreated were examined with phase-contrast optics, glial cells, axons, and myelinated fibers were not broken or distorted (Fig. 1A, inset). Deposits of reaction product were present in a few myelin sheaths and oligodendroglial processes located along split surfaces of optic nerve prepara-

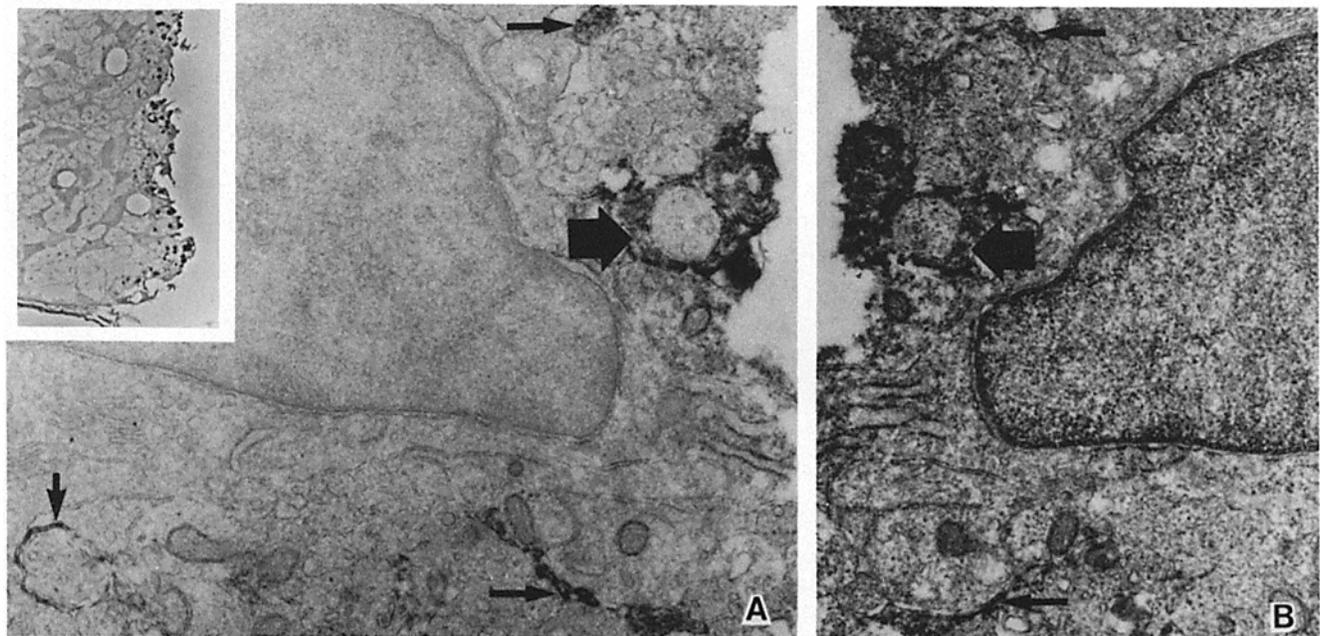


FIGURE 1 Light (inset) and electron micrographs of the same transversely sectioned 7-d optic nerve preparation. 0.05% glut. mixture; 1:5,000 BP antiserum. *Inset* - Immunostaining along the right edge; cellular relationships well maintained.  $\times 180$ . (A) Immunostained oligodendroglial processes (upper arrow, wide arrow) and membranes (lower arrows). Thin section not stained with heavy metals.  $\times 26,000$ . (B) Adjacent thin section treated with uranyl acetate and lead citrate contains the same immunostained oligodendroglial processes (upper arrow, wide arrow) and membranes (lower arrow). Cellular and membrane fine structure is well preserved even along the teased edge.  $\times 26,000$ .

tions and at depths up to  $\sim 20 \mu\text{m}$ . Generally, the depth of BP immunostaining was greater in younger pups. Tissue architecture also was well-maintained in the "brush" preparations of trigeminal nerves and they contained some intensely immunostained myelin sheaths and Schwann cells.

In electron micrographs of thin sections that had been stained with uranyl acetate and lead citrate, myelin sheaths, cell membranes, cytoplasmic organelles, and nuclei were well-preserved even along edges of teased surfaces (Fig. 1*B*). Although there were some minor focal distortions, loose myelin spirals and the characteristic lamellar structure of compact myelin were easily identified. As many investigators have shown (reviewed in reference 33, illustrated in Fig. 4), central and peripheral myelin sheaths occur around axons as paired, spirally wrapped extensions of oligodendroglial or Schwann cell surface membranes. Extracellular faces of membranes are adjacent to each other in mesaxons and loose spirals and they continue as the less dense intraperiod lines of compact myelin. Dense line regions are only present in compact myelin and are formed during conventional processing by the fusion of cytoplasmic faces of oligodendroglial and Schwann cell membranes.

After fixation in the solution that contained 0.05% glutaraldehyde, the distribution of BP immunostaining was studied in adjacent thin sections that had not been stained with uranyl acetate and lead citrate. Reaction product was present in some oligodendroglial processes that surrounded axons (Fig. 1*A*). Some ribosomes, microtubules, and profiles of granular endoplasmic reticulum were immunostained. More intense staining was located on cytoplasmic faces of surface membranes and extended along membranes that formed loose myelin spirals (Fig. 2*A*). In regions where two cytoplasmic faces fused, there was a wider, even more intense band of staining that corresponded to a dense line region. In multilayered compact sheaths, outer tongue processes were stained and a wider band of intense staining began where their membranes fused to form myelin's outermost major dense line (Fig. 2*B*). At higher magnification, the widths of the immunostained bands and the control unstained dense lines were 7.5 nm (Fig. 2*D*) and 4.0 nm (Fig. 2*C*), respectively. The bands' center-to-center distance measured  $\sim 13$  nm and was the same as that of dense lines in control myelin in nerves that were not teased or in teased preparations that were treated with preimmune or absorbed serum instead of BP antiserum (Fig. 2*D, C*). Only a small

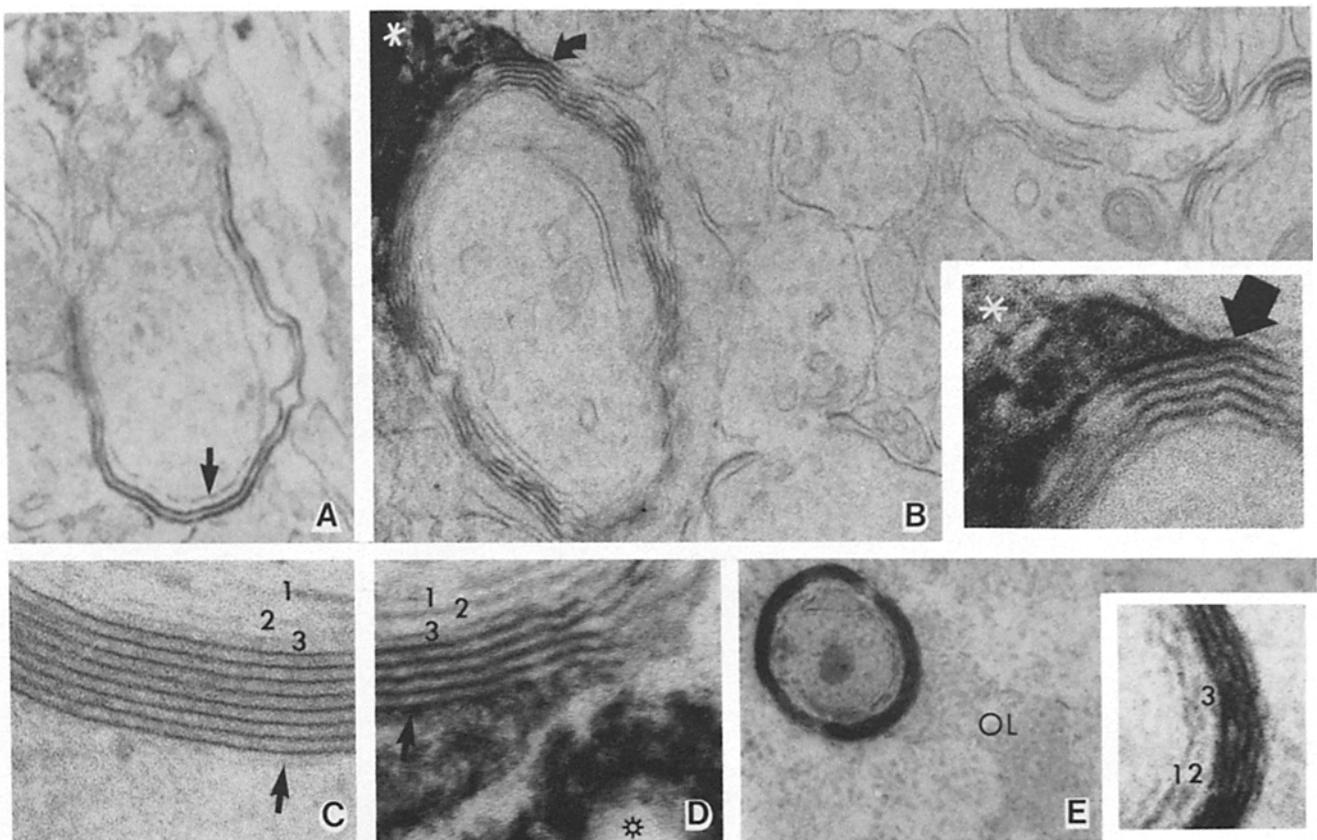


FIGURE 2 Electron micrographs of transversely sectioned 7d (A) and 11d (B–E) optic nerve preparations. 0.2% glut. mixture; 1:500 BP antiserum (A, B, D, E); 1:500 preimmune serum (C); sections not stained with uranyl or lead salts. (A) Newly formed myelin sheath has two immunostained loosely wrapped membranes; axolemma (arrow) is unstained.  $\times 83,000$ . (B) Immunostained oligodendroglial outer tongue process membranes fuse (arrow) forming outermost dense line. A deposit of reaction product (\*) is located on the teased surface.  $\times 66,000$ . (Inset) fusion site at higher magnification (see Fig. 4).  $\times 154,000$ . (C) No immunostaining. Axolemma (1), two inner oligodendroglial membranes (2, 3), eight dense lines of compact myelin, and outer oligodendroglial membrane (arrow) clearly shown.  $\times 143,000$ . (D) Same area of immunostained sheath. Unstained axolemma (1) and oligodendroglial membranes (2, 3) are above four immunostained dense line regions. Below the last dense line, an immunostained oligodendroglial membrane (arrow) and outer tongue process cytoplasm extend to lower left. Reaction product deposit (\*) is on teased surface.  $\times 143,000$ . (E) Ethanol pretreatment. Intense immunostaining of myelin sheath; cell membranes poorly preserved.  $\times 38,000$ . (Inset) Gaps in axolemma (1), oligodendroglial membranes (2, 3). Immunostained bands in dense line regions present but less well-defined. Myelin periodicity decreased and more variable.  $\times 120,000$ .

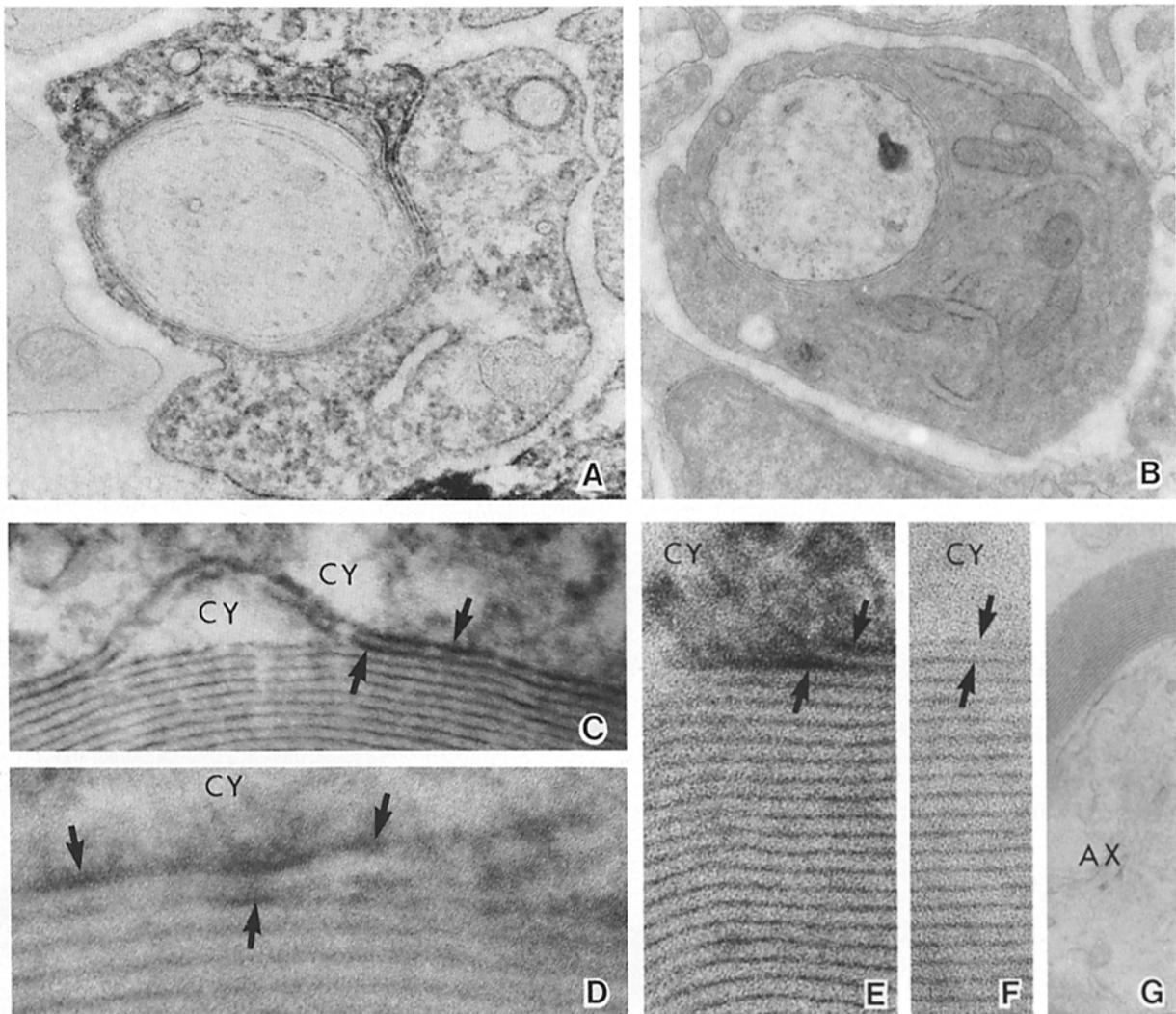


FIGURE 3 Electron micrographs of transversely sectioned 7d (A, B) and 11d (C-G) trigeminal nerve preparations. 0.05% glut. mixture; 1:5,000 (A) and 1:500 (C, E) BP antiserum; 1:500 preimmune serum (B and F); 1:500 BP absorbed serum (C). (A) BP immunostaining along cytoplasmic surfaces of membranes forming a loosely wrapped myelin spiral with two turns. Patchy immunostaining of ribosomes, Schwann cell surface membrane.  $\times 40,000$ . (B) No immunostaining; mesaxon, Schwann cell and axon are well preserved.  $\times 27,000$ . (C) BP immunostaining of outermost dense line (lower arrow) and Schwann cell membrane (upper arrow) next to cytoplasm (CY).  $\times 130,000$ . (D and E) Same staining pattern as in C shown in different sheaths; see Fig. 4 for relationships of outermost dense line (lower arrows) and Schwann cell membrane (upper arrows) next to cytoplasm (CY).  $\times 300,000$  and  $\times 210,000$ , respectively. (F) No immunostaining of Schwann cell membrane (upper arrow), cytoplasm (CY) or outermost dense line (lower arrow); myelin periodicity same as in E.  $\times 210,000$ . (G) No immunostaining of Schwann cell cytoplasm (top left), myelin or axon (AX).  $\times 40,000$ .

number of compact sheaths near the teased surface had several immunostained lamellae and these lamellae were located next to the fibers' axonal or extracellular surface. Restriction of immunostaining to dense line regions was confirmed by examining these fibers in a few serial sections. When compared at different levels, the distribution of dense line staining was about the same, shifted to nearby dense line regions, or became more patchy along some lamellae. Cytoplasmic faces of oligodendroglial membranes that surrounded axons and formed inner mesaxons also were not stained or were less intensely stained than dense line regions of compact myelin. BP antiserum did not stain axons or astrocytes. Preimmune and absorbed sera did not stain myelin, glial cells, or axons.

When BP immunostaining was compared in optic nerve preparations fixed in aldehydes containing 0.05 and 0.2% glu-

taraldehyde, staining in the latter was limited to plasma membranes of oligodendrocytes and to myelin sheaths along the teased surface. On the teased surface there were some focal nonspecific deposits of reaction product which were not seen in preparations fixed with the 0.05% glutaraldehyde mixture. No cytoplasmic immunostaining was observed in preparations fixed with the 0.2% glutaraldehyde mixture. Also, the staining of oligodendroglial membranes, mesaxons, and myelin dense line regions was more patchy and less uniform in intensity. Under the conditions tested, this higher concentration of glutaraldehyde did not improve the electron microscopic preservation of cells and myelin significantly.

When optic nerve preparations that had been fixed in the mixture containing 0.2% glutaraldehyde were pretreated with ethanol, BP antiserum stained myelin sheaths and patches of

oligodendroglial membranes farther from the surface. Staining of myelin's dense line regions appeared more intense and uniform, but when examined at higher magnification, membranes were less well-preserved, reaction product was present between deposits in dense line regions, and the myelin periodicity was reduced to ~12 nm (Fig. 2E).

The fine structure of Schwann cells, their organelles, and developing myelin sheaths was well-preserved in thin sections of control trigeminal nerve "brush" preparations that had been treated with absorbed or preimmune serum (Fig. 3B). In thin sections not treated with heavy metals, BP antiserum stained cytoplasmic faces of Schwann cell surface membranes, mesaxons, and loose myelin spirals (Fig. 3A). There also was patchy staining of ribosomes and microtubules. As in the CNS, more intense immunostaining was present on dense line regions of compact myelin but since most sheaths were thicker, fewer lamellae usually were stained than in the CNS; they were located at the outer margins of sheaths, and the staining was more patchy (Fig. 3C-E). The myelin periodicity measured ~15 nm and was the same in electron micrographs of unteased controls or teased preparations treated with control serum (Fig. 3F). Schwann cells, myelin, and axons were not immunostained by preimmune (Fig. 3F) or BP absorbed (Fig. 3G) sera.

Teased optic and trigeminal nerve preparations also were immunostained with a rabbit antiserum to rat BP. In electron micrographs, the pattern of oligodendroglial, Schwann cell, and myelin dense line staining was the same as we observed in experiments using goat antiserum to rabbit BP. The distribution of BP immunostaining observed in central and peripheral compact myelin is summarized diagrammatically in Fig. 4.

## DISCUSSION

For more than 15 years, investigators have used electron microscopic immunocytochemical methods in efforts to obtain direct evidence on the localization of BP in the lamellar structure of myelin. Conflicting results have been described in studies handicapped by problems with reagent penetration (16, 21, 27), preservation of myelin fine structure (16, 21, 39), and antibody specificity (21).

In two reports (16, 21), reaction product was only observed in myelin areas where the layered structure was broken or severely distorted. In the first study (21), limitation of staining to badly disrupted myelin precluded lamellar localization of BP: neuronal constituents also were immunostained by the

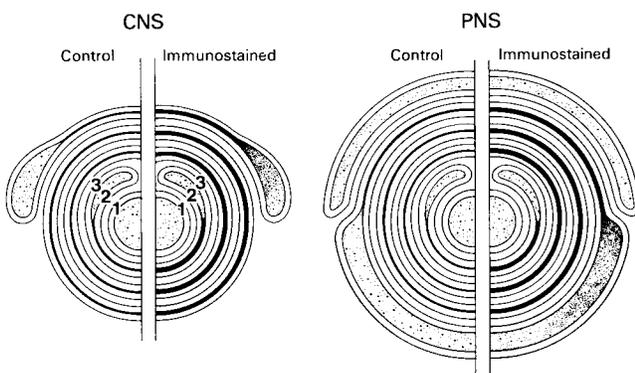


FIGURE 4 Diagram by Peters (reference 32) modified to show the lamellar structure of CNS and PNS myelin. Basic protein is located in dense line regions (see text). Regions of the axolemma and adjacent oligodendroglial membranes shown in Fig. 2 C-E are numbered.

antisera used in these experiments. Although Herndon and his collaborators (16) described dense line localization of BP in adult CNS myelin, this conclusion has been questioned because reaction product was found only in severely distorted lamellae and they also observed some myelin staining in tissue treated with control serum. Dense line localization has also been demonstrated in developing CNS tissue containing many preparative artifacts (39). In a careful investigation of many technical variables, Mendell and Whitaker (27) processed adult tissue so that the lamellar structure of compact myelin was well-preserved. They found that BP antiserum stained intraperiod line regions of PNS myelin, but only after it had been pretreated with ethanol, a solvent known to extract myelin lipids (20, 28, 50). Even after pretreatment, they could not locate BP in CNS myelin; reagents still did not penetrate and react well enough to define staining localization unequivocally (27). Intraperiod line staining has also been observed when central (43) and peripheral (Sternberger, N. H., personal communication) myelin fractions were treated with BP antiserum according to the PAP method. However, the myelin had to be disrupted by the isolation procedure (29) before it could be immunostained (46). Other publications that include electron microscopy observations do not describe the localization of BP in myelin lamellae (14, 46).

Therefore, this study, which shows that BP is located in dense line regions of CNS and PNS myelin, adds important new evidence to published data. It contains observations on both central and peripheral myelin and demonstrates for the first time that BP immunostaining can be achieved in compact myelin without pretreating it or grossly distorting its layered structure. Our immunostaining method produced substantially better electron microscopic preservation of myelin, mesaxons, oligodendroglia, and Schwann cells than has been described previously. In early tests, we compared the preservation of fine structure in vibratome sections, surfaces of tissue slices and teased preparations; it was significantly better in the last, so they were selected. Compact myelin was studied in immature sheaths for several reasons. When the PAP method was used for light-microscope observations, BP antiserum stained developing myelin intensely but only minimal staining was observed in adult myelin unless it was pretreated with ethanol or osmium (45). This suggested that more immunoreactive sites in BP might be exposed in compact lamellae of immature sheaths and that PAP reagents might also penetrate better. In developing nervous tissue, extracellular spaces are larger, membranes are more permeable, and fewer diffusion barriers are present (33); these features probably also helped us achieve immunostaining in myelin lamellae without pretreating them or grossly altering their periodicity. Finally, our main goal was to localize BP in compact CNS myelin. A major previous effort with adult tissue had not been successful (27) and we thought that the position of BP in compact lamellae would be established early in development. During subsequent maturation, it seemed unlikely that BP would move to a different location on the other side of the lipid bilayer. Since we used immature tissue and only used ethanol in a few experiments before obtaining immunoreactivity without pretreatment, we did not test ethanol concentrations and time intervals used by Mendell and Whitaker (27).

Can the PAP technique be used to localize a protein in myelin when the resolution of the method is ~80 nm (41)? Sternberger (41) states that the layered structure of myelin could increase this substantially by restricting entry of antibody and PAP molecules to those oriented edgewise (thickness of

antibody and of PAP is 3–4 nm). Are dense line regions large enough for antibodies and disc-shaped PAP molecules to enter and react? Peterson and Pease (35) summarized evidence on myelin measurements after various preparative procedures. In their combined data for peripheral myelin, each of the apposed cytoplasmic leaflets was ~1.5 nm and the gap between them was 2.5 nm, giving a total width of 5.5 nm for dense line regions (35). Since they demonstrated this gap and similar dimensions in aldehyde fixed myelin (35), we believe that our dense line regions were large enough to contain antibodies and PAP oriented edgewise. To produce immunostaining in compact myelin, these macromolecules would have to cross several lamellae. We think that this penetration and exchange probably occurred through small gaps created in some surface membranes and lamellae by tissue teasing. The gaps allowed entry and patchy specific immunostaining of a few dense line regions but were not large or numerous enough to grossly distort the fine structure of cells or compact myelin. Although the myelin period in immunostained areas may have been increased by deposition of reaction product, the increase was not noted after postfixation, dehydration, and embedding. During these processing steps, the gap between apposed cytoplasmic leaflets disappears, a single dense line is formed, and the myelin period is reduced from 19.5 nm to 13 nm (35). Probably, enough myelin constituents are extracted and/or rearranged so that any increase that may have been present initially was eliminated.

Several lines of indirect evidence also have suggested that BP is located in dense line regions of compact myelin. BP is not labeled when the lactoperoxidase-catalyzed iodination method is used to incorporate radioactive iodine into proteins on external (extracellular) surfaces of CNS (36) and PNS (34) myelin sheaths. When ammonium acetate-Triton X-100 solutions are used to remove BP from nerve myelin sheaths and purified myelin preparations, the dense lines are swollen and split (34). BP has not been detected in myelin isolated from young mld (22, 26) or shiverer (22) mutant mice. Fewer CNS myelin sheaths are present, they are thinner, and their lamellar structure is abnormal; many dense lines are interrupted or missing (17, 26, 38). In x-ray diffraction studies of PNS myelin in shiverer, the dense line region of the electron density profile is slightly altered (19). This finding is of interest because myelin sheaths are normal in number and thickness; there are no widespread changes in dense or intraperiod lines and their spacing (19, 37). Some PNS myelin lamellae surround cytoplasmic pockets, project into axons, end in loops or have other focal abnormalities (37), but these would not affect the electron density profile. Electron microscope and x-ray diffraction studies of lipid protein mixtures also favor locating BP in dense line regions (25); this localization also has been described in histochemical experiments that use phosphotungstic acid hematoxylin (1) and ethanolic phosphotungstic acid (47) as stains.

As noted above, evidence for BP localization in these studies is indirect and other interpretations have been proposed by the authors and others. Absence of labeling with the lactoperoxidase-iodination method could reflect lack of exposed amino acid residues needed for radiolabel attachment. Dense lines in electron micrographs of myelin are the result of tissue processing procedures (2, 20, 31, 35). During myelin isolation and/or conventional processing, lamellar spacing changes and some constituents may be rearranged or removed (11, 20, 35). After constituents are extracted, myelin's appearance in electron micrographs may be highly variable and difficult to interpret; also, data in one electron microscopy and biochemical study of

isolated myelin suggested that BP was located in intraperiod line regions (13). In the shiverer and mld mutants, levels of other CNS myelin constituents are decreased and this also could affect the density and spacing of myelin lamellae. X-ray diffraction observations are useful but must be combined with data provided by other methods to suggest localizations for myelin proteins. Histochemical results can be hard to interpret because of problems with staining specificity.

Thus, there are alternate interpretations for the above data; still, almost all of it favors locating BP in dense line regions where we have localized it immunocytochemically. Our observations have provided the same localization for BP in both central and peripheral myelin without procedures required previously for immunostaining - disruption (16, 21, 43) or pretreatment (27). In addition, nonspecific staining (16, 21,) was not observed. Thus, this study provides important new direct evidence for the concept that BP exists in vivo as a membrane protein interacting with lipids on the cytoplasmic side of the bilayer in the spirally wrapped compact myelin membrane (3, 23, 24, 36, 40, 48).

The authors thank Dr. Dale McFarlin for the rabbit anti-rat BP serum and Ms. Trudy Nicholson for drawing Figure 4, and Drs. Martenson and Stoner for reading the manuscript.

This research was supported in part by a Fellowship from Janggen-Pöhn-Stiftung and Schweizerische Stiftung für med. biol. Stipendien, Switzerland and by a Research Grant from the Kroc Foundation.

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