Myelin-associated glycoprotein demonstrated immunocytochemically in myelin and myelin-forming cells of developing rat

(central nervous system/peripheral nervous system/oligodendroglia/Schwann cells/peroxidase–antiperoxidase)

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ABSTRACT The unlabeled peroxidase–antiperoxidase method has been used with antiserum against "myelin-associated glycoprotein" to establish the presence of the glycoprotein in myelin and myelin-forming cells of the developing rat nervous system. Myelin-associated glycoprotein is found in oligodendroglial cytoplasm before the beginning of myelination. Staining intensity of oligodendroglia increases during early development and slowly declines during the period of rapid myelination. Myelin staining is confined to the periaxonal region of the myelin sheath and does not increase as large, compact sheaths are formed. Antiserum to central nervous system myelin-associated glycoprotein also stains Schwann cells in developing trigeminal ganglia and the periaxonal region of peripheral myelin sheaths.

Highly purified myelin isolated from rat brain contains glycoproteins of high molecular weight (1). Biochemical studies have shown that the most prominent glycoprotein (approximately 100,000 daltons) is closely associated with myelin or related structures in normal brains (1–3). This "myelin-associated glycoprotein" (MAG) is present in small amount and is probably not a major structural component of the central nervous system (CNS) myelin sheath.

In peripheral nervous system (PNS) myelin, the major protein is a 30,000-dalton glycoprotein (P_0). The large MAG of CNS myelin has not been demonstrated in PNS myelin (4). However, several minor glycoproteins with M_rs higher than that of P_0 have recently been detected in preparations of purified peripheral myelin (5, 6).

Glycoproteins are believed to participate in establishing specific interneuronal and glial–neuronal connections in the developing nervous system. Although MAG is quantitatively a minor protein component of CNS myelin, it may play an important role in the initial formation, compaction, or maintenance of the spiraled myelin sheaths surrounding axons.

In this report we describe a light-microscopic, immunocytochemical method for detecting MAG in myelin and myelin-forming cells of the developing rat nervous system. In the CNS, oligodendroglia contain MAG before myelination begins. MAG antiserum also stains myelin sheaths during the early stages of their formation. Later, MAG is localized in periaxonal regions of myelineated CNS fibers. The same region of PNS myelinated fibers is also stained by MAG antiserum.

MATERIALS AND METHODS

MAG was purified from myelin isolated from the brains of 200- to 250-g Osborne–Mendel rats. The first step in MAG purification was lithium diiodosalicylate extraction of the myelin fraction and subsequent partitioning of the extract with phenol

as described (7). The glycoprotein preparation obtained by this procedure was dissolved in a solution containing 2% (wt/vol) sodium dodecyl sulfate, 1.5% (wt/vol) dithiothreitol, and 8% (wt/vol) sucrose for application to a preparative 5% polyacrylamide gel. The procedure for preparative electrophoresis and extraction of MAG from appropriate gel slices was as described (8). The glycoprotein extract was lyophilized, suspended in a small volume of saline, and emulsified with an equal volume of complete Freund's adjuvant for rabbit immunization.

The emulsion containing about 0.5 mg of MAG was injected intradermally in multiple sites on the rabbit's back. Similar injections were repeated twice at 3-week intervals, except that the final injection utilized incomplete Freund's adjuvant. Also, a control rabbit was injected on the same time schedule with analogous extracts of preparative polyacrylamide gels to which no glycoprotein samples were applied.

A double antibody radioimmunoassay was used for measuring the level of antibodies to the glycoprotein. The MAG antigen used in the assay was purified by the lithium diiodosalicylate/phenol procedure (7) after radiolabeling in vivo by injecting adult rats intracranially with [^{3}H]fucose or 13-day-old rats with [^{14}C]fucose (1). The incubation mixture contained 1 μg of MAG (1500 dpm ^{3}H or 900 dpm ^{14}C) and 10 μl of normal rat serum and was made up to a total volume of 150 μl with phosphate-buffered saline (pH 7.4). The serum to be tested for MAG antibodies was added as 20 μl of a 1:20 dilution in phosphate-buffered saline, and the assay tubes were incubated for 1 hr at 37°C. Then, 100 μl of goat anti-rabbit IgG (Cappel Laboratories, Cochranville, PA) was added, and the tubes were incubated for another 30 min at 37°C followed by 1 hr at 4°C. The mixtures were centrifuged at 2000 × g for 30 min, and the precipitates were washed with phosphate-buffered saline. The precipitates were dissolved with 1% sodium dodecyl sulfate and NCS solubilizer (Amersham) for liquid scintillation counting.

Newborn to adult Osborne–Mendel rats were anesthetized with chloral hydrate and fixed by intracardiac perfusion for 10 min with a solution containing 76 ml of $HgCl_2$ (saturated at 0°C) and 20 ml of 37% (vol/vol) formaldehyde. Twenty-micrometer coronal sections of brainstem and spinal cord, cross sections of trigeminal ganglia, or sagittal midline sections of the anterior commissure were cut on a Vibratome.

Sections were stained immunocytochemically by using the unlabeled antibody method (9) and described staining procedures (10). Sections were postfixed briefly in 2% (wt/vol) OsO_4 before addition of antiserum. Specificity of staining was shown by incubating sections with preimmune serum, gel control

Abbreviations: MAG, myelin-associated glycoprotein; MBP, myelin basic protein; CNS, central nervous system; PNS, peripheral nervous system.

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serum, or MAG antiserum absorbed with excess purified antigen.

An Optomax image analyzer attached to a Zeiss microscope was used to measure optical densities of oligodendroglia stained with antiserum to MAG according to the procedure (11) used earlier for myelin basic protein. Measurements were made of the highest density of cytoplasmic staining and the highest density of the surrounding unstained background. The OD of the background was subtracted from the OD of the cytoplasm. Data were expressed as the mean OD of 10 cells minus the background readings ± SEM.

RESULTS

MAG was purified by a combination of the selective lithium diiodosalicylate/phenol extraction procedure (7) and preparative polyacrylamide gel electrophoresis. The appearance of MAG purified by this procedure on analytical sodium dodecyl sulfate gels is shown in Fig. 1. The preparation shows a single major band except for some very minor components that were also in the extracts of control preparative gels. In order to assure that these minor extraneous components coming from the polyacrylamide gels were not influencing the immunological results, we immunized a control rabbit with extracts from blank gels. Serum from this rabbit as well as prebleeds of the MAG-immunized rabbit were used as controls in the immunological experiments described below.

Antibodies to MAG were demonstrated by both Ouchterlony immunodiffusion and a double antibody radioimmunoassay by using MAG that had been radiolabeled *in vivo* in adult rats
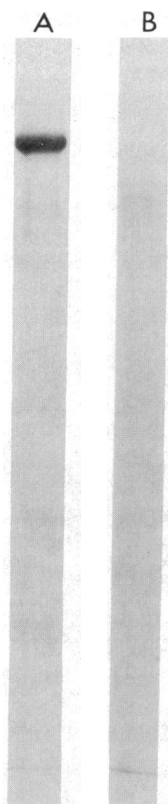

FIG. 1. Electrophoresis in an analytical sodium dodecyl sulfate/12% polyacrylamide gel of MAG that had been purified by preparative gel electrophoresis after extraction from myelin by the lithium diiodosalicylate/phenol procedure. Gels: A, purified MAG; B, an equal proportion of an extract from a control preparative gel to which no glycoprotein sample was applied. It can be seen that MAG is the only significant protein in the preparation, except for some very minor components that were extracted from the gels themselves.

with [³H]fucose. The antisera raised against MAG gave a single precipitin line when diffused against purified MAG, but gave no indication of reactivity with the P₀ glycoprotein of peripheral myelin. The radioimmunoassay indicated that 1 ml of immune serum contained sufficient antibody to react with about 0.2 mg of MAG. Preincubation of the antiserum with excess unlabeled MAG eliminated the reactivity in this assay in parallel with the loss of immunostaining described below. Because most of the immunostaining was done on immature rats, it was important to determine whether our antisera prepared to adult rat MAG also reacted with the immature MAG, which has been shown to have a higher apparent M_r on sodium dodecyl sulfate gels (3). This was tested by using [¹⁴C]fucose-labeled MAG purified from brains of 14-day-old rats as test antigen in the double antibody assay. The antisera reacted with the immature [¹⁴C]MAG to about the same extent as with the mature [³H]MAG.

The MAG antiserum stained oligodendroglial cytoplasm and myelin of newborn rat spinal cord and brainstem (Fig. 2A). As development proceeded, increased numbers of myelin sheaths and oligodendroglia were stained by MAG antiserum and staining appeared in more rostral brain regions. Intensity of oligodendroglial staining also increased during early development (Fig. 2B). As shown in Fig. 3, optical densities of MAG-containing oligodendroglia in the pontine tectospinal tract reached a maximum at 5–7 days and slowly decreased. Rapid accumulation of myelin begins at approximately 7 days in this tract.

The anterior commissure becomes myelinated later than the brainstem and is a tract well suited for studying the appearance of myelin components in oligodendroglia before myelination (12). Before 5 days no reaction with MAG antiserum was detected. At 5 and 7 days (Fig. 2C) only 2–4 small, faintly stained oligodendroglia and no stained myelin sheaths were found. By 12 days the number of oligodendroglia reacting with antiserum to MAG increased as did the intensity of staining. Myelin sheaths containing MAG were also present (Fig. 2D). Changes in MAG staining intensity in oligodendroglia with age are shown in Fig. 4.

There was no reaction product seen in sections incubated with preimmune serum (Fig. 2E), control gel serum, or antiserum absorbed with highly purified MAG (Fig. 2F). In sections incubated with MAG antiserum, no staining was seen in oligodendroglial nuclei, axons, astrocytes, and other cells. Faint staining of occasional neurons was sometimes observed which was not absorbed by MAG.

Oligodendroglial cytoplasm and processes were diffusely stained by MAG antiserum. In addition, granular staining of cytoplasm and processes occurred (Fig. 2G). This pattern was particularly predominant at times of greatest staining intensity (5 and 7 days in the pontine tectospinal tract) but could be seen at all ages. The granules were also irregularly distributed along the myelin sheath (Fig. 2H). Myelin sheath staining did not increase as the myelin sheaths grew longer and thicker. Staining was confined to a narrow ring surrounding the axon (Fig. 2I).

PNS myelin also reacted with MAG antiserum. As seen in Fig. 2J, heavily stained Schwann cell cytoplasm surrounds a lightly stained myelin sheath in the trigeminal ganglia from a 7-day-old rat. As in the CNS, myelin staining was confined to a periaxonal ring (Fig. 2K). Neurons, their satellite cells, Schwann cell nuclei, and axons were unstained. Again, no reaction was seen in sections incubated with preimmune serum, gel control serum, or MAG-absorbed serum.

MAG was not detected along surfaces of dendrites or unmyelinated axons. As seen in Fig. 5, there was no staining in
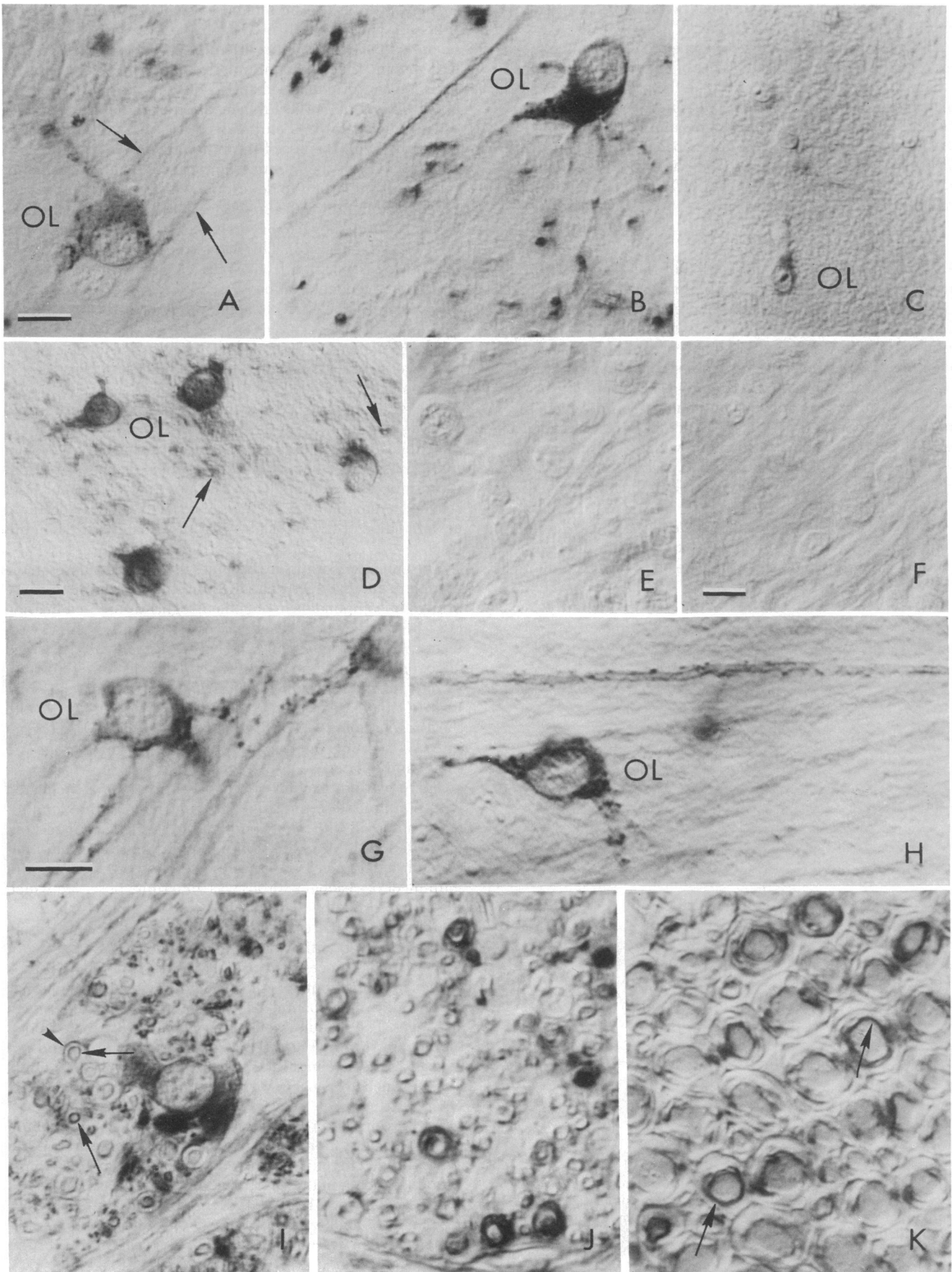
FIG. 2.   (*Legend appears at bottom of the next page.*)

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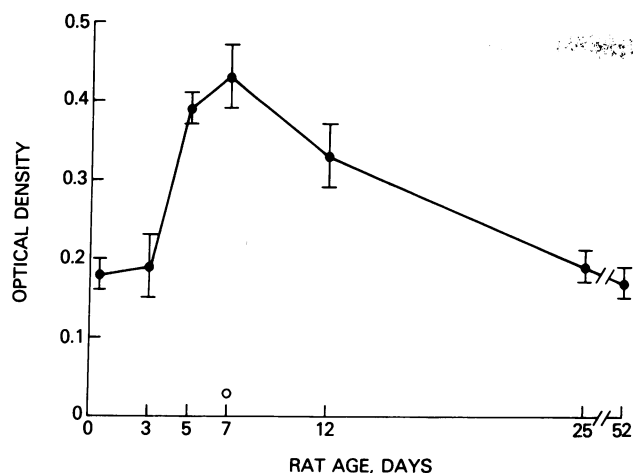*Proc. Natl. Acad. Sci. USA 76 (1979)*   1513

FIG. 3. Optical densities of oligodendroglia in the pontine tectospinal tract during early development that were stained with MAG antiserum (●) or preimmune control serum (○).
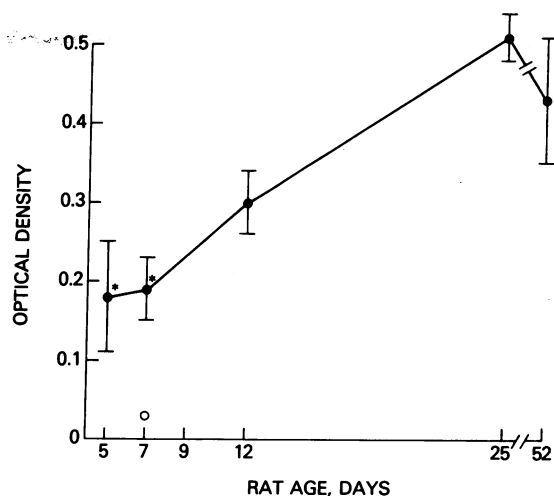

FIG. 4. Optical densities of oligodendroglia in the anterior commissure during early development that were stained with MAG antiserum (●) or preimmune control serum (○). Ages at which there were less than 10 stained oligodendroglia in the anterior commissure at the level sectioned are indicated with an asterisk.

the cerebellar molecular layer, which contains Purkinje cell dendrites, granule cell axons that are unmyelinated, a few astrocytes, and no oligodendroglia.

DISCUSSION

Experiments showing the presence of MAG in highly purified preparations of isolated CNS myelin (1, 3), regional and developmental studies (1, 3), and biochemical experiments with myelin-deficient mutant mice (2) have all strongly suggested that MAG is a component of CNS myelin or closely associated structures. This morphological study utilizing antiserum to purified MAG now shows conclusively that MAG is located specifically in oligodendroglia and myelin of the developing rat CNS.

During development, MAG is detected first in oligodendroglia before the onset of myelin formation, a finding described previously for myelin basic protein (MBP) (10). Like MBP, MAG staining of oligodendroglia and myelin sheaths is found in the newborn rat spinal cord and brainstem and oligodendroglial staining increases during early development. The time of maximal MAG staining also depends on the tract being studied. When oligodendroglia in the anterior commissure and pontine tectospinal tract were stained with MAG and MBP antisera and their optical densities were compared, MAG was detected slightly later in development than MBP. Relative to initial levels, the peak level for MAG was lower and also occurred a little later than the maximal MBP level. MAG staining intensity declined more slowly and still could be measured at 52 days of age, whereas little or no MBP could be detected in oligodendroglia by 25 days (11). Unlike MBP staining, MAG staining of oligodendroglial cytoplasm was often granular. The granules frequently extended from the tips of processes along newly formed stained myelin sheaths.

As compact myelin sheaths grow in thickness and length, only

their periaxonal portions are stained by MAG antiserum. This localization is consistent with the available biochemical evidence. Investigations of CNS myelin subfractions (13) and membrane fractions released when crude myelin is osmotically shocked (14, 15) show that MAG is not uniformly distributed in multilamellar myelin, but is concentrated in fractions consisting primarily of single membranes. Localization of MAG in the axolemma or axon seems unlikely, because staining is detected first in perinuclear regions of oligodendroglia. Also, surfaces of unmyelinated axons that we have studied are not stained by MAG antiserum. Finally, the above results and recent

FIG. 5. Purkinje cell dendrites and unmyelinated granule cell axons in the molecular layer of the cerebellum from a 52-day-old rat are unstained by MAG antiserum.

FIG. 2 (*on preceding page*). Micrographs of coronal sections of the pons (*A, B, E, F,* and *I*) and midbrain (*G* and *H*), midline sagittal sections of the anterior commissure (*C* and *D*), and cross sections of trigeminal ganglia (*J* and *K*). All sections were incubated with a 1:500 dilution of MAG antiserum and photographed with differential interference contrast optics. Lightly stained oligodendroglia (OL) and myelin sheaths (arrows) are present in the newborn pons (*A*). Staining of OL is much denser at 7 days (*B*). The anterior commissure contains small, faintly stained OL at 7 days (*C*). By 12 days (*D*), immunostaining of OL is increased and stained myelin sheaths (arrows) are also present. Preimmune serum (*E*) and MAG-absorbed antiserum (*F*) do not stain myelin sheaths or cells in sections of 7-day pons. OL at 5 days (*G*) and 7 days (*H*) exhibit granular staining of cytoplasm and processes that extend along myelin sheaths (*H*). Cross sections of myelin sheaths at 14 days (*I*) are stained periaxonally (arrows). Axons and compact portions of myelin sheaths are unstained. Thin, less dense rings are present at the upper outer margins of some sheaths (arrowheads). They are produced by the differential interference contrast illumination, because they shift or disappear when the section is rotated or the Wollasten prism is moved. In 7-day trigeminal ganglia, Schwann cells are heavily stained and surround lightly stained myelin sheaths (*J*). In adult PNS myelin (*K*), only a periaxonal ring of staining is present (arrows). Bars, 10 μm.

evidence that periaxonal MAG is altered in the early stages of multiple sclerosis (16) suggest that MAG has a role in glial-axonal interactions involved in the formation, maintenance, and breakdown of CNS myelin.

It is of interest that our MAG antiserum also stains developing Schwann cells and PNS myelin. Reaction of MAG antiserum with PNS myelin was also confined to a rim of periaxonal staining, suggesting that this peripheral myelin component is MAG or a similar large glycoprotein. The P_0 glycoprotein does not react with MAG antiserum. The large amount of P_0 may have masked the presence of MAG in previous biochemical studies of the PNS (4), and it is possible that one of the large glycoproteins recently reported in PNS myelin (5, 6) is MAG or a similar glycoprotein. Failure to detect MAG biochemically in the PNS may also be due to the use of less sensitive methods than the one used here.

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