MYELIN-SPECIFIC PROTEINS AND GLYCOLIPIDS IN RAT SCHWANN CELLS AND OLIGODENDROCYTES IN CULTURE

RHONA MIRSKY, JANET WINTER, ERIKA R. ABNEY, REBECCA M. PRUSS, JELENA GAVRILOVIC, and MARTIN C. RAFF

From the Medical Research Council Neuroimmunology Project, Department of Zoology, University College London, London WC1E 6BT, England

ABSTRACT

We have used antibodies to identify Schwann cells and oligodendrocytes and to study the expression of myelin-specific glycolipids and proteins in these cells isolated from perinatal rats. Our findings suggest that only Schwann cells which have been induced to myelinate make detectable amounts of galactocerebroside (GC), sulfatide, myelin basic protein (BP), or the major peripheral myelin glycoprotein (P_0). When rat Schwann cells were cultured, they stopped making detectable amounts of these myelin molecules, even when the cells were associated with neurites in short-term explant cultures of dorsal root ganglion. In contrast, oligodendrocytes in dissociated cell cultures of neonatal optic nerve, corpus callosum, or cerebellum continued to make GC, sulfatide and BP for many weeks, even in the absence of neurons. These findings suggest that while rat Schwann cells require a continuing signal from appropriate axons to make detectable amounts of myelin-specific glycolipids and proteins, oligodendrocytes do not.

Schwann cells and oligodendrocytes also displayed very different morphologies in vitro which appeared to reflect their known differences in myelinating properties in vivo. Since these characteristic morphologies were maintained when Schwann cells and oligodendrocytes were grown together in mixed cultures and in the absence of neurons, we concluded that they are intrinsic properties of these two different myelin-forming cells.

KEY WORDS Schwann cells · oligodendrocytes · myelin · basic protein galactocerebroside · dissociated cell cultures

One of the most remarkable examples of cell-cell interaction in the nervous system is the formation of myelin around neuronal axons by myelin-forming glial cells. Schwann cells in the peripheral nervous system (9) and oligodendrocytes in the central nervous system (16, 21) wrap their plasma membranes concentrically around axons to form multilayered myelin sheaths. Although myelin has been the subject of intensive biophysical and biochemical study, remarkably little is known about the molecular events involved in myelination. It seems likely that a detailed understanding of these events will require studying the process in vitro, preferably in dissociated cultures of purified myelin-forming cells mixed with purified neurons.

Recently, we have defined surface antigenic markers which allow Schwann cells and oligodendrocytes to be unambiguously identified in dissociated cell cultures of peripheral and central nervous tissues, respectively. Rat neural antigen-1

(Ran-1), which is defined by a mouse antiserum produced against a rat neural cell line (7), is expressed by rat Schwann cells but not fibroblasts, neurons, astrocytes, oligodendrocytes, or microglial cells (3, 8, 27). Galactocerebroside (GC), the major glycolipid in myelin (20), has been found only on oligodendrocytes in a wide variety of neural cultures (26, 27). Now that we have specific markers for the two myelin-forming cells and can purify (4) and grow (4, 25) Schwann cells in large numbers, we have begun to study some of the myelin-related properties of these cells in vitro.

We have previously presented evidence that rat Schwann cells in vivo only begin to make detectable amounts of the peripheral myelin basic proteins (P_1 and P_2) and glycoprotein (P_0) once they have been signalled to myelinate (5), and that purified secondary Schwann cells in culture do not make detectable amounts of these myelin proteins (5) or the myelin-specific glycolipids, GC (26) or sulfatide (27). In the studies reported here, we show that a proportion of Schwann cells freshly isolated from neonatal rat sciatic nerve and dorsal root ganglion (DRG) express these myelin-specific molecules but lose them over several days in culture. In contrast, oligodendrocytes dissociated from neonatal tissues, days before myelination is due to begin, make myelin-specific glycolipids and basic protein (BP) for many weeks in culture, even in the absence of neurons. This suggests that a continuing signal from an appropriate axon is required for rat Schwann cells to begin and continue making detectable amounts of myelin-specific molecules, while this is not the case for oligodendrocytes. In addition, we demonstrate striking intrinsic morphological differences between cultured Schwann cells and oligodendrocytes which reflect their differing myelinating characteristics in vivo (6, 32).

MATERIALS AND METHODS

Cell Cultures

Wistar/Furth (W/Fu) rats were obtained from the Imperial Cancer Research Fund animal colony, Mill Hill, England. Embryos were taken from pregnant rats which had been caged with males for 16 h and then separated; the day of separation was taken as day 0.

Cells from embryonic or neonatal sciatic nerve, dorsal root ganglion (DRG), optic nerve, corpus callosum, and cerebellum were dissociated in trypsin and collagenase and cultured on glass coverslips as previously described (3, 8, 26, 27). Cultures of superior cervical ganglion and adjacent sympathetic trunk were prepared in the same way as DRG cultures. Purified secondary Schwann cells were prepared as previously described (4). In some experiments dissociated cells from newborn sciatic nerve were plated together with dissociated cells from 5- to 6-d-old optic nerve or corpus callosum or on top of optic nerve or corpus callosum cells which had been in culture for 5–8 d. In other experiments cells from optic nerve or corpus callosum were grown for 2–10 wk in Falcon tissue culture flasks (Falcon Labware, Div. Becton, Dickinson Co., Oxnard, Calif.), removed from the flasks with 0.05% trypsin/0.16% EDTA in Tris-buffered saline, and then cultured on glass coverslips.

Explant DRG cultures from newborn W/Fu rats were prepared by dissecting out DRGs as previously described (8), cleaning the individual ganglia free of roots, and incubating them in collagenase (0.5 mg/ml) in Minimal Eagle's Medium with 0.02 M *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid buffer (MEM-HEPES) containing 0.6% glucose for 5 min. Individual ganglia were then mounted in Rose chambers, with a small piece of dialysis tubing holding down the explants, and cultured in Dulbecco's Modified Eagle's Medium containing 10% foetal calf serum.

Antisera

The preparation and specificity of the following antisera have been described previously (27): mouse anti-Ran-1 (supplied by K. L. Fields) (3, 7); mouse anti-tetanus toxin (supplied by K. L. Fields) (27); rabbit anti-tetanus toxin (8); rabbit antigalactocerebroside (27); rabbit antisulfatide (supplied by S. Hakomori) (10); rabbit anti-rat P_0 (supplied by J. Brockes) (15); immunoadsorbent-purified rabbit antibodies against rat central myelin basic proteins (BP) (supplied by D. McFarlin) (5); guinea pig anti-guinea pig central myelin BP (supplied by R. Lisak) (12, 13) and rabbit anti-glial fibrillary acid protein (GFAP) (3, 27). Tetanus toxin was the same as previously described (19). All of the antisera were heated at 56°C for 30 min.

Antibody binding was visualised by the use of goat anti mouse IgG conjugated to rhodamine (Nordic Immunological Laboratories, Tilburg, Holland, batch No. 2-773) adsorbed with rabbit IgG coupled to Sepharose 6B, goat anti-rabbit IgG conjugated to rhodamine (Nordic, batch No. 5-874) or to fluorescein (Nordic, batch No. 27-175) or rabbit anti-guinea pig IgG coupled to fluorescein (N. L. Cappel Laboratories Inc., Cochranville, Penn.).

All reagents were centrifuged at 100,000 *g* for 30 min and stored at 4°C in the presence of 0.02% (wt/vol) sodium azide and were used at final dilutions which were shown to be on the plateaus of titration curves in indirect immunofluorescence assays. These dilutions were the same as previously reported for most of the reagents (27). The rabbit anti-BP antibodies were used at 100 μg/ml, the guinea pig anti-BP serum at 1:10, the rabbit anti-P_0 serum at 1:20, the rabbit anti-GFAP serum at 1:100 and the rabbit anti-guinea pig IgG conjugated to fluorescein at 1:100. As described previously (27), only macrophages were labeled when the fluorescent conjugates were used on their own or when normal mouse or rabbit serum was used in place of the various antisera with living cells. In all cases, reagents used in double labeling experiments were checked to be certain that they did not react with each other or detect the inappropriate immunoglobulin in indirect immunofluorescence assays.

Immunofluorescence

Living cells on coverslips were labeled by indirect immunofluorescence as previously described (3); coverslips were incubated in 30–50 μl of antiserum followed by the appropriate fluorescent conjugate. Reagents were diluted in MEM-HEPES with 10% foetal calf serum. All incubations were for 25–30 min at room

temperature and cells were washed by dipping coverslips in 4–5 beakers of MEM-HEPES. In double labeling experiments on living cells, cells were exposed to two reagents (mouse and rabbit antisera) simultaneously, followed by a mixture of goat anti-mouse IgG and goat anti-rabbit IgG conjugated to two different fluorochromes. After washing, cells were fixed in 5% acetic acid in ethanol (acid-alcohol) at −20°C for 10 min. For demonstrating BP and P_0, cells were prefixed with acid-alcohol before being exposed to anti-BP or anti-P_0 antibodies. In double labeling experiments involving BP or P_0, cells were first labeled while alive with appropriate antisera and conjugates, then fixed in acid-alcohol and stained for BP or P_0. Coverslips were mounted in glycerol containing 10^{-4}M *p*-phenylenediamine (Sigma Chemical Co., St. Louis, Mo.) to inhibit fluorescein inactivation by UV light, and sealed with nail varnish.

In some experiments, freshly dissociated cells were labeled in suspension, washed, cytocentrifuged (Shandon Scientific Co. Ltd., London) onto a glass slide, and fixed in acid-alcohol. In some cases, such cells were then labeled with anti-BP or anti-P_0 antibodies followed by the appropriate conjugate. These cells were then mounted under coverslips and sealed as described above.

All preparations were examined with a ×63 objective on a Zeiss Universal Fluorescence microscope equipped with phase contrast and Nomarski interference optics, filters, and dichroic mirrors for viewing fluorescein and rhodamine fluorescence, and epi-illumination with an Osram HBO-mercury arc lamp (Osram Gesellschaft, Munich, W. Germany) as a light source. Photographs were taken with Kodak Ektachrome 200 color slide film.

RESULTS

Some Schwann Cells Isolated from Neonatal Sciatic Nerve and Dorsal Root Ganglion Express Myelin-specific Proteins and Glycolipids

When newborn sciatic nerves were dissociated in trypsin and collagenase and the resulting cell suspension was labeled with anti-GC or anti-Thy-1 antibodies, 30–40% of the cells were GC^+ and 20–30% were Thy-1^+. In double labeling experiments, the GC^+ and Thy-1^+ cells belonged to nonoverlapping populations. When dissociated cells were cytocentrifuged onto a glass slide, fixed, and stained with anti-BP or anti-P_0 antibodies, 2–8% of the cells were labeled with either reagent. The staining usually occurred diffusely over the nonnuclear pole of the cell which often had a scalloped contour (Fig. 1). In double labeling experiments all of the BP^+ and P_0^+ cells were GC^+.

Since Ran-1 is trypsin sensitive, anti-Ran-1 serum could not be used to identify the Schwann cells in these suspensions. Therefore, cells dissociated from newborn sciatic nerves and DRG were cultured on coverslips for 16–20 h before being studied by indirect immunofluorescence, using mouse anti-Ran-1 to identify the Schwann cells

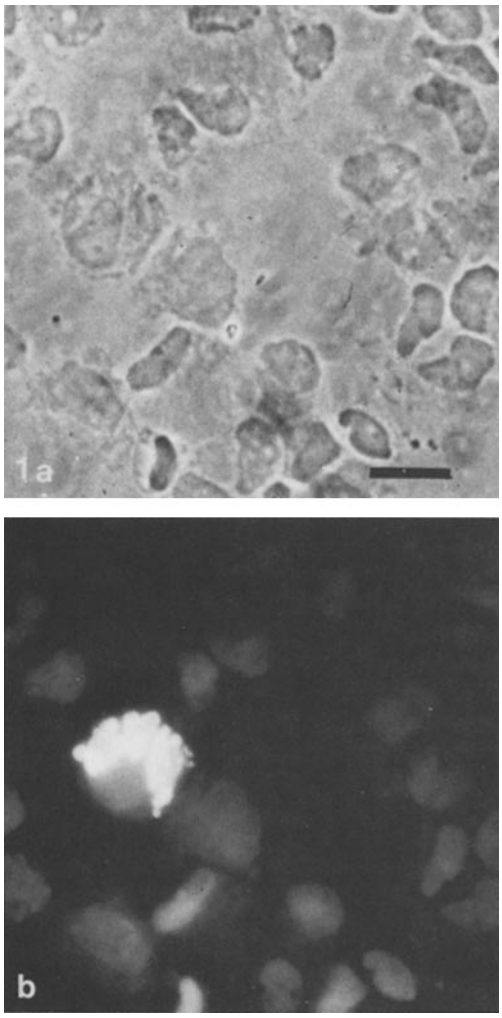


FIGURE 1 BP^+ cell in a cytocentrifuge preparation of a freshly prepared suspension of cells from 1-day-old sciatic nerve. The cells were fixed before labeling and were viewed with phase contrast (*a*) or fluorescence (*b*) optics. A single cell is BP^+ with the fluorescence located over the nonnuclear pole of the cell. Bars, 10 μM.

and rabbit antibodies to localize the myelin-specific proteins and glycolipids. In these cultures, 25–55% of the Ran-1^+ Schwann cells were labeled by anti-GC and antisulfatide sera (Table I) while none of the fibroblasts (Thy-1^+, Ran-1^-) or neurons (tetanus $toxin^+$, Thy-1^+, Ran-1^-) were labeled by these sera. Since anti-GC and antisulfatide sera always labeled a similar proportion of the Schwann cells and this proportion was not increased when cells were treated with the two sera

TABLE I

Myelin Specific Glycolipids and Proteins in Cultured Schwann Cells

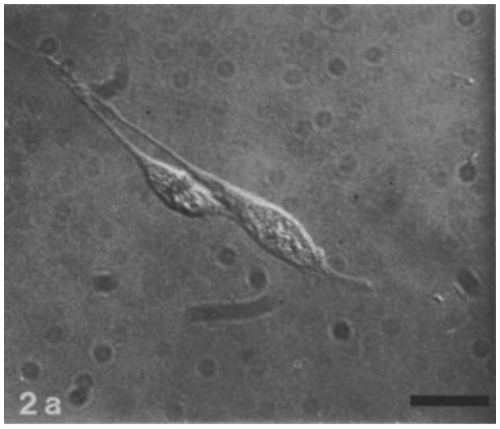
Tissue	Age of rat	Time in culture	% Ran-1$^+$ Schwann cells expressing			
			GC	Sulfatide	BP	P_0
	d	*h*				
Sciatic nerve	Embryonic 16, 17, 18	16–20	0	0	0	0
Sciatic nerve	Embryonic 19	16–20	2 ± 2	—	0	0
Sciatic nerve	Newborn	16–20	45 ± 11	43 ± 15	5 ± 2	8 ± 6
Sciatic nerve	Newborn	40–46	25 ± 8	27 ± 9	2 ± 2	4 ± 2
Sciatic nerve	Newborn	65–70	4 ± 2	—	<1	<1
Sciatic nerve	Newborn	90–96	0	0	0	0
Sciatic nerve	5–7	16–20	61 ± 9	59 ± 10	38 ± 8	49 ± 11
Sciatic nerve	5–7	40–46	36 ± 7	34 ± 9	20 ± 5	33 ± 8
Sciatic nerve	5–7	65–70	11 ± 6	9 ± 6	—	25 ± 6
Sciatic nerve	5–7	110–120	0	0	<1	<1
DRG	Embryonic 18	16–20	0	—	—	—
DRG	Embryonic 19	16–20	8	—	0	<1
DRG	Newborn	16–20	28 ± 7	—	2 ± 0.5	6 ± 2
DRG	5–7	16–20	39 ± 7	—	9 ± 1	34 ± 9
DRG	5–7	40–46	8 ± 2	—	8 ± 2	21 ± 4
DRG	5–7	65–70	0	—	2	12
DRG	5–7	90–96	0	—	0	1.5
DRG	5–7	184–190	—	—	—	2
SCG	Newborn	16–20	1	—	0	2 ± 1
SCG	5–7	16–20	3 ± 2	—	0	4 ± 2
DRG explant	Newborn	9 d	0	—	—	0

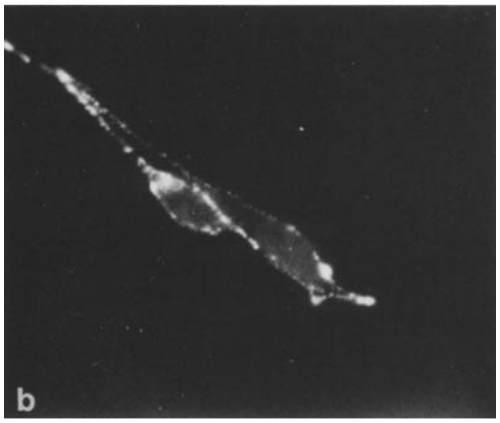
simultaneously, it is likely that they labeled the same subpopulation of Schwann cells.

After fixation, 2–10% of the Ran-1$^+$ cells were labeled by either anti-BP or anti-P_0 antibodies (Table I). All of the BP$^+$ cells and P_0^+ cells were Ran-1$^+$ and >95% of them were GC$^+$. While GC and sulfatide were diffusely distributed on the surface of living Schwann cells (Fig. 2), both anti-BP and anti-P_0 antibodies only labeled fixed cells and the staining was usually confined to one or more vesicles on one or both sides of the nucleus (Fig. 3), although occasional cells were more diffusely labeled (Fig. 4). The patterns of staining seen with anti-BP and anti-P_0 antibodies were indistinguishable.

More Schwann Cells Express Myelin-specific Molecules in Sciatic Nerve and DRG from 5- to 6-d-old Rats than from Newborns while no such Cells are Seen in 16- to 18-d Embryonic Nerves or Ganglia

Of the Ran-1$^+$ cells in 16- to 20-h-old cultures prepared from 5- to 6-d-old rat sciatic nerve or DRG, 40–70% were GC$^+$ and sulfatide$^+$, 35–50% were P_0^+ and 10–40% were BP$^+$ (Table I). The patterns of glycolipid, BP, and P_0 labeling were similar to that seen in cultures of newborn nerve although, in general, the labeling was more intense in the older Schwann cells (Fig. 4). The proportion of cells that was labeled with anti-BP antibodies was always less than that labeled with anti-P_0 serum, especially in DRG cultures (Table I). Since the proportion of cells labeled when anti-BP and anti-P_0 antibodies were used together was not greater than that labeled with anti-P_0 alone, it is likely that all of the BP$^+$ cells were also P_0^+. A small proportion (<10%) of Thy-1$^+$/Ran-1$^-$ fibroblasts contained one or two intracellular vesicles which were labeled with anti-BP or anti-P_0 antibodies. Since such Thy-1$^+$/BP$^+$ and Thy-1$^+$/P_0^+ cells were not detected in cell suspensions freshly isolated from 5- to 6-d-old sciatic nerves, the cultured fibroblasts probably acquired these myelin proteins by ingesting fragments of degenerating myelin during the 16–20 h of culture. In freshly prepared suspensions of 5- to 6-d-old sciatic nerve, 40–50% of the Thy-1$^-$ cells were BP$^+$ and P$^+$, and all BP$^+$/P_0^+ cells were GC$^+$, suggesting that at least some Schwann cells had made these myelin





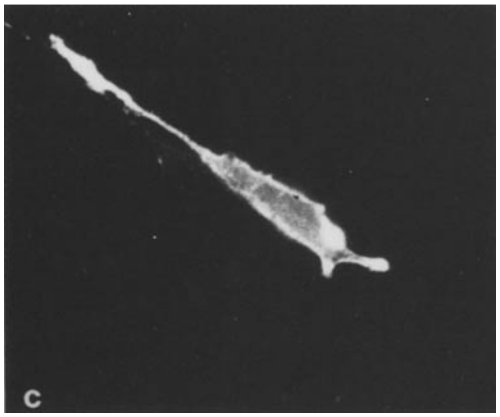
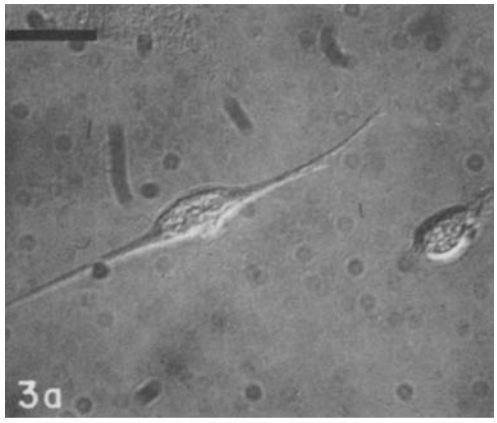
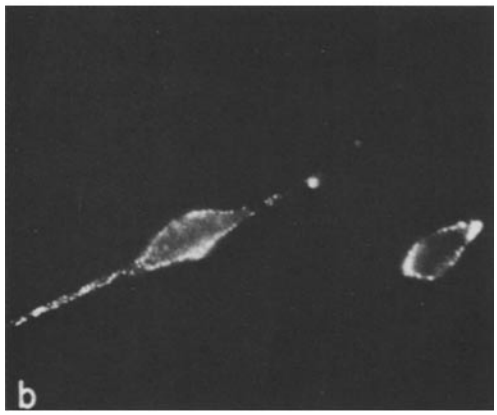


FIGURE 2 Two Schwann cells in a 16- to 20-h culture of newborn sciatic nerve. The cells were incubated simultaneously with mouse anti-Ran-1 and rabbit anti-GC followed by a mixture of goat anti-mouse Ig-rhodamine and goat anti-rabbit-Ig-fluorescein and then viewed with Nomarski (*a*), rhodamine (*b*), and fluorescein (*c*) optics. Both cells are Ran-1$^+$ but only one is GC$^+$. Bar, 10 μM.





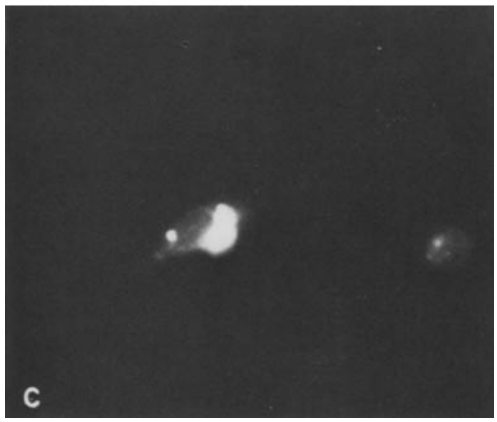
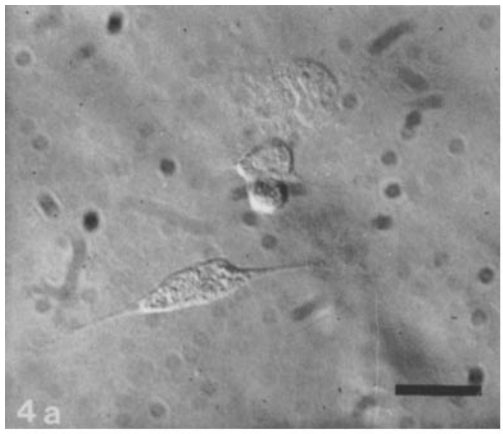
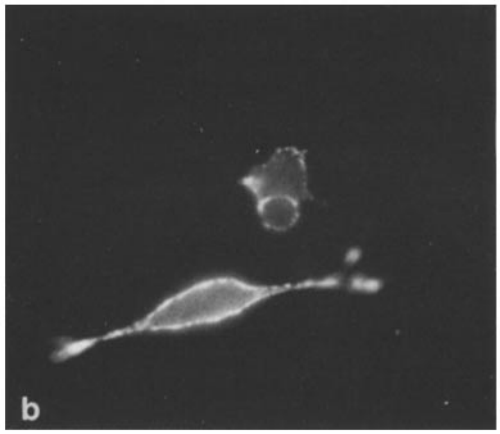


FIGURE 3 Cells in a 16- to 20-h culture of newborn sciatic nerve incubated with mouse anti-Ran-1 followed by goat anti-mouse-Ig-rhodamine and, after fixation, with rabbit anti-BP followed by goat anti-rabbit-Ig-fluorescein. The cells were viewed as in Fig. 2. Two Schwann cells (but not the fibroblastic cell) are Ran-1$^+$ and one of these is BP$^+$. Bar, 10 μM.





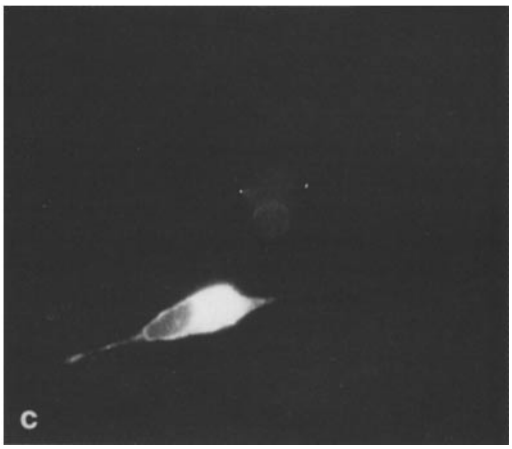


FIGURE 4 Cells in a 16- to 20-h culture of 5-day-old sciatic nerve were labeled with mouse anti-Ran-1 and goat anti-mouse Ig-rhodamine, fixed, and then labeled with rabbit anti-P_0 and goat anti-rabbit Ig-fluorescein. The cells were viewed as in Fig. 2. Three of the cells are Ran-1^+ Schwann cells and one of these is P_0^+. Bar, 10 μM.

proteins in vivo. However, in 16- to 20-h sciatic nerve cultures, 10–20% of the BP^+/P_0^+ Schwann cells were GC^-, which may indicate that some Schwann cells in culture had also injested degenerating myelin fragments.

Cultures prepared from 16-, 17-, or 18-d embryonic sciatic nerves or DRGs did not contain cells which were labeled by any of the antibodies directed against the myelin-specific proteins or glycolipids when studied at 16, 40, or 64 h, although >50% of the cells in these cultures were Ran-1^+ Schwann cells. The first GC^+ cells were detected in cultures prepared from 19-d embryonic sciatic nerves and DRGs, while BP and P_0 were first detected in nerves and ganglia prepared from 20-d embryos or newborns (Table I).

Only Occasional Schwann Cells Dissociated from Newborn or 5- to 6-d-old Sympathetic Ganglia Express Myelin-specific Glycolipids or Proteins

Unlike the case in sciatic nerve and DRG, where the majority of axons become myelinated, most of the axons which arise from neurons in sympathetic ganglia never become myelinated. In cultures prepared from newborn or 5- to 6-d-old superior cervical ganglion and adjacent sympathetic trunk, only occasional cells expressing these myelin-specific molecules were seen (Table I), even though 50% of the cells were Ran-1^+.

Schwann Cells Stop Making Detectable Amounts of Myelin-specific Molecules when Put into Culture

The proportions of Ran-1^+ Schwann cells in sciatic nerve or DRG cultures that were labeled by anti-GC or antisulfatide progressively decreased with time in culture so that by 72 h only rare cells expressed these glycolipids and no such cells were found at 96 h or thereafter (Table I). To exclude the possibility that, with time in culture, GC and sulfatide were progressively masked on the Schwann cell surface by glycoproteins or other glycolipids, secondary Schwann cells were treated with pronase (Koch-Light Laboratories Ltd., Colnbrook, Buckinghamshire, England; 1 mg/ml for 60 min at 37°C) and/or neuraminidase (*Vibrio cholerae*; lot No. 500284 at 20 U/ml at pH 6.5 for 30 min at 37°C; Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.). Such cells still were not labeled by anti-GC or antisul-

fatide sera. The fact that the intensity with which Schwann cells were labeled with these antibodies progressively decreased with time in culture suggested that the positive cells did not die but progressively lost these glycolipids. Moreover, the absolute number of Schwann cells in these cultures increased rather than decreased with time and there was no evidence for large-scale Schwann cell death.

The proportions of Ran-1$^+$ Schwann cells that expressed BP and P_0 also progressively decreased with time in culture. In cultures of sciatic nerve and DRG from 5- to 6-d-old rats, the loss of BP and P_0 occurred more slowly than the loss of GC and sulfatide (Table I), presumably reflecting a slower rate of catabolism of the proteins. Nonetheless, BP$^+$ or P_0^+ cells were rarely seen after 5–6 d in culture.

Myelin glycolipids and proteins were also lost in explant cultures of neonatal DRG. After 1 wk in culture, Ran-1$^+$ cells associated with neuronal cell bodies (satellite cells) as well as those associated with tetanus toxin$^+$ neurite bundles growing out from the edges of explants (Fig. 5) were unlabeled by antibodies against GC, BP, or P_0.

Oligodendrocytes Express Galactocerebroside, Sulfatide, and Basic Protein for Many Weeks in Culture

We have previously shown that oligodendrocytes in short-term culture express readily detectable amounts of GC and sulfatide on their surface (26, 27). We have now followed such cells for up to 10 wk in dissociated cell cultures of neonatal optic nerve and corpus callosum and have found that cells with typical oligodendrocyte morphology were GC$^+$ and sulfatide$^+$ even when no tetanus toxin$^+$ neurons were present in the cultures and after they had been passaged one or more times. 2–5% of the cells in 1-wk-old cultures are oligodendrocytes, and the absolute number per culture does not change from 1 to 8 wk. Since the oligodendrocytes are not dividing (R. Pruss and P. Bartlett, manuscript in preparation), it is assumed that there is no significant death of these cells in long-term cultures. In long-term cultures, astrocytes (identified by their intracellular staining with antibody against the GFAP [2, 26]) and tetanus toxin$^+$ neurons (19) were not labeled by anti-GC or antisulfatide sera, as was previously found in short-term cultures (27).

When cultures of optic nerve, corpus callosum,

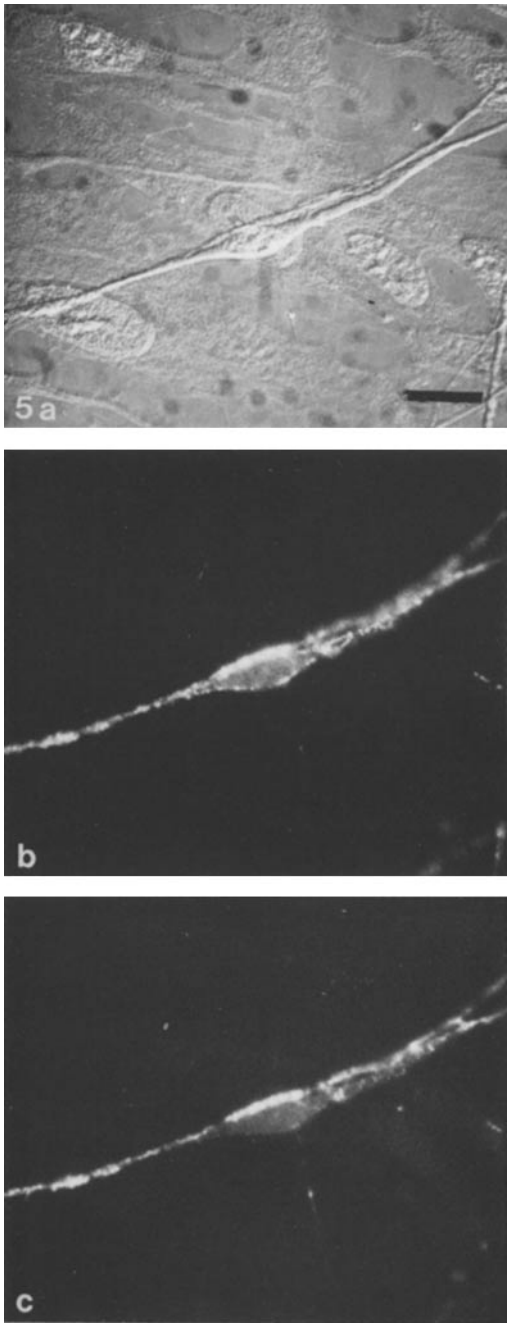


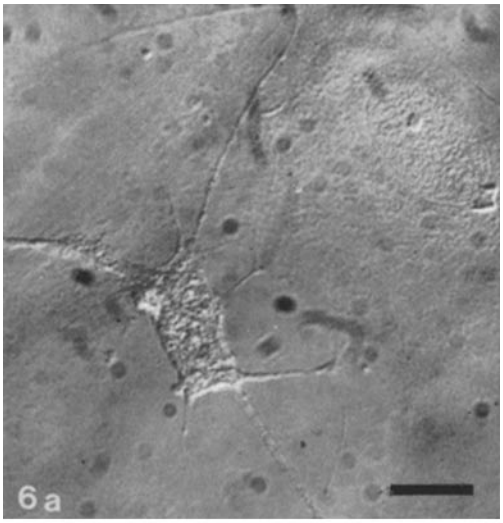
FIGURE 5 Newborn DRG explant, 9 d in culture, was incubated with tetanus toxin and then simultaneously with mouse anti-Ran-1 and rabbit anti-tetanus toxin, followed by a mixture of goat anti-mouse-Ig-rhodamine and goat anti-rabbit-Ig-fluorescein. The cells were viewed as in Fig. 2. A Ran-1$^+$ Schwann cell is closely associated with a tetanus toxin$^+$ neurite. The flat background cells are unlabeled. Bar, 10 μM.

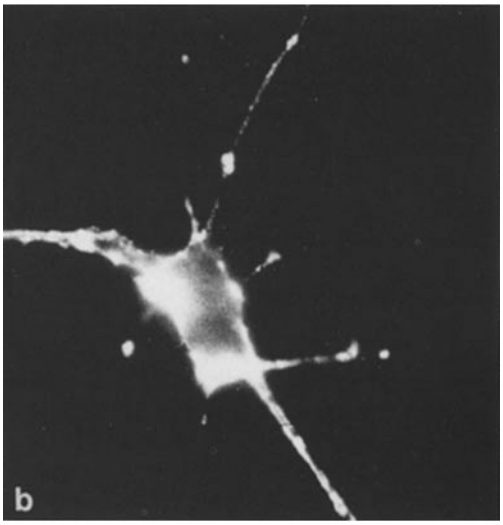
or cerebellum, prepared from 5- to 8-d-old rats, were fixed after 1–10 wk in culture and stained with anti-BP antibody, only cells with the characteristic appearance of oligodendrocytes were labeled. In double labeling experiments with rabbit anti-GC and guinea pig anti-BP antibodies, all BP^+ cells were GC^+ and all GC^+ cells were BP^+. The BP staining was most intense in the cell bodies of oligodendrocytes (Fig. 6) but extended into the processes of some of these cells. In some cells, which appeared to be degenerating, BP^+ vesicles bubbled from the cell surface. No staining was seen when unfixed oligodendrocytes were exposed to anti-BP antibodies.

In cultures of newborn optic nerve, a small number of GC^+, $sulfatide^+$ oligodendrocytes were seen as early as 1 d in culture. Such cells did not label with anti-BP antibodies until 5–6 d in culture, and by 10 d all GC^+ cells were BP^+. The development of BP in cultured oligodendrocytes from newborn optic nerve mirrored in vivo development. When freshly prepared suspensions of optic nerve were studied from rats during the first week of life, BP^+ cells were first seen at 5–6 d.

The Characteristics of Schwann Cells and Oligodendrocytes are Unchanged in Mixed Cultures of Central and Peripheral Nervous Tissues

The majority of Schwann cells in sciatic nerve, DRG, and SCG cultures were bipolar (Figs. 2, 3, 4, 5, 8) while most oligodendrocytes in central neural cultures had many processes which were often long and extensively branched (Figs. 6, 7, 8). The processes of some oligodendrocytes occasionally ended in large membranous expansions, which were striking when viewed by fluorescence microscopy (Fig. 7), but often barely visible by phase contrast microscopy. To exclude the possibility that these characteristic morphologies were a result of the different environments in peripheral and central neural cultures, cells dissociated from





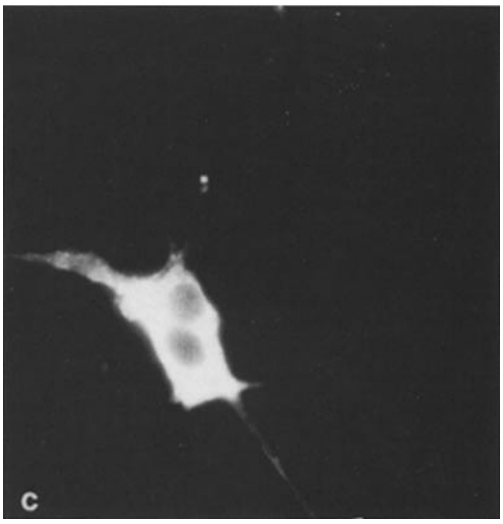


FIGURE 6 Cells in a 10-d-old culture of optic nerve prepared from 5- to 6-d-old rats, incubated with rabbit anti-GC and goat anti-rabbit-Ig-rhodamine, fixed, and incubated with guinea pig anti-BP and goat anti-guinea pig-Ig-fluorescein. The cells were viewed as in Fig. 2. The two oligodendrocytes are GC^+ and BP^+. Oligodendrocytes in much older cultures (up to 10 wk) stain in a similar manner with anti-GC and anti-BP sera. Bar, 10 μM.

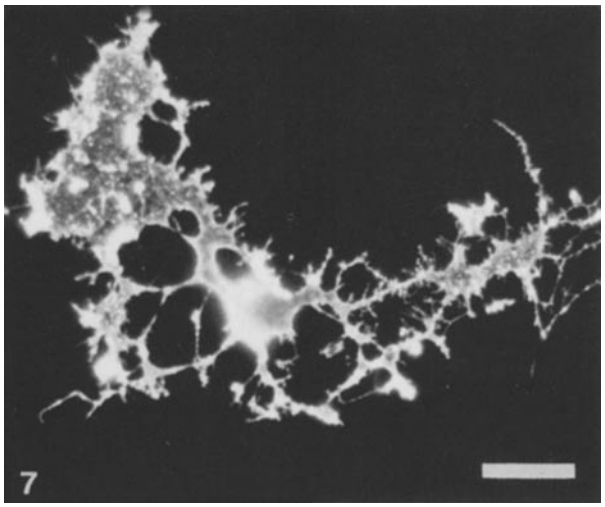


FIGURE 7 Fluorescence micrograph of an oligodendrocyte labeled with anti-GC in an 8-d culture of 5-d-old optic nerve. Several processes end in large membranous expansions. Bar, 10 μM.

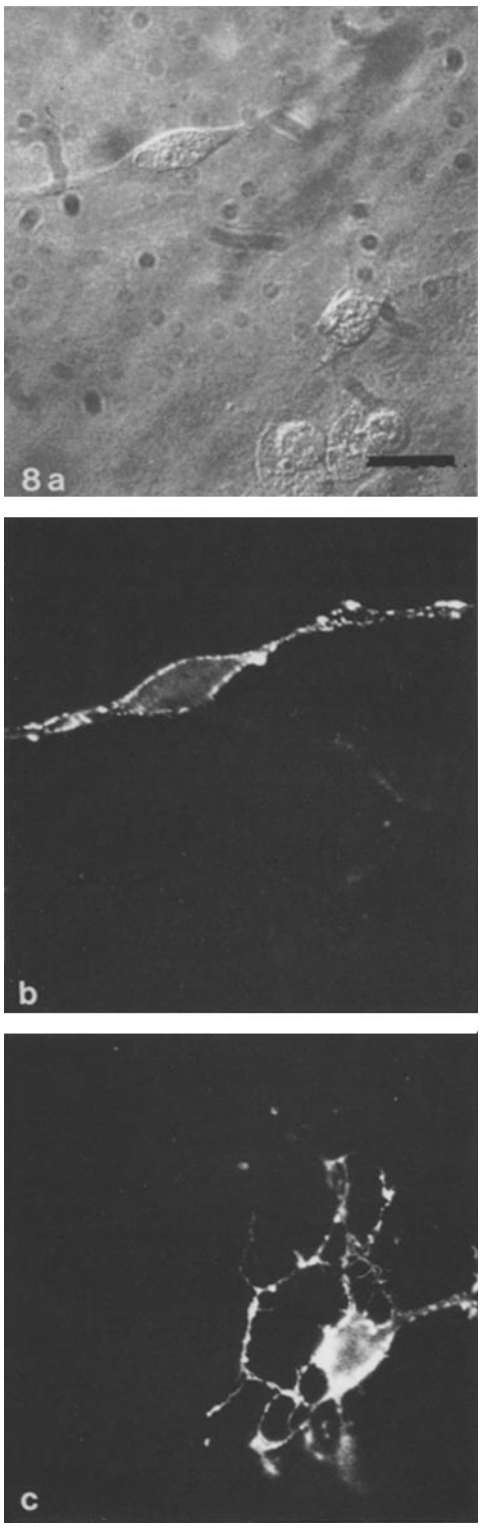
newborn sciatic nerve were co-cultured for up to 2 wk with cells dissociated from 5- to 7-old optic nerve or corpus callosum. In such mixed cultures, Ran-1$^+$ Schwann cells maintained their bipolar morphology and GC$^+$ oligodendrocytes were indistinguishable from those in unmixed central cultures (Fig. 8). Moreover, Ran-1$^+$ Schwann cells in such mixed cultures lost the expression of GC (Fig. 8) sulfatide, BP, and P_0 with the same time course as they did in unmixed cultures of sciatic nerve and DRG.

DISCUSSION

While all axons in peripheral nerves are at least partly enclosed by Schwann cell plasma membrane (32), only certain axons (usually those with diameters >1 μM) (15) are surrounded by multiple layers of Schwann cell membrane in the form of myelin sheaths. Nerve cross-anastomosis (33) and nerve transplantation (1) studies have shown that axons regenerating from myelinated nerves cause Schwann cells derived from unmyelinated nerves to myelinate, indicating that the decision of the Schwann cell to myelinate is determined by the axon. Our present findings suggest that only those Schwann cells that have been signaled by appropriate axons to myelinate make detectable amounts of myelin-specific glycolipids (GC and sulfatide) and myelin proteins (P_0 and BP). This is suggested by the fact that (*a*) these myelin-specific molecules were first detectable in sciatic nerve and DRG Schwann cells isolated from 19- to 20-d embryos, which is close to the time that the first axons are beginning to be myelinated (31), (*b*) the proportion of Schwann cells expressing these molecules in sciatic nerve and DRG increased with age in the first week of life, during which time the proportion of axons being myelinated in vivo is known to be rapidly increasing (31), and (*c*) Schwann cells isolated from the newborn or 5- to 6-d-old sympathetic ganglia and adjacent sympathetic trunk (the great majority of which do not myelinate axons in vivo) did not express these myelin-specific molecules. The previous findings that unmyelinated nerve fibres in frozen sections of peripheral nerve are unlabeled by anti-BP (5, 18) or anti-P_0 (5) antibodies in immunofluorescence (5, 18) and immunoperoxidase (18) assays also support this conclusion.

Two findings suggest that Schwann cells begin making myelin-specific glycolipids before myelin-specific proteins in the early stages of myelination: (*a*) GC and sulfatide were detectable 1 d earlier in ontogeny than BP and P_0 (19 d gestation compared to 20 d), and (*b*) a higher proportion of Schwann cells expressed GC and sulfatide than BP and P_0 in the first week of life. Similarly, the fact that more Schwann cells expressed P_0 than BP in neonatal sciatic nerve and DRG raises the possibility that P_0 is made before BP during myelinogenesis.

The distribution of the myelin glycolipids in Schwann cells appeared to be different from that of the myelin proteins. While the glycolipids were diffusely distributed on the surface of Schwann



cells, none of the proteins were detected on the cell surface. Since both BP and P_0 are known to be trypsin sensitive (34), if either were exposed on the Schwann cell surface they could have been digested during the enzymatic dissociation procedure. In freshly isolated Schwann cells which had been cytocentrifuged onto a glass slide and fixed, so that antibodies could enter the cell, the myelin proteins were found to occupy one pole of the cell; they were not found in or under the plasma membrane overlying the nucleus at the other pole of the cell. After Schwann cells had been in culture for 16 h or more, the myelin proteins were usually confined within single or multiple vesicles. This may have resulted from the cultured Schwann cells' ingesting those parts of their plasma membranes which were involved in myelinating axons in vivo.

The fact that neonatal rat sciatic nerve and DRG Schwann cells, which initially expressed myelin proteins and/or glycolipids, lost these molecules within days of being placed in culture, explains our previous failure to demonstrate these myelin-specific molecules in cultured secondary Schwann cells (5, 26, 27). It suggests that a continuing signal is required from myelin-inducing axons for Schwann cells to continue to make readily detectable amounts of myelin glycolipids or proteins. This is supported by the observation that >95% of Ran-1^+ Schwann cells were GC^- in 20-h dissociated cell cultures of adult rat sciatic nerves which were undergoing Wallerian degeneration 11 d after the nerves were cut (R. Lisak and M. C. Raff, unpublished observations). Previous studies have demonstrated marked decreases in myelin-specific proteins in rabbit distal nerve stumps weeks and months after nerve transection (17); such experiments are difficult to interpret in view of the massive decrease in Schwann cell membrane in demyelinated nerve stumps compared to myelinated nerve. Our studies on isolated, single Schwann cells avoid this problem.

Since Schwann cells which were in contact with

FIGURE 8 Cells in mixed culture of newborn sciatic nerve and 6-d corpus callosum were incubated after 8 d in culture with a mixture of mouse anti-Ran-1 and rabbit anti-GC followed by a mixture of goat anti-mouse-Ig-rhodamine and goat anti-rabbit-Ig-fluorescein. The cells were viewed as in Fig. 2. The Ran-1^+/GC^- Schwann cell shows characteristic bipolar morphology while the Ran-1^-/GC^+ oligodendrocyte has several, branched processes. Bar, 10 μM.

DRG neurons or neurites in short-term dissociated or explant cultures of newborn DRG stopped making detectable amounts of myelin glycolipids and proteins, it is clear that mere contact with such neurons or their neurites is insufficient to provide the necessary signal to rat Schwann cells in culture. This could be related to the diameter of the DRG neurites in such cultures or to some other property of neurons undergoing (or recovering from) an axonal reaction. The fact that myelination occurs only after weeks in rat DRG cultures (22) presumably reflects the same phenomenon.

It has been previously shown that oligodendrocytes isolated from calf and lamb white matter (14, 24) and neonatal rat oligodendrocytes in culture (26, 27) express GC and sulfatide. In this study we have shown that rat oligodendrocytes in culture also make BP. While GC and sulfatide were diffusely distributed on the surface of cultured oligodendrocytes, BP could only be visualized in fixed cells and was concentrated in the cell body. Our failure to detect BP on the surface of living oligodendrocytes is consistent with previous studies which have failed to detect BP on the surface of intact central myelin (11, 23, 35); together, these studies suggest that BP is confined to the cytoplasmic side of the oligodendrocyte and myelin membrane.

Unlike Schwann cells, oligodendrocytes isolated from neonatal optic nerve, corpus callosum, or cerebellum continued to make GC, sulfatide, and BP for up to 10 wk in vitro, even when tetanus toxin$^+$ neurons were not present in the cultures. This difference between Schwann cells and oligodendrocytes was maintained in mixed cultures of sciatic and optic nerves, suggesting that it is intrinsic to the myelin-forming cells themselves and not related to differences in other cells in peripheral and central cultures. Clearly, oligodendrocytes do not require a continuing signal from neurons to continue synthesizing myelin-specific molecules in culture. Although myelination does not begin in the optic nerve until 6 d after birth (28), oligodendrocytes in dissociated cell cultures of newborn optic nerve were labeled with anti-GC and anti-sulfatide antibodies after 1 d in culture and with anti-BP antibodies after 6–10 d in culture. If oligodendrocytes require a signal from axons to begin making myelin glycolipids and proteins, the signal must be given many days before myelination commences. Recently, Sternberger et al. (30) have convincingly visualized BP in oligodendrocytes before morphological myelin sheath formation using immunoperoxidase labeling of neonatal rat brain sections.

Schwann cells and oligodendrocytes had very different morphologies in culture. While Schwann cells were usually bipolar, most oligodendrocytes had a variable number of long, branching processes, some of which occasionally ended in large membranous expansions. These different morphologies mimicked the different myelinating behaviour of these two cell types in vivo, where each Schwann cell myelinates only one axon (32), while each oligodendrocyte normally myelinates many different axons (6, 29). These characteristic morphologies were seen even in the absence of neurons. They were also seen when Schwann cells were cultured from 16-d embryonic sciatic nerve and when oligodendrocytes were cultured from newborn optic nerve, days before myelination would normally begin in these tissues. Moreover, these morphological differences between Schwann cells and oligodendrocytes were maintained in mixed cultures of sciatic nerve and optic nerve or when the cells were grown on a monolayer of 3T3 cells (unpublished observations). This strongly suggests that these different morphologies and myelinating characteristics are intrinsic properties of these two different cell types.

There is one practical implication of our findings. The fact that rat Schwann cells stop making detectable amounts of myelin-specific glycolipids and proteins in culture means that these molecules can serve as convenient immunological markers for studying Schwann cell myelination in vitro, while this is not the case for studies of oligodendrocytes myelination.

We are grateful to Drs. J. Brockes, K. L. Fields, S. Hakomori, R. Lisak, and D. McFarlin for kindly providing antisera.

E. Abney was supported by a Medical Research Council Project grant to M. C. Raff and N. A. Mitchison, R. Mirsky by a fellowship from Action Research for the Crippled Child, and R. M. Pruss by a fellowship from the Jane Coffin Childs Memorial Fund for Medical Research.

Received for publication 17 April 1979, and in revised form 18 September 1979.

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 autoradiographic study. *J. Neurocytol.* 5:565–573.
2. BIGNAMI, A., L. F. ENG, D. DAHL, and C. T. UYEDA. 1972. Localization

of the glial fibrillary acidic protein in astrocytes by immunofluorescence. *Brain Res.* **43**:429–435.

3. BROCKES, J. P., K. L. FIELDS, and M. C. RAFF. 1977. A surface antigenic marker for rat Schwann cells. *Nature (Lond.).* **266**:364–366.
4. BROCKES, J. P., K. L. FIELDS, and M. C. RAFF. 1979. Studies on cultured rat Schwann cells. I. Establishment of purified populations from cultures of peripheral nerve. *Brain Res.* **165**:105–118.
5. BROCKES, J. P., M. C. RAFF, J. NICHIGUCHI, and J. WINTER. 1979. Studies on cultured rat Schwann cells. II. Assays for peripheral myelin proteins. *J. Neurocytol.* In press.
6. BUNGE, R. P. 1968. Glial cells and the central myelin sheath. *Physiol. Rev.* **48**:197–251.
7. FIELDS, K. L., C. GOSLING, M. MEGSON, and P. L. STERN. 1975. New cell surface antigens in rat defined by tumours of the nervous system. *Proc. Natl. Acad. Sci. U. S. A.* **72**:1286–1300.
8. FIELDS, K. L., J. P. BROCKES, R. MIRSKY, and L. M. B. WENDON. 1978. Cell surface markers for distinguishing different types of rat dorsal root ganglion cells in culture. *Cell.* **14**:43–51.
9. GEREN, B. B. 1954. The formation from the Schwann cell surface of myelin in the peripheral nerves of chick embryos. *Exp. Cell Res.* **7**:558–562.
10. HAKOMORI, S. 1974. Preparation and properties of anti-sulfatide serum. *J. Immunol.* **112**:424–426.
11. HERNDON, R. M., H. C. RAUCH, and E. R. EINSTEIN. 1973. Immunoelectron microscopic localization of the encephalitogenic basic protein in myelin. *Immunol. Commun.* **2**:163–172.
12. LISAK, R. P., G. A. FALK, R. G. HEINZE, M. W. KIES, and E. C. ALVORD. 1970. Dissociation of antibody production from disease suppression in the inhibition of allergic encephalomyelitis by myelin basic protein. *J. Immunol.* **104**:1435–1447.
13. LISAK, R. P., R. G. HEINZE, and M. W. KIES. 1970. Relationship between antibodies and experimental allergic encephalomyelitis. *Int. Arch. Allergy Appl. Immunol.* **37**:621–629.
14. LISAK, R. P., O. ABRAMSKY, S. H. DORFMAN, J. GEORGE, M. C. MANNING, D. E. PLEASURE, T. SAIDA, and D. H. SILBERBERG. 1979. Antibodies to galactocerebroside bind to oligodendroglia in suspension culture. *J. Neurol. Sci.* **40**:65–73.
15. MATHEWS, M. A. 1968. An electron microscopic study of the relationship between axon diameter and the initiation of myelin production in the peripheral nervous system. *Anat. Rec.* **161**:337–351
16. MATURANA, H. R. 1960. The fine anatomy of the optic nerve of Anurans—an electron microscopic study. *J. Biophys. Biochem. Cytol.* **7**:107–120.
17. MCDERMOTT, J. R., and H. M. WISNIEWSKI. 1977. Studies on the myelin protein changes and antigenic properties of rabbit sciatic nerves undergoing Wallerian degeneration. *J. Neurol. Sci.* **33**:81–94.
18. MENDELL, J. R., and J. N. WHITTAKER. 1978. Immunochemical localization studies of myelin basic protein. *J. Cell Biol.* **76**:502–511.
19. MIRSKY, R., L. M. B. WENDON, P. BLACK, C. STOLKIN, and D. BRAY. 1978. Tetanus toxin: a cell surface marker for neurons in culture. *Brain Res.* **148**:251–259.
20. NORTON, W. T., and L. A. AUTILIO. 1966. The lipid composition of purified bovine brain myelin. *J. Neurochem.* **13**:213–222.
21. PETERS, A. 1960. The formation and structure of myelin sheaths in the central nervous system. *J. Biophys. Biochem. Cytol.* **8**:431–446.
22. PETERSON, E. R., and M. R. MURRAY. 1955. Myelin sheath formation in cultures of avian spinal ganglia. *Am. J. Anat.* **96**:319–355.
23. PODUSLO, J. F., and P. E. BRAUN. 1975. Topographical arrangement of membrane proteins in intact myelin sheaths. *J. Biol. Chem.* **250**:1099–1105.
24. PODUSLO, S. E., and G. M. MCKHANN. 1977. Synthesis of cerebrosides by intact oligodendroglia maintained in culture. *Neurosci. Lett.* **5**:159–163.
25. RAFF, M. C., E. ABNEY, J. P. BROCKES, and A. HORNBY-SMITH. 1978. Schwann cell growth factors. *Cell.* **15**:813–822.
26. RAFF, M. C., R. MIRSKY, K. L. FIELDS, R. P. LISAK, S. H. DORFMAN, D. H. SILBERBERG, N. A. GREGSON, S. LIEBOWITZ, and M. KENNEDY. 1978. Galactocerebroside: a specific cell surface antigenic marker for oligodendrocytes in culture. *Nature (Lond.).* **274**:813–816.
27. RAFF, M. C., K. L. FIELDS, S. HAKOMORI, R. MIRSKY, R. M. PRUSS, and J. WINTER. 1979. Cell type-specific markers for distinguishing and studying neurons and the major classes of glial cells in culture. *Brain Res.* **174**:283–308.
28. SKOFF, R. P., D. L. PRICE, and A. STOCKS. 1976. Electron microscopic autoradiographic studies of gliogenesis in rat optic nerve. II. Time of origin. *J. Comp. Neurol.* **169**:313–333.
29. STERNBERGER, N. H., Y. ITOYAMA, M. W. KIES, and H. DE F. WEBSTER. 1978. Immunochemical method to identify basic protein in myelin-forming oligodendrocytes of newborn rat C. N. S. *J. Neurocytol.* **7**:251–263.
30. STERNBERGER, N. H., Y. ITOYAMA, M. W. KIES, and H. DE F. WEBSTER. 1978. Myelin basic protein demonstrated immunochemically in oligodendroglia prior to myelin sheath formation. *Proc. Natl. Acad. Sci. U. S. A.* **75**:2521–2524.
31. WEBSTER, H. DE F. 1971. The geometry of peripheral myelin sheaths during their formation and growth in rat sciatic nerves. *J. Cell Biol.* **48**:348–367.
32. WEBSTER, H. DE F. 1975. Development of peripheral myelinated and unmyelinated nerve fibres. *In* Peripheral Neuropathy. P. J. Dyck, P. K. Thomas, and E. H. Lambert, editors. Saunders, Philadelphia.
33. WEINBERG, H., and P. S. SPENCER. 1976. Studies on the control of myelinogenesis. II. Evidence for neuronal regulation of myelination. *Brain Res.* **113**:363–378.
34. WOOD, J. G., and R. M. C. DAWSON. 1974. Some properties of a major structural glycoprotein of sciatic nerve. *J. Neurochem.* **22**:627–630.
35. WOOD, D. D., R. M. EPAND, and M. A. MOSCARELLO. 1977. Localization of the basic protein and lipophilin in the myelin membrane with a non-penetrating reagent. *Biochim. Biophys. Acta.* **467**:120–129.