# MYELINOGENESIS IN OPTIC NERVE

## A Morphological, Autoradiographic, and Biochemical Analysis

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ABSTRACT

Morphological, autoradiographic, and biochemical methods were used to study the time of appearance, distribution, and nature of sulfated constituents in the developing rat optic nerve. Electron microscope studies showed that myelination begins (6 days postnatal) shortly after the appearance of oligodendroglia (5 days postnatal). Over the ensuing 3 wk, myelination increased rapidly. During the 1st postnatal wk, mucopolysaccharides and glycoproteins were labeled with $^{35}S$ and autoradiographs showed grains over arachnoidal cells, astroglia, and the glia limitans. These results indicated that astroglia synthesize sulfated mucopolysaccharides of the glia limitans. After the onset of myelination, however, the major portion of [$^{35}S$]sulfate was incorporated into sulfatide. Autoradiographs showed a shift of radioactive grains from astroglia and arachnoidal cells to myelin, indicating that actively myelinating oligodendroglia incorporate [$^{35}S$]sulfate into myelin sulfatide; there was a concomitant increase in the activity of cerebroside sulfotransferase. In addition, the increasing amounts of proteolipid protein and myelin basic protein corresponded with the morphological appearance of myelin.

These results point to a strict correlation between the structural and biochemical changes occurring during myelination. This system provides a useful model for studies designed to evaluate the effects of various perturbations on the process of myelination.

The process of myelination in the central nervous system (CNS) has been studied morphologically (50, 51, 53) or biochemically (32, 33, 37), but in only one study have the two approaches been combined (25). Morphological investigations can define the onset and, to some extent, estimate the rate of myelination in specific single pathways. However, biochemical studies have usually been done on CNS tissue containing several asynchronously myelinating systems, so that it has been difficult to relate biochemical findings with morphological observations. We have studied the development of rat optic nerve, a system in which there is a single anatomical pathway being myelinated. This investigation is part of a larger body of work designed to define parameters of gliogenesis (50, 51) and myelinogenesis in a specific myelinating system.

In the study of gliogenesis in rat optic nerve (50, 51), [$^{3}H$]thymidine autoradiography showed that astroglia and oligodendroglia are derived from astroblasts and oligodendroblasts, respectively, and not from undifferentiated glioblasts. Astrocytes are formed throughout late embryonic and postna-

tal development, while oligodendroglia begin their final division at 5 days postnatal, i.e., a day or two before the onset of myelination (50, 51).

Using biochemical and high resolution autoradiographic methods, the present study focuses on the changes in the pattern of incorporation and the cellular distribution of sulfate before and after the onset of myelination. At 2 days postnatal, sulfate is incorporated into glycoproteins and mucopolysaccharides, whereas at 12 and 16 days the $^{35}S$ appears predominantly in sulfatide. High resolution autoradiography shows a corresponding change in grain density from arachnoidal elements and glia limitans to myelin sheaths. These findings are related to changes in the activity of cerebroside sulfotransferase and the amounts of proteolipid protein and myelin basic protein.

The purpose of these studies is twofold: first, to provide quantitative data on both gliogenesis and myelination in a single system; second, to establish a framework which can be used to study pathological processes in which myelination is affected (24).

## MATERIALS AND METHODS

Sprague-Dawley rats (Charles River Farms, Boston, Mass.) were obtained between 7 and 10 days postnatal. For pups of younger ages, 13-day gestation mothers were obtained and, on the day of delivery, each litter was reduced to 10 pups. The adult animals were fed ad lib. on a standard laboratory rodent diet.

[$^{35}S$]Sodium sulfate ($Na_2SO_4$) and [$^{35}S$]phosphadenosine phosphosulfate (PAPS) were obtained from New England Nuclear (Boston, Mass.). Imidazole was obtained from Calbiochem (San Diego, Calif.); galactocerebroside from Supelco, Inc. (Bellefonte, Pa.); and Triton X-100 from Schwarz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). All solvents were reagent grade.

### *Histology and Autoradiography*

For microscope examination, specimens were fixed by immersion or by intra-aortic perfusion with the following fixative solution: 3% glutaraldehyde, 1.5% formaldehyde, 0.6% acrolein, 0.6% dimethyl sulfoxide, and 0.001% calcium chloride in 0.1 M cacodylate buffer (pH 7.3). Animals were sacrificed 48 h after injection of isotope. After overnight fixation at 4°C, the intracranial portion of the optic nerve was removed, and rinsed in 0.1 M cacodylate buffer (pH 7.3) with 10% sucrose. The specimens were postfixed in 2% solution of buffered osmium tetroxide for 90 min at room temperature, dehydrated in a graded series of ethanols, and embedded in Epon 812. Sections for light microscopy were cut at 1 $\mu$m and stained with toluidine blue; thin sections were stained with uranyl acetate and lead citrate.

For autoradiography, thick (1.5 $\mu$m) sections were dipped in Kodak NTB-2; thin sections (pale gold) were coated with Ilford L4 emulsion (Ilford Ltd., Ilford, Essex, England) (purple interference color) which was maintained at 40°C and diluted 1:5 with water. Exposure took place in light-tight boxes at 4°C for 10–80 days. Light and electron microscope autoradiographs were developed in Kodak D19, stained with toluidine blue or uranyl acetate-lead citrate, respectively, and examined in a Zeiss photomicroscope (Carl Zeiss, Inc., New York) or an AEI 801 electron microscope.

### *Analysis*

The percentage of myelinating fibers at different postnatal stages was obtained by counting the number of myelinated and unmyelinated axons in electron micrographs (× 23,500). At 5 days of age, a total of 249 axons were counted; at 7 days, 2,232; at 10 days, 1,683; at 14 days, 389; at 21 days, 1,379; and at 28 days, 588. The same electron micrographs were used to document the number of myelin lamellae per axonal diameter. The axonal diameters were determined on micrographs of 28-day optic nerves. Axons that appear as circular profiles in cross sections were selected, and the mean of the longest and shortest diameters was estimated.

The distribution of $^{35}$-labeled materials in animals sacrificed at 2, 10, and 14 days postnatal was determined by a grain density analysis of electron microscope autoradiograph (22, 26, 46, 47). Each grain was localized to one of five anatomic compartments (axons, myelin, glial cells, blood vessels, and subarachnoid space including glia limitans) by placing a transparent plastic mask with a series of concentric radii over the developed grain. The center of the smallest circle that could circumscribe the grain was punched through the micrograph and considered the grain location. Since the half distance for $^{35}S$ in the high resolution autoradiograph is 230 nm (46), a circle of 230 nm was drawn around each grain center. The grain was considered compartment associated if the major part of the circle was within that compartment (36, 46). Using autoradiographs printed at a magnification of 10,000, the anatomic compartments (axons, myelin, glial cells, blood vessels, and subarachnoid space, including glia limitans) were cut out and weighed. The total unit area analyzed was 5351.77 $\mu m^2$ at 2 days, 8596.8 $\mu m^2$ at 10 days, and 3677.38 $\mu m^2$ at 14 days. Grain densities expressed as grains/1.0 $\mu m^2$ were obtained by dividing the number of grains within a compartment by the weight of that compartment (Table II).

### *Biochemical Studies*

WET AND DRY WEIGHTS: 10 optic nerves were removed and placed in a few drops of phosphate-buffered saline on a glass slide. The slide with the optic nerves was weighed. After removal of the optic nerves the slide was reweighed and thus a wet weight determination made. The optic nerves were then homogenized in chloroform:methanol (1:1, vol/vol). The precipitate was dried and weighed. The chloroform:methanol-solu-

ble material was partitioned and the lower phase was dried and weighed. By the addition of the lower phase weight to the weight of the precipitate, a dry weight determination was made.

LIPID-SOLUBLE PROTEINS: The amount of proteolipid protein present in the optic nerve was measured by the method of Lowry et al. (27) on the lower phase after partitioning.

PHOSPHOLIPID PHOSPHORUS: Phosphorus was assayed on aliquots of the lower phase by the method of Fiske and SubbaRow (10).

$^{35}$S-LABELING IN VIVO: The [$^{35}$S]$Na_2SO_4$ was given intracranially, using a precalibrated Hamilton syringe. The injections were made to the right of the midline and anterior and medial to the ear. The needle penetrated the intracranial space, and upon injection there was minimal leakage from the site of the injection. Each animal was given 500 $\mu$Ci of the isotope. 48 h later, the rats were sacrificed by decapitation. The amount of [$^{35}$S]$Na_2SO_4$ was standardized according to the wet weight of the brain. The intracranial portion of the optic nerve was removed and a wet weight obtained. The nerves were then homogenized in 0.9% sodium chloride (pH 7.3) and aliquots were taken for protein determination. Subsequently, 1.0 ml of 10% label-free carrier brain homogenate was added to the optic nerve homogenate and mixed with 12 vol of chloroform:methanol (1:1, vol/vol). After overnight extraction, the mixture was centrifuged at 1,400 rpm for 10 min. The supernate was then adjusted to 2:1 chloroform:methanol (vol/vol) and, after 1 h, it was partitioned with 0.2 vol of 0.45% $K_2SO_4$. The emulsion was clarified by centrifugation and the upper phase was removed. The lower phase was then washed four more times with theoretical upper phase containing 0.75% KCl (15, 37). The lipid extract was dried under nitrogen and the sample counted with 10.0 ml of scintillation fluid (toluene ± 2,5-diphenyloxazole [PPO]). The identity of the compounds in the lower phase was determined by thin-layer chromatography on Analtech G plates (Analtech Inc., Newark, Del.) activated at 110° for 1 h and then cooled in a desiccator. The samples were spotted with carrier sulfatide (40 $\mu$g) and the plates were run in a neutral solvent system (chloroform:methanol:water, 70:30:4).

DETERMINATION OF [$^{35}$S]MUCOPOLYSACCHARIDES AND GLYCOPROTEINS: The optic nerves of animals given [$^{35}$S]$Na_2SO_4$ were analyzed for glycoproteins and mucopolysaccharides (30). Label-free carrier brain homogenate was added to optic nerve homogenate and then extracted first with chloroform: methanol (2:1, vol/vol) and then with chloroform: methanol (1:2, vol/vol). The lipid-free residue was dried in a vacuum, suspended at a concentration of 2% in 0.2 M boric acid-borax buffer (pH 7.8) containing 0.005 M calcium chloride, and digested with pronase (1 mg/ml) for a total of 72 h at 55°C. Cold TCA at a concentration of 10% was added to the digest. The supernatant solution was neutralized and then dialyzed for 4 days, initially against tap water, followed by deionized water. The retained material was lyophilized and then made up in 0.04 M sodium chloride. Mucopolysaccharides were precipitated with 10% cetylpyridinium chloride, leaving a supernatant solution containing glycopeptides. Aliquots of the supernatant solution were counted and the precipitate of mucopolysaccharides was dissolved in 2.5 M NaCl:methanol (2:1, vol/vol) and again aliquots were taken and counted.

MEASUREMENT OF CEREBROSIDE SULFOTRANSFERASE (EC 2.8.2.1): Optic nerves from 10 animals were homogenized in 0.9% NaCl. The assay, previously used in our laboratory (12), was scaled down for small quantities of protein. The final reaction volume was 100 $\mu$l containing 0.1 M imidazole-HCl buffer (pH 7.0) with 0.02 M magnesium chloride. Galactocerebroside acceptor was prepared by homogenizing and then sonicating 8 mg of cerebroside with 0.1% Triton X-100 in 10 ml of water. To each assay 40 $\mu$l of buffer, 8 $\mu$l of galactocerebroside, 2 $\mu$l of [$^{35}$S]PAPS, 10 $\mu$l of ATP (2.5 mM), and 40 $\mu$l of enzyme source were added. The mixture was incubated at 37°C for 15 and 30 min. The reaction was stopped with chloroform:methanol (1:1, vol/vol) and lipids were extracted (15, 37). The counts in the washed lipid-soluble phase represent sulfatide.

RADIOIMMUNOASSAY FOR MYELIN BASIC PROTEIN: For each assay, the optic nerve from one rat was homogenized in 0.5 ml of 0.2 M Tris-acetate (pH 7.3). Aliquots were taken for protein determination and for the radioimmunoassay for myelin basic protein (7). All proteins were assayed by the method of Lowry et al. (27).

## RESULTS

### *Morphology*

At 2 days postnatal the neuroglial elements of the optic nerve consist of undifferentiated cells, astroblasts, and astrocytes (51, 53). Oligodendrocytes are not present. The axons are small (0.1–0.2 $\mu$m) and not myelinated (Fig. 1 *a*). The astroglial processes tend to be arranged radially and divide the optic axons into bundles. Some of these processes terminate as expansions around blood vessels; these end-feet are separated from capillary endothelial cells by the basal lamina. At the pial surface, astrocytic processes give rise to the glia limitans which separates the neural elements from the subarachnoid space.

During the latter part of the 1st postnatal wk, the unmyelinated axons increased in size (0.2–0.3 $\mu$m) and it is at this time that oligodendroglia can first be recognized (50, 51). Extending among the axons, oligodendroglia processes became applied to the surface of the axons of larger diameter. Lamellae of myelin were first visible at 6 days

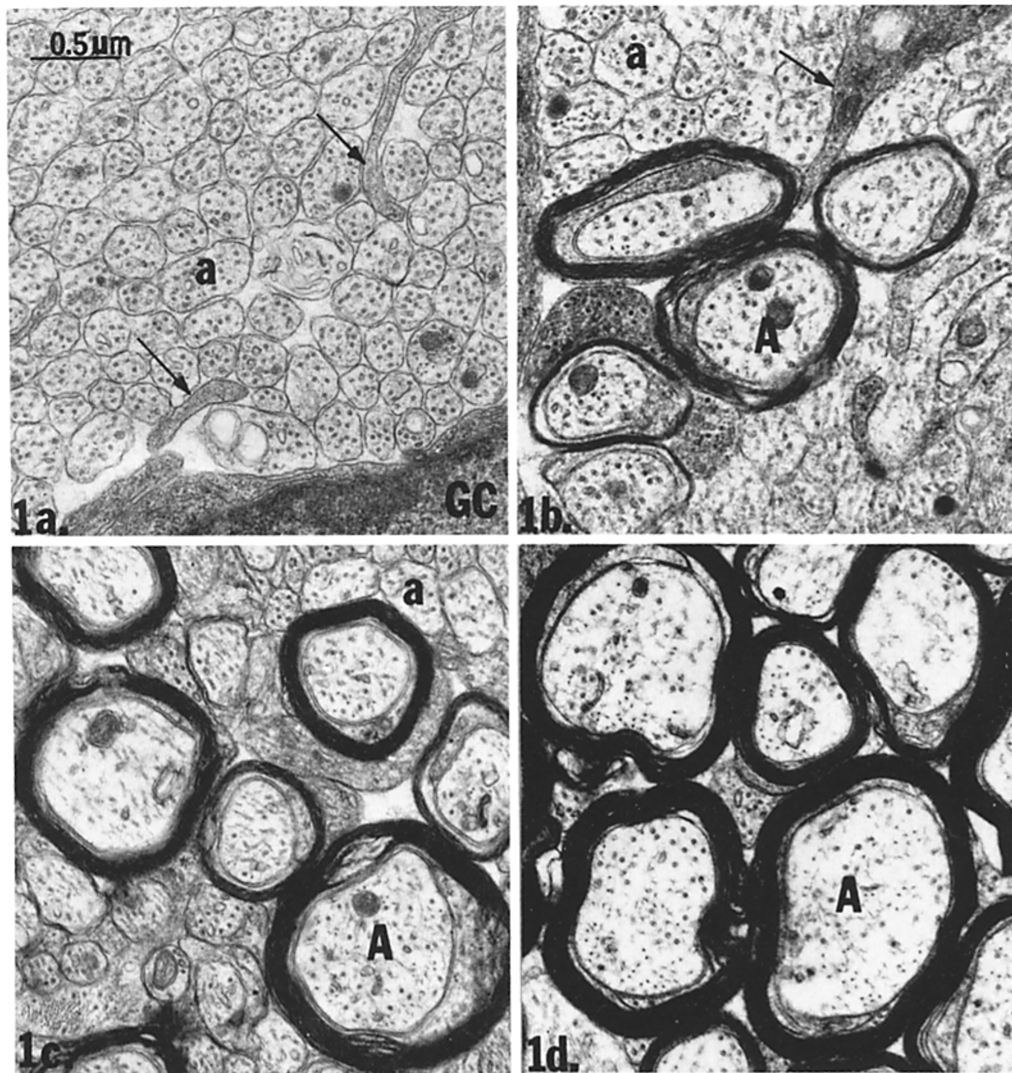


FIGURE 1 Electron micrographs of rat optic nerve at four postnatal time intervals. (a) 2 days. Optic axons (*a*) are small and not myelinated. They contain microtubules and occasional mitochondria. Note glial cell (*GC*) and processes (arrows). (b) 10 days. Largest axons (*A*) show various degrees of myelination. Unmyelinated axons, (*a*). Note oligodendroglial processes (arrows). (c) 14 days. The majority of axons are not myelinated (*a*), but several large axons (*A*) are surrounded by well-compacted myelin sheaths. (d) 28 days. Large axons (*A*), containing microtubules and neurofilaments, show compact myelin sheaths. 85% of axons are myelinated at this stage. Bar, 0.5 $\mu$m. All magnifications × 25,000.

postnatal. The axons with the largest diameter (0.4 $\mu$m) were the first to be ensheathed, usually showing one to three turns of loosely wrapped myelin membrane. By the 10th postnatal day, compact myelin was evident around some axons (Fig. 1 *b*); at this time, approximately 15% of he axons were in the process of being myelinated (Fig. 2). At 15 days postnatal, about 30% of the axons are myelinated. It is at this time that both $^{35}$S incorporation in sulfatide and the activity of cerebroside sulfotransferase reached a peak. Furthermore, the rate of increase of dry weight, myelin basic protein, and proteolipid protein was maximal. Thus, this probably represents the period of maximal myelination as can be determined by biochemical data. Over the ensuing weeks (Fig. 1

*c* and *d*), myelination increased rapidly so that by 21 days, 50% of axons were myelinated. By the 28th postnatal day (Fig. 1 *d*), 85% of the axons were myelinated. At this stage, axon diameters showed a broad spectrum, with some fibers reaching diameters of up to 1.3 μm; in addition, some of the small axons (0.2–0.3 μm) were now being myelinated. There was a linear correlation between the axon diameter and the number of myelin lamelle (Fig. 3).

## *Sulfate Incorporation In Vivo*

48 h after injection of [$^{35}$S]sodium sulfate (500 μCi/animal), only a small percentage (0.01%) of the counts were incorporated into the optic nerve. The distribution of $^{35}$S-labeled compounds was significantly different at 2 and 12 days postnatal.

At 2 days postnatal, 96% of the $^{35}$S-labeled compounds were sulfated mucopolysaccharides and glycoproteins, while only 4% of the counts

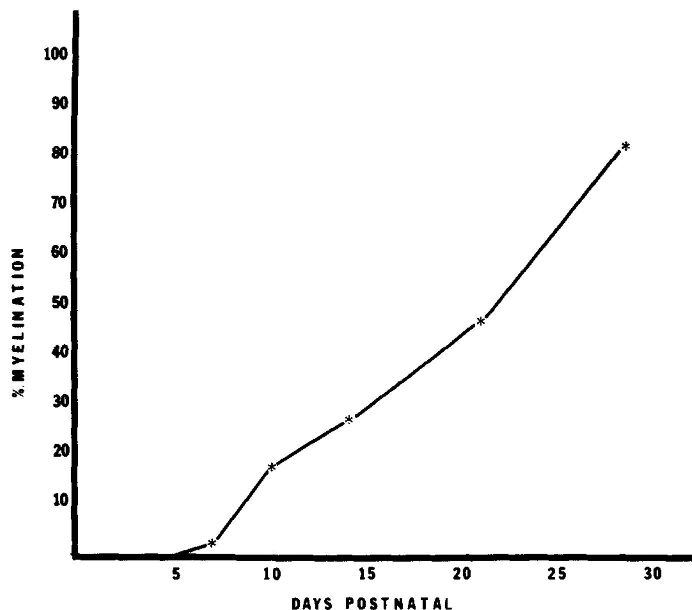


FIGURE 2 Quantitative analysis of the number of myelinated axons as a function of age in rat optic nerve. The experimental details are described in the text.

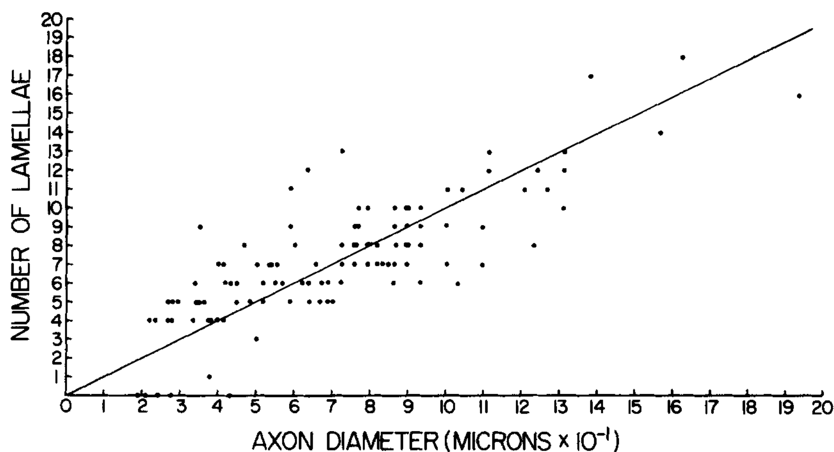


FIGURE 3 Correlation of axon diameter with the number of myelin lamellae in rat optic nerves (28 days postnatal). The correlation coefficient for the linear plot was 0.76.

were found in sulfatide (Table I). The sulfated compounds presumably reflect in part the biosynthetic activity of astroglia, which are the only differentiated glial cells present at this time.

The incorporation of $^{35}S$ into sulfatide starts on the 6th postnatal day when myelin first appears (Fig. 4). At 12 days postnatal, 84% of the counts were in sulfatide and 16% in sulfated mucopolysaccharides and glycoproteins (Table I). Incorporation into sulfatide reached a peak about the 16th postnatal day, after which the incorporation rate dropped, paralleling decreasing activity with the sulfotransferase.

### *Loss of Labeled Compounds during Tissue Processing*

During the process of fixation for electron microscopy, there was no significant loss of lipid labeled with $^{35}S$. However, 33% of the total counts were lost during the steps of dehydration. The major portion (62%) of the counts lost during gradual dehydration partitioned in the lower phase of a chloroform:methanol extraction, suggesting that these counts were in sulfatide. Optic nerves processed as for electron microscopy were then analyzed for the distribution of $^{35}S$-labeled compounds (Table I).

### *Autoradiography of $^{35}S$-Compounds*

Since the percentage of $^{35}S$ label in specific compounds differed at 2, 10, and 14 days postnatal, light and electron microscope autoradiography was used to localize label to specific tissue components (Table II) at these times. There was no evidence of latent image fading or positive chemography.

At 2 days postnatal, the grain density was greatest over the subarachnoid compartment, particularly the glia limitans (Table II, Fig. 5 *a*), whereas at 10 and 14 days postnatal the grain density was greatest over myelin (Table I, Fig. 5 *b* and *c*). On the basis of these observations and biochemical data, it is suggested that the label over the subarachnoid compartments and axons of 2-days-postnatal animals represents radioactivity in sulfated mucopolysaccharides and/or glycoproteins, whereas the radioactivity over myelin sheaths in animals at 10 and 14 days postnatal is predominantly sulfatide.

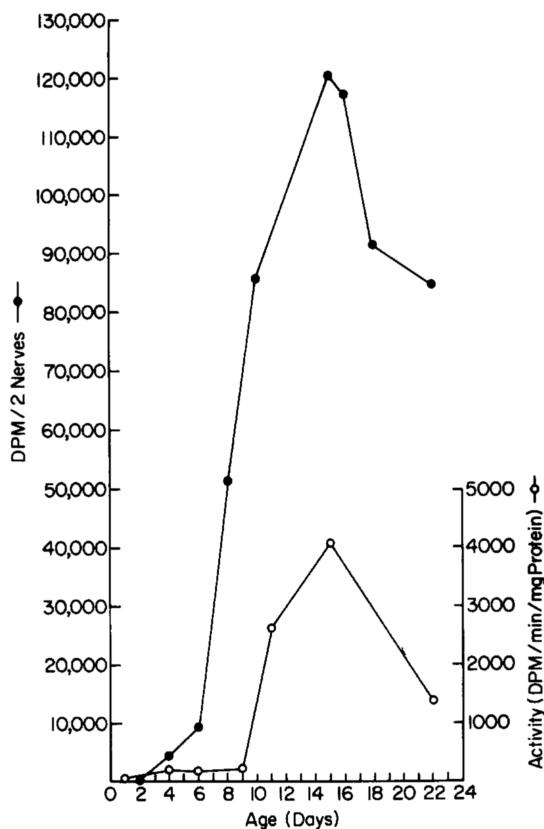


FIGURE 4 The incorporation of [$^{35}S$]sodium sulfate into sulfatide (●- - -●). The animals were injected intracranially with [$^{35}S$]sodium sulfate at varying ages and sacrificed 48 h later. The optic nerves were removed and sulfatide determined as described under Materials and Methods. The activity of cerebroside sulfotransferase (O- - -O) was assayed in optic nerves as a function of age.

TABLE I

*Percent of Counts in Sulfatide, Sulfated Glycoproteins, and Sulfated Mucopolysaccharides at Two Postnatal Ages*

| | | Counts (dpm)/nerve | |
|---|---|---|---|
| Labeled compounds | Expt. | 2 Days | 12 Days |
| Sulfatide | I | 260 (3%) | 22,200 (76%) |
| | II | 150 (8%) | 31,915 (80%) |
| | III | 70 (6%) | 12,810 (90%) |
| Sulfated glycoprotein and mucopolysaccharide | I | 7,750 (97%) | 6,000 (24%) |
| | II | 1,990 (92%) | 5,165 (14%) |
| | III | 1,130 (94%) | 1,490 (10%) |

The distribution of sulfated compounds in the rat optic nerve at two different ages. Animals were given 500 μCi of [$^{35}S$]sulfate intracranially and sacrificed 48 h later. The optic nerves were removed, fixed, and dehydrated. They were then analyzed for these sulfated compounds (see Materials and Methods).

TABLE II
*Percent Total Area and Grain Densities in Each Compartment at Three Different Ages*

| Anatomic compartment | 2 Days | | 10 Days | | 14 Days | |
|---|---|---|---|---|---|---|
| | Total area | Grains/$\mu m^2$ | Total area | Grains/$\mu m^2$ | Total area | Grains/$\mu m^2$ |
| | % | | % | | % | |
| Subarachnoid tissue (including glia limitans) | 26 | *0.05** | 7.6 | 0.08 | 1.1 | 0.02 |
| Glial cells | 32.5 | *0.02* | 38.4 | 0.02 | 31.4 | 0.004 |
| Axons | 31.3 | 0.009 | 45.6 | 0.006 | 45.7 | 0.004 |
| Myelin | – | – | 5.0 | *0.32* | 20.1 | *0.22* |
| Blood vessels | 10.2 | 0.006 | 3.3 | 0.02 | – | – |

* 73% of the total number of grains in this compartment was associated with the glia limitans. Electron microscope autoradiographs were obtained from optic nerves at different ages. Grains were then allocated to various compartments and counted. The area of each of these compartments was determined and grain density was calculated. The numbers in italics are the highest grain densities at the ages indicated.

### *Assay of Sulfotransferase Activity In Vitro*

The rate of incorporation of [$^{35}$S]sulfate into sulfatide during development may be altered by a variety of factors, such as availability of the delivered isotope, changes in the sulfate pool, and changes in the extracellular space. To supplement the data obtained in experiments in vivo, parallel studies of isotope incorporation were carried out in vitro. In this system, the transfer of [$^{35}$S]PAPS to galactocerebroside to form sulfatide is accomplished by the enzyme cerebroside sulfotransferase (Fig. 4). In the developing optic nerve, the activity of this enzyme appeared on the 9th postnatal day (197 dpm/min per mg protein) and reached a peak about the 15th postnatal day (4,100 dpm/min per mg protein). The activity of the enzyme was maximal during the period of most active myelinogenesis. Subsequently, the activity declined, but there was a continual low level of sulfatide synthesis. Thus, the developmental profile for the activity of the enzyme paralleled the $^{35}$S incorporation into sulfatide in vivo.

### *Wet and Dry Weight Estimations*

During development of the optic nerve, the rate for the changes of wet weight with age was maximal about the 10th postnatal day (Fig. 6), the time of maximal generation of oligodendroglia. The alterations in dry weight were maximal around the time the biochemical changes associated with myelination were at their peak (Fig. 6).

### *Myelin-Specific Proteins*

Myelin basic protein appeared at the 6th postnatal day (5 ng/$\mu$g protein) (Fig. 7), with a rapid increase in amount beginning on the 9th postnatal day. At the crest of the $^{35}$S incorporation into sulfatide, the quantity of basic protein present was 160 ng/$\mu$g protein. The curve reached a plateau at 210 ng of myelin basic protein per microgram of protein (21 $\mu$g of myelin basic protein per milligram of tissue) at the 4th postnatal wk. A similar curve was obtained for proteolipid protein. The rise started at the 10th postnatal day (500 $\mu$g of protein/nerve) with a plateau reached at the 4th postnatal wk (9.3 mg/nerve).

## DISCUSSION

The optic nerve of the rat provides an excellent model for combined ultrastructural and biochemical studies of gliogenesis (25, 50, 51, 53) and myelinogenesis. The maximum postnatal growth of the optic nerve occurred during the first 3 postnatal wk (32, 33). In this system, both the development of oligodendroglia and the formation of myelin were postnatal events (53). In the intracranial portion of the nerve, myelin was laid down synchronously and was of homogeneous composition. Since the myelin components of the adult optic nerve are identical to myelin constituents in other parts of the CNS (28), it should be possible to extrapolate data from this system to other pathways in white matter. Moreover, this approach can be used to analyze experimental models in which myelinogenesis is abnormal.

### *Glial Cells in Optic Nerve*

The optic nerve is free of neuronal cell bodies, and glial cell types can be readily identified (53), thus permitting studies of proliferation and differentiation of oligodendroglia (50, 51). The use of [$^3$H]thymidine for autoradiographic studies (50,

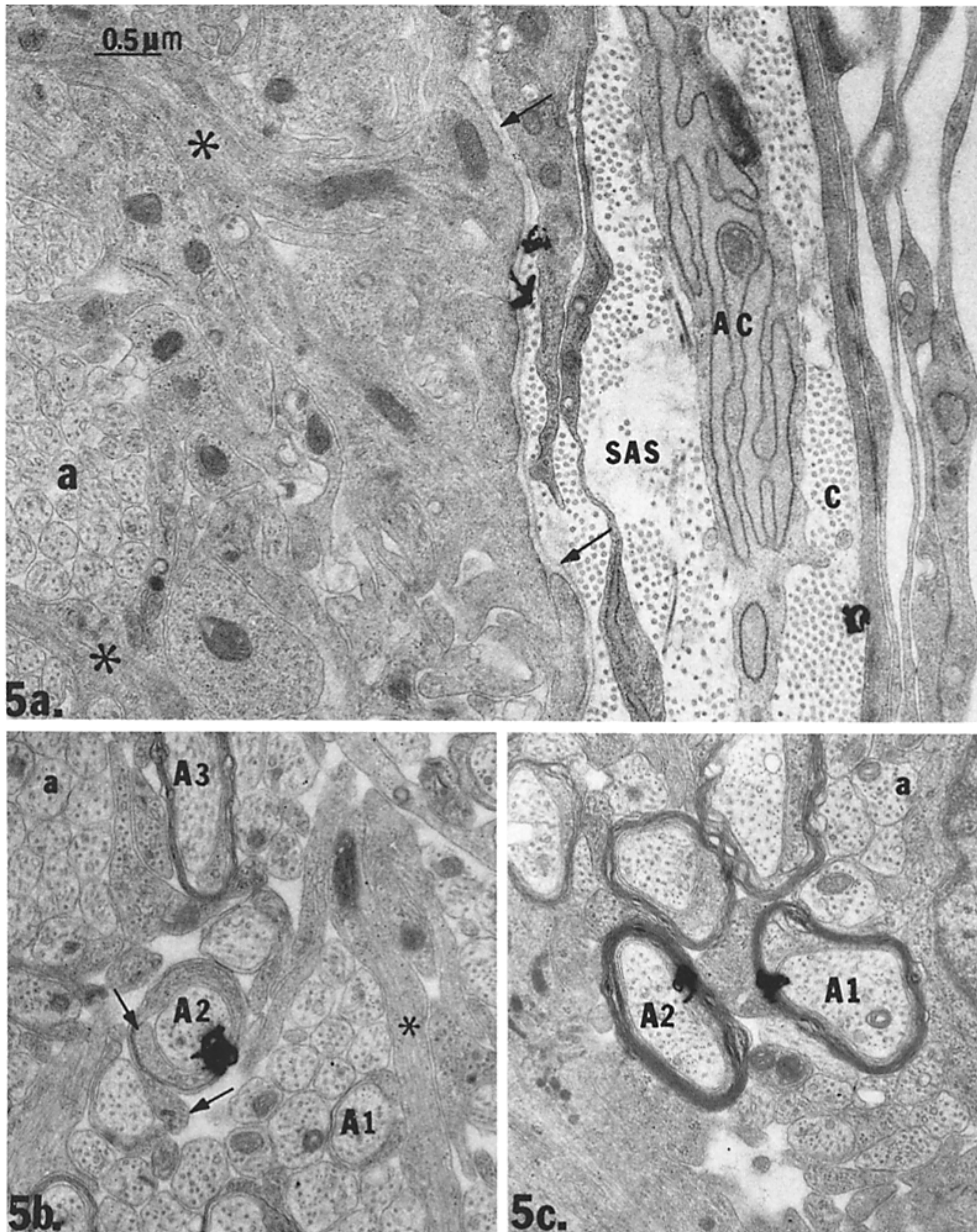


FIGURE 5 Electron microscope autoradiographs from animals injected with [$^{35}$S]sulfate 48 h before death. (a) 2 days postnatal. The optic nerve contains small unmyelinated axons (*a*) interspersed with glial cell processes (*). The basal lamina of the glia limitans (arrows) separates the optic nerve from the subarachnoid space (*SAS*). Two silver grains overlie the glia limitans. Note collagen (*C*) and arachnoidal cells (*AC*). × 18,500. (b) 10 days postnatal. Axons (*A*) show several stages of ensheathment: A1, oligodendroglial process partially surrounds axon; A2, myelin is loosely compacted; A3, compaction more advanced. A silver grain is present over a process ensheathing A2; the inner and outer mesaxons are indicated by arrows. An oligodendroglial process (*) contains microtubules. × 19,500. (c) 10 days postnatal. Several axons (*a*) are unmyelinated. Silver grains are present over myelin sheath surrounding A1 and A2. Bar, 0.5 µm. × 18,500.

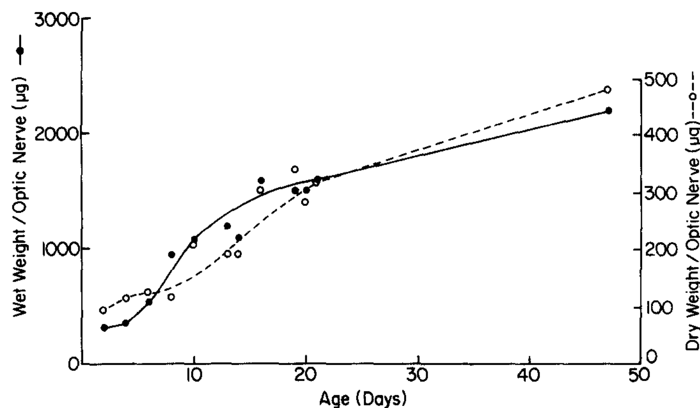


FIGURE 6 Changes in the dry (O- - -O) and wet (●- - -●) weight of the optic nerve as a function of age.

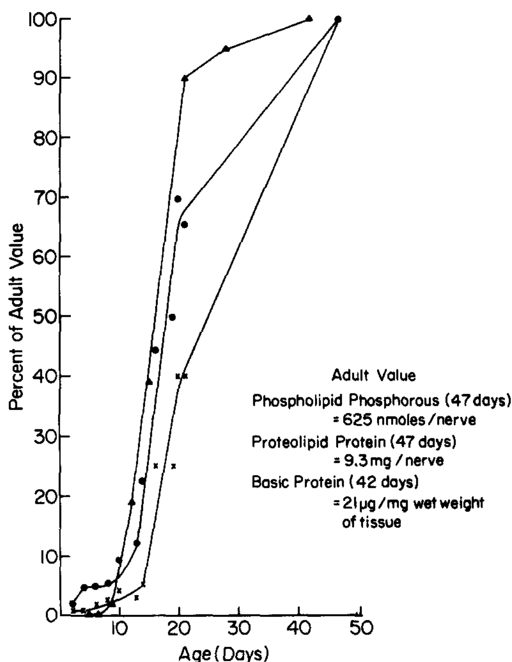


FIGURE 7 The developmental appearance of myelin basic protein (▲), proteolipid protein (●), and phospholipid phosphorus (x) in rat optic nerves. The values are expressed as a percentage of the value found in a 42- and 47-day-old animal. The assays are described under Materials and Methods.

51) of developing optic nerve showed that astroglia are formed throughout late embryonic and early postnatal development, while oligodendroglia are generated only during the postnatal period. Thus, at 2 days postnatal, astroglia represent 85% of the cells in the optic nerve. The processes of these cells form the framework for the ingrowing axons of the retinal ganglion cells. Expansions of these processes around blood vessels and at the pial surface are associated with the glia limitans which separates neural elements from both endothelial cells and the subarachnoid space.

Oligodendroglia begin to undergo their final cell division at 5 days of age (50, 51), 1–2 days before the onset of myelin formation. In the optic nerve, the great majority of these cells are produced after the onset of myelination with the peak period occurring between 10 and 14 days postnatal (50, 51). At 12 days, 75% of the labeled cells are oligodendrocytes and approximately 20–30% of the axons are myelinated. After the 2nd wk, the rate of production of oligodendrocytes declines. The number of myelinated axons continues to increase so that by the 4th postnatal wk, 85% of the axons are myelinated. By this time, mature oligodendrocytes constitute 60–70% of the total number of cells in the rat optic nerve (50, 51, 53). In adult rat optic nerve, each oligodendrocyte myelinated 30–50 internodes (41).

Through some as yet unknown mechanism, oligodendroglia are signaled to begin to make myelin during the latter part of the 1st postnatal wk. Previous studies of myelination in the CNS have shown that the first axons to be ensheathed are those with diameters measuring 0.2–0.7 $\mu$m (14, 20, 34, 42). At the onset of myelination in the rat optic nerve, the processes of the oligodendrocytes appear to wrap selectively about those axons with the largest diameters (mean 0.4 $\mu$m), an observation supporting the suggestion that axons must reach a certain diameter before they begin to be myelinated (34). However, once myelination is

well advanced, axons with smaller diameters become myelinated (see Results). In addition, there was a linear correlation between diameters of optic nerve axons and the number of lamellae of myelin, with the largest fibers having the greatest numbers of lamellae. Similar data have been obtained on myelin/axon ratios in both the peripheral nervous systems and other parts of the CNS (16–18)

### *Biochemical Components of Optic Nerve*

SULFATIDES AND SULFATED GLYCOPROTEINS: In the CNS, the important sulfated compounds include sulfatide which is enriched in myelin (39, 40), sulfated glycoproteins which are present in small quantities in myelin (35, 43), and sulfated mucopolysaccharides which appear to be associated with neurons (31) rather than myelin (52). Sulfate is donated to these compounds by PAPS (6, 45). The group transfer of sulfate to specific acceptor molecules, either cerebrosides (5), glycoproteins (29), or mucopolysaccharides (21), is catalyzed by specific enzymes. In the CNS, cerebroside sulfotransferase (5) is responsible for the transfer of sulfate from PAPS to galactocerebroside to form sulfatide. The rate-limiting factor in the synthesis of sulfatide appeared to be the activity of this enzyme rather than the availability of substrates for the reaction.

Our studies in developing optic nerves showed two peaks of [$^{35}$S]sulfate incorporation. The first peak (2 days postnatal) was associated with incorporation of [$^{35}$S]sulfate into glycoproteins and mucopolysaccharides. At the end of the 1st wk, $^{35}$S began to be incorporated into sulfatide. The incorporation of $^{35}$S into sulfatide was taken as a direct index of the synthesis of sulfatide (19, 49) and an indirect index of myelination both in vivo and in myelinating explants. At 16 days postnatal there was a second peak of $^{35}$S incorporation which corresponded to the maximal incorporation of label into sulfatide. Since sulfate was incorporated into different compounds at these ages, we were able to use autoradiography to localize the sulfated compounds to structural elements at these two developmental stages. In interpreting the autoradiographs, we recognized that some of the lipid label (i.e. [$^{35}$S]sulfate) was lost during processing. To make biochemical-structural correlations meaningful, chemical and autoradiographic studies were both performed on tissue already processed through graded ethanol steps.

AUTORADIOGRAPHIC LOCALIZATION OF LABEL: At 2 days postnatal, the basal lamina of the glia limitans showed a high grain density. This structure, a surface product of astroglia, was thought to be composed in part of sulfated mucopolysaccharides (52). Since astroglia are the major cell type in the first few postnatal days (50, 51), it is probable that these cells were active in the sulfation of mucopolysaccharides. Arachnoidal cells were presumably capable of carrying out similar sulfation reactions. The few grains within axons at 2 days postnatal are thought to be sulfated glycoproteins and/or mucopolysaccharides carried by anterograde axonal transport (4, 11). The time interval between injection and death was sufficient for rapidly transported axonal constituents to be present within the intracranial portion of a 2 days postnatal rat optic nerve (48). During the 2nd and 3rd wk, when myelinogenesis was most active, the electron microscope autoradiographs showed the grain density to be highest over myelin. At this time, [$^{35}$S]sulfate was in sulfatide and sulfated glycoproteins. Since the amount of sulfated glycoproteins in myelin is very low (35, 43), it is highly likely that the grains over myelin represent label in sulfatide.

The increase in rate of incorporation of [$^{35}$S]sulfate into sulfatide was paralleled by the appearance of differentiated oligodendrocytes (50, 51). It was likely that the cells became increasingly active in the synthesis of sulfatide during the most active phase of myelination. During this period, oligodendroglia show conspicuous Golgi complexes. It was of interest that autoradiographic studies (36, 56) of other systems showed that [$^{35}$S]sulfate label first appeared over the Golgi complexes, and that this organelle, isolated from kidney (13), contained sulfotransferase activity.

MYELIN PROTEINS: The amounts of myelin proteins change during development (1, 9, 57). Wolfgram protein and other high molecular weight proteins were the first to appear. Glycoproteins were also present early, but their time of sulfation has not been determined. Basic protein and proteolipid protein are predominantly, if not exclusively, in myelin or premyelin membranes (2, 23, 54). In rat optic nerve, myelin basic protein appeared a little earlier than proteolipid protein.

### *Myelinogenesis in Optic Nerve*

During the 1st postnatal wk, a population of glial cells in the optic nerve was stimulated to divide, with the result that oligodendrocytes were formed. The signal for the generation of these

cells is unknown, but it is likely that oligodendroglia enter their final cell division due to neuronal influences (3, 55) mediated via retinal cell axons. The processes of oligodendroglia extend among optic axons and during the initial phases of myelination they wrap loosely around the large axons. The early phase of myelin formation was associated with an increased synthesis of lipids including cholesterol, cerebroside, and sulfatide. It appears likely that the latter two compounds are synthesized in the Golgi complexes of oligodendroglia. The mechanism by which these lipids are transported from site of synthesis in the cell body to their final destination in myelin membrane is not clearly established. Although the role played by these lipids in myelin membranes is unknown, they appear to be very important and if they are not synthesized myelination will be delayed (44).

After the 10th postnatal day, there was a rapid increase in the amount of tightly compacted myelin. Basic protein and proteolipid protein appear at this time and it had been suggested that these macromolecules may be necessary for the compaction of myelin (8, 38). Our studies suggest that the appearance of myelin basic protein more closely correlates with compaction of myelin than does proteolipid protein.

The present investigation of myelinogenesis, in conjunction with the autoradiographic studies of gliogenesis (50, 51) in rat optic nerve, demonstrates some of the relationships which exist between the structural and biochemical events occurring during the development. Our intention was to provide normative data on gliogenesis and myelination which could be used to analyze the mechanisms by which pathologic processes interfere with myelinogenesis. This approach is presently being used to study the pathological changes in oligodendroglia and myelin in two experimental models: hypomyelination associated with malnutrition in infant rats, and remyelination occurring after virus-induced demyelination in mice (24). Our goal is to develop strategies which will allow us to ask specific questions concerning some of the pathogenetic mechanisms associated with disorders of myelin.

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