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The Differentiation of Oligodendrocytes in the Rat Optic Nerve

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The time of appearance and the rate of accumulation of specific myelin lipids and proteins were measured in the rat optic nerve during the period from birth to 18 days. The appearance of the activities of several enzymes involved in the synthesis of these lipids was also monitored. Correlation of these biochemical data with previously known morphological findings indicated that the "active" oligodendrocytes (detected between 5 and 15 days after birth) displayed maximal levels of synthesis of the components of myelin, and that these cells appeared to be responsible for the initial synthesis of myelin. Both young and mature oligodendrocytes showed limited capacity to synthesize these compounds. Furthermore, induction of the enzymes involved in the synthesis of myelin components appeared to take place in a simultaneous, rather than a sequential manner.

INTRODUCTION

Correlative morphological and biochemical studies can provide considerable insight into developmental mechanisms. We have previously examined the processes of gliogenesis and myelination in developing rat optic nerve by such an approach (Tennekoon *et al.*, 1977). Morphological, biochemical, and autoradiographic examination showed that the levels of protein components of myelin and enzymes associated with myelin, including myelin basic protein, proteolipid protein, 2′,3′-cyclic nucleotide phosphodiesterase, and ^{35}S incorporation into sulfolipids correlated with the morphological appearance of myelin in this system (Sprinkle *et al.*, 1978; Tennekoon *et al.*, 1977). These studies have now been extended to investigate differentiation in the oligodendrocyte by determining levels of glycolipids and the enzymes involved in their synthesis, and correlating these with known morphological changes in oligodendroglial cells during development. Furthermore, studies on the appearance of myelin-associated lipids and proteins provide information on the sequence of gene expression during differentiation.

The developing optic nerve of the rat was chosen for these studies for several reasons. In this system gliogenesis and myelination take place postnatally; the nerve is easily separated from adjacent central nervous system tissues; it is free of neuronal cell bodies; and myelination occurs synchronously. By studying the developing rat optic nerve we were able to show that a single cell, the glioblast (which is a multipotential cell), gives rise to cells that are "committed" to become either astrocytes or oligodendrocytes (Skoff *et al.*, 1976a,b). These can then divide to give rise to daughter astrocytes and oligodendrocytes, or they may differentiate to a more mature cell. During the process of differentiation three types of oligodendrocyte have been identified, based on morphological characteristics such as size, density of cytoplasm, and disposition of organelles (Mori and Leblond, 1970; Sturrock, 1974; Vaughn, 1969). These cells have been classified as "young" (the most immature), "active," and "mature" oligodendrocytes. Myelin starts to appear

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in the optic nerve at 6 to 7 days after birth and increases rapidly in amount until 28 days, when 80% of the axons are well myelinated.

The present studies were concerned with measuring the levels of glycolipids which are major constituents of myelin, and the enzymes involved in their synthesis, including those responsible for the formation of basic components (e.g., the formation of ceramide from sphingosine) and those involved in completing the complex molecule (e.g., cerebroside sulfotransferase). Our findings indicate that myelin is largely synthesized by active oligodendrocytes, and that all the enzyme activities can be detected at about the same time in this cell.

MATERIALS AND METHODS

ANIMALS

Sprague–Dawley rats (Charles River Farms, Boston, Mass.) were obtained between 7 and 10 days postnatal. For younger pups, mothers were obtained at Day 13 of their gestation and on delivery each litter was reduced to 10 pups. The adult animals were fed *ad libitum*.

For each estimation a whole litter (10 pups) was killed. The cranium of each animal was opened and the optic nerves were dissected as previously described. Prior to 6 days postnatal, a dissecting microscope was used to aid in the removal of the nerves. Unless otherwise stated, each assay (pooled material from 10 optic nerves) was performed in duplicate and repeated at least on two different litters.

DETERMINATIONS OF GLYCOLIPIDS

The following glycolipids were measured simultaneously by the use of high-performance liquid chromatography: glucocerebroside, galactocerebrosides (both HFA[1] and NFA), sulfatides (both HFA and NFA), and monogalactosyl diglyceride (Nonaka and Kishimoto, 1979). The lipids were extracted from the optic nerves by the method of Folch *et al.* (1957). The lower phase was evaporated to dryness under nitrogen and further dried in a desiccator over phosphorus pentoxide. The total dried lipids were heated with benzoyl chloride in pyridine (1:5, v/v) at 60°C for 1 hr. The reaction mixture was then dried under nitrogen and the residue was dissolved in 0.2 *M* perchloric acid in acetonitrile and *n*-hexanes to remove the sulfate moiety from sulfatide. The products were left for 2 hr at room temperature and the hexane extract was washed with 3.0% sodium bicarbonate, followed by two extractions with 1 ml each of an acetonitrile:water mixture (4:1, v/v). The final hexane extract was dried and analyzed by high-performance liquid chromatography. A stainless-steel tube (3 (i.d.) × 25 mm) packed with 5 μm Spherisorb silica was used. After the column was equilibrated with hexane–isopropanol (99.5:0.5), the sample was introduced and subjected to isocratic elution with hexane–isopropanol and then to gradient elution such that the isopropanol concentration changed from 0.5% initially to 10% at the end of the run. The flow rate was maintained at 1.2 ml/min, and the effluent was monitored at 230 nm with a spectromonitor. The peak areas were measured by the "cut and weigh" method. Figure 1 shows the separation obtained with the glycocerebrosides from an 18-day rat optic nerve. Glucocerebroside and galactocerebrosides were well separated from each other, and there was good separation among NFA and HFA galactocerebrosides, sulfatides, and monogalactosyl diglyceride.

ENZYME ASSAYS

Sphingosine acyltransferase (EC 2.3.1.24)

Sphingosine + acyl-CoA →

ceramide + CoA

This enzyme was assayed as described by Morell and Radin (1970). The incubation medium contained 0.25 mg of sphingosine,

[1] Abbreviations used: HFA, hydroxy fatty acid; NFA, nonhydroxy fatty acid; CNS, central nervous system.

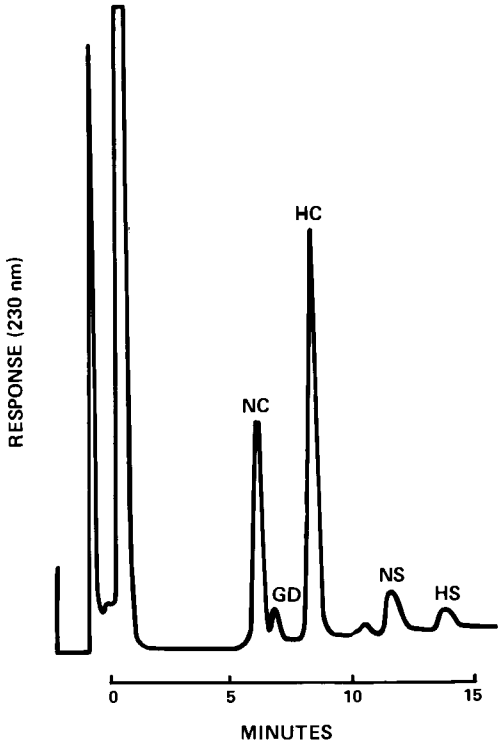

FIG. 1. The elution profile obtained by high-performance liquid chromatography (gradient elution with isopropranol (0.5 to 10%) with a flow rate of 1.2 ml/min). The effluent was monitored at 230 nm. The peaks identified are nonhydroxy fatty acid galactosylceramide (NC); monogalactosyl diglyceride (GD); hydroxy fatty acid galactosylceramide (HC); nonhydroxy fatty acid sulfatide (NS); and hydroxy fatty acid sulfatide (HS).

25 μmol of phosphate buffer, 0.5 μmol of dithiothreitol, 1 μmol of ATP, 0.2 ml of tissue (3–4 mg of protein), and 0.16 μmol of [^{14}C]lignoceroyl-CoA in a total volume of 0.5 ml. The amine was coated on 25 mg of Celite, the ATP was neutralized before use, and the buffer was added as a 0.5 *M* solution, pH 7.4. The mixture was incubated at 34°C for 10 min and the reaction was stopped by the addition of 8.5 ml of chloroform:methanol (2:1, v/v). After filtration to remove the Celite and protein, 2 ml of 2 *M* KCl were added. The layers were separated and the lower phase was collected and washed three times with theoretical upper phase. Carrier ceramide (0.1 mg) was added and the lower phase was dried under nitrogen. The lipids were separated by thin-layer chromatography, identified by exposure to iodine vapor, and the ceramide spot was scraped off. The counts per minute in lignoceroylceramide were determined by liquid scintillation counting.

Microsomal fatty acid elongation activity

Stearyl-CoA + malonyl-CoA →

very long-chain fatty acids

The method described by Bourre (Bourre *et al.*, 1975, 1973; Murad and Kishimoto, 1978) for determining the elongation of stearyl-CoA by malonyl-CoA was used. The assay mixture contained 50 μ*M* [1,3-^{14}C]-malonyl-CoA (2 mCi/mmol), 500 μ*M* NADPH, 0.05 m*M* stearyl-CoA, 0.08 *M* potassium phosphate buffer (pH 6.9), 0.32 *M* sucrose, 0.94% NaCl, 2 μg of phosphotransacetylase, and about 1 mg of protein. The final volume was 1.0 ml and the mixture was incubated at 37°C for 1 hr, after which the reaction was stopped by the addition of 0.5 ml of 4.5 *N* alcoholic KOH. The mixture was saponified for 15 min in a boiling water bath and then acidified with 0.5 ml of 5.5 *N* HCl. This mixture was extracted twice with petroleum ether and then dried under nitrogen. After methylation of the residue, fatty acid methyl esters were identified by thin-layer and gas chromatography.

UDPgalactose:Ceramide galactosyltransferase (EC 2.4.1.47)

Ceramide + UDPgalactose →

galactosylceramide + UDP

This enzyme was determined by the method of Costantino-Ceccarini (1973, 1975). The standard incubation medium contained, in a final volume of 0.13 ml, 75 μmol of Tris-HCl (pH 7.4), 0.3 μmol of EDTA (adjusted to pH 7), 0.3 μmol of $MgCl_2$, 12.5 μg of α-hydroxyceramide, 25 mg of Celite, and 250 μg of phosphatidylethanolamine. Each tube contained 200–300 μg of protein. The tubes were sonicated for 20 sec and then incubated at 37°C for 2 hr

with vigorous shaking. The reaction was stopped with chloroform–methanol (1:1, v/v), followed by a lipid extraction. The washed lower phases were transferred into scintillation vials, dried, and then 10.0 ml of scintillation fluid was added. The mixtures were counted in a liquid scintillation counter (Beckman LS-250) with a ^{14}C counting efficiency of 70%.

Galactocerebroside sulfotransferase (EC 2.8.2.11)

Galactosylceramide + phosphoadenosine phosphosulfate → sulfatide + adenosine diphosphate

The assay was performed as previously described (Tennekoon and McKhann, 1978). The incubation medium contained 100 m*M* imidazole buffer (pH 7.0); 20 m*M* $MnCl_2$; 4 m*M* dithiothreitol; 2.5 m*M* ATP, [^{35}S]phosphoadenosine phosphosulfate, 3 nmol (3×10^5 dpm); 40 μl of enzyme preparation containing 100 μg of protein; 8 μl of cerebroside (80 μM); and 0.5% Triton X-100. The mixture was incubated at 37°C for 1 hr and the reaction was stopped by the addition of 10 vol of chloroform:methanol (1:1, v/v). The lipids were extracted and partitioned, after which the lower phase was washed five times (Folch *et al.*, 1957; McKhann and Ho, 1967), transferred to scintillation vials, and dried. To each vial, 10.0 ml of scintillation mixture were added, and the samples were counted in the ^{14}C channel of a Beckman LS-250.

PROTEIN DETERMINATION

For all these studies proteins were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. Myelin basic protein was measured by radioimmunoassay as described by Cohen *et al.* (1975).

RESULTS

MEASUREMENT OF GLYCOLIPIDS IN RAT OPTIC NERVE DURING DEVELOPMENT

The use of high-performance liquid chromatography permitted the detection of very small amounts of glycolipid. With this technique the changes in concentration of monogalactosyl diglyceride as well as cerebrosides and sulfatides containing both hydroxy and nonhydroxy fatty acids were measured in rat optic nerves isolated from animals at various ages (Fig. 2). Cerebrosides containing nonhydroxy fatty acids were detected as early as 4 days after birth; the levels remained low until about 10 days, when the rate of synthesis began to increase. Between 14 and 18 days the amounts rose from 14 to 64 nmol/mg total lipid, after which the rate of synthesis decreased. The pattern for the hydroxycerebroside was similar, except that none was detectable until the 8th postnatal day and the increase between 14 and 18 days was even more marked (18 to 100 μmol/mg total lipid). From the 16th day the amount of HFA cerebroside exceeded the amount of NFA cerebroside, which could be accounted for, in part, by the higher affinity of the galactosyltransferase for HFA-ceramide as the substrate.

Sulfatides containing both HFA and NFA were detected at 8 days after birth (0.43 nmol/mg total lipid), and began to increase in amount after 10 days, with the most rapid rate of synthesis being between 16 and 18 days. For these lipids the NFA sulfatides exceed their HFA counterparts, and this could be observed as early as the 14th postnatal day. After 18 days the rate of synthesis decreased slightly.

These results clearly show that all glycolipids with the exception of NFA-galactocerebroside (which was detected a little earlier) began to increase at about the 8th day after birth, and that all showed a maximal rise between the 14th and 18th day after birth. To complement these studies, a series of enzymes involved in the synthesis of lipids was examined as described below.

GLYCOLIPID SYNTHESIZING ENZYMES

Sphingosine:acyl-CoA acyltransferase (Fig. 3) was detectable at 4 days after birth.

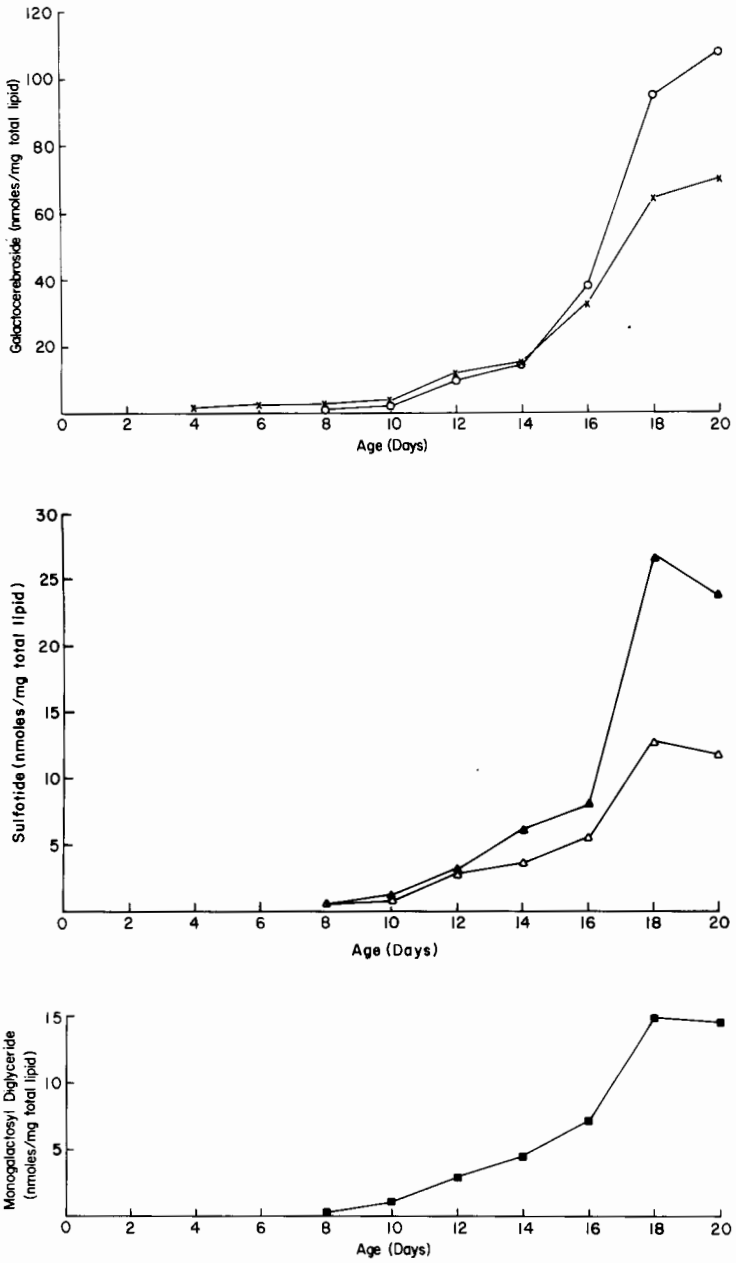

FIG. 2. Glycolipid accumulation in the rat optic nerve. Optic nerves were obtained from rats at the specified ages as described under Materials and Methods. The lipids were extracted according to the method of Folch *et al.* (1957). The organic phase was dried, benzoylated, and the products were analyzed by high-performance liquid chromatography. The peak areas were measured by the "cut and weigh" method. Upper panel, net synthesis of NFA-galactocerebrosides (x) and HFA-galactocerebrosides (○); middle panel, net synthesis of NFA-sulfatide (▲) and HFA-sulfatide (△); lower panel, net synthesis of monogalactosyl diglyceride (■).

This activity increased slightly to reach a peak at 9–10 days after birth, after which the synthesis of ceramide decreased. Fatty acid elongation activity (Fig. 4) was detectable at 4 days after birth. This activity increased quite rapidly to reach a peak at about 13 days after birth, after which the activity gradually decreased. UDPgalac-

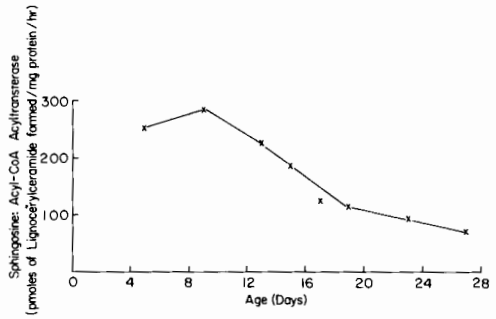

FIG. 3. Activity of sphingosine:acyl-CoA acyltransferase in the developing rat optic nerve. For each estimation the optic nerves from 10 rats were pooled and assayed for the acyltransferase as described under Materials and Methods. The reaction product was identified by thin-layer chromatography and the counts in lignoceryl ceramide were determined by liquid scintillation counting. The assays were run in duplicate and the profile was repeated twice. The values are averages from the two profiles.

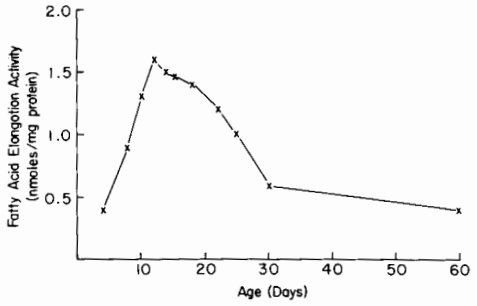

FIG. 4. Fatty acid elongation activity in the developing optic nerve. For each estimation the optic nerves from 10 rats were pooled and assayed for elongation activity as described under Materials and Methods. After incubation the reaction products were extracted and methylated, and then identified by thin-layer and gas chromatography. Each assay was run in duplicate and the profile was repeated twice. The values are the averages from the two profiles.

tose:ceramide galactosyltransferase (Fig. 5) activity began to increase after 4 days and reached a peak at 12 days after birth. Cerebroside sulfotransferase (Fig. 5) had a similar profile with activity starting to rise after the 4th day, but the activity continued to rise, reaching a peak at 16 days after birth. The pattern of activity for each of these enzymes was, therefore, similar; activity was first detected at about 4 days after birth, rose to a peak between 9 and 16 days, and then declined although all the enzymes studied were still active at 20 days. The developmental profiles of each enzyme activity were obtained at least twice, and were remarkably consistent (variation for activities except the sulfotransferase >10%).

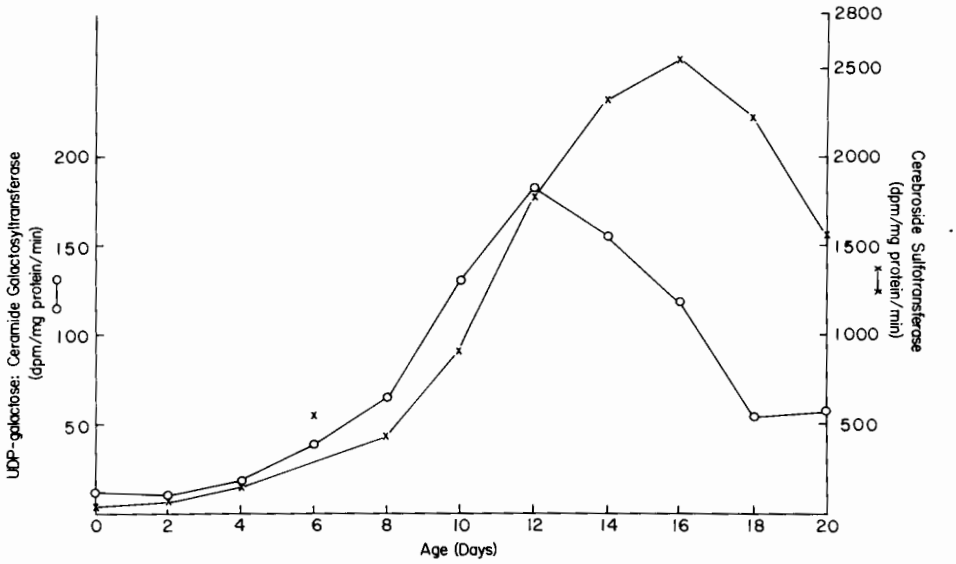

FIG. 5. Activity of ceramide:UDPgalactose galactosyltransferase (○) and cerebroside sulfotransferase (×) in the rat optic nerve. Both of these enzymes were assayed as described under Materials and Methods on optic nerves pooled from 10 rats at each time. After incubation the reaction mixture was extracted and counted by liquid scintillating counting. Each assay was done in duplicate and the profile has been repeated three times.

With cerebroside sulfotransferase, which has been repeated several times, there was some variation in amount of activity in different samples, but the time at which this enzyme was detected was always the same, as well as the general shape of the profile. In Fig. 6 activities of some of the enzymes have been plotted, together with

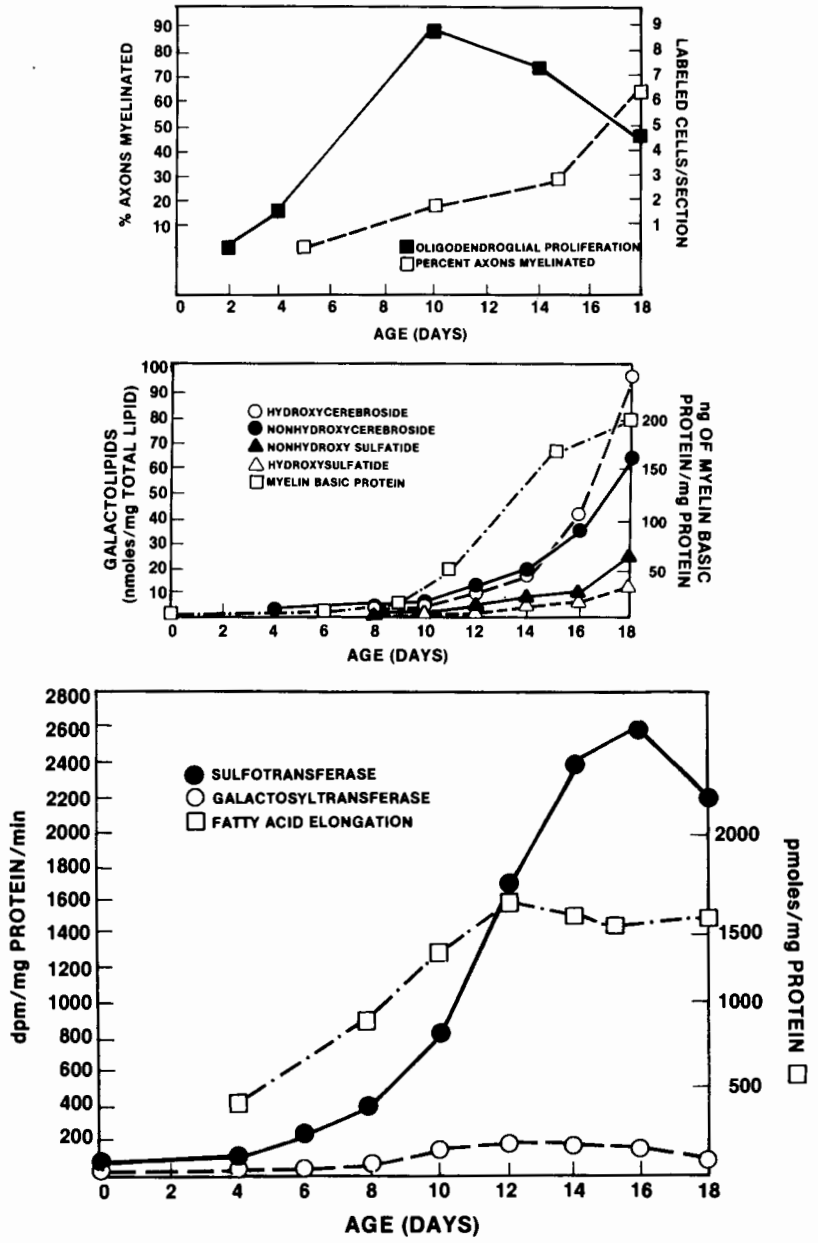

FIG. 6. Comparison of amounts of glycolipids, myelin basic protein, enzyme activities, proliferation of oligodendroglia, and myelination of axons in developing rat optic nerve. Upper panel, production of oligodendroglia was measured by [^{3}H]thymidine autoradiography as described by Skoff *et al.* (1976b). The percentage of axons myelinated was measured as described by Tennekoon *et al.* (1977). Values in the middle and lower panels are taken from Figs. 2–5 except for myelin basic protein which was measured by radioimmunoassay (Cohen *et al.*, 1975).

information on the proliferation of oligodendroglia, the amount of myelin basic protein, and the percentage of axons being myelinated.

DISCUSSION

Differentiation of a population of cells may be considered as the transition of a clone of cells through various morphological and functional stages during which different groups of genes are expressed. In eukaryotic cells there are three main stages of differentiation (Fantoni *et al.*, 1977). In the first stage the multipotential precursor cell (glioblast) becomes "committed" to differentiate into a single cell type (oligodendrocyte). During the second stage the committed cells are induced to express the genes for specialized lipids and protein. The third stage is usually referred to as "terminal differentiation" where the mature cells have the capacity to maintain synthesis at a rate to keep up with the turnover of these specialized lipids and protein. Studies of this process in a number of diverse tissues including red blood cells, lens, silk glands, chick oviduct, and skeletal muscles show that in each system, each event was carefully programmed (Boland *et al.*, 1977; Boland and Martonosi, 1974; Fantoni *et al.*, 1977; Kent *et al.*, 1974; Merlie *et al.*, 1977). In the mammalian central nervous system there is no information on differentiation of glial cell population, although much is known about changes in composition during myelinogenesis, both in isolated myelin and homogenates of the CNS (Morell *et al.*, 1972; Norton and Poduslo, 1973; Hirose and Bass, 1973; Oulton and Mezei, 1976). Similarly biochemical data on the composition of isolated oligodendrocytes from mature CNS are available (Fewster *et al.*, 1975; Poduslo and Norton, 1972), but there are no biochemical studies of oligodendroglia during differentiation. Any attempts to correlate the morphological changes with biochemical analyses in the CNS have been hampered by the heterogeneity of cell types and the varying rates of myelination of different tracts within this tissue. At the present time the optic nerve appears to be the most suitable *in vivo* model system in which to study gliogenesis.

Previous morphological studies have identified the multipotential cell, the glioblast, which then undergoes the first stage of differentiation (Skoff *et al.*, 1976a,b). The young oligodendrocytes, previously described by several groups (Mori and Leblond, 1970; Skoff *et al.*, 1976a,b; Sturrock, 1974; Vaughn, 1969), are rarely seen at birth, but start to appear about the third postnatal day. These cells are clearly distinguished from their more mature counterparts by their larger size and different morphology. They are capable of cell division, based on evidence of mitotic indices determined by [^{3}H]thymidine incorporation, but the pale cytoplasm, short cisternae of rough endoplasmic reticulum, and small Golgi complexes are not characteristic of metabolically active cells. Indeed neither biochemical markers nor enzymes that synthesize these compounds are detectable at this time by the assays used (Fig. 6), indicating that these cells do not express specialized biochemical functions.

Before myelin formation begins, histochemical studies showed the appearance of lipid-laden oligodendroglia—the "myelination glia." In the optic nerve this cell type increases in number from the onset of myelination (5 days after birth) until the time of maximal myelination (14–16 days after birth) is reached (Skoff *et al.*, 1976a,b). The cells represent the active oligodendrocytes. These cells appear metabolically active with dense cytoplasm, cisternae of the rough endoplasmic reticulum elongated and arranged in stacks, prominent distended Golgi saccules, and numerous rosettes of free ribosomes. The active oligodendrocyte has lost some of its potential to undergo mitotic cell division (Skoff *et al.*, 1976a,b), but the appearance of these cells correlates with the increases in proteins and glycolipids (Figs. 2–6) indicating that it is this cell type that is vigorously synthesiz-

ing and assembling the components of myelin.

The mature oligodendrocyte appears at about 16 days (Skoff *et al.*, 1976a,b), toward the end of the period of maximal myelination. Under normal circumstances, these cells do not undergo mitotic division, and have much less prominent Golgi complexes and saccules. It is clear from Figs. 3–6 that in these cells the rates of enzyme synthesis have either reached a peak and are starting to decline, or are being maintained at a plateau level, suggesting that these cells are merely maintaining the myelin that has been synthesized by the active oligodendrocytes. Thus the biochemical data obtained from the rat optic nerve support the concepts that were previously suggested based on ultrastructural studies of oligodendroglia.

Determination of the sequence of appearance of lipids was also an objective of this study. The developmental profiles for the appearance of myelin basic protein and 2′,3′-cyclic nucleotide phosphodiesterase have been previously obtained (Cohen and Guarnieri, 1976; Sprinkle *et al.*, 1978). Galactocerebroside, sulfatide, monogalactosyl diglyceride, myelin basic protein, and 2′,3′-cyclic nucleotide phosphohydrolase can be detected at almost the same time (Fig. 6). Moreover the enzymes that synthesize the galactolipids also start to show activity at the same time, a little before the accumulation of the galactolipids. These data imply that in the active oligodendrocyte, differentiation, and in turn, expression of specialized functions takes place almost simultaneously. This form of differentiation has been shown to occur in muscle cells, in contrast to red blood cells where expression of specialized functions appears to take place in a sequential manner (Fantoni *et al.*, 1977; Merlie *et al.*, 1977).

The evidence we have presented for our proposed model of oligodendrocyte differentiation is indirect. Proof of this model awaits either the isolation of active oligodendrocytes and their maintenance in culture, or the production of antisera directed against specific molecules on these cells and demonstration of the presence of such molecules in defined cell populations.

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