# Characterization of myelin of chick sciatic nerve during development

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**Abstract** Myelin was isolated from the sciatic nerves of chicks of ages 18-day embryonic, 1-day, 4-day, 7-day post-hatch, and adult to study developmental changes in lipid composition of this structure. The yield of myelin increased throughout the early stages of development and the preparations were of high purity. Although the lipid content of the myelin did not change, significant changes took place in lipid composition during development. The most significant changes were a relative increase in cerebrosides, phosphatidalethanolamine and long-chain fatty acids of cerebrosides, and a relative decrease in the content of phosphatidylserine and phosphatidylethanolamine.

A second fraction ("lower band") was obtained during the isolation procedure. This "lower band" was present at all developmental stages and layered consistently at the interface of 1.2 and 0.8 M sucrose on a discontinuous gradient. The quantity of this fraction did not change during development and it differed from myelin in electron microscopic appearance. Its lipid composition, which did not change, resembled that of 18-day embryonic myelin in its high phospholipid:cholesterol ratio and low galactolipid content.

The enzyme, 2′:3′-cyclic-nucleosidemonophosphate phosphodiesterase was found to be present in both the myelin and "lower band" fractions; however there was no enrichment of this enzyme in purified myelin.

**Supplementary key words** nerve subcellular fraction • cholesterol • cerebrosides • sulfatides • plasmalogen • phospholipids • 2′:3′-cyclic-nucleosidemonophosphate phosphodiesterase • peripheral nervous system

Fundamental to an understanding of the mechanism by which the myelin sheath is organized and maintained is knowledge of the chemical properties of this structure. Most of the available data in this area have been obtained from studies of the central nervous system (CNS). Such investigations have established that the chemical composition of CNS myelin is altered throughout development until a mature form is attained (1–5). Although data are available on the chemical composition of adult peripheral nervous system (PNS) myelin from many species (6–9), little is known about the composition of developing PNS myelin. Preliminary results from this laboratory indicated that the lipid composition of chick sciatic nerve myelin changed significantly during development and that these changes were qualitatively similar to those reported for CNS myelin (10).

Recently a number of investigators have described a myelin-like fraction that they obtained during the preparation of myelin from brain tissues of certain rodents (11–13). In our preliminary study, we noticed the presence of a second fraction in the peripheral nerve of chicks after sucrose gradient fractionation of the post-nuclear supernatant of this tissue and suggested that it may be similar to the myelin-like material obtained from the CNS (10). To further characterize the chemical properties of myelin and this second fraction in the PNS, we carried out a more extensive study of the lipid composition of these components of the sciatic nerve during development. Furthermore, we explored the possibility of establishing the enzyme, 2′:3′-cyclic-nucleosidemonophosphate phosphodiesterase (E.C. 3.1.4.16) as a marker for PNS myelin. This enzyme has been shown to be enriched in the myelin fraction of CNS (14–16).

## MATERIALS AND METHODS

### Chemicals

Lipid standards, "lipopure" solvents and Absorbosil-4 (silica gel of extra high purity containing 10% magnesium silicate binder) were purchased from Applied Science Labs., Inc., State College, Pa. or from Serdary Research Labs., London, Ontario. Alkaline phosphatase, adenosine 2′:3′-cyclic monophosphoric acid (sodium salt) were obtained from the Sigma Chemical Company, St. Louis, Mo. [U-$^{14}$C]sucrose was purchased from New England Nuclear, Boston, Mass. Orcinol was purchased from Fisher Scientific Co., Ltd., Montreal, Quebec and was recrystallized twice from benzene before use. The twice recrystallized orcinol was stored in a desiccator in the dark. All other chemicals and solvents were

Abbreviations: LDH, lactate dehydrogenase; CYTc, Ox cytochrome$_c$ oxidase; CNPD, 2′:3′-cyclic-nucleosidemonophosphate phosphodiesterase; GLC, gas–liquid chromatography; CNS, central nervous system; PNS, peripheral nervous system; TLC, thin-layer chromatography.

reagent grade and were purchased from Canadian Laboratory Supplies, Ltd., Halifax, N.S., or from Fisher Scientific Co., Ltd., Montreal, Quebec.

### Animals

Fertile eggs (Shaver Starcross No. 288 line of White Leghorn Strain) were purchased from Lone Pine Farm, Ltd., Berwick, N.S., and incubated in a rotating incubator (Humidaire Co., New Madison, Ohio) at 37°C. At 20 days of age, the eggs were transferred to a hatching incubator (Leahy Manufacturing Co., Higginsville, Mo.) maintained at 37°C. The hatched chicks were given only water for 3 days. At this age chicks were transferred to open containers at 22°C and maintained on a diet of water and chick starter (Maritime Co-operative Services, Ltd., Moncton, N.B.). Adult hens (White Leghorn Strain) were purchased by The Animal Care Centre, Dalhousie University. Developmental stages investigated included 18-day embryos, 1-day, 4-day, 7-day chicks and adult hens.

### Dissection of the sciatic nerve

The dorsal and leg skin of the embryo or hatched chick was excised and the sciatic nerve was exposed by severing the superficial muscles of the upper thigh. The nerve was severed proximally just below the spinal ganglia, and severed distally just above the knee joint.

### Preparation and purification of myelin and "lower band" fractions

The sciatic nerves, immediately after dissection, were soaked in 0.05 M Tris–0.7 M glycine buffer, pH 6.0, for 1 hr. Myelin was isolated from 60–180 embryos or chicks and three hens for each experiment according to the procedure of Adams et al. (17) with some modifications. The nerves were homogenized in 6 volumes (w/v) of 0.32 M sucrose for 2 min at 2500 rpm in a Fisher Dyna-Mix homogenizer (Fisher Scientific). The nerve homogenate was centrifuged for 20 min at 600 *g* in a Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.) to remove nuclei and cellular debris. A 5.2 ml aliquot of the supernatant was transferred to an ice-cold homogenizer together with 5.2 ml of 1.2 M sucrose and the mixture was homogenized 10 strokes by hand. Discontinuous sucrose gradients were prepared by layering 4.8 ml of this homogenate (which would be approximately 0.8 M in sucrose) over 5.4 ml of 1.2 M sucrose in a 12-ml polycarbonate centrifuge tube, followed by 1.8 ml of 0.32 M sucrose on the top. The gradients were centrifuged at 100,000 *g* for 90 min in a Beckmann SW 41 Ti rotor (Beckmann Instruments, Spinco Div., Palo Alto, Calif.).

The myelin fraction, which concentrated at the interface between the 0.8 M and 0.32 M sucrose layers, was removed and subjected to osmotic shock by suspending it in 10 volumes of ice-cold water for 15 min. The suspension was then homogenized 10 strokes by hand and centrifuged at 32,800 *g* for 30 min.

The myelin pellet was washed once with 20 ml of water, suspended in 2.6 ml of 0.32 M sucrose and transferred to an ice-cold homogenizer together with 2.6 ml of 1.2 M sucrose and homogenized 10 strokes by hand. A discontinuous gradient was prepared and centrifuged at 100,000 *g* as described above. The purified myelin fraction, which formed a band at the interface between the 0.8 M and 0.32 M sucrose layers, was removed and washed five times with 20 ml of water. The washed myelin was then freeze-dried and stored under nitrogen at −20°C until used for chemical analysis.

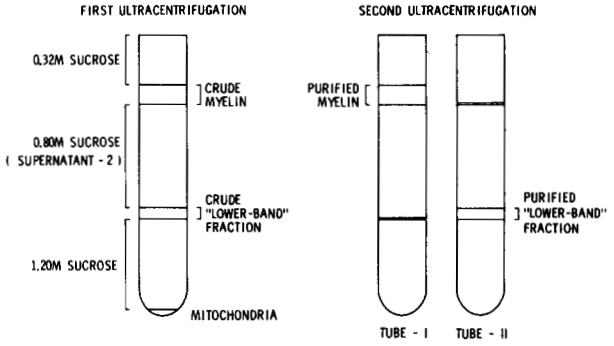


**Fig. 1.** Preparation of myelin and "lower band" fractions by centrifugation of a density gradient at 100,000 *g* for 90 min. The fractions obtained are identified in the figure.

After centrifugation of the first discontinuous sucrose gradient, a second band of material that was present at all developmental stages layered at the interface between the 1.2 M and 0.8 M sucrose. This fraction, designated as "lower band" (**Fig. 1**) was removed and attempts were made to purify it for chemical analysis. When this fraction was subjected to osmotic shock treatment as described for the crude myelin fraction or was washed with water, the resulting opaque solution could not be sedimented at 32,800 *g* for 30 min and, therefore, this approach was abandoned. For routine analysis, the fraction was washed three times with 30 ml of 0.25 M sucrose followed by centrifugation at 32,800 *g* for 30 min, and chemical analysis was carried out on the washed fraction. In one set of experiments the washed fraction was recentrifuged on a sucrose density gradient. However, this procedure resulted in low recoveries and no apparent change in lipid composition of the recovered fraction was observed.

### Electron microscopy

Aliquots of the purified myelin and "lower band" pellets were fixed overnight in 2–3 ml of 3% glutaraldehyde, stained with 1% osmium tetroxide, and embedded in eponresin for electron microscopic examination.

### Content of nucleic acids, proteins, and marker enzyme activities of the subcellular fractions

The nucleic acid content of the previously delipidated myelin and "lower band" fractions was determined according to the two wavelength method of Santen and Agranoff (18).

Total protein content was determined by a micro biuret method (19) after solubilization of 5–10 mg portions of the

freeze-dried myelin preparations in 1 M NaOH containing 3% (v/v) Triton-x-100 and 0.2% (w/v) sodium deoxycholate (20). Bovine serum albumin (dissolved in the same detergent-containing solution as the myelin samples) was used as a standard. Accurate measurements could be made in samples containing 50–400 $\mu$g of protein.

The activities of lactate dehydrogenase (E.C. 1.1.1.27) (21), cytochrome$_c$ oxidase (E.C. 1.9.3.1) (22) and 2′:3′-cyclic-nucleosidemonophosphate phosphodiesterase (E.C. 3.1.4.16) (23) were determined immediately for all fractions after centrifugation of the sucrose density gradient (Fig. 1). One unit of cytochrome$_c$ oxidase (Cyt$_c$ Ox) is defined as that amount of enzyme which changes the absorbance of reduced cytochrome$_c$ at 550 nm by 0.001 in 1 min in a 3 ml assay mixture at 24°C. One unit of lactate dehydrogenase (LDH) is defined as that amount of enzyme which changes the absorbance due to NADH at 340 nm by 0.001 in 1 min in a 3 ml assay mixture at 24°C. One unit of 2′:3′-cyclic-nucleoside-monophosphate phosphodiesterase (CNPD) is defined as that amount of enzyme that produces 1 $\mu$mole/min of adenosine 2′-phosphate from adenosine 2′:3′-cyclic phosphate. Sufficient enzyme protein was added to each reaction mixture to produce 0.002–0.100 $\mu$mole adenosine 2′-phosphate/min.

### Lipid analysis of the myelin and "lower band" fractions

Lipids were extracted according to Folch, Lees, and Sloane Stanley (24). Methods for determining the contents of cholesterol, cerebrosides, and both total and individual phospholipids were described in a preliminary report (10).

Molar contents of the individual lipid classes were determined by assuming mean molecular weights of 387 (cholesterol), 846 (galactolipids), and 775 (phospholipids) (25). Total lipid content of myelin was calculated by summing the values determined for the individual classes.

The values previously reported by us for cerebroside content (10) were in fact representative not only of cerebrosides but of total galactolipids. Sulfatides, determined by the method of Kean (26), represented the only other galactolipids present. The total galactolipid content would therefore represent the sum of the sulfatides and cerebrosides. Cerebrosides were therefore estimated by difference between total galactolipids and sulfatides. Total galactolipid content of both myelin and "lower band" fractions was routinely measured by the orcinol method (27). Sucrose contamination in the "lower band" fraction was corrected for as described in Results. In some experiments the galactolipid content of the myelin fraction was determined by the enzymic method described by Finch et al. (28) after hydrolysis of the galactose moiety according to Svennerholm (29). The two methods gave essentially the same results.

The values previously reported by us for phosphatidylethanolamine were representative of the contents of both phosphatidylethanolamine and its plasmalogenic form, phosphatidalethanolamine (10). The content of the individual forms of these phospholipids was determined by an adaptation of the reaction-thin-layer chromatography method of Viswanathan et al. (30). By this procedure, aliquots of the phospholipid fraction of the myelin lipid extract were spotted on four separate channels of a thin-layer plate coated with Absorbosil-4. Two of the channels were treated for 2 min with 20 $\mu$l of a solution containing 12% (by wt) HCl in methanol. The plate was developed in chloroform–methanol–28% $NH_4OH$ 14:6:1 (v/v/v) for 1 hr. The phosphorus content of the individual spots was determined (31). The difference in phosphorus content between the ethanolamine phospholipid spot of the untreated and that of the treated channel was taken as being representative of the content of ethanolamine plasmalogen.

### Fatty acid analysis of myelin cerebrosides

The glycolipid fraction of myelin obtained by silicic acid column chromatography (32) was further fractionated into cerebrosides and sulfatides by TLC according to Singh, Spritz, and Geyer (33). The cerebrosides were removed and interesterified with 14% $BF_3$ in methanol at 100°C for 90 min (34). The methyl esters of unsubstituted and hydroxy fatty acids were separated by TLC and extracted from the silica gel with ether (33). Hydroxy acids, after conversion to trimethylsilyl derivatives (33) and unsubstituted acids were analyzed by GLC, using a Varian-Aerograph Model 1200 gas chromatograph with a stainless steel column (size 8′ × 1/8″) and equipped with a hydrogen flame ionization detector (Varian Assoc., Palo Alto, Cal.). The stationary phase was 10% EGSS-X on Gas-Chrom Q 100–120 mesh. The column was run isothermally at 180°C. The peaks were identified by comparison of relative retention times with those of standard esters. The product of peak height and retention time was used as a measure of peak area (35). The relative detector response was checked using quantitative mixtures of methyl esters (Applied Sci. Lab., Inc.) and was found to be within a 5% range. The identity of $C_{22:0}$ and $C_{24:0}$ fatty acids of myelin cerebrosides was also checked by GLC and mass spectrometry by Dr. O. A. Mamer (Mass Spectrometry Unit, Royal Victoria Hospital, Montreal, Que.).

## RESULTS

### Yield and Purity

The yield of the myelin and "lower band" fractions, based on their protein content, is shown in **Fig. 2.** The yield of myelin increased significantly from 18-day embryonic stage to 4-day post hatch, after which there was no further increase. The yield of myelin, when determined on the basis of the dry weight of isolated myelin/g wet weight of nerve showed a similar developmental pattern (results not shown). The yield of the "lower band" fraction did not change significantly throughout all stages of development.

Electron microscopy of the myelin fraction **(Fig. 3A)** indicated the presence of mainly typical myelin lamellae with minimal contamination by other subcellular particles. The "lower band" fraction, however, (Fig. 3B) contained mostly single membrane vesicles with other unidentifiable fragments present. Nucleic acids comprised a low 0.09 ± 0.05% of the dry weight of myelin. This value represents the mean ± S.D.

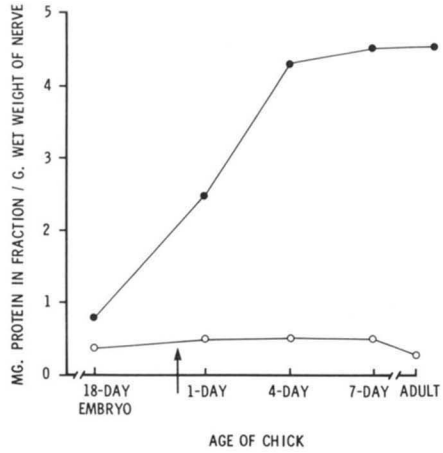


**Fig. 2.** Yield of myelin and "lower band" fractions as determined from the protein content of these fractions for each developmental stage. (●—●) Myelin; (○—○) "lower band" fraction. Arrow represents 21st day of embryonic age and hatching of chicks.

of the values obtained for each developmental stage and suggests negligible contamination of myelin by nucleic acid-containing organelles. Based on the wet weight of nerve, the value obtained for myelin was 7.2 ± 4.7 ng nucleic acids/mg wet weight of nerve, while that for the "lower band" fraction was 41.2 ± 11.6 ng nucleic acids/mg wet weight of nerve.

Results with marker enzymes LDH and CYTc Ox indicated that both of these enzymes were present in the crude myelin and "lower band" fractions. These enzymes were completely removed from myelin after osmotic shock treatment and recentrifugation of this fraction. The "lower band" fraction, however, retained an average of 0.34 ± 0.15 units/mg protein of LDH and 0.29 ± 0.15 units/mg protein of CYTc Ox activity even after a second ultracentrifugation purification step.

CNPD activity was found to be present in the myelin and "lower band" fractions of nerve from all developmental stages even after rigorous purification procedures (**Table 1**). However, it was not enriched in the purified myelin of PNS but was distributed over all fractions of the gradient. For comparison with CNS, 7-day chick brain was similarly fractionated and the fractions assayed for CNPD activity. In contrast to nerve, this enzyme was enriched in the purified myelin fraction of chick brain.

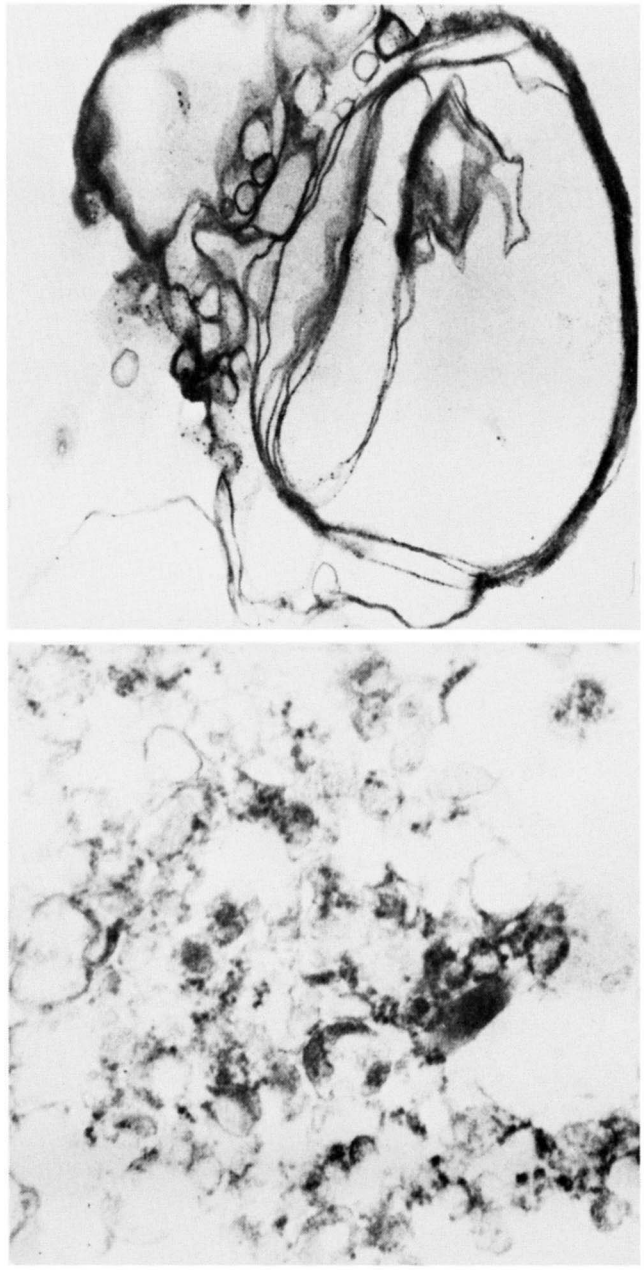

**Fig. 3.** Electron micrographs of purified myelin prepared from sciatic nerves of 4-day chicks (A; magnification × 69,000) and purified "lower band" fraction isolated from 18-day embryonic nerves (B; magnification × 11,400).

### Chemical composition of myelin

The total lipid content of purified, freeze-dried myelin preparations from all age groups (**Table 2**) did not change significantly but remained at approximately 70% of the dry weight of myelin. Therefore any change observed in the content of individual myelin lipids must be due to actual changes within the individual lipid classes and not to a change in total lipid content. The total protein content of myelin was found to be 25.4 ± 1.8% of the dry weight of myelin.

Cholesterol content did not change significantly during development of chick sciatic nerve myelin. The content of total galactolipids showed significantly large increases, particularly in the early developmental stages. This increase could be attributed to an increasing cerebroside content; sulfatides showed no significant change. Total phospholipids decreased, the major relative decrease occurring in phosphatidylcholine content with a small but significant relative decrease in phosphatidylserine. Sphingomyelin showed no change when expressed as molar percent of total lipids. However, it represented increasing proportions of the total phospholipids when the individual phospholipids were expressed as molar percent of total phospholipids (10). Although total ethanolamine phospholipids did not change significantly, the content of the

TABLE 1. CNPD activity of total homogenates of sciatic nerve and brain and of fractions obtained after ultracentrifugation of nerve and brain homogenates on a sucrose gradient

| | Enzyme Activity[a] in Nerve | | | | | Enzyme Activity in Brain |
|---|---|---|---|---|---|---|
| Age of chick | 18-Day Embryo | 1-Day | 4-Day | 7-Day | Adult | 7-Day |
| Distribution | | | | | | |
| Whole homogenate[b] | 0.298 | 0.286 | 0.191 | 0.274 | 0.227 | 0.131 |
| Crude myelin | 0.374 | 0.305 | 0.344 | 0.330 | 0.430 | 0.241 |
| Purified myelin | 0.116 | 0.309 | 0.192 | 0.170 | 0.260 | 1.700 |
| Crude "lower band" | 0.200 | 0.173 | 0.262 | 0.358 | 0.148 | 0.086 |
| Purified "lower band" | 0.311 | 0.173 | 0.351 | 0.462 | not determined | 0.089 |

[a] Enzyme activity is expressed as units/mg protein. One unit of CNPD is described in Methods.
[b] Whole homogenate represents a 17% (w/v) nerve or brain homogenate in 0.32 M sucrose. The remainder of fractions are designated in Fig. 1.

TABLE 2. Lipid composition of chick sciatic nerve myelin during development

| | Age of Chick | | | | | |
|---|---|---|---|---|---|---|
| Lipid Content[a] | 18-Day Embryo 70.71 ± 2.29 | 1-Day 72.31 ± 2.32 | 4-Day 71.70 ± 3.39 | 7-Day 66.57 ± 3.72 | Adult 72.60 ± 6.04 | Analysis $P > 0.05$ |
| Component | Lipid composition, molar % of total lipid | | | | | |
| Cholesterol | 37.1 ± 3.4 | 34.8 ± 0.7 | 35.8 ± 2.5 | 33.7 ± 1.7 | 35.0 ± 1.6 | $P > 0.05$ |
| Total galactolipid | 14.6 ± 1.0[b] | 19.2 ± 1.2[b] | 21.9 ± 2.7 | 24.7 ± 1.2 | 26.1 ± 0.5 | $P < 0.01$ |
| Cerebroside | 9.1 ± 2.0 | 13.6 ± 0.9 | 16.6 ± 0.7 | 18.7 ± 2.5 | 21.0 ± 2.7 | $P < 0.01$ |
| Sulfatide | 5.5 ± 0.6 | 5.6 ± 0.5 | 5.3 ± 0.3 | 6.0 ± 0.8 | 5.1 ± 0.7 | $P > 0.05$ |
| Total phospholipids | 48.3 ± 4.2 | 46.0 ± 0.7 | 42.3 ± 0.7 | 41.5 ± 2.0 | 39.3 ± 1.8 | $P < 0.05$ |
| Phosphatidylserine | 10.3 ± 0.7 | 8.2 ± 0.5 | 7.6 ± 0.5 | 7.0 ± 1.0 | 7.5 ± 0.3 | $P < 0.01$ |
| Sphingomyelin | 10.5 ± 1.4 | 10.2 ± 0.1 | 9.7 ± 0.6 | 10.1 ± 0.9 | 12.0 ± 0.7 | $P > 0.05$ |
| Phosphatidylcholine | 12.0 ± 0.7 | 10.0 ± 0.5 | 8.4 ± 0.4 | 7.6 ± 0.9 | 4.8 ± 0.2 | $P < 0.01$ |
| Phosphatidylethanolamine | 10.4 ± 0.5 | 9.5 ± 0.3 | 7.5 ± 0.1 | 6.4 ± 0.2 | 4.7 ± 0.2 | $P < 0.01$ |
| Phosphatidalethanolamine | 5.1 ± 0.5 | 8.1 ± 0.3 | 9.3 ± 0.1 | 10.4 ± 0.2 | 10.4 ± 0.2 | $P < 0.01$ |

[a] Lipid content is expressed as per cent of dry weight of myelin and was calculated by summing the values determined for the individual lipid classes. Results are expressed as molar percent of total lipids and represent the mean ± S.D. of determinations for three separate preparations at each developmental stage.
[b] Values underscored by a common line are significantly different. The $P$ values were determined from the difference in lipid composition in the 18-day embryonic and adult myelin.

individual forms of this class of phospholipids showed large significant changes, with phosphatidylethanolamine decreasing and the plasmalogenic form increasing during development.

The composition of the cerebroside fatty acids was determined for three developmental stages only. Significant changes were observed in the composition of both unsubstituted (**Table 3**) and hydroxy (**Table 4**) fatty acids. There was an increase in the content of $C_{22}$–$C_{24}$ in both cases. The most significant increase occurred in the saturated fatty acids 22:0 and 22 h:0. These two fatty acids were the most abundant for each developmental stage. The content of unsubstituted monoenes increased significantly during development, 24:1 showing the most significant increase. Due to large standard deviations, no significant changes were observed in total hydroxy monoenes at adjacent developmental stages although the overall change (from the 18-day embryonic stage to the 7-day chick stage) was a significant decrease. The most significant decrease took place in 20 h:1 whereas 24 h:1 showed no change.

### Lipid composition of the "lower band" fraction

Two types of experiments were carried out to assess the amount of sucrose contamination of the "lower band" fraction which would be carried over from the density gradient and washing steps and would contribute to erroneously high galactolipid values as determined by the orcinol method. In the first experiment, [U-$^{14}$C]sucrose was used throughout all steps of a preparation of this fraction from a known amount of 1-day chick nerve tissue. As a control, this fraction was also prepared from a known amount of 1-day chick nerve tissue on a nonradioactive sucrose gradient. Lipid extracts

TABLE 3. Composition of unsubstituted fatty acids of cerebrosides of chick sciatic nerve myelin during development

| Fatty Acid | Age of Chick | | | Analysis |
|---|---|---|---|---|
| | 18-Day Embryo | 1-Day | 7-Day | |
| | *Relative Percentage*[a] | | | |
| 14:0[b] | 8.3 ± 0.7 | 3.3 ± 1.1 | 4.7 ± 1.8 | $P = 0.05$ |
| 16:0 | 9.7 ± 1.4 | 7.4 ± 2.6 | 2.7 ± 0.7 | $P < 0.05$ |
| 18:0 | 21.3 ± 2.0 | 15.7 ± 1.2 | 10.6 ± 0.6 | $P < 0.01$ |
| 20:0 | 5.3 ± 1.0 | 5.7 ± 0.2 | 5.9 ± 1.0 | $P > 0.05$ |
| 20:1 | 2.3 ± 0.4 | 2.1 ± 0.0 | 1.0 ± 0.2 | $P < 0.05$ |
| 22:0 | 27.4 ± 0.5 | 30.9 ± 0.7 | 32.0 ± 3.0 | $P = 0.05$ |
| 22:1 | N.D.[c] | 0.7 ± 0.2 | 2.1 ± 0.7 | $P < 0.05$ |
| 24:0 | 18.4 ± 1.8 | 18.7 ± 1.3 | 23.8 ± 2.3 | $P > 0.05$ |
| 24:1 | 8.8 ± 1.5 | 15.7 ± 1.0 | 17.3 ± 2.3 | $P < 0.01$ |
| % $C_{22} - C_{24}$ | 55.1 ± 2.9 | 66.0 ± 2.8 | 75.2 ± 1.4 | $P < 0.01$ |
| % Monoenes | 11.0 ± 1.9 | 18.5 ± 0.8 | 20.4 ± 3.1 | $P < 0.05$ |

[a] Results are expressed as relative percentages of total unsubstituted fatty acids and represent the mean ± S.D. of determinations for three separate preparations at each developmental stage. Statistical analysis was carried out as described in Table 2. Values underscored by a common line show significant differences.
[b] Number of carbon atoms of fatty acids: number of double bonds.
[c] No detectable peak (< 0.2% of the mixture).

TABLE 4. Composition of hydroxy fatty acids of cerebrosides of chick sciatic nerve myelin during development

| Fatty Acid | Age of Chick | | | Analysis |
|---|---|---|---|---|
| | 18-Day Embryo | 1-Day | 7-Day | |
| | *Relative Percentage*[a] | | | |
| 14h:0[b] | 9.4 ± 3.1 | 4.4 ± 1.9 | 4.9 ± 0.8 | $P > 0.05$ |
| 16h:0 | 4.3 ± 0.1 | 3.0 ± 1.4 | 3.1 ± 1.1 | $P > 0.05$ |
| 18h:0 | 6.1 ± 1.9 | 6.9 ± 1.9 | 4.6 ± 0.4 | $P > 0.05$ |
| 20h:0 | 11.9 ± 3.3 | 9.1 ± 1.1 | 7.9 ± 3.6 | $P > 0.05$ |
| 20h:1 | 11.6 ± 0.6 | 4.4 ± 2.1 | 1.2 ± 0.5 | $P < 0.01$ |
| 22h:0 | 23.6 ± 1.1 | 38.3 ± 5.8 | 46.4 ± 4.7 | $P < 0.01$ |
| 22h:1 | 5.2 ± 1.7 | 5.2 ± 0.9 | 2.5 ± 0.5 | $P > 0.01$ |
| 24h:0 | 18.5 ± 2.4 | 18.7 ± 0.5 | 19.2 ± 4.8 | $P > 0.05$ |
| 24h:1 | 9.4 ± 0.4 | 10.3 ± 1.0 | 10.1 ± 2.9 | $P > 0.05$ |
| % $C_{22} - C_{24}$ | 56.7 ± 2.2 | 75.7 ± 6.1 | 78.3 ± 5.8 | $P < 0.01$ |
| % Monoenes | 26.1 ± 1.9 | 19.8 ± 2.7 | 13.8 ± 2.9 | $P < 0.01$ |

[a] Results are expressed as relative percentages of total hydroxy fatty acids and represent the mean ± S.D. of determinations for three separate preparations at each developmental stage. Statistical analysis was carried out as described in Table 2. Values underscored by a common line show significant differences.
[b] Number of carbon atoms of hydroxy fatty acids: number of double bonds.

were prepared from both of these fractions and, from a determination of the apparent galactose content (27) of the control and the amount of [$^{14}$C]sucrose in the radioactive fraction, the contribution made by sucrose to the galactose value obtained by the orcinol method was determined.

In the second type of experiment, which was carried on in conjunction with the first, a nonradioactive sucrose gradient (containing only sucrose and no nerve tissue) was prepared. The region of the gradient that would correspond to the region where the "lower band" fraction forms, was removed. This fraction, named "lower band" blank, was carried through the complete procedure used for lipid extraction and washing. This blank was then assayed by the orcinol method.

By these two types of experiments, it was found that the

"lower band" fraction was contaminated with sucrose by such an amount that would contribute 29% of the value for galactose as determined by the orcinol method. Corrections for galactose content were therefore made using this factor. Because of the small quantities of this fraction available, particularly at later developmental stages, only the major lipid classes were determined (**Table 5**). No significant changes in lipid composition of this fraction took place during development. In fact, the composition resembles that of 18-day embryonic myelin in its high phospholipid:cholesterol ratio and low galactolipid content.

## DISCUSSION

Several methods are available for isolation of myelin; however, they all involve differential and density gradient centrifugation steps. After extensive investigation of the preparation of myelin from rat brain, Norton and Poduslo (5) showed that, in order to collect all of the myelin at a step-gradient interface, the dense layer used in the gradient must be approximately 0.8 M sucrose (1.103 g/ml). This concentration of sucrose was used in the present investigation. In every experiment the myelin fraction banded at the interface between the 0.8 M and 0.32 M sucrose. Therefore the myelin isolated at each developmental stage represented myelin populations of consistently similar densities. This was further indicated by the lipid content of myelin which remained at approximately 70% throughout all developmental stages.

One of the criteria of myelin purity is its electron microscopic appearance. In the present study, electron microscopy showed the myelin preparations to consist of typical lamellae with minimal contamination by other subcellular particles (Fig. 3A). The nucleic acid content of myelin would be expected to be low because compact myelin contains no nuclei, cytoplasm or mitochondria. Norton and Poduslo (36) reported a nucleic acid content for highly purified rat brain myelin of 0.1–0.5% of the dry weight of myelin. The values obtained for chick sciatic nerve myelin, which range from 0.01 to 0.18% of the dry weight of myelin, are indicative of the high purity of the preparations at each developmental stage. A further indication of the purity of myelin would be a minimization of marker enzymes for other subcellular fractions and a maximization of myelin marker enzymes. Both LDH (cytoplasmic marker) and CYTc Ox (mitochondrial marker) were removed from the myelin fractions of all developmental stages after osmotic shock treatment. Marker enzymes for myelin are not so well characterized, although several lines of evidence suggest that CNPD is localized in the CNS myelin sheath. This enzyme has been found to be enriched in CNS myelin (ref. 14 and Table 1 of present investigation) and to be diminished in mutant mice with deficient myelin (37) and in plaque areas of brains of multiple sclerosis patients (16). More recent studies, however, have shown CNPD activity in glial and Schwann cell tumors (38–40) and in cloned glial cell plasma membranes (41). These results indicate that the enzyme is not exclusively localized in the myelin sheath.

CNPD activity has been shown to increase during myelination of chick sciatic nerve (42, 43). Mezei, Mezei, and Hawkins (44) have demonstrated loss of activity during Wallerian degeneration. These findings suggest some type of association of this enzyme with PNS myelination. In contrast, Braun and Barchi (16) found no increase in activity in myelinating rat sciatic nerve. It was therefore of interest to determine if CNPD activity was enriched in PNS myelin. The results show that, although activity was present in the extensively purified myelin and "lower band" fractions, there was no enrichment in this enzyme in purified PNS myelin (Table 1). It is possible that the properties of CNPD are different from its counterpart in the CNS. The enzyme might be more labile or might not be as strongly bound to the myelin membrane and could have been partially inactivated or removed during osmotic shock treatment and re-centrifugation of the crude PNS myelin. The results of our present investigation, therefore, could not establish CNPD as a marker for PNS myelin in the chicken.

Although the total lipid content of chick sciatic nerve myelin did not change during development, significant changes in lipid composition were observed. As a proportion of total lipids, the content of cerebrosides increased and that of phospholipids decreased, with the major decrease occurring in phosphatidylcholine. These changes are qualitatively similar to those reported for developing rat brain myelin (2, 3, 5) and rabbit brain and cord myelin (4). Although Dalal and Einstein (4) report an increase in cholesterol for both rabbit brain and cord myelin, no such change has been reported for CNS myelin of other species. The cholesterol content of

**TABLE 5. Lipid composition of the "lower band" fraction obtained from chick sciatic nerve during development**

| Component | Age of Chick | | | | |
|---|---|---|---|---|---|
| | 18-Day Embryo | 1-Day | 4-Day | 7-Day | Adult |
| Cholesterol | 26.0 ± 1.0 | 33.6 ± 5.6 | 33.7 ± 2.4 | 31.2 ± 3.4 | 33.0 |
| Phospholipid | 57.5 ± 5.8 | 53.5 ± 6.2 | 51.9 ± 3.8 | 52.4 ± 2.2 | 48.9 |
| Galactolipid | 16.5 ± 4.8 | 12.9 ± 0.9 | 14.4 ± 2.7 | 16.4 ± 1.6 | 18.1 |

Results are expressed as molar percent of total lipid and represent the mean ± S.D. of determinations for three separate preparations of 18-day embryos, 1, 4 and 7-day old chicks. Statistical analysis indicated no significant differences ($P > 0.05$) between the lipid composition of the 18-day embryonic and 7-day chick "lower band" fraction.

developing chick sciatic nerve myelin did not change. The contents of the individual ethanolamine phospholipids changed significantly. These changes are in agreement with those found by Eng and Noble (3) for rat brain, although Norton and Poduslo (5) found no changes in these lipids in rat brain. No other data on the chemical development of PNS myelin are available for comparison with the data obtained for chick sciatic nerve. However, the composition of adult chick sciatic nerve myelin was found to be comparable to adult PNS myelin from other species (6–9).

Shaikh and Palmer[1] have found that the lipid composition of whole chick sciatic nerve changes during development. The changes were similar to those found in our laboratory for isolated myelin. This indicates that changes found in lipid composition of isolated myelin are not due to preferential removal or enrichment of certain lipid classes during the isolation and purification procedure. Norton and Poduslo (5) have shown similar patterns of lipid composition for developing rat whole brain and isolated myelin.

Nervous tissue is characterized by an abundance of long-chain fatty acids (more than 20 carbon atoms) that are mostly associated with the sphingolipids. Significant increases in the content of both unsubstituted and $\alpha$-hydroxy long-chain fatty acids of cerebrosides were found. The most significant increase occurred in behenic acid ($C_{22:0}$) and $\alpha$-hydroxy behenic acid ($C_{22h:0}$). Svennerholm and Stallberg-Stenhagen (45) have shown that the content of cerebroside long-chain fatty acids of developing human whole brain also increases during development. However, they found very low levels of $C_{22:0}$ or $C_{22h:0}$; the major increase occurred in the content of lignoceric acid ($C_{24:0}$), $\alpha$-hydroxy lignoceric acid ($C_{24h:0}$), and their monounsaturated analogues. High levels of the $C_{22}$ fatty acids have been found in adult mouse brain (33, 46) and Baumann et al. (46) have shown that the content of $C_{22:0} + C_{22:1}$ fatty acids of mouse brain cerebrosides increased from 2.1% in the one-day mouse to 23.4% in the 12-day mouse and that this value decreased to 5.1% in the adult. For chick sciatic nerve myelin cerebrosides, the content of fatty acids was only determined up to the 7-day post-hatch developmental stage. The possibility therefore exists that further changes would take place after this stage.

The significance of a changing lipid composition of developing myelin is not fully understood. It is not known, for example, whether an immature form of myelin is deposited and subsequently altered by insertion of lipid components or whether the myelin sheath is formed by the sequential addition of presynthesized myelin layers of differing compositions. Myelin subfractions of different compositions have been isolated from the CNS (47–49), a finding which suggests that the mature myelin sheath consists of many layers of different lipid compositions and that the lipid composition of mature myelin represents the total composition of the individual layers. Agrawal et al. (49) have shown that one of these subfractions, which they named membrane fraction, is a precursor of more mature myelin.

In addition to myelin, sciatic nerve homogenates contained a more dense, "lower band" fraction. The electron microscopic appearance of this subcellular fraction resembled the so-called myelin-like material isolated from the CNS (11–13). The lipid composition of the "lower band" fraction from each developmental stage resembled that of purified myelin isolated from the 18-day chick embryo (compare results in Tables 2 and 5). Since myelin from the 18-day embryo contains mainly early deposited lammelar structures, in the developing peripheral nerve, it is possible that the "lower band" represents an immature, loosely-bound form of myelin.

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[1] Shaikh, N. A., and F. B. St. C. Palmer. Personal communication.

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