PRESERVATION OF MYELIN LAMELLAR STRUCTURE IN THE ABSENCE OF LIPID

A Correlated Chemical and Morphological Study

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ABSTRACT

The fine structure of myelin was studied in glutaraldehyde-fixed rat sciatic nerves depleted of lipid by acetone, chloroform:methanol (2:1 v/v), and chloroform:methanol:concentrated HCl (200:100:1, v/v/v). One portion of each of these nerves, plus the extracts, was saponified and analyzed by gas–liquid chromatography for fatty acids. The remainder of each nerve was stained in osmium tetroxide in CCl_4 (5g/100cc) and was embedded in Epon 812. Thin sections, examined in the electron microscope, revealed the preservation of myelin lamellar structure with a 170 A periodicity in nerves depleted of 98% of their lipids. Preservation of myelin lamellar structure depended on glutaraldehyde fixation and the introduction of osmium tetroxide in a nonpolar vehicle (CCl_4) after the lipids had been extracted. It is concluded that the periodic lamellar structure in electron micrographs of myelin depleted of lipid results from the complexing of osmium tetroxide, plus uranyl and lead stains, with protein.

INTRODUCTION

Myelin, a complex of lipid and protein, has been the subject of intense investigation during the past 15 yr. A variety of techniques including X-ray diffraction, electron microscopy, and chemical analyses have produced a large body of information on this unique biological structure (see reviews in references 1–3). From electron microscopic observations of myelinated nerves, Robertson developed the concept of the unit membrane, which he later postulated to be common to all cell-membrane systems. The unit membrane in his hypothesis consists of two electron-opaque zones separated by a region of lesser density. Each of the three components has a dimension of approximately 25 A in $KMnO_4$-fixed tissues. In many respects, this description bears a resemblance to the classic membrane model postulated by Danielli and Davson (4). However, the densities observed in electron micrographs of membranes have been variously interpreted as being due to the protein component of the membrane, the lipid in the membrane, or the resulting complex between protein and lipid (5).

Recent studies in which lipids have been extracted or enzymatically digested from either tissues or cell organelles prior to treatment with osmium tetroxide have resulted in discordant observations with respect to the role of lipids in the preservation and/or visualization of membrane structure. The unit membrane was preserved in the cristae of lipid-depleted mitochondria (6) and in muscle microsomes treated with phospholipase

C (7). Membrane structures were not observed, however, in lipid-extracted lung (8) or liver (9).

Studies from our laboratory on the small intestine of cats (10) indicate that the fine structure of cell membranes and cell organelles can indeed be maintained and visualized after lipid extraction. The nature of this organ (variations of cell populations in the mucosa and submucosa) did not lend itself to a critical, correlative chemical and morphological study. In view of (*a*) the existing biophysical and biochemical studies of myelinated nerve, (*b*) the relative homogeneity of the tissue, and (*c*) the demonstration that myelin is developed as an elaboration of the Schwann-cell membrane, a correlated biochemical and morphological study of rat sciatic nerve was undertaken. In order that the chemical and morphological studies could be correlated as closely as possible, the chemical analysis to determine extent of removal of lipids was performed on the same nerve from which electron micrographs were prepared.

This report will be concerned with the following: (*a*) techniques for preserving the fine structure of myelin in rat sciatic nerve depleted of lipid (*b*) the thesis that membrane protein can be the binding site of osmium tetroxide when the lipids are essentially removed from the tissue.

METHODS

Extraction of Lipids and Tissue Preparation

FRESH NERVES: (*a*) Rats weighing 300–350 g were anesthetized with ether. Sciatic nerves were exposed, dissected free from surrounding soft tissues, excised, and placed in tared, ground glass, stoppered weighing bottles; and the fresh weight was obtained. Chloroform:methanol (2:1 v/v) was added to the bottle and the nerves allowed to soak 24 hr at room temperature in the stoppered weighing bottles. After that time, the nerves were transferred to a second tared weighing bottle containing the same solvent and were agitated briefly; the rinsing solvent was pipetted back into the first bottle. This rinsing was repeated four times, and then an acidified solvent (chloroform:methanol:concentrated hydrochloric acid; 200:100:1 v/v/v) was added to the nerve in the second bottle. After soaking for 1 hr at room temperature the nerve was rinsed with acidified chloroform–methanol in a manner similar to the previous rinsings with the neutral solvent. Both extracted nerves and extracts were dried to constant weight at room temperature with a stream of N_2.

FIXED NERVES: (*a*) Rats weighing 300–350 g were anesthetized with ether. Sciatic nerves were exposed and were gently dissected free from surrounding soft tissues; the region was flooded with cold 0.2 M cacodylate-buffered glutaraldehyde (pH 7.2). The fixative was 315 milliosmols as determined by freezing-point depression (Advanced Osmometer, Advanced Instruments, Inc., Newton Highlands, Mass.). After a 5 min period, the nerve was excised and transferred to a fresh vial of fixative for 24 hr. The nerves were then washed in cacodylate buffer for a total time of 30 min (five changes of 5 min each). Dehydration was accomplished in acetone, 30, 50, 70, 85, and 95%, and finally anhydrous acetone, a total elapsing time of 20 min. The nerves were then transferred to tared, ground glass, stoppered weighing bottles and were extracted in chloroform:methanol followed by acidified chloroform:methanol as described above for the fresh nerves. The acetone used in dehydration was pooled and stored for further analysis (see below). After the last acidified chloroform–methanol rinsing each nerve was cut in half. One portion was transferred to osmium tetroxide dissolved in CCl_4 (5 g/100 cc) for 10 min. These nerves were then rinsed in absolute ethyl alcohol (two changes of 10–15 min each) and transferred to anhydrous propylene oxide. After 30 min the tissues were placed in a solution of propylene oxide and a complete Epon 812 mixture (1:1) and were rotated for 30 min. The tissue was finally infiltrated in a complete Epon mixture on a rotator for 60 min, after which it was flat embedded and polymerized in a 60°C oven overnight. The blocks were trimmed, and thick (1–2 μ) and thin (silver-gold interference color) sections were cut on a diamond knife with a Huxley-Cambridge ultramicrotome. The thick sections were examined in a Zeiss photomicroscope with phase optics. The thin sections were picked up on unsupported, 300 mesh, athene grids, and were stained in aqueous or alcoholic uranyl acetate (11), 3 min, followed by lead citrate (12), 3 min. In addition, nonstained grids were also examined in a Philips EM 200 electron microscope at an accelerating voltage of 60 kv. Electron micrographs were taken at magnifications of 12,500–41,000 on Kodak contrast plates (Eastman Kodak Co., Rochester, N.Y.).

The other halves of the nerves and corresponding extracts were dried to constant weight and analyzed for fatty acids (see below).

In addition to the correlated chemical and morphological studies on nerves, fine structural analyses were attempted after modifications of the above regime. Descriptions of these modifications follow.

(*b*) After the final extraction in acidified chloroform–methanol the tissues were gently rehydrated in decreasing concentrations of acetone, ending in a phosphate buffer (0.2 M, pH 7.4). After two to three changes of phosphate buffer the tissues were placed in 1% osmium tetroxide buffered with phosphate (pH 7.4) for 1 hr. Following this, the nerves were

dehydrated and embedded in the manner already described.

(*c*) Sciatic nerves not fixed in glutaraldehyde were extracted in the same manner as described above. After exposure to 5% osmium tetroxide in CCl_4 for 10 min they were embedded; similar nerves, after the extraction procedure, were gently rehydrated and then placed in a solution of 1% osmium tetroxide buffered with phosphate (pH 7.4) for 1 hr. The nerves were then dehydrated and embedded in Epon 812.

(*d*) Sciatic nerves were fixed in cacodylate-buffered glutaraldehyde (pH 7.2; 315 milliosmol) for 2–24 hr, washed in buffer (five changes of 5 min each) and then placed in 1% osmium tetroxide in phosphate buffer (0.2 M, pH 7.4) for 1–2 hr. The tissues were then dehydrated, embedded, and examined in a manner similar to the extracted nerves.

Fatty-Acid Analysis by Gas Chromatography

Prior to analysis by gas-liquid chromatography the "lipid depleted" tissues and the lipid extracts derived from evaporated chloroform:methanol, acidified chloroform:methanol, and pooled acetone were subjected to saponification by refluxing for 2 hr in a 2 N KOH–CH_3OH mixture. The resulting mixtures, extracted once with *n*-hexane to remove nonsaponifiable material, were acidified to approximately pH 3.0 and extracted four times with *n*-hexane (20 ml per extraction) and once with 20 ml diethyl ether. The combined organic phase, washed once with equal volumes of water, was evaporated to dryness and subjected to esterification with diazomethane (13).

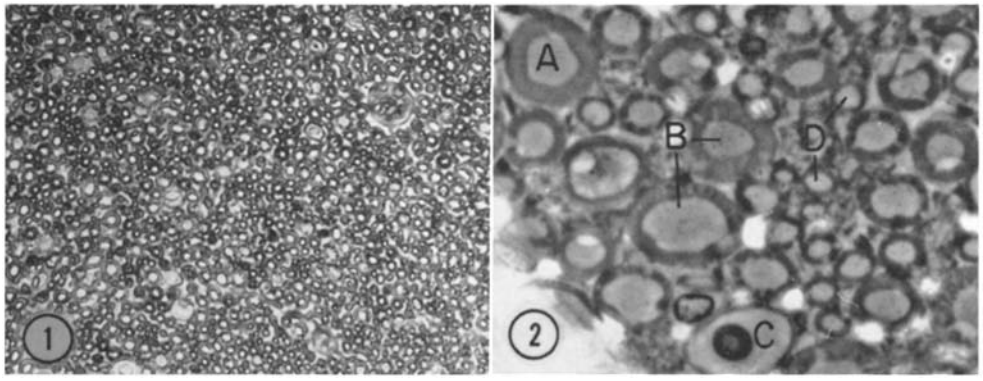
Methylesters of the fatty acids of the various fractions were analyzed with a Beckman GC-4 instrument employing a hydrogen flame ionization detector and helium-carrier gas. The instrument was operated at near maximum sensitivity (attenuation 2×10^2) and minimum noise level. A 12 ft, stainless steel column ⅛ inch OD packed with 15% butanediolsuccinate on Chromosorb W (80–100 mesh) (Perkin-Elmer Corp., Norwalk, Conn.) was employed at 215°C with a flow rate of 60–100 ml/min. Detector response and column efficiency were quantitatively calibrated by interspersing a standard mixture, K-102, with each group of samples (K-102

TABLE I

Extent of Removal of Fatty Acids from Rat Sciatic Nerves

Extraction procedure	Sample No.	Fatty acids remaining in extracted nerves: Fatty acids per mg dry wt of residue	Fatty acids remaining in extracted nerves: Total fatty acids of fresh tissue found in extracted residue
		μg	%
Extraction without fixation*	1	5.62	0.8
	2	7.37	1.7
Extraction after glutaraldehyde fixation‡	3	22.9	3.1
	4	31.5	4.7
	5	26.5	4.2
	6	45.1	6.3
	7	24.4	4.9
	8	18.8	3.6

* Groups of three whole nerves processed together.
‡ Individual half nerves analyzed. The remaining one-half of each nerve was processed for electron microscopy.



FIGURES 1 and 2 Thin section (1–2 μ) of Epon-embedded rat sciatic nerve photographed with phase optics. The tissues were fixed in glutaraldehyde, lipid extracted, and stained in osmium tetroxide in CCl_4 (5 g/100). The lettering in Fig. 2 is explained in the text. Fig. 1, × 200; Fig. 2, × 1500.

Applied Science Laboratories Inc., State College, Pa.; composed of methyl esters of C14:0, C16:0, C16:1, C18:0, and C18:1). Quantitative results with Applied Science Laboratories, Standard K-102 agreed with the stated composition data with a relative error less than 6% for major components (>10% of total mixture) and less than 3% for minor components (<10% of total mixture). The carbon numbers of the fatty acids were determined from semilogarithmic curves as described by Woodford and Van Gent (14).

RESULTS

Extent of Extraction of Lipids

The extent of residual lipids in the nerves extracted by our procedure was determined by analysis for fatty acids, this parameter being certainly the most characteristic and specific for lipids. In both the fresh nerves and the glutaraldehyde-fixed half-nerves from which morphological observations were made, the fatty acids were

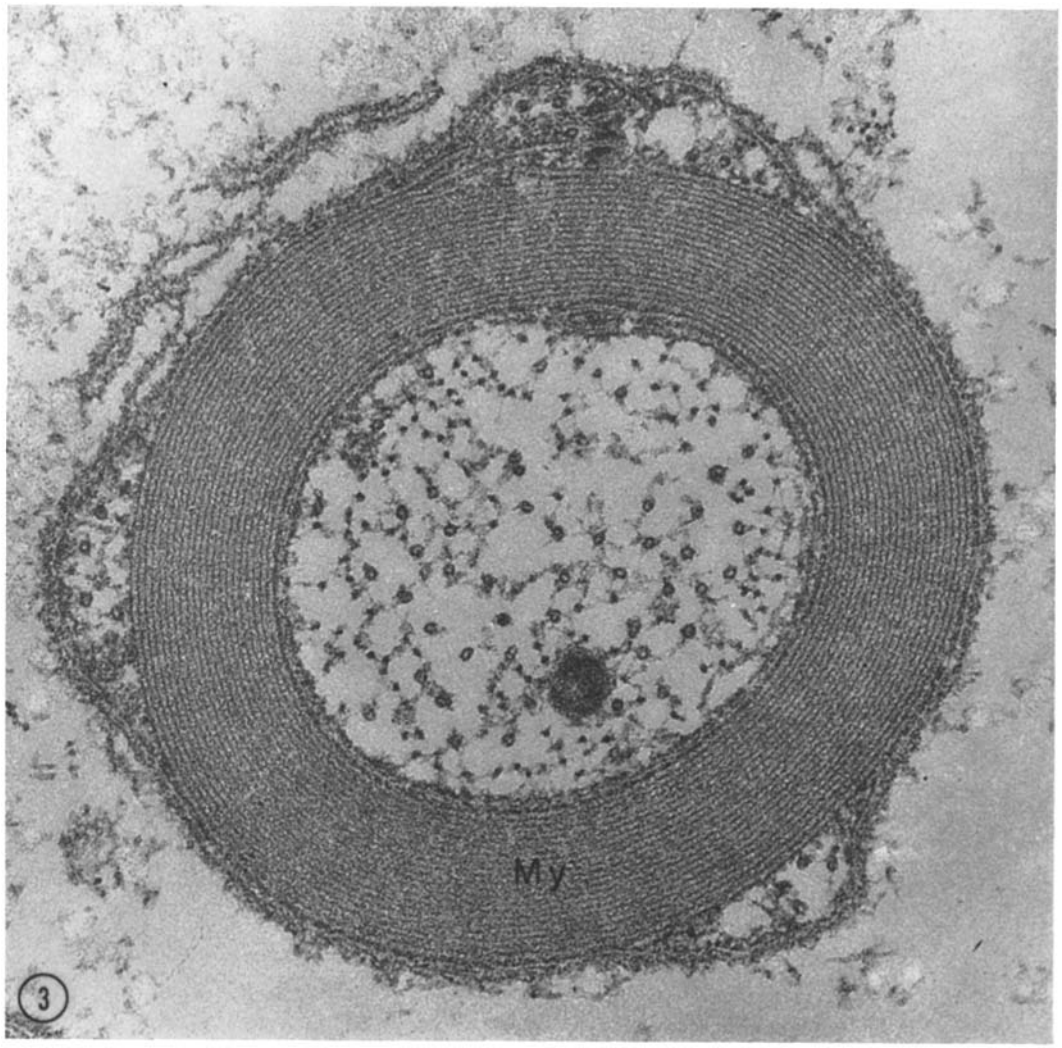


FIGURE 3 An electron micrograph of a myelinated fiber in a nerve (No. 3) depleted of its lipid. The periodic lamellar structure of myelin (*My*) has been sectioned at nearly right angles. Glutaraldehyde fixed; lipid extracted; osmium tetroxide in CCl_4. × 41,500.

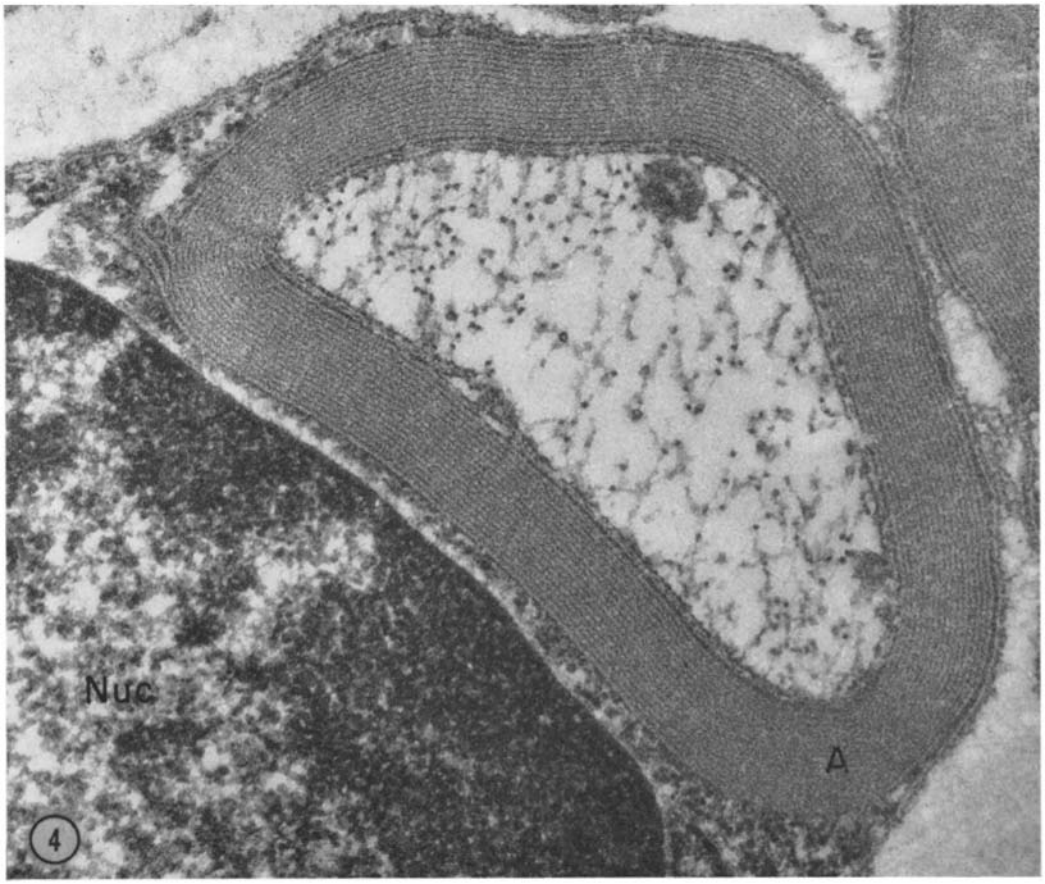


FIGURE 4 An electron micrograph of a lipid-depleted nerve. The lower left margin is occupied by the Schwann cell nucleus (*Nuc*). The lamellar structure of myelin at *A* has been sectioned at a plane slightly tangential to the normal. The major and minor periods are both preserved. Glutaraldehyde fixed; lipid extracted; osmium tetroxide in CCl_4, × 41,000.

determined directly after total saponification. All the extracts from nerves were also analyzed, and a value for total tissue fatty-acids was calculated as the sum of all these fractions. As shown in Table I, better than 98% of the total fatty acids had been removed during the extraction of unfixed nerve and about 1% of the dried tissue residue was fatty acids. The glutaraldehyde-fixed nerves contained slightly more fatty acids, 1.8–4.5% of the dried tissue residue. Nevertheless it is certain that at least 97% of the total fatty acids were removed from the tissue in the best extracted nerve (Table I, nerve 3).

In the course of the analysis for fatty acids in the various tissue residues and extracts, the proportions of the constituent fatty acids in each sample were determined. These proportions were not the same for all samples. For example, the patterns in the acidified chloroform:methanol extracts were similar for the different tissues but were different from the patterns in the neutral chloroform:methanol extracts from the same source. Of more pertinence to the present discussion, the proportions of the fatty acids remaining in the half-nerves analyzed after glutaraldehyde fixation were similar to each other but differed from the proportions of the fatty acids in the lipid extracts which had been obtained from these same nerves. Consequently, while the amount of lipid remaining in these nerves from which the electron micrographs were made was minimal, it possibly represents a residue of a particular type of lipid which is more difficult to

extract than the bulk of the tissue lipids. Since the primary purpose of this study was the quantitative removal of fatty acids, the most exacting precautions to minimize lipid peroxidation were not employed. It is possible that the qualitative patterns observed may differ in studies, now in progress, which utilize more sophisticated techniques.

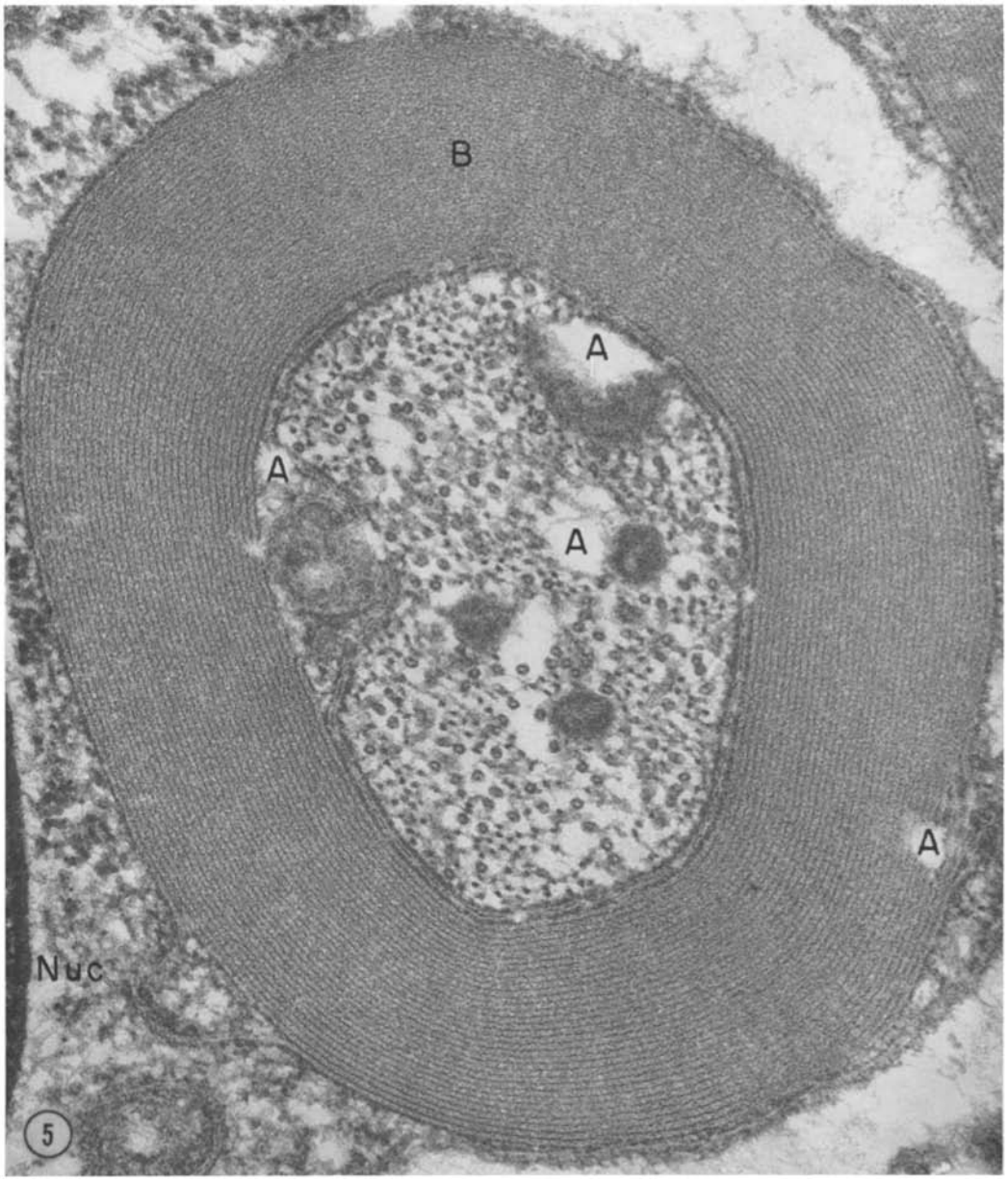


FIGURE 5 An electron micrograph of a myelinated axon from a lipid-extracted rat sciatic nerve. Imperfections at *A* probably result from damage in either polymerization or microtomy. The Schwann cell nucleus (*Nuc*) borders the left margin of the figure. The approximately 34 major periods become ill-defined at the top of the micrograph (*B*) owing to their plane of section. Glutaraldehyde fixed; lipid extracted; osmium tetroxide in CCl_4. × 59,000.

Morphology

LIGHT MICROSCOPY: Thin sections (1–2 μ) of lipid-extracted nerve stained with osmium tetroxide in CCl_4 were examined with phase optics (Figs. 1 and 2). The contrast between myelin and the axons was significantly reduced in lipid-extracted nerve compared to nonextracted nerve fixed in glutaraldehyde followed by phosphate-buffered 1% osmium tetroxide (Methods). The appearance of myelin in the extracted nerves of the larger fibers (above 8 μ) was variable. Myelin appeared as a homogeneous, uniform layer (*A* in Fig. 2) or had a mottled appearance (*B* in Fig. 2). In some cases (Fig. 2) a circumferential cleft (*C*) was observed between the myelin applied to the axon and the most peripheral lamellae. The majority of fibers below 8 μ in diameter (*D* in Fig. 2) possessed a myelin sheath of uniform density and appeared well preserved.

ELECTRON MICROSCOPY:[1] It was more difficult to obtain thin sections of lipid-extracted nerves than of nerves prepared with routine procedures. Shearing artifacts were not uncommon, especially of the large fibers. However, the preservation of large fibers with no imperfections in any myelin lamellae was difficult regardless of the preparative procedures employed. Microtomy and polymerization damage were the major contributors to these imperfections (Fig. 5).

Thin sections of lipid-extracted nerves not stained with uranyl and lead salts prior to examination in the electron microscope possessed low contrast. At visual magnifications above × 60,000 the periodicity of myelin could be observed but the inherent problem of contrast and focus made adequate photography difficult. Equivalent sections stained with uranyl acetate followed by lead citrate are illustrated in Figs. 3–5. The lamellar structure of myelin indicated by the regular repeating densities was readily observed. The morphological preservation and contrast were independent of the amount of lipid remaining in the tissue. In fact, some of the best preparations were obtained from nerves with the smallest per cent of fatty acids remaining after the extraction procedures (Fig. 3).

The appearance of the major and minor periods (densities) in myelin is influenced by the plane of section. If the plane of section is nearly perpendicular to the orientation of the lamellae, then the components are clearly defined (compare Figs. 3–5). However, even in ideal sections the major and minor periods are not so sharply defined in lipid-extracted nerves as in nonextracted nerves. In this study, the major period had a corded appearance, but no uniformly sized subunits were discerned, at least at the resolutions afforded. Each minor period density, although uniformly present, was less distinct than the major period. It was frequently interrupted at points for varying distances, but the apparent discontinuities may merely have reflected the rigorous procedures employed in the preparation of the tissues. As in the major period no fine structural subunit of uniform size was observed.

Measurements of a series of electron micrographs were made using a × 10 eye micrometer and micrographs enlarged 100,000–200,000 times. The initial magnification on the plates was calibrated by a diffraction grating (28,800 lines per inch, Ladd Research Industries, Inc., Burlington, Vt.) at the same objective-lens current and in series with the plates of the tissues to obviate significant alterations in lens voltage.

Owing to the quality of the image inherent in our material, the measurements are given in terms of maxima. A summary line drawing indicating

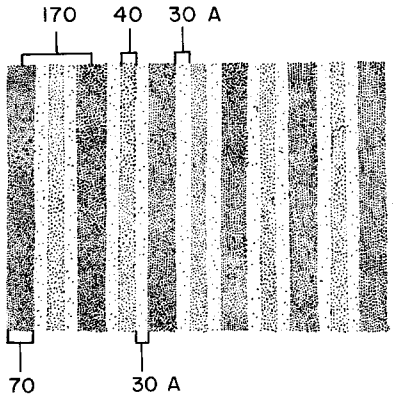


FIGURE 6. A drawing indicating the relative sizes of the components observed in lipid-depleted myelin. The distance of 170 A from the middle of one major period to an adjacent one is the most reliable figure. The measurements were made on electron micrographs enlarged to ×100,000–200,000. Dense stipple, major period; moderate stipple, minor period; occasional stipple, intraperiod spacing.

[1] This report will be confined to a description of the lamellar structure of myelin depleted of lipids. A detailed description of the fine structure of other cell membrane systems in both nerve and other tissues is in preparation.

the relative sizes of the components comprising the myelin sheath is depicted in Fig. 6. The most precise and reliable value, 170 A, is the distance from the center of one major period to the center of an adjacent major period. The other values, 70 A major period width, 40 A minor width period, and 30 A intraperiod width, are dependent upon the precise point at which measurements were obtained. The figures, other than the 170 A major period repeating unit, are an approximation and are not construed to be a precise unequivocal demonstration of the components comprising the fine structure of the myelin sheath.

Thin sections of sciatic nerves prepared as described in Methods (steps *b* anc *c*) displayed no periodic structure in the zone occupied by myelin, regardless of the plane of section. There were gross distortions in the region occupied by myelin and in most cases, where a compact segment of myelin was present, that segment appeared as a homogeneous density characterized by an indistinct granularity.

DISCUSSION

This study, combining chemical analysis with electron microscopy, offers an approach to a more precise interpretation of electron micrographs. Specifically, the application of the procedures outlined in this paper has (*a*) elaborated a technique for preserving the structure of myelin in rat sciatic nerve essentially depleted of lipid, and, (*b*) as a consequence, provided evidence that myelin protein constitutes a binding site for osmium tetroxide. It must be emphasized that the preservation of myelin lamellar structure was dependent on the stabilization of the protein with glutaraldehyde and the introduction of osmium tetroxide in a nonpolar (CCl_4) vehicle after the lipids were extracted from the tissue. This is evident from the observations that the lamellar structure of myelin was not preserved in either (*a*) glutaraldehyde-fixed nerves, depleted of lipid, rehydrated, and placed in a polar solution (phosphate buffer) of osmium tetroxide, or (*b*) nonfixed sciatic nerve, depleted of lipid, and placed in either a polar or nonpolar vehicle containing osmium tetroxide. These latter observations are in agreement with Finean and Rumsby et al. (1, 15) who found that the fine structure of myelin was essentially lost in electron micrographs of lipid-extracted nerves. The conflicting data (6–10) with respect to membrane preservation in cell organelles and organs subjected to various methods of lipid depletion may merely reflect the unique requirements necessary for the proper visualization of the membranes in those systems. Clearly, after lipid extracting procedures, the methodology employed in preparing tissues for electron microscopy becomes an important factor in attempts to correlate fine structure with chemical composition.

Korn (16) has recently emphasized the metabolic and functional dissimilarities between myelin and other cell membrane systems. However, it has clearly been established that myelin is elaborated by the Schwann cell (17) and is morphologically continuous with its plasma membrane. Indeed, Robertson (18) has demonstrated that the major and minor periods in myelin are derived from and are continuous respectively with the inner and outer leaflets of the unit membrane of the Schwann cell plasmalemma. Thus, myelin may still offer advantages as a system in which to correlate chemical and morphological observations as applied to membrane.

In the present study, chemical analysis revealed that the fatty acids constitute only 1.8–4.5% dry weight of the nerves examined in the electron microscope. The proposed area ratio of protein-to-lipid in normal myelin was approximately 0.43 (16). On this basis, removal of some 98% of the lipid would yield an approximate area ratio of protein-to-lipid of >14. Thus the contribution to the total area of our sections by lipid molecules was minimal. In addition, the actual content of residual lipid, although small in amount, varied from one sample to the other. Nevertheless, there was no significant variation in densities among the specimens. In view of these findings it appears justified to assume that the densities in the myelin lamellar structures in our electron micrographs are the result of complexing of osmium tetroxide (plus the uranyl and lead stains) with protein rather than lipid.

When preserved by the procedure employed in this report, the protein structure (major period) in myelin had a spacing of 170 A. This is in agreement with X-ray diffraction studies of fresh myelin (1) and is larger by some 50 A than previously reported electron micrograph measurements (19). The data support the notion that in three-dimensional arrangement fresh myelin contains spiral sheets of protein (representing the major periods) separated by approximately 170-A spacings. Since glutaraldehyde fixation was found to be essential

for the preservation and visualization of the protein structure, it seems likely that a chemical alteration of the protein had occurred. This could involve intramolecular and/or intermolecular linkages between amino, amide, or guanidyl groups on the proteins which stabilize the associations of adjacent units (20). The arrangement of the lipids within these spacings, whether globular or lamellar, cannot be directly deduced from our studies. Presumably the lipid–protein macromolecules are disrupted by the glutaraldehyde fixative and the lipid constituents are subsequently removed from the interstices between the protein layers by the chloroform:methanol.

Various authors have proposed both hydrophobic and electrostatic bonding as the principal means by which the lipids and proteins are associated in the myelin macromolecule. In all probability both types of bonding occur to varying degrees, and there is evidence (21) that the binding is electrostatic for at least one minor myelin lipid constituent. Our results could be interpreted as supporting the theory that this type of bonding may also be the more quantitatively significant. Thus, in contrast to the results obtained with mitochondria (6) we found it necessary to maintain a nonpolar medium in order to introduce osmium tetroxide after the removal of the lipids, otherwise the lamellar protein arrangement was not preserved. One could postulate that this nonpolar medium would, in fact, facilitate the aggregation of proteins of adjacent layers if they had been associated with intervening lipid by hydrophobic groups oriented radially. On the other hand, if the protein surface facing the intervening lipids was charged and associated with charged groups on the lipid, the maintenance of a hydrophobic (nonpolar) medium in this area after lipid removal could prevent the association of charges in adjacent protein layers and thus preserve the integrity of the fine structure.

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