Vol. 97, No. 1, 1980
November 17, 1980

BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS
Pages 139 - 147

LIPID ORGANIZATION IN PERIPHERAL NERVE: NATURAL ABUNDANCE CARBON-13 NMR SPECTROSCOPY ON INTACT TISSUES

Yuri Wedmid[a], Alfred C. Lais[b], Peter J. Dyck[b], and Wolfgang J. Baumann[a]

The Hormel Institute, University of Minnesota, Austin, MN 55912[a], and Department of Neurology, Mayo Clinic and Mayo Foundation, Rochester, MN 55901[b]

Received September 25,1980

SUMMARY

Native structures of peripheral nerve myelin can directly be observed by carbon-13 nuclear magnetic resonance (NMR) spectroscopy after dissecting epineurial and perineurial tissue sheaths from the endoneurium. NMR spectra of the intact endoneurial tissue describe a highly ordered and largely immobilized lipid phase in the myelin membrane which is in marked contrast to the fluid-like lipid distribution in the intracellular lipid droplets of epineurium and perineurium. Membrane reconstitution experiments with total endoneurial lipids, but without protein, suggest a primary role of the lipids in myelin bilayer assembly. Reconstitution experiments also indicate that polar lipid-protein interactions in native myelin involve the CH_2-N function of choline phospholipids, in particular.

Peripheral nerve function is affected by the molecular composition and the supramolecular architecture of nerve myelin. Such structures and their specific molecular interactions can, in principle, directly be studied by nuclear magnetic resonance (NMR) spectroscopy (1,2) on intact tissue. Earlier attempts to examine nerve myelin by proton (3) and carbon-13 (4) NMR on whole sciatic nerves were hampered, however, by the occurrence of intense and interfering resonances due to the adipose tissue-type fat droplets (4) present in the nerve tissue sheaths.

Peripheral nerve consists of morphologically and functionally distinct tissues, namely the endoneurium, perineurium, and epineurium (5,6). The endoneurium contains fascicles of myelinated and unmyelinated nerve fibers, associated with their supporting cells, and longitudinally oriented collagen fibrils; these in turn, are ensheathed by a lamellar arrangement of flattened neuroepithelial cells, the perineurium. The epineurium is the connective tissue matrix containing blood vessels that surrounds the perineurially ensheathed fascicles.

0006-291X/80/210139-09$01.00/0
Copyright © 1980 by Academic Press, Inc.
All rights of reproduction in any form reserved.

The present study shows that after careful separation of the intact opalescent endoneurium from the gel-like epineurium and perineurium, natural abundance ^{13}C NMR on the native neural tissues can provide information on the lipid organization in peripheral nerve myelin which is distinct from that of the connective tissue sheaths. Moreover, myelin lipid reconstitution followed by NMR can shed light on inherent lipid properties and specific interactions that prevail in myelin membranes.

MATERIALS AND METHODS

Sciatic nerves (2-3 cm in length) were excised from male Sprague-Dawley rats (250-400 g; Biolab Corp., St. Paul, MN) after intraperitoneal injection of sodium pentabarbital. The epineurial and perineurial sheaths were stripped from small nerve segments (1 cm) by forceps under a stereomicroscope (x 10) in glucose-free Tyrode solution (7) on a cooling stage (5-10°C) to obtain the intact endoneurium containing the nerve fibers.

Lipids were extracted from epineurium plus perineurium, and from endoneurium, after mincing and homogenization using chloroform:methanol, 2:1 (v/v) (8). Thin-layer chromatography (TLC) on 0.3-mm layers of silica gel H (Merck) and densitometry after charring showed that the epi- plus perineurial lipids almost exclusively consisted of triglyceride (> 95%, R_f 0.71) with traces of fatty acid (R_f 0.58), cholesterol ester (R_f 0.94) and polar lipids (R_f 0.0) present (developing solvent, hexane:diethyl ether:acetic acid, 80:20:1, v/v/v). TLC of the endoneurial lipids (developing solvent, chloroform:methanol:water, 65:25:4, v/v/v) showed the presence of sphingomyelin (R_f 0.05), phosphatidylcholine (R_f 0.13), cerebroside sulfate (R_f 0.24), phosphatidylethanolamine (R_f0.40), hydroxy fatty acid-containing (R_f 0.56) and normal cerebrosides (R_f 0.62), and cholesterol (R_f 0.89). Quantitative data were in line with those reported in the literature (9,10). Unilamellar liposomes were prepared from total lipids of rat sciatic nerve endoneurium. The lipid extract was lyophilized, sonicated as a 2% solution (11) in modified Tyrode, and the vesicular suspension was brought to the final concentration by ultrafiltration. Thin-layer chromatography followed by densitometry showed that the lipid composition of the liposomes was identical to that of the initial lipid extract from endoneurium.

Natural abundance ^{13}C NMR spectra were measured at 20 MHz using a Varian FT-80 pulse Fourier transform instrument. Proton-decoupled spectra were recorded at 37 ± 1°C using 10-mm sample tubes; the tip angle was 90°; the acquisition time and repetition rate were 1.023 sec. The epi- plus perineurial tissue and the endoneurial tissue were uniformly packed in modified Tyrode solution, the tubes were centrifuged at 100 x g for 5 min to insure tight packing, and Teflon plugs were used to prevent vortexing. Modified Tyrode solution (7) was prepared glucose-free, and 99.5% D_2O instead of water was used for NMR field-frequency locking purposes; 1 mM NaN_3 was added to retard microbial activity. Chemical shifts are given relative to the ω-1 CH_2 resonance (peak 13, Fig. 1) of the long aliphatic chains (δ = 22.85 ppm) (12). Halfheight line widths were measured on spectra that were obtained without applying an exponential time constant to the free induction decay.

RESULTS AND DISCUSSION

The 20 MHz ^{13}C NMR spectrum of intact epineurial plus perineurial tissue from rat sciatic nerve shows distinct and narrow resonances which are readily assigned to specific constituent carbons of unsaturated triglyceride (Fig. 1, A). The signals correspond to C=O (peak 1) of the ester groups, CH=CH (peaks 2) of unsaturated aliphatic chains, CHO (peak 3) and CH_2O (peak 4) of glycerol, and to CH_2 (peaks 8-13) and terminal CH_3 (peak 14) of the long chains. The spectrum of triolein is given for comparison (Fig. 1, A, insert). The well-defined resonances produced by sciatic nerve epineurium/perineurium (Fig. 1, A) are only slightly broadened relative to those of neat triolein indicating a rather similar molecular mobility in both systems. Hence, the epineurium/perineurium spectrum is consistent with a nearly isotropic triglyceride distribution within the lipocyte-type lipid droplets in this tissue. Such a lipid distribution permits a high degree of free rotation and rapid reorientation of the triglyceride molecules.

The great similarity of the spectrum (Fig. 1, A) of the epi-plus perineurial tissue portions from rat sciatic nerve with that previously reported for whole canine sciatic nerve (4) confirms the contention of Williams *et al.* (4) that NMR observations on entire nerves (3,4) can provide information only on the organizational state of the triglyceride-rich epineurial matrix support tissue, whereas the motionally more restricted myelin structures remain invisible.

The structureless lipid droplets (1-2 μm diameter) in epineurial cells can, in fact, be readily observed by electron microscopy (Fig. 2, A). Thus, the ultrastructural information obtained by ^{13}C NMR complements rather well the electron microscopic evidence.

Fig. 1, B shows the ^{13}C NMR spectrum of intact rat sciatic nerve endoneurium containing the myelin-rich nerve fibers. The spectrum is characterized by its simplicity, low intensity, and by generally broadened

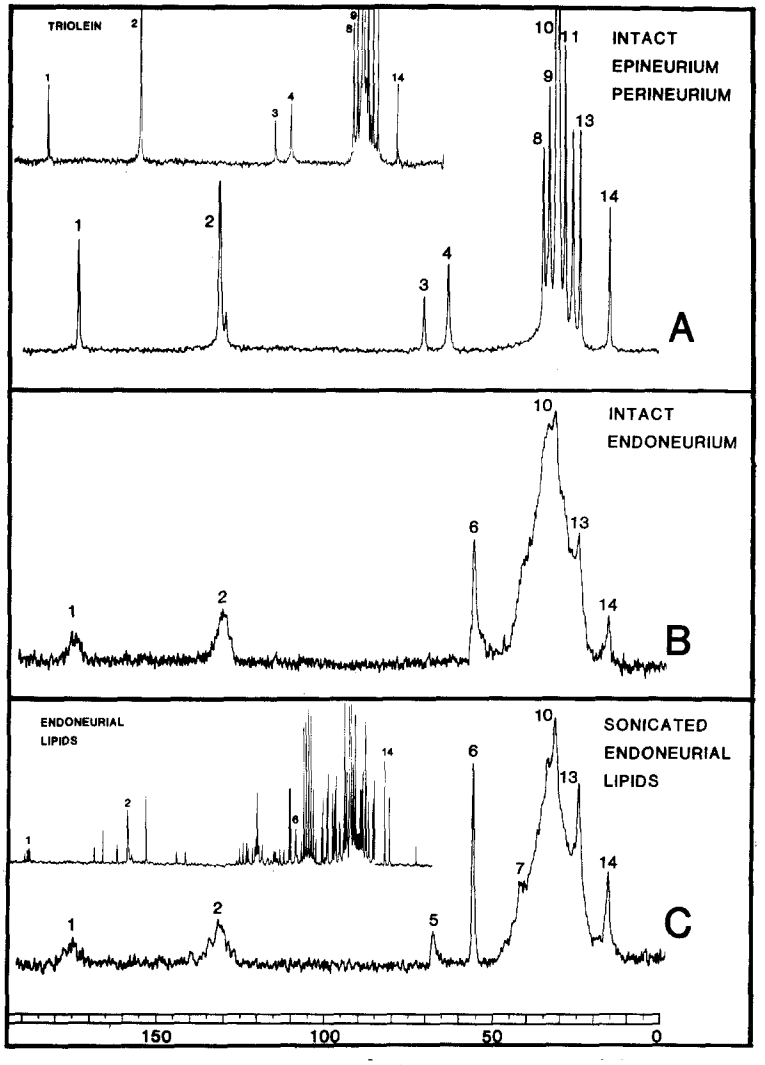

Figure 1. 20 MHz carbon-13 NMR spectra of rat sciatic nerve tissues and lipid preparations. - (A). Intact epineurium/perineurium; wet weight, 0.39 g; 100,000 transients. Resonances are observed at δ=171.84 ppm (peak 1, C=O), 129.76 and 128.13 ppm (peaks 2, $(CH_2)_n$-CH= and =CH-CH_2-CH=, respectively, 69.19 ppm (peak 3, CHO of glycerol), 62.00 ppm (peak 4, CH_2O of glycerol), 33.86 ppm (peak 8, $OCOCH_2$), 32.16 ppm (peak 9, ω-2 CH_2), 29.90 ppm (peak 10, $(CH_2)_n$), 27.35 ppm (peak 11, CH_2-CH=), 25.00 ppm (peak 12, $OCOCH_2CH_2$), 22.85 ppm (peak 13, ω-1 CH_2) (12), and 14.10 ppm (peak 14, ω CH_3). - (A, insert). Triolein (neat), using a coaxial D_2O capillary for field-frequency stabilization; 275 transients. Assigned peaks and chemical shifts agree with those of spectrum A within ± 0.08 ppm. - (B). Intact endoneurium; wet weight, 2.0 g, containing 0.355 g of lipid; 52,000 transients. Resonances are observed near 173.3 ppm (peak 1, C=O), 128.9 ppm (peak 2, CH=CH), 54.07 ppm (peak 6, $N(CH_3)_3$ of choline), 29.9 ppm (peak 10, $(CH_2)_n$), 22.85 ppm (peak 13, ω-1 CH_2) (12), and 14.13 ppm (peak 14, ω CH_3). - (C). Sonicated endoneurial lipids (132 mg lipid/ml) in modified Tyrode solution; 56,000 transients. Resonances are observed near 173.3 ppm (peak 1, C=O), 129.2 ppm (peak 2, CH=CH), 66.17 ppm (peak 5, CH_2-N of choline), 54.32 ppm (peak 6, $N(CH_3)_3$ of choline), 40.2 ppm (peak 7, tentatively assigned to CH_2-N of ethanolamine), 29.7 ppm (peak 10, $(CH_2)_n$), 22.85 ppm (peak 13, ω-1 CH_2) (12), and 14.06 ppm (peak 14, ω CH_3. - (C, insert). Endoneurial lipids dissolved in $CDCl_3$:CD_3OD:D_2O, 5:4:1 (v/v/v) at 100 mg/ml; 118,000 transients.

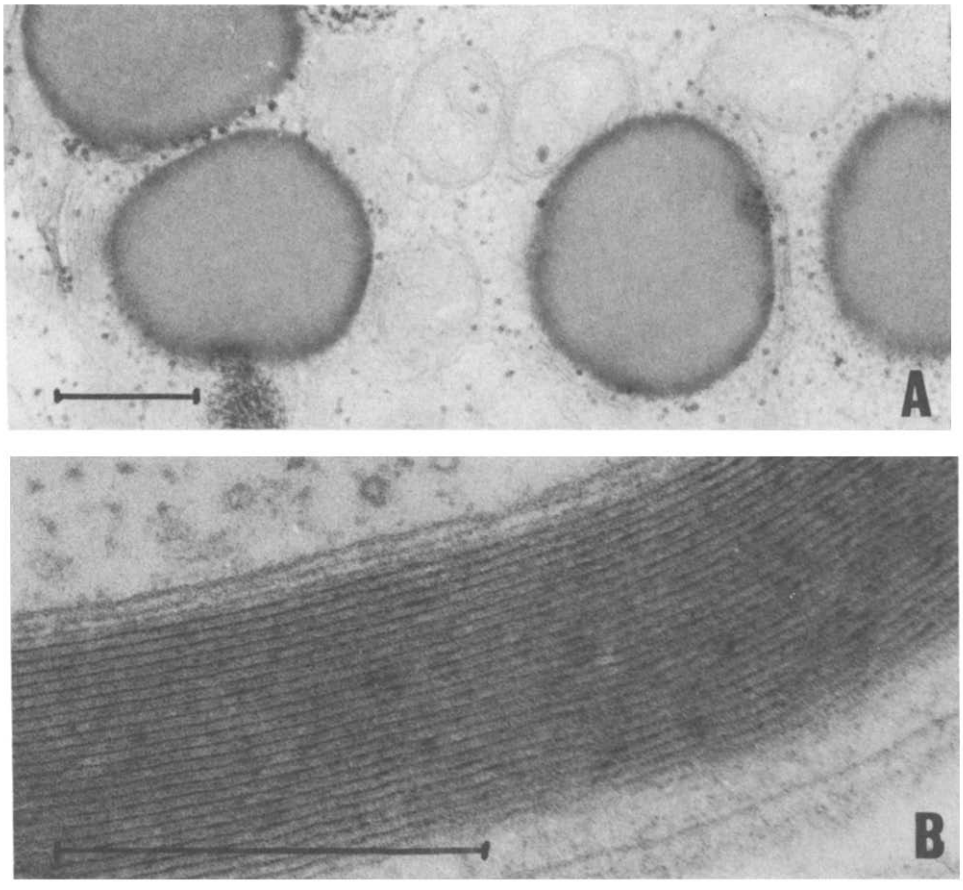

Figure 2. Electron micrographs of typical epineurial (A) and endoneurial (B) regions of rat sciatic nerve illustrating the droplet-like lipid distribution in mast cells (A) and the highly organized bilayered structures of myelin (B). The inserted scale equals 0.5 μm. Tissues were fixed in 3% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, washed, osmiated, dehydrated, and embedded into epoxy resin.

bands; signals characteristic of triglyceride are absent. Broad bands are observed in the C=O (peak 1), CH=CH (peak 2) and aliphatic (peaks 8-14) regions; a prominent and sharp resonance (peak 6) can be assigned to the trimethylammonium carbons of choline phospholipids. The spectrum (Fig. 1, B) of the intact endoneurium does not reflect the abundance and complex variety of lipids present in this tissue. Nerve myelin contains high percentages of cholesterol (34% of total lipids), phosphatidylcholine (21%), cerebrosides (20%), phosphatidylethanolamine (15%) and sphingomyelin (10%), but with the notable exception of $N(CH_3)_3$ (peak 6), signals characteristic for these

complex lipids are generally absent. The complexity and heterogeneity of the constituent myelin lipids become apparent, however, when the spectrum of the total lipid extract is measured in solution (Fig. 1, C, insert) where restrictive molecular interactions are minimized and signal intensities of the narrow line-width resonances largely reflect the relative abundance of the monomeric lipid species in the mixture. The low intensity of $N(CH_3)_3$ (peak 6) in the solution spectrum (Fig. 1, C, insert) in comparison to the relative prominence of the signal in the tissue spectrum (Fig. 1, B) is noteworthy and allows estimation of the extent of general line broadening that occurs for the signals of the highly ordered but motionally restricted hydrophobic myelin regions. In fact, the endoneurium spectrum in its simplicity and with its broad bands agrees with a lipid organization in peripheral nerve myelin in which rotational reorientation of lipid components is severely limited, segmental motions and the amplitudes of chain flexing are restricted (13), and in which lipid domains assume a physical state intermediate between liquid crystalline and gel (14). In this arrangement, only the choline $N(CH_3)_3$ function of phosphatidylcholine and sphingomyelin would retain the spatial separation and the degree of mobility necessary to produce a narrow resonance.

The concept of a highly ordered lipid organization within a rather rigid membrane arrangement in myelin, as can be deduced from NMR data (Fig. 1, B) , is quite consistent with the ultrastructural evidence. The electron micrograph (Fig. 2, B) of a myelinated nerve fiber illustrates quite well the high degree of order that extends into the multilamellar bilayer membrane system which appears to be a prerequisite for rapid nerve impulse transmission.

To assess the role of the lipids of myelin in the process of membrane assembly, and to define specific myelin lipid properties and interactions, the total lipids from rat sciatic nerve endoneurium were sonicated (11) and the ^{13}C NMR spectrum was measured. We observed (i) that the total endoneurial lipids readily formed, without selective lipid losses, unilamellar liposomes

as would lecithin (11,15) or a lecithin/cholesterol mixture (16), and (ii) that the ^{13}C NMR profile of the protein-free, artificially assembled myelin lipid vesicles (Fig. 1, C) was remarkably similar to that of native endoneurial tissue (Fig. 1, B). Both spectra (Figs. 1B and 1C) show broadened carbonyl bands (peaks 1) and broadened carbon chain resonances (peaks 2, and 8-14), indicating that a similarly organized and motionally restricted state exists in both systems, and both show a relatively sharp $N(CH_3)_3$ signal and lack signals due to cholesterol (13,15,16) and other major lipid constituent. This suggests that the high degree of order that exists within the bilayers of normal peripheral nerve myelin is foremost a lipid property, and that the constituent endoneurial lipids play a crucial role in myelin membrane assembly.

Despite the general similarity of the endoneurium spectrum (Fig. 1, B) and the spectrum of the reconstituted membrane preparation (Fig. 1, C), subtle but informative differences do exist in the phospholipid polar headgroup and in the aliphatic regions. Noteworthy is the emergence of a prominent signal (peak 5; relative intensity, ~35% of $N(CH_3)_3$) in the spectrum of the protein-free membrane preparation which can be assigned to $\underline{C}H_2$-N of the choline phospholipid headgroup; significantly, the appearance of CH_2-N (peak 7) from ethanolamine phospholipids is also indicated. Furthermore, the $N(CH_3)_3$ signal (peak 6) produced by the reconstituted membrane is considerably sharper (halfheight line width, $\nu_{1/2}$ = 8.5 Hz), than that in the endoneurium spectrum ($\nu_{1/2}$ = 21 Hz). This strongly suggests that the CH_2-N functions of choline phospholipids, as well as $N(CH_3)_3$, are quite exposed in the protein-free membrane and hence retain a higher degree of mobility than they do in native myelin. Relative immobiliztion of specific phospholipid polar headgroup regions in the intact endoneurium can well be rationalized by specific binding to structural myelin proteins. These proteins would to a considerable extent be located along the polar phospholipid bilayer surface where they can exert specific bonding upon the CH_2-N function of lecithin and sphingomyelin, in

particular. Further evidence in support of the concept of specific choline phospholipid CH_2-N to myelin protein binding has recently emerged from our experiments in which binding was disrupted by exposure of nerve myelin to sublytic amounts of lysolecithin (0.5 mg/100 mg myelin lipid). Such interference with lipid-protein binding resulted in a more than five-fold increase in CH_2-N signal intensity relative to $(CH_2)_n$.

In addition to such strong evidence for specific polar lipid-protein interactions in peripheral nerve myelin, more subtle differences in spectral line width of the aliphatic signals (peaks 10, 13, 14) for the native (Fig. 1, B) and the protein-free systems (Fig. 1, C) point toward nonpolar interactions involving the aliphatic lipid chains and possibly the more hydrophobic protein regions. Thus, halfheight line widths measured were $\nu_{1/2}$ = 215 Hz for $(CH_2)_n$ (peak 10) and $\nu_{1/2}$ = 12 Hz for $\omega\, CH_3$ (peak 14) for the endoneurium, and $\nu_{1/2}$ = 165 Hz for $(CH_2)_n$ and $\nu_{1/2}$ = 9 Hz for $\omega\, CH_3$ for the protein-free, reconstituted myelin.

We realize that signal sharpening in the spectrum of the protein-free vesicles could at least in theory be interpreted in terms of higher tumbling rates (17); however, because similar results were obtained when multilayered liposomes instead of single-walled vesicles were used, aggregate size can be ruled out as a major determinant in causing line width differences between the native and reconstituted systems.

ACKNOWLEDGEMENTS

This study was supported in part by U.S. Public Health Service Research Grant NS 14304 from the National Institute of Neurological and Communicative Disorders and Stroke, by USPHS Research Grant HL 08214 from the Program Projects Branch, Extramural Programs, National Heart, Lung and Blood Institute, and by The Hormel Foundation.

REFERENCES

1. Opella, S. J. (1977) Science 198, 158-165.
2. Shulman, R. G., Brown, T. R., Ugurbil, K., Ogawa, S., Cohen, S. M., and den Hollander, J. A. (1979) Science 205, 160-166.
3. Dea, P., Chan, S. I., and Dea F. J. (1972) Science 175, 206-209.
4. Williams, E., Hamilton, J. A., Jain, M. K., Allerhand, A., Cordes, E. H., and Ochs, S (1973) Science 180, 869-871.

5. Key, A., and Retzius, G. (1876) Studien in der Anatomie des Nervensystems und des Bindegewebes, Samson and Wallin, Stockholm.
6. Thomas, P. K., and Olsson, Y. (1975) Peripheral Neuropathies, pp. 168-189, W. B. Saunders Co., Philadelphia.
7. Stedman's Medical Dictionary (1966) p. 1475, The Williams and Wilkins Co., Baltimore.
8. Folch, J., Lees, M., and Sloane Stanley, G. H. (1957) J. Biol. Chem. 226, 497-509.
9. Sheltawy, A., and Dawson, R. M. C. (1966) Biochem. J. 100, 12-18.
10. Klein, F., and Mandel, P. (1976) Lipids 11, 506-512.
11. Huang, C., and Thompson, T. E. (1974) Methods Enzymol. 32, 485-489.
12. Batchelor, J. G., Cushley, R. J., and Prestegard, J. H. (1974) J. Org. Chem. 39, 1698-1705.
13. Williams, E. C., and Cordes, E. H. (1976) Biochemistry 15, 5792-5799.
14. Oldfield, E., and Chapman, D. (1972) FEBS Lett. 23, 285-297.
15. Metcalfe, J. C., Birdsall, N. J. M., Feeney, J., Lee, A. G., Levine, Y. K., and Partington, P. (1971) Nature 233, 199-201.
16. Lundberg, B. (1977) Chem. Phys. Lipids 18, 212-220.
17. McLaughlin, A. C., Cullis, P. R., Hemminga, M. A., Hoult, D. I., Radda, G. K., Ritchie, G. A., Seeley, P. J., and Richards, R. E. (1975) FEBS Lett. 57, 213-218.