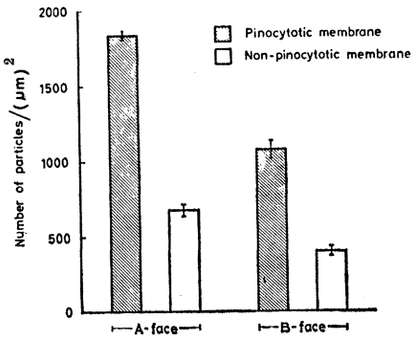
Fig. 2. Results of counting the membrane-associated particles on fracture faces A and B, within and outside the pinocytotic rows. The vertical bar at the top of each column indicates two standard errors of the mean. For both faces, the difference between the number of particles in the pinocytotic and nonpinocytotic membrane has a probability $P < .005$.



an area of 0.25 nm² on pictures magnified 100,000 times. This grid was placed alternately over pinocytotic and nonpinocytotic membrane, and a total of 2700 particles were counted. The results are presented in Fig. 2, which indicates that there are approximately twice as many particles in regions that show pinocytotic events as in regions that do not. This difference also holds for the B-face of the membrane (*8*), which has fewer particles than the A-face. Within the rows of pinocytosis, many particles are preferentially localized as necklaces around the craters (indicated by the dark arrows in Fig. 1B). This disposition is emphasized in Fig. 1C, which illustrates a zone near the border of one pinocytotic row. In addition, one sees a peculiar "necklace" arrangement of particles which is associated with an incompletely formed crater (dark arrow).

The orderly disposition of pinocytotic caveolae in smooth muscle cell has enabled us to show that (i) membrane-associated particles are increased in pinocytotic regions of the cell membrane compared to nonpinocytotic ones; (ii) in pinocytotic regions the particles often gather in a necklace or rosette formation around the individual caveolae; and (iii) in some cases necklaces of particles can be identified before clear-cut craters are seen. These observations might suggest that the distribution of membrane particles is related in some way to the formation of caveolae. Assuming that the particles represent specific functional sites in the membrane, we propose that the accumulation of such sites in one particular area of the membrane of the smooth muscle cell might be a necessary prelude to pinocytosis. The finding of particle aggregation at specific sites of the membrane (other than the well-known gap junctions) has precedents in other studies of freeze-fractured tissues. For example, longitudinal arrays of particles, the "ciliary necklaces," have been described at the base of the motile cilia in vertebrates and invertebrates (*10*). Even more relevant to our work are the studies by Satir *et al.* (*11*), who reported "rosette" arrangements of particles on the plasma membrane as a prelude to mucocyst discharge in the protozoan *Tetrahymena*. From their results and ours, it seems therefore that rosette figures occur at sites where membrane fragments are to be added to the preexisting plasma membrane (as in mucocyst discharge), or subtracted from it (as in pinocytosis).

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12. Supported in part by grants 3.553.71 and 3.8080.72 from the Swiss National Science Foundation. We thank M. Amherdt and M. Siperstein for help.

3 April 1973

Natural Abundance Carbon-13 Nuclear Magnetic Resonance Spectra of the Canine Sciatic Nerve

Abstract. *The proton-decoupled natural abundance carbon-13 nuclear magnetic resonance spectrum of the canine sciatic nerve is virtually identical to that of canine adipose tissue and markedly similar to that of liquid triolein. No resonances assignable to cholesterol, glycolipids, or sphingolipids are detectable in the sciatic nerve spectrum despite their abundance in the myelin sheath of this nerve. However, many such resonances are observed in lipid extracts of the nerve. Chromatographic analysis of specimens of canine and rabbit sciatic nerve has revealed that these contain sufficient triglyceride to account quantitatively for the observed spectrum. Proton nuclear magnetic resonance and spin-labeling results for preparations containing myelin, especially those derived from the peripheral nerve, should be critically examined for experimental artifacts reflecting the triglyceride content.*

Recent investigations into the structure and properties of biological membranes have made considerable use of the techniques of proton nuclear magnetic resonance (NMR) and electron spin resonance spectroscopy, particularly in order to study the dynamic properties of the alkyl chains of lipid molecules. Among these studies are several which present proton NMR spectra of myelin-containing preparations from the central nervous system (*1, 2*) and peripheral nerve (*3*). Two of these studies (*2, 3*) conclude, on the basis of line widths of proton resonances, that myelin contains lipids with alkyl chains in a fluid-like or liquid crystalline state. Other physical studies on myelin, however, suggest that the presence of cholesterol in the myelin sheath restricts the segmental motions of the lipid chains, thereby creating a physical state which is intermediate between liquid crystal and gel (*1, 4*). Here we explore the possibility that the reported proton NMR spectra of the myelin-containing preparations may actually reflect a mobile nonpolar lipid fraction rather than the lipids of the myelin sheath itself.

We have examined the natural-abundance ^{13}C Fourier transform NMR spectrum of canine sciatic nerve, which contains mostly myelinated fibers. All measurements were carried out at both 27°C and 38°C and several were done at 32°C as well. The results obtained were entirely independent of temperature.

Female mongrel dogs weighing between 10 and 20 kg were killed by intravenous injection of a 2.5 percent solution of sodium thiopental at a dosage of approximately 100 mg/kg. Portions 10 to 12 cm long of both sciatic nerves were excised and stripped of the surrounding fat and connective tissue. Both nerves were then packed as tightly as possible into a 13-mm NMR tube (inner diameter 11.7 mm) and covered with Ringer solution containing sodium oxacillin (0.5 mg/ml). The proton-decoupled ^{13}C NMR spectrum of such a preparation is shown in Fig. 1A. Except for a very weak resonance at 138.2 parts per million (ppm) (peak 7 in Fig. 1A), the spectrum is indistinguishable from that of a sample of canine adipose tissue (Fig. 1B). It is also nearly identical to that of neat liquid triolein (Fig. 1C), except for the presence of two additional weak resonances in the spectrum of the sciatic nerve (peaks 4 and 13 in Fig. 1A) which are readily assigned to a small fraction of linoleyl groups (*5*). The resonance at 138.2 ppm (peak 7 in Fig. 1A) can be assigned to the choline trimethylammonium carbons of phospholipids or sphingolipids. Other assignments and some integrated intensities are shown in Table 1. Conspicuously absent, even after 25,000 spectral accumulations, are resonances characteristic of cholesterol (*6*), of the sugar moieties of glycolipids, and of the sphingosine moiety of sphingolipids, all of which are present in appreciable quantities in myelin (*7*).

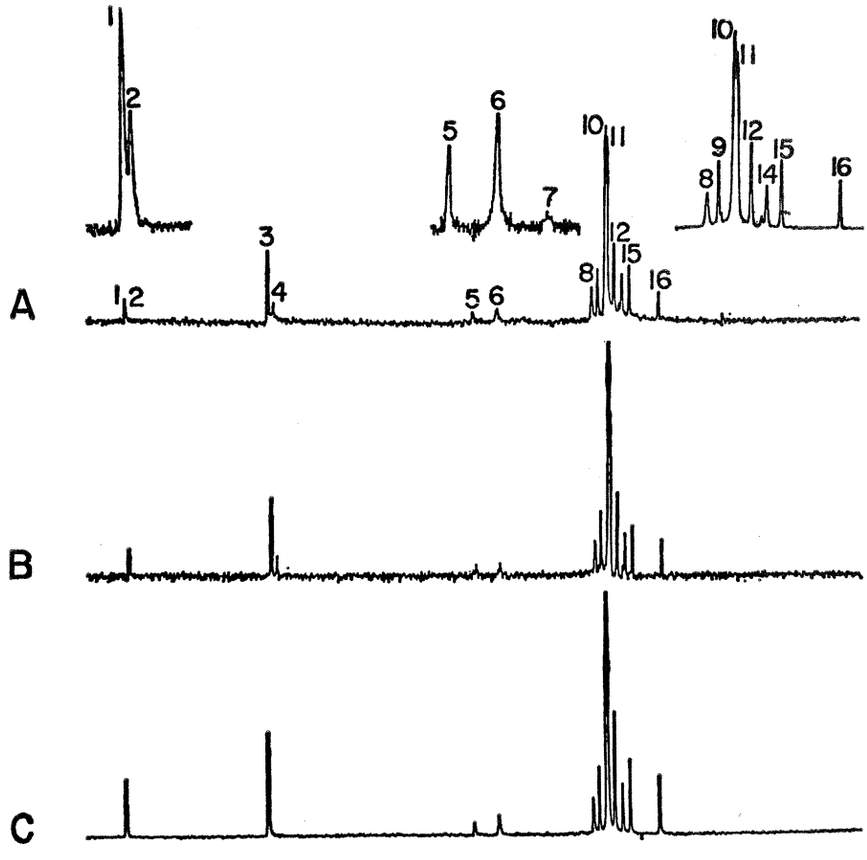

Fig. 1. Proton-decoupled natural abundance ^{13}C Fourier transform NMR spectra at 15.18 Mhz. (A) Canine sciatic nerve (dry weight 0.72 g, two nerves). Chemical shifts are given in Table 1. The main spectrum was recorded at 32°C with a digital resolution of 2 hertz, after 2048 accumulations with a recycle time of 0.8 second. The expanded scale insert of the carbonyl resonances (peaks 1 and 2) was recorded at 27°C with a digital resolution of about 0.3 hertz, 2751 accumulations, and a recycle time of 16 seconds. The other expanded scale inserts were recorded at 38°C with a digital resolution of 1 hertz, 26,000 accumulations, and a recycle time of 1.2 seconds. (B) Canine adipose tissue (dry weight 0.28 g), recorded at 32°C with a digital resolution of 2 hertz, after 256 accumulations, with a recycle time of 0.8 second. (C) Neat liquid triolein, recorded at 40°C with a digital resolution of 2 hertz, after 4 accumulations with a recycle time of 32 seconds. A sample tube 13 mm in diameter was used to get spectra A and B. Spectrum C was obtained in a 20-mm tube.

Two features in the observed spectrum are particularly significant. First, the carbonyl region contains two resonances, one about twice as intense as the other (peaks 1 and 2 in Fig. 1A). This is consistent with a triglyceride, but not with a phospholipid, sphingolipid, or glycolipid structure. Second, in the glycerol region there are also two resonances (peaks 5 and 6) with intensities in the ratio 1 : 2 (Table 1). Moreover, the integrated intensity of the single glycerol CH_2 peak is about two-thirds that of each fatty acyl carbon. On the basis of known chemical shifts (*5*, *8*) these results are only consistent with a triglyceride structure. A rough calculation based on the relative intensities in ^{13}C spectra of neat liquid triolein and canine sciatic nerve indicates a triglyceride content in the latter of about 10 to 15 percent of the dry weight. The integrated intensities (Table 1) indicate the presence of about 15 percent linoleyl-type fatty acyl chains, about 65 percent oleyl-type chains, and about 20 percent saturated species.

To establish the full complement of ^{13}C NMR signals potentially observable in the canine sciatic nerve, one such nerve, which had been preserved in ethanol, was homogenized in equal volumes of 1-pentanol and water. The layers were separated and the organic one was dried with sodium sulfate. The ethanol in which the nerve had been preserved was added to the 1-pentanol layer and the solvents were removed by evaporation under reduced pressure; the residue was dissolved in a 5 : 1 mixture of chloroform and methanol. A ^{13}C NMR spectrum of this solution revealed a total of 54 distinguishable peaks, about two-thirds of which we have tentatively assigned, on the basis of known chemical shifts, to various carbon atoms of cholesterol, phospholipids, and glycolipids. If these components had been present in a mobile phase in the intact sciatic nerve, their ^{13}C resonances would have been observed.

To determine whether the nerve preparation contained appreciable quantities of triglyceride, a lipid extract was prepared by soaking the nerves in a 2 : 1 chloroform-methanol mixture for several days at 4°C. The extract was subjected to thin layer chromatography

on alumina plates with a chloroform-methanol-water (64 : 24 : 4) solvent system and developed with iodine. Comparison with commercially obtained reference compounds showed that the extract contained significant amounts of triglyceride and cholesterol, and somewhat smaller amounts of sphingomyelin, phosphatidylcholine, phosphatidylethanolamine, and cerebroside. This is in general agreement with the results of Berry *et al.* (*7*), who found that triglyceride and cholesterol were the most prominent lipids in the cat sciatic nerve, comprising 35 and 17 percent by weight, respectively, of the total nerve lipids. These investigators also found that triglyceride comprised about 7 percent of the wet weight of the nerve, which on the basis of our experience would correspond to about 14 percent of the dry weight, since the fresh nerve contains about 50 percent water, as shown by weight loss after lyophilization.

Previously published proton NMR spectra of rabbit sciatic nerve (*3*) strongly resemble a proton spectrum of liquid triolein that we recorded at 220 Mhz. To evaluate the possibility that the reported proton spectra of rabbit sciatic nerve reflect the presence of triglyceride in the nerve, we determined the amount of triglyceride present in a comparable sample. Four female New Zealand white rabbits were stunned by a blow to the head and killed by exsanguination. Segments of sciatic nerve 3 to 4 cm long were excised and cleaned under a dissecting microscope to remove as much of the surrounding adipose and connective tissue as possible. A 220-Mhz proton NMR spectrum of a sample of nerve was obtained on a Varian HR-220 spectrometer operating at a probe temperature of 16°C. The spectrum was similar to that previously published (*3*), except that the lines were somewhat narrower in the published spectrum. The combined nerves were then lyophilized and weighed, and extracted twice with 20-ml portions of petroleum ether. The extract, when evaporated to dryness, was found to comprise about 30 percent of the dry weight of the nerves. The extract was redissolved in petroleum ether, chromatographed on alumina-coated plates with a chloroform-methanol-water solvent system (90 : 10 : 2), and developed with iodine vapor. The chromatogram revealed two spots of roughly equal intensity, one of which had an R_F value of 0.9, identical to that of commercially obtained triolein, and the other an R_F of 0.75, identical to that of cholesterol. To separate the two components, the extract was passed through a column of Florisil according to the procedure of Carroll (*9*). Fractions were obtained which contained material corresponding to each of the spots on the thin-layer plates. The fraction containing triglyceride was evaporated to dryness and weighed; its weight was 20 percent of the dry weight of the nerves. When dissolved in carbon tetrachloride this fraction gave a 220-Mhz NMR spectrum nearly identical to that of commercial triolein.

Our results suggest that the spectra of rabbit sciatic nerve obtained by Dea *et al.* (*3*) may not represent the lipids of the myelin sheath, but rather triglycerides which are present in the nerve interstices presumably in fat cells. The failure to observe resonances attributable to cholesterol, which comprises 17 percent by weight of sciatic nerve lipids (*7*), or to other lipids present in appreciable amounts in peripheral nerve myelin, indicates that these are immobilized to a marked degree and thus yield very broad undetectable resonances. Fast rotational reorientations in the triglyceride component yield narrow resonances, so that even though there is less than 20 percent triglyceride, it produces the only easily detectable NMR signals.

Table 1. Chemical shifts, assignments, and integrated intensities of the ^{13}C resonances of canine sciatic nerve at 38°C. Chemical shifts are expressed in parts per million upfield from CS_2, and have an estimated accuracy of ± 0.3 ppm. The terminal methyl group of fatty acyl chains at 178.5 ppm (*5*) was used as an internal reference. Assignments are based on known chemical shifts of triolein (*5*), trilinolein (*5*), and lecithin (*8*). Specific carbon assignments are indicated by **C**. Integrals were obtained digitally from a spectrum recorded with a digital resolution of 1 hertz, 26,000 accumulations, and a recycle time of 1.2 seconds (expanded scale inserts in Fig. 1A). Relaxation time measurements established that this recycle time was sufficiently long to get equilibrium intensities for all carbons except those of the terminal CH_3CH_2 groups. The integrated intensity of peak 8 (carbon 2) was normalized to unity. The estimated accuracy is ± 0.1 unit. Unless otherwise stated, one carbon from every fatty acyl chain contributes to the intensity. The integrated intensities were corrected for the frequency-dependent gain of our fourth-order Butterworth filter.

Peak in Fig. 1A	Chemical shift (ppm)	Assignment	Integrated intensity
1	21.0	Carbonyl*	
2	21.3	Carbonyl†	
3	62.9	$-CH=CH-CH_2-CH_2-$	1.6‡
4	64.5	$-CH=CH-CH_2-CH=CH-$	0.3§
5	123.5	Glycerol CH	0.3
6	130.6	Glycerol CH_2	0.6
7	138.2	$+$ $-N(CH_3)_3$	
8	158.8	$-CH_2-CH_2-CO-$	1.0
9	160.4	$CH_3-CH_2-CH_2-CH_2-$	1.0
10	162.6	$(CH_2)_n$	8.1‖
11	163.1		
12	165.3	$-CH_2-CH_2-CH=CH-$	1.3‡
13	167.0	$-CH=CH-CH_2-CH=CH-$	0.2¶
14	167.6	$-CH_2-CH_2-CO-$	0.9
15	169.8	$CH_3-CH_2-CH_2-$	#
16	178.5	CH_3-CH_2-	#

* Carbonyl group attached to CH_2 group of glycerol. † Carbonyl group attached to CH group of glycerol. ‡ Two carbons per oleyl-type chain and two per linoleyl-type chain contribute to this intensity. § Two carbons from each linoleyl-type chain contribute to this intensity. ‖ Multiple-carbon resonance. ¶ One carbon from each linoleyl-type chain contributes. # Not measured because the recycle time was too short to yield equilibrium intensities.

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10. Contribution No. 2202 from the Department of Chemistry, Indiana University. Supported by NIH grants AM-08232-09 and NS-10977-01.

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