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Natural Abundance Carbon-13 Nuclear Magnetic Resonance Studies of Bovine White Matter and Myelin†

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ABSTRACT: Whole bovine white matter yields a poorly resolved natural abundance ^{13}C nuclear magnetic resonance (NMR) spectrum. The spectrum principally reflects carbon atoms of the constituent membrane lipids: several resonances could be specifically assigned but no resonances attributable to cholesterol are detectable. Except for the methyl group at the terminus of fatty acyl chains, lipid carbons giving rise to the ^{13}C NMR spectrum have values of spin–lattice relaxation time between 140 and 500 ms, indicating significant restrictions on segmental and rotational mobilities but consistent with a generally fluid structural organization. The ^{13}C NMR spectrum of myelin isolated from bovine white matter is similar to that for the whole white matter itself. In both white matter and isolated meylin, the integrated intensities for several carbon atoms are considerably less than those for the same carbon atoms in total lipid extracts. The data for white matter and myelin are consistent with a model in which observed line broadening is due to restrictions in the amplitude of chain flexing rather than to severe restrictions on chain segmental motion. Failure to detect resonances of cholesterol ring system carbon atoms may reflect marked anisotropy of rotational reorientation.

Both ^{1}H and ^{13}C NMR[1] studies of myelin-containing preparations derived from peripheral nerve have been previously undertaken in an effort to derive information concerning the physical state of the lipids of the membranes comprising the myelin sheath (Dea et al., 1972; Williams et al., 1973). In neither of those studies, however, was it possible to detect many resonances arising from the lipids of the myelin itself, reflecting in part the broadness of these resonances and in part the interference from adventitial triglyceride (Williams et al., 1973). The studies on bovine brain white matter and central nervous system myelin reported herein represent an attempt to circumvent the latter problem by using myelin-containing preparations which are triglyceride free. Moreover, these preparations are obtainable in large quantities, thereby

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[1] Abbreviations used: NMR, nuclear magnetic resonance; ESR, electron spin resonance; NOE, nuclear Overhauser enhancement.

overcoming one aspect of the signal-to-noise problem associated with very broad resonances. The focal point of this work is to obtain new information pertinent to the molecular dynamics of the lipids of biological membranes.

Materials and Methods

White Matter. Whole fresh beef brains were obtained from a local slaughterhouse. These were packed in ice and used as soon as possible. White matter from the subcortical region and the corpus callosum was obtained by slicing the brains and removing grey matter with a Pasteur pipet attached to an aspirator. Chunks of white matter obtained in this fashion ranged in size from a few millimeters to as much as 3 cm in their longest dimension. These were prepared for NMR studies by rinsing in Ringer's solution and were placed whole into 20-mm NMR tubes to a volume of about 13 ml and covered with a few milliliters of Ringer's solution containing 1 mM sodium azide, and a Teflon vortex plug was inserted into the tube.

Myelin. Myelin was prepared from bovine white matter, isolated as described above, according to the method of Autilio et al. (1964). Electron micrographs of myelin preparations yielded the multilamellar structure typical of myelin. Chemical analysis indicated 74 wt % lipid and 24 wt % protein, in accord with literature values. For NMR studies, the loosely packed pellets from the final distilled water wash were resuspended in 0.4% NaCl containing 1 mM sodium azide and recentrifuged; the pellet was pipetted directly into a 20-mm NMR tube to a volume of about 13 ml, and a Teflon vortex plug was inserted into the tube.

Lipid Extracts. Lipid extracts of brain white matter were prepared according to the procedure of Folch et al. (1957) or in the following manner.

A sample of white matter having a wet weight of 34 g was lyophilized and 7.5 g (dry weight) was homogenized in 700 ml of 1:1 pentanol–water in a Waring Laboratory Blendor. The homogenate was filtered and the insoluble material from the filtration was washed with 200 ml of 1:1 chloroform–methanol and refiltered. The pentanol phase was combined with the filtrate from this wash and dried with sodium sulfate, and the solvent was removed on a rotary evaporator. Residual solvent was removed by placing the sample on a lyophilizer for several hours. However, it was difficult to remove all the pentanol from the extracted lipids (see Results). A lipid extract of myelin was prepared according to Autilio et al. (1964).

Carbon-13 NMR Measurements. The apparatus used to obtain most of the ^{13}C NMR spectra has been described previously (Allerhand et al., 1971, 1973). Unless otherwise indicated, measurements were made at 14.2 kG (^{13}C resonance frequency = 15.182 MHz) in spinning 20-mm (o.d.) sample tubes, with a sample volume of 10–13 ml. Probe temperatures used were between 30 and 60 °C. Line broadening due to magnetic field inhomogeneity in this system is usually on the order of 0.3 Hz (Allerhand et al., 1973). All spectra were recorded under conditions of noise-modulated proton decoupling. Typically on the 20-mm probe system, 5 W of decoupling power were used (higher powers gave rise to sample heating problems) with a noise bandwidth of 600 Hz, centered 4 ppm downfield from the proton resonance of tetramethylsilane. Integrals of peaks in the spectra of white matter, myelin, and their lipid extracts were obtained by tracing the peaks on heavy paper, cutting the tracings out, and weighing them.

Spin–lattice relaxation data were obtained using the inversion–recovery method, with a 180°–τ–90° pulse sequence, where τ refers to the time spacing between the 180 and 90° pulses (Vold et al., 1968).

Results

The natural abundance ^{13}C NMR spectrum of bovine white matter is shown in Figure 1A. Most of the peaks in the spectrum are attributable to carbons of the various lipids present in the sample; only two resonances, the arginine guanidino carbon and histidine C4 (numbers 5 and 8, in Figure 1A), represent carbons of the protein constituents. The broad peaks which dominate the spectrum are assigned to carbons in the fatty acyl chains of the various phospholipids and glycolipids of myelin and to the other lipid-containing elements of white matter. A detailed list of assignments is not provided since many of the assignments have been previously published and others are only tentative. The only narrow peaks which can definitely be assigned to lipid carbons are the large peak (number 16) reflecting the choline methyl carbons, and the smaller peak (number 25) contributed by the terminal methyl group of the fatty acyl chains, and a peak (number 23) appearing as a shoulder on the broad main methylene resonance attributable to the penultimate methylene of the fatty acyl chains. The five narrow peaks in the region between 62 and 75 ppm downfield from Me_4Si (peaks 9, 10, 11, 12, and 15) are tentatively assigned to cerebroside galactose carbon atoms. These may, however, reflect a soluble cytoplasmic or extracellular component of the white matter. The assignments were made on the basis that the chemical shifts are more nearly consistent with those of methyl β-D-galactoside (taken as a model for the galactose moiety of glycolipids) than with those of glucose, which is the most likely soluble substance to give rise to resonances in this region. No resonances attributable to cholesterol, which comprises about 40 mol % of bovine white matter lipids (Norton and Autelio, 1966), are seen, with the possible exception of peak 17. In the absence of any other resonances attributable to cholesterol, it seems quite unlikely that a relatively large peak such as this arises from a cholesterol carbon.

The poorly resolved nature of the white matter spectrum, as well as the long time (about 10 h) required to obtain spectra with suitably high signal/noise ratios, precluded accurate determination of spin–lattice relaxation times (T_1's) of the various resolved carbons. An attempt was made to get an approximate idea of the spin–lattice relaxation behavior of the system. In Figure 2 are shown partially relaxed spectra which were obtained with τ values of 100 and 350 ms. It can be seen that, although at 100 ms the entire spectrum with the exception of peak 15 (arrow) is inverted, at 350 ms the only peak which remains inverted is peak 25 (arrow) arising from the terminal methyl of the fatty acyl chains. Thus the bulk of the carbons giving rise to the spectrum have a null point between 100 and 350 ms. Since $T_1 = t_{null}/\ln 2$, these carbons must have values of T_1 between 140 and 500 ms, whereas the methyl groups giving rise to peak 25 have an average T_1 longer than 500 ms.

The natural abundance ^{13}C NMR spectrum of bovine brain myelin is shown in Figure 1B. This spectrum resembles that of whole white matter, with the exception of the peaks due to sucrose trapped in the myelin vesicles during preparation (these are designated with an "S" in the figure). The intensity of the peak representing the fatty acyl chain terminal methyl group is decreased relative to other peaks in the spectrum due to the short recycle time used (0.55 s). This also may be true for the peaks representing the choline methyl carbons and the fatty acyl carbonyl. In addition there is a loss of sharpness of some of the shoulders on the bulk methylene peak, compared with the spectrum of white matter.

The effect of temperature on the ^{13}C NMR spectrum of

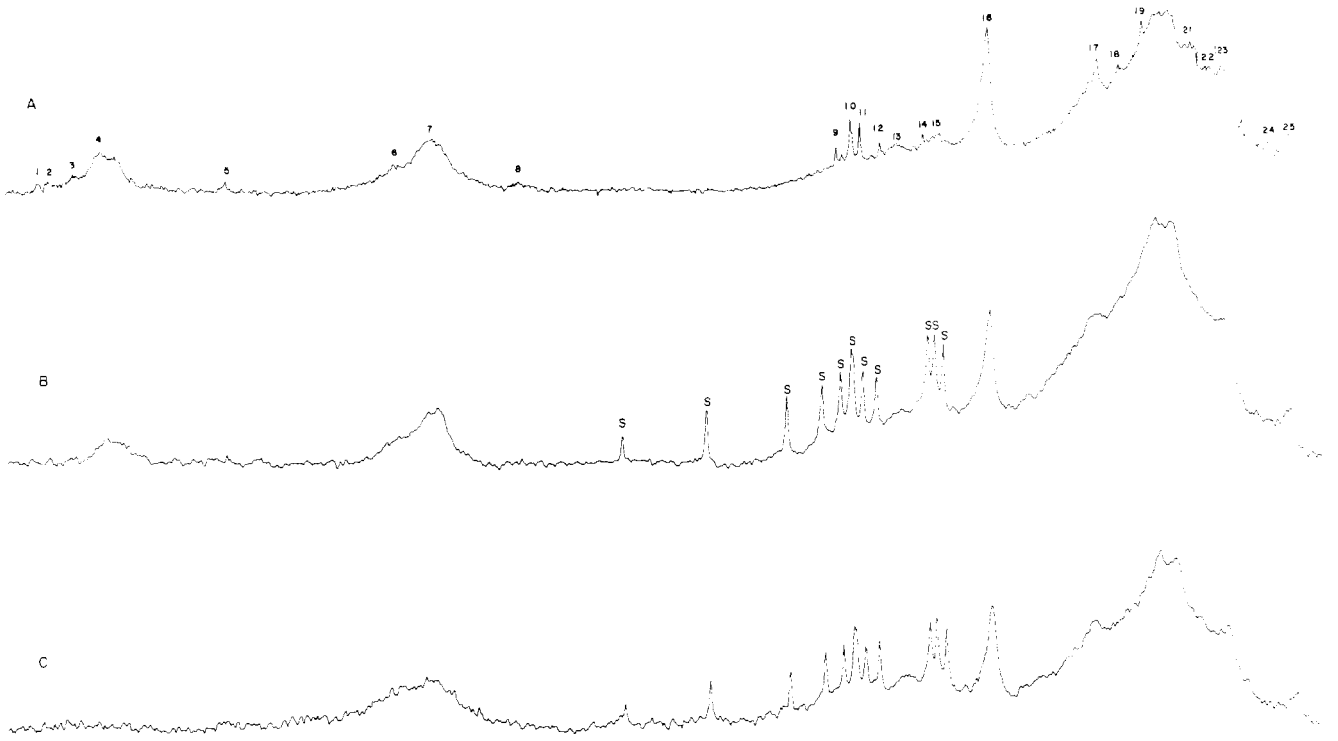

FIGURE 1: Proton-decoupled natural abundance ^{13}C Fourier transform NMR spectra at 15.18 MHz. (A) Bovine whole white matter (dry weight, 3.0 g). The spectrum was recorded at 34 °C with a digital resolution of 1.8 Hz, after 32:678 accumulations with a recycle time of 1.0 s. (B) Bovine myelin (1.3 g dry weight). Peaks marked "S" are due to sucrose trapped in the vesicles during preparation. The spectrum was recorded at 42 °C with a digital resolution of 1.8 Hz, after 65 536 accumulations with a recycle time of 0.55 s. (C) Myelin lipids (dry weight ca. 0.8 g) in a hand-shaken aqueous dispersion. The spectrum was recorded at 42 °C with a digital resolution of 1.8 Hz, after 65 536 accumulations with a recycle time of 0.55 s. In processing each of these spectra, the signal/noise ratio was enhanced by the application to the free induction decay of an exponential function with a time constant of −6, resulting in a line broadening of 3.5 Hz.

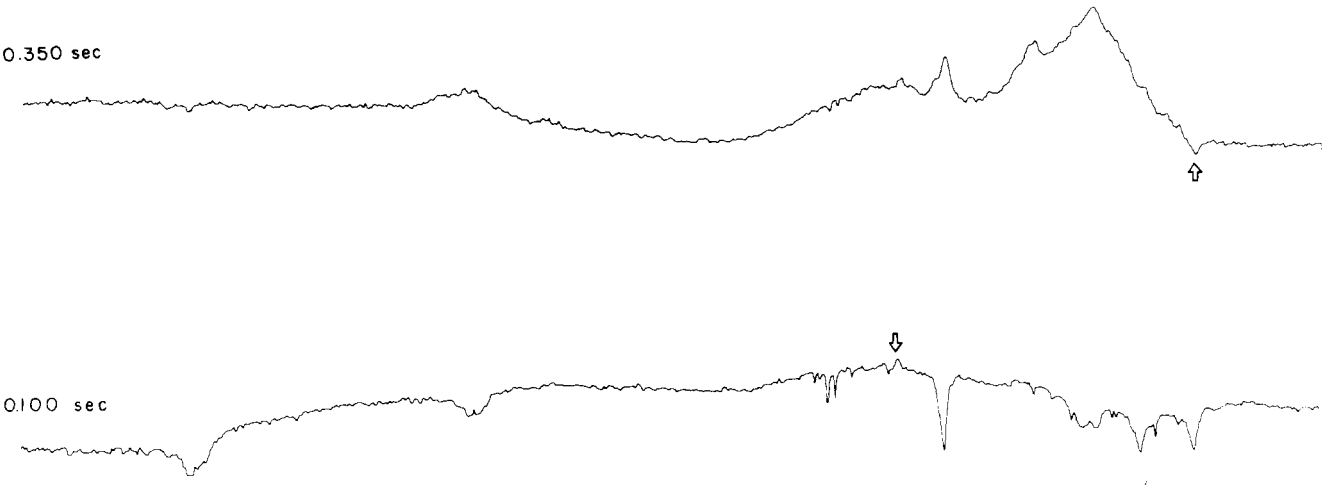

FIGURE 2: Partially relaxed ^{13}C Fourier transform NMR spectra of bovine whole white matter. Both spectra were recorded at 34 °C with a digital resolution of 1.8 Hz, after 32:678 accumulations with a recycle time of $1 + \tau$ s, where τ is the delay between the 180 and 90° pulses. Both spectra were recorded under conditions of proton decoupling. The τ values used in each case are indicated next to each spectrum.

bovine myelin is shown in Figure 3. Raising the temperature from 33 to 57 °C results in some narrowing of the choline methyl resonance and of those which are shoulders on the bulk methylene resonance. The bulk methylene resonance itself is not appreciably narrower at the higher temperature, but it has increased in intensity with respect to the other peaks in the spectrum. It is apparent that some irreversible changes take place upon heating, because the heat-treated sample yielded a spectrum at 33 °C which retained many of the characteristics of the spectrum recorded at 57 °C (see Figure 3).

The natural abundance ^{13}C NMR spectrum of a hand-shaken aqueous dispersion of lipids extracted from bovine myelin is shown in Figure 1C. This spectrum is similar to the spectrum of whole myelin, except that the olefinic resonance is somewhat broader and the carbonyl resonance has disappeared completely.

In order to determine what lipid resonances are potentially observable in the ^{13}C NMR spectra of white matter and myelin, total lipid extracts were prepared from both in chloroform–methanol and their spectra obtained. These are presented in Figure 4. The spectra of lipid extracts of white matter and myelin are similar: variations in the relative intensity of the

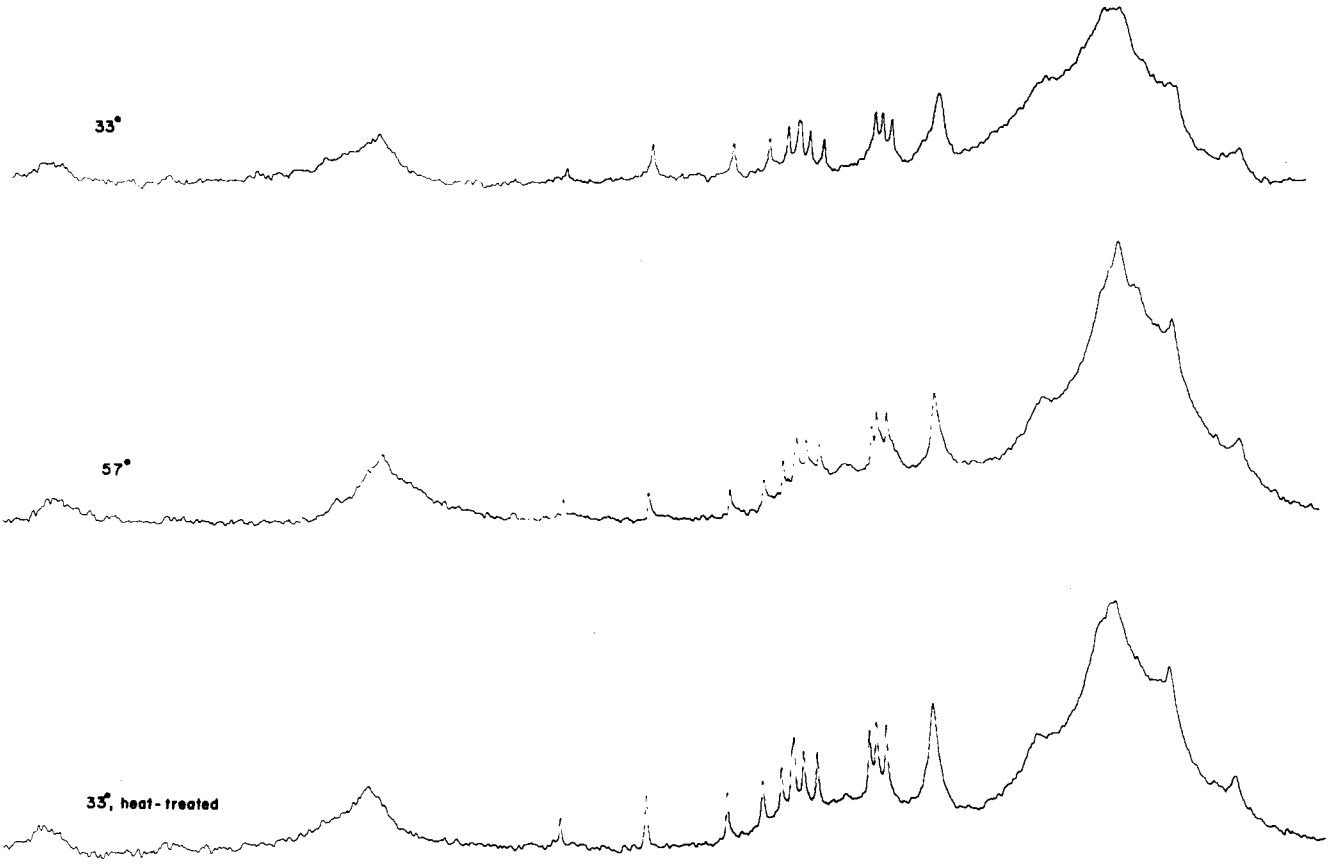

FIGURE 3: The effect of increasing temperature on the ^{13}C NMR spectrum of bovine myelin. All spectra were recorded with a digital resolution of 1.8 Hz, after 65 536 accumulations with a recycle time of 0.55 s, under conditions of proton decoupling. The temperature at which each spectrum was recorded is indicated. The same sample was used for all three spectra. The lower spectrum was recorded at 33 °C after heat-treating the sample by immersion in boiling water for an hour.

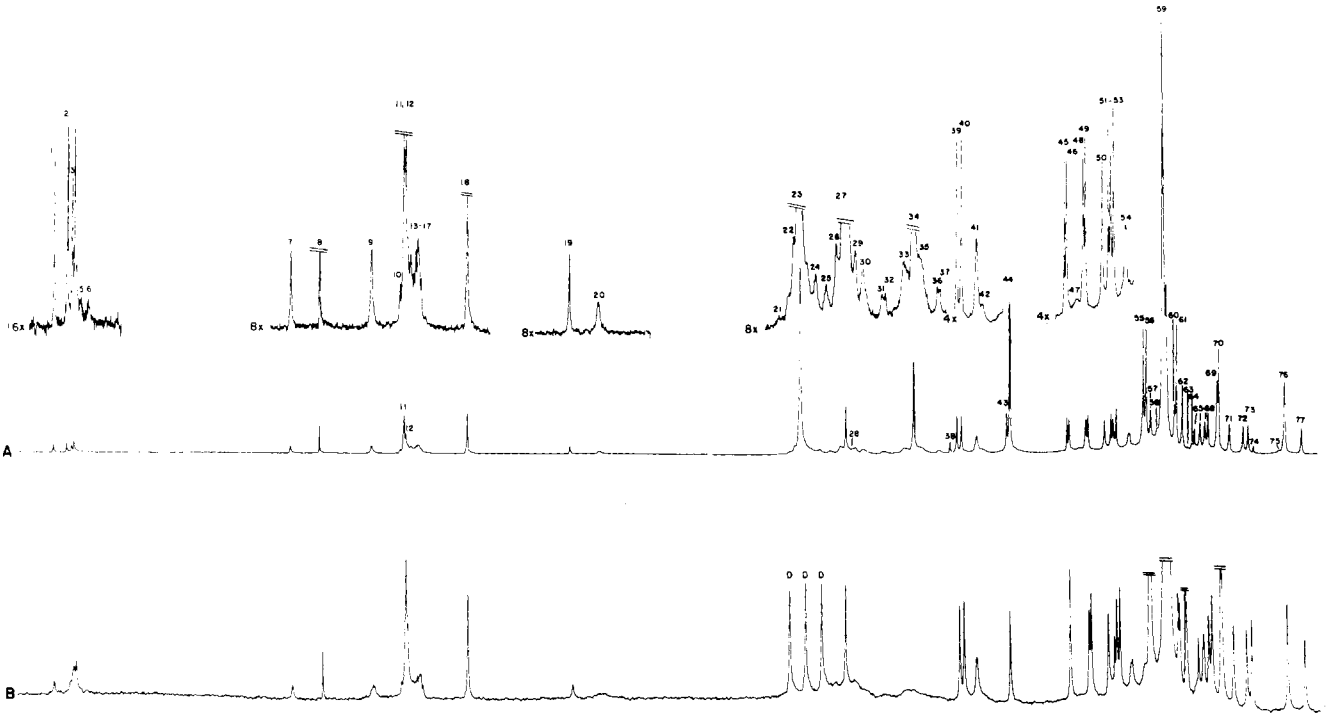

FIGURE 4: Proton-decoupled natural abundance ^{13}C Fourier transform NMR spectra at 15.182 MHz. (A) Total lipid extract of bovine white matter. The extract was prepared by pentanol extraction (see Materials and Methods) and consisted of about 4 g of lipid dissolved in 9:1 chloroform–methanol to give a total volume of 12 ml. The spectrum was recorded at 34 °C with a digital resolution of 0.9 Hz, after 4096 accumulations with a recycle time of 2 s. Peak 28 is absent from the insert due to a recorder artifact. (B) Total lipid extract of bovine myelin. The extract consisted of 0.9 g of lipid dissolved in deuteriochloroform to give a total volume of 13.2 ml. The spectrum was recorded at 34 °C with a digital resolution of 0.45 Hz, after 16 384 accumulations with a recycle time of 2.2 s.

TABLE I: Approximate Integrated Intensities of ^{13}C Resonances in the Proton-Decoupled Spectra of Bovine White Matter and Myelin, Relative to the Intensities of the Corresponding Resonances in Spectra of Total Lipid Extracts Prepared from These Materials.[a]

Material	Peak(s)[b]	Intensity Rel to Peak in Corresponding Extract
White matter	Choline $N^+(CH_3)_3$ (#41)	0.84
	Olefin (#s 9-17)	0.23
	Terminal $-CH_3$ (#76)	0.10
Myelin	Choline $N^+(CH_3)_3$	0.58
	Olefin	0.21
	Terminal $-CH_3$	0.06

[a] In each case the number given is the ratio of the integrated intensity of the resonance arising from the lipid of myelin or white matter to that of the peak or peaks arising from the lipid in the extract. Integrals were obtained from spectra of samples of white matter and myelin, and total lipid extracts were prepared from the same samples and dissolved in $CDCl_3$ to give the same volume in the NMR sample tube as the material from which the particular extract was obtained. The lipid extract of white matter was prepared by the method of Folch et al. (1957). The integrals were corrected for any differences in absolute intensity introduced as a result of differences in number of spectral accumulations, digital processing, or printout conditions. The recycle times used in obtaining each spectrum were as follows: 1.5 s for the white matter spectrum, 0.55 s for the myelin spectrum, and 5 s for the spectra of both lipid extracts. The shortened recycle time (necessitated by the smaller sample size and consequent diminution of signal strength) in the case of the myelin spectrum probably resulted in the production of peaks which did not attain their full equilibrium intensities, particularly in the case of the fatty acyl terminal methyl group. This peak is in fact probably not at its equilibrium intensity in the white matter spectrum either since relaxation studies (see Figure 2) indicate that it has a T_1 somewhat longer than 0.5 s. (In order for a peak to attain equilibrium intensity, the recycle time used should be at least three times the T_1 for that peak.) The estimated accuracy of these integrals is ±20%. [b] The numbers indicate which peaks in the white matter lipid extract spectrum were included in the integrals obtained from that spectrum. Corresponding peaks were used to obtain integrals from the myelin lipid extract spectrum.

resonances occur which may reflect differences in chemical composition. Peaks arising from carbons in the more polar regions of certain lipids have different linewidths, presumably reflecting changes in the state of solvation and aggregation as the result of changes in solvent composition.

In order to estimate the proportion of ^{13}C nuclei which give rise to observable resonances in the spectra of white matter and myelin, integrals of several of these resonances were measured and compared with integrals of the corresponding peaks in the lipid extracts. These integrals are collected in Table I.

Discussion

There have been numerous attempts to understand the dynamic properties of lipids in organized phases on the basis of NMR measurements of relaxation behavior. Such studies may eventually lead to information concerning dynamic properties of lipids in biological membranes (Lee et al., 1974). Such efforts have been hampered by the fact that biological membranes themselves have not yielded high-resolution NMR spectra which are of adequate quality to allow an accurate determination of relaxation parameters. In addition, there are questions regarding the interpretation of these parameters (the longitudinal and transverse relaxation times, T_1 and T_2) in terms of molecular motion in organized phases.

The interpretation of the ^{13}C NMR spectra of bovine white matter and myelin presents several problems. They are not high-resolution spectra in the sense that each resonance observed can be assigned to a specific carbon, although such assignments are possible in several cases. The origin of the line broadening must be explained, and, if possible, related to the physical state of the lipids of the myelin membrane. In addition, an explanation must be sought for the apparent loss of intensity in the resonances arising from the carbons of the lipid hydrocarbon chains, as well as the absence of any resonances definitely assignable to the carbons of cholesterol.

There is considerable evidence to indicate that the NMR line broadening observed in aqueous dispersions of phospholipids is dipolar in nature, i.e., is due to incomplete rotational averaging of nuclear dipole-dipole coupling (Chan et al., 1971, 1972; Oldfield et al., 1971; Finer et al., 1972; Kohler et al., 1972; Seiter and Chan, 1973). T_1 measurements on these systems, which in ^{13}C NMR reflect rates of segmental motion of the lipid chains (Doddrell and Allerhand, 1971; Levine et al., 1972; Williams et al., 1973), indicate that such motion around the long chain axis is relatively rapid, with correlation times for the aliphatic chain carbons of lecithin on the order 0.1 ns having been reported (Levine et al., 1972; Sears, 1971). For an *isotropically* tumbling molecule, such a correlation time would indicate that molecular motion is fast enough to average out all dipolar interactions and a high resolution NMR spectrum with $T_2 \simeq T_1$ would be observed. The motion of lipid chains in vesicular or other organized systems has been shown by spin labeling and fluorescence studies to be highly *anisotropic* (Hubbell and McConnell, 1971; Shinitzky et al., 1971; Boggs and Hsia, 1973), however, and in these systems some residual dipolar interactions will remain even if segmental motion is quite rapid, resulting in a ^{13}C NMR spectrum having T_2 values which are less than the corresponding values of T_1. The decrease in T_2 (that is, the extent of line broadening) will depend on the degree of motional anisotropy; if chain segmental motion is fairly rapid (if the correlation time for such motion is significantly less than $1/\omega_0$ where ω_0 is the ^{13}C resonant frequency in rad/s), the linewidth may be almost solely determined by the degree to which motion perpendicular to the chain long axis is restricted (Seiter and Chan, 1974). In the case of motion which is highly restricted in one or more planes, the resultant large line broadening may result in a resonance which is not detectable by a high resolution NMR spectrometer, even though chain segmental motion is rapid enough to satisfy the extreme narrowing limit ($\omega_c^2\omega_0 \ll 1$).

In ^{13}C NMR spectra of aqueous dispersions of phospholipids above their transition temperatures, significant dipolar broadening is only observed if the dispersion is unsonicated. Sonication has been shown to substantially reduce the size of the phospholipid vesicles, with a consequent increase in the rate of vesicle tumbling which appears to be great enough to at least partially average out the relatively slow local fluctuations in $\overline{H}_{eff}$ due to residual dipolar interactions (Finer, 1974). In addition, there may be some alteration in packing of the phospholipids upon sonication which also affects T_2 (Sheetz and Chan, 1972; Lichtenberg et al., 1975), although there is not a corresponding change in chain segmental motion as reflected by T_1 values (Sears, 1971; Sears et al., 1974).

The dipolar broadening seen in NMR spectra of phospholipid dispersions is substantially increased by the addition of cholesterol to the system. This phenomenon has been best characterized in unsonicated lecithin-cholesterol dispersions,

but a substantial amount of line broadening is also observed in the ^{13}C spectrum of sonicated lecithin–cholesterol mixtures (Sears, 1971; Keough et al., 1973). The line broadening due to cholesterol is observed in both ^{1}H and ^{13}C NMR spectra and has been seen with mixtures of cholesterol with phospholipids other than lecithin (Oldfield and Chapman, 1972; Chapman and Penkett, 1966; Phillips et al., 1970). The T_1 values for the carbons of lecithin in a sonicated 2:1 lecithin–cholesterol mixture do not seem to be markedly less then those observed in sonicated pure lecithin, indicating that cholesterol does not cause a significant slowing of segmental chain motion (Sears, 1971).

It seems reasonable to conclude that the line broadening observed upon the addition of cholesterol to phospholipid dispersions is mainly due to the rigid steroid nucleus restricting the amplitude of cooperative chain flexing motions, resulting in decreased motional averaging of dipolar interactions. Evidence indicating that cholesterol does indeed restrict chain motion may be drawn from studies with spin labels or fluorescent probes, in which the addition of cholesterol to a phospholipid dispersion containing the probe results in an increase in the anisotropy of probe motion, as reflected by changes in the ESR hyperfine splitting (Hubbell and McConnell, 1971; Boggs and Hsia, 1973) or the rate and wavelength dependence of fluorescence depolarization (Shinitzky et al., 1971). Inhibition of chain flexing by cholesterol is also implicated by the condensing effect of cholesterol on phospholipid monolayers; the addition of cholesterol to such systems can be shown to decrease the average area occupied by each phospholipid molecule (Chapman et al., 1969; Ghosh et al., 1971). X-ray studies on phospholipid bilayers have shown that the addition of cholesterol causes an increase in bilayer thickness, as well as a decrease in the average area/lipid chain (Lecuyer and Dervichian, 1969; Levine and Wilkins, 1971). The increase in bilayer thickness is presumably due to straightening of the phospholipid chains. Disruption of cooperative chain interactions by cholesterol is also evidenced by a diminution (and eventual disappearance at a cholesterol content of 33–50 mol %) of the enthalpy change associated with the gel-to-liquid crystal phase transition observed in phospholipid aggregates (Ladbrooke et al., 1968; Hinz and Sturtevant, 1972).

The T_1 data for bovine white matter are consistent with the theory that the observed line broadening is due to restrictions on the amplitude of chain flexing rather than to severe restrictions on chain segmental motion. The approximate T_1 values observed for the protonated alkyl chain carbons of white matter are similar to those measured by Sears (1971) for model systems containing lecithin and cholesterol, and not markedly different from published T_1 values observed for pure lecithin liposomes (Levine et al., 1972). The fact that the chain terminal methyl carbon, which due to its extra degree of motional freedom would be expected to have a shorter τ_{eff} than the other chain carbons, relaxes more slowly than these carbons indicates that the motions contributing to longitudinal relaxation are on the fast side of the T_1 minimum, i.e., $1/\tau_{eff} > \omega_0$ (Doddrell et al., 1972). The range of T_1 values observed for the chain methylene carbons is consistent with a τ_{eff} of 10^{-10} s, which is not markedly different from rates of segmental motion seen in lecithin liposomes (Levine et al., 1972).

The absence of any observable ^{13}C resonances arising from the cholesterol ring can be rationalized; the rigid steroid nucleus would not be expected to undergo flexing at all. Motional anisotropy to this degree could result in sufficient dipolar broadening to cause the disappearance of any NMR resonances. That the hydrocarbon chain tail of cholesterol does not give rise to any observable resonances may be an indication that the steroid nucleus acts as an anchor, restricting flexing of the tail to such a degree that no observable ^{13}C resonances arise from this portion of the molecule either. It is possible, however, that the tail does in fact contribute to the very broad resonance complex in the alkyl region of the myelin spectrum.

The diminished intensities of the ^{13}C resonances of the alkyl chain carbons relative to the intensities of corresponding peaks in the total lipid extract are difficult to explain. A decrease in intensity would occur if a resonance from a homogeneous population of nuclei were partially "broadened out" by the dipolar mechanism discussed above, or if the resonances from a portion of a heterogenous population of nuclei (e.g., a membrane "domain") were completely broadened out. Another factor which might give rise to differences in resonance intensity is the nuclear Overhauser enhancement (NOE), which might cause as much as a threefold difference in intensity between resonances from groups of nuclei present in equal numbers. If all these factors were operative, the net effect would be to make differences in resonance intensities very difficult to interpret in terms of the motional characteristics of the parent carbon atoms.

If the observed differences in relative intensity between the various resonances in the white matter spectrum are not due to differences in the NOE, then they are very likely due to differential dipolar broadening, with a relatively greater portion of some resonances than others being lost in the dipolar wings. If the extent of dipolar broadening of a resonance is in fact related to the extent to which motion of the parent carbon atom is restricted, then the pronounced difference in relative intensity between the choline methyl carbons and the alkyl chain carbons would seem to indicate that the motion of the head groups is much less restricted than that of the chains. That the increased mobility of the choline methyls does not stem entirely rom the extra degree of motional freedom allowed any group can be seen from the fact that the chain terminal methyl resonance is substantially diminished in intensity, despite the fact that it apparently has the shortest τ_1 of any of the nuclei contributing to the spectrum as judged from the T_1 data.

The aqueous dispersion of myelin lipids whose spectrum is shown in Figure 1C differs from whole myelin in that it contains little or no protein. The degree to which the removal of protein contributes to the relatively small differences between the spectra of myelin and myelin lipids is uncertain. The disappearance of the carbonyl peak might indicate that this peak in the myelin spectrum represents protein carbonyls. This seems unlikely since protein contributes little to other portions of the myelin spectrum (i.e., the differences in other portions of the spectrum upon removal of protein are slight). An alternative explanation might be that in the absence of protein the environment of the polar ends of the lipids changes so as to cause either broadening of the carbonyl peak via dipolar broadening or chemical shift nonequivalence, or loss of intensity due to slowing of longitudinal relaxation of the carbonyls or a decrease in their NOE. It would seem, however, that no drastic change in lipid head group configuration or mobility is occurring since changes in either portion of the spectrum (e.g., the choline methyl resonance) are slight.

The picture of myelin which emerges from a consideration of the relaxation and intensity data derived from the ^{13}C NMR spectra of bovine white matter and myelin, then, is one of the bilayer system with lipid hydrocarbon chains which are relatively free to move within a limited region, but which are pre-

vented from undergoing cooperative, high-amplitude flexing motions due to the high cholesterol content of the bilayers. The charged head groups of the phospholipids and sphingolipids are more free to flex and/or rotate, possibly facilitated by the fact that the hydroxyl group which serves as the cholesterol "head group" is small and leaves a good deal of room in which they can do so. Myelin proteins apparently cause few if any constraints on lipid mobility.

What are the implications of this (admittedly incomplete) picture of myelin structure for theories of the "fluidity" of cell membranes? First of all, myelin is hardly typical of most biological membranes, having an unusually high content of cholesterol and a low protein content. In the second place, it is not completely clear what "fluidity" means in terms of the motion of individual lipid molecules. It is possible to envisage a system in which restrictions on segmental motions of individual chains are relatively slight, but where restrictions on chain translational movements are sufficient to prevent the rapid movement of large molecules such as proteins through the lipid matrix. On the other hand, it is conceivable that a system could contai constraints on lipid chain flexing which are sufficient to cause considerable broadening or even disappearance of the NMR signal, but still allow a significant degree of translational freedom for lipids and proteins. At any rate, this NMR data does not rule out the existence of a translationally "fluid" lipid matrix, and in fact indicates that, from the standpoint of chain segmental motions, the lipids of myelin are not greatly restricted compared with those in model systems which contain no cholesterol. With regard to the possible existence of fluid "domains" within an otherwise solid-like membrane, the observation that only a fraction of the possible intensity is seen for some resonances does not necessarily mean that only a relatively fluid fraction of the nuclei of a given type in the sample contributes to these resonances. Seiter et al. (1972) have observed that, in chemically homogeneous dipalmitoyllecithin dispersions above the phase transition, only 35 and 45% respectively of the possible intensity of the terminal methyl and choline methyl resonances are observed in delayed Fourier transform experiments.

It is apparent that the spectra of the total lipid extracts of myelin and white matter offer a considerably more accurate picture of myelin lipid composition than do the spectra of the native materials. The apparent relative amounts of phospholipid, sphingolipid, and cholesterol are in accord with published data on the chemical composition of myelin and white matter (Autilio et al., 1964; Norton and Autilio, 1966; O'Brien and Sampson, 1965; Cuzner et al., 1965; Soto et al., 1966). Further work with model compounds is needed in order to completely and unambiguously assign the ^{13}C resonances in the spectra of these extracts, but it is clear that ^{13}C NMR is a potentially valuable analytical tool in the investigation of lipid composition of biological materials since it offers a method for performing such analyses without the need for chromatographic separation or chemical alteration of the various lipid components.

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Study of Abnormal Plasma Low-Density Lipoprotein in Rhesus Monkeys with Diet-Induced Hyperlipidemia†

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ABSTRACT: Male rhesus monkeys were divided into three groups: five were fed a regular primate chow diet and were used as controls; four received an "average" American diet; and five a special low-fat primate chow diet supplemented with 25% coconut oil and 2% cholesterol. In all of these animals, the plasma low-density lipoproteins (LDL) were isolated by ultracentrifugal flotation between densities of 1.019 and 1.050 g/ml. The LDL of the five control monkeys had variable molecular weights, with a mean value of $3.12 \pm 0.21 \times 10^6$ (range: 2.92×10^6 to 3.45×10^6), and an average partial specific volume of 0.969 ± 0.003 ml/g; both were assessed by flotation equilibrium analysis in the analytical ultracentrifuge. In the individual animals, however, the physical properties of LDL were invariant with time. The administration of either an "average" American diet or a coconut oil–cholesterol diet was accompanied by hypercholesterolemia associated with changes in LDL which were characterized by increases in molecular weight to $3.52 \pm 0.21 \times 10^6$ (average of nine monkeys) and in partial specific volume to 0.973 ± 0.002 ml/g. These changes were particularly evident when the molecular weight of LDL from monkeys in the normolipidemic state was compared with that obtained from the same monkeys during the hyperlipidemic state. Chemical analyses revealed that the particles from the hyperlipidemic animals had a relatively higher cholesteryl ester content, a slight increase in phospholipids, and a marked decrease to nearly complete absence of triglycerides. The other lipoprotein components, protein, carbohydrate, free cholesterol, and fatty acids, did not vary significantly from those of control LDL. It is concluded that the administration of atherogenic diets causes structural changes in LDL which appear to be accounted for, at least in part, by changes in the composition of the lipid moiety. The changes in physical and chemical properties noted in the LDL of rhesus monkeys with experimentally induced hypercholesterolemia contrast with the apparent structurally normal LDL from rhesus monkeys with spontaneous hypercholesterolemia reported previously.

The rhesus monkey is a good model for the study of experimental atherosclerosis since advanced atheromatous lesions closely resembling those observed in human subjects accompany the hyperlipidemias induced by diets rich in fat and cholesterol (Taylor et al., 1962, 1963; Wissler, 1968). It was recently observed that hyperlipidemic sera, particularly the low-density lipoproteins (LDL)[1] from rhesus monkeys, stimulate proliferation and cholesteryl ester accumulation in stationary cultures of aortic smooth muscle cells, whereas sera or LDL from normolipidemic animals do not exhibit such an effect (Fischer-Dzoga et al., 1971, 1974; Bates and Wissler, 1976). This important difference in cellular response prompted us to examine the structural relationship between the LDL of control monkeys and that of animals fed atherogenic diets. In this report, we describe some of the physicochemical properties of rhesus plasma LDL (ρ 1.019–1.050 g/ml) during diet-induced hyperlipidemia. A description of the properties of LDL from normolipidemic monkeys has already appeared (Fless and Scanu, 1975).

Materials and Methods

Animals and Diets. Male rhesus monkeys were obtained from the Food Research Institute, University of Wisconsin, Madison. Five animals serving as controls were fed a regular Purina primate chow diet. Four monkeys were placed on a human diet which represents an "average" American diet as described by Wissler and Vesselinovitch (1975). Five other animals were fed a diet that consisted of 70.5% of a modified, low-fat Purina primate chow supplemented with 25% coconut oil, 2% cholesterol, 1% vitamin mix, and 1.5% gelatin. This diet was modified slightly from that published previously by Wissler et al. (1962). Each animal was fasted 16–18 h before 30

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[1] Abbreviations: LDL, low density lipoprotein; n-LDL, LDL from normolipidemic monkeys; hyp-LDL, LDL from monkeys fed an "average" American diet or a coconut-oil-supplemented diet; apo-B, apolipoprotein B from apo-LDL; Na_2EDTA, disodium ethylenediaminetetraacetate.