Interpretation of 100- and 360-MHz Proton Magnetic Resonance Spectra of Retinal Rod Outer Segment Disk Membranes†

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ABSTRACT: Well resolved proton nuclear magnetic resonance (^{1}H NMR) spectra of bovine retinal rod outer segment (ROS) disk membranes have been obtained at 100 and 360 MHz. The resolved ^{1}H resonances of the ROS membranes are due to phospholipids, with little contribution from rhodopsin. The spectra of both the ROS membranes and bilayer vesicles prepared from purified ROS phospholipids (liposomes) appear to represent a superposition of relatively sharp resonance components and a broad, underlying background. The distribution between sharp and broad spectral components is sensitive to sonication and temperature. The percentage of choline methyl protons which are resolved in the ROS membrane spectra as sharp resonance components increases from approximately 35 to 100% and the average of the lipid hydrocarbon chain protons from approximately 20 to 40% over the temperature range 5–50 °C. The motional state of terminal CH_3 groups on the polyunsaturated docosahexenoic acid (C22:6ω3) side chains cannot be very different from those on the less unsaturated side chains, since the observed terminal CH_3 resonance consists of components from the C22:6ω3 and other side chains in proportions which reflect their composition ratios. The observation of a comparable fraction of phospholipids yielding high-resolution spectral components and similar resonance line widths for the ROS membranes and ROS liposomes suggests that rhodopsin does not greatly alter the lower frequency segmental motions of phospholipids in the ROS membrane. The NMR data are discussed in terms of models for the organization of phospholipids in the disk membrane and their interaction with rhodopsin.

Nuclear magnetic resonance (NMR)[1] has been used to elucidate details of motion (Horwitz et al., 1972; 1973b; Levine et al., 1972), phase behavior (Chapman, 1975), and asymmetry (Michaelson et al., 1974) in pure and mixed phospholipid bilayer membranes. In addition, well resolved NMR spectra have been obtained of several natural membranes (see, e.g., Lee et al., 1974a; Davis et al., 1976). A relatively simple membrane system which appears to be particularly amenable to magnetic resonance studies is the rod outer segment (ROS) disk membrane from the vertebrate retina (for reviews see Hagins, 1972; Daemen, 1973; Hubbell, 1975; Ebrey and Honig, 1975; Dratz, 1977). A single protein, rhodopsin, comprises at least 85% of the total protein content. The ROS phospholipids are particularly rich in polyunsaturated fatty acids, with docosahexenoic acid (C22:6ω3) comprising at least 31–37% of the total fatty acid composition of bovine ROS disk membranes[2] (Nielson et al., 1970; Anderson and Sperling, 1971). The visual pigment rhodopsin rotates and translates freely in the ROS disk membrane (Brown, 1972; Cone, 1972; Poo and Cone, 1974; Liebman and Entine, 1974), so that a considerable fraction of the ROS phospholipids must be relatively fluid.

In this communication, we present a comparison of ^{1}H NMR spectra of ROS disk membranes and liposomes of purified, extracted ROS phospholipids obtained at 100 and 360 MHz. The spectra of these preparations are similar and suggest that the ROS disk membrane phospholipids are organized in mixed domains which may be broadly categorized as "fluid" and "less fluid". We interpret the similar resonance line widths of the ROS membrane and ROS liposome spectra to mean that rhodopsin does not markedly inhibit the large amplitude/low frequency segmental motions and self-diffusive motions of the most fluid phospholipids in the disk membrane.

Experimental Methods

ROS disk membranes were isolated by a step float centrifugation procedure as described by Raubach et al. (1974), with additional precautions to avoid oxygen damage to the polyunsaturated fatty acids (Farnsworth and Dratz, 1976; W. L. Stone, T. C. Huffaker, C. C. Farnsworth, and E. A. Dratz, in preparation). The ROS phospholipids were extracted according to a modification of the Folch et al. (1957) procedure and purified by silicic acid chromatography to remove retinal, retinol, and cholesterol. Egg phosphatidylcholine (PC) was obtained from Sigma (type III-E) and further purified on silicic acid. The NMR samples were sonicated with a 20-kHz Branson Model W140 sonifier equipped with a microtip. Further details of the purification and preparation of ROS membranes and ROS phospholipids for ^{1}H NMR spectroscopy are described elsewhere (Brown, 1975; Brown et al., 1976).

The 100-MHz ^{1}H NMR spectra were obtained in the Fourier transform (FT) mode on a JEOLCO PFT-100 spectrometer interfaced to a Varian 620/i computer. Unless otherwise noted, the acquisition parameters were a 2-kHz frequency range, 4K data points, 300-μs trigger delay, a 2.5-s

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[1] Abbreviations used are: NMR, nuclear magnetic resonance; ROS, rod outer segment; FT, Fourier transform; PC, phosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; ESR, electron spin resonance.

[2] A recent reinvestigation of the fatty acid composition of minimally oxidized bovine ROS membranes indicates that the C22:6 content is at least 42% (W. L. Stone, C. C., Farnsworth, L. de Ghetaldi, and E. A. Dratz, submitted for publication).

TABLE I: Chemical Shifts and Assignments[a] of Principle Resonances in 360-MHz ^{1}H NMR Spectra of ROS Membranes and Phospholipids.

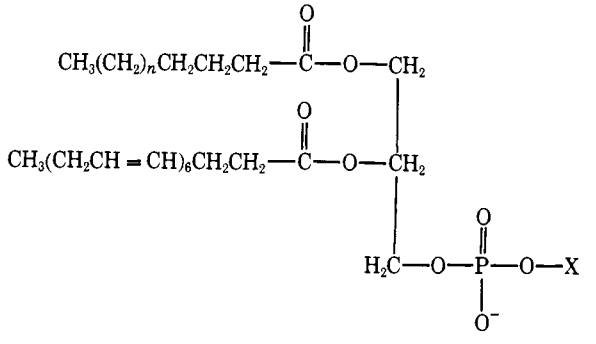
Assignment	Chemical shift[b]		
	ROS phospholipids in 19:1 $CDCl_3$-CD_3OD	ROS membranes[c]	ROS liposomes[c]
CH=CH	5.37	5.27	5.31
$N^+(CH_3)_3$	3.26	3.23	3.25
$CH{=}CHCH_2{*}CH{=}CH$	2.84	2.73	2.77
$CH{=}CHCH_2CH_2{*}CO$	2.39	2.35	2.41
$(CH_2)_nCH_2{*}CO$	2.30	2.35	2.41
$CH{=}CHCH_2{*}$	2.08	1.97	2.02
$CH_2{*}CH_2CO$	1.58		
$CH_2{*}CH_2CH_2CO$	1.34		
$(CH_2)_n$	1.25	1.22	1.26
$CH{=}CHCH_2CH_3{*}$	0.98	0.88	0.93
$(CH_2)_nCH_3{*}$	0.88	0.82	0.86

[a] Resonances were assigned by spin decoupling and comparison to the literature for other phospholipids and membranes (Finer et al., 1972a; Dea et al., 1972). [b] ppm relative to external sodium 3-trimethylsilylpropanesulfonate (TSP) at 20 °C. [c] Sonicated 15–20 min.

repetition rate between successive 90° pulses, and an exponential line broadening of ~8–10 Hz. The 360-MHz spectra were obtained on the Bruker HXS-360 spectrometer at the Stanford Magnetic Resonance Laboratory. The 360-MHz FT studies employed 5000–10 000-Hz frequency ranges (50–100 μs dwell times), 8K data points, and a 15-s repetition rate. The 360-MHz correlation NMR spectra were recorded over a 24-kHz frequency range, employing 16K data points with a dwell time of 1.25 μs/data point.

Results

ROS Phospholipids in Organic Solvents. An "average" ROS phospholipid contains a saturated fatty acid at the 1 position of the glycerol moiety and a polyunsaturated fatty acid (predominantly C22:6ω3) at the 2 position (Anderson and Sperling, 1971), as indicated in structure I, where X indicates



a specific head group. An ^{1}H NMR spectrum of total extracted ROS phospholipids in 19:1 $CDCl_3$-CD_3OD at 360 MHz is shown in Figure 1. Resonances are observed from the choline methyl head group (N^+Me_3) and the side chain methylene ($(CH_2)_n$), singly allylic ($CH{=}CHCH_2{*}$), doubly allylic ($CH{=}CHCH_2{*}CH{=}CH$), vinyl (CH=CH), and terminal CH_3 protons (Table I). The resonances from the first two side-chain methylene groups next to the ester linkages are well resolved from the bulk methylene resonance at 1.25 ppm, with the $CH_2{*}CO$ resonance split into two components of similar area. The downfield resonance at 2.39 ppm is tentatively assigned to the $CH{=}CHCH_2CH_2{*}CO$ groups of the C22:6 fatty acid side chains and the upfield resonance at 2.30 ppm to the $(CH_2)_nCH_2{*}CO$ protons of the other side chains. The second

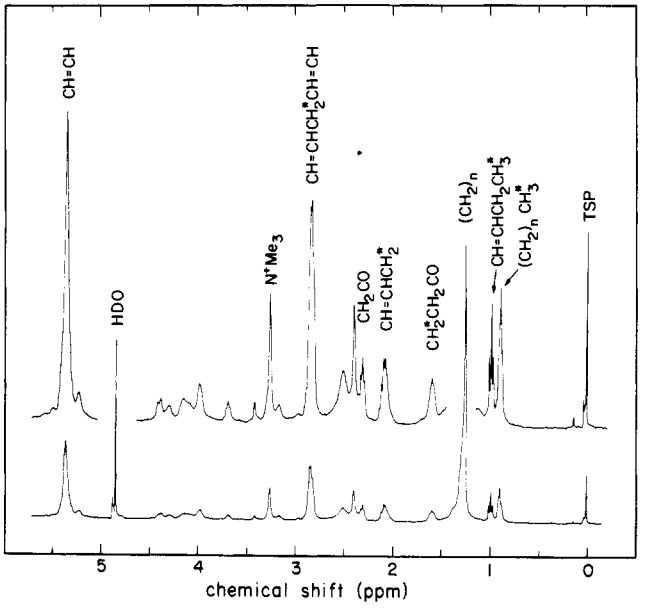


FIGURE 1: The 360-MHz ^{1}H NMR spectrum of total extracted ROS phospholipids in 19:1 $CDCl_3$-CD_3OD at 20 °C.

side chain methylene appears as a single resonance at 1.58 ppm.

An unusual feature of the ^{1}H NMR spectra of ROS phospholipids is the observation of two distinct terminal CH_3 resonances with characteristic spin–spin couplings. The relative areas of these resonances are approximately 2:3 (downfield: upfield). The downfield CH_3 appears as a well resolved triplet (J = 7.4 Hz), whereas the upfield CH_3 exhibits more complex spin–spin couplings. These resonances were assigned by spin decoupling from the directly bonded side-chain methylenes to fatty acids with a double bond in the ω position with respect to the CH_3 group (predominantly docosahexenoic acid, C22:6ω3) (downfield) and CH_3 groups on the other fatty acid side chains (upfield) (Figure 2). The downfield shoulder of the methylene envelope is probably due to the third side chain methylene groups ($CH_2{*}CH_2CH_2CO$), and is quite well resolved upon irradiation of the bulk $(CH_2)_n$ protons.

ROS Liposomes and ROS Membranes. ^{1}H NMR spectra of unsonicated dispersions of ROS phospholipids and ROS

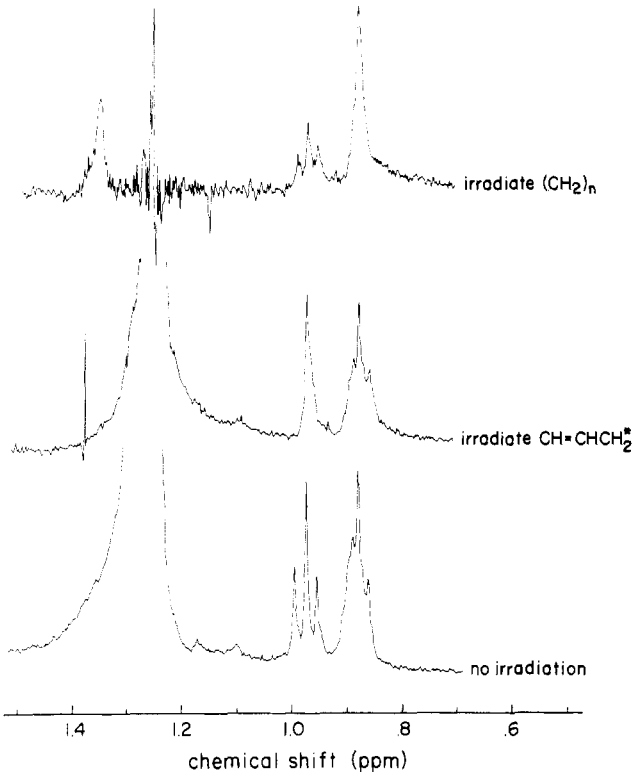


FIGURE 2: Spin-decoupling of the terminal CH_3 resonances from the directly bonded side-chain methylene resonances of total extracted ROS phospholipids in 19:1 $CDCl_3$-CD_3OD, 20 °C, at 360 MHz.

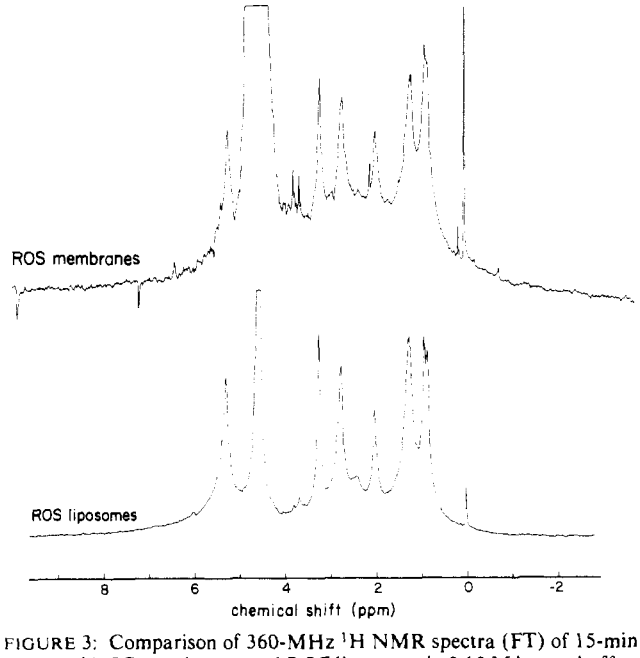


FIGURE 3: Comparison of 360-MHz ^{1}H NMR spectra (FT) of 15-min sonicated ROS membranes and ROS liposomes in 0.10 M borate buffer, 0.10 M KCl, pH 7, at 40 °C. Trigger delay = 100 μs.

membranes are relatively broad and poorly resolved. The effect of sonication is a reduction in the resonance line widths and an increase in the resolved areas of all resonances. After 15–20 min of sonication, little further spectral changes occur. ROS membranes and ROS phospholipids do not appear to form ~300-Å diameter "limit" vesicles even upon prolonged sonication, since both preparations elute near the column void volume when chromatographed on Sepharose 2B or 4B.

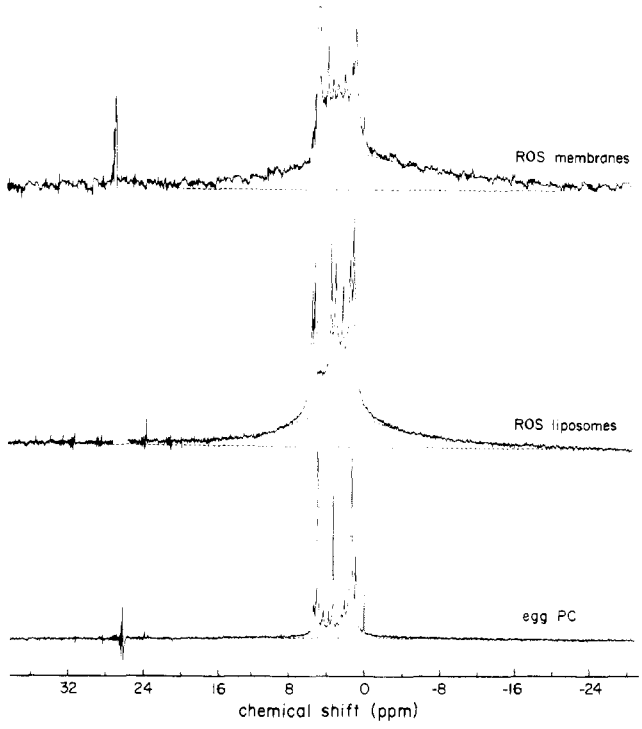


FIGURE 4: Correlation NMR spectra of ROS membranes, ROS liposomes, and egg PC. All samples were sonicated for 15 min and are in 0.10 M borate buffer, 0.10 M KCl, pH 7. Note that the sample concentration and spectrometer gain of each spectrum is different.

Electron microscopy reveals an average vesicle diameter of ~1000 Å in both cases (Brown et al., 1976). These observations are in essentially complete accord with a previous study of the effect of sonication on retinal rod membranes (Shichi and Shelton, 1974).

The CH=CH, N^+Me_3, $CH{=}CHCH_2{*}CH{=}CH$, $CH{=}CHCH_2{*}$, $(CH_2)_n$, and both terminal CH_3 resonances are clearly resolved in 360-MHz ^{1}H NMR spectra of sonicated ROS membranes and liposomes of extracted ROS phospholipids (Figure 3; Table I). The effect of aggregation of ROS phospholipids into bilayer membranes is a decrease in resonance intensity and substantial broadening of all side-chain resonances. This is particularly evident for the $(CH_2)_n$ resonance, as shown by a comparison of Figures 1 and 3. The spectra of both the ROS membrane vesicles and ROS liposomes appear to represent a superposition of relatively sharp, well-resolved resonances and a broad, underlying spectral component. This is most apparent in 360-MHz continuous wave correlation NMR spectra, as shown in Figure 4, where there is no attenuation of broad, rapidly relaxing components during the spectrometer trigger delay time.[3] An underlying component of apparent line width ~6 kHz is evident in the spectra of the sonicated ROS membranes and ROS liposomes, but not in the spectrum of sonicated egg PC liposomes. The observed line width of the broad component appears to be field/frequency dependent (~2 kHz in 100-MHz FT spectra with trigger delays $\leq$50 μs), which implies a contribution from several chemically shifted resonances.

[3] From the relation $I/I_0 = e^{-\Delta\tau/T_2}$, where I is the apparent resonance amplitude in a delayed FT spectrum obtained after a trigger delay of $\Delta\tau$ and I_0 is the continuous wave amplitude, it can be shown that the use of data acquisition trigger delays in excess of 50 μs results in >10% attenuation of fast relaxing spectral components with T_2 values shorter than 0.5 ms ($\Delta\nu_{1/2}$ > 600 Hz, assuming a Lorentzian line shape).

TABLE II: Percentage of Total Phospholipid Protons Resolved as Sharp Resonance Components in ^{1}H NMR Spectra of Sonicated[a] ROS Membranes and ROS Liposomes at 360 MHz.[b]

Resonance	ROS membranes				ROS liposomes			
	5 °C	20 °C	35 °C	50 °C	5 °C	20 °C	35 °C	50 °C
CH=CH		17	29	40		37	54	60
N^+Me_3	37	64	85	100	79	97	100	100
$CH{=}CHCH_2{*}CH{=}CH$	10	17	23	28	20	31	37	40
$CH{=}CHCH_2{*}$	24	35	42	47	28	41	49	52
$(CH_2)_n$	15	20	25	30	15	27	35	38
CH_3	23	39	45	50	33	56	62	64

[a] 15-20 min, to maximum increase in spectral intensity. [b] The estimated error is ±10%.

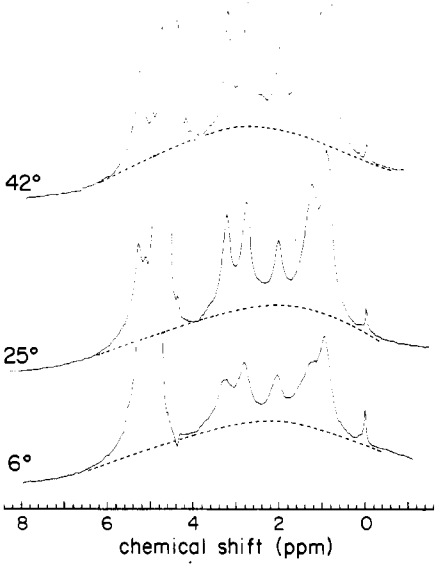


FIGURE 5: Effect of temperature on 100-MHz ^{1}H NMR spectra (FT) of 15-min sonicated ROS liposomes in 0.10 M borate buffer, 0.10 M KCl, pH 7. Trigger delay = 300 μs.

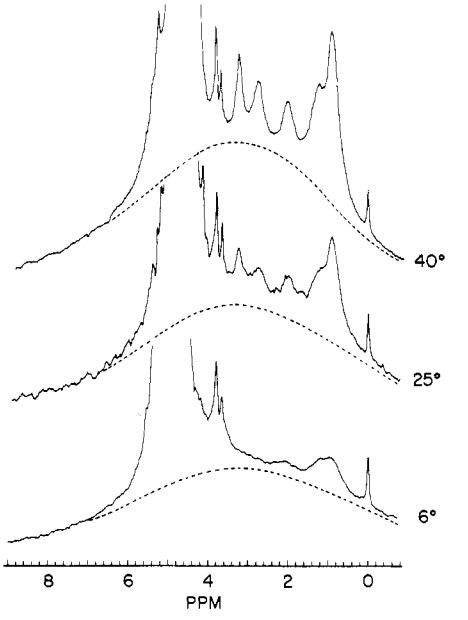


FIGURE 6: Effect of temperature on 100 MHz ^{1}H NMR spectra (FT) of 15-min sonicated ROS membranes in 0.068 M phosphate buffer, pH 7. The sharp resonances near 4 ppm are due either to ROS sugar residues or sucrose from the membrane preparation. Trigger delay = 300 μs.

An increase in intensity and decrease in the line widths of all the sharp resonances of the ROS membrane vesicles and ROS liposomes are apparent with an increase in temperature over the range 5–50 °C (Figures 5 and 6). The temperature-induced intensity changes are fully reversible to at least 50 °C. The percentage of total phospholipid protons resolved as sharp components (at 360 MHz) was estimated by choosing a curved baseline to approximate the broad, underlying spectral component and using the spectrum of extracted ROS phospholipids in 19:1 $CDCl_3$–CD_3OD as an area standard. The results are summarized in Table II. The experimental accuracy is limited by baseline uncertainties to ±10%. Essentially 100% of the N^+Me_3 head-group protons are resolved at temperatures above 45 °C in ^{1}H NMR spectra of both the ROS membrane vesicles and ROS liposomes. The areas of the unsaturated and saturated side-chain resonances correspond to significantly less than the full intensity. The resolved area of the $CH{=}CHCH_2{*}CH{=}CH$ resonance from the C22:6 fatty acid side chains is surprisingly low and approximately equal to that of the $(CH_2)_n$ protons. The percentage of the $CH{=}CHCH_2{*}$ protons yielding sharp resonances is consistently greater than that of the $CH{=}CHCH_2{*}CH{=}CH$ protons. Approximately 50–60% of the terminal CH_3 protons are resolved above 45 °C. Excepting the N^+Me_3 head-group resonance at lower temperatures, a similar fraction of phospholipid protons contribute to the sharp resonance components of both the ROS membrane vesicles and ROS liposomes.

The estimated line widths of the resolved sharp resonances of the ROS liposomes and ROS membrane vesicles are summarized in Table III. Within the present level of experimental error, it appears that there are rather minimal differences in the resonance line widths of the ROS membranes compared to the protein-free ROS liposomes at 40 °C. The line widths of the sharp components are somewhat broader in the ROS membrane vesicles at lower temperatures.

Discussion

^{1}H NMR spectra of sonicated ROS disk membranes and liposomes of extracted ROS phospholipids contain well-resolved sharp and broad resonance components. In contrast, spectra of sonicated model PC bilayer membranes in the liquid-crystalline phase contain only sharp resonance components. The observed sharp resonances correspond to the protons of the N^+Me_3 groups and a fraction of the saturated and unsaturated side chains of the ROS disk membrane phospholipids. A distinctive feature of the ^{1}H NMR spectra of the ROS membrane vesicles and ROS liposomes is the presence of two

TABLE III: Estimated ^1H Resonance Line Widths of Sharp Components of ROS Membranes and ROS Liposomes.[a]

Sample	Frequency (MHz)	Temp (°C)	$\Delta\nu_{1/2}$(Hz) CH=CH	N^+Me_3	CH=CHCH$_2$*-CH=CH	CH=CHCH$_2$*	$(CH_2)_n$	CH_3
ROS membranes	100	40	46 ± 18	32 ± 6	42 ± 8	54 ± 14	85 ± 21	29 ± 4
	360	40	65 ± 6	36 ± 2	75 ± 10	74 ± 19	118 ± 20	56 ± 4[b]
ROS Liposomes	100	42	37 ± 5	26 ± 6	36 ± 12	44 ± 12	82 ± 18	25 ± 2
	360	45	72 ± 4	26 ± 4	60 ± 4	48 ± 3	86 ± 12	54 ± 3[b]

[a] Considerable experimental error arises from uncertainties in the choice of spectral baselines, particularly for the ROS membrane spectra where there is an uncertain protein contribution. The limits of error were estimated from line shape and baseline choices representing a range of reasonable values. [b] Composite line width of both spectral components. The CH=CHCH$_2$CH$_3$* and $(CH_2)_nCH_3$* line widths are approximately the same.

terminal CH_3 resonances which are assigned to the fatty acids with a double bond in the $\omega 3$ position (predominantly docosahexenoic acid, C22:6ω3) and to the other, predominantly saturated fatty acid side chains. These resonances have similar line widths and are observed in proportions which reflect their composition ratios, suggesting that the motional state of the docosahexenoic acid terminal CH_3 groups is similar to those on predominantly saturated side chains. The spectra of the ROS membrane vesicles contain little resolved contribution from rhodopsin. Protein resonances are not observed in the aromatic spectral region, where there are no interfering phospholipid resonances. Since the disk membrane consists of ~35% rhodopsin by weight and rhodopsin contains a typical fraction of aromatic amino acids (see, e.g., Abrahamson and Fager, 1973), it appears that the rhodopsin resonances must be very broad, in spite of its rotational and translational mobility in the disk membrane (Brown, 1972; Cone, 1972; Poo and Cone, 1974; Liebman and Entine, 1974). The aliphatic and other protein resonances appear to contribute to the broad underlying component in the ^1H NMR spectra of the ROS membranes.

Interpretation of the Sharp and Broad Components in Terms of the Phospholipid Organization of the ROS Disk Membrane. The major contribution to the resonance line widths of phospholipid bilayers and lamellar soaps is dipolar in origin (Finer et al., 1972b; Chapman et al., 1972). The distinct sharp and broad components present in the ^1H NMR spectra of the ROS membrane vesicles and ROS liposomes must correspond to phospholipid protons which are subject to different degrees of motional averaging of magnetic dipolar interactions. In the following discussion, we consider three possible origins of these different degrees of motion: (1) a distribution of sizes of sonicated liposomes and membrane vesicles; (2) a gradient of motion proceeding from one end of the fatty acid chains to the other; (3) clustered or laterally separated domains of phospholipids with different degrees of motional freedom.

The precise motional origin of the sharp ^1H resonances observed from phospholipids in sonicated vesicles has been the subject of some contention. Contrary to earlier interpretations (Sheetz and Chan, 1972; Horwitz et al., 1973b; Seiter and Chan, 1973; Feigenson and Chan, 1974; Lichtenberg et al., 1975), evidence has been recently obtained that sonication does not produce significant increases in intramolecular structural disorder (Mendelsohn et al., 1976; Stockton et al., 1976; B. P. Gaber and W. L. Peticolas, submitted). Wennerström (1973) and Bloom and co-workers (1973; 1975) have shown that the broad, super-Lorentzian line shape characteristic of unsonicated lipid dispersions (Lawson and Flautt, 1968) can be explained by the presence of nonzero time-averaged dipolar interactions due to restricted segmental motions of the hydrocarbon side chains. Such residual static dipolar interactions are most generally characterized by the second moment M_2 of the absorption line shape (see, e.g., Abragam, 1961; Carrington and McLachlan, 1967), which in principle includes both *static* and *motional* contributions to the line width. In sonicated vesicles, rapid angular reorientation of phospholipids occurs by lateral diffusion about the vesicle surface and by vesicle rotation. This angular motion leads to further reduction of the static contribution to the second moment, so that in the limit of rapid motion defined in eq 1

$$M_{2r}\tau_c'^2 \ll 1 \tag{1}$$

one obtains a single Lorentzian line shape with an intrinsic line width governed by the fluctuating matrix elements of the dipolar Hamiltonian.[4] τ_c' is the correlation time for angular reorientation of phospholipids in spherical vesicles and is given by (Bloom et al., 1975)

$$\frac{1}{\tau_c'} = \frac{1}{\tau_r} + \frac{1}{\tau_d} = \frac{3kT}{4\pi\eta r_v^3} + \frac{6D_T}{r_L^2} \tag{2}$$

where τ_r and τ_d are the correlation times for vesicle rotation and lateral diffusion, respectively, r_v and r_L are the hydrodynamic vesicle radius and the effective radius for lateral diffusion of protons at different depths in the bilayer ($r_L \leq r_v$), D_T is the translational diffusion coefficient, and all other symbols have their usual meaning. In the long correlation time limit ($M_{2r}\tau_c'^2 \gg 1$) appropriate to unsonicated phospholipid dispersions, the time-averaged part of the dipolar Hamiltonian is nonzero and results in a super-Lorentzian line shape. In the above expressions, it is essential to note that M_{2r} is the *residual* second moment of the vesicular phospholipids, rather than the rigid lattice second moment previously invoked by Horwitz et al. (1973b) to evaluate the manifestations of rotational Brownian motion on the ^1H line shapes of phospholipids in multibilayers and vesicles. A lower limit to the frequency of rotational diffusion of phospholipids in vesicles can be calculated from eq 2 using $r_L = r_v$, and $D_T \approx 10^{-8}$ cm^2 s^{-1} (Devaux and McConnell, 1972; Träuble and Sackmann, 1972; Brûlet and McConnell, 1975). The average residual second moment can be estimated from the line width of unsonicated

[4] Equation 5 of the original paper by Bloom et al. (1975) is erroneous and has been subsequently corrected by these authors. In the short correlation time limit, a single Lorentzian line shape is obtained, rather than the superposition of Lorentzians given by eq 5 of Bloom et al. (1975).

phospholipid dispersions (Lichtenberg et al., 1975) or from the magnitude of the residual dipolar interactions (Charvolin and Rigny, 1971; Boden et al., 1976a) to be about (3 kHz)^2. Taking these values for M_{2r} and D_T, it follows that for vesicles with an average diameter $\lesssim 10^3$ Å the line widths become independent of vesicle size, whereas for significantly larger vesicles and multibilayers residual dipolar interactions are not completely averaged to zero, leading to a line width which is theoretically dependent on the overall vesicle tumbling rate.

From the above discussion, it is apparent that the observed non-Lorentzian ^{1}H NMR spectra of the ROS membrane vesicles and ROS liposomes (i.e., sharp and broad components) may, in principle, reside in differences in macroscopic vesicle tumbling, due to the differences in r_v, or in differences in internal phospholipid motions, characterized by M_{2r} and D_T. Electron microscopic examination of fully sonicated ROS membrane vesicles and ROS liposomes indicates a relatively narrow size distribution of vesicles approximately 10^3 Å in diameter (Brown et al., 1976). Furthermore, NMR spectra of sonicated vesicles subjected to sedimentation velocity centrifugation to fractionate them by size are essentially identical to the spectra of unfractionated vesicles (G. P. Miljanich and A. J. Deese, unpublished). That approximately 100% of the N^+Me_3 resonance is observed as a single, sharp line is additional evidence that significant amounts of large vesicles are not present in these preparations. We therefore conclude that the sharp and broad components primarily manifest differences in the internal motions of the ROS phospholipids, either due to a gradient of motion along the fatty acid chains (i.e., intramolecular differences in M_{2r}) or the presence of lateral domains of phospholipids in different motional states (i.e., intermolecular differences in M_{2r} and/or D_T).

There is ample evidence of increased molecular motion and disorder upon proceeding towards the CH_3 chain termini of phospholipids (see, e.g., Lee et al., 1972, 1976; Seelig and Seelig, 1974). Assuming that dipolar interactions between methylene proton pairs are dominant, Seiter and Chan (1973) have explained the observation of a fraction of the $(CH_2)_n$ protons of unsonicated phospholipid multilayers in delayed FT experiments by incomplete motional averaging of the angularly dependent geminal proton dipolar coupling. Those CH_2 groups nearest the chain terminus are relatively disordered, so that the residual dipolar splittings are expected to be small and the total resonance line widths sharp, whereas those near the glycerol moiety are comparatively well ordered, with greater residual dipolar splittings and hence very broad total line widths. Given this interpretation, superposition of resonances from all the $(CH_2)_n$ groups would result in a non-Lorentzian line shape, with sharp components superimposed upon a broad, underlying background. This work has led Davis et al. (1976) to suggest that a motional gradient accounts for the sharp and broad components in the ^{1}H NMR spectra of sarcoplasmic reticulum vesicles of average diameter $\sim 10^3$ Å, where any residual dipolar couplings are expected to be further modulated by the rotational motion of the vesicles.

The theory of Seiter and Chan is based on the assumption that, to a first approximation, the methylene groups of phospholipid bilayers can be treated as magnetically isolated spin pairs. A case can be made against significant spin diffusion among the hydrocarbon chain protons of sonicated vesicles (Horwitz et al., 1972; Lee et al., 1974a); however, it is clear that the CH_2 protons of unsonicated lipid dispersions are highly coupled and, most likely, cannot be treated as isolated spin pairs (Boden et al., 1976a,b). It is therefore debatable whether the model of Seiter and Chan can be used to explain the presence of broad and sharp components in the ^{1}H NMR spectra of the ROS membrane vesicles and sarcoplasmic reticulum. The recent work of Boden et al. (1976a,b), however, has also shown that the side-chain methylene protons of soaps and phospholipids in the lamellar fluid (L_α) phase can be divided into two distinct spin subsystems with different residual second moments, so that, in any case, the presence of a motional gradient must be considered as a possible explanation for the distinct broad and sharp components of the methylene resonances in the ROS liposomes and membrane vesicles.

The resolution of significantly less than 100% of the terminal CH_3 intensity in the ROS membrane vesicles and liposomes is difficult to explain by a motional gradient interpretation. The observation of different spin-lattice (T_1) relaxation rates for the methylene and terminal CH_3 groups of sonicated egg PC and DPPC liposomes (Horwitz et al., 1972; Lee et al., 1972; McLaughlin et al., 1973) precludes extensive coupling between these spin systems, so that the effects of segmental motion on the CH_3 resonance may be treated in a relatively simple manner. The fraction of the terminal CH_3 intensity resolved in the ^{1}H NMR spectra of unsonicated bilayers has been proposed by Seiter and Chan (1973) to correspond to the central $\frac{1}{2} \leftrightarrow -\frac{1}{2}$ transition in the powder spectrum for a spin $\frac{3}{2}$ system undergoing rapid reorientation about the C_3 symmetry axis, with restricted motion of this axis. The wings from the angularly dependent satellite $|\frac{3}{2}| \leftrightarrow |\frac{1}{2}|$ spin transitions are presumed to be of low amplitude and not easily observed. It is possible to experimentally estimate the average amplitude of the terminal CH_3 group motion from the segmental order parameters of sonicated and unsonicated dispersions of egg PC derived from ^{2}H NMR studies (Stockton et al., 1976) and thereby gain a rough estimate of the residual dipolar splittings between the $|\frac{3}{2}| \leftrightarrow |\frac{1}{2}|$ spin transitions to be expected in the absence of vesicle rotation. For a terminal CH_3 group, the order parameter of the C_3 symmetry axis is given by (Saupe et al., 1965)

$$S_{C_3} = \frac{1}{2} \langle 3 \cos^2 \theta'(t) - 1 \rangle \tag{3}$$

where the brackets denote either a time or ensemble average and $\theta'(t)$ is the time-dependent displacement of the C_3 axis from the lowest energy chain conformation ($\theta' = 0°$). It follows that

$$S_{C_3} = \frac{1}{2} \int_0^{\Delta\theta'} (3 \cos^2 \theta' - 1) \sin \theta' d\theta' \Big/ \int_0^{\Delta\theta'} \sin \theta' d\theta'$$

$$= \frac{1}{2} (\cos^2 \Delta\theta' + \cos \Delta\theta') \tag{4}$$

where $\Delta\theta'$ is defined as the average amplitude of the motion of the C_3 axis assuming a rectangular probability distribution (Seiter and Chan, 1973). The segmental order parameter S_{mol} is related to that of the C_3 axis by $S_{mol} = 2 S_{C_3}$. Using the values for S_{mol} given by Stockton et al. (1976), we estimate that $\Delta\theta' > 85°$, in which case the $|\frac{3}{2}| \leftrightarrow |\frac{1}{2}|$ dipolar splitting is expected to be negligible (C. H. A. Seiter, personal communication) and all of the CH_3 intensity resolved as a single sharp resonance. This is in agreement with our ^{1}H NMR spectra of sonicated limit vesicles of egg PC, which show that 90–100% of the $(CH_2)_n$ protons and 100% of the terminal CH_3 protons are resolved as single sharp resonances, with little or no underlying broad component (Figure 4). The effectiveness of rotational diffusion of larger vesicles in averaging inter- and intramolecular dipolar interactions (Lee et al., 1973; Bloom et al., 1975; Kroon et al., 1976) can be empirically estimated from the fraction of the terminal CH_3 resonance area resolved

in the published ^{1}H NMR spectra of $\sim 10^3$-Å diameter DPPC vesicles (Sheetz and Chan, 1972; Lichtenberg et al., 1975). While it is difficult to accurately integrate the relatively broad terminal CH_3 resonance of the larger DPPC vesicles, we estimate that ~70–100% of the terminal CH_3 intensity is observed. The terminal CH_3 line width of the ROS membrane vesicles and ROS liposomes is somewhat sharper than that of the $\sim 10^3$-Å DPPC vesicles, so that it is possible to obtain a relatively more accurate estimate of the resolved CH_3 area. Since only ~50% of the terminal CH_3 intensity of the ROS membrane vesicles (and sarcoplasmic reticulum vesicles) is observed in the sharp resonances, the remaining fraction of the terminal CH_3 protons, which appear in the broad component, must possess significantly greater order. In addition, since the order parameter of the terminal CH_3 groups is lower than that of the N^+Me_3 groups in the liquid crystalline phase of egg PC (Stockton et al., 1976), the observation of 100% of the N^+Me_3 protons implies that 100% of the terminal CH_3 protons would also be resolved if all the ROS phospholipids were in a liquid-crystalline phase similar to that of egg PC. It is therefore likely that some of the ROS phospholipids are in the gel phase or a somewhat less fluid state.

We feel that the simplest explanation of the presently available NMR data for the ROS membrane vesicles and ROS liposomes that some of the phospholipid side chains are fluid and yield sharp NMR spectral components, and some are less fluid and yield the broad, underlying components. In relatively homogeneous bilayers, such as egg PC, a single liquid-crystalline phase is present (Chapman, 1975) and only sharp resonance components are observed upon sonication. Given this interpretation of the spectra, it follows that the fluid and less fluid ROS phospholipids are organized into laterally segregated domains, as found for model phospholipid mixtures (Shimshick and McConnell, 1973; Wu and McConnell, 1975), or, possibly, into short-lived clusters of different average microviscosity (Lee et al., 1974b). Lateral diffusion of phospholipids between any mosaic domains would have to be too slow to average their effective transverse relaxation rates, since distinct sharp and broad components are observed. Since a similar fraction of broad and sharp components are observed in the ^{1}H NMR spectra of the ROS membrane vesicles and ROS liposomes, the presence of lateral domains must be a consequence of lipid-lipid interaction and cannot be greatly affected by the interaction of phospholipids with rhodopsin. Pontus and Delmelle (1975b) have concluded from studies of 2,2,6,6-tetramethylpiperidinyl-1-oxy (Tempo) and stearic acid spin-probe solubility in bovine ROS membranes that about $\frac{2}{3}$ of the phospholipids are fluid at 37 °C, which is in substantial agreement with the fraction of the terminal CH_3 intensity resolved in the ^{1}H NMR spectra of both the ROS membrane vesicles and ROS liposomes.

Effect of Temperature on the ^{1}H NMR Spectra of ROS Disk Membranes. The observation of a reversible, temperature-induced redistribution of the resonance intensity between the broad and sharp spectral components of ROS membranes and ROS liposomes can be simply explained in terms of the domain interpretation. The appearance of an increasing fraction of sharp NMR spectral intensity with temperature is accompanied by increasing solubility of Tempo or di-*tert*-butyl nitroxide in ROS membranes and ROS liposomes (Pontus and Delmelle, 1975a; M. F. Brown, G. P. Miljanich, R. W. Becker, and E. A. Dratz, unpublished). The solubility of these molecules in the hydrophobic regions of biological membranes is thought to be governed by the fraction of phospholipids which are sufficiently fluid to accommodate bulky probe molecules (McConnell et al., 1972; Shimshick and McConnell, 1973). Similarly, the increasing proportion of broad spectral components with decreasing temperature may be related to the sharp increase in the fluorescence quantum yield of β-parinaric acid (Sklar et al., 1977a), which partitions selectively into rigid lipid domains (Sklar et al., 1977b). These observations are consistent with differential scanning calorimetric experiments, which reveal endothermic transitions involving a fraction of the ROS phospholipids over the temperature range 5–50 °C (Miljanich et al., 1976). Chabre (1975) has observed a $(4.15\ \text{Å})^{-1}$ equatorial x-ray reflection of cattle ROS disk membranes at low temperatures, which is characteristic of the gel phase of saturated hydrocarbon chains. The intensity of the $(4.15\ \text{Å})^{-1}$ reflection decreases gradually with increasing temperature, suggesting that at least part of the observed ^{1}H NMR spectral changes may arise from a gel to liquid-crystalline phase transition of some of the saturated chains of the membrane phospholipids. The PC fraction is probably involved in the membrane structural changes, since large intensity changes are observed in the N^+Me_3 resonance. Although the majority of the ROS phospholipids have a saturated chain at the 1 position of the glycerol moiety and a polyunsaturated chain at the 2 position (Anderson and Sperling, 1971), 20–30% of the PC fraction appears to be disaturated. These disaturated phospholipids constitute 8–12% of the total ROS phospholipids and may be implicated in the observed temperature-dependent behavior.

Interaction of Rhodopsin and Phospholipids. We now wish to briefly discuss the question of the motional state of those ROS phospholipids which are in direct hydrophobic contact with rhodopsin. These lipids have been referred to as "annular" or "boundary" lipids by other workers and we will use these terms interchangeably. Since residual static dipolar interactions in liquid crystalline phospholipids are averaged to very small values in vesicles of diameter $\lesssim 10^3$ Å, the line widths of the Lorentzian resonance components are determined predominantly by molecular motions and can be used as a measure of the transverse (T_2^*) relaxation rates.[5] The differences in the line widths of the sharp resonance components of the ROS membranes and the ROS liposomes are rather small, so that the transverse relaxation rates of the protons in these two preparations must be very similar. The spin-lattice (T_1) relaxation rates of the sharp resonance components, however, indicate a large effect of protein-lipid interaction in the ROS membranes (Brown et al., 1976; 1977). We have concluded that the effect of interaction with rhodopsin is to inhibit the more frequent small amplitude segmental motions of some of the ROS phospholipids, leaving the less frequent large amplitude motions relatively unaffected. These effects could result if the relatively large amplitude motions are coupled to lateral jumps of phospholipids to adjacent bilayer lattice sites and if rhodopsin does not immobilize the fluid phospholipids for periods of time longer than the life-time for a jump mechanism of lateral diffusion ($\sim 10^{-7}$ s; Devaux and McConnell, 1972). This would suffice to account for the similar T_2^* relaxation rates observed for the ROS membrane vesicles and ROS liposomes. The motions which govern T_1 most likely involve interconversion between highly β-coupled gauche$^+$-gauche$^-$ conformations (kinks) (Träuble, 1971; Horwitz et al., 1972; Horwitz et al., 1973a), which require relatively small lateral displacements and probably occur at relatively high frequencies about given lattice sites in the bilayer. The differences in the

[5] $T_2^* = 1/\pi\Delta\nu_{1/2}$ for a Lorentzian line shape, where $\Delta\nu_{1/2}$ is the resonance line width at half-height.

T_1 relaxation rates of the ROS membrane vesicles and ROS liposomes suggest that while a phospholipid is interacting with rhodopsin (i.e., between jumps) its kink motions are considerably inhibited. Thus, at least some of the ROS phospholipids may be translationally fluid, yet segmentally hindered by interaction with rhodopsin. The question is whether these lipids are in direct hydrophobic contact with rhodopsin or whether the T_1 effects are propagated indirectly from the annular to the nonannular lipids.

The bulk of previous evidence has been interpreted in terms of a model of immobilized boundary lipids surrounding integral membrane proteins (see, e.g., Jost et al., 1973a,b,c; Griffith et al., 1973; Lee, 1976). However, the precise nature of this "immobilization" is not well defined at the present time. The observation of immobilized components in the electron-spin resonance spectra of 16-doxyl stearate bound to membrane proteins (Jost et al., 1973b; Hesketh et al., 1976) requires only that the rotational correlation time of the nitroxide moiety is in the long correlation time regime ($\tau_r > 3 \times 10^{-8}$ s) and does not by itself preclude the possibility of lateral exchange of spin labels between boundary and nonboundary lipid environments at frequencies approaching that estimated for lateral diffusive jumps in pure bilayers ($\sim 10^7$ Hz). Since the fluid lipid component appears to be in the short correlation time regime ($\tau_R < 3 \times 10^{-8}$ s), the effects of chemical exchange between lipid environments are not amenable to simple calculation. It is also very difficult to distinguish unequivocally between exchanging and nonexchanging boundary lipids on the basis of reconstitution or lipid-depletion experiments (Warren et al., 1975; Pontus and Delmelle, 1975b; Hesketh et al., 1976), since exchange is impossible when all lipids are annular and it is difficult to observe the effects of immobilized lipids in the presence of excess extra-annular lipids. The ^{1}H NMR data for the ROS membranes could be explained in terms of an immobile annulus of boundary lipids by assuming that rhodopsin is primarily interacting with the phospholipids giving rise to the underlying broad components of the ROS membranes and the ROS liposomes. A similar fraction of fluid phospholipids is observed in both cases and the effect of rhodopsin on the T_1 relaxation of the fluid lipids would then be indirect. The alternative explanation is that rhodopsin is directly interacting with the phospholipids giving rise to the sharp resonance components. We are not able at this time to determine which of these interpretations is correct. However, we note that (1) Hong and Hubbell (1972) found a linear dependence of the order parameters of spin-labeled phospholipids on lipid/protein ratio in recombinant ROS membranes, which suggests the possibility of exchange between interacting and noninteracting lipid environments, (2) freeze-fracture electron microscopy of rhodopsin-PC recombinant membranes indicates that rhodopsin is excluded from the phospholipid gel phase and partitions into the fluid liquid crystalline phase (Chen and Hubbell, 1974). At least for the ROS disk membrane, therefore, it seems that translational exchange of annular lipids should be seriously considered as an alternative to the immobile boundary lipid model. The presently available NMR data suggest that domains of phospholipids in different motional states exist in the ROS disk membrane and that rhodopsin may be preferentially interacting with the more fluid phospholipids.

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