

# PROTON NMR $T_1$ , $T_2$ , AND $T_{1\rho}$ RELAXATION STUDIES OF NATIVE AND RECONSTITUTED SARCOPLASMIC RETICULUM AND PHOSPHOLIPID VESICLES

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**ABSTRACT** The phospholipid protons of native and reconstituted sarcoplasmic reticulum (SR) membrane vesicles yield well-resolved nuclear magnetic resonance (NMR) spectra. Resonance area measurements, guided by the line shape theory of Bloom and co-workers, imply that we are observing a large fraction of the lipid intensity and that the protein does not appear to reduce the percent of the signal that is well resolved. We have measured the spin-lattice ( $T_1$ ) and spin-spin ( $T_2$ ) relaxation rates of the choline, methylene, and terminal methyl protons at 360 MHz and the spin-lattice relaxation rate in the rotating frame ( $T_{1\rho}$ ) at 100 MHz. Both the  $T_1$  and  $T_2$  relaxation rates are single exponential processes for all of the resonances if the residual water proton signal is thoroughly eliminated by selective saturation. The  $T_1$  and  $T_2$  relaxation rates increase as the protein concentration increases, and  $T_1$  rates decrease with increasing temperature. This implies that the protein is reducing both high frequency (e.g., *trans-gauche* methylene isomerizations) and low frequency (e.g., large amplitude, chain wagging) lipid motions, from the center of the bilayer to the surface. It is possible that spin diffusion contributes to the effect of protein on lipid  $T_1$ 's although some of the protein-induced  $T_1$  change is due to motional effects. The  $T_2$  relaxation times are observed to be near 1 ms for the membranes with highest protein concentration and  $\sim 10$  ms for the lipids devoid of protein. This result, combined with the observation that the  $T_2$  rates are monophasic, suggests that at least two lipid environments exist in the presence of protein, and that the lipids are exchanging between these environments at a rate  $> 1/T_2$  or  $10^3 \text{ s}^{-1}$ . The choline resonance yields single exponential  $T_{1\rho}$  relaxation in the presence and absence of protein, whereas the other resonances measured exhibit biexponential relaxation. Protein significantly increases the single  $T_{1\rho}$  relaxation rate of the choline peak while primarily increasing the  $T_{1\rho}$  relaxation rate of the more slowly relaxing component of the methylene and methyl resonances.

## INTRODUCTION

The sarcoplasmic reticulum (SR) membrane is well suited for the study of protein-lipid interactions. It is a highly specialized membrane consisting mainly ( $> 90\%$ ) of one transmembrane protein, the calcium pump protein (Ca pump) (Fleischer et al., 1979a). Membranes that are fully functional and of defined composition may be reconstituted (Meissner and Fleischer, 1974; Wang et al., 1979; Hymel and Fleischer, 1980). Thus, the protein:lipid ratios can be varied in these reconstituted membranes to study the effects of the protein on the motion of the native or synthetic lipids.

We have used proton NMR relaxation to examine the influence of the Ca pump protein on the motions of membrane lipids. Specifically, we have measured the spin-lattice ( $T_1$ ), spin-spin ( $T_2$ ) and spin-lattice in the rotating frame ( $T_{1\rho}$ ) relaxation rates of the lipid molecules

in the presence and absence of protein. The  $T_1$  relaxation parameter is sensitive to high frequency lipid motions such as *trans-gauche* methylene isomerizations, whereas the  $T_2$  time is sensitive to low frequency (e.g., large amplitude chain wagging) lipid motions.  $T_{1\rho}$  can provide information about lipid motions whose correlation times are between those monitored by  $T_1$  and  $T_2$ .

Increasing protein concentration decreases the lipid  $T_1$ . The protein may act as a spin polarization sink which contributes to the protein-dependent decrease in the lipid  $T_1$  apart from effects on lipid motion. However, similar effects of protein on lipid  $T_1$  have been observed using natural abundance  $^{13}\text{C}$  NMR, implying that spin diffusion between protein and lipid is not a dominant path for lipid-proton relaxation. The ability to resolve lipid resonances in the headgroup region (the choline methyl protons), those from along the hydrocarbon chain (e.g., the

methylene and vinyl protons), and the terminal methyl groups, has allowed us to study the effects of a membrane protein on lipid motions from the surface to the center of the membrane.

## MATERIALS AND METHODS

Sarcoplasmic Reticulum (SR) was isolated as described by Meissner et al. (1973). SR lipids were extracted with chloroform-methanol (2:1), and nonlipid materials were removed by back-extraction according to Folch et al. (1957), as described by Rouser and Fleischer (1967). Any neutral lipids were removed by silicic acid chromatography according to Rouser and Fleischer (1967).

Sarcoplasmic reticulum was dissociated and reconstituted by the method of Meissner and Fleischer (1974) as modified by Hymel and Fleischer.<sup>1</sup> The amount of deoxycholate used for dissociation was adjusted to give 60–85% solubilization of protein and 80–90% solubilization of phospholipid. Insoluble fragments were removed by centrifugation. The time of dialysis was varied from 30 min to 4 h to obtain membranes with lipid content from approximately half to slightly greater than that of the original SR. The membranes were sedimented and washed to remove remaining detergent. The functional activity of the reconstituted vesicles was typical of that reported by Hymel and Fleischer,<sup>1</sup> and depended on the solubilization procedure used and the lipid to protein ratio of the resultant reconstituted membranes. The calcium pumping rate and ATPase activity were found to be stable in the experimental buffer at 25°C for > 24 h. Exchange of D<sub>2</sub>O for H<sub>2</sub>O was correlated with a 30–50% decrease in the ATPase activity and the calcium pumping rate, whether the exchange was carried out by centrifugation or a gentle dialysis procedure. Sonication for > 10 min also reduced the calcium pumping activity. Sonication did not affect the T<sub>1</sub> time within experimental error (data not shown).

Native and reconstituted SR membranes were pelleted and allowed to soak for ~ 12 h at 4°C in deuterated buffer (0.01 M phosphate, 0.15 M KCl, pH 7.0), to allow <sup>1</sup>H ↔ <sup>2</sup>H exchange. The membrane suspensions were then pelleted (27,000 g, 60 min), and resuspended in deuterated buffer. The pelleting and resuspension was repeated five to six times. The final concentration of the samples ranged from ~ 15–40 mg phospholipid/ml. Alternatively, the <sup>1</sup>H ↔ <sup>2</sup>H exchange was accomplished by dialysis of the membranes against deuterated buffer (40 ml, five to six changes) at 4°C.

The SR lipids were dried to a thin film by rotary evaporation, hydrated in deuterated buffer, and dispersed by gentle swirling with several glass beads. All manipulations were performed under an argon atmosphere as a precaution against lipid peroxidation.

Most of the samples for NMR were sonicated for 20 min in 2 min on, 2 min off cycles under an argon atmosphere at 4°C, using a well-tuned Branson model W 140 sonifier equipped with a microtip (Branson Sonic Power Co., Danbury, CT). Some samples were sonicated for 0, 5, 10 or 20 min to assess the effect of sonication on T<sub>1</sub>.

The solvent water proton resonance was saturated during T<sub>1</sub> and T<sub>2</sub> measurements only. This was accomplished by continuous wave homonuclear decoupling or by gating the decoupler on for short periods just before acquisition of the FID. The residual sharp rf spike in the water region was eliminated by alternate 90<sub>x</sub> and 90<sub>y</sub> excitation of the free induction decay (FID). The FID resulting from 90<sub>x</sub> was subtracted from the FID resulting from 90<sub>y</sub> (Krishna, 1976).

T<sub>1</sub> and T<sub>2</sub> were obtained at 360 MHz on a Nicolet NTC-360 spectrometer (Nicolet Instrument Corp., Madison, WI) equipped with an Oxford Instruments solenoid (Oxford Instruments, Inc., Columbia, MO). All spectra were acquired using the Fourier transform technique. The acquisition parameters were: 5 kHz frequency range, 8 K data points, 150 μs trigger delay, 15.0 s repetition rate. All free induction decays were

filtered with a 1 Hz exponential line broadening before Fourier transformation. T<sub>1</sub> relaxation rates were measured using the inversion recovery (180°, τ, 90°) pulse sequence. T<sub>2</sub> relaxation rates were measured using the Carr-Purcell-Meiboom-Gill pulse echo sequence (90°, τ, 180°, τ, [echo], τ, 180°, τ, [echo], τ, ...). T<sub>1ρ</sub> values were obtained at 100 MHz using the spin locking technique in the Fourier transform mode. Acquisition parameters were: 1 KHz frequency range, 8 K data points, 150 μs trigger delay, 1 s repetition rate, and a locking field strength of ~ 1.7 gauss.

The T<sub>1</sub> and T<sub>2</sub> relaxation data was analyzed using a three-parameter, nonlinear least squares fitting routine (Sass and Ziessow, 1977) available on the Nicolet 1180 minicomputer, and by linear least squares fitting of the data plotted semilogarithmically.

## Relative Intensity Measurements

Sodium dodecyl sulfate (SDS) was accurately weighed into several 5-mm NMR tubes. D<sub>2</sub>O buffer was added to give final concentrations of SDS ranging from 10–30 mM. These samples, of known concentration, were used to calibrate the NMR signal intensity of sodium 3-trimethylsilylpropionate-2,2,3,3-d<sub>4</sub> (TSP, 5–25 mg/ml in CCl<sub>4</sub>) contained in a Wilmad coaxial capillary insert (10 μl, 32-mm stem length) (Wilmad Glass Co., Buena, NJ). The calibration was achieved by recording the 360 MHz <sup>1</sup>H-NMR spectrum of one of the SDS samples in the presence of the capillary insert. The main methylene peak (18 protons) was integrated and the integral was set to the appropriate mM proton concentration. Then the TSP resonance was integrated and its value recorded. The calibrated TSP insert accurately reproduced the known proton concentration of the methylene and methyl peaks of the other SDS samples. Spectra of SR membranes and SR lipids were recorded when the samples contained the calibrated TSP insert.

The percent of the NMR resonance intensities observed in the spectra were defined in the following operational manner. The relative intensity of the choline proton resonances to the TSP insert was obtained directly from these spectra by computer integration. The intensities of the fatty acid methyl and methylene resonances were estimated using a lineshape guided by the theoretical analysis of Bloom et al. (1978). To estimate the methylene resonance area, the spectra were folded over the peak methylene resonance frequency and a symmetrical, smooth lineshape was drawn. The lineshape smoothly excluded the methyl resonance as well as some low field asymmetry in the methylene peak due to low amplitude resonances poorly resolved from the main methylene peak. This lineshape is very similar to the *ad hoc* shape which Bloom et al. (1978) found adequately accounted for the methylene area of egg PC vesicles. The areas of the methylene and methyl resonances were determined by cutting out and weighing in duplicate. The percent of the signal observed in the membranes was calculated, using the membrane-bound phosphorus concentration (i.e., phospholipid) and assuming an average fatty acid chain length of 18 carbons (Morai and Kuksis, 1973) and a phosphatidyl choline content of 60% (Meissner et al., 1972).

## RESULTS

<sup>1</sup>H-NMR spectra obtained for pure SR lipids, native SR membranes, and recombinant SR membranes at 360 MHz are shown in Fig. 1. These samples yield high resolution spectra. The terminal methyl, methylene, single and double allylic, choline methyl, and vinyl proton lipid resonances are clearly resolved. A significant broadening of the various resonances can be observed with increasing protein content. This is most evident in the methylene and terminal methyl region, judging from the ratio of the peak height of either resonance to the trough between them. The broadening of the choline peak is accompanied by a decrease in peak height. In the spectra of the protein-

<sup>1</sup>Hymel, L., and S. Fleischer. Manuscript in preparation.

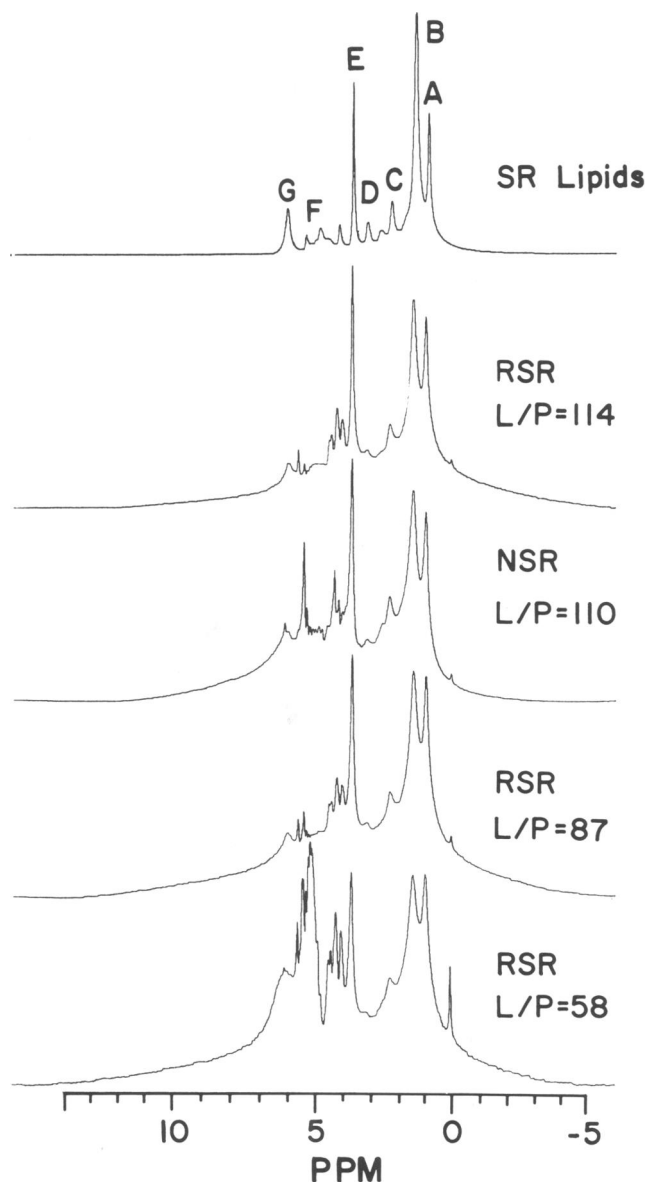


FIGURE 1 Proton NMR spectra (360 MHz) of SR lipids, reconstituted SR membranes, and native SR membranes, at 25°C. The protein content of the various samples increases from top to bottom and the L/P values are the molar ratios of phospholipid to Ca pump protein. The letters (A–G) in the pure lipid spectrum correspond to the following lipid proton resonances: A, terminal methyl; B, saturated chain methylenes; C, single allylic methylenes; D, double allylic methylenes; E, choline methyls; F, residual HDO signal after suppression by selective saturation; G, vinyl methylenes.

containing samples, a broad underlying component, which grows with increasing protein content, is apparent. It is likely that this broad component largely represents unresolved protein resonances, as suggested by Inesi and co-workers (Davis et al., 1976). Not all of the SR lipid proton groups contribute to the high resolution spectra of the pure lipids and membranes. We have measured the percent of the total possible intensity observed using a calibrated

TABLE I  
PERCENT OF TOTAL LIPID SIGNAL OBSERVED AS  
SHARP  $^1\text{H}$ -NMR LINES

Native SR Membranes			SR Lipid Vesicles		
Resonance	%	Total	Resonance	%	Total
	25°C	35°C		25°C	35°C
Choline	79	94	Choline	81	96
Methylene	57	71	Methylene	44	44
Methyl	103	125	Methyl	98	102

capillary insert containing TSP at 25° and 35°C (see Methods section for details). Table I indicates that 80–95% of the *N*-methyl group protons of the phosphatidylcholine components, at least 40% of the chain methylene and ~ 100% of the terminal methyl protons are observed as high resolution signals at these temperatures whether or not the protein is present. Therefore, although we do not see all of the lipid proton intensity, we observe at least as large a percentage of the signal, within experimental error, in the presence of protein as in the pure lipid vesicles.

Initially, the  $T_1$  relaxation rates of SR membranes were measured in the presence of a rather large residual solvent water proton peak. The size of the water resonance was reduced by extensive exchange of the SR membranes against 99.8%  $\text{D}_2\text{O}$  followed by 100%  $\text{D}_2\text{O}$  buffer. Although much HDO remained no attempt was made initially to reduce its resonance further since we wanted to compare our data with previous ROS membrane  $T_1$  relaxation results (Brown et al., 1977). Under these conditions, the  $T_1$  relaxation rates of the SR lipids are biexponential for the protein-containing samples, and single-exponential for the pure lipids.<sup>2</sup> The amplitude of the slow  $T_1$  component was proportional to the protein concentration in the membranes. We initially interpreted this as a protein-induced perturbation of the motions of the lipids in contact with the Ca pump protein. However, when the water signal was thoroughly suppressed by selective saturation techniques (Krishna, 1976), the membrane lipid resonances exhibited single-exponential  $T_1$  relaxation behavior. The slowly relaxing  $T_1$  component appears to be a water-related effect instead of a direct protein influence, and the amount of residual water proton signal observed after extensive exchange is dependent upon the protein concentration.<sup>2</sup>

A plot of the  $T_1$  data obtained for the methyl, methylene, and choline resonances of pure SR lipids and recombinant SR membranes shows that the  $T_1$  relaxation rates are single exponential processes in the presence of solvent water proton saturation (Fig. 2). The slopes increase with increasing protein concentration; so that the lipid  $T_1$  relaxation times decrease with increasing protein concen-

<sup>2</sup>Deese, A. J., L. Hymel, S. F. Fleischer, and E. A. Dratz. Manuscript in preparation.

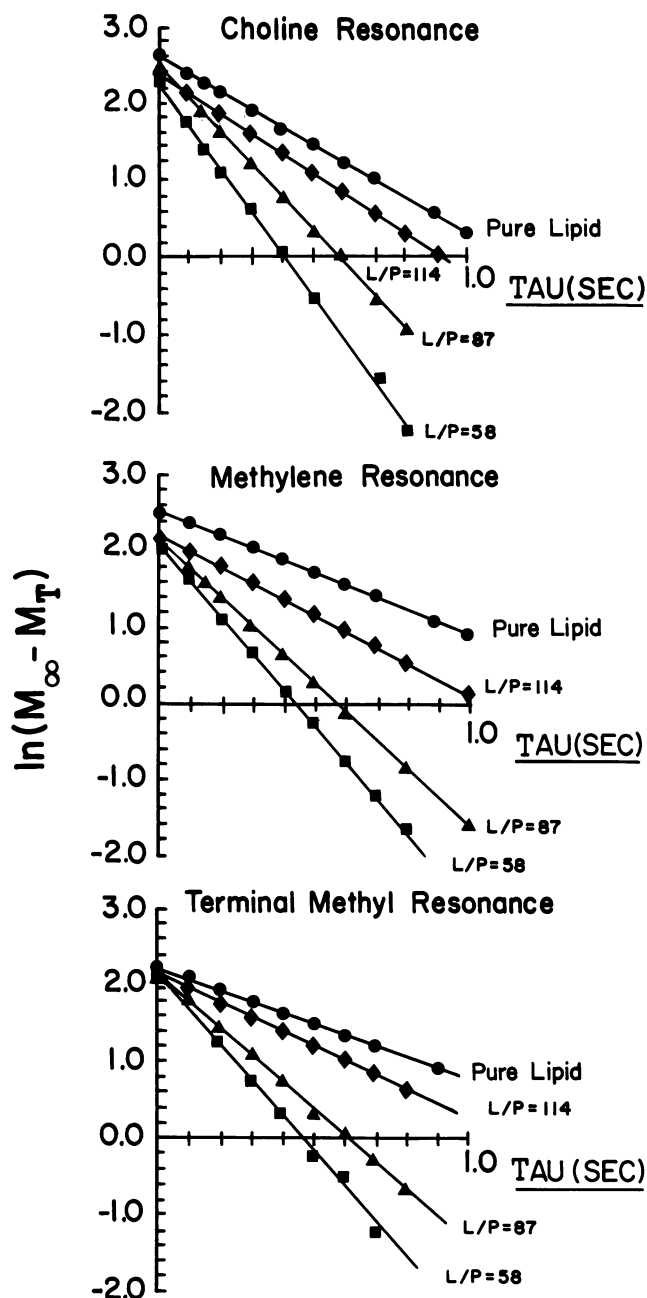


FIGURE 2 Comparison of 360 MHz inversion recovery vs.  $\tau$ -plots for the methyl, saturated methylene, and choline resonances of the SR lipids and recombinant SR membranes (25°C). The lipid:protein ratios of the various samples are shown next to the respective plot. The slopes of the plots increase with increasing protein concentration.

tration of the membranes. The effect is largest for the methyl resonance, decreasing from 0.74 s in the pure lipids to 0.21 s in the highest protein recombinant sample investigated. The  $T_1$  data are summarized in Table II.

$T_1$  relaxation times were also measured as a function of temperature and sonication time. Shown in Fig. 3 is a 360 MHz  $^1\text{H}$ -NMR spectrum of unsonicated SR membranes at 25°C. Without sonication, the spectrum consists mainly

TABLE II  
EFFECT OF PROTEIN ON LIPID PROTON  $T_1$

Sample	L/P $\ddagger$	Choline	Single allylic	Methylene	Methyl
RSR 1	58	0.18	0.16	<sup>s</sup> 0.21	0.21
RSR 2	87	0.22	0.26	0.28	0.28
NSR	110	0.24	0.30	0.31	0.32
RSR 3	114	0.32	0.37	0.47	0.47
SR lipids	—	0.43	0.59	0.62	0.74

\*The average error in the fit of these measurements is 0.01 s.

$\ddagger$ Lipid-to-protein molar ratio.

of a large broad peak lying beneath sharp choline and rather small hydrocarbon chain resonances. It should be noted that we observe a sharp choline resonance in the absence of sonication. Other workers (Davis and Inesi, 1971; Robinson et al., 1972) did not observe a well-resolved choline peak in their unsonicated membranes. Robinson et al. (1972) attributed the absence of the choline signal in unsonicated SR membranes to the interaction with membrane protein. However, we find no evidence for such an effect. Furthermore,  $T_1$  does not significantly depend on sonication time in native or reconstituted SR membranes (data not shown).  $T_1$  vs. temperature data (Table III) show that  $T_1$  increases with increasing temperature in both SR membranes and SR lipid vesicles. The lipid motions dominating  $T_1$  relaxation, therefore, seem to be faster than the observational frequency of 360 MHz.

In Table IV, we show that  $T_2$  decreases with increasing protein content in the three lipid resonances studied. For example, in the case of the methylene resonance,  $T_2$  decreases from a value of 2.4 ms in the pure lipids to 0.64 ms in the reconstituted membranes with the highest protein content. The  $T_2$  relaxation is observed to be a single exponential process for all resonances (there is a faster component to the  $\text{CH}_2$   $T_2$  relaxation which arises from the broad fraction, ~50% of the total  $\text{CH}_2$  area,

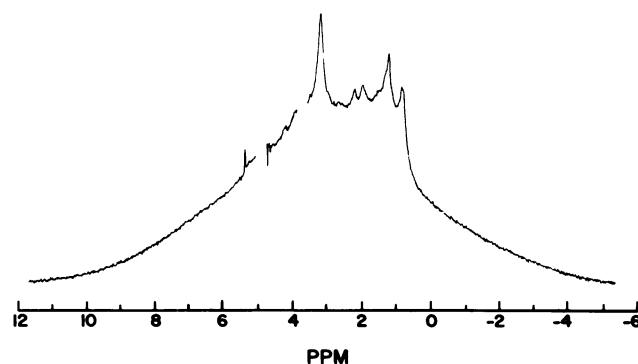


FIGURE 3 360 MHz spectrum of unsonicated native SR membranes at 25°C. Note the sharp choline resonance occurring at ~ 3.2 PPM on the arbitrary scale used.

TABLE III  
TEMPERATURE DEPENDENCE OF  $T_1$

Native SR Membranes, $T_1$ (s)				
Temp. (°C)	Choline	Single Allylic	Methylene	Methyl
10	0.18 ± 0.006	0.27 ± 0.010	0.21 ± 0.010	0.22 ± 0.010
25	0.21 ± 0.006	0.30 ± 0.010	0.28 ± 0.002	0.31 ± 0.003
35	0.25 ± 0.002	0.34 ± 0.010	0.33 ± 0.003	0.37 ± 0.008

SR Lipid Vesicles, $T_1$ (s)				
Temp. (°C)	Choline	Single Allylic	Methylene	Methyl
10	0.30 ± 0.010	0.32 ± 0.010	0.41 ± 0.010	0.55 ± 0.010
25	0.43 ± 0.010	0.59 ± 0.010	0.62 ± 0.003	0.74 ± 0.002
45	0.51 ± 0.002	0.65 ± 0.010	0.72 ± 0.010	1.01 ± 0.010

which relaxes before the first data point in the  $T_2$  experiment).

Semilog plots of  $T_{1\rho}$  relaxation data obtained from native SR membranes and pure SR lipids are shown in Fig. 4. The choline protons exhibit single exponential  $T_{1\rho}$  relaxation in the absence and presence of protein, whereas the methylene and methyl protons relax biexponentially in both cases. The protein increases the  $T_{1\rho}$  relaxation rate of the choline resonance by a factor of 1.6 while primarily reducing the relaxation rate of the slowly-relaxing component of the methylene and methyl resonances.

#### DISCUSSION

We have shown that the presence of solvent water protons can add a slow component to the lipid  $T_1$  relaxation. Solvent water protons have relatively long  $T_1$ 's and can apparently act as a long-lived source of spin polarization, as is indicated by a number of experiments. (Direct observation of proton saturation transfer [i.e., spin diffusion] within lipid molecules and between water and lipids has been carried out. The saturation transfer effects are not larger than ~ 30%, but are easily measurable. Furthermore, the addition of 3mM  $\text{Cr}^{+++}$  to the aqueous medium sharply reduces the solvent water  $T_1$  and eliminates the water protons as a long-lived source of spin polarization. The  $\text{Cr}^{+++}$  abolishes the slow  $T_1$  component in the lipid relaxation [Deese et al., 1981].) Reduction of solvent water protons by lyophilization and resuspension in  $\text{D}_2\text{O}$

TABLE IV  
EFFECT OF PROTEIN ON LIPID PROTON  $T_2^*$

Sample	L/P‡	Choline	Methylene	Methyl
		(ms)		
RSR 1	58	0.60	0.64	0.67
RSR 2	87	2.38	0.96	1.40
NSR	110	2.75	0.99	1.45
RSR 3	114	3.20	1.06	1.59
SR Lipids	—	7.40	2.45	3.24

\*The average error in the fit of these measurements is 0.02 (ms).

‡Lipid to protein molar ratio.

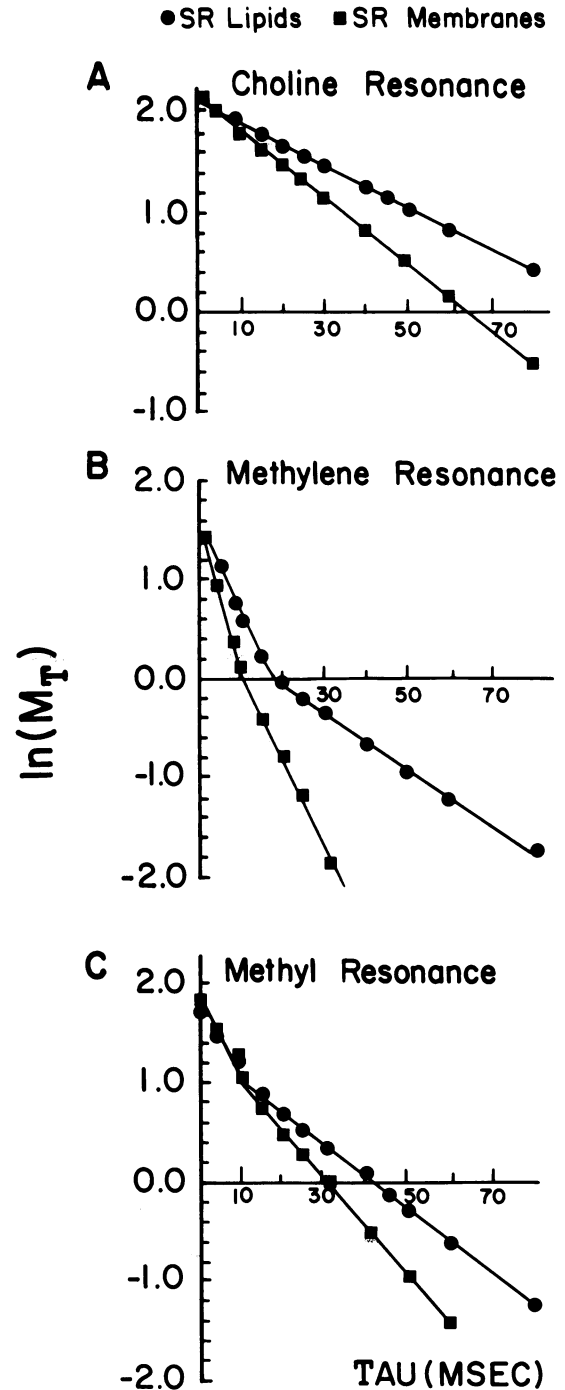


FIGURE 4 Comparison of 100 MHz  $T_{1\rho}$  semilog plots for the choline methylene and methyl resonances of native SR membranes and pure SR lipid vesicles at 25°C.

essentially removes the slow  $T_1$  component from all the lipid resonances. Readdition of  $\text{H}_2\text{O}$  restores the slow lipid  $T_1$  component. Lyophilization may be used in some membrane systems such as the retinal rod disk membrane without apparent damage to the system but the Ca pump protein will not survive lyophilization.

Elimination of the interference of the solvent water spin

polarization has allowed us to observe protein-dependent reductions of the lipid  $T_1$  rates. The extent of this reduction depends on the amount of protein present. We believe that the effect of protein on membrane lipid  $T_1$ 's is due to protein restricting the lipid motions. It is possible that the protein acts as a spin polarization sink and speeds lipid  $T_1$  relaxation by a spin diffusion mechanism. The protein-lipid spin diffusion which can be detected is not a strong effect. Natural abundance  $^{13}\text{C}$   $T_1$  relaxation of SR lipids increases substantially in the presence of the Ca pump protein (unpublished results). Furthermore, natural abundance  $^{13}\text{C}$   $T_1$  relaxation rates of ROS lipids also increase in the presence of rhodopsin (Deese, et al. 1981). Spin diffusion cannot contribute to the  $^{13}\text{C}$   $T_1$ 's, which suggests that the influence of the proteins observed here must largely be due to motional effects.

Deuterium NMR studies of specifically labeled lipids (Seelig and Seelig, 1974; Stockton et al., 1974) show that the choline headgroup, fatty acid methylenes near the terminus, and the terminal methyl group have a low degree of average order in model membranes. Bloom et al. (1978) have shown that proton order parameters are essentially equivalent to those determined by  $^2\text{H}$  NMR. Therefore, it seems reasonable to assume that the  $T_1$  behavior of these groups can be approximated by an isotropic model.  $T_1$  vs. temperature experiments of Robinson et al. (1972) and our group (Table III) indicate that the  $T_1$  relaxation rates of the SR lipid protons decrease with increasing temperature in the presence and absence of protein. These data suggest that the motions which govern  $T_1$  relaxation are more rapid than the observational frequency (360 MHz) and that the protein is slowing those lipid motions responsible for spin-lattice relaxation. The higher the protein content, the larger the observed decrease in  $T_1$ . In lipid bilayers, high frequency motions, such as *trans-gauche* methylene isomerizations, are thought to be the most effective in causing  $T_1$  relaxation (Lee et al., 1974).  $T_1$  is not affected by vesicle size differences. These studies imply a decrease of high frequency lipid motions by the calcium pump protein.

Unlike  $T_1$ ,  $T_2$  is sensitive to vesicle size. The most satisfactory theory developed to date for interpreting  $^1\text{H}$ -NMR lineshapes of sonicated membrane lipids is that of Bloom et al. (1978) and Wennerström and Ulmius (1976). The vesicle lineshape is a superposition of Lorentzian lines that arise from different parts of the spectra of an oriented bilayer. The contribution to a spectral component at a frequency  $\omega$  has a value of  $T_2$  given by:

$$\frac{1}{T_2} = \frac{\omega^2 \tau_v}{5} + \Delta. \quad (1)$$

The parameter  $\Delta$  represents all contributions to the NMR linewidth which are approximately independent of vesicle reorientation, such as magnetic field inhomogeneity or unresolved differences in chemical shift.  $\tau_v$  is the correla-

tion time for vesicle reorientation. The shortest values of  $\sim 1 \mu\text{s}$  are obtained for vesicles having a diameter of  $\sim 250 \text{ \AA}$ , while  $\tau_v$  is essentially infinite for multilamellar systems. There are two contributions to  $1/\tau_v$ , one associated with the rate of vesicle tumbling,  $1/\tau_R$ , and the other,  $1/\tau_D$ , due to the rate of lateral diffusion of the lipid molecules. The appropriate expression as given by Bloom et al. (1978) is

$$\frac{1}{\tau_v} = \frac{1}{\tau_R} + \frac{1}{\tau_D} = \frac{3kT}{4\pi\eta r^3} + \frac{6D}{r^2}. \quad (2)$$

Here  $\eta$  is the viscosity of the vesicle medium and  $r$  is the vesicle radius.  $D$  is the translational diffusion constant of the lipids in the membrane;  $k$  and  $T$  have their usual meaning. It can be seen from Eqs. 1 and 2 that one must consider the contribution of the vesicle size to  $1/T_2$  (and, hence, the width of the  $^1\text{H}$ -NMR lines), in addition to the contribution of local lipid motions. Thus, the linewidth changes observed might be partly due to vesicle size differences in the various samples. That is, one effect of increasing the protein:lipid ratio might be to increase the size of the limit vesicles formed by sonication. Larger vesicles would increase  $\tau_v$ , which would increase  $1/T_2$  and hence the linewidths. Electron microscopy studies of sonicated ROS membranes and protein-free lipids indicated that protein does not significantly change vesicle size (Brown et al., 1976). We tentatively propose that the protein is slowing low frequency lipid motions that are monitored by  $T_2$ .

One important observation involving the  $T_2$  experiments which is not dependent upon vesicle size differences is that the  $T_2$  relaxation is a single exponential process. The Ca pump protein has been shown to span the membrane with a portion of its mass embedded in the bilayer; clearly, some of the membrane lipids are in contact with the protein.  $1/T_2$  is observed to increase with increasing protein content of the membranes, suggesting that lipids influenced by the Ca pump protein have different  $T_2$  relaxation rates. These data are consistent with the existence of at least two lipid domains in the protein-containing samples. We expect that lipids next to intrinsic membrane proteins will be restricted in large amplitude motions because of the relatively rigid protein surface. The simplest picture envisions two lipid domains, one surrounding the protein and another similar to bulk lipid. Lipids in these two domains would have to be in rapid exchange with each other, interchanging at a rate greater than or equal to the observed spin-spin relaxation rate of  $\sim 10^3 \text{ s}^{-1}$ . If this were not the case, one would expect to observe a biexponential or more complex  $T_2$  relaxation process for the lipids in the presence of protein. These  $T_2$  effects are corroborated by the increased broadening of the various lipid resonances, when the protein concentration increases (Deese et al., 1981). Recent  $^2\text{H}$  NMR studies of Seelig et al. (1981) and phosphorus NMR studies of McLaughlin et al. (1980) find no

evidence for long-lived protein:lipid interactions in the SR system on the NMR time scale.

We have estimated the fraction of the lipid resonances observed using an internal standard and integration guided by the lineshape theory of Bloom et al. (1978). We see a relatively large fraction of the lipid hydrocarbon resonances (50–60%), but a substantial fraction of the signal is unresolved. Restricted motion of the lipid chains in the membranes leads to residual dipole-dipole interactions that broaden the signal, especially near the head-group region. Also, lipids in larger, slower-tumbling vesicles broaden lines. We find no evidence for protein immobilizing lipids; rather, we appear to see larger lipid signal in the presence of protein.

ESR studies of reconstituted SR has recently been reinvestigated (McIntyre et al., 1981). Some immobilized phospholipid is detected. Computer subtraction to estimate constrained lipids requires assuming a model for constrained lipid motion. Even so, there is not a unique solution for the amount of immobilized lipid. Assuming a model with a maximum  $2A$  of 60 gauss, we can estimate ~8 mol of constrained phospholipid per mole of calcium pump protein at 25°C. This amount of immobilized lipid is less than one-third that reported by Hesketh et al. (1976), and is insufficient to constitute a boundary of immobilized phospholipid surrounding the Ca pump protein. Similar SR preparations were used in the studies presented here for  $^1\text{H}$ -NMR, as well as for the  $^2\text{H}$ -NMR (Seelig et al., 1981),  $^{31}\text{P}$ -NMR (McLaughlin et al., 1981; Seelig et al., 1981) and ESR (McIntyre et al., 1981). These are well-defined and functional membranes. McIntyre et al. (1981) used a spin label covalently linked to phospholipid molecules (*sn*-2-16-doxyl-1-phosphatidylcholine) in contrast to the free fatty acid spin labels employed by Hesketh et al. (1976). The basis for the immobilized lipid observed on the ESR time scale ( $\sim 10^{-8}$  s) is intriguing. Studies by many investigators (cf., Fleischer et al., 1979b; Inesi et al., 1976; Martinosi et al., 1975; and Wang et al., 1979) suggest that the Ca pump unit exists as an oligomer. The small immobilized lipid component recently observed with ESR may thus represent phospholipid trapped within the Ca pump unit between protein monomers.

We have initiated  $T_{1\rho}$  studies in these systems. The  $T_{1\rho}$  relaxation of the methyl and methylene resonances is sensitive to more than one motion, since the decays are biexponential. Protein affects predominately the slower relaxation process. We anticipate that further  $T_{1\rho}$  measurements as a function of spin lock field strength may be able to reveal correlation times for some of the lipid motions in the presence and absence of protein.

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## DISCUSSION

Session Chairman: V. Adrian Parsegian Scribe: Barbara A. Lewis

YEAGLE: Is it possible to rule out any unusual or peculiar behavior with respect to the  $T_1$  that might be related to the non-Lorentzian nature of the lines?

DRATZ: Myer Bloom, would you like to speak to this point?

BLOOM: I don't see why the non-Lorentzian behavior of the lines should affect  $T_1$ . There are two kinds of spectroscopy being done here, NMR spectroscopy measuring the line shape and relaxation spectroscopy in which each relaxation time ( $T_1$ ,  $T_2$ ,  $T_{1\rho}$ , etc.) gives a measure of the intensity of the fluctuating interactions at the frequency to which the relaxation measurement is sensitive. For example, a measurement of  $T_1$  gives the intensity of the fluctuating field at the Larmor frequency of the nuclei, and the non-Lorentzian nature of the lines is related to motions at frequencies much slower than the Larmor frequency. The  $T_1$  measurements should not be affected by the non-Lorentzian nature of the line. In the case of vesicles, the motions which lead to narrowing of the line and ultimately to line shape can be divided into two classes corresponding to motions which are fast and slow compared with the vesicle tumbling time.

There is a subtlety in the theory of the line shape which caused a lot of difficulty about five years ago but which is now understood (Wennstrom and Ulmius. 1976. *J. Magn. Reson.* 23:431–435; Bloom et al. 1978. *Biochemistry* 17:5750). This was the effect of vesicle tumbling on the residual width of the line after taking into account the effect of the faster motions.

The difference between vesicle systems and almost every other spin system is that the fast motions in the vesicles have an axis of symmetry which projects all of the averaged dipolar or quadrupolar interactions along a simple axis, the normal to the bilayer. Thus there is one principal direction in the system, and all the interactions are projected along it by the fast motions. The complete interaction averaged over the fast motion scales with  $(3 \cos \theta - 1)$ , where  $\theta$  is the angle between the bilayer normal and the external magnetic field; every part of the NMR line which would be observed in the absence of tumbling is separately motionally-narrowed. The result is a superposition of Lorentzians, and instead of a

Lorentzian line shape, one gets lines with wider wings and a narrow central portion.

This problem is now solved, and I regret that the people who have done this, including myself, have not done the further work to develop simpler analytical formulae which would facilitate utilization of the results of the rigorous theory. At present it is necessary to go through a messy numerical analysis to account for the line shape.

DRATZ: We have taken an empirical approach to eliminating the influence of complex, non-Lorentzian line shapes on relaxation parameters. We have measured  $T_1$  at the peaks of the spectra, at the wings of the spectra, and between the lines. We have also measured  $T_1$  as a function of sonication. To obtain the best spectra one must sonicate, but one can get useful spectra (see Fig. 3) without any sonication.  $T_1$  and the effect of protein on  $T_1$  appear to be unaffected by sonication. Also, the  $T_1$  values obtained differ little whether measured in the center or on the sides of the lines.

For completeness we should note the potential value of  $T_{1\rho}$  in measuring the influences of protein on lipid motion. Different techniques have different time windows:  $T_2$  provides a fairly slow time window for lipid systems in the range of  $10^{-3}$ – $10^{-4}$  s, whereas  $T_1$  depends on motions in the time range of  $10^{-9}$ – $10^{-10}$  s. With  $T_{1\rho}$  it is possible, at least in principle, to sweep out the frequency range between  $T_2$  and  $T_1$ , where presumably a lot of the action in lipid-protein interaction is happening (i.e., in the  $10^{-7}$  s time range). With  $T_{1\rho}$  we have the potential to see any frequency up to  $\sim 10^7$  Hz, by changing the strength of  $H_1$ . However, there are also many possible problems with using this approach in lipid systems. I hope that Myer Bloom will comment on this point.

BLOOM:  $T_{1\rho}$  is a relaxation time in the rotating reference frame; you are measuring the time a spin takes to undergo reorientation in the radio-frequency field. The virtue of this measurement is that you can vary the radio-frequency field from zero to values which correspond to frequencies of the order of 1 MHz; you can thus probe motions having correlation times down to the microsecond range. If you use vesicles or measure proteins in solution, then unfortunately there is a range of correlation times, larger than the tumbling time of the vesicle, that is inaccessible to this measurement. Whenever some motion averages the open interactions, then the energy or square of the interaction that is modulated by the motions gets put into a frequency spectrum capable of inducing spin