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

ALAN J. DEESE AND EDWARD A. DRATZ
Department of Chemistry, University of California at Santa Cruz, Santa Cruz, California 95064 U.S.A.

LIN HYMEL AND SIDNEY FLEISCHER
Department of Molecular Biology, Vanderbilt University, Nashville, Tennessee 37235 U.S.A.

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 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., 1979*a*). 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}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.[1] 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,[1] 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_2O for H_2O 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_1 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 $^1H \rightleftharpoons {}^2H$ 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 $^1H \rightleftharpoons {}^2H$ 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_1.

The solvent water proton resonance was saturated during T_1 and T_2 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_x and 90_y excitation of the free induction decay (FID). The FID resulting from 90_x was subtracted from the FID resulting from 90_y (Krishna, 1976).

T_1 and T_2 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: 5kHz 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_1 relaxation rates were measured using the inversion recovery (180°, τ, 90°) pulse sequence. T_2 relaxation rates were measured using the Carr-Purcell-Meiboom-Gill pulse echo sequence ($90°_x$, τ, $180°_y$, τ, [echo], τ, $180°_y$, τ, [echo], τ, ...). $T_{1\rho}$ 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_1 and T_2 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_2O 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-d4 (TSP, 5–25 mg/ml in CCl_4) 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 ^{1}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

^{1}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-

[1]Hymel, L., and S. Fleischer. Manuscript in preparation.

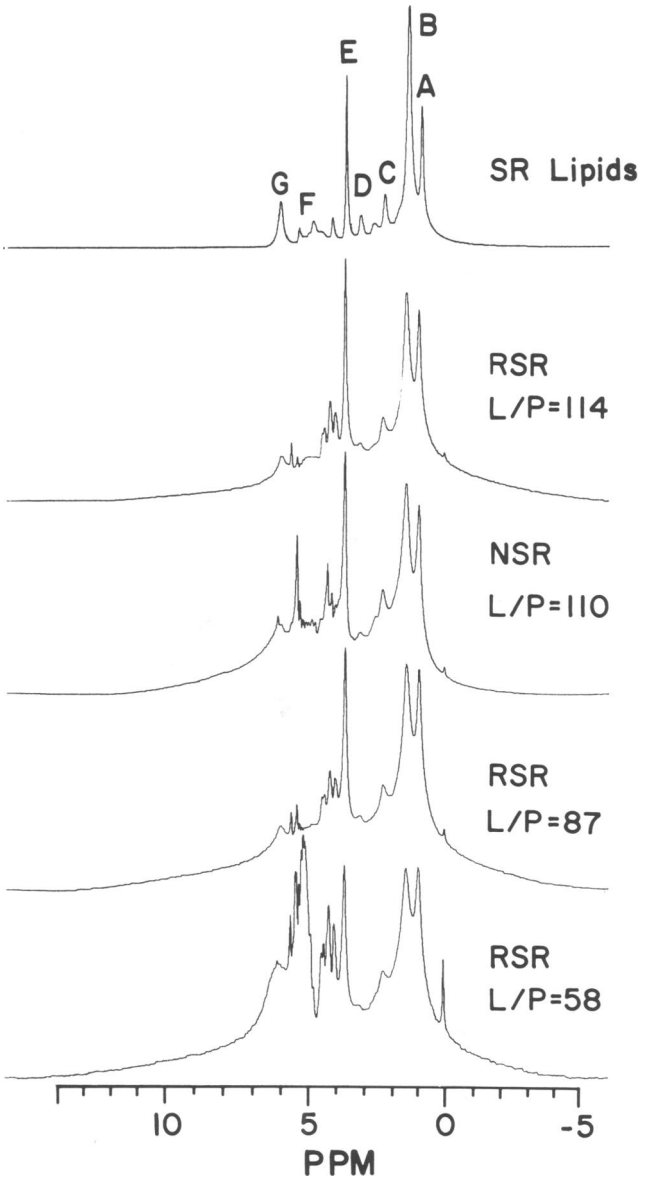

FIGURE 1 Proton NMR spectra (360 MHz) of SR lipids, reconstituted SR membranes, and native SR membranes, at 25°C. The protein content of the various samples increases from top to bottom and the L/P values are the molar ratios of phospholipid to Ca pump protein. The letters (*A–G*) in the pure lipid spectrum 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 coworkers (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}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% D_2O followed by 100% D_2O 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.[2] 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.[2]

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-

[2]Deese, A. J., L. Hymel, S. F. Fleischer, and E. A. Dratz. Manuscript in preparation.

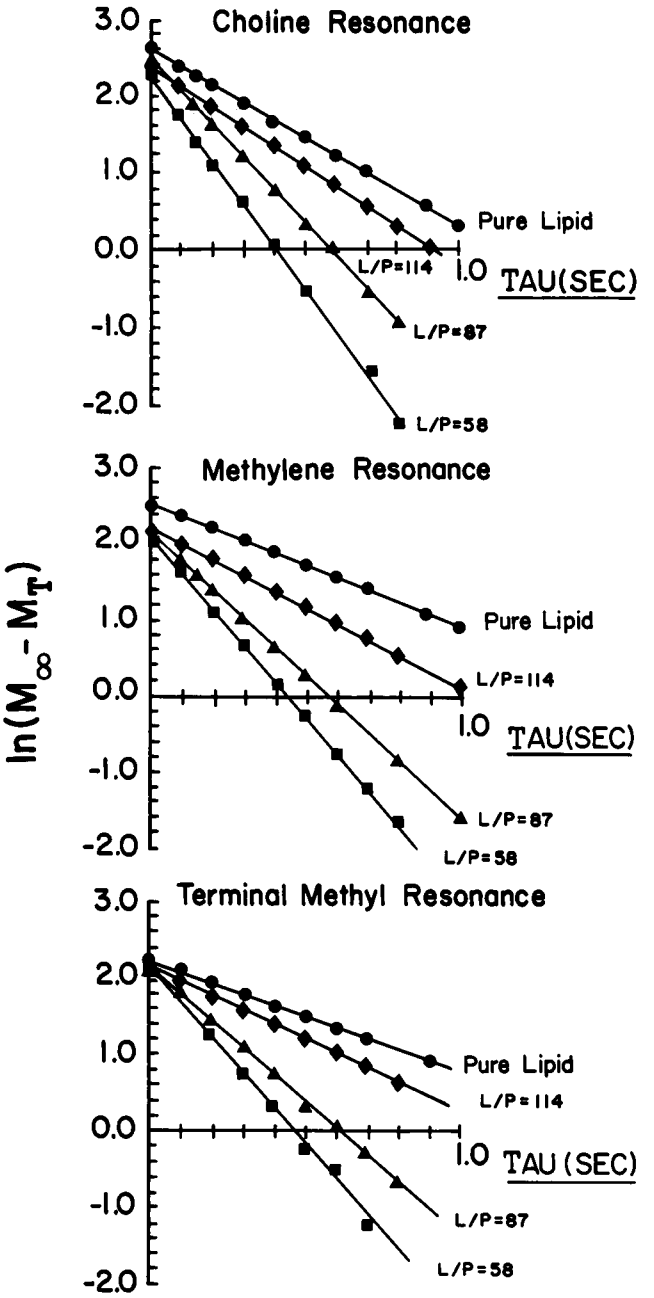

FIGURE 2 Comparison of 360 MHz inversion recovery vs. τ-plots for the methyl, saturated methylene, and choline resonances of the SR lipids and recombinant SR membranes (25°C). The lipid:protein ratios of the various samples are shown next to the respective plot. The slopes of the plots increase with 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}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‡	Choline	Single allylic	Methylene	Methyl
			s		
RSR 1	58	0.18	0.16	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.
‡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 CH_2 T_2 relaxation which arises from the broad fraction, ~50% of the total CH_2 area,

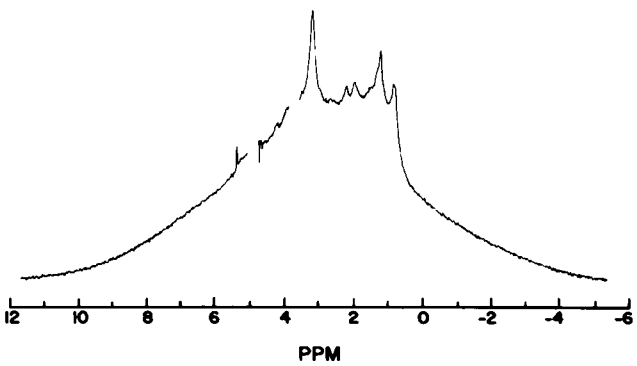

FIGURE 3 360 MHz spectrum of unsonicated native SR membranes at 25°C. Note the sharp choline resonance occurring at ~ 3.2 PPM on the arbitrary scale used.

TABLE III
TEMPERATURE DEPENDENCE OF T_1

Temp. (°C)	Choline	Single Allylic	Methylene	Methyl
	Native SR Membranes, T_1 (*s*)			
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 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 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 D_2O essentially removes the slow T_1 component from all the lipid resonances. Readdition of H_2O 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

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.

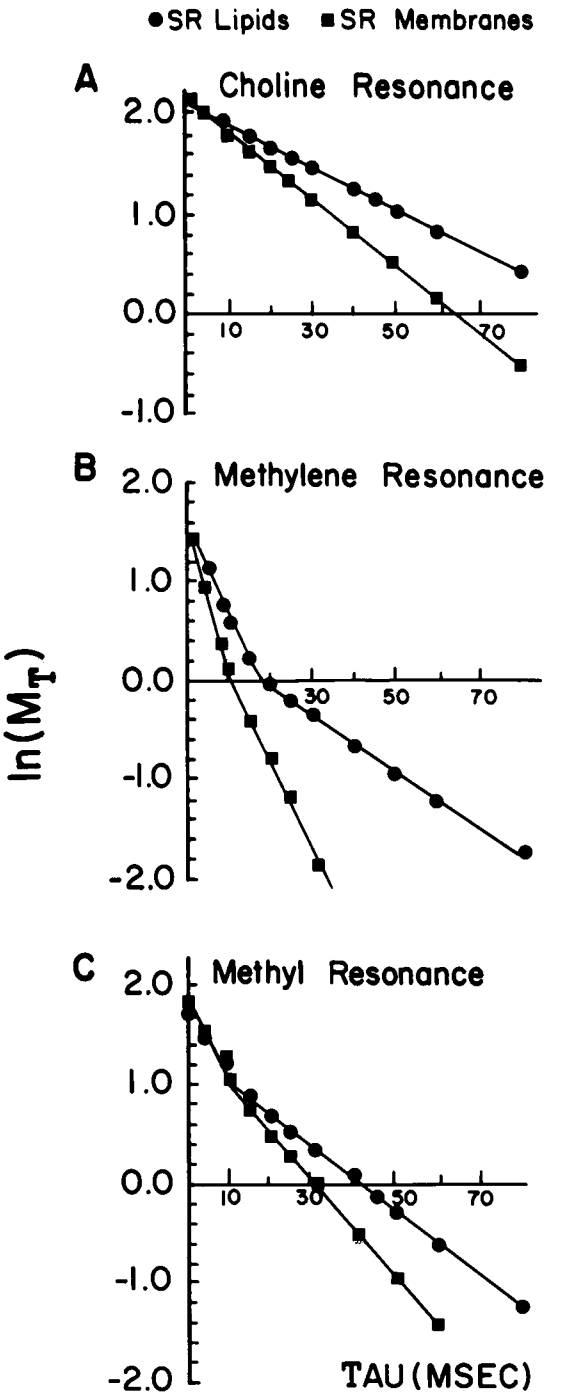

FIGURE 4 Comparison of 100 MHz $T_{1\rho}$ semilog plots for the choline methylene and methyl resonances of native SR membranes and pure SR lipid vesicles at 25°C.

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}C T_1 relaxation of SR lipids increases substantially in the presence of the Ca pump protein (unpublished results). Furthermore, natural abundance ^{13}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}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 ^2H 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 ^1H-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 from a spectral component at a frequency ω has a value of T_2 given by:

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

The parameter Δ 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. τ_v is the correlation time for vesicle reorientation. The shortest values of ~ 1 μs are obtained for vesicles having a diameter of $\sim$ 250 Å, while τ_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

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

Here η 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 ^1H-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 τ_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 $\sim 10^3$ 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 ^2H 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}H-NMR, as well as for the ^{2}H-NMR (Seelig et al., 1981), ^{31}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-l-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 (~ 10^{-8} s) is intriguing. Studies by many investigators (cf., Fleischer et al., 1979*b*; 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.

We wish to thank Drs. Gerald Matson and Jerry Dallas of the University of California at Davis Nuclear Magnetic Resonance facility for their help with the spectrometer and Drs. Morton Bradbury and Gerd LaMar for their hospitality. Mrs. Christine Dettbarn provided valuable technical assistance.

This research was supported by grants R01 EY 00175 to Dr. Dratz; 5SO7 RR 07135-10 to Dr. Deese; AM 14632 and AM 21987 to Dr. Fleischer from the National Institutes of Health and a grant to Dr. Fleischer from the Muscular Dystrophy Association of America.

Received for publication 25 April 1981.

REFERENCES

Blasie, J. K., L. Herbette, P. H. DeFoor, and S. Fleischer. 1981. Profile structure of the calcium pump protein molecule in the isolated sarcoplasmic reticulum membrane. *Biophys. J.* 33:228 Abstr.

Bloom, M., E. Burnell, A. MacKay, C. Nichol, M. I. Valic, and G. Weeks. 1978. Fatty acyl chain order in lecithin model membranes determined from proton magnetic resonance. *Biochemistry.* 17:5750–5762.

Brown, M. F., G. P. Miljanich, and E. A. Dratz. 1976. Proton NMR studies of protein-lipid interactions in retinal rod outer segment disc membranes. *Fed. Eur. Biochem. Soc. Lett.* 70:56–60.

Brown, M. F., G. P. Miljanich, and E. A. Dratz. 1977. Proton spin-lattice relaxation studies of retinal rod outer segment membranes and liposomes of extracted lipids. *Proc. Natl. Acad. Sci. U.S.A.* 74:1978–1982.

Davis, D. G., G. Inesi, and T. Gulik-Krzywicki. 1976. Lipid molecular motion and enzyme activity in sarcoplasmic reticulum membrane. *Biochemistry.* 15:1271–1276.

Deese, A. J. 1981. NMR studies of protein-lipid interactions in ROS and SR membranes. PhD thesis, University of California at Santa Cruz, Santa Cruz, California.

Deese, A. J., L. Hymel, S. Fleischer, and E. A. Dratz. 1981. Proton NMR spin-lattice and spin-spin relaxation studies of native and reconstituted sarcoplasmic reticulum membrane and pure lipids. *Fed. Proc.* In press.

Fleischer, S., C. T. Wang, L. Hymel, J. Seelig, M. F. Brown, L. Herbette, A. Scarpa, A. C. McLaughlin, and J. K. Blasie. 1979*a*. Structural studies of the sarcoplasmic reticulum membrane using the reconstitution approach. *In* Function and Molecular Aspects of Biomembrane Transport. E. Quagliariello et al., editors. Elsevier/North-Holland Biomedical Press, Amsterdam. 465–485.

Fleischer, S., C-T. Wang, A. Saito, M. Pilarska, and J. O. McIntyre. 1979*b*. Structural studies of sarcoplasmic reticulum in vitro and in situ. *In* Cation Flux Across Biomembranes. Y. Mukohata and L. Packer, editors. Academic Press, Inc., New York. 193–205.

Folch, F., M. Lees, and G. H. Sloane-Stanley. 1957. A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* 226:497–509.

Hesketh, T. R., G. A. Smith, M. D. Houslay, K. A. McGill, N. J. M. Birdsall, J. C. Metcalfe, and G. B. Warren. 1976. Annular lipids determine the ATPase activity of a calcium transport protein complexed with dipalmitoyllecithin. *Biochemistry.* 15:4145–4151.

Jilka, R. L., A. N. Martonosi, and T. W. Tillact. 1975. Effect of the purified [Mg^{++} + Ca^{++}]-activated ATPase of sarcoplasmic reticulum upon the passive Ca^{++} permeability and ultrastructure of phospholipid vesicles. *J. Biol. Chem.* 250:7511.

Krishna, N. R. 19 . A method for solvent peak suppression in FTNMR spectra by double resonance. *J. Magn. Res.* 22:555–559.

Lee, A. G., N. J. M. Birdsall, and J. C. Metcalfe. 1974. Nuclear magnetic relaxation and the biological membrane. *In* Methods in Membrane Biology. E. D. Korn, editor. Plenum Press. 1–157.

McIntyre, J. O., P. Samson, S. C. Brenner, L. A. Dalton, and S. Fleischer. 1981. EPR studies of spin labeled phospholipids incorporated into functional reconstituted sarcoplasmic reticulum membranes vesicles (RSR). *Biophys. J.* 37:49–50.

McLaughlin, A. C., J. K. Blasie, L. Herbette, C-T. Wang, L. Hymel, and S. Fleischer. 1981. ^{31}P-NMR studies of oriented multilayers formed from isolated sarcoplasmic reticulum: evidence for rapid motion of "boundary" layer phospholipid. *Biochim. Biophys. Acta.* 643:1–16.

Meissner, G., and S. Fleischer. 1972. The role of phospholipid in Ca^{++} stimulated ATPase activity of sarcoplasmic reticulum. *Biochim. Biophys. Acta.* 255:19–33.

Meissner, G., G. E. Conner, and S. Fleischer. 1973. Isolation of sarcoplasmic reticulum by zonal centrifugation and purification of Ca^{++}-pump and Ca^{++}-binding proteins. *Biochem. Biophys. Acta.* 298:246–269.

Meissner, G., and S. Fleischer. 1974. Dissociation and reconstitution of functional sarcoplasmic reticulum vesicles. *J. Biol. Chem.* 249:302–309.

Morai, L., and A. Kuksis. 1973. Molecular species of glycerolipids of adensine triphosphatase and sarcotubular membranes of rabbit skeletal muscle. *Can. J. Biochem.* 51:1248–1379.

Robinson, J. D., N. J. M. Birdsall, A. G. Lee, and J. C. Metcalfe. 1972. Carbon-13 and proton nuclear magnetic resonance relaxation measurements of the lipids of sarcoplasmic reticulum membranes. *Biochemistry.* 11:2903–2906.

Rouser, G., and S. Fleischer. 1967. Isolation, characterization, and determination of polar lipids of mitochondria. *Methods Enzymol.* 10:385–406.

Sass, M., and D. Ziessow. 1977. Error analysis for optimized inversion recovery spin-lattice relaxation measurements. *J. Mag. Res.* 25:263–276.

Scales, D., and G. Inesi. 1976. Assembly of ATPase protein in sarcoplasmic reticulum membranes. *Biophys. J.* 16:735–751.

Seelig, A., and J. Seelig. 1974. The dynamic structure of fatty acyl chains in a phospholipid bilayer measured by deuterium magnetic resonance. *Biochemistry.* 13:4839–4845.

Seelig, J., L. T. Tamm, L. Hymel, and S. Fleischer. 1981. Deuterium and phosphorus NMR and fluorescence depolarization studies of functional reconstituted sarcoplasmic reticulum membrane vesicles. *Biochemistry.* 20:3922–3932.

Stockton, G. W., C. F. Polnaszek, L. C. Leitch, A. P. Tulloch, and I. C. P. Smith. 1974. A study of mobility and order in model membranes using ^2H NMR relaxation rates and quadrupole splittings of specifically deuterated lipids. *Biochem. Biophys. Res. Commun.* 60:844–850.

Wang, C. T., A. Saito, and S. Fleischer. 1979. Correlation of ultrastructure of reconstituted sarcoplasmic reticulum vesicles with variation in phospholipid to protein ratio. *J. Biol. Chem.* 254:9209–9219.

Wennerström, H., and J. Ulmius. 1976. Proton NMR bandshape in phospholipid bilayer vesicles. *J. Mag. Reson.* 23:431–435.

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 (Wennestrom and Ulmias. 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 θ 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, nonLorentizan 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

transitions, and this can be determined from relaxation time measurements. Now the only part of the interaction you can observe is the part that has been averaged, and because the vesicle tumbling is isotropic, it averages everything. Once the tumbling has taken place, there is nothing more that can be measured by relaxation. The correlation time for vesicle tumbling is ~1 –10 ms.

DRATZ: It depends on how big the vesicles are. We have made many of our measurements on unsonicated systems with average diameter of 1 μm or so; these tumble quite slowly.

BLOOM: If you use big vesicles, then you get broad lines, and you might as well go to unsonicated dispersions. If you have very small vesicles you can use a high-resolution spectrometer, but then very slow motions are inaccessible to you by relaxation spectroscopy.

T_1 and T_2 measurements imply that the lipid-protein interaction induces motions in the acyl chains of the phospholipid molecule that have correlation times shorter than 1 μs, $\sim 10^{-7}$ s (Paddy et al. 1981. *Biochemistry*. 20:3152 –3162. For the reasons just stated, the effects of these motions should not be modulated by the vesicle tumbling. The contribution of these motions to the line width should be included in the term Δ that you have written for $1/T_2$.

SYKES: I want to return to the initial question by Philip Yeagle and the interpretation of spin-lattice relaxation times. You have said that the spin-lattice times are determined by fast motion, and implicated *trans-gauche* isomerization as that fast motion. But since your methylene and methyl relaxation times T_1 are absolutely equal, and looking at all the early polymer work by people like W. Slichter, one would wonder about the role of spin diffusion along the polyene chain. Is it possible that one is measuring the relaxation of the methyl sink and that the only parameter one is perturbing is the rotation of the methyl group, and that one doesn't really have a handle on *trans-gauche* isomerization? Is that reasonable?

DRATZ: We have worried about this point. First, the interpretation of the effects of protein on the terminal methyl T_1 is not influenced by CH_2–CH_3 spin exchange. The methyl and methylene T_1 is rather close, although the methylene T_1 is usually a bit faster than the methyl. The choline and single allylic are always considerably faster than the methyl.

There is spin diffusion going on in the system, but a methyl sink is not a sufficient explanation because the methyl has the slowest T_1. We have made some quantitative measurements of how effective spin diffusion is by selectively placing spin polarization, using long, soft, pulses, into the methylne, methyl, or other resonance, and watching it show up in other places. You do see spin diffusion, as you would expect, but we believe that that spin diffusion is not enough quantitatively to account for all of the effects of protein. Probably the best experiments to show this are natural-abundance ^{13}C - T_1 on the lipids in the presence and absence of the protein.

FEIGENSON: You have talked about lipid, and you have lipid and protein present; how do you resolve the protein contribution in the hydrocarbon region?

DRATZ: The protein adds a broad component which is proportional to the amount of protein. We have considered the possibility that the protein may have some sharp resonances due to local motion, and we plan to reconstitute the system with perdeuterated lipids to remove the lipid hydrocarbon resonances and to see if the protein had sharp resonances in the CH_2 and CH_3 region. Myer Bloom told me that he did that experiment with rhodopsin and found no sharp methyl and methylene resonances from the protein in unsonicated reconstituted dispersion with perdeuterated lipid.

FEIGENSON: But I don't think you can compare the different protein systems, because this Ca^{++}-ATPase has the bulk of its signal coming from the part that is out of the membrane. You could certainly have significant local mobility giving rise to hydrocarbon contributions, and then you have trouble resolving the protein contribution from the lipid contribution. With other polypeptides we see signals that are fairly sharp, 40 –50 Hz wide, in the proton spectrum.

DRATZ: To the extent that different integral membrane proteins behave differently, that is a useful criticism. It really would be best to do that experiment with the calcium pump protein. We see similar proton T_1 effects of rhodopsin as those we have reported here for the Ca^{++} pump. The ^{13}C relaxation data illuminate this question further because we are seeing very similar effects on the methylenes and the vinyl signals, and the protein has no vinyl signals at all. The same kind of effect occurs on vinyl relaxation as on methylene relaxation. It would be ideal to do the experiment you suggest, but the carbon data indicate that it is not going to be a big effect in Ca^{++} pump.

FEIGENSON: A corollary question: could you have a significant fraction of the lipid signal actually immobilized by the protein, yet see an increase in that region of the spectrum because of the protein contribution?

DRATZ: Yes, that is possible, but I don't think it will happen, judging from Bloom and co -workers' experiments with rhodopsin in perdeuterated membranes.

FEIGENSON: In your paper you describe an increased area in the signal when protein is present. How can you distinguish protein and lipid?

DRATZ: It is very difficult to get accurate measurements of areas under these conditions, and probably the increases in area that we see are a little bit above the error of those areas, so there may be a small protein contribution to the methylene signals, ~10 –20%.

FEIGENSON: You mean the protein signal is much broader?

DRATZ: Most of the protein signal is broad but there might be a small contribution to the sharp signal from the protein.

FEIGENSON: Do all your T_1 measurements refer only to the sharp part?

DRATZ: We have also measured between the sharp resonances and out in the wings. In the unsonicated state, as we mentioned in the paper, we find the same quantitative effects.

FEIGENSON: To measure T_1 on the slope of a line is certainly difficult. When you measure at the peak you are disproportionately weighting the most mobile components, and it may not be characteristic of the whole lipid signal, most of which is present in the broad component of the line.

DRATZ: No, not most. Less than half under most of the resonances signals. Protein interference can be ruled out for the vinyl, methyl, and choline signals but cannot be ruled out in the methylene region.

McLAUGHLIN: Can one use the T_1 data, which would be most sensitive to correlation times of $\sim 10^{-9}$ s, to bridge the gap between the EPR time scale of $\sim 10^{-9}$ s and NMR time scales? For phosphorous and deuterium there are usually $\sim 10^{-6}$ s. Do your data reflect the time scale of the EPR type of experiment?

DRATZ: T_1 measures the intensity of the fluctuations at the Larmor frequency, which in these experiments is 10^{-9} –10^{-10} s, and so the protein is reducing the intensity of the fluctuations at that frequency apparently from the center of the bilayer right out to the head groups. The

interpretation of the T_2 measurements is that the protein speeds up the T_2 as well. The vesicles don't seem to change size greatly in the presence of protein. Thus, whatever the vesicles' tumbling effects are, they are reasonably constant, although we have not adequately shown this point. We believe that the speeding up of the T_2 in the presence of the protein is also caused by the restriction of slower motions, but that because the T_2 is single-exponential (except for a very rapidly decaying fraction of broad methylenes), the exchange time between more hindered motions and the bulk lipid motions is fast compared to the T_2 time scale, $\sim 10^{-4}$ s.

We are hoping to exploit the $T_{1\rho}$ measurements to get into the 10^{-3}–10^{-4} s time window; potentially we can do that, but for technical reasons we haven't done much more with it yet.

JOST: We find from electron micrographs from reconstituted samples that there is a variation in vesicle sizes and the average size can be dependent on the lipid:protein ratio.

T. THOMPSON: It is true that the tumbling rate of the vesicles is important in the interpretation of your data. In my experience, the vesicle size distribution is almost always a function of the protein composition; in your system you clearly have a distribution of sizes. The question is whether that distribution changes with lipid:protein ratio. Electron microscopy is only one way to assess the size distribution. I wonder if you have really carefully assessed that parameter?

FLEISCHER: That is an important and valid point. Ed Dratz may have overstated the homogeneity, which is difficult to evaluate. Leo Herbette carried out light-scattering studies on the vesicles with different lipid:protein ratios; the size does change with change in lipid:protein ratio, but not very much. (Herbette et al. 1981. *Biophys. J.* 36:27–72). That may not be so relevant for these studies. Dratz did two types of experiments, one with vesicles which were sonicated to a uniform, small size, and the other with vesicles as they were prepared without sonication. In both cases the T_1 times were very similar. Your comment would be more valid for the T_2 time data.

DRATZ: I did not claim that the vesicles had a homogeneous size distribution. What I said was that the size distribution does not seem to shift very much with protein dose.

T. THOMPSON: It depends on how you measure the distribution; the average values can remain the same, but the distribution can become skewed. You have to know what the distribution function looks like. It could be an important parameter.

LENTZ: Could spin diffusion possibly reflect a site heterogeneity induced by the protein? If there were many different proton environments, would there be more opportunity to transfer polarization to a slightly different environment?

DRATZ: The group-transferring species would have to be very close, because the fields fall off very rapidly. Thus we may be able to use spin diffusion to measure the ability of proteins and lipids to interact and possibly pick up site heterogeneity as you suggest.

M. BROWN: What direct evidence is there for *trans-gauche* isomerization of the lipid acyl chains as the source of the spin-lattice relaxation?

DRATZ: There isn't any direct evidence. It is just a plausible mechanism. The activation energy is in the right ball park, but this is not the only possible kind of motion.

M. BROWN: Certainly the activation energies are consistent with rotational isomerization as a source of the T_1 relaxation, but one really has to do frequency-dependent T_1 relaxation measurements to gain a better handle on the molecular mechanism of the relaxation; otherwise, one is fitting an unknown correlation function to a single data point. I think it's very dangerous to ascribe a single mechanism as a source of the relaxation.

DRATZ: This is only a suggested mechanism and the detailed mechanism seems to be a rather secondary point. The basic point is that the protein is restricting lipid motions that occur at high frequencies.

WESTERMAN: In these SR vesicles, is it known if there is exchange of lipids between vesicles? If so, how fast is it, and is this exchange facilitated by the protein?

FLEISCHER: We don't have any experiments that speak to this. For phospholipid vesicles, the exchange is very slow.

WESTERMAN: But it may be facilitated by the protein.

FLEISCHER: That is not known.

HIDALGO: You stated that your SR vesicles are fully functional. However, in order to measure spectra you have to replace water by D_2O, which interferes with Ca^{++}-pumping activity, and you have to sonicate them for periods up to 20 min, which you also say interferes with Ca pumping. How active *are* your vesicles during your experiment?

DRATZ: Their function is impaired by some of these treatments. D_2O does inhibit the pumping activity by up to 50%, but usually not quite that much. Sonication is probably the worst thing for pump activity; we have measured T_1 as a function of time of sonication, and see no changes. At 10 min of sonication there is a small effect on activity while with 20 min there is a substantial effect. This does not seem to affect the measurements which we are making. The activity that we have is very stable over the time of the measurement.

ONG: In your relaxation measurements you remove the water resonance either by presaturation or by adding chromium ions. Did you check the effect of chromium on your lipid control sample? Chromium removed the slow-relaxation component in your membranes containing protein, but in your text you did not mention that slow component in your lipid sample. Do you have a good control for the addition of chromium to your sample?

DRATZ: If we work with pure lipid vesicles, we see no effect of chromium, at the level we have used, on the lipid relaxation. If we add H_2O to our pure lipid samples, we can induce the slow T_1 relaxation component that we can then remove with chromium, just as in the regular membrane system.

STEITZ: I would like to introduce some unpublished data of Richard Henderson, David Agard, and Joyce Baldwin on bacteriorhodopsin. They have extended their study to ~3.5-Å resolution. They can see in their electron-density map-ordered lipid head groups. Inside the trimer there are three lipids on each side of the bilayer, and outside there is a layer of phospholipids. This might be relevant to this morning's discussion.

DRATZ: That is very interesting, but we have to keep in mind that the case of bacteriorhodopsin is not very typical; it is a two-dimensional crystal, while most membranes we are talking about are two-dimensional fluids. So highly ordered lipids in the bacteriorhodopsin two-dimensional crystal may not be relevant to a more typical kind of membrane. Do you agree?

STEITZ: Well, all crystals of soluble proteins have bound water. Some people might say that is irrelevant to solution, but I would certainly disagree.