Deuterium and Phosphorus Nuclear Magnetic Resonance and Fluorescence Depolarization Studies of Functional Reconstituted Sarcoplasmic Reticulum Membrane Vesicles†

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ABSTRACT: Sarcoplasmic reticulum membrane vesicles were reconstituted in functional form with a lipid matrix composed of either 1,2-di[9,10-$^2H_2$]oleoyl-, 1,2-di[2,2-$^2H_2$]oleoyl-, or 1,2-di[9,10-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine. In some of the preparations, the native phospholipids were exchanged with a single lipid environment to the extent of 99%. The Ca^{2+} pump protein accounted for >90% of the proteins in these membranes, and the lipid-to-protein ratio varied from 50 to 130 mol of phospholipid per mol of Ca^{2+} pump protein. This high protein content is comparable to that in natural sarcoplasmic reticulum (approximately 110 mol of phospholipid per mol of Ca^{2+} pump protein) and is a favorable range to detect the influence of the protein on the motional characteristics of the phospholipids. Deuterium nuclear magnetic resonance (NMR) spectra were obtained at 46 MHz with the quadrupole echo technique; proton-decoupled ^{31}P NMR spectra were recorded at 36 and 121 MHz. The deuterium and phosphorus spectra were characteristic of a homogeneous liquid-crystalline bilayer. The structure-related NMR parameters such as the residual deuterium quadrupole splitting constant, $\Delta\nu_Q$, and the phosphorus chemical shielding anisotropy, $\Delta\sigma$, were found to be 10–20% smaller in reconstituted sarcoplasmic reticulum membranes than in protein-free liposomes. This indicates a small but uniform disordering of the lipids, presumably to conform to the uneven shape of the Ca^{2+} pump protein. The dynamic properties of reconstituted sarcoplasmic reticulum membranes were studied by measuring the phosphorus and deuterium spin–lattice relaxation times (T_1). The relaxation process followed a single exponential for both the phosphate group and the deuterated cis and trans double bonds of the phosphatidylcholine, a result supporting the conclusion of a single homogeneous lipid environment, i.e., the same for boundary layer and bulk bilayer phospholipid. Measurement of the temperature dependence of the ^{31}P spin–lattice relaxation time at 121 MHz revealed a distinct T_1 minimum which was not observed at 36 MHz. The quantitative analysis of the phosphorus T_1 relaxation time measurements showed that (1) the correlation time of the reorientation of the phosphate group was ~1 ns in pure dioleoylphosphatidylcholine vesicles at 4 °C, (2) the reorientation rate was slowed down by not more than 10–20% in reconstituted sarcoplasmic reticulum membranes, and (3) the ^{31}P spin–lattice relaxation at 121 MHz was determined to an equal extent by dipole–dipole relaxation and relaxation via chemical shielding anisotropy. The analysis of the deuterium T_1 relaxation times led to similar conclusions for the hydrocarbon part of the lipid bilayer. The correlation time for the reorientation of the cis double bond was 0.17 ns in pure dioleoylphosphatidylcholine liposomes and increased to 0.21 ns in reconstituted sarcoplasmic reticulum membranes (at 24 °C). These data suggest that the incorporation of protein leads to only a minor reduction of the rate of the internal modes of phospholipid motions, i.e., the segmental fluctuations and reorientations. Steady-state fluorescence depolarization anisotropy measurements were made with 1,6-diphenyl-1,3,5-hexatriene (DPH) as a fluorescent probe. The depolarization anisotropy, $\bar{r}$, was distinctly larger in reconstituted sarcoplasmic membrane vesicles than in pure lipid vesicles. This can be explained by an increase in the orientational correlation time of the probe molecule and/or by an increase in the probe ordering. The results obtained with the DPH fluorescence label tend to contradict those obtained with ^2H and ^{31}P NMR, possibly because DPH, a relatively rigid molecule, does not reflect correctly the motion of the phospholipids which are highly flexible.

An interesting application of magnetic resonance techniques during the last few years has been the investigation of lipid–protein interactions in reconstituted membranes and intact biological membranes [for a review, see Chapman et al. (1979)]. The early studies were performed almost exclusively with spin-labeled phospholipids. The electron paramagnetic resonance (EPR)[1] spectra of the spin-labeled membranes consistently contained two components, indicating different membrane environments. Whereas one spectral component was almost identical with the spectrum of a pure lipid bilayer, the second spectral component was much broader and characteristic of "immobilized" spin-label. As a result of these studies, a distinction was made between the phospholipids in immediate contact with the hydrophobic protein surface—the so-called boundary lipids—and those farther away from it. The boundary lipids were thought to be immobilized on the protein surface, at least on the time scale of the EPR experiment ($\gtrsim 10^8$ Hz), whereas the bulk lipid remained disordered and highly mobile as in a pure phospholipid membrane [cf. Jost et al. (1973)].

This physical concept of lipid–protein interactions was modified to suggest interesting biochemical consequences (Warren et al., 1975). On the basis of the temperature and lipid dependence of sarcoplasmic reticulum ATPase reconstituted with synthetic lipids, it was suggested that this protein is surrounded by a "lipid annulus", i.e., a single shell of tightly bound phospholipids which regulates the functional properties of the protein and can exclude, for example, cholesterol from

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[1] Abbreviations used: EPR, electron paramagnetic resonance; NMR, nuclear magnetic resonance; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DEPC, 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; SR, sarcoplasmic reticulum; R-SR, reconstituted sarcoplasmic reticulum; DPH, 1,6-diphenyl-1,3,5-hexatriene; Hepes, *N*-2-(hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid.

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the lipid–protein interface (Hesketh et al., 1976; Warren et al., 1975).

In the present study, we have investigated the problem of lipid–protein interactions in functional reconstituted sarcoplasmic reticulum membrane vesicles with deuterium and phosphorus NMR and fluorescence depolarization spectroscopy. In the reconstituted sarcoplasmic reticulum (R-SR), the lipids were exchanged with a single lipid environment to the extent of 99%, of which at least 90% were selectively deuterated. Two different lipids, namely, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC) and 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC), were employed in order to investigate the possible influence of the gel-to-liquid crystal phase transition, T_c (DOPC, $T_c = -22$ °C; DEPC, $T_c \simeq 10$–13 °C). The lipids were selectively deuterated in either the 9,10 or the 2,2 positions of the fatty acyl chains.

The use of ^2H NMR in combination with deuterium-labeled lipids has the following advantages. (1) Deuterium substitution does not perturb the system since the van der Waals radii of the two isotopes, ^1H and ^2H, are identical. (2) The natural abundance of deuterium is low, thus allowing a straightforward assignment of ^2H NMR signals. (3) Finally, the quantitative interpretation of ^2H NMR spectra is simple and provides unique structural and motional information which is difficult to obtain otherwise. In particular, the deuterium quadrupole splitting, $\Delta\nu_Q$, can be related to the average lipid conformation (a structural property) whereas the deuterium T_1 relaxation rate is determined by the rate of motion (a dynamic property). Compared to the spin-label EPR technique, ^2H NMR is less sensitive but has the advantage of a larger frequency range [cf. Jost & Griffith (1980)]. Motions with frequencies slower than 10^8 Hz appear completely immobilized by conventional EPR spectroscopy whereas ^2H NMR has the potential to resolve motions down to 10^5 Hz [for a review on ^2H NMR, see Seelig (1977)].

The time scale of ^{31}P NMR is similar to that of ^2H NMR. Structural information is derived from the chemical shielding anisotropy, $\Delta\sigma$, whereas T_1 relaxation time measurements shed light on the motional properties. No synthetic labeling is necessary due to the 100% natural abundance of ^{31}P [cf. Seelig (1978)].

Finally, the fluorescent probe 1,6-diphenyl-1,3,5-hexatriene (DPH) was introduced into the same sarcoplasmic reticulum membrane vesicles as used for the NMR studies, in order to measure the steady-state fluorescence depolarization. It has recently been shown that the depolarization ratio contains information not only about the membrane "microviscosity" but also about membrane order (Heyn, 1979; Jähnig, 1979). We therefore applied the fluorescence method to a system where the molecular structure had been characterized in detail by NMR spectroscopy.

Materials and Methods

Lipid Synthesis. [2,2-$^2H_2$]Oleic acid, [9,10-$^2H_2$]oleic acid, and [9,10-$^2H_2$]elaidic acid were prepared as described earlier (Seelig & Waespe-Šarčevič, 1978). DOPC and DEPC were synthesized by reacylation of *sn*-glycero-3-phosphocholine with the corresponding fatty acid anhydrides. 1-Oleoyl-2-[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine was prepared by reacylation of 1-oleoyl-2-lyso-*sn*-glycero-3-phosphocholine with deuterated oleic acid anhydride (Cubero Robles & Van den Berg, 1969). Protonated phospholipids were prepared by acylation of *sn*-glycero-3-phosphocholine according to Warner & Benson (1977).

Preparation of Lipid Dispersions. For the NMR measurements, the lipids were dispersed by vortexing in excess water or deuterium-depleted water to form large multilayered vesicles (coarse liposomes). Lipids were also dispersed in buffer (0.3 M sucrose, 100 mM KCl, and 1 mM Hepes, pH 7.1). Within experimental error, the addition of buffer had no effect on the NMR parameters. For the phosphorus relaxation time measurements, 1 mM EDTA was added. Deuterium T_1 relaxation times are insensitive to the presence of small concentrations of paramagnetic ions, and addition of EDTA was omitted. Fluorescence depolarization measurements were made with single-layered vesicles prepared by the ethanol injection method, using a lipid concentration of 100 mg of DOPC/mL of ethanol (Batzri & Korn, 1973; Kremer et al., 1977). The vesicles had a diameter of 60–100 nm as determined by dynamic light scattering and negative-stain electron microscopy.

Reconstituted Sarcoplasmic Reticulum Membrane Vesicles. Sarcoplasmic reticulum (SR) was prepared by the method of Meissner et al. (1973). Reconstituted SR vesicles were enriched with deuterated DOPC up to about 40% by dialyzing mixtures of solubilized SR with DOPC–deoxycholate suspensions by use of the method described by Meissner & Fleischer (1974) as modified by Wang et al. (1979) to vary the lipid content of the membrane. Exchange of phospholipids in SR for DOPC or DEPC was accomplished with the technique of Wang et al. (C. T. Wang, L. Hymel, G. Meissner, and S. Fleischer, (unpublished experiments). This incubation mixture was layered over a step gradient containing an exchange layer composed of lecithin (3.0 equiv) and deoxycholate. The gradients were centrifuged 18–22 h at 25 000 rpm in a Beckman SW 25.2 rotor at 20 °C. The reconstituted membranes were recovered from the bottom interface, diluted to about 0.1 mg of protein per mL with "dialysis buffer" without sucrose, and sedimented to remove residual deoxycholate. The vesicles were finally suspended in 0.3 M sucrose, 0.1 M KCl, and, 1 mM Hepes (pH 7.1 at 0 °C) and were stored in this medium at −70 °C or in liquid nitrogen until measurement. The above procedure results in at least 90% exchange of the phospholipids. This procedure was first performed with protonated DOPC or DEPC. A second exchange with deuterated DOPC or DEPC was carried out, yielding reconstituted vesicles which were at least 99% of a single species of lipid, and at least 90% of the lipid was deuterated. The homogeneity of the preparations was demonstrated by the fact that they sedimented as a thin single band upon isopycnic sucrose density gradient centrifugation. A small amount of deoxycholate remained in the membrane vesicles after reconstitution, equivalent to or less than 1 mol of deoxycholate per 15 mol of phospholipids. This amount of detergent when added to normal SR did not seem to adversely affect Ca^{2+} pumping function or efficiency.

Freeze–fracture electron microscopy revealed that the samples contained single-walled small vesicles with closely packed particles on both fracture faces. The vesicle size increased somewhat as a function of the lipid content. The vesicles with the lowest lipid content were the smallest; i.e., reconstituted SR containing 99% DEPC with a lipid phosphorus content of 10.3 μg of phosphorus/mg of protein averaged 94 ± 22 nm as determined by freeze–fracture electron microscopy. The calcium pumping characteristics were typical of those described by Wang et al. (C. T. Wang, L. Hymel, G. Meissner, and S. Fleischer, unpublished experiments). Nearly all of the protein (>90%) in reconstituted SR membrane vesicles is the Ca^{2+} pump protein (Wang et al., 1979). The molecular weight of the Ca^{2+} pump protein was taken to be 119 000 (Rizzolo et al., 1976).

For the NMR measurements, the SR membrane vesicles were centrifuged at 200000g for 30 min, resuspended in deuterium-depleted buffer, and again centrifuged. The pellet was transferred into a NMR sample tube. The use of EDTA during measurement was avoided with reconstituted SR. However, reconstituted membranes were prepared in the presence of EDTA, which would be expected to remove paramagnetic contaminants.

NMR Measurements. The ^2H NMR spectra were measured at 46.1 MHz with a Bruker-Spectrospin CXP-300 pulse spectrometer using the quadrupole echo technique (Davis et al., 1976). Pulses (90°) of ~5 μs were used with an echo pulse separation of 30 μs. All spectra were recorded with quadrature phase detection, and the spectral width was varied between 100 and 500 kHz. Deuterium T_1 relaxation times were measured by the conventional 180°–τ–90° sequence modified to include the quadrupole echo. Since deuterium T_1 relaxation times of lipid bilayers are generally $\lesssim$30 ms, a relaxation delay of 200 ms was normally sufficient for a full recovery of the magnetization. In all ^2H NMR experiments, a phase alternating sequence was employed to reduce coherent noise and phase errors.

^{31}P NMR measurements were performed at 36.4 (Bruker-Spectrospin HX-90 FT spectrometer) and 121.4 MHz (CXP-300). At 36.4 MHz, the spectra were recorded with 10-W continuous proton decoupling. At 121.4 MHz, inverse gated decoupling was employed. Phosphorus T_1 relaxation times were also measured with the 180°–τ–90° method under inverse gated decoupling conditions.

Steady-State Fluorescence Depolarization Experiments. Measurements were carried out as described in more detail by Heyn et al. (1981). 1,6-Diphenyl-1,3,5-hexatriene (DPH) was used as the fluorescence probe. Samples were labeled with DPH at a concentration of 1 probe molecule/500 lipid molecules. The steady-state fluorescence anisotropy $\bar{r}$ defined as

$$\bar{r} = \frac{I_\parallel - I_\perp}{I_\parallel + 2I_\perp} \quad (1)$$

was measured with a Schoeffel RRS 1000 fluorometer, equipped with two photomultipliers in the T configuration for simultaneous recording of the parallel and perpendicularly polarized emission. Excitation was at 360 nm and emission at 428 nm.

Results

Sarcoplasmic Reticulum Membrane Vesicles Reconstituted with 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC). The gel-to-liquid crystal transition temperature of DOPC is −22 °C. The ^2H NMR spectrum of coarse DOPC liposomes deuterated at the 9,10 positions of both oleic acyl chains consists of three overlapping "powder-type" spectra [cf. Seelig (1977)] as is illustrated in Figure 1A. The assignment of the splittings can be made by comparison with DOPC labeled in the *sn*-2 chain only (Figure 1B) and by analogy with 1-palmitoyl-2-[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (Seelig & Waespe-Šarčevič, 1978). The smallest deuterium quadrupole splitting comes from the C-10 deuteron in the *sn*-2 oleic acyl chain, the intermediate from the corresponding deuteron in the *sn*-1 chain, while the largest is a superposition of the two remaining C-9 deuterons. The analysis of the ^2H NMR spectrum of DOPC is interesting for two reasons: (1) The two fatty acyl chains assume different average conformations in the membrane, and (2) the cis double bond is tilted with respect to the bilayer normal. Indeed, the quantitative analysis of the quadrupole splittings reveals that the average orientation of the C=C bond vector is not exactly parallel to the bilayer normal but is tilted by 5–10° (Seelig & Waespe-Šarčevič, 1978).

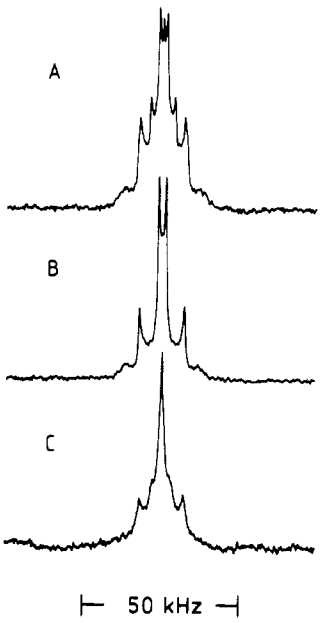

FIGURE 1: ^2H NMR spectra at 46.1 MHz of functional reconstituted sarcoplasmic reticulum membrane vesicles (R-SR) exchanged with 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine ([9,10-$^2H_2$]DOPC). Spectra were recorded with the quadrupole echo technique with quadrature phase detection. Spectral width, 100 kHz; measuring temperature, 4 °C (liquid-crystalline phase). (A) Pure [9,10-$^2H_2$]-DOPC (60 mg) dispersed in water (160 mg), 2000 scans at 4 scans/s. (B) 1-Oleoyl-2-[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (~70 mg) in water (~100 mg), 1000 scans at 4 scans/s. (C) R-SR in buffer. Total protein 53 mg, containing 23 mg of lipid. Lipid-to-protein ratio, L/P, = 0.43 (w/w), corresponding to about 64 mol of phospholipid per mol of Ca^{2+} pump protein, 40 000 scans at 4 scans/s. The lipid was 99% DOPC of which at least 90% was [9,10-$^2H_2$]DOPC.

Figure 1C shows a ^2H NMR spectrum of reconstituted sarcoplasmic reticulum vesicles exchanged with [9,10-$^2H_2$]DOPC. The spectrum is characteristic of a single homogeneous lipid phase and does not provide evidence for two discrete lipid environments. Qualitatively, we observe the same type of spectrum for R-SR as for the pure DOPC bilayers, although with some differences. The lines are broader and the quadrupole splittings are smaller than those observed for pure DOPC. These effects are most conspicuous for the innermost splitting (C-10 deuteron of the *sn*-2 chain) which is well-resolved in pure DOPC but collapsed to a single broad line in R-SR. The quadrupole splittings of pure DOPC liposomes and R-SR membranes as a function of temperature are summarized in Figure 2A. The quadrupole splittings are found to decrease monotonically with increasing temperature.

Deuterium T_1 relaxation times were measured in order to compare the rate of fatty acyl chain motion in DOPC vs. R-SR. Figure 3 shows relaxation experiments with pure DOPC (3A) and with R-SR (3B). The recovery of the magnetization after a 180° pulse followed a single exponential, and no differences were observed in the relaxation rates of the C-9 and C-10 deuterons, respectively. The temperature dependences of the T_1 relaxation times are represented as Arrhenius plots in Figure 4. Straight lines were obtained for coarse DOPC liposomes and for R-SR yielding activation energies of 4.7 and 2.9 kcal/mol, respectively.

Proton-decoupled ^{31}P NMR spectra (at 121.4 MHz) are shown in Figure 5 for coarse DOPC liposomes (5A) and for R-SR (5B). The shape of the spectra is determined by the anisotropic motion of the phosphate group: from the edges

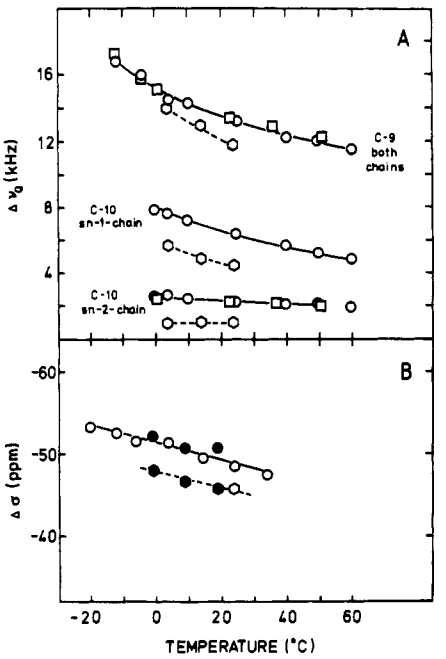

FIGURE 2: Reconstituted sarcoplasmic reticulum membranes exchanged with 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine. Temperature dependence of ^2H and ^{31}P NMR parameters. (A) ^2H NMR. Temperature dependence of the residual quadrupole coupling constant, $\Delta\nu_Q$. (O) 1,2-Di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine liposomes in excess water; (□) 1-oleoyl-2-[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine; (○) reconstituted sarcoplasmic reticulum membranes. The smallest splitting of R-SR is estimated and is certainly smaller than 1 kHz (same sample as in Figure 1C). (B) ^{31}P NMR. Variation of the chemical shielding anisotropy, $\Delta\sigma$, with temperature. Measurements were made at 121.4 MHz (open symbols) and at 36.4 MHz (solid symbols). (O, ●) 1,2-Dioleoyl-*sn*-glycero-3-phosphocholine in excess water (same sample as in Figure 1A); (○, ●) reconstituted sarcoplasmic reticulum membranes. The reconstituted SR was the same sample as used in Figure 1C.

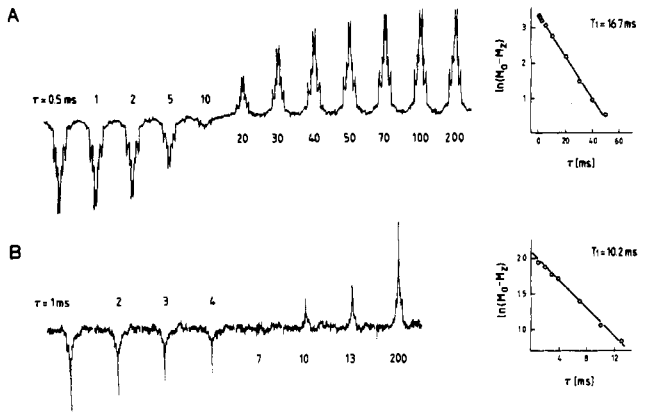

FIGURE 3: Measurements of the deuterium T_1 relaxation time (at 46.1 MHz) with the inversion recovery technique. (A) DOPC. 1,2-Di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (~60 mg) was dispersed in excess water (~160 mg). Spectra were measured with a total spectral width of 325 kHz. Only the relevant part of the spectra is shown in the figure (31 °C). (B) SR reconstituted with [9,10-$^2H_2$]DOPC and dispersed in deuterium-depleted buffer (15 °C).

of the powder-type spectrum, it is possible to derive the chemical shielding anisotropy, $\Delta\sigma$, which can be used as a quantitative measure for the motional restrictions imposed on the phosphate segment [cf. Seelig (1978)]. Due to the asymmetry of the static chemical shielding tensor of phospholipids [cf. Kohler & Klein (1976) and Griffin (1976)], the quantitative interpretation of $\Delta\sigma$ requires the determination of *two* order parameters and is therefore more complicated than the analysis of the deuterium quadrupole splittings (Niederberger

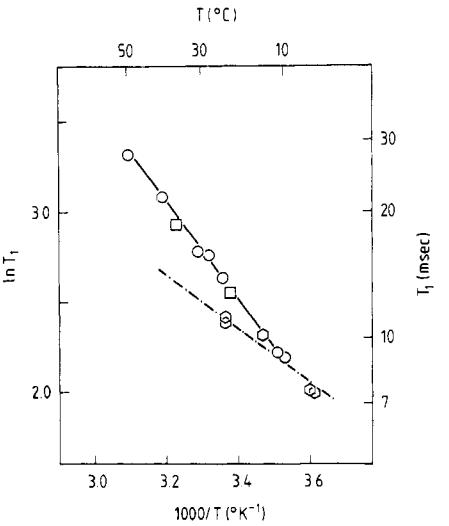

FIGURE 4: Arrhenius plots of the deuterium T_1 relaxation times (at 46.1 MHz). (O) 1,2-Di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine dispersed in excess water; (□) 1-oleoyl-2-[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine in excess water; (○) sarcoplasmic reticulum membrane vesicles reconstituted with 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine dispersed in buffer (same sample as in Figure 1C).

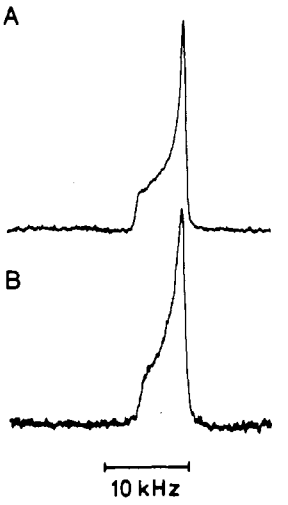

FIGURE 5: Proton-decoupled ^{31}P NMR spectra at 121.4 MHz. Measuring temperature, 4 °C. (A) DOPC liposomes; 116 mg of phospholipid in 0.3 M sucrose buffer, 500 scans. (B) R-SR exchanged with DOPC (same sample as in Figure 1C); 6700 scans, 31.25-kHz spectral width.

& Seelig, 1976). Inspection of Figure 5 reveals that the low- and high-field edges are relatively well-defined for DOPC whereas they are slightly broader for SR reconstituted with DOPC. Figure 2B shows the temperature dependence of the chemical shielding anisotropy for DOPC and R-SR. The variation of $\Delta\sigma$ with temperature was the same for DOPC and R-SR, but the numerical value of the chemical shielding anisotropy was consistently 3–6 ppm smaller in R-SR than in pure DOPC. The data are also in good agreement with ^{31}P NMR studies on oriented natural SR membranes (Mc Laughlin et al., 1981).

The result of a phosphorus T_1 relaxation time experiment on R-SR (at 121 MHz) is reproduced in Figure 6. No anisotropy in the T_1 relaxation time was observed; i.e., the T_1 relaxation time was the same for all parts of the "powder-type" spectrum. Furthermore, the relaxation process could again be described by a single exponential. However, a surprising and unexpected result was obtained for the temperature dependence of the phosphorus T_1 relaxation time (Figure 7). In

Table I: ^2H and ^{31}P NMR Measurements on Reconstituted Sarcoplasmic Reticulum Membranes (R-SR); Comparison between Pure Lipids and Functional Membranes[a]

phospholipid employed for exchange	temp (°C)	^2H NMR (at 46.1 MHz): quadrupole splitting, $\Delta\nu_Q$ (kHz)		^2H NMR (at 46.1 MHz): T_1 relaxation time (ms)		^{31}P NMR (at 121.4 MHz): chemical shielding, anisotropy, $\Delta\sigma$ (ppm)		^{31}P NMR (at 121.4 MHz): T_1 relaxation time (s)	
		DEPC	R-SR	DEPC	R-SR	DEPC	R-SR	DEPC	R-SR
[9,10-$^2H_2$]DEPC	14	23.6	20.0			−55.0	−48.9	0.87	1.09
(lipid/protein	24	21.8	19.0	20.1	17.1	−51.0	−46.8	1.26	1.20
ratio 0.34 w/w)	34	20.0	17.5				−44.7	1.35	1.43
		DOPC[b]	R-SR[b]	DOPC[b]	R-SR[b]	DOPC	R-SR	DOPC	R-SR
[9,10-$^2H_2$]DOPC	4	15.8	14.1	7.7	7.4	−51.0	−47.5	1.00	1.30
(lipid/protein	14	14.0	13.0	10.6	10.2	−49.0	−46.5	1.02	1.23
ratio 0.43 w/w)	24	13.2	11.7	13.8	11.0	−48.5	−45.6	1.07	1.25
	34					−47.4		1.24	1.39
[2,2-$^2H_2$]DOPC		DOPC[c,d]	R-SR[c]						
(lipid/protein	21	25.5	26.0						
ratio 0.61 w/w)		15.9	16.1						
		10.4	11.2						

[a] Some data are interpolated. [b] Three quadrupole splittings are observed (see text). Only the largest is listed in the table. [c] C-2 segments produce three quadrupole splittings. All three are listed. [d] The pure lipid was measured at 24 °C.

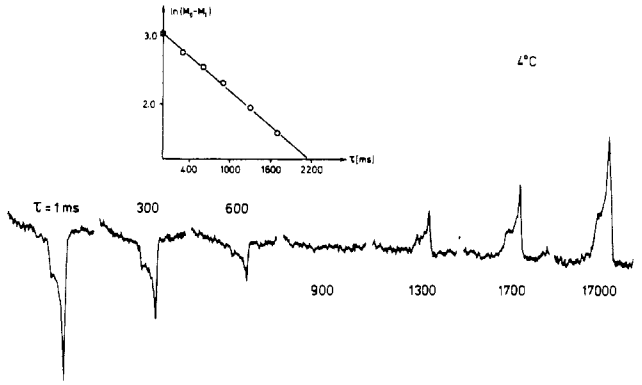

FIGURE 6: Measurement of the phosphorus T_1 relaxation time at 121.4 MHz with the inversion recovery technique. Reconstituted sarcoplasmic reticulum membrane vesicles exchanged with 1,2-di[9,10-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine (same sample as in Figure 1C), 31.25-kHz spectral width. Proton-decoupled spectra with inverse gated decoupling conditions, 300 scans/spectrum, T_1 = 1.30 s, temperature 4 °C.

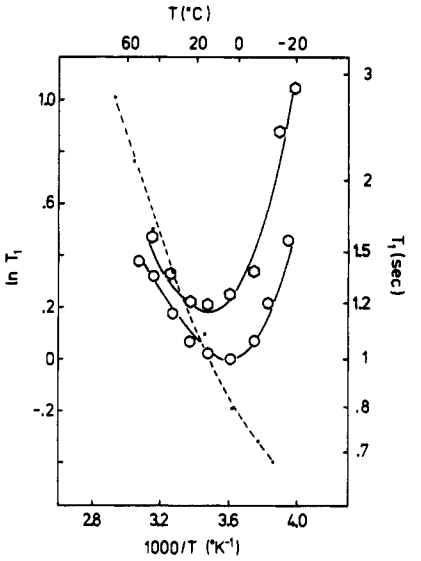

FIGURE 7: Variation of the phosphorus T_1 relaxation time with temperature. (O) DOPC liposomes measured at 121.4 MHz resonance frequency; (○) sarcoplasmic reticulum membrane exchanged with DOPC measured at 121.4 MHz (same sample as in Figure 1C); (---) DOPC liposomes at 36.4 MHz without proton decoupling (measurements were made by Dr. J. L. Browning).

contrast to ^2H NMR where the Arrhenius plots resulted in straight lines, the phosphorus T_1 relaxation time in the same temperature interval was characterized by a distinct minimum. For pure DOPC, this minimum occurred at 277 K with a relaxation time of ~1.0 s, whereas for R-SR, the minimum was shifted toward higher temperatures (287 K) and had a longer T_1 of 1.2 s. Also included in Figure 7 is the result of a phosphorus T_1 relaxation time study on DOPC liposomes at 36.4 MHz (dashed line). At this resonance frequency, the phosphorus T_1 relaxation time behaved similarly to the ^2H NMR relaxation time; i.e., the relaxation time increased smoothly with temperature. However, it is clear from inspection of Figure 7 that the data would not fit a single straight line in the Arrhenius representation. In passing, it should also be noted that the 36.4-MHz T_1 relaxation times of DOPC were in good agreement with comparable data on egg yolk lecithin (Horwitz & Klein, 1972; Yeagle et al., 1975).

The sarcoplasmic reticulum membrane was also exchanged with 1,2-di[2,2-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine ([2,2-$^2H_2$]DOPC) in order to probe the fatty acyl chain conformation close to the lipid–water interface. In these experiments, only ~38% of the natural lipids were replaced by [2,2-$^2H_2$]DOPC. Accordingly, the protein-free control was made with a lipid bilayer composed of 38% [2,2-$^2H_2$]DOPC and 62% SR lipids. Figure 8 shows a comparison of ^2H NMR spectra of pure lipid vesicles and R-SR. Qualitatively, the results were consistent with those obtained from other pure lipid bilayers [cf. Seelig & Browning (1978)] as well as from intact biological membranes (Gally et al., 1979). The ^2H NMR spectrum consisted of three doublets, i.e., one large splitting (~26 kHz at 21 °C) from the C-2 segment of the *sn*-1 chain and two smaller splittings (16 and 11 kHz) from the C-2 segment of the *sn*-2 chain. From the size of the splittings, it can be concluded that the *sn*-1 chain is straight while the *sn*-2 chain assumes a bent conformation [cf. Seelig & Seelig (1975)]. This average conformation is not modified by the presence of SR protein, since within experimental error no quantitative differences were observed between the protein-free liposomes and the reconsituted membrane (cf. Table I).

Sarcoplasmic Reticulum Membrane Vesicles Reconstituted with 1,2-Dielaidoyl-sn-glycero-3-phosphocholine (DEPC). Since the gel-to-liquid crystal phase transition of DEPC is

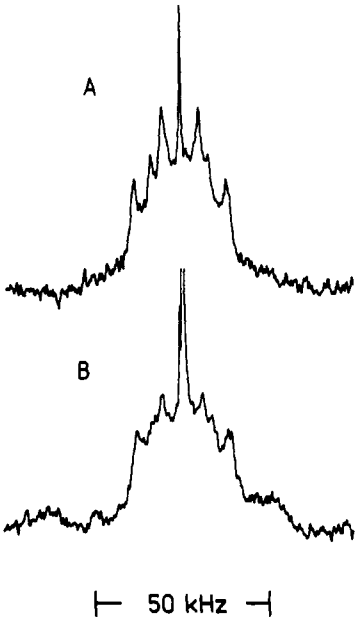

FIGURE 8: ^2H NMR spectra at 41.6 MHz of reconstituted sarcoplasmic reticulum membrane vesicles exchanged to 38 wt % with 1,2-di[2,2-$^2H_2$]oleoyl-*sn*-glycero-3-phosphocholine. Spectral width 125 kHz. (A) Liposomes composed of 38% [2,2-$^2H_2$]DOPC and 62% SR-lipids: 24 °C; 32000 scans; $\Delta\nu_Q$ = 25.5, 15.9, and 10.4 kHz. (B) R-SR in buffer. Total protein 33.4 mg, containing 20.4 mg of lipid (~7.8 mg of [2,2-$^2H_2$]DOPC and 12.7 mg of SR-lipids). Lipid-to-protein ratio was ~0.61 (w/w), i.e., a molar ratio of 91 phospholipids per Ca^{2+} pump protein. The measurement was made at 21 °C. The central signal is due to the natural abundance of deuterium in water; 250000 scans at 4 scans/s, $\Delta\nu_Q$ = 26.0, 16.1, and 11.2 kHz.

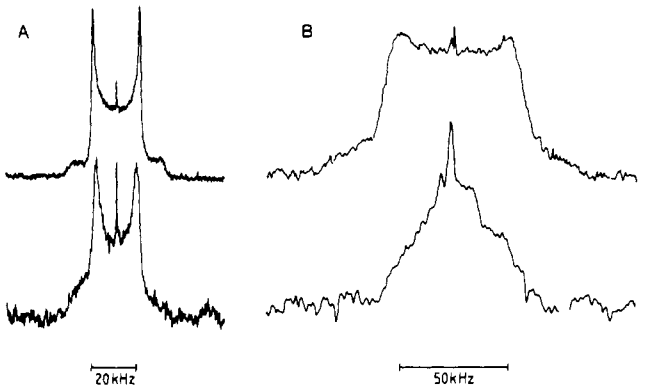

FIGURE 9: ^2H NMR spectra (at 46.1 MHz) of reconstituted functional sarcoplasmic reticulum. The natural lipids are exchanged to the extent of 99% with 1,2-dielaidoyl-*sn*-glycero-3-phosphocholine (DEPC). At least 90% of the DEPC is deuterated in both chains at the 9,10 position, i.e., at the trans double bond. The phase transition of DEPC occurs at about 10 °C. Total membrane protein and phospholipid are 68 and 23 mg, respectively. Lipid-to-protein ratio is 0.34 (w/w). The mole ratio of phospholipid to Ca^{2+} pump protein is 51. (A) Measurements above the phase transition temperature. Measuring temperature 25 °C. The upper spectrum corresponds to pure 1,2-di-[9,10-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine, and the quadrupole splitting is 21.4 kHz. The lower spectrum is due to reconstituted sarcoplasmic reticulum exchanged with the same lipid. The quadrupole splitting is reduced to 18.8 kHz. (B) Measurement below the phase transition temperature. Measuring temperature 4 °C. Upper spectrum, pure DEPC. Lower spectrum, reconstituted sarcoplasmic reticulum membrane vesicles.

about 10 °C, the ^2H NMR spectra above and below T_c can readily be obtained. Representative results for pure DEPC vesicles deuterated at the trans double bond ([9,10-$^2H_2$]DEPC) and for reconstituted sarcoplasmic reticulum membrane vesicles (R-SR) are shown in Figure 9. Above the T_c, the R-SR spectra were again characteristic of a single homogeneous lipid

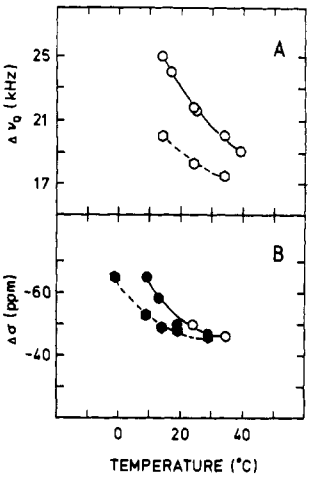

FIGURE 10: Reconstituted sarcoplasmic reticulum membranes exchanged with 1,2-di[9,10-$^2H_2$]elaidoyl-*sn*-glycero-3-phosphocholine (same sample as in Figure 9). (A) ^2H NMR. Temperature dependence of the deuterium quadrupole splitting, $\Delta\nu_Q$. (B) ^{31}P NMR. Temperature dependence of the chemical shielding anisotropy, $\Delta\sigma$. (●) Reconstituted sarcoplasmic reticulum membranes at 36.4 MHz; (○, ●) pure DEPC liposomes in excess water. ^{31}P NMR measurements at 121.4 (○) and 36.4 (●) MHz.

phase (Figure 9A). Only one quadrupole splitting was observed for the two deuterons of the trans double bond due to the different symmetry of this structure compared to the cis double bond (Seelig & Waespe-Šarčevič, 1978). The quadrupole splittings of R-SR were found to be 10–15% smaller than those of pure DEPC liposomes at the same temperature, which is consistent with the DOPC reconstitution experiment (cf. Figure 10A). Deuterium T_1 relaxation times were compared at 24 °C only. Again, the relaxation time was found to be shorter in R-SR (17.1 ms) than in pure DEPC (20.1 ms).

Gel-state ^2H NMR spectra of reconstituted sarcoplasmic reticulum and DEPC bilayers at 4 °C are shown in Figure 9B. The interpretation of such spectra is more complex since they can no longer be described by a single order parameter. At present, it is unclear if the spectral shape results from slow motional effects [cf. Meirovitch & Freed (1979) and Huang et al. (1980)] or from a distribution of order parameters [cf. Davis et al. (1979)]. If the assumption of an order parameter distribution should turn out to be the correct quantitative approach, then the spectrum of reconstituted sarcoplasmic reticulum membrane certainly comprises a larger distribution of quadrupole splittings than that of the pure lipid. This, in turn, would imply a larger spectrum of chain conformations in the reconstituted membrane. Alternatively, a comparison of Figure 9B with the slow motional spectral simulations of Huang et al. (1980) suggests that the spectral differences between DEPC and R-SR below T_c could also be caused by a somewhat increased rate of segmental motions in R-SR compared to the pure lipid phase. No decision between the two possibilities can be made at present.

Qualitatively, the ^{31}P NMR spectra of DEPC liposomes and R-SR have the same shape as those shown in Figure 5. However, the temperature dependence of the chemical shielding anisotropy was somewhat different as indicated by Figure 10B. Phosphorus T_1 relaxation times were measured at 121.3 MHz. For both pure DEPC and R-SR, the T_1 relaxation experienced a minimum approximately at the phase transition. A few selected T_1 values for temperatures above T_c are given in Table I, and the results are qualitatively similar to those found for DOPC and the corresponding R-SR.

Steady-State Fluorescence Depolarization. The variation of the anisotropy parameter $\bar{r}$ with temperature is shown in

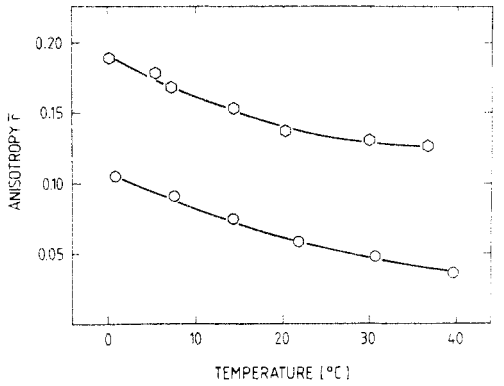

FIGURE 11: Variation of the fluorescence anisotropy, $\bar{r}$, with temperature. (O) DPH incorporated into unilamellar vesicles of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine. (○) DPH incorporated into sarcoplasmic reticulum membrane vesicles exchanged with DOPC. The natural lipids were exchanged with DOPC to at least 99%, 90% of which was deuterated in the 9,10 position. The molar ratio was 64 mol of phospholipid/mol of Ca^{2+} pump protein.

Figure 11. At all temperatures, the fluorescence anisotropy $\bar{r}$ was found to be almost twice as large in reconstituted sarcoplasmic membrane vesicles (lipid-to-protein ratio, 64:1 mol/mol) as in pure lipid vesicles; e.g., at 25 °C, the anisotropy $\bar{r}$ values were 0.134 and 0.054, respectively. A similar increase in the anisotropy $\bar{r}$ of DPH was noted when bacteriorhodopsin was incorporated in unilamellar vesicles of 1,2-dimyristoyl-*sn*-glycero-3-phosphocholine (DMPC) (Heyn, 1979; Heyn et al., 1980). At 40 °C (i.e., 17 °C above the phase transition of DMPC), $\bar{r}$ increased from ~0.07 to 0.21 for vesicles with a molar phospholipid/bacteriorhodopsin ratio of 49. Our results are also consistent with DPH labeling studies of natural sarcoplasmic membrane vesicles (Moore et al., 1978) and of recombinants of sarcoplasmic ATPase with two synthetic lipids (Gomez-Fernandez et al., 1979, 1980). In the latter experiments, the polarization, $\bar{p} = (I_\parallel - I_\perp)/(I_\parallel + I_\perp)$, has been reported which is related to the anisotropy $\bar{r}$ according to $\bar{r} = 2\bar{p}/(3 - \bar{p})$.

Discussion

Membrane Organization and Lipid Conformation. Natural sarcoplasmic reticulum (SR) membrane contains ca. 40 wt % lipid, corresponding to a lipid-to-protein weight ratio of 0.67 (Meissner & Fleischer, 1974). This is approximately 110 mol of phospholipid/mol of Ca^{2+} pump protein. Sarcoplasmic reticulum membranes can be molecularly dispersed with the use of detergents, and the membrane can be reassembled upon removal of these perturbants; the vesicles which are formed are capable of energized Ca^{2+} accumulation. In the present study, the SR membrane was solubilized, the phospholipid was exchanged, and the membrane was reconstituted to form functional membrane vesicles (R-SR). Conditions were used which ensured complete exchange with added phospholipid (C. T. Wang, L. Hymel, G. Meissner, and S. Fleischer, unpublished experiments). The key studies were carried out with functional reconstituted membrane vesicles which contained 99% DOPC or DEPC of which at least 90% of the lipid contained deuterium label in specific positions. In these reconstituted membrane vesicles, the Ca^{2+} pump protein is the major protein constituent (>90% by weight) of the membrane. Therefore, the reconstituted membranes studied consisted mainly of one protein and one phospholipid type. Reconstituted membranes were prepared which varied in lipid content from about 50 to 130 mol of phospholipid per mol of Ca^{2+} pump protein; i.e., their phospholipid content was in the same range as that of the original SR membrane. Ten preparations were studied, and qualitatively similar results were obtained for each. The use of DOPC and DEPC for SR reconstitution mimics the natural phospholipid environment of the SR insofar as function, including Ca^{2+} pumping, is concerned (C. T. Wang, L. Hymel, G. Meissner, and S. Fleischer, unpublished experiments).

The NMR experiments address aspects of lipid molecular conformation and their rate of motion. From the shapes of the ^2H and ^{31}P spectra and from the size and sign of the residual quadrupole splittings and chemical shielding anisotropies, it can be concluded that the lipids in R-SR vesicles are organized exclusively in a bilayer structure. The additional isotropic signal sometimes seen in the ^2H NMR spectra is due to the natural abundance of deuterium in water. No such isotropic signal is observed in the phosphorus spectra. Furthermore, the present data provide no evidence for a strong, long-lived interaction between the membrane protein and the lipid. In the extreme case, such an interaction should produce two different sets of quadrupole splittings which are not observed. In addition, the observation of a single T_1 relaxation time in both the ^{31}P and ^2H spin–lattice relaxation time experiments strongly corroborates the conclusion of a homogeneous lipid phase.

Nevertheless, it cannot be ruled out completely that a small number of lipid molecules is tightly bound to the membrane protein. At the present signal-to-noise level, a conservative estimate leads to a maximum of 5–10 lipid molecules per monomer protein which could escape detection due to exaggerated line broadening. However, this amount of immobilized lipid would not be sufficient to form a coherent and discrete lipid annulus around the Ca^{2+} pump protein. The latter has been estimated to be 25–30 molecules of phospholipid per molecule of Ca^{2+} pump protein (Metcalfe & Warren, 1977).

The structural parameters such as the chemical shielding anisotropy and the residual quadrupole coupling constant are similar for pure lipid vesicles and R-SR, at least for temperatures above the phase transition (cf. Figures 2 and 10). We therefore conclude that the average lipid conformation at the level of the phosphate group, the C-2 segment, and the cis or trans double bond is not appreciably changed in spite of the high protein content in the membrane. This finding is in agreement with results obtained for intact biological systems such as *Escherichia coli* cells (Gally et al., 1979, 1980; Davis et al., 1979; Nichol et al., 1980) and membranes of *Acholeplasma laidlawii* (Stockton et al., 1977; Rance et al., 1980). The molecular details of this time-averaged lipid conformation have been discussed elsewhere (Seelig & Browning, 1978; Gally et al., 1979; Seelig & Seelig, 1980). It may also be noted that the $\Delta\nu_Q$ and $\Delta\sigma$ parameters are 10–20% smaller in R-SR than in pure lipid vesicles, indicating that the lipids are distorted by the proteins in the membrane and become more disordered. Similar results have been obtained in other membrane reconstitution studies (Seelig & Seelig, 1978; Oldfield et al., 1978; Rice et al., 1979).

Dynamic Aspects of the Lipid–Protein Interaction. In order to discuss in a more quantitative way how the spin–lattice relaxation time T_1 is related to the molecular motions, we recall a basic result of magnetic relaxation theory.

$$\frac{1}{T_1} \propto \tau_c/(1 + \omega_0^2\tau_c^2) \qquad (2)$$

Here, ω_0 is the NMR resonance frequency (in rad/s) and τ_c the molecular correlation time. If the value of τ_c is such that $\omega_0\tau_c \simeq 1$, then T_1 relaxation is most efficient; i.e., the T_1 relaxation time is minimized. If the molecular motions are slower or faster than ω_0, the T_1 times must become longer.

Since the rate of the molecular fluctuations depends on the viscosity, η, and the temperature, T (in K)

$$\tau_c \propto \eta/T \tag{3}$$

a plot of the spin–lattice relaxation time T_1 vs. the reciprocal temperature should exhibit a minimum if at a certain temperature the condition $\omega_0\tau_c \simeq 1$ is satisfied (cf. Appendix for a more quantitative discussion).

Earlier ^1H, ^2H, ^{13}C, and ^{31}P NMR spin–lattice relaxation time studies on liquid-crystalline membranes consistently showed an increase of T_1 with increasing temperature, indicating that for the specific experimental conditions employed the molecular motions were much faster than the resonance frequency ($\omega_0\tau_c \ll 1$, fast correlation time regime). The phosphorus T_1 measurements reported here are the first example of a T_1 minimum for a lipid bilayer. This can be traced back to a particularly fortunate combination of experimental conditions. First, the NMR experiments were performed at relatively high field so that ω_0 was large ($\omega_0 = 7.6 \times 10^8$ rad/s). Second, since the DOPC bilayer remained in the liquid-crystalline state down to −22 °C, it was possible to slow down the molecular motions sufficiently without freezing the molecules into a rigid bilayer crystal. Finally, the phosphate group has a larger volume than, e.g., a CH_2 segment, and its molecular reorientation rate is therefore reduced by the increased size. It should be appreciated that the importance of the T_1 minimum rests on the fact that it is not essential to know the exact relaxation mechanism(s) in order to evaluate τ_c at the T_1 minimum. At least to a close approximation, the relation $\tau_c = 1/\omega_0$ holds for all types of T_1 relaxation mechanisms at the T_1 minimum. Hence, we conclude from Figure 7 that the correlation time in DOPC bilayers at the relaxation time minimum (277 K) is $\simeq$1 ns. This information may be used to evaluate the phosphorus data more quantitatively as shown in the appendix. In the temperature interval of −20 to +47 °C, the correlation time τ_c of pure DOPC bilayers follows a single exponential law (cf. Appendix)

$$\tau_c = (5.41 \times 10^{-4}) \exp(4076/RT) \tag{4}$$

where τ_c is given in nanoseconds, and the activation energy is $E_a = 4.08$ kcal/mol. In reconstituted sarcoplasmic membrane vesicles, the T_1 minimum is shifted by 10 °C toward higher temperatures, which means that the high frequency motions dominating the T_1 process are slowed by 10–20% in the presence of the Ca^{2+} pump protein. The more detailed analysis of ^{31}P T_1 relaxation times via the T_1 minimum furthermore demonstrates that a simple numerical comparison of two T_1 times of two different systems is not sufficient and may lead to incorrect conclusions about the motional changes involved.

We may now turn to the analysis of the deuterium T_1 relaxation times. Extensive T_1 relaxation time studies have been reported for bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (Brown et al., 1979; Davis, 1979). The dominant relaxation pathway is the quadrupolar interaction, which is generally much stronger than the dipolar coupling involving adjacent nuclei. This unique feature of deuterium NMR greatly simplifies the interpretation of ^2H T_1 relaxation times and avoids the problems encountered in ^{31}P NMR. For an ordered system such as a lipid bilayer, the following T_1 formula has been derived in the fast correlation time limit (Brown et al., 1979):

$$\frac{1}{T_1} = \frac{3\pi^2}{2}\left(\frac{e^2qQ}{h}\right)^2\left(1 + \frac{1}{2}S_{CD} - \frac{3}{2}S_{CD}^2\right)\tau_c \tag{5}$$

e^2qQ/h is the static quadrupole coupling constant and S_{CD} the deuterium order parameter. S_{CD} is related to the residual quadrupole splitting of a powder-type pattern according to

$$\Delta\nu_Q = \frac{3}{4}(e^2qQ/h)S_{CD} \tag{6}$$

For pure DOPC bilayers as well as for R-SR, the T_1 relaxation time increases with increasing temperature (cf. Figure 4), from which it can be concluded that the rate of reorientation of the cis double bond falls into the short correlation time regime ($\omega_0\tau_c \ll 1$, $\omega_0 = 2.9 \times 10^8$ rad/s). Figure 4 also shows that the deuterium T_1 relaxation times are shorter in R-SR than in pure DOPC vesicles at ambient temperature. It is the reciprocal of the T_1 relaxation time which is proportional to the motional parameter, i.e., the rotational correlation time τ_c. For DOPC bilayers at 24 °C, one measures $\Delta\nu_Q = 13.2$ kHz and $T_1 = 13.8$ ms, leading to $S_{CD} \sim 0.10$ and $\tau_c = 0.17$ ns. For R-SR at the same temperature, the T_1 relaxation time is 11.0 ms with $\Delta\nu_Q = 11.7$ kHz; hence, $S_{CD} \sim 0.09$ and $\tau_c = 0.21$ ns. Thus, the analysis of the deuterium T_1 relaxation time yields the same conclusion as reached above for the phosphate group; i.e., the rate of segmental reorientation is slowed by about 20% due to the presence of protein.

Reorientation Rate of the Phosphocholine Head Group. Correlation with Earlier Work. The reorientation rate of the phosphocholine dipole in bilayers of 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine (DPPC) has been determined by dielectric measurements. At 50 °C, a correlation time of 2.3 ns and an activation enthalpy $\Delta H^* = 4.0$ kcal/mol were obtained (Shepherd & Büldt, 1978). A correlation time of 1.4 ns (at 23 °C) was derived from nuclear Overhauser enhancement experiments on ^{31}P in the head groups of egg phosphatidylcholine vesicles (Yeagle et al., 1975). Finally, deuterium T_1 relaxation time studies on the $-POCD_2CH_2N-$ head group of DPPC bilayers yielded $T_1 = 22$ ms at 45 °C (Gally et al., 1975). The reorientation rate of the α-CD_2 segment is 0.1 ns with an activation energy of $E_a = 5 \pm 2$ kcal/mol. The present results for DOPC with $\tau_c \simeq 1$ ns (at 4 °C), $E_a = 4.0$ kcal/mol, and $\Delta H^* = 4.7$ kcal/mol (cf. Appendix) are in general agreement with these studies. In fact, the agreement between the dielectric measurements and the magnetic resonance techniques can even be improved by taking into account the different nature of the relaxation process. For a rotational diffusion process characterized by a diffusion constant D_r, the experimentally accessible correlation time τ_l depends on the rank l of the relaxing tensor according to $1/\tau_l = l(l+1)D_r$; for dielectric relaxation, $l = 1$, whereas for magnetic relaxation, $l = 2$. From the dielectric relaxation time of 2.3 ns, one thus calculates $D_r = 2.2 \times 10^8$ s^{-1} whereas the phosphorus correlation time yields $D_r = 1.7 \times 10^8$ s^{-1}.

As discussed above, the reorientation rate of the phosphate group does not change dramatically in R-SR when compared to pure DOPC bilayers. Under the present experimental conditions, the τ_c value of the phosphate group is increased by not more than about 20%. This finding is consistent with a ^{13}C NMR study by Stoffel et al. (1977), where 80% of the native SR lipids were exchanged against [*N*-$^{13}CH_3$]lecithin. The T_1 relaxation time of the choline methyl group in pure lipid vesicles was 510 ms compared to 480 ms in exchanged SR (molar lipid/protein ratio, 180:1).

Fluorescence Depolarization Anisotropy vs. ^2H and ^{31}P NMR. An increase in the fluorescence depolarization anisotropy has generally been interpreted in terms of an increase in the bilayer "viscosity" (Shinitzky et al., 1971; Cogan et al., 1973). However, this approach is too simple for an ordered

system such as a lipid bilayer (Kawato et al., 1977, 1978; Heyn, 1979; Jähnig, 1979). It has been shown recently that the steady-state fluorescence anisotropy, $\bar{r}$, depends at least on four different factors (Heyn, 1979).

$$\bar{r} = r_\infty + (r_0 - r_\infty)\frac{\tau_c}{\tau_c + \tau_F} \quad (7)$$

In a single-flash experiment, r_0 is the fluorescence depolarization anisotropy at time zero whereas the nonvanishing end value r_∞ expresses the fact that the equilibrium distribution of DPH is anisotropic. τ_c is the rotational correlation time for the approach to the equilibrium distribution, and τ_F is the fluorescence lifetime. r_∞ is a measure of the ordering of the DPH molecules in the membrane and can be related to the conventional order parameter S of the long molecular axis according to (Heyn, 1979)

$$S^2 = r_\infty/r_0 \quad (8)$$

In the present DPH labeling experiments, we observe an increase of the fluorescence depolarization anisotropy from pure DOPC vesicles with $\bar{r} = 0.054$ (at 25 °C) to $\bar{r} = 0.134$ for R-SR. Since the fluorescence lifetime τ_F is not significantly altered by the presence of the Ca^{2+} pump protein ($\tau_F \simeq 7.5$ ns; Gomez-Fernandez et al., 1980), the observed increase in $\bar{r}$ can be caused by (1) a change in the rotational correlation time τ_c (viscosity increase) and/or (2) a change in the ordering of the DPH labels as indicated by r_∞. In a steady-state experiment, these two contributions are not separable.

However, a semiquantitative discussion is possible since nanosecond fluorescence data have been reported at least for pure DOPC multilayers (Stubbs et al., 1981). At 25 °C, the following parameters have been measured: $r_\infty = 0.013$, $\tau_c = 1.84$ ns, $\tau_F = 7.7$ ns. With $r_0 = 0.39$ (Kawato et al., 1977), one calculates from eq 7 a steady-state fluorescence anisotropy $\bar{r} = 0.086$. The experimental steady-state values are $\bar{r} = 0.089$ for DOPC multilayers (Stubbs et al., 1981) and $\bar{r} = 0.054$ for single-shelled vesicles (this work). According to eq 8 the order parameter of DPH in DOPC multilayers is $S = 0.18$, which is in agreement with ^2H NMR studies on a related unsaturated system (Seelig & Seelig, 1977) if the DPH label is assumed to be positioned in the central part of the bilayer [cf. also Heyn (1979)].

No comprehensive nanosecond fluorescence spectroscopy data are as yet available for reconstituted sarcoplasmic reticulum membranes. Nevertheless, it is useful to consider the following two extreme cases. First, by analogy with the ^2H and ^{31}P NMR results, we may assume that the ordering of the DPH probe is not affected by the protein. With $\bar{r} = 0.134$ and r_∞ and τ_F unchanged, we calculate $\tau_c = 3.64$ ns. Compared to the pure lipid bilayer with $\tau_c = 1.84$ ns, this corresponds to a 2-fold increase in the correlation time and the apparent membrane "microviscosity". This result cannot be correlated with the only small increase in the correlation time of the segmental motions as evidenced by ^2H and ^{31}P NMR. Alternatively, we may consider the other extreme where τ_c remains unaltered ($\tau_c = 1.84$ ns) but r_∞ increases. Using again eq 7, we calculate from the experimental $\bar{r} = 0.134$ an end value of $r_\infty = 0.073$, which means that the DPH order parameter would increase from $S = 0.18$ for pure DOPC vesicles to $S = 0.43$ for R-SR. However, this is at variance with the deuterium order parameters, which are found to be *smaller* in R-SR than in DOPC. A combination of an ordering effect and a viscosity effect would lead to intermediate values for τ_c and S. Hence, it is safe to conclude that the changes sensed by the DPH molecule upon addition of protein are different from those of the lipid molecules. This may be the result of the rodlike character of the DPH molecule since in contrast to the flexible phospholipids the stiff DPH molecule cannot adapt its shape to the protein surface.

Nanosecond fluorescence spectroscopy of DPH-labeled biological membranes has yielded correlation times of 2.6 ns (at 25 °C) for mouse leukemic cells (Senē et al., 1978), 4 ns (at 30 °C) for erythrocyte ghosts (Glatz, 1978), and ~2 ns (at 25 °C) for a variety of different membrane systems (Hildenbrand & Nicolau, 1979). These data are in broad agreement with those mentioned above for R-SR (1.8–3.6 ns, depending on which extreme is considered) but do not allow any decision between the two alternative interpretations presented. The molecular information gained from steady-state fluorescence depolarization measurements of membranes thus appears to be rather limited and ambiguous.

Conclusions

The present investigation underscores the close structural similarity of SR membranes and pure lipid bilayers as far as the phospholipid conformation is concerned. This is in agreement with related studies on other biological membranes [cf. Seelig & Seelig (1980)]. Equally important, the conbination of ^2H and ^{31}P NMR with membrane reconstitution techniques provides conclusive evidence that the Ca^{2+} pump protein is *active in a normal bilayer environment*. The NMR spectra of reconstituted sarcoplasmic membranes are characteristic of a single homogeneous bilayer phase even at the lowest phospholipid-to-protein ratio investigated (i.e., ~50 mol of phospholipid per mol of Ca^{2+} pump protein). This excludes the possibility of a discrete and tightly bound lipid annulus, at least in the reconstituted sarcoplasmic reticulum membranes. Measurement of the T_1 relaxation times allowed a precise quantitative analysis of the internal, high-frequency modes of phospholipid motion. The observation of a minimum in the ^{31}P spin–lattice relaxation time (at 121 MHz) of DOPC and R-SR demonstrates unambiguously that motions with frequencies in the relatively narrow interval of 10^9–10^{10} Hz have a high probability of occurrence in the bilayer head-group region. These motions must be identified as intramolecular segmental reorientations. In addition, the data demonstrate that the high-frequency motions of the polar groups are only slightly slowed (by 10–20% at most) in the presence of protein, even at very high protein contents. The interpretation of the deuterium T_1 relaxation times leads to the same conclusion for the hydrocarbon region of the membrane. The cis and trans double bonds exhibit very fast reorientation rates of again 10^9–10^{10} Hz which are reduced by 10–20% in the presence of protein. Estimates of membrane "microviscosities" (η) derived from translational and rotational diffusion constants of phospholipid molecules and membrane proteins yield values on the order of 1–10 P [cf. Edidin (1974) and Cherry (1979)]. Moreover, the few data available on reconstituted systems indicate a strong increase of η with increasing protein concentration [cf. Cherry (1979) and Heyn et al. (1980)]. By contrast, evaluation of the above NMR correlation times in terms of a "microviscosity" yields much lower values of ca. 0.1–0.5 P which are furthermore only little dependent on the protein concentration. This suggests that the fast internal modes of motions of a phospholipid molecule are much less influenced by membrane proteins than are translocation and also, perhaps, the reorientation of the molecule as a whole.

Appendix

Analysis of Phosphorus T_1 Relaxation Times of Pure DOPC Bilayers. The most important mechanisms contributing to ^{31}P spin–lattice relaxation are dipole–dipole relaxation (T_{1D})

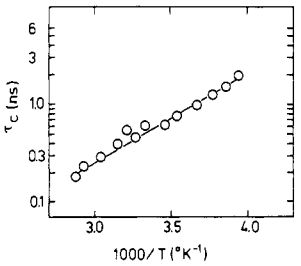

FIGURE 12: Temperature dependence of the correlation time, τ_c, of the phosphate polar group in bilayers of 1,2-dioleoyl-*sn*-glycero-3-phosphocholine. Data calculated from phosphorus T_1 relaxation time measurements at 36.4 MHz as described in the Appendix.

and relaxation via chemical shielding anisotropy (T_{1CSA}). The two relaxation mechanisms can be differentiated by their dependence on the magnetic field strength, H_0. Dipole–dipole relaxation is independent of H_0, while relaxation via chemical shielding anisotropy increases according to $1/T_{1CSA} \propto H_0^2$. Previous work has led to the conclusion that up to a field strength of 2.25 T the spin–lattice relaxation time is dominated by dipolar interactions and that contributions from other mechanisms are negligible (Yeagle et al., 1975, 1977). This result no longer holds true for the present study where the measurements were performed at a much higher magnetic field (7.05 T). Figure 7 demonstrates that in the fast correlation time regime (high temperatures) the T_1 relaxation times measured at 121.4 MHz are shorter than those measured at 35.4 MHz, suggesting additional contributions from chemical shielding anisotropy relaxation.

Quantitatively, the dipolar contribution to the total relaxation time may be estimated according to (Doddrell et al., 1972)

$$\frac{1}{T_1} = \frac{1}{10}|F_0|^2[J(\omega_H - \omega_P) + 3J(\omega_P) + 6J(\omega_H + \omega_P)] \quad \text{(A1)}$$

with

$$J(\omega_i) = \tau_c/(1 + \omega_i^2\tau_c^2)$$

Equation A1 is strictly valid only for an isotropic motion characterized by a single correlation time τ_c. If a single proton at a distance r from the phosphorus atom is involved in the relaxation process, F_0 can be calculated according to

$$F_0 = h\gamma_H\gamma_P/r^3$$

where γ is the gyromagnetic ratio of the corresponding nucleus. The situation is more complex, however, for the phosphocholine head group where intra- as well as intermolecular dipole–dipole interactions are involved and where the head group is furthermore flexible. Fortunately, by combining the measurements at 121 and 36 MHz, it is possible to evaluate the amplitude factor F_0^2 directly from the experimental results. For this analysis, we make use of two pieces of information. First, we know from the minimum in the T_1 curve at 121 MHz that $\tau_c = 0.93$ ns at 277 K.[2] Second, we recall that at 36 MHz the relaxation process is exclusively dipolar. The T_1 relaxation time at 36 MHz and 277 K is found to be 0.85 s. Inserting both values into eq A1 leads to $|F_0|^2 = 1.65 \times 10^9$ s^{-2}. ^{2}H NMR and neutron diffraction studies on the phosphocholine head group have led to the conclusion that the phosphocholine head-group conformation is relatively insensitive to temperature [cf. Büldt et al. (1978, 1979)]. To a first approximation, it is thus safe to assume that $|F_0|^2$ remains unchanged as long as the bilayer is in the liquid-crystalline state. Using the T_1 relaxation times at 36 MHz and the above amplitude factor, we can then determine τ_c for all other temperatures measured. The result of this analysis is shown in Figure 12. A straight line is obtained in a semilogarithmic plot of τ_c vs. the reciprocal absolute temperature. A least-squares fit to this line gives an activation energy of $E_a = 4.08$ kcal/mol. Alternatively, the correlation time τ_c can be analyzed with the Eyring equation:

$$\tau_c = \frac{h}{kT}\exp(-\Delta S^*/R)\exp(\Delta H^*/RT)$$

A plot of ln $(\tau_c T)$ vs. $1/T$ also leads to a straight line, and an activation enthalpy of $\Delta H^* = 4.7$ kcal is evaluated from the slope.

[2] The T_1 minimum of eq A1 does not occur exactly at $\tau_c = 1/\omega_0$ but at $\tau_c \approx 0.7/\omega_0$. This has been taken into account in the calculation.

Table II: Analysis of ^{31}P Spin-Lattice Relaxation Times (at 121 MHz) of DOPC Bilayers

temp (°C)	τ_c (ns)	$1/T_1$ (s^{-1})	$1/T_{1D}$ (s^{-1})	ΔR (s^{-1})	$1/T_{1CSA}$ (s^{-1})
−12	1.44	0.81	0.48	0.33	0.56
−6	1.21	0.93	0.50	0.43	0.56
4	0.91	1.01	0.51	0.50	0.53
14	0.71	0.98	0.50	0.48	0.47
24	0.55	0.93	0.47	0.46	0.40
34	0.44	0.84	0.44	0.40	0.34
44	0.36	0.73	0.40	0.33	0.29
54	0.29	0.69	0.36	0.33	0.24

We may now proceed to an analysis of the relaxation times obtained at 121 MHz. The observed relaxation time T_1 contains contributions from T_{1D} and T_{1CSA} according to

$$\frac{1}{T_1} = \frac{1}{T_{1D}} + \frac{1}{T_{1CSA}}$$

Using the correlation times of Figure 12 and the amplitude factor $|F_0|^2 = 1.65 \times 10^9$ s^{-2}, we can calculate $1/T_{1D}$ by means of eq A1. The difference $\Delta R = 1/T_1 - 1/T_{1D}$ yields the contribution of chemical shielding anisotropy relaxation. Table II summarizes the results. It may be seen that both relaxation pathways participate to approximately equal extents in the T_1 relaxation. For isotropic reorientation, the following expression is obtained for T_{1CSA} [cf. Abragam (1961)]:

$$\frac{1}{T_{1CSA}} = \frac{3}{10}\omega_P^2\Delta\sigma^2\frac{\tau_c}{1 + \omega_P^2\tau_c^2} \quad \text{(A2)}$$

With $\Delta\sigma = 70$ ppm and $\omega_P = 7.6 \times 10^8$ rad/s, we calculate the relaxation rates $1/T_{1CSA}$ given in the last column of Table II. The agreement between the semiexperimental ΔR and the theoretical $1/T_{1CSA}$ is reasonable, but not perfect. This is not surprising in view of the simplifying assumption made in the calculations, notably the assumption of isotropic reorientation with a single correlation time, and also in view of the experimental errors involved in measuring T_1.

Acknowledgments

We are indebted to Dr. M. P. Heyn and M. Rehorek for their help with the fluorescence depolarization measurements. Dr. J. L. Browning is gratefully acknowledged for the phosphorus T_1 relaxation measurements at 36.4 MHz. We thank Dr. R. Ghosh for critically reading the manuscript.

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