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^{31}P-NMR STUDIES OF ORIENTED MULTILAYERS FORMED FROM ISOLATED SARCOPLASMIC RETICULUM AND RECONSTITUTED SARCOPLASMIC RETICULUM

EVIDENCE THAT 'BOUNDARY-LAYER' PHOSPHOLIPID IS NOT IMMOBILIZED

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Summary

^{31}P-NMR spectra were obtained from oriented multilayer preparations of normal sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum with lipid to protein ratios varying between 42 : 1 and 110 : 1. The dependence of the ^{31}P-NMR spectra on the alignment of the membranes with respect to the magnetic field was used to draw two conclusions about the motion of the phospholipid molecules that contribute to the observed spectra. First, the phosphate group and the two adjacent methylene groups are able to rapidly rotate (i.e., $\tau_R << 10^{-5}$ s) around the normal to the plane of the membrane. Second, the restricted internal motion of the phosphate group and the glycerol CH_2OP group is very similar to that found in liposomes formed from sarcoplasmic reticulum phospholipids. Calibration experiments showed that all (100 ± 7%) of the phospholipid molecules in the membrane can be accounted for in the observed spectra. Thus, essentially all the phospholipid molecules in the sarcoplasmic reticulum and the reconstituted sarcoplasmic reticulum membranes have the same motion in the polar headgroup region as found in model bilayer membranes. Since a large fraction of the phospholipid molecules (between one-quarter and one-half, depending on the lipid to protein ratio) are immediately surrounding the calcium-pump protein, we conclude that the calcium-pump protein does not perturb the motion of these 'boundary-layer' lipids.

Abbreviation: Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid.

Introduction

Sarcoplasmic reticulum, a membranous network that surrounds each sarcomere unit [1], mediates muscular contraction and relaxation by regulating the intracellular calcium concentration (for recent reviews see Refs. 2—6). Excitation-contraction coupling results in the release of calcium from the sarcoplasmic reticulum, thereby triggering muscle contraction [7,8]. Active uptake of calcium back into the sarcoplasmic reticulum reduces the concentration in the sarcoplasm, enabling the muscle fiber to relax.

Sarcoplasmic reticulum is one of the most intensively studied membrane systems. As isolated in highly purified form, it is capable of energized calcium uptake and has a relatively simple composition. The major protein constituent (greater than 90%) of the sarcoplasmic reticulum membrane is the calcium-pump protein [9,10]. This protein has been dissociated from the sarcoplasmic reticulum membrane and reconstituted to form functional membrane vesicles that have a lipid to protein ratio similar to the original membrane (115 : 1) [11]. Also, methodology has recently been developed to form reconstituted sarcoplasmic reticulum vesicles with lipid to protein ratios both lower and higher than that of the original membrane [12]. Such membranes of defined lipid content make possible detailed studies aimed at correlating membrane composition with structure and structure with function [12—15].

^{31}P-NMR has been used to study the motion of the polar head group region of model phospholipid membranes [16—18]. Oriented multilamellar systems have proved particularly useful for this purpose [16,17,19]. The angular dependence of the position of the ^{31}P-NMR signal from oriented membranes can be used to calculate the phosphorus chemical shift anistropy and the direction of the symmetry axis for the motion of the phosphate group. The angular dependence of the width of the ^{31}P-NMR signal can be used to calculate the dipolar interaction between the phosphorus nucleus and the protons on the two adjacent methylene groups, and the direction of the symmetry axis for the motion of these two groups. The ^{31}P-NMR spectrum of an unoriented dispersion of membranes gives some of the information obtained from the ^{31}P-NMR spectra of oriented membranes, i.e., the phosphorus chemical shift anisotropy. However, it does not, in general, give information about the dipolar interaction between the phosphorus nucleus and the methylene protons, or about the direction of the symmetry axes for the motion of the phosphate or the methylene groups.

In the work presented here ^{31}P-NMR has been used to study the motional characteristics of the polar head group region of phospholipid molecules in normal sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum membranes of varying lipid to protein ratios. Our major conclusion is that the calcium-pump protein does not perturb the motion of the polar head group region of phospholipid molecules in the sarcoplasmic reticulum membrane.

Materials and Methods

Materials

Dipalmitoylphosphatidylcholine was obtained from Koch-Light Laborato-

ries, Colnbrook, Bucks, U.K. Other materials are described in Herbette et al. [20] or Wang et al. [12].

Isolation and reconstitution of sarcoplasmic reticulum membranes

Isolation of sarcoplasmic reticulum and sarcoplasmic reticulum phospholipids. Highly purified sarcoplasmic reticulum membrane vesicles were isolated from fast (white) skeletal muscle from rabbit hind leg [9]. Sarcoplasmic reticulum phospholipids were extracted three times from sarcoplasmic reticulum with 20 vol. of $CHCl_3/CH_3OH$ (2/1), and non-lipid materials were removed by back-extraction, according to the method of Folch et al. [21], as described by Rouser and Fleischer [22]. The neutral lipids were removed by silicic acid chromatography [22].

Dissociation and reconstitution of sarcoplasmic reticulum vesicles. Dissociation and reconstitution of sarcoplasmic reticulum was carried out according to the method of Meissner and Fleischer [23] as modified by Wang et al. [12] to vary the lipid to protein ratio of the membrane. The functional characteristics of reconstituted sarcoplasmic reticulum membrane vesicles with varying lipid to protein ratios were similar to that described previously [12]. The reconstituted sarcoplasmic reticulum and normal sarcoplasmic reticulum were stored in 300 mM sucrose, 100 mM KCl and 1 mM Hepes (pH 7.1) at −70°C until use.

Analytical procedures

The protein concentration of the sample was determined according to the method of Lowry et al. [24], using bovine serum albumin as a standard, and phospholipid phosphorus was estimated by the Fiske-SubbaRow method [11]. The phospholipid to protein ratio calculated from these measurements is expressed on a mol/mol basis, using 119 000 for the molecular weight of the calcium-pump protein [25]. Sucrose gradient centrifugation studies showed that the vesicles were homogeneous with respect to the phospholipid to protein ratio [12].

Preparation of samples for ^{31}P-NMR studies

Dispersions of sarcoplasmic reticulum phospholipids, sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum. 70 mg sarcoplasmic reticulum phospholipids in $CHCl_3/CH_3OH$ (2 : 1) were dried under argon and resuspended in 4 ml of a medium containing 100 mM KCl, 10 mM $MgCl_2$, 2 mM Tris/maleate (pH 7.0) and 15% ^2H_2O. The suspension was centrifuged at 41 300 × *g* for 2 h to remove small vesicles and the pellet was resuspended in the same medium to a final concentration of 18 mg/ml. Sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum in sucrose solutions were diluted into a medium containing 100 mM KCl, 10 mM $MgCl_2$, 2 mM Tris/maleate (pH 7.0) and 15% ^2H_2O. They were then centrifuged and resuspended in the same medium to a final concentration of 10—25 mg protein/ml. All ^{31}P-NMR spectra were taken at 8 ± 1°C.

Oriented multilayers of sarcoplasmic reticulum phospholipids, sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum. A $CHCl_3/CH_3OH$ (2 : 1) solution of sarcoplasmic reticulum phospholipids was dried under argon and suspended in a medium containing 100 mM KCl, 10 mM $MgCl_2$, 2 mM Tris/maleate (pH 7.0). Oriented multilayers were prepared by centrifugation of the

suspension for 3 h at 100 000 × *g* in a Beckman SW 27 swinging bucket rotor onto a mylar disc. The multilayers were then dehydrated at 93% relative humidity, 5°C, for 24 h. Sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum in sucrose solutions (15—20 mg protein) were diluted into a medium containing 100 mM KCl, 10 mM $MgCl_2$, 2 mM Tris/maleate (pH 7.0). Oriented multilayers were prepared by centrifugation (see above) and dehydration at 93% relative humidity, 5°C, for 24 h. Several mylar discs were stacked on top of each other, and the resulting 'sandwich' was placed in a sample holder which allowed the 'sandwich' to be aligned at any desired angle with respect to the magnetic field. The sample holder contained a reservoir of saturated salt solution and was sealed to ensure a constant relative humidity. The sample holder allowed rapid (less than 5 s) changes of the alignment of the multilayer sandwich, which eliminated any significant humidity or temperature 'shock' to the specimens. These precautions were taken to preserve the functional and structural integrity of the oriented sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum samples [14,15].

Oriented multilayers of dipalmitoylphosphatidylcholine. Oriented multilayers of dipalmitoylphosphatidylcholine were prepared by gentle shearing of a small amount of partly hydrated phospholipid between two microscope cover slips. The resulting sample was incubated at 100% relative humidity, 50°C, for 24 h, and was shown to be well oriented by polarization microscopy. The sample was maintained at 100% relative humidity for the duration of the experiment.

^{31}*P-NMR measurements*

^{31}P-NMR spectra of the various sarcoplasmic reticulum preparations were taken at 145 MHz in a Bruker WH-360 spectrometer utilizing quadrature detection and operating in the Fourier transform mode. The spectrometer was equipped with a BST-100/700 temperature control accessory which maintained the temperature constant to within ±1°C. All spectra were taken at 8°C. The NMR sample tube (15 mm outer diameter) was aligned so that the long axis was parallel to the main magnetic field. The oriented 'sandwich' rested on a Teflon disc which was able to rotate about an axis perpendicular to the long axis. The normal to the plane of the Teflon disc could thus be aligned at any angle, θ, with respect to the magnetic field. The angle θ was fixed by resting the Teflon disc on the surface of an obliquely cut Teflon cylinder which was positioned in the bottom of the sample tube. Four different Teflon cylinders, which gave values for θ of 0°, 30°, 55° and 90°, respectively, were employed.

When it was not necessary to quantitate the intensity of the ^{31}P-NMR signals a 25 μs radiofrequency pulse (approx. 45° precession angle) and a 0.3 s delay between consecutive free induction decays were employed. These conditions gave the optimal signal-to-noise for the oriented membrane samples. When it was necessary to quantitate the intensity of the ^{31}P-NMR signals a 50 μs radiofrequency pulse (approx. 90° precession angle) and a delay time of 4 s were employed. The probe was tuned for each sample. A total sweep-width of 50 kHz was employed. No proton decoupling was used and no deuterium lock was employed for the oriented samples. The drift of the magnetic field or the transmitter frequency over the time course of the experiments (approx. 3 h per spectrum) was minimal.

^{31}P-NMR spectra for oriented dipalmitoylphosphatidylcholine multilayers were obtained on an instrument at the Biochemistry Department, Oxford University, which operated at 129 MHz for phosphorus. These spectra were taken at 50°C to ensure that the lipid was above the gel-liquid crystalline phase transition [26]. All other experimental details were similar to those mentioned above for spectra of the sarcoplasmic reticulum preparations taken on the Bruker WH-360 spectrometer.

Theory

The position of the ^{31}P-NMR signal (i.e., the chemical shift) of phospholipid molecules in oriented model membranes is determined by the anisotropy of the phosphorus chemical shift tensor [16—19]. If the rotation of the phosphate group around the bilayer normal is much faster than 10 μs, the position of the ^{31}P-NMR signal, ν, is given by the expression:

$$\nu = \nu_0 + 2/3\ \Delta\sigma H_0 \left(\frac{3\cos^2\theta - 1}{2}\right) \qquad (1)$$

where θ is the angle between the bilayer normal and the magnetic field, H_0. $\Delta\sigma$ is the average value of the anistropy of the chemical shift tensor, taken over all internal motions that are faster than 10 μs [18] *. $\nu_0 = \bar{\sigma}H_0$, where $\bar{\sigma}$ is the isotropic average value of the chemical shift tensor.

Fig. 1 shows the position of the ^{31}P-NMR signal from oriented dipalmitoylphosphatidylcholine membranes as a function of θ. ν is linearly dependent on $\frac{1}{2}(3\cos^2\theta - 1)$, and is equal to ν_0 for $\theta = 55°$ where $\frac{1}{2}(3\cos^2\theta - 1) = 0$ **. It can be concluded that the phosphate group is able to rapidly rotate (i.e., faster than 10 μs) around the bilayer normal. Using Eqn. 1, $\Delta\sigma$ for dipalmitoylphosphatidylcholine was calculated to be 46 ppm at 50°C (i.e., above the phase transition).

The interpretation of $\Delta\sigma$ in terms of the explicit details of the motion of the phosphate group is difficult. Although some models have been proposed [27], they are not necessarily unique [28]. However, since $\Delta\sigma$ is very sensitive to the details of the rapid internal motion of the phosphate group it is useful on a comparative basis.

The lineshape of the ^{31}P-NMR signal from phospholipid molecules in oriented model membranes is gaussian [18,29] and the width, Δ, is primarily determined by the dipolar interaction between the phosphorus nucleus and the protons on the two adjacent methylene groups. If the rotation of this segment of the phospholipid molecule around the bilayer normal is much faster than 10

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* Different symbols have previously been used for these parameters [19]. However, to allow comparison we have used symbols which are generally consistent with those used in a recent review [18]. The major difference is that we use θ (instead of δ) for the angle between the normal to the plane of the membrane and the magnetic field.

** In the analysis of the data ν_0 for the phospholipids was assumed to be the same as the position of the ^{31}P-NMR signal from 85% phosphoric acid. In aqueous solutions of membranes these two values do not exactly coincide, but the difference is small (less than 1 ppm). The spectra for the oriented membranes were not obtained in aqueous solutions, so the exact value for the correction is not known. However, since it is quite small it was ignored.

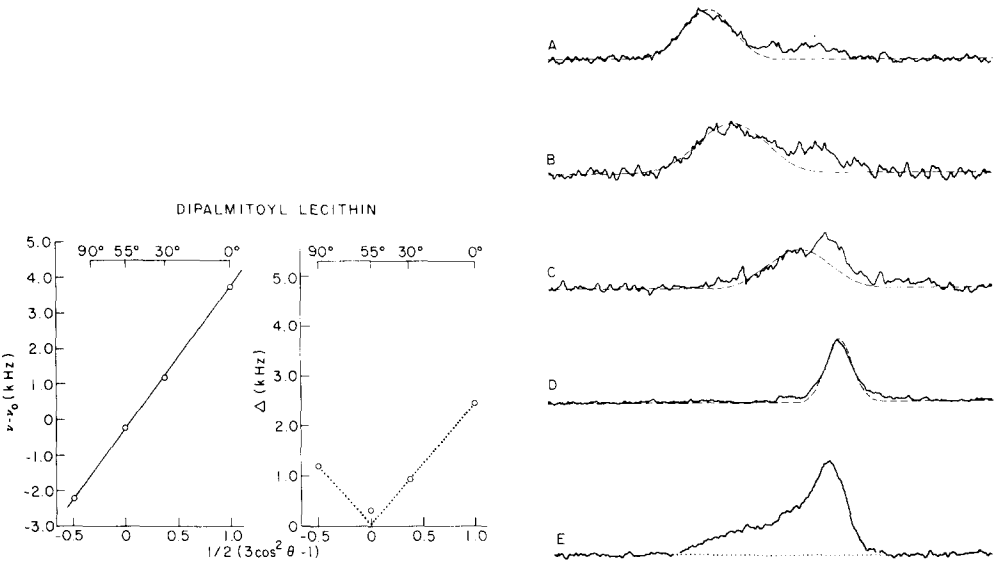

Fig. 1. The angular dependence of the position, ν (left), and width, Δ (right), of the ^{31}P-NMR signal from oriented multilayers formed from dipalmitoylphosphatidylcholine. θ is the angle between the magnetic field and the normal to the plane of the oriented bilayer membranes. The position of the signal was measured with respect to the position of an external 85% phosphoric acid sample, ν_0. ———, the best fit of the angular dependence of the position to Eqn. 1. . . . . ., the best fit of the angular dependence of the width to Eqn. 2. The membranes were oriented between glass discs by a shearing procedure (see Materials and Methods) as contrasted to the centrifugation and dehydration procedure employed for the sarcoplasmic reticulum (SR), reconstituted sarcoplasmic reticulum (RSR) and sarcoplasmic reticulum lipid samples. The spectra were taken at 129 MHz and the probe temperature was 50°C. ^{31}P-NMR spectra of all other samples were obtained at 145.7 MHz. The linewidth data are directly comparable to the linewidth data taken at the higher magnetic field strength (see Fig. 6). The relative position of the ^{31}P-NMR signal must be multiplied by 1.12 to compare it with the data taken at the higher field strength.

Fig. 2. The angular dependence of the ^{31}P-NMR signal from oriented sarcoplasmic reticulum membranes. The spectra shown in traces A—D were taken with membranes aligned at $\theta = 0°$, 30°, 55° and 90°, respectively, where θ is the angle between the magnetic field and the normal to the plane of the membrane. . . . . ., theoretical gaussian curves which were used to define the position and the width of the major component of the signals (see Results). Trace E shows the spectrum of an aqueous dispersion of sarcoplasmic reticulum membranes. The spectra were run at 145.7 MHz and the total sweep width is 20 kHz. Each spectrum was accumulated for approx. 2 h. The probe temperature was 8 ± 1°C.

μs, this dipolar contribution to Δ is given by the expression:

$$\Delta = \Delta_1\left(\frac{3\cos^2\theta - 1}{2}\right) \qquad (2)$$

where θ is the angle between the magnetic field and the bilayer normal [18, 29]. Δ_1 is the average value of the phosphorus-proton dipolar interaction, and is determined by all of the internal motions of the phosphodiester segment that are faster than 10 μs. From the ^{2}H order parameters for the two methylene groups adjacent to the phosphate group [27] it may be inferred that the dominant part of the dipolar broadening of the ^{31}P-NMR signal is due to the interaction with the glycerol methylene group.

Fig. 1 shows the width of the ^{31}P-NMR signal from oriented dipalmitoylphosphatidylcholine membranes as a function of θ. Δ is directly proportional to

the absolute value of $\frac{1}{2}(3\cos^2\theta - 1)$, and approaches zero when $\theta = 55°$. It can be concluded that, above the transition temperature, both methylene groups adjacent to the phosphate group can rapidly rotate (i.e., faster than 10 μs) around the bilayer normal. Using Eqn. 2, Δ_1 for dipalmitoylphosphatidylcholine was calculated to be 2500 Hz above the phase transition (50°C). As with $\Delta\sigma$, the interpretation of Δ_1 in terms of the explicit details of the motion of the phosphodiester segment is difficult. However, since Δ_1 is very sensitive to the details of these motions, it may also be used on a comparative basis.

The ^{31}P-NMR spectrum of a dispersion of unsonicated membranes is simply the superposition of the signals from all the different orientations [16]. For example, the dispersion spectrum in Fig. 2E is the superposition of the signals in Fig. 2A—D, and also the signals for membranes aligned at all the intermediate angles. The preferential weighting of the perpendicular orientation (i.e., θ = 90°) produces an apparent peak at the position of the signal from membranes aligned at $\theta = 90°$ and makes the spectrum asymmetric. $\Delta\sigma$ may be estimated from the separation between the two inflection points in the dispersion spectrum, although the dispersion spectrum provides no information on the direction of the axis of symmetry of the rapid internal motion of the phosphate group. In some cases Δ_1 can be estimated from the shape of the dispersion spectrum by a curve-fitting procedure [18]. However, this procedure is only valid when the membrane contains a single species of phospholipid, which is not the case for biological membranes.

Results

^{31}P-NMR spectra from oriented sarcoplasmic reticulum membranes are shown in Fig. 2. The spectrum for membranes aligned at $\theta = 90°$ is symmetrical and gaussian in shape, while the spectra from membranes aligned at the other angles are more complex. The dashed curves in Fig. 2 show the best-fit of these spectra to symmetrical gaussian curves. The component of the spectra which could be simulated by a symmetrical gaussian curve was designated as the 'major' component. It contained approx. 78% of the intensity in the spectrum. The remaining signal was designated the 'minor' component. The accuracy of this deconvolution procedure was clearly best when the two components were most widely separated (i.e., $\theta = 0°$) or when the minor component did not significantly contribute to the observed spectrum (i.e, $\theta = 90°$).

The position of the major component of the ^{31}P-NMR spectrum from oriented sarcoplasmic reticulum membranes is very sensitive to the alignment of the membranes with respect to the main magnetic field. When the membranes were rotated from the parallel ($\theta = 0°$) to the perpendicular ($\theta = 90°$) alignment the signal shifted approx. 6000 Hz upfield. The position of the major component (as determined from the fit of the gaussian curves to the spectra) is plotted as a function of $\frac{1}{2}(3\cos^2\theta - 1)$ in Fig. 3. To within the experimental error, the position of the major component obeys Eqn. 1. This implies that the phosphate group is able to rapidly rotate (i.e., faster than 10 μs) around the normal to the plane of the membrane (see Theory). Using Eq. 1, the average value of the chemical shift anistropy, $\Delta\sigma$, was calculated to be 43.2 ± 1.3 ppm. The error in $\Delta\sigma$ is the S.D. calculated from the least-squares fit to Eqn. 1.

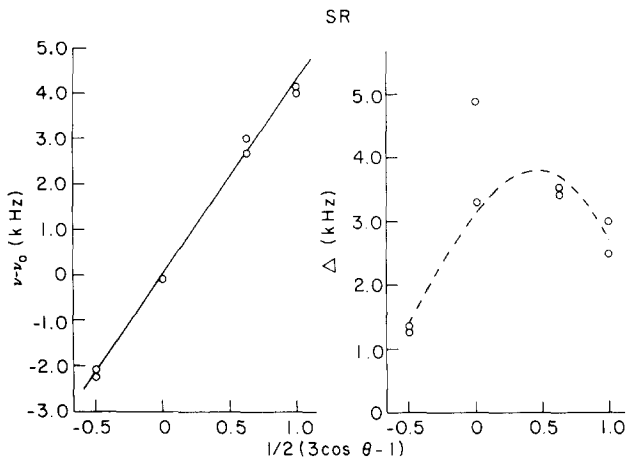

Fig. 3. The angular dependence of the position, ν (left) and the width, Δ (right) of the major component of the ^{31}P-NMR signal from oriented sarcoplasmic reticulum (SR) membranes (see Fig. 2). θ is the angle between the magnetic field and the normal to the plane of the membrane. The position of the signal was measured with respect to the position of an external phosphoric acid sample. ν_0. ———, the least-squares fit of the angular dependence of the position to Eqn. 1. - - - - - -, the best fit of the angular dependence of the linewidth to Eqn. 2, taking into account the mosaic spread of the sample (see Results).

The width of the major component of the ^{31}P-NMR spectrum from oriented sarcoplasmic reticulum membranes is plotted as a function of $\frac{1}{2}(3\cos^2\theta - 1)$ in Fig. 3. While the determination of the width is inaccurate for $\theta = 55°$, the accuracy is sufficient to conclude that the observed values do not obey Eqn. 2. This is probably due to the fact that all the membranes in the sarcoplasmic reticulum sample do not have exactly the same orientation (see Fig. 4). The distribution of orientations can be described by a gaussian curve, the width of which is defined as the mosaic spread of the sample. The effect of mosaic spread on the observed linewidth of the ^{31}P-NMR signal can be calculated by convoluting Eqn. 1 with this distribution function. Since the mosaic spread ($\Delta\theta$) is independently determined from X-ray diffraction experiments ($\Delta\theta = \pm 18°$), the only adjustable parameter in this procedure is Δ_1. The theoretical curve that best fits the linewidth data for oriented sarcoplasmic reticulum membranes is shown on the right-hand side of Fig. 3. The value of Δ_1 used to generate the curve is 1740 Hz. While the observed values of the linewidth for $\theta = 0°$, 30° and 90° agree reasonably well with the theoretical curve, the observed value at $\theta = 55°$ does not. However, given the inherent errors in the measured value of the linewidth at this angle (see Fig. 2), the overall agreement between the experimental data and the theoretical curve is considered reasonable.

The minor component of the ^{31}P-NMR signal from oriented sarcoplasmic reticulum membranes is best visualized in the signal for the $\theta = 0°$ alignment (Fig, 2, top) where the major component is shifted farthest downfield. The minor component probably arises from phospholipid molecules in the edge of the oriented membrane sandwich. The oriented sarcoplasmic reticulum membranes are made by centrifuging spherical single-walled vesicles (approx. 1250 Å in diameter (Saito, A., Hymel, L. and Fleischer, S., unpublished results)) onto a mylar disc. While the two membranes in the center of the flattened vesicle are

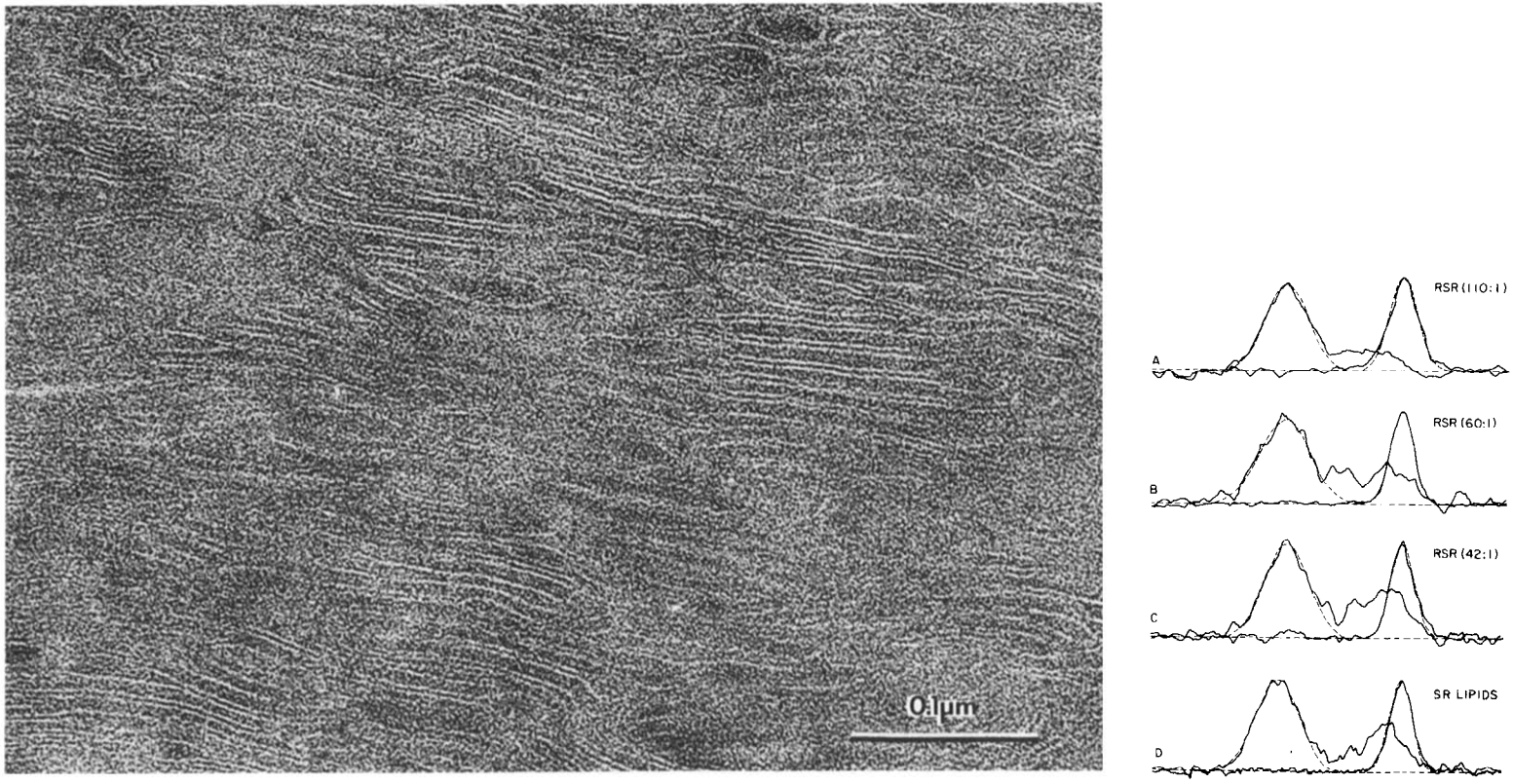

Fig. 4. Electron micrograph of oriented multilayers formed by centrifugation and partial dehydration of spherical sarcoplasmic reticulum vesicles (see Materials and Methods). The sarcoplasmic reticulum membrane is highly asymmetric, the outer face being much wider than the inner face. The alternating thick and thin bands observed in this micrograph reflect this asymmetry. The thick and thin bands are derived from the apposition of two outer and inner faces, respectively. Occasionally a loop with electron-dense contents is observed. This electron-dense material is assigned to the contents of heavy sarcoplasmic reticulum. Light sarcoplasmic reticulum is devoid of such contents. The fraction of sarcoplasmic reticulum shown here is a mixture of both types [31,32].

Fig. 5. The angular dependence of the ^{31}P-NMR signal from oriented bilayer membranes made from sarcoplasmic reticulum (SR) phospholipids and oriented reconstituted sarcoplasmic reticulum (RSR) membranes with various lipid to protein ratios. The spectra were taken with membrane aligned at $\theta = 0^{\circ}$ (left) and $\theta = 90^{\circ}$ (right), where θ is the angle between the magnetic field and the normal to the plane of the membrane. - - - - - -, theoretical gaussian curves which were used to define the position and the width of the major component of the signal (see Results). The spectra were run at 145.7 MHz and the total spectral width is 20 kHz. The probe temperature was $8 \pm 1^{\circ}$C. Each spectrum was accumulated for approx. 2 h.

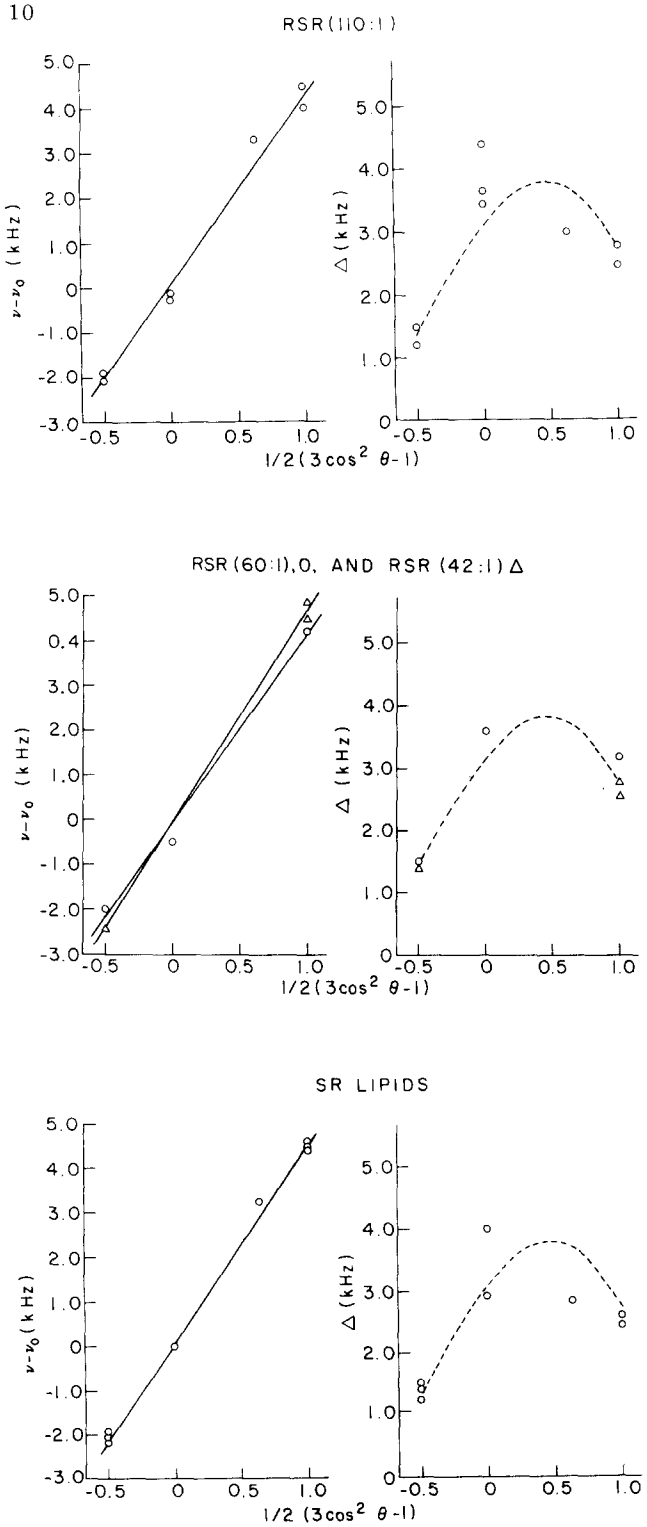

Fig. 6. The angular dependence of the position, ν (left), and the width, Δ (right), of the major component of the ^{31}P-NMR signal from oriented bilayer membranes formed from sarcoplasmic reticulum (SR) phospholipids and oriented reconstituted sarcoplasmic reticulum (RSR) membranes with various lipid to protein ratios. ———, least-squares fits of the data to Eqn. 1. - - - - - -, curve derived from Eqn. 2 assuming that $\Delta_1 = 1740$ Hz and $\Delta\theta = \pm 18^\circ$ (see Results).

coplanar with the mylar disc, the membrane at the edge forms a semicircular torus (see Fig. 4). The membrane in this torus will have a random distribution with respect to the magnetic field, regardless of the orientation of the mylar discs. It would be expected to display a ^{31}P-NMR spectrum similar in shape to the signal for the unoriented dispersion of sarcoplasmic reticulum membranes shown in Fig. 2E. While the poor signal-to-noise of the minor component precludes an accurate determination of the lineshape, it does have a distribution throughout the expected region of the spectrum. Also, the apparent intensity of the minor component (approx. 22% of the total intensity) is consistent with theoretical calculations based on the size of the vesicles and with determinations of the fraction of membrane in the torus from the electron micrographs.

The ^{31}P-NMR spectrum of an unoriented dispersion of sarcoplasmic reticulum membranes is shown in Fig. 2E. The spectrum is broad and asymmetric, with a pronounced low-field shoulder. The position of the inflection points on the low and the high-field sides of the dispersion spectrum in Fig. 2E correspond closely to the position of the signals for $\theta = 0°$ (Fig. 2A) and $\theta = 90°$ (Fig. 2D). We conclude (see Theory) that the value of $\Delta\sigma$ is very similar in the aqueous sarcoplasmic reticulum dispersions and in the partly hydrated sarcoplasmic reticulum multilayers. This implies that partial dehydration does not affect the motion of the polar head group region of the sarcoplasmic reticulum membrane.

^{31}P-NMR spectra from oriented membranes formed from sarcoplasmic reticulum phospholipids (Fig. 5D) and reconstituted sarcoplasmic reticulum with various phospholipid to protein ratios (Fig. 5A—C) were very similar to the spectra from oriented sarcoplasmic reticulum membranes (Fig. 2). The spectra were fitted to gaussian curves (dashed curves) and the position and width of the major components are plotted as a function of $\frac{1}{2}(3\cos^2\theta - 1)$ in Fig. 6. The position of the major component of all of the membrane preparations obeys Eqn. 1, and the calculated values of $\Delta\sigma$ are shown in Table I. Within the accuracy of the measurements, $\Delta\sigma$ is the same for the phospholipid bilayer membranes, sarcoplasmic reticulum membranes and reconstituted sarcoplasmic reticulum membranes.

The angular dependence of the width of the major component of ^{31}P-NMR signals from oriented reconstituted sarcoplasmic reticulum membranes and bilayer membranes formed from sarcoplasmic reticulum phospholipids agrees qualitatively with the modified form of Eqn. 2 that incorporates the effects of mosaic spread. The dashed curves in Fig. 6 were calculated assuming the same value of Δ_1 (1740 Hz) and $\Delta\theta$ (±18°) found for oriented sarcoplasmic reticulum membranes. As discussed above, the observed linewidths of samples aligned at $\theta = 30°$ and $\theta = 55°$ contain substantial errors, and they were not used in the quantitative fit to the data. The reasonable quantitative fit of the theoretical curve to the observed linewidths for $\theta = 0°$ and $\theta = 90°$ implies that Δ_1 is very similar for sarcoplasmic reticulum membranes, the various reconstituted sarcoplasmic reticulum membranes and bilayer membranes formed from sarcoplasmic reticulum phospholipids.

A series of ^{31}P-NMR spectra of oriented reconstituted sarcoplasmic reticulum (110 : 1) and reconstituted sarcoplasmic reticulum (60 : 1) membranes and oriented bilayer membranes made from sarcoplasmic reticulum phospholipids

TABLE I

$\Delta\sigma$ FOR THE MAJOR COMPONENT OF THE ^{31}P-NMR SPECTRA FROM VARIOUS SARCOPLASMIC RETICULUM PREPARATIONS

$\Delta\sigma$, the average value of the phosphorus chemical shift anisotropy, was determined from the least-squares fit of Eqn. 1 to the data shown in Figs. 3 and 6. The error in $\Delta\sigma$ is the S.D. calculated from the least-squares analysis.

	$\Delta\sigma$ (ppm)
Sarcoplasmic reticulum lipids	45.5 ± 1.1
Sarcoplasmic reticulum	43.2 ± 1.3
Reconstituted sarcoplasmic reticulum	
110 : 1	44.5 ± 2.1
60 : 1	43.8 ± 4.4 *
42 : 1	49.2 ± 2.4 *

* The relatively large S.D. of these two values arises from the fact that they were calculated from only three experimental points (see Fig. 6).

were taken with a 4 s delay time between consecutive free induction decays. The T_1 relaxation time of the ^{31}P-NMR signal from oriented reconstituted sarcoplasmic reticulum (60 : 1) membranes aligned at $\theta = 0°$ was determined to be 1.1 ± 0.1 s by the inversion recovery method. Assuming the T_1 relaxation times for the oriented reconstituted sarcoplasmic reticulum (110 : 1) and the oriented sarcoplasmic reticulum phospholipid membranes are not significantly longer than this value, the 4 s delay time ensured that the observed intensities of the ^{31}P-NMR signals were directly proportional to the number of phospholipid molecules contributing to the signal. The relative intensities of the ^{31}P-NMR signals and the total amount of lipid phosphorus in each sample are shown in Table II. The intensity of the signal from oriented sarcoplasmic reticulum phospholipid membranes was arbitrarily scaled to a value of 1.0. The

TABLE II

THE FRACTION OF THE TOTAL PHOSPHOLIPID MOLECULES DETECTED IN THE OBSERVED ^{31}P-NMR SIGNAL FROM ORIENTED RECONSTITUTED SARCOPLASMIC RETICULUM MEMBRANES

The relative intensities of the ^{31}P-NMR signals were determined under non-saturating conditions (see Materials and Methods and Results) from oriented membranes aligned at $\theta = 90°$. A and B show the results of two separate sets of experiments. SR, sarcoplasmic reticulum; RSR, reconstituted sarcoplasmic reticulum.

	Relative ^{31}P-NMR signal intensity	Total lipid phosphorus (μg)	Fraction of phospholipid molecules detected
(A) Sarcoplasmic reticulum lipids	1.00	465	1.00
RSR			
110 : 1	0.675	352	0.94
60 : 1	0.576	287	0.90
(B) Sarcoplasmic reticulum lipids	1.00	497	1.00
RSR			
110 : 1	0.67	310	1.08
60 : 1	0.67	318	1.05

observed intensity of the ^{31}P-NMR signals from reconstituted sarcoplasmic reticulum membranes was calibrated by assuming that the ^{31}P-NMR signal from oriented sarcoplasmic reticulum phospholipid membranes arose from all the phospholipid molecules in the sample. The fraction of phospholipid molecules contributing to the observed signal from oriented reconstituted sarcoplasmic reticulum membranes was calculated by comparing the amount of lipid phosphorus contributing to the ^{31}P-NMR signal with the total amount of lipid phosphorus in the sample. These results are shown in the right-hand column of Table II. The experiment was performed in duplicate and the second set of results is shown in the bottom half of Table II. It may be concluded from Table II that the ^{31}P-NMR spectra are detecting essentially all (100 ± 7%) of the phospholipid molecules in the oriented membranes samples.

Discussion

The major conclusion of the ^{31}P-NMR studies described here is that the motional characteristics of the phospholipid molecules in the normal and reconstituted sarcoplasmic reticulum membrane are very similar to that of phospholipid molecules in model bilayer membranes (liposomes) formed from sarcoplasmic reticulum phospholipids. That is to say, the presence of the calcium-pump protein in the membrane does not perturb the motion of the polar head group region of the phospholipid molecules.

The ^{31}P-NMR studies were performed on partially hydrated oriented multilayers. However, since the average value of the phosphorus chemical shift anistropy, $\Delta\sigma$, was the same for oriented sarcoplasmic reticulum membranes and fully hydrated dispersions of sarcoplasmic reticulum, the partial dehydration procedure did not affect the motion of the phosphate group. Also, oriented sarcoplasmic reticulum multilayers [20] and reconstituted sarcoplasmic reticulum multilayers which are resuspended in aqueous solution [14] still retain calcium-pumping activity. The conclusion reached above is thus relevant to the functional, intact, sarcoplasmic reticulum membrane.

^{31}P-NMR spectra from oriented sarcoplasmic reticulum multilayers were resolved into two components. The major component, which contained approx. 70% of the total intensity, was sensitive to the alignment of the sample with respect to the magnetic field and is interpreted to arise from the flattened portion of the oriented vesicles (see Fig. 4). The minor component, containing approx. 30% of the total intensity, was relatively insensitive to the alignment of the sample and is interpreted to arise from the torous region at the edge of the flattened vesicles. The lineshape of the minor component is consistent with a rapid lateral diffusion of phospholipid molecules around the highly curved torous region (McLaughlin, A.C., unpublished results).

The position of the major component of the ^{31}P-NMR spectra from oriented sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum membranes and oriented bilayer membranes formed from sarcoplasmic reticulum phospholipids obeys Eqn. 1. When the mosaic spread of the samples is taken into account, the width of the major component is consistent with Eqn. 2. The average value of the phosphorus chemical shift anistropy, $\Delta\sigma$, and the average value of the dipolar interaction, Δ_1, are the same (to within the experimental

error) for sarcoplasmic reticulum, reconstituted sarcoplasmic reticulum (100 : 1), (60 : 1), and (42 : 1) membranes and bilayer membranes formed from sarcoplasmic reticulum phospholipids. We can thus draw two important conclusions about the motion of the phospholipid molecules that give rise to major component of the ^{31}P-NMR spectra from oriented sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum membranes. First, the phosphate group and the two adjacent methylene groups can rotate rapidly around the normal to the plane of the membrane (i.e., $\tau_R << 10$ μs). Second, the restricted internal motion of the phosphate group and the glycerol CH_2OP group * is very similar to that found in model phospholipid membranes.

In order to quantitate these conclusions it is necessary to determine the fraction of phospholipid molecules that contribute to the observed ^{31}P-NMR signals. Calibration experiments showed that the total intensity in the ^{31}P-NMR spectra from oriented reconstituted sarcoplasmic reticulum (110 : 1) and (60 : 1) membranes accounted for essentially all (100 ± 7%) of the phospholipid molecules in the membranes. These measurements imply that the observed intensity of the major component accounts for the same fraction of phospholipid molecules in the oriented regions of the membrane. We can thus extend the two conclusions in the previous paragraph to all the phospholipid molecules in the oriented regions of these reconstituted sarcoplasmic reticulum multilayers.

The term boundary lipid [33] or lipid annulus [34] have been used to discriminate between phospholipid molecules immediately surrounding the protein and the remainder of the phospholipid molecules in the membrane. It has been estimated [33] that approx. 30 mol of phospholipid surround 1 mol of calcium-pump protein. From calculations of the dimensions of calcium-pump protein obtained from recent X-ray and neutron diffraction experiments, we also estimate the number to be at least 30 (Herbette, L., et al., unpublished results). Using this estimate, the 'boundary-layer' of lipid would constitute 27% of the phospholipid in reconstituted sarcoplasmic reticulum (110 : 1) membranes, and 50% of the phospholipid in reconstituted sarcoplasmic reticulum (60 : 1) membranes. Yet, in these membranes essentially all (100 ± 7%) of the polar head groups have the same $\Delta\sigma$ and Δ_1 as found in model bilayer membranes, and are free to rotate around the normal to the plane of the membrane. We thus conclude that the motion of the phosphate group and the glycerol CH_2OP group of 'boundary-layer' phospholipid molecules is not constrained by the adjacent protein molecule on a time scale of 10 μs or shorter, and is the same as that found in phospholipid molecules in the 'bilayer' regions of the membrane.

Recent experiments using ^{2}H-NMR to study the motion of the hydrocarbon region of phospholipids in reconstituted sarcoplasmic reticulum membranes [13,35,36] or other membranes [37,38] also do not detect a separate domain of immobilized lipid. However, EPR results indicate that a significant fraction of phospholipid molecules in reconstituted sarcoplasmic reticulum and other membranes could be immobilized [33,34,39]. Recent studies by McIntyre et al. [40] were carried out to see whether immobilized phospholipid could be

* CH_2OP designates the methylene group adjacent to the phosphate group.

detected in reconstituted sarcoplasmic reticulum membrane vesicles prepared by the procedures used in the present study. A small amount of immobilized spin-labelled phosphatidylcholine (spin label on C- 16 of the *sn*-2-acyl stearic acid) could be detected; however, the amount appears insufficient to constitute a boundary layer or annulus of immobilized phospholipid surrounding the calcium-pump protein. Thus, for sarcoplasmic reticulum, the EPR results [40] are in qualitative agreement with the ^{31}P-NMR results reported here and with the ^{2}H-NMR results [35,36] that there is insufficient immobilized phospholipid to constitute a complete boundary layer.

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