Rapid Lateral Diffusion of Phospholipids in Rabbit Sarcoplasmic Reticulum

( membrane/lipids/model/spin label/NMR)

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ABSTRACT Phospholipid spin labels incorporated in the sarcoplasmic reticulum from rabbit-skeletal muscle undergo rapid lateral diffusion within the plane of the membrane. The diffusion constant, $D$, is $6 \times 10^{-8}$ cm$^2$/sec at $37^\circ$. With this diffusion constant, a phospholipid molecule can diffuse a distance of the order of 5000 nm in 1 sec.

Phospholipid diffusion in model membranes was first studied by utilization of nuclear magnetic resonance (NMR) and phosphatidylcholine vesicles containing about 1% of spin-label I. The rate of lateral diffusion was so rapid that only a

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\begin{align*}
\text{CH}_3&-\text{CH}_2 & O & \text{C} & \text{O} & \text{CH}_2 & \text{CH}_2 & \text{N} & (\text{CH}_3) \\
& & & & & \text{H} & \text{C} & \text{O} & \text{N} & (\text{CH}_3) \\
& & & & & \text{H} & \text{C} & \text{O} & \text{CH}_2 & \text{CH}_2 & \text{N} & (\text{CH}_3) \\
\end{align*}
\]

lower limit could be placed on the diffusion rate by these techniques (1).§ By another method, a diffusion constant, $D = 1.8 \times 10^{-8}$ cm$^2$/sec, was obtained for I in phosphatidylcholine multilayers at 25° (2). Sackmann and Trauble measured a diffusion constant, $D = 1 \times 10^{-8}$ cm$^2$/sec, for an androstan spin label in phosphatidylcholine model membranes at 40° by an entirely different method of analysis (3). Rapid lateral diffusion clearly occurs in model membranes, but it is by no means obvious that components of natural membranes also enjoy this freedom of motion.

Here we describe a method for measuring phospholipid diffusion in natural membranes. In particular, we report that a spin-label analog of phosphatidylcholine (II) diffuses at nearly the same rate in sarcoplasmic reticulum as in lipid model membranes. We have used a resonance lineshape analysis, similar to that of Sackmann and Trauble, (to measure the diffusion rates of II in sarcoplasmic reticulum of rabbit-skeletal muscle, in egg lecithin, and in the lipids extracted from sarcoplasmic reticulum. The spectral characteristics of this label are suitable for measurement of large rates of lateral diffusion in membranes where the hydrocarbon chains are highly flexible. Sarcoplasmic reticulum of rabbit-skeletal muscle was selected for this study because earlier investigations of this tissue indicated that a large fraction of the membrane lipid has very flexible fatty-acid chains (4, 5). Phosphatidylcholine is the major phospholipid of sarcoplasmic reticulum (6).

The sarcoplasmic reticulum controls muscle contraction by sequestering calcium from the myoplasm (7). After a homogenization procedure and differential centrifugation, these membranes can be obtained as closed vesicles of 60–300 nm diameter (7), which retain the ability to pump calcium. We found that phospholipid II could be incorporated into these membranes by simply mixing a sonicated dispersion of II with the membrane vesicles. The in vitro assay for calcium uptake provides a convenient measure of the integrity of spin-labeled membranes (8).

Incorporation of spin-label II into sarcoplasmic reticulum

When phospholipid II is dispersed in buffer, the unpaired electrons interact strongly, giving rise to a single broad peak in the resonance spectrum (Fig. 1a) After addition of sarcoplasmic-reticulum vesicles to this dispersion, three sharp lines appear superimposed upon the initial broad-absorption peak (Fig. 1b, c). The mixture of dispersed phospholipid and sarcoplasmic-reticulum vesicles was resolved by centrifugation into liposome ($\rho < 1.1$) and sarcoplasmic membrane fractions (Table 1). The resonance spectrum of the liposome fraction consisted of a single broad-absorption peak. After extraction of the lipid from the sarcoplasmic-reticulum fraction, and thin-layer chromatography, at least 95% of the spin label

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§ An analogous unpublished experiment has been done by H. M. McConnell, P. Devaux, and A. A. Bothner-By, who found a strong broadening of all the N-methyl signals of endogeneous phosphatidylethanolamine in sarcoplasmic reticulum containing about 3% of a (7, 6) phospholipid spin label analogous to the (1, 14) label identified in the text as II. Label II at a comparable concentration does not broaden this N-CH$_3$ signal, since the oxazolidine ring is too far removed from the headgroups. This experiment also shows that lateral diffusion in this membrane is rapid, but does not yield a numerical value for the diffusion constant.
migrated as phosphatidylcholine. From this result, together with the resonance spectrum of II in the sarcoplasmic-recticulum fraction (Fig. 3), we conclude that the spectral changes seen in Fig. 1b, c represent the incorporation of some of the intact spin label into the lipid phase of the sarcoplasmic membranes. Centrifugation to equilibrium in a linear sucrose-density gradient separates the sarcoplasmic vesicles into fractions of higher and lower density (6). The ratio of spin label to lipid phosphorus was nearly constant when the spin-labeled sarcoplasmic-recticulum vesicles were analyzed in this manner. From this result, together with the observation that the spectra of spin-labeled vesicle preparations do not change under conditions that promote the transfer of label between vesicles (see below), we conclude that the label is uniformly distributed among the membrane vesicles. Label II is stable in sarcoplasmic-recticulum vesicles (<6% loss of spin-label paramagnetism) for at least 1 week at 0–2°, or for at least 15 min at temperatures up to 70°. The resonance spectra showed no hysteresis effects; low-temperature spectra were unchanged after incubation at 70°. We conclude that the concentration of II is constant throughout the lipid phase of these membranes. The lineshape analysis provides a critical test of this assumption.

A satisfactory rate of incorporation was attained by simply mixing the membrane vesicles with a sonicated dispersion of II. Table 1 shows that the amount of II transferred is roughly proportional to the concentrations of sarcoplasmic-recticulum vesicles and of II. The tendency of II to reaggregate after sonication presented some technical problems. A small amount of phosphatic acid included in the spin-label dispersion (see legend to Table 1) retarded aggregation. Even so, the spin-label dispersion quickly turned cloudy at 4°. Aggregation was particularly severe in the presence of sarcoplasmic-recticulum vesicles. We found that complete resolution of membranes from unincorporated label could only be achieved if the mixing was done above 35°.

Phospholipid II, up to a concentration of 2.5% of the total phospholipid, does not diminish the rate at which membrane vesicles accumulate calcium (Fig. 2a). The inhibition associated with higher levels of incorporation could be a consequence of alteration of the membrane composition, or it might result from the release of a small amount of fatty acid from hydrolysis of the exogenous phospholipid [free-fatty acids inhibit calcium pumping by these vesicles (8)]. A comparable loss of activity is observed after sarcoplasmic-recticulum vesicles are incubated with a sonicated dispersion of egg lecithin instead of II. The calcium pumping activity of these vesicles withstood a 15-min exposure to temperatures as high as 50–55° (Fig. 2b) in the presence of 30% sucrose. In the absence of sucrose, the activity declines rapidly at 40° and the vesicles

<table>
<thead>
<tr>
<th>Table 1. Incorporation of II into sarcoplasmic reticulum</th>
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<tr>
<td>Initial conditions</td>
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<tr>
<td>Sarcoplasmic reticulum, µmol phospholipid</td>
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<tr>
<td>------------------------------------------------------</td>
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<tr>
<td>1.0 0.80</td>
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<tr>
<td>2.0 1.6</td>
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<tr>
<td>3.0 1.6</td>
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<tr>
<td>4.0 2.4</td>
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<tr>
<td>5.0 3.2</td>
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<tr>
<td>6.0 6.4</td>
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<tr>
<td>7.0 9.6</td>
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Sarcoplasmic-recticulum vesicles and a sonicated dispersion of spin-label II were mixed, in a total volume of 0.6 ml containing 10% sucrose (Mann special enzyme grade), 0.1 M KCl, 0.05 M histidine-HCl (pH 7.0). After incubation for 1 hr at 40°, the mixture was applied to a step gradient consisting of 40% (w/v) sucrose, 0.1 M KCl, 0.05 M histidine-HCl (pH 7.0)(0.2 ml) and 20% sucrose, 0.1 M KCl, 0.05 M histidine-HCl, (pH 7.0)(3.7 ml), then centrifuged for 40 min at 300,000 × g at 30° in an SW56 rotor of a Beckman L265B ultracentrifuge. The sarcoplasmic-recticulum vesicles were recovered in nearly quantitative yield (about 90% of lipid phosphorus) at the 20–40% sucrose boundary. Liposomes that remain at the top of the gradient, and most of the 20% sucrose, were removed by aspiration, leaving the sarcoplasmic-recticulum band in 0.4 ml (measured gravimetrically) of buffer containing 30% sucrose. These samples were homogenized by several passages through a small-bore Pasteur pipet, then transferred to 50-µl capillary tubes (24) for resonance measurements. Spectra were recorded at 9.0 GHz on a Varian E-12 EPR spectrometer equipped with a temperature control accessory and interfaced to an IBM 360/50 computer (24). Spin-label incorporation was determined by twice integrating the spectra.

Sarcoplasmic-recticulum vesicles were prepared from rabbit skeletal muscle, essentially as described by Maritono and Feretos (22), except that the isolation procedure was done with argon-saturated buffers in an inert atmosphere. This precaution enabled us to obtain resonance spectra at elevated temperatures (45° ≤ T ≤ 70°), probably by preventing the formation of lipid peroxidation products that react with nitroxides. The pellet after the centrifugation was taken up in buffer containing 25% sucrose. Membrane vesicles prepared in this way retained >95% of their calcium-pumping activity for at least 1 week when stored at 0–2°.

Label II was prepared by the acylation of egg yolk lecithin with spin-label fatty-acid anhydride, by the procedure of Hubbell and McConnell (25). A sonic dispersion was prepared as follows: 6 µmol of II and 0.024 µmol of phosphatidic acid derived from egg lecithin (a gift of Dr. R. Kornberg) were dried under reduced pressure, then 4 ml of buffer [0.01 M Tris-HCl (pH 7.5)–0.02 M NaCl; saturated with argon] was added. Sonication was for 60 min, in a bath maintained at 25°, with a Branson model W18SD sonifier (power output, 65 W). 85 ± 5% of the initial spin-label paramagnetism survived this sonication procedure. Titanium particles from the probe tip, and a small amount of undispersed lipid, were removed by centrifugation for 30 min at 150,000 × g at 25°.

**Fig. 1.** The resonance spectrum of II changes when a sonic dispersion of II is mixed with rabbit-sarcoplasmic reticulum. Rabbit-sarcoplasmic reticulum vesicles (0.32 µmol of lipid phosphorus) were added to 0.12 ml of buffer (0.1 M NaCl–0.05 M histidine-HCl, pH 7) containing 0.04 µmol of a sonicated dispersion of label II. The resonance spectra were recorded at 37° (a) before mixing (——), (b) after 30 min at 37° (——), (c) after 60 min at 37° (——).
clump at high temperatures. A high concentration of spin-label phospholipid (5.4%) renders the activity slightly more susceptible to thermal inactivation (Fig. 2b).

Other examples of permissible variations in the composition of membranes include the reports of Luck (9) and Mindich (10) that culture conditions influence the ratio of lipid to protein in membranes of mutant strains of Neospora crassa and Bacillus subtilis, respectively. Several studies (11-13) have shown that fatty-acid auxotrophs of Escherichia coli tolerate a wide variety of unnatural acyl chains, even bromine-substituted chains, in their phospholipid. The oxazolidine ring of II should perturb the bilayer structure of a membrane minimally when it is located near the methyl terminus, where the chains are packed less tightly (14).

In one experiment (Table 1, sample 6) unincorporated spin label was recovered from the liposome fraction after centrifugation. This material was analyzed by gas-liquid chromatography and found to contain less than 1% contamination by rabbit lipid, as judged by the concentrations of C18:0, C18:1, and C18:2 present (15). These data show that less than one molecule of sarcoplasmic-reticulum phospholipid appears in the liposome fraction for every 50 molecules of II transferred to the sarcoplasmic reticulum. Clearly the transfer is not due to a one-to-one exchange protein, as described by Wirtz and Zilversmit (16).

**Several factors influence resonance line shapes**

The paramagnetic resonance spectrum of a spin-label phospholipid contains information about translational motions of the molecule, because these motions influence the electron-spin-electron-spin interactions between pairs of radicals. In order to make diffusion measurements from resonance spectra, one must first evaluate the contributions of the several factors that influence resonance line shapes. The following discussion outlines the method of analysis that we have used. A detailed description of the spectral analysis will be given elsewhere (manuscript in preparation).

The resonance lines become broadened when the mole fraction of label II exceeds about 0.01 in pure phospholipid bilayers and in sarcoplasmic-reticulum vesicles (see Fig. 3b). Two types of interactions between electron spins contribute to this broadening: (magnetic) dipole–dipole and (coulombic) spin-exchange interactions. The spin-exchange interaction (analogous to chemical exchange effects in NMR) requires that the nitroxide groups be virtually in van der Waals contact and, therefore, increases as the diffusion rate increases. The dipole–dipole interaction is relatively long range, and tends to be averaged out in the presence of rapid diffusion. The concentration-dependent line broadening can be used to measure the rate of diffusion once the type of interaction (dipole–dipole or spin exchange) is known. The relative contributions of these two types of interaction can be estimated from a plot of linewidth against temperature (see Fig. 3a). At low temperatures (T < 20°C) the dipole–dipole contribution to the linewidth is dominant, and decreases with increasing temperature. We have not used the dipole–dipole interaction to measure diffusion rates, because there is evidence for a

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**Table 2. Diffusion constants (cm²/sec × 10⁶)**

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<tr>
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<th>Egg phosphatidyl choline vesicles</th>
<th>Sarcoplasmic reticulum vesicles</th>
<th>Lipid extract</th>
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<tr>
<td>°C</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>20 ± 1.5</td>
<td>12 ± 1.5</td>
<td>10 ± 3</td>
</tr>
<tr>
<td>60</td>
<td>17 ± 1.5</td>
<td>9.5 ± 1.5</td>
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</tr>
<tr>
<td>50</td>
<td>15 ± 1.5</td>
<td>7.5 ± 1.5</td>
<td></td>
</tr>
<tr>
<td>40 (measured)</td>
<td>15 ± 2</td>
<td>7.5 ± 2</td>
<td></td>
</tr>
<tr>
<td>(extrapolated)</td>
<td>(12 ± 2)</td>
<td>6 ± 2</td>
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</table>

These values were calculated from the spin-label collision rates by the relation given in the text, using a value of 60 Å² for a². The values given in parenthesis at 40° were obtained by extrapolation of the values at higher temperatures. The stated error limits reflect the uncertainty in the values of k obtained by comparison of experimental and calculated spectra. The larger uncertainty for the lipid extract is due to the larger intrinsic linewidths, which may arise from domains of differing lipid composition. All of these values are subject to an uncertainty of about 2- to 3-fold, which arises from the assumptions introduced in converting collision rates to diffusion constants.

* The ratio of phosphatidylcholine–cholesterol was 4:1.

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* We have observed the transfer of II from sarcoplasmic-reticulum vesicles containing 5% II to unlabeled sarcoplasmic vesicles by observing changes in the shape of the resonance spectrum. The label did not transfer from sarcoplasmic-reticulum vesicles to egg-lecithin vesicles under the same conditions. Homogeneous phospholipid exchange protein specific for phosphatidylcholine (generously provided by Dr. Wirtz) stimulated the transfer of II from labeled sarcoplasmic-reticulum vesicles to unlabeled sarcoplasmic-reticulum vesicles, as well as to egg-lecithin vesicles. In contrast, the phospholipid exchange protein did not promote the transfer of label from the sonic dispersion of pure II to sarcoplasmic-reticulum vesicles, although it stimulated the transfer of radioactive-dipalmityl lecithin or II from egg-lecithin vesicles to the sarcoplasmic-reticulum vesicles by at least 10-fold under the same conditions.
phase separation of II below 20°. At higher temperatures \((T > 40°)\) the spin-exchange contribution to the linewidth dominates. Our estimates of lateral diffusion in sarcoplasmic reticulum vesicles are limited to the high-temperature region, which fortunately borders the physiological temperature.

Concentration-broadened spectra were calculated from the intrinsic spectrum by the use of modified Bloch equations. The Bloch equations were modified by addition of a single new parameter, corresponding either to an isotropic broadening of the resonance lines (dipole–dipole interaction), or to a given frequency of spin exchange. Dipole broadening and spin-exchange frequencies were measured by comparison of the calculated and observed spectra.

**Calculation of diffusion constants from high-temperature spectra**

The spin-label collision frequency can be calculated from spin-exchange broadening of the resonance spectra in the temperature range 50–70°, neglecting the dipole–dipole interaction. Spectra of II in the concentration range 0.01–0.025 can be accounted for equally well by assuming either dipole–dipole or spin-exchange contributions to the linewidth. However, for concentrations >0.03 the spin-exchange calculation clearly gives a better representation of the observed spectra, as we expect from the plot in Fig. 3. Fig. 4 shows the calculated collision rates for various concentrations of II at 70, 60, and 50° in sarcoplasmic reticulum vesicles. The linear dependence of collision rate of concentration shows that the rate of phospholipid diffusion is independent of spin-label concentration. The slopes of the corresponding straight lines increase as a function of the temperature, supporting the validity of these calculated collision rates in the 70–50° temperature range.

At 40°, the calculated collision rates are not very different from those calculated at 50°. The agreement between experimental spectra at 40° and the spectra calculated using only a spin-exchange interaction is less satisfactory for the higher concentrations of II. This result is consistent with the data of Fig. 3, which show roughly equal contributions of dipole broadening and spin-exchange broadening at 40°.

Table 2 lists some diffusion constants obtained from the high-temperature (40–70°) spectra. In order to calculate these diffusion constants from collision-rate constants, one must introduce assumptions about the organization of lipids in the membrane. For a hexagonal lattice of cylindrical lipids, one can derive the relation

\[
D = k a^2/12C \quad \text{(valid for } C \ll 1)\]

where \(C\) is the mole fraction of phospholipid II, \(k\) is the collision-rate constant, and \(a\) is the distance between nearest neighbors (the area per molecule, \(\sqrt{3}/2 a^2\), is 0.58–0.65 nm² for lipid bilayers (17]). Better values for diffusion constants at 40° were obtained by extrapolation of the diffusion constants determined at 70, 60, and 50°.

**Spectra at lower temperatures show evidence of a phase separation**

The spin–spin contribution to the resonance linewidth increases below 20° because the larger dipole interaction more than compensates for the smaller spin-exchange interaction. This effect is evident in the linewidth plots of Fig. 3a for the egg lecithin, egg lecithin–cholesterol, and sarcoplasmic-
reticulum systems. For the first two systems, there is good agreement between the spectra observed below 20° and those calculated by use of a single dipolar broadening parameter acting equally on all three lines. Moreover, the dipole broadening parameters increase approximately linearly with spin-label concentration. In contrast, the sarcoplasmic-reticulum spectra at higher concentrations of II below 20° are not as well represented by the simple dipole-dipole calculation, and the dipole-broadening parameter reaches a plateau at a spin-label concentration of about 0.02. This behavior suggests that a phase of pure II, or at least concentrated II, separates out below 20° (The fraction of spin label present in concentrated patches should give a resonance spectrum similar to that in Fig. 1a; thus, it should have little influence on the linewidth of the three-line spectrum of the remainder of the spin label). Two additional observations support this interpretation: the spectra of II in sarcoplasmic reticulum below 20° deviate from the calculated spectra in the manner predicted, and resonance spectra of the neat phospholipid II in water show clear evidence of a phase transition centered at 15°.

Discussion

At 40°, the diffusion constants of II in egg lecithin–cholesterol 4:1, and a lipid extract from sarcoplasmic reticulum are \( D \approx 10^{-7} \text{ cm}^2/\text{sec} \). Evidently the diffusion constant of a phospholipid in a model membrane is not strongly dependent on the composition of the membrane. In sarcoplasmic-reticulum vesicles, the diffusion constant of II at 40° is \( D \approx 0.6 \times 10^{-7} \text{ cm}^2/\text{sec} \). The exact values of these diffusion constants are subject to some uncertainty, particularly at lower temperatures, but the qualitative result is clear: phospholipids diffuse at nearly the same rate in the sarcoplasmic reticulum as in phospholipid-model membranes. These diffusion constants correspond to a high degree of freedom for lateral motion. For comparison, the diffusion constant of ribonuclease (molecular weight about 14,000) in water at 20° is \( D = 1 \times 10^{-4} \text{ cm}^2/\text{sec} \) (18).

We expect to find much slower rates of phospholipid diffusion in some biological membranes. For instance, the hydrocarbon chains in the purple membranes of Halobacterium halobium are extended and rigid, as judged by x-ray diffraction (19) and spin-label studies (W. L. Hubbell and W. D. Stoeckenius, personal communication). Phospholipid-diffusion constants in this membrane must be smaller by several orders of magnitude.

The evidence for lateral mobility of other membrane components has been reviewed recently (11). Lateral diffusion of larger membrane components (glycolipids, glycoproteins, and proteins that penetrate the lipid phase) may also occur at a rapid rate, since diffusion depends weakly on molecular weight. For the case of a globular protein in water, the diffusion constant varies as the cube root of the protein’s molecular weight. Thus, it is not surprising that the lateral-diffusion rates estimated for surface antigens in mouse-human cell hybrids (21) are compatible with the diffusion constants that we measure for phospholipids.

We are indebted to Dr. Betty G. McFarland for help in the synthesis of spin-label II, for help with the computer programs, and for the initial observation that some phospholipid spin labels are readily incorporated into sarcoplasmic reticulum. We are also indebted to Dr. Roger Kornberg for advice concerning the sarcoplasmic-reticulum preparation and for a gift of spin-label I. This work was supported by NIH NB-08058-04. CJ S was a NSF postdoctoral fellow from 1970–71. Unpublished work cited was supported by the NMR Facility for Biomedical Studies, NIH Grant RR 00292. This work has benefited from facilities made available by the Advanced Research Projects Agency through the Center for Materials Research at Stanford University.