Similar studies have been carried out using $^{31}$P-NMR which probes the polar region of the PL molecule. In a membrane where the PL molecules are not able to diffuse isotropically, the phosphorus signal is not a sharp line but exhibits a characteristic asymmetric lineshape which can be calculated theoretically. In presence and absence of the CPP, the $^{31}$P NMR spectra (proton decoupled) exhibit the same lineshape; no broad component indicative of a strongly bound phospholipid class can be detected. The distance between the two edges of the spectrum, called the chemical shift anisotropy, $\Delta\sigma$, is a measure of the structural order of the phosphate segment. In the presence of protein, a small but consistent decrease in order was observed; the headgroup order decreased with increasing temperature (Fig. 2 *B*) (4, 6). $^{31}$P-NMR $T_1$ relaxation measurements lead to the conclusion that the motion of the polar moiety of the PL is slowed by 10–20% in the presence of the CPP.

In summary, the D-NMR and $^{31}$P-NMR measurements detect motional parameters of the hydrophobic and polar moieties of the PL, respectively, and lead to similar conclusions: (*a*) in the presence of the CPP, there is in RSR, to a first approximation and with respect to the NMR time domain, a single homogeneous PL environment typical of a PL bilayer; (*b*) the ordering of the PL is decreased (10–15%) (more orientational disorder) in the presence of CPP as evidenced by the decrease in quadrupole splitting (deuterium NMR) and decrease in chemical shift anisotropy ($^{31}$P NMR); and (*c*) the motion of the PL is decreased (10–20%) by the presence of CPP in RSR as detected by the changes in $T_1$ times (4).

This work was supported by grants AM 14632 and AM 21987 from the National Institutes of Health, by the Muscular Dystrophy Association of America and by grant 3.309.78 from the Swiss National Science Foundation.

*Received for publication 27 May 1981.*

## REFERENCES

1. Fleischer, S., C. T. Wang, A. Saito, M. Pilarska, and J. O. McIntyre. 1979. *In* Cation Flux Across Biomembranes. Structural Studies of Sarcoplasmic Reticulum *in vitro* and *in situ*. Y. Mukohata and L. Packer, editors. Academic Press, Inc., New York. 193–205.
2. Wang, C. T., A. Saito, and S. Fleischer. 1979. Correlation of ultrastructure of reconstituted sarcoplasmic reticulum membrane vesicles with variation in phospholipid to protein ratio. *J. Biol. Chem.* 254:9209–9219.
3. Seelig, J., and A. Seelig. 1981. Lipid conformation in model membranes and biological membranes. *Q. Rev. Biophys.* 13:19–61.
4. Seelig, J., L. Tamm, L. Hymel, and S. Fleischer. 1981. Deuterium and phosphorus NMR and fluorescence depolarization studies of functional reconstituted sarcoplasmic reticulum membrane vesicles. *Biochemistry.* 20:3922–32.
5. Jost, P. C., O. H. Griffith, R. A. Capaldi, and G. Vanderkooi. 1973. Evidence for boundary lipids in membranes. *Proc. Natl. Acad. Sci. U.S.A.* 70:480–484.
6. McLaughlin, A. C., L. Herbette, J. K. Blasie, C-T, Wang, L. Hymel, and S. Fleischer. 1981. $^{31}$P NMR studies of oriented multilayers formed from isolated sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum: evidence that "boundary-layer" phospholipid is not immobilized. *Biophys. J.* 37:49–50.

# EPR STUDIES OF THE MOTIONAL CHARACTERISTICS OF THE PHOSPHOLIPID IN FUNCTIONAL RECONSTITUTED SARCOPLASMIC RETICULUM MEMBRANE VESICLES

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Functional reconstituted sarcoplasmic reticulum membrane vesicles (RSR) were prepared containing spin-labeled lecithins. The spin probe (doxyl) was located at either carbon 5 (SL-I) or carbon 16 (SL-II) of the *sn*-2 stearoyl moiety to monitor the motional parameters at different locations within the phospholipid (PL) bilayer. Membranes were reconstituted with protein content greater than or similar to that of normal SR (L/P [molar ratio PL:CPP] 110) to facilitate the study of the influence of the protein on the motion of the PL.

## RESULTS AND DISCUSSION

The EPR spectra of SL-I (5-doxyl) in SR PL and RSR were similar. There was no indication of a signal referable to very slow motion ($\tau_c < 10^{-7}$ s) in the presence of calcium

pump protein (CPP), although there was a slight increase in the hyperfine splitting (61 G in SR PL at 2°C) with increasing CPP content of the vesicles (to 62.5 G at 2°C for L/P = 60). The increase in hyperfine splitting indicates a small decrease in the average motion and/or disorder of the PL in the presence of CPP in RSR as monitored by the nitroxide probe at position 5 of the *sn*-2 acyl chain (not shown).

The EPR spectra at 4°C of SL-II (16-doxyl) in SR PL (Fig. 1 *B*1) and RSR (Fig. 1 *A*) are similar but there is a decrease in the hyperfine splitting of SL-II in RSR throughout the temperature range studied (2°–32°C) Fig. 2). This decrease probably reflects a small increase in the motion and/or disorder of the bulk PL in the presence of CPP. Deuterium and $^{31}P$ NMR studies showed both a small increase in disorder and a small decrease in the motion of the phospholipid (1,2[9,10 $D_2$] DOPC) due to the presence of CPP (1, 6).

The EPR spectra of SL-II (16-doxyl) in SR PL (Fig. 1 *B*1) and RSR (Fig. 1 *A*) show a significant broadening of the EPR signal in the presence of the CPP as seen in both the more typical EPR and first integral (absorption) spectra. This broadening is indicative of PL whose motion is perturbed by the CPP in RSR. Immobilized PL has been reported previously in other membrane preparations (2–4) and forms the basis for the concept of "boundary" lipid (2). Immobilized lipid in such preparations was quantitated by spectral subtraction using a reference sample for either the bound (2) or mobile bilayer PL (3) spectral components.

The composite spectrum of SL-II (16-doxyl) in RSR (L/P = 60) at 4°C (Fig. 1 *A*) was analyzed using spectral subtraction (4). Four different reference spectra were used and proportions of each reference spectrum (normalized to the same second integral value) were subtracted from the composite spectrum until the residual spectrum corresponded to a selected "end-point." In the first procedure (*B*1), the EPR spectrum of SL-II in SR PL (at the same temperature) was subtracted from the composite spectrum yielding a residual spectrum (*C*1) referable to the "constrained" lipid. The other three reference spectra (Fig. 1 *B*2, *B*3, and *B*4) used in the spectral subtractions reflect spin-labeled lipid with increasing motional constraint. Each of these spectra was subtracted from the composite spectrum of SL-II in RSR, until the first integral (absorption spectrum) of the difference spectrum started to give negative values, indicating oversubtraction. This "end-point" criterion yields only an upper limit for constrained PL as can be demonstrated by noting that smaller subtractions also yield difference spectra without negative absorption values and with characteristics similar to the free component. The end-point difference spectra (*C*2–*C*4) should have characteristics similar to the spectrum of the

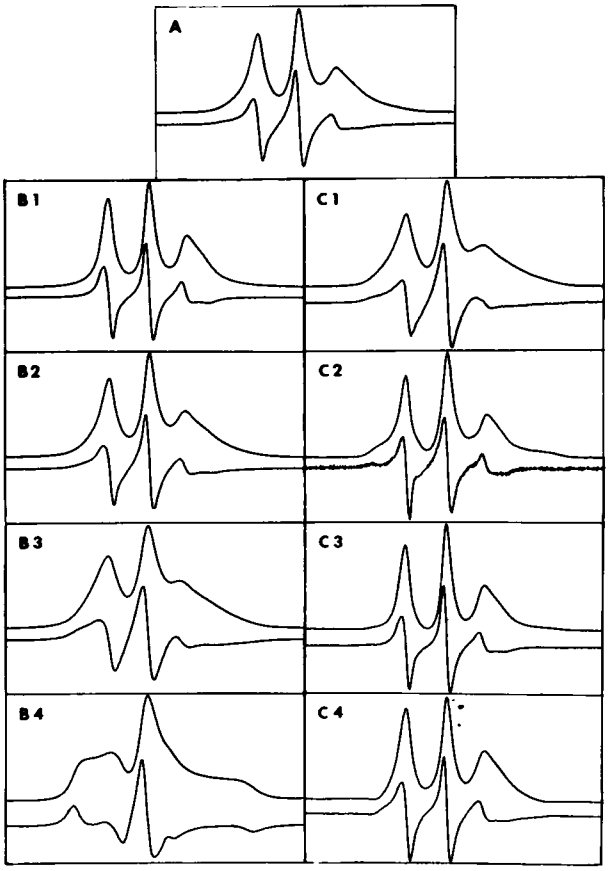


FIGURE 1 EPR spectra of spin-labeled 16-doxyl stearate *sn*-2 lecithin (SL-II) in RSR at 4°C (*A*) and examples of spectra involved in spectral analysis. Reference spectra of phospholipid (*B*1 to *B*4) were subtracted from the composite spectrum (*A*) to yield the corresponding residual spectra (*C*). The reference PL spectra (*B*) are lecithin with: 1) 16-doxyl at 4°C; 2)16-doxyl at −5°C; 3) 16-doxyl at −10°C and 4) 5-doxyl at 4°C. The first integral spectra (top) and the more typical EPR spectra (bottom) are shown for each. The L/P (molar ratio of PL/CPP) for the RSR is 60.

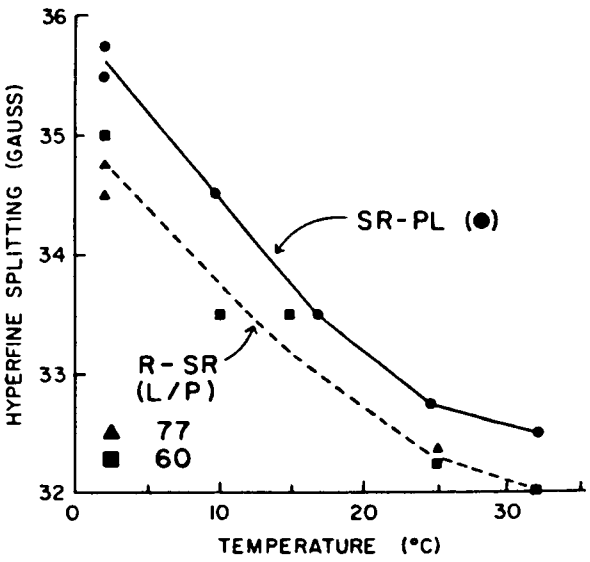


FIGURE 2 Temperature dependence of the hyperfine splitting of spin-labeled 16-doxyl stearate *sn*-2 lecithin (SL-II) in either SR PL (●) or RSR of either L/P = 77 (▲) or L/P = 60 (■).

free component, i.e. bilayer phospholipid (*B*1). With the three different reference spectra of constrained PL (Fig. 1, *B*2, *B*3, and *B*4) three different end-point percentages for the amount of immobilized PL and three different residual spectra are obtained. As the motion of the constrained PL is assumed to be slower, the proportion of this component in the composite decreases from 87% (*B*2) to 65% (*B*3) to 36% (*B*4) in RSR (L/P = 60) at 4°C (Figs. 1 and 3).

The amount of constrained PL in RSR (L/P = 60), estimated by spectral subtractions using the four different reference spectra, is given as a function of temperature in Fig. 3. Using free PL at the same temperature as the RSR sample (method *B*1, Fig. 1), the amount of constrained PL decreases somewhat with increasing temperature (up to 37°C). The other three spectral subtraction procedures show more marked decreases in the amount of constrained PL at higher temperatures, so that at 37°C only a maximum of 20% (12 mol/mol CPP) of the label is constrained using method 4 to model for constrained lipid. Similar results were obtained with RSR of higher PL content (L/P = 77 or 101).

The results of spectral subtraction methods one and three are compatible with one another (Fig. 1). Method one subtracts bilayer PL at 4°C (*B*1) from the composite spectra at 4°C (*A*) to give constrained PL (*C*1) which matches the assumed constrained PL at −10°C (*B*3). Method three subtracts "immobilized" PL of −10°C (*B*3) from (*A*) to give a spectrum (*C*3) which appears to be equivalent to bilayer PL at 4°C (*B*1). That is to say, constrained PL in RSR at 4°C approximates the reduced motion of bilayer PL at −10°C. The innate problem with the spectral subtraction method is that possible solutions are not unique solutions but are based on an assumed model for "immobilized" PL. By this method a smaller percentage of PL is perturbed when a reference spectrum with a more highly constrained PL motion is assumed for the model. For example, when a model with $2A_{Max}$ of 60G is assumed for the constrained species, we estimate ~8 mol of constrained phospholipid/mol calcium pump protein at 25°C. This amount of constrained lipid is less than one-third that estimated to constitute the boundary layer surrounding the calcium pump protein.

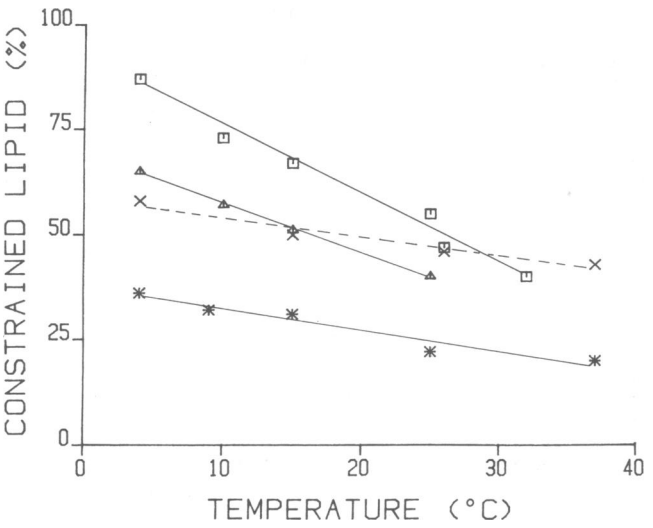


FIGURE 3 Constrained lipid as a function of temperature. Four different spectral subtraction procedures were applied to the EPR spectrum of RSR (L/P = 60). The spin label was PC SL-II. In each method a different spectrum was subtracted from the composite of the RSR. Method 1 (×), SL-II of PC at corresponding temperatures with the composite RSR. Method 2, (□) SL-II of PC at −5°C; method 3, (△) same as method 2 but at −10°C; method 4, (*) SL-I of PC at corresponding temperatures with the RSR. Method 1 subtractions make use of the inversion of the high field band, referable to free lipid, to indicate the end-point and as such aims for the best estimate of the constrained lipid. The spectral subtractions used in methods 2–4, involve negative absorption end-points and thus yield only an upper limit of the amount of constrained lipid.

Both EPR and NMR have been applied to study the motion of PL in well-defined RSR membrane vesicles and lead to the following conclusions: (*a*) both deuterium and $^{31}P$ NMR demonstrate that, to a first approximation, the PL environment in the RSR membrane is homogeneous and typical of a PL bilayer with only minimal perturbations observed in the presence of CPP (1, 6). In this context, homogenous means that there is a single phospholipid environment or that there are two or more environments which exchange rapidly within the time-resolution of the NMR technique; (*b*) proton NMR studies lead to similar conclusions (5); (*c*) constrained PL can be observed by EPR which is sensitive to motions faster than $10^{-7}$ s. Such motion is not detected by NMR which is sensitive to motions of $10^{-5}$ s or slower. (*d*) The amount of constrained PL, as determined by EPR spectral subtraction techniques, is critically dependent on a number of assumptions and the reference spectra used. (*e*) The constrained motion of the PL, detected by EPR, has motional characteristics considerably faster than the motion of the protein (for the CPP, $10^{-5}$ s) in the membrane.

Our goal is to ascertain whether motional characteristics of the lipid and protein components of the membrane are related to the exercise of function.

We thank Patricia C. Jost for helpful discussions with regard to spectral subtraction.

This work was supported inpart by grants AM 14632 and AM 21987 from the National Institutes of Health and by the Muscular Dystrophy Association of America.

*Received for publication 27 May 1981.*

## REFERENCES

1. Fleischer, S., L. Hymel, L. Tamm, and J. Seelig. 1981. NMR studies of the motional characteristics of the phospholipid in reconstituted sarcoplasmic reticulum membrane vesicles. *Biophys. J.* 37:
2. Jost, P. C., O. H. Griffith, R. A. Capaldi, and G. Vanderkooi. 1973. Evidence for boundary lipid in membranes. *Proc. Natl. Acad. Sci. U.S.A.* 70:480–484.
3. Watts, A., I. D. Volotovski, and D. Marsh. 1979. Rhodopsin-lipid

associations in bovine rod outer segment membranes. Identification of immobilized lipid by spin-labels. *Biochemistry.* 18:5006–5013.
4. Jost, P. C., and O. H. Griffith. 1978. The spin labeling technique. *Methods Enzymol.* 49:369–418.
5. Deese, A. J., E. A. Dratz, L. Hymel, and S. Fleischer. 1982. Proton NMR $T_1$, $T_2$, and $T_{1rho}$ relaxation studies of native and reconstituted sarcoplasmic reticulum and phospholipid vesicles. *Biophys. J.* 37:207–216.
6. McLaughlin, A. C., L. Herbette, J. K. Blasie, C. T. Wang, L. Hymel, and S. Fleischer. 1981. $^{31}P$ NMR studies of oriented multilayers formed from isolated sarcoplasmic reticulum and reconstituted sarcoplasmic reticulum. *Biophys. J.* 37:49–50.

# $Ca^{2+}$-ATPase–DETERGENT INTERACTIONS

## A Good Model for Protein-Lipid Interactions

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The $Ca^{2+}$-ATPase from sarcoplasmic reticulum has been demonstrated to have an obligatory requirement for bound amphiphiles, either phospholipids or detergents (1, 2). The results from several different approaches have suggested that 30–40 molecules of phospholipid are associated with the ATPase in native or reconstituted membranes (3, 4). However, it has not been possible to determine the affinity for phospholipid, or to observe directly the interaction of phospholipid monomers with the protein because of aggregation of phospholipid molecules at very low concentrations. In the present report, the interaction of detergent molecules below the critical micelle concentration (c.m.c) with delipidated $Ca^{2+}$-ATPase is described. In addition, the effect of temperature on detergent-ATPase interactions is investigated.

### METHODS

Binding of [$^3$H]Triton X-100 to the delipidated ATPase (3, 5) was observed on a Sepharose 6B column equilibrated with 0.01 M TES buffer, pH 7.5, containing 0.1 M KCl, 2.74 M glycerol, 1.0 mM dithiothreitol, and the desired concentration of [$^3$H]Triton X-100 at 6°C (6). Binding was calculated from the difference in radioactivity between column fractions containing ATPase protein, and fractions free of protein. $Ca^{2+}$-ATPase activity was determined by a coupled assay (4). Steady-state anisotropy of 1,6-diphenyl-1,3,5-hextriene (DPH) was estimated using a Glan polarizer in conjunction with an Aminco-Bowman spectrophotofluorometer with a water-jacketed cell compartment.

### RESULTS AND DISCUSSION

Binding of [$^3$H]Triton X-100 to the delipidated ATPase occurred in two steps. First, from $1 \times 10^{-5}$M to $12 \times 10^{-5}$M Triton, binding of the detergent occurred at 35–40 equivalent sites. Near the c.m.c ($20 \times 10^{-5}$M), cooperative binding of an additional 70 mol of Triton was observed. About half of the $Ca^{2+}$-ATPase activity was recovered during the noncooperative binding process and the remaining activity was observed after the cooperative binding was complete. The value of 35–40 Triton molecules bound per ATPase molecule is in good agreement with the result reported for the number of phospholipid molecules associated with the $Ca^{2+}$-ATPase in native or reconstituted membranes (3). The cooperative binding of additional Triton molecules is probably associated with micelle formation and incorporation of the ATPase into detergent-protein aggregates. The affinity of the ATPase for Triton molecules below the cmc is probably at least several hundred-fold lower than the affinity of the ATPase for phospholipid molecules, based on the 300-fold greater molar ratio of Triton needed for complete reactivation of the delipidated ATPase as compared with phosphatidylcholine (2, 5). However, the affinity constant reported here may not apply directly to the binding of Triton to individual ATPase molecules because of the highly aggregated state of the protein under these conditions. These results support the hypothesis that distinct binding sites occur on the $Ca^{2+}$-ATPase for phospholipid molecules in the native membrane and that these sites must be occupied for the ATPase to be active. However, the degree of immobilization of the lipid cannot be predicted from these results since no kinetic measurements were attempted.

It is well documented that a break occurs in the Arrhenius plot of ATPase activity in native membranes near 20°C (3, 5) and that this transition temperature can be changed by substituting different lipids in reconstitution experiments (3). However, the nature of the lipid-protein interactions that affect the enzymatic behavior of the ATPase is a subject of controversy. In the present study, the temperature dependence of ATPase activity has been determined for the delipidated ATPase in three different detergents as shown in Table I. The observation that the Arrhenius plots exhibit different discontinuity temperatures in different detergents suggests that the micelle environment can affect this property of the ATPase; this differs from the initial conclusion of Dean and Tanford (6). Table I also shows the results of steady-state fluorescence polarization measurements of DPH in the same three detergents. These results suggest that some type of structural perturbation is occurring in the detergents at the same temperature at which ATPase activity is affected. This alteration can also be detected by paranaric acid fluorescence and differential scanning calorimetry


BIOPHYS. J. © Biophysical Society · 0006-3495/82/01/56/02 $1.00
Volume 37 January 1982 56–57