negligible effect on the transfer efficiency. The difference between a planar monolayer and planar bilayer is larger, especially for R_0 values larger than about 40 Å. This effect is expected, since energy transfer across the bilayer becomes more effective when the R_0 is comparable to or larger than the distance between the layers of donors and acceptors. For example, for R_0 = 50 Å, σ_{50} decreases from 0.055 for a planar monolayer to 0.044 acceptor per phospholipid for a planar bilayer. Again, there is little difference between the transfer efficiency in a planar bilayer and a spherical bilayer.

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Effects of Sarcoplasmic Reticulum Ca^{2+}-ATPase on Phospholipid Bilayer Fluidity: Boundary Lipid†

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ABSTRACT: Sarcoplasmic reticulum membrane vesicles have been isolated from rabbit skeletal muscle and delipidated to varying degrees by treatment with cholate. The structure within the hydrophobic region of cholate-free membranes has been probed by measuring the anisotropy of diphenylhexatriene fluorescence. The Ca^{2+}-ATPase activity of these samples was determined as a function of both temperature and Ca^{2+}-ATPase content. Arrhenius plots of the Ca^{2+}-ATPase activity could not be fit to straight lines over the entire range of temperature. However, Arrhenius plots of the Ca^{2+}-ATPase activity and the fluorescence-derived "microviscosity" both fit well to straight lines in the range of 20–40 °C. Both plots gave activation energies of 7–9 kcal/mol, supporting the contention that membrane fluidity is the rate-determining factor in determining Ca^{2+}-ATPase activity in the physiological temperature range. Calculations based on a precise mathematical statement of the "lipid annulus" model supported the contention that this model may offer a reasonable description of Ca^{2+}-ATPase–lipid interactions at low temperatures but is probably an oversimplified description at physiological temperatures. The dependence of Ca^{2+}-ATPase activity on membrane protein content has also been interpreted as qualitatively supporting this conclusion. Alternative explanations of the data have been explored.

Studies of the lipid dynamics within phospholipid bilayers have led to increased interest in the details of lipid–protein interactions within membrane bilayers. It is believed that intrinsic proteins inserted into or through the membrane tend to disrupt the region of the lipid bilayer in their vicinity [see Gennis and Jonas (1977) for a review]. At least two intrinsic membrane proteins (glycophorin and the acetylcholine receptor) have been reported to loosen the adjacent bilayer structure (Brûlet and McConnell, 1976; Bienvenüe et al., 1977). On the other hand, several investigators have reported results purporting to demonstrate the coexistence of normal and restricted bilayer domains within model membranes reconstituted from isolated proteins and lipids (Grant and McConnell, 1974; Faucon et al., 1976; Brown et al., 1977; Almeida and Charnock, 1977). The most convincing evidence for the existence of a restricted lipid "annulus" in the neighborhood of an intrinsic membrane protein results from studies of beef heart mitochondrial cytochrome oxidase (Jost et al., 1973a,b, 1977). Complexes of the membrane protein with

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varying amounts of mitochondrial lipid were probed by electron-spin resonance spectroscopy, and the resulting spectra were shown to be consistent with the coexistence of both a rigid and "bulk" lipid bilayer environment, the amount of rigid environment being in direct relation to the amount of protein present. Dahlquist et al. (1977) have recently qualitatively confirmed these results using deuterium nuclear magnetic resonance and reconstituted lipid/cytochrome oxidase complexes. Aside from the studies of cytochrome oxidase, successive delipidation studies carried out on sarcoplasmic reticulum (SR[1]) membrane vesicles (Nakamura and Onishi, 1975; Hesketh et al., 1976) have also been interpreted, although less quantitatively, as supporting the model of a lipid annulus associated with the Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase (Ca^{2+}-ATPase).

We report here an attempt to quantitatively test the lipid annulus model as a description for the interaction of SR-membrane lipids with the Ca^{2+}-ATPase within isolated vesicles. We have used the fluorescence depolarization of 1,6-diphenyl-1,3-5-hexatriene (DPH), a hydrophobic membrane probe, to report the average motional state of phospholipid acyl chains (Kawato et al., 1977) as a function of membrane protein content and temperature. We make use of the fortunate property of DPH that it distributes rapidly (Lentz et al., 1976a,b) and, in most cases, equally (Lentz et al., 1976a,b; Stubbs et al., 1976; Lentz and Barrow, 1978) between membrane regions of vastly different composition and fluidity. Thus, the observed fluorescence properties of DPH should be simply calculable from the fluorescence properties of DPH in the various domains between which it distributes in a membrane. In this study, we use this observation to test whether DPH distributes between bulk lipid bilayer and protein-associated, annular domains in the SR membrane.

Materials and Methods

Materials. All materials used were reagent grade. Cholic acid was purchased from Sigma (St. Louis, Mo.) and was recrystallized before use (Meissner et al., 1973). [^{3}H]Cholate was obtained from New England Nuclear (Boston, Mass.). [γ-^{32}P]ATP was the generous gift of Dr. J. Wilson's laboratory. Quinine sulfate for use as a fluorescence standard was Baker Ultrex grade. The ionophore A 23187 was the generous gift of Dr. R. Hamill (Lilly Research Laboratories, Indianapolis).

Preparation of SR Vesicles. Membranes were isolated from an intermediate-density vesicle fraction of rabbit skeletal muscle SR (Meissner, 1975). Membrane vesicles [in 0.3 M sucrose, 0.45 M KCl, 1.5 mM $MgCl_2$, 1.0 mM ethylenediaminetetraacetic acid, 0.1 mM $CaCl_2$, 10 mM Tris (pH 8.0)] were delipidated according to the method of Meissner et al. (1973). Different amounts of sodium cholate (2–4 mg/mL) were added to suspensions of membrane vesicles (4.5 mg of protein/mL) in order to obtain the desired range of phospholipid removal. The partially delipidated vesicles were then recovered by centrifugation for 2 h at 33 000 rpm in a Beckman 35 rotor. Pellets were resuspended in a Potter-Elvejehem hand homogenizer in 0.3 M sucrose–50 mM KCl–0.5 mM Hepes (pH 7.5) and dialyzed against several changes of the same buffer for 2 days. One series of delipidated vesicles was prepared with [^{3}H]cholate ($\sim 10^6$ cpm/mg of cholate) and analyzed to confirm that greater than 99% of the detergent was removed from the vesicle preparations by this procedure. For every series of delipidations, an untreated original sample of intermediate-density vesicles was maintained as a control. The control sample was dialyzed separately to prevent contamination with cholate. Protein content of each delipidated sample was estimated by the procedure of Lowry et al. (1951).

Lipids and Lipid Analysis. Lipids were extracted from SR membrane vesicles by the method of Bligh and Dyer (1959), using argon-saturated solvents to prevent oxidation. To test whether this procedure removed all the phospholipids that might be more tightly associated with the membrane proteins, two modifications of this procedure were tested. In one, 1% sodium dodecyl sulfate was added to the membrane suspension before extraction with chloroform/methanol. In the other, the aqueous phase that remained after the initial chloroform/methanol extraction was adjusted to pH 12–13 with concentrated NH_4OH and extracted again. The resulting aqueous phase was then acidified with concentrated HCl and extracted twice more. The phosphorus contents of the organic phases obtained from these modified procedures and from the original Bligh and Dyer (1959) procedure were identical within experimental error.

Analysis of phospholipid content in the membrane extracts was made by one-dimensional thin-layer chromatography, using silica gel G plates prepared by Quantum Industries (250 μm). Plates were developed in chloroform/methanol/15 N NH_4OH (65:35:5, v/v) and were visualized by I_2 vapor staining. The spots were identified with appropriate standards and scraped from the plates for quantitation by phosphorus determination by the method of Chen et al. (1956).

The fatty acid methyl esters of extracted phospholipids were prepared by transmethylation with methanolic HCl (Stoffel et al., 1959). Methyl esters were chromatographed on 10% Silar 10C coated onto Gas Chrome Q, 100/120 mesh (Applied Science) at a column temperature of 180 °C, using a Perkin-Elmer 900 gas–liquid partition chromatograph. Peaks were identified with standards (Applied Science) and quantified by cutting and weighing.

Chromatographically pure egg-yolk phosphatidylcholine was prepared from fresh hen eggs as previously described (Lentz and Barrow, 1978).

Phospholipid Vesicles. Large, multilamellar vesicles (Bangham et al., 1967) were prepared from extracted phospholipids by procedures described in detail elsewhere (Lentz et al., 1976a,b). The aqueous medium contained 50 mM KCl (ultra high purity, Alpha Ventron) and water that was deionized, distilled from alkaline $KMnO_4$, and, finally, glass distilled. Small, single-walled vesicles were prepared by subjecting the large, multilamellar structures to sonic radiation, using a Branson 300W sonicator. A small, homogeneous population was isolated from residual large structures by chromatography on a Sepharose 4B (Pharmacia, Inc.) column by methods described previously (Huang, 1969).

Fluorescence Measurements. A modified Perkin-Elmer MPF-3 spectrofluorometer was used to record the fluorescence depolarization of DPH-labeled lipid vesicles as a function of temperature. A detailed description of the instrumental protocol has been given elsewhere (Lentz et al., 1978a). DPH was introduced into membrane vesicles by injecting sufficient DPH, dissolved in tetrahydrofuran, into membrane suspensions so as to arrive at a final ratio of 500–1000 lipid molecules per fluorescent probe molecule. At least 2 h were allowed for DPH to become incorporated into the membrane bilayers before any fluorescence measurements were made. This was found to be sufficient to allow a steady level of fluorescence intensity to be reached, indicating equilibration of DPH uptake. Measure-

[1] Abbreviations used: DPH, 1,2-diphenyl-1,3,5-hexatriene; SR, sarcoplasmic reticulum; Ca^{2+}-ATPase, Ca^{2+}-stimulated, Mg^{2+}-dependent adenosine triphosphatase; Tris, tris(hydroxymethyl)aminomethane; Hepes, *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; EGTA, [ethylenebis(oxyethylenenitrilo)]tetraacetic acid.

ments of fluorescence polarization were made during heating scans performed at 18–30 °C/h. The general theory of the fluorescence probe depolarization technique as applied to membrane systems has been presented in detail by Shinitzky et al. (1971). The use of DPH as a fluorescent probe specific for the hydrophobic region of lipid bilayers is discussed in detail elsewhere (Lentz et al., 1976a,b). Kawato et al. (1977) have given a theoretical treatment of the motion of DPH in a membrane which depicts the way in which the motion of DPH reflects the hydrocarbon chain motion in the bilayer. As a measure of DPH rotational motion, the anisotropy of DPH fluorescence was recorded as described previously (Lentz et al., 1976a,b), and the "microviscosity" and "microviscosity activation energy" were calculated from the anisotropy and estimated fluorescence lifetime (Lentz et al., 1976a,b; Lentz et al., 1978b). The fluorescence lifetime of DPH was assumed at low temperatures to be 10 ns, based on previous experience. Errors in this estimate affect only the absolute value of the "microviscosity", which is not used in any of the results presented here. Values of fluorescence anisotropy were corrected for scattering of the polarized exciting and emitted light, as outlined in the theory of Teale (1969). These corrections are discussed in more detail elsewhere (Lentz et al., 1978a).

The relative quantum yield of DPH in different membranes was determined as described by Lentz and Barrow (1978), using quinine sulfate in 0.1 N H_2SO_4 as a standard.

Zone-purified DPH was obtained as a gift from M. Shinitzky and Y. Barenholz.

The partitioning of DPH between highly delipidated membranes and pure phospholipid vesicles was determined by a modification of the method of Stubbs et al. (1976). The fluorescence intensity and antisotropy of DPH incorporated into a delipidated sample (about 40 lipids per Ca^{2+}-ATPase) were measured. A small aliquot of concentrated, single-lamellar vesicles (9.88 mM), containing egg yolk phosphatidylcholine was then added to the delipidated sample, with continuous stirring. DPH reequilibrated between the two lipid environments essentially instantaneously, and new measurements of intensity and anistropy were made. Additional aliquots of egg phosphatidylcholine vesicles were added until the sample contained roughly 90 mol % of this lipid component. To correct for instrument fluctuations during the course of the experiment, intensities were corrected relative to the intensity of a standard quinine sulfate solution (1 mg/mL in 0.1 N H_2SO_4). The data were analyzed as described in the Appendix.

Activity of the Ca^{2+}-ATPase. The Ca^{2+}-stimulated ATPase activity of the SR membrane vesicles was determined by directly measuring the initial velocity of inorganic phosphate release. The assay medium (2 mL total volume) contained 0.1 M KCl, 5 mM $MgCl_2$, 0.02 mM $CaCl_2$, 10 mM Hepes (pH 7.3), 8–60 μg of protein (mainly Ca^{2+}-ATPase), and the Ca^{2+} ionophore A 23187 (10 μg/mL). The reaction was initiated by the addition of 30 μL of 0.2 M adenosine triphosphate and terminated after a short time interval by the addition of 0.7 mL of 1.5 M $HClO_4$. The samples were immediately cooled to 0 °C and centrifuged in a clinical centrifuge to yield a clear supernatant. Inorganic phosphorous was determined from a 1-mL aliquot of this supernatant by the procedure of Fiske and Subbarow (1925).

The phosphoenzyme-forming ability of the Ca^{2+}-ATPase was determined using [γ-^{32}P]ATP. The ^{32}P phosphorylated intermediate of the Ca^{2+}-ATPase was formed under steady-state conditions at 0 °C in 0.2 mL of a medium containing 100 μg of protein, 0.1 M KCl, 5 mM Mg^{2+}, 0.2 mM Ca^{2+} or 1 mM EGTA (Blank), and 10 mM Hepes (pH 7.6). The reaction was initiated by the addition of 10 μL of 3 mM [γ-^{32}P]ATP and was stopped after 6 s by addition of 3 mL of ice-cold 4.25% trichloroacetic acid containing 0.5 mM ATP and 1 mM P_i. An aliquot (1 mL) was placed on a 0.45-μm Millipore filter and rinsed with the above trichloroacetic acid solution. The radioactivity retained on the filter was counted in 4.5 mL of a scintillation liquid, which completely dissolved the filter. The fluid contained 60 g of naphthalene, 4.2 g of 2,5-diphenyloxazole, 180 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene, and 70 mL of water in 900 mL of dioxane. Counting was carried out in a Nuclear Chicago Mark II liquid scintillation system using minivials.

TABLE I: Analysis of SR Membrane Lipid Content.[a]

	mol % total			
	org.	1	2	3
phospholipid[b]				
PC	68.4 ± 4.8	72.6 ± 1.9	70.2 ± 3.8	70.5 ± 4.7
PE	14.5 ± 1.9	14.2 ± 1.8	15.2 ± 2.0	15.5 ± 2
PI + PS	12.3 ± 3.3	11.4 ± 1.3	11.2 ± 2.9	12.1 ±1.3
fatty acid				
$C_{14:1}$	7.9	9.6	6.6	5.7
$C_{16:0}$	30.4	30.8	31.7	30.4
$C_{16:1}$	3.1	4.1	3.4	2.0
$C_{18:0}$	6.4	6.0	5.3	9.0
$C_{18:1}$	14.9	14.4	15.5	15.6
$C_{18:2}$	22.7	22.6	24.6	22.4
$C_{22:0}$	0.8			0.7
$C_{22:1}$	8 7	7.2	7.8	8.7
$C_{24:1}$	0.9	0.9	0.5	1.0
$C_{24:2}$	0.6	0.6	1.0	0.7
$C_{24:3}$	1.9	1.2	2.2	2.4
$C_{26:1}$	0.8	0.6	0.3	0.7
unident	0.8	1.8	1.0	0.7

[a] Errors represent standard deviations calculated from multiple analyses of the same sample. [b] Abbreviations used: PC, phosphatidylcholine; PE, phosphatidylethanolamine; PI, phosphatidylinositol; PS, phosphatidylserine.

Results

Composition of Delipidated Vesicles. Polyacrylamide gel electrophoresis of the original or undelipidated sample in the presence of sodium dodecyl sulfate showed the existence of three major bands: one dominant band previously identified as the Ca^{2+}-ATPase and two bands which correspond to a Ca^{2+}-binding protein (calsequesterin) and M_{55} protein (Meissner et al., 1973). Densitometer tracings of these gels indicated that the ATPase content of samples not treated with cholate was 75–80%, while in those samples which were treated with sodium cholate the Ca^{2+}-ATPase accounted for 90–95% of the protein. Thus, treatment with cholate resulted in an enrichment of the Ca^{2+}-ATPase in the vesicles.

Assuming a molecular weight of 100 000 for the Ca^{2+}-ATPase and an average molecular weight of 770 for a phospholipid, we calculated from our phosphorus and protein determinations that the molar ratio of phospholipid to protein ranged from 100:1 to 33:1 for the different delipidated samples. The data of Table I indicate that no selective removal of any phospholipid class occurred as a result of delipidation with cholate. The values for phospholipid composition are in good agreement with those previously reported by Meissner and Fleischer (1971). It should be noted that we have reported combined values for some pairs of phospholipids, since these pairs were not well separated by one-dimensional thin-layer chromatography. The data in Table I also indicate that cholate

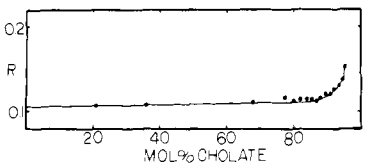

FIGURE 1: DPH fluorescence anisotropy as a function of increasing content of sodium cholate in suspensions of SR membrane vesicles.

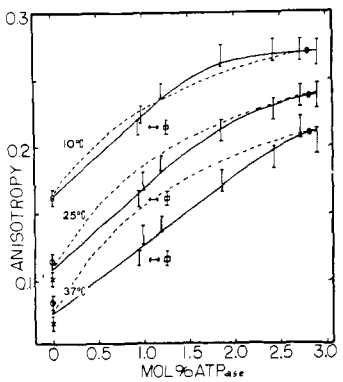

FIGURE 2: Variation of DPH fluorescence anisotropy with Ca^{2+}-ATPase content of SR membranes at three temperatures: (□) data derived from undelipidated SR membrane vesicles and plotted as mol % protein. The solid lines are drawn through the data. The dashed lines represent the anisotropies predicted by the simple model presented under Discussion. The anisotropy for DPH in a pure lipid bilayer environment was measured in large, multilamellar vesicles (X) and small unilamellar vesicles (O) composed of egg phosphatidylcholine. Error bars represent ±1 standard deviation.

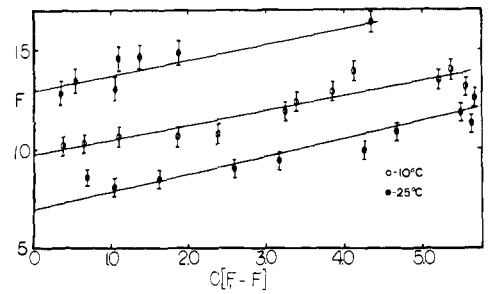

FIGURE 3: Derivation of DPH partition coefficients. The total fluorescence intensity (F) is plotted in arbitrary units as a function of the fluorescence-weighted relative concentrations of membrane environments ($C[F_i - F]$; see Appendix A): (○) 10 °C; (●) two sets of data obtained at 25 °C are arbitrarily displaced from one another on the ordinate. Error bars represent ±1 standard deviation in five individual intensity measurements.

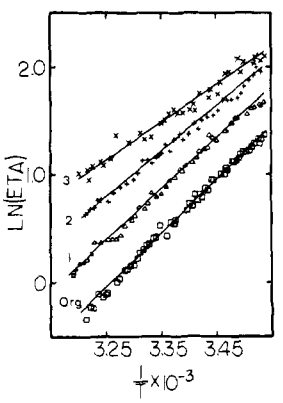

FIGURE 4: Arrhenius plots of DPH-derived "microviscosity" (ETA) for the untreated (□) and three delipidated membranes (△, +, X). Membranes were estimated to contain 105 (□), 83 (△), 54 (+), and 34 (X) molecules of phospholipid per molecule of Ca^{2+}-ATPase.

treatment did not preferentially remove phospholipids containing acyl chains of different degrees of saturation. This would suggest that the major difference between the various delipidated samples is in the lipid to Ca^{2+}-ATPase ratio.

Fluorescence Studies. In order to confirm that any residual sodium cholate was not affecting the depolarization of DPH fluorescence in delipidated membranes, we explored the effect of sodium cholate on DPH anisotropy in the SR membrane vesicles. Figure 1 demonstrates that even very large amounts of sodium cholate have an insignificant effect on DPH fluorescence anisotropy and suggests that a possible contamination of our samples by less than 1 mol % cholate should not affect our results or conclusions.

In Figure 2, the directly measured anisotropy of DPH fluorescence is presented as a function of membrane Ca^{2+}-ATPase content, for 10, 25, and 37 °C. Since an increase in anistropy is thought to reflect a decrease in the motional freedom of phospholipid acyl chains within the bilayer (Kawato et al., 1977), it appears that the intrinsic membrane protein has an ordering effect on the phospholipid bilayer. The partition coefficient for the distribution of DPH between free lipid bilayers and highly delipidated membrane samples has been measured to allow us to test the effect of ATPase on bilayer structure in a more quantitative manner (see Discussion and Appendix). Determination of the DPH partition coefficient is illustrated in Figure 3, where data obtained at both 10 and 25 °C are plotted. The derivation of partition coefficients from these plots is described in the Appendix. The values of the partition coefficient obtained from these plots were 0.82 ± 0.08 and 0.75 ± 0.07 at 25 and 10 °C, respectively. While these measurements could indicate some temperature dependence of the partition coefficient, this dependence is within the experimental error of the data and has, therefore, been ignored in the calculations presented below (see Discussion).

Arrhenius plots of the DPH-derived "microviscosity" are given in Figure 4 for three delipidated membrane preparations and the untreated or "original" sample from which the delipidated samples are derived. Several points can be made about these plots. First, the data in all four plots are reasonably well approximated by straight lines over the experimental range of temperature. There are no discernable "breaks" or inflection points to indicate temperature-induced phase separations in the membranes. Second, the "microviscosity" reported by DPH fluorescence increases as the ratio of lipid to protein decreases (curves "org" to "3" in Figure 4). This is in agreement with the general view that proteins have an ordering or rigidifying effect on membrane bilayers (Hong and Hubbell, 1972; Faucon et al., 1976). Finally, the slopes of the lines in Figure 4 are seen to decrease slightly with decreasing lipid to protein ratios, a fact which is depicted in more detail in Figure 5, in which we have plotted the "microviscosity activation energy" as a function of Ca^{2+}-ATPase content.

Ca^{2+}-ATPase Activity. In Figure 6, the temperature dependence of Ca^{2+}-ATPase activity of the delipidated and original membranes is presented in the form of Arrhenius plots. The data could not be fit well to a straight line over the entire temperature range (5–40 °C). This result is in reasonable agreement with previous reports of slight "breaks" in Arrhenius plots of Ca^{2+}-ATPase activity around 20 °C (Inesi et al., 1973; Deamer, 1973; Lee et al., 1974; Hidalgo et al., 1976;

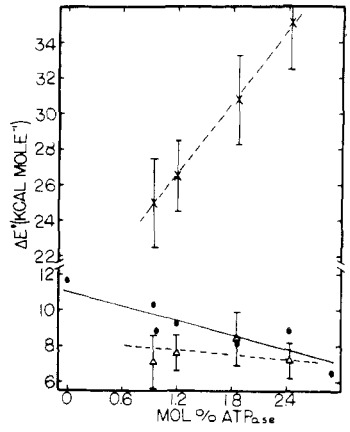

FIGURE 5: Dependence of activation energies on the ATPase content of the membrane: (●) "microviscosity activation energy", taken from the data in Figure 4; (X) Ca^{2+}-ATPase activation energy at low temperature, taken from a linear approximation to the 5–20 °C data in Figure 6; (Δ) Ca^{2+}-ATPase activation energy at high temperature, taken from a linear approximation to the 20–40 °C data in Figure 6. Error bars (point size for ●) represent ±0.5 standard deviation derived from linear least-square fits of the Arrhenius plots shown in Figures 4 and 6.

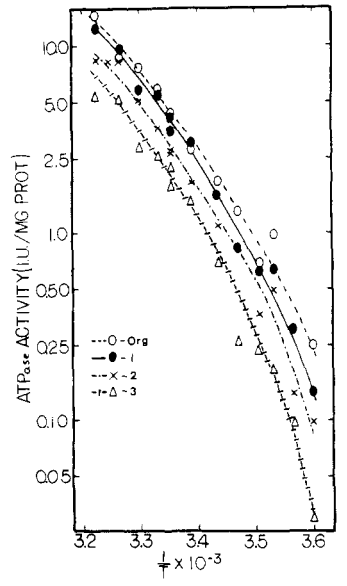

FIGURE 6: Arrhenius representation of the temperature dependence of Ca^{2+}-ATPase activity. Data for original, undelipidated SR membrane vesicles (○) and three delipidated samples (●, X, Δ) are presented. Membranes were estimated to contain 105 (○), 83 (●), 54 (X), and 34 (Δ) molecules of phospholipid per molecule of Ca^{2+}-ATPase. Error bars are not presented, since they would obscure the data. Errors generally ranged between ±10 to 20%, as estimated from linear least-square fits of plots of phosphate released with time.

Dean and Tanford, 1978). In order to obtain estimates of the extremes of activation energies that can be associated with the Arrhenius plots of Figure 6, we have made linear least-square fits of the data between 5 and 20 °C and between 20 and 40 °C. The activation energies so derived are plotted in Figure 5 as a function of Ca^{2+}-ATPase content, along with the activation energy of the fluorescence-derived microviscosity. The agreement between the high-temperature Ca^{2+}-ATPase activation energies and those of the microviscosity is striking. By contrast, the activity activation energies obtained from the low-temperature Ca^{2+}-ATPase data bear no resemblence to the "microviscosity" activation energies.

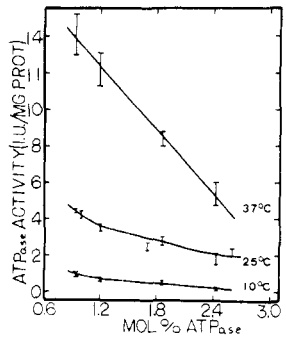

FIGURE 7: Variation in the Ca^{2+}-ATPase activity of SR membranes with Ca^{2+}-ATPase content within the membrane. Upper curve 37 °C; middle curve, 25 °C; lower curve, 10 °C. Error bars represent ±1 standard deviation, as described in Figure 6.

From Figure 7, it can be seen that the Ca^{2+}-ATPase activity decreased appreciably as the number of phospholipid molecules per Ca^{2+}-ATPase polypeptide chain was lowered from about 105 to 40. This result is in contrast to the relatively constant activity reported by Hesketh et al. (1976) and by Knowles et al. (1976) but agrees with the result of Nakamura and Onishi (1975). As yet, we have no explanation for these discrepancies.

In order to test whether there occurred significant enzyme inactivation during delipidation, formation of a [^{32}P]phosphoenzyme intermediate of the Ca^{2+}-ATPase from [γ-^{32}P]-ATP was determined in the presence of Ca^{2+}. Formation of the [^{32}P]phosphoenzyme intermediate is considered to be largely independent of membrane lipid content and composition [e.g., see review by MacLennan and Holland (1975)]. The phosphoenzyme levels of the original membrane, first delipidated, second delipidated, and limiting-delipidated membranes were 0.6 ± 0.1, 0.57 ± 0.07, 0.63 ± 0.08, and 0.53 ± 0.11 mol of ^{32}P/mol of Ca^{2+}-ATPase, respectively. In order to derive these values on a mol/mol basis, the Ca^{2+}-ATPase was assumed to account for 90% of the protein in all but the original sample (75% therein) and was assigned a molecular weight of 100 000. These results suggest that part of the decrease in ATPase activity observed for the fully delipidated sample (Figure 7) might be due to an irreversible inactivation of Ca^{2+}-ATPase during the delipidation procedure. However, the data are not sufficiently precise to allow an unequivocal statement at this point. At any rate, no more than about 10% of the loss in ATPase activity seen for the most delipidated membrane can be accounted for in this way.

Discussion

Breaks in Arrhenius plots of the SR Ca^{2+}-ATPase activity have been suggested to be due to lateral-phase separations in the membrane lipid bilayer (Inesi et al., 1973; Lee et al., 1974; Hidalgo et al., 1976). Recently, however, Davis et al. (1976) have concluded on the basis of X-ray scattering and nuclear magnetic resonance data that all SR membrane lipids are in the liquid-crystalline state at 20 °C, where the Arrhenius plot breaks are observed. These authors showed by proton nuclear magnetic resonance studies that as the temperature was increased from 5 to 40 °C an increasing fraction of the lipid molecules acquired isotropic motion. They attributed the nonlinear Arrhenius behavior of Ca^{2+}-ATPase activity to a shift from anisotropic to isotropic lipid motions, rather than to lateral lipid-phase separation. On the other hand, Dean and Tanford (1978) have observed a 20 °C break in an Arrhenius plot of the ATPase activity of a nearly lipid-free, detergent-solubilized SR Ca^{2+}-ATPase. This report calls into question

the association of the nonlinear Arrhenius behavior of Ca^{2+}-ATPase activity with specific changes in lipid motion.

A clue to the reason for the nonlinear Arrhenius behavior of Ca^{2+}-ATPase activity is found in the very similar behavior of membrane fluidity and Ca^{2+}-ATPase activity between 20 and 40 °C. Figure 5 illustrates that Arrhenius plots of the "microviscosity" and of the Ca^{2+}-ATPase activity yielded similar activation energies in this temperature range. These observations suggest that the rate-limiting process of the Ca^{2+}-ATPase reaction at physiological temperatures is dependent on lipid-bilayer fluidity. By contrast, the ATPase activation energy at low temperature is much larger than the "microviscosity" activation energy and increases with decreased membrane lipid content, again unlike the "microviscosity" activation energy. This suggests that additional reaction steps increasingly contribute to the observed rate of ATPase activity as the temperature is decreased below 20 °C. We suggest that these additional steps may be rate limited by protein-dependent processes and not only related to bilayer fluidity. This is not to suggest that the Ca^{2+}-ATPase activity is unaffected by the presence of a fluid lipid bilayer (indeed, see Figure 7), but rather that the temperature-induced changes in bilayer fluidity cease to have a major influence on the rate-determining step at low temperature. Such a shift in the nature of the rate-determining step would explain the observed nonlinear Arrhenius behavior.

A major aim in beginning the study reported here was to test the usefulness of the "annular-lipid" concept in describing the interactions of the Ca^{2+}-ATPase with the lipid bilayer in the SR membrane. In the following, the "lipid annulus" will be understood as a layer of lipid adjacent to an intrinsic membrane protein, in which layer acyl-chain motions are restricted relative to acyl-chain motions in the bulk lipid bilayer. This immobilized layer is assumed to coexist with bulk lipid bilayer and to have a structure independent of the presence of the bulk lipid bilayer—i.e., interactions between the two domains do not perturb the structure of either domain (Jost et al., 1973a,b). With this definition of the "lipid annulus", we would expect Ca^{2+}-ATPase activity to remain unaffected by delipidation as long as annular lipid was not disturbed. This is the behavior observed by Hesketh et al. (1976), who offered their results as evidence for the "lipid annulus" model as applied to the Ca^{2+}-ATPase. By contrast, our results reveal a rather dramatic decrease in Ca^{2+}-ATPase activity with decreasing lipid content, well before the level of delipidation that would be expected to cause removal of lipid from the annulus (see Figure 7). Thus, these results cannot support the "lipid annulus" model, as stated above.

In Figure 2, we have presented the variation in DPH fluorescence anisotropy that occurs with protein content, according to the model outlined above. The details of this calculation are given in the Appendix. Reference to these details reveals that several parameters are required for the calculation. The DPH fluorescence anisotropy characteristic of the bulk bilayer domain has been taken to be that observed in pure lipid bilayers (0 mol % ATPase, in Figure 4), while that characteristic of the "annular" domain has been taken as that observed in limiting-delipidated samples (roughly 3.0 mol % ATPase, in Figure 2). The ratio of the quantum yields of DPH in pure lipid bilayer to that in limiting-delipidated samples has been measured to be 0.29. The only remaining parameter is the number of phospholipid molecules interacting with the protein, i.e., the number in the annular domain (see Appendix B). We have taken this parameter, α, to be 34 phospholipids per protein for two reasons. First, our DPH fluorescence anisotropy data leveled off at roughly this value for the three temperatures examined (see Figure 2). Second, a value of roughly 30 lipids per protein is fairly well accepted to be the limiting composition of SR membrane that will maintain reasonable activity of the Ca^{2+}-ATPase (Meissner et al., 1973; Le Maire et al., 1976; Hesketh et al., 1976). Variation of α produced no significantly better fit at 10 °C. A reasonably good representation of the data at either 25 or 37 °C could not be obtained for any single value of α. Our conclusion is that the "annular lipid" model, as defined here, may provide a roughly accurate description of protein–lipid interactions in the SR membrane vesicles at low temperatures. However, it is clear that the nature of the lipid–Ca^{2+}-ATPase interactions is less satisfactorily described by this simple model at physiological temperatures.

We feel it worthwhile to speculate on the possible reasons for the failure of the "lipid annulus" model at higher temperatures in SR membrane vesicles. Two alternative explanations exist. First, and most likely, is the possibility that the assumption of noninteracting domains holds reasonably well at low temperature when acyl-chain motions are slight but does not hold at higher temperatures when the acyl chains should be undergoing increased random motions. Thus, at low temperatures, the extent of acyl-chain motions in the bulk and annular domains should be similar and the two domains should not greatly perturb each other at their interface. At higher temperatures, when the annular domain should be considerably more rigid than the bulk domain, it is difficult to imagine a sharp interface between the two, with the result that the assumption of independent, noninteracting domains would break down. However, we do not want to propose that the lipids experience no restrictions of packing or motion in limiting-delipidated SR membranes. Clearly, our results (ATPase activity, Figure 7; DPH fluorescence anisotropy, Figure 2; DPH partition coefficient) are inconsistent with such a proposal. Our results do imply, however, that the restricted lipid environment adjacent to the Ca^{2+}-ATPase is less well defined at high temperatures than previously assumed (Hesketh et al., 1976), at least when substantial amounts of bulk lipid bilayer are available to interact with the restricted region. This picture can also account for the marked variation of Ca^{2+}-ATPase activity with protein content at higher temperatures (Figure 7).

The second possibility to explain the deviation of our anisotropy data from the calculated curves at high temperatures (Figure 2) is that protein lateral aggregation may occur at low temperatures and result in different behaviors for the membrane at low and high temperatures. Thus, the possibility exists that closely packed protein domains separate from the bulk bilayer and increasingly dominate the DPH fluorescence anisotropy at low lipid levels. Such a situation would be adequately fit by our model calculations. We feel that this is not a likely possibility, since Davis et al. (1976) have concluded from their X-ray diffraction results that nearly all the lipid in SR membrane vesicles is in the liquid-crystalline phase above 1 °C. Nonetheless, we are investigating this possibility further using freeze–fracture electron microscopy.

In addition to its implications for the validity of the "lipid-annulus" model, Figure 2 offers insight into the location of proteins that are removed during cholate treatment (e.g., calsequestrin). When the data for the original or cholate-free sample were plotted in terms of mol % protein (□, in Figure 2) rather than in terms of mol % Ca^{2+}-ATPase (indicated by arrows in Figure 2), the points did not follow the pattern established by the data for delipidated samples. This implies that the proteins removable by cholate treatment do not affect the fluorescence anistropy of DPH and, therefore, that these proteins do not significantly modify the properties of the hydrophobic core of the bilayer.

Further insight into the nature of protein–lipid interactions in the SR membrane may be obtained by commenting on the unequal distribution of DPH between a pure lipid bilayer and a delipidated SR membrane vesicle. In previous studies, the partition coefficient of DPH between lipid bilayers of different structure has been found to be near unity (Lentz et al., 1976a,b; Stubbs and Litman, 1976; Lentz and Barrow, 1978). The anomalous behavior of the SR system (partition coefficient = 0.8, in favor of the pure lipid bilayer) would indicate that the free volume available for DPH in the neighborhood of the Ca^{2+}-ATPase is less than that available in the pure lipid bilayer environment. Such a reduction in free volume would be reasonably presumed to result from the close juxtaposition of protein and lipid in delipidated SR membrane vesicles. One possible interpretation is that phospholipid acyl chains or DPH molecules directly adjacent to protein would experience less freedom of motion in the direction of the protein, simply due to a steric interaction with a protein "wall". The possibility also exists that roughly 20% of the lipids may not be accessible to DPH due to specific binding of phospholipid to the Ca^{2+}-ATPase. Our methods cannot distinguish between these two possibilities. In this regard, it is worth noting that the study of DPH distribution does make it unlikely that specific DPH binding sites occur on the Ca^{2+}-ATPase.

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Appendix A

Determination of DPH Partition Coefficient. The total fluorescence intensity of DPH incorporated into the mixture of SR membrane vesicles and egg phosphatidylcholine small vesicles is given by:

$$F = f_1F_1 + f_2F_2 \tag{A1}$$

where f_1 and F_1 are the mole fraction and intensity, respectively, of DPH in SR membrane vesicles; f_2 and F_2 are the corresponding quantities for the egg phosphatidylcholine small vesicle environment. If the mole fractions of total phospholipid present in SR and egg phosphatidylcholine vesicles are respectively Y_1 and Y_2, then the partition coefficient for the distribution of DPH between the two environments is given by:

$$K = (f_1/Y_1)/(f_2/Y_2) \tag{A2}$$

Using eq A1 and A2:

$$F = K(Y_1/Y_2)f_2F_1 + f_2F_2 \tag{A3}$$

Using

$$f_1 + f_2 = 1 \tag{A4}$$

and eq A2, we obtain

$$f_2 = [1 + K(Y_1/Y_2)]^{-1} \tag{A5}$$

Substituting eq A5 into A3 yields:

$$F = (F_1 - F)K(Y_1/Y_2) + F_2 \tag{A6}$$

which is the expression plotted in Figure 3, with $C = Y_1/Y_2$. The intercept of a straight line drawn through such a plot is F_2, which can be compared with a directly measurable value of fluorescence intensity resulting from the pure egg phosphatidylcholine environment as an independent check on the procedure. The partition coefficient for DPH between the two environments is given by the slope of the line.

Appendix B

Calculation of DPH Fluorescence Anisotropy from the "Lipid-Annulus" Model. For a membrane in which DPH is distributed between two coexisting microscopic domains, the average observed anisotropy for the ith experiment can be expressed in terms of the anisotropies in the two domains and the intensity-weighted mole fractions of probe in the two environments (Lentz et al., 1976):

$$r(i) = Z_1(i)(r_1 - r_2) + r_2 \tag{B1}$$

where r_1 and Z_1 will be understood to be the anisotropy and intensity-weighted mole fraction, respectively, of DPH in the protein-associated "annular" domain; r_2 is the anisotropy of fluorescence resulting from DPH in the "bulk lipid bilayer" domain. The problem of calculating the expected anisotropy resulting from membranes containing different mole fractions of Ca^{2+}-ATPase, $Y_p(i)$, thus becomes the problem of expressing $Z_1(i)$ in terms of $Y_p(i)$. An expression for Z_1 in terms of the mole fractions of probe in the "annular" and "bulk" domains (f_1 and f_2, respectively) may be obtained from the treatment of Lentz et al. (1976a,b):

$$Z_1(i) = f_1(i)/[f_1(i) + \{1 - f_1(i)\}q_2/q_1] \tag{B2}$$

where q_1 and q_2 are the relative quantum yields of DPH in the two domains.

We identify the "lipid annulus" domain as the lipid environment experienced by DPH in the limiting-delipidated vesicles, as described in Appendix A. The model assumes that α lipid molecules per protein polypeptide chain are associated with the annular domain, independent of the total lipid content of a given SR membrane vesicle. The remaining lipid is assumed to be in a "bulk bilayer" domain with properties identical to those of the egg phosphatidylcholine small vesicles discussed in Appendix A. It can be easily shown that the mole fractions of "annular" (Y_1) and "bulk" (Y_2) environments are given by:

$$\begin{aligned} Y_1 &= \alpha Y_p(i) \\ Y_2 &= 1 - Y_p(i)(1 + \alpha) \end{aligned} \tag{B3}$$

Using eq B3, A4, and A5, we have for the mole fraction of DPH in the annular domain:

$$f_1(i) = 1 - [1 + K\alpha Y_p(i)/\{1 - Y_p(i)(1 + \alpha)\}]^{-1} \tag{B4}$$

In summary, given α, we can calculate the anisotropy predicted by the "annular lipid" model for SR membrane vesicles containing Ca^{2+}-ATPase at a mole fraction of $Y_p(i)$ by using eq B4, B2, and B1, in succession.

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