

# LIPID PHASE OF TRANSVERSE TUBULE MEMBRANES FROM SKELETAL MUSCLE

## An Electron Paramagnetic Resonance Study

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**ABSTRACT** The lipid phase of transverse tubule membrane was probed with a variety of fatty acid spin labels. The motion of the probe increased as the distance between the spin label and polar head group increased, in agreement with results reported in other membranes. The value of the order parameter at 37°C for a fatty acid spin label containing the label attached to its fifth carbon atom was closer to values reported for bacterial membranes than to the lower values reported for other mammalian membranes. Order parameters for spin labels containing the label nearer to the center of the bilayer were closer to the values reported in other mammalian membranes than to values reported for bacterial membranes. These results indicate that the lipid segments in the vicinity of the polar head group, and less so those near the center of the bilayer, are motionally more restricted in transverse tubules than in other mammalian membranes. In particular, the lipid phase of the transverse tubule membrane is less fluid than that of the sarcoplasmic reticulum membrane. A possible role of the high cholesterol content of transverse tubules in generating the lower fluidity of its lipid phase is discussed.

### INTRODUCTION

The transverse tubule (T-tubule)<sup>1</sup> membrane of skeletal muscle plays a crucial role in the process of excitation-contraction coupling. The action potential elicited at the neuromuscular junction propagates through the external plasma membrane into the T-tubules (1, 2). By a mechanism yet unknown, the depolarization of the T-tubule membrane triggers release of calcium from the terminal cisternae of the sarcoplasmic reticulum (SR), and the ensuing increase in intracellular calcium produces muscle contraction.

Physiological evidence obtained with whole muscle fibers, and biochemical, pharmacological, and structural information obtained with isolated T-tubule membrane preparations, indicate that the T-tubule and the external plasma membrane differ in many ways. They have different passive permeability properties (3, 4), different densities of both calcium (5, 6) and sodium channels (7), different characteristics of the sodium channels (8), and different lipid compositions, with a higher cholesterol

content of T-tubules relative to that of the plasma membrane (9, 10, 11).

In view of its role in the physiology of muscle contraction, it is important to characterize the T-tubule membrane. Recent studies with isolated T-tubule membrane preparations have established some of their enzymatic (10, 12, 16), transport (16–18), and pharmacological properties (5, 6, 8, 19). However, there is no information about the structural organization of the membrane.

This report describes a study of the lipid phase of the T-tubule membrane, as probed with a variety of lipid spin labels. The results indicate that at a physiological temperature the lipid phase of the T-tubules is less fluid than that of other mammalian plasma membranes, a finding that might be related to its high cholesterol content.

### METHODS

Transverse tubule membranes were prepared from rabbit skeletal muscle as described in detail elsewhere (10, 16). Briefly, microsomes were isolated by differential sedimentation of a rabbit muscle homogenate. After sedimentation at 10,000 *g* to discard a fraction enriched in mitochondria, contaminating actomyosin was removed by extraction with 0.6 M KCl, and the microsomal fraction was collected by sedimentation at 100,000 *g*. Separation of T-tubules from sarcoplasmic reticulum was accomplished by sedimentation through discontinuous sucrose density gradients as previously described (10, 16). If the isolated T-tubules were still contaminated with SR, further purification was achieved by loading the contaminating SR with calcium phosphate followed by removing the loaded SR by sedimentation in discontinuous sucrose gradients (10). The usual yield of purified T-tubules was 5–10 mg of protein from 500 g of muscle. T-tubule vesicles were resuspended in 0.3 M sucrose, 20 mM

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<sup>1</sup>*Abbreviations used in this paper:* T-tubule (s), transverse-tubule (s); SR, sarcoplasmic reticulum; SDS, sodium dodecylsulfate; doxyl, 4,4-dimethyl-3-oxazolidinyl-oxy; E-C coupling, excitation-contraction coupling.

Tris-maleate, pH 7.0 and stored at  $-20^{\circ}\text{C}$  at a concentration of 5–10 mg of protein per milliliter. The SR vesicles used in this work were obtained free of T-tubule contamination from the 35–40% interface of the sucrose gradient (10).

Stearic acid spin labels with the 4,4-dimethyl-3-oxazolidinyloxy (doxyl) group attached to either carbon atoms 5 (5-doxylstearic acid), 7 (7-doxylstearic acid), 12 (12-doxylstearic acid), or 16 (16-doxylstearic acid) were purchased from the Syva Company (Palo Alto, CA). Stearic acid spin label with the doxyl group attached to carbon 14 (14-doxylstearic acid) was a gift from D. Marsh. The fatty acid spin labels were incorporated into the T-tubule membrane by adding a small aliquot of an ethanol solution; the final concentration of spin label was 0.5–1% (mol/mol) relative to the membrane phospholipids. Ethanol concentration was  $<1\%$ .

Electron paramagnetic resonance (EPR) spectra were obtained with a Varian E-109 spectrometer with a V4550 variable temperature controller (Varian Associates, Palo Alto, CA) as described previously (20). EPR spectra were recorded at 9.1 GHz, at a peak-to-peak modulation amplitude of 2 G, a microwave power setting of 10 mW, and a field modulation of 100 kHz. All spectra were obtained from samples containing 5–10 mg of protein per ml, in 0.3 M sucrose, 20 mM Tris-maleate pH 7.0.

For the spin labels undergoing rapid anisotropic motion, the order parameter  $S$  was calculated (21, 22) as

$$S = \frac{1}{2} (3[\cos^2 \theta] - 1) = \frac{a_0}{a'} \frac{(T_{\parallel} - T_{\perp})}{(T_{zz} - T_{xx})},$$

where  $\theta$  is the angular deviation of the nitrogen  $2p\pi$  orbital from the

bilayer normal (the brackets denote an ensemble average), and  $T_{\parallel}$  and  $T_{\perp}$  are half the separation of the outer and inner hyperfine splittings, respectively; the factor  $a_0/a'$  is a correction of the polarity dependence of the hyperfine splitting, with  $a_0 = 1/3 (T_{xx} + T_{yy} + T_{zz})$  and  $a' = 1/3 (T_{\parallel} + 2 T_{\perp})$ . The values of  $T_{xx} - T_{yy} = 5.8$  G and  $T_{zz} = 30.8$  G (21) were used. It was not possible to determine  $S$  at all temperatures for all spin labels used, since neither the inner nor the outer splitting was well defined in all cases.

## RESULTS

The EPR spectra for the fatty acid spin labels incorporated into T-tubule membranes and measured at  $37^{\circ}\text{C}$  showed that, as seen in other membranes, the motion of the label increases as the position of the doxyl group is removed from the polar head region (Fig. 1). The inner and outer splittings were clearly defined for 5-, 7-, 12-, and 14-doxylstearic acid, indicating rapid but anisotropic motion; in contrast, the spectrum of 16-doxylstearic acid at  $37^{\circ}\text{C}$  showed rapid and nearly isotropic motion, without clear-cut resolution of inner and outer splittings.

The EPR spectra were recorded as a function of temperature for all five spin labels, and the corresponding order parameters were determined. For 5-doxylstearic acid and 7-doxylstearic acid, order parameters could only be

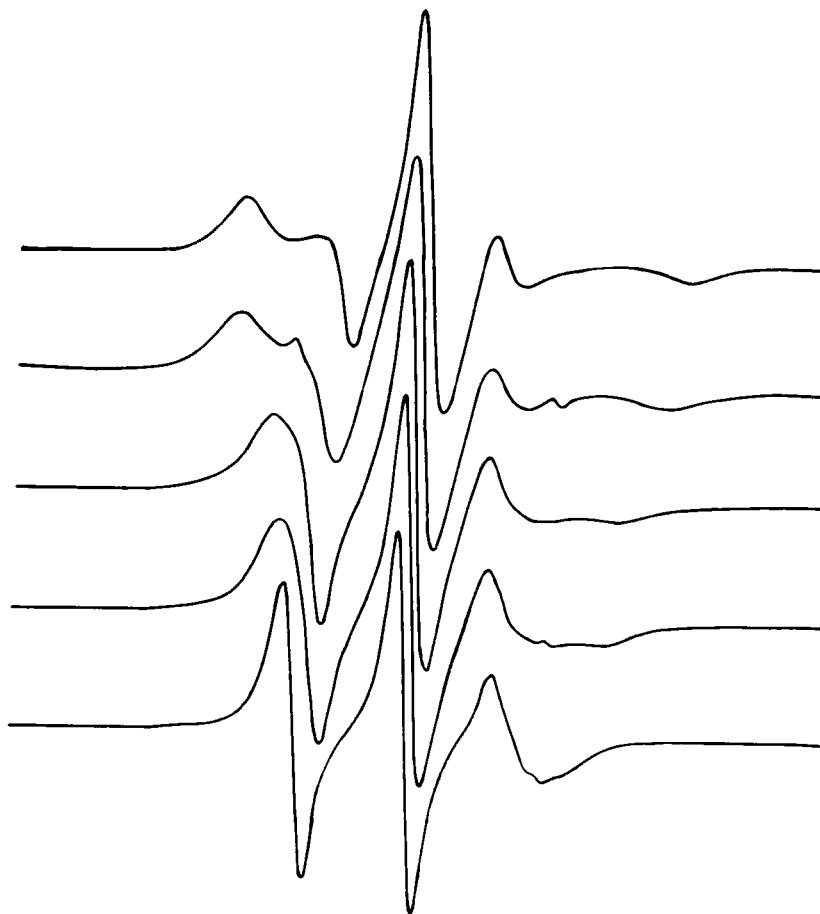


FIGURE 1 EPR spectra of 5-, 7-, 12-, 14-, and 16-doxylstearic acid (from top to bottom) incorporated into T-tubule membranes. All spectra were recorded at  $37^{\circ}\text{C}$ . Experimental conditions are given in the text. The spectral width in this and subsequent figures corresponds to 100 G.

determined above 15°C. A linear decrease of order parameter with increasing temperature from 15 to 40°C was observed (Fig. 2). Below 15°C the spectra indicated little or no motion in the  $10^{-7}$  s range, with the value of the outer splitting approaching  $2 T_{zz}$  and the inner splitting not being resolved.

For 12-doxylstearic acid and 14-doxylstearic acid, order parameters could be accurately calculated from below 10°C to 38–40°C, with a linear decrease of  $S$  on increasing temperature (Fig. 2). For 16-doxylstearic acid,  $S$  values could only be calculated from 2 to 16°C (Fig. 2), since above 16°C the spectra became nearly isotropic.

At any given temperature, the order parameters decreased as the distance between the doxyl group and the polar head group increased (Fig. 3), indicating, in common with other membranes studied, that there is a flexibility gradient in the T-tubule membrane lipid phase at all temperatures. At 16°C, the changes in  $S$  were slightly more marked the farther along the fatty acyl chain the doxyl group was located, whereas at 25 and 37°C,  $S$  changed in an almost linear fashion with the position of the reporter group.

A comparison of the order parameter values obtained at 37°C for 5-doxylstearic acid and 12-doxylstearic acid in a variety of biological membranes was presented by Hauser

et al. (23). A summary of that data plus the  $S$  values obtained in the present work are given in Table I. In general, for all mammalian membranes the order parameters of 5-doxylstearic acid lie between 0.53 and 0.60 at 37°C. The value of 0.68 measured for the T-tubule membrane at 37°C is significantly higher, with differences in order parameter values in the range of 0.08 to 0.15, well outside the error of the determination. In fact, the order parameter for T-tubules approaches the value of  $S$  of 0.71 described for *Halobacterium cutirubrun* (29, Table I). This finding indicates that the lipid segments in the vicinity of the polar head group are more motionally restricted in T-tubules than in other mammalian membranes.

The 12-doxylstearic acid spin label incorporated into the T-tubule membrane showed an intermediate behavior between that of mammalian and procaryote membranes, with order parameters of 0.44 for T-tubules and 0.62 for *Halobacterium* at 37°C (Table I). The value of  $S = 0.44$  is closer to the  $S$  values of other mammalian membranes, which lie in the range of 0.33–0.38, than those of procaryote membranes (Table I). Thus, on approaching the center of the bilayer, the T-tubule membrane becomes considerably more fluid than the procaryote membranes, but still remains less fluid than the other mammalian membranes shown in Table I.

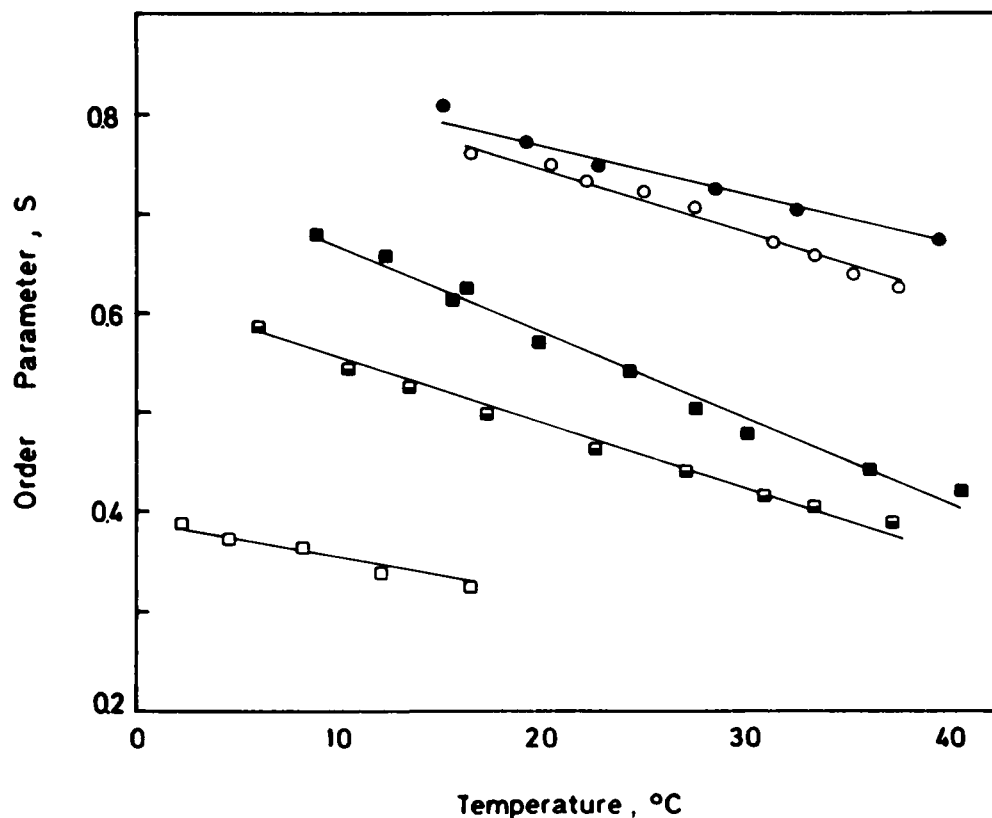


FIGURE 2 Order parameter  $S$  as a function of temperature for stearic acid spin labels incorporated into T-tubule membranes. ●, 5-doxylstearic acid; ○, 7-doxylstearic acid; ■, 12-doxylstearic acid; ■, 14-doxylstearic acid; □, 16-doxylstearic acid.

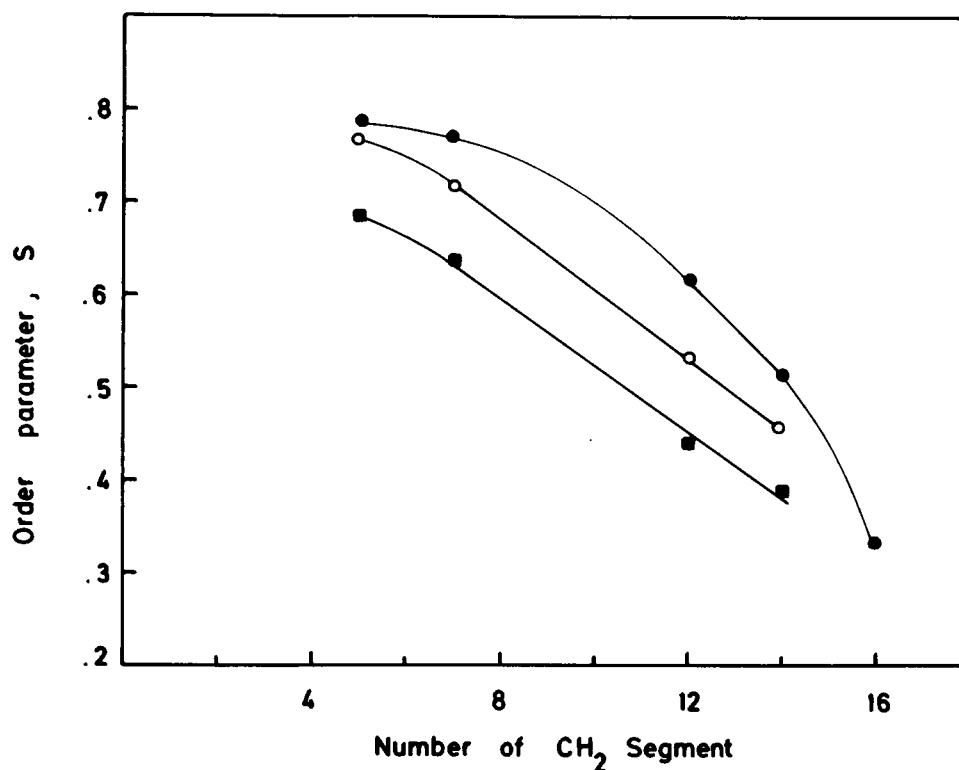


FIGURE 3 Order parameters  $S$  measured at different temperatures and plotted as a function of the position of the doxyl group along the fatty acyl chain. ●, measured at 16°C; ○, measured at 25°C; and ■, measured at 37°C.

To probe the region closer to the center of the lipid bilayer, 16-doxylstearic acid was used. As described above, at 37°C the spectra showed nearly isotropic motion (Fig. 1), so that order parameters could not be calculated. Accordingly, an effective correlation time was calculated as described (30).

The value of the effective rotational correlation time at 37°C for 16-doxylstearic acid incorporated in T-tubules was  $14 \times 10^{-10}$ s, lower than the value of  $20.2 \times 10^{-10}$ s reported for rabbit small intestinal brush border vesicles (23), but higher than the value of  $10 \times 10^{-10}$ s determined in this work for sarcoplasmic reticulum. These results

TABLE I  
ORDER PARAMETERS\* FOR SPIN-LABELED STEARIC ACIDS INCORPORATED  
INTO A VARIETY OF BIOLOGICAL MEMBRANES

Membrane	5-doxylstearic acid	12-doxylstearic acid	Reference
Mammalian membranes			
transverse-tubules‡	$0.68 \pm 0.01$	$0.44 \pm 0.01$	this work
sarcoplasmic reticulum‡	$0.58 \pm 0.01$	§	this work
small intestinal brush‡			
border vesicles	0.60	0.38	23
Chinese hamster ovary	0.53	—	24
chromaffin granule membranes	0.55	0.37	25
plasma membrane of sarcoma 180 mouse ascites tumor	0.55	0.33	26
Procaryote cell membranes			
<i>Tetrahymena pyriformis</i> , microsomes	—	$0.56-0.61$ ¶	27
<i>Halobacterium cutirubrum</i>	0.72	0.62	28

\*All values were measured at 37°C.

‡From rabbit.

§Order parameters could not be measured since at 37°C the motion became nearly isotropic.

||From cow.

¶These values were obtained from organisms grown at 15°C and 40°C, respectively.

indicate that in the center of the bilayer the fluidity of the T-tubule membrane is intermediate between the fluidity of these two other mammalian membranes.

A comparison of the EPR spectra of 12-doylestearic acid and 14-doylestearic acid incorporated either in T-tubule or in SR vesicles at different temperatures (Figs. 4 and 5) revealed that while the EPR spectra of SR visually showed two components at low temperatures, only one component was visually apparent in the T-tubules at all temperatures.

A plot of  $S$  as a function of temperature (Fig. 6) for 5-doylestearic acid incorporated in sarcoplasmic reticulum and in T-tubules shows that in the temperature range studied the  $S$  values at any given temperature are higher for T-tubules than for SR. A plot of  $2T_1$  vs. temperature for 12-doylestearic acid (it was not possible to measure  $2T_1$  in SR above 8°C, due to the presence of two components; see Fig. 5) shows that all temperatures studied the values for the inner splitting are lower for T-tubules (Fig.

6), indicating less motional freedom of the probe in this region of the T-tubule bilayer than in the corresponding region of the SR membrane. In addition, at 37°C the effective correlation time determined with 16-doylestearic acid was higher for T-tubules than for SR (see above). Accordingly, these results suggest that the T-tubule membrane is less fluid than the SR membrane along the entire lipid bilayer width.

## DISCUSSION

As shown in this work, the EPR spectra for a variety of stearic acid spin labels incorporated into T-tubule membranes are characteristic of anisotropic motion, with unrestricted high frequency (subnanosecond) rotation about the bilayer normal and restricted high frequency (subnanosecond) reorientation of the long fatty acyl chain axis with respect to its average orientation normal to the bilayer. The order parameter is a measure of the amplitude of rapid

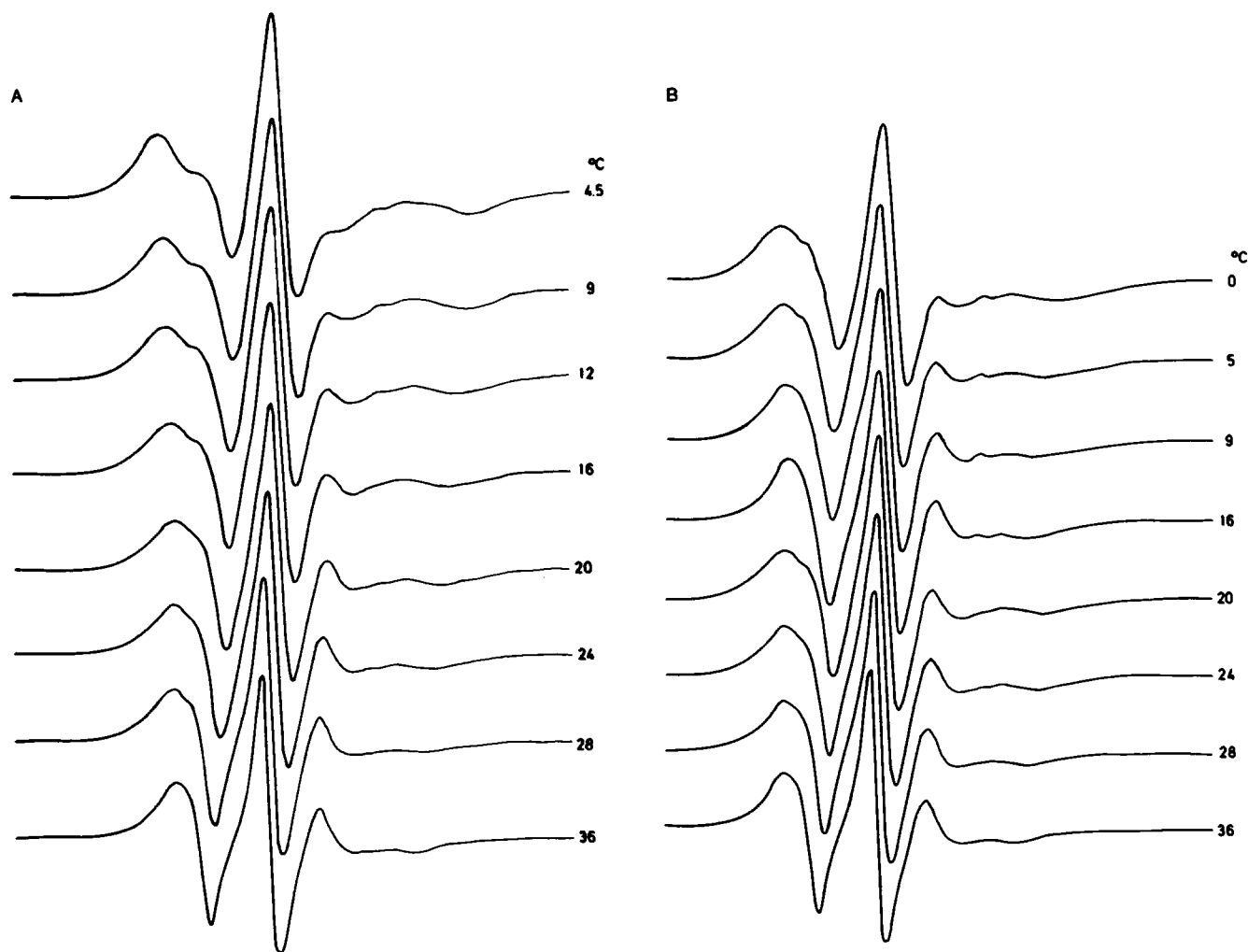


FIGURE 4 EPR spectra of 12- and 14-doylestearic acid incorporated into T-tubule membranes as a function of temperature. (A) 12-doylestearic acid; (B) 14-doylestearic acid.

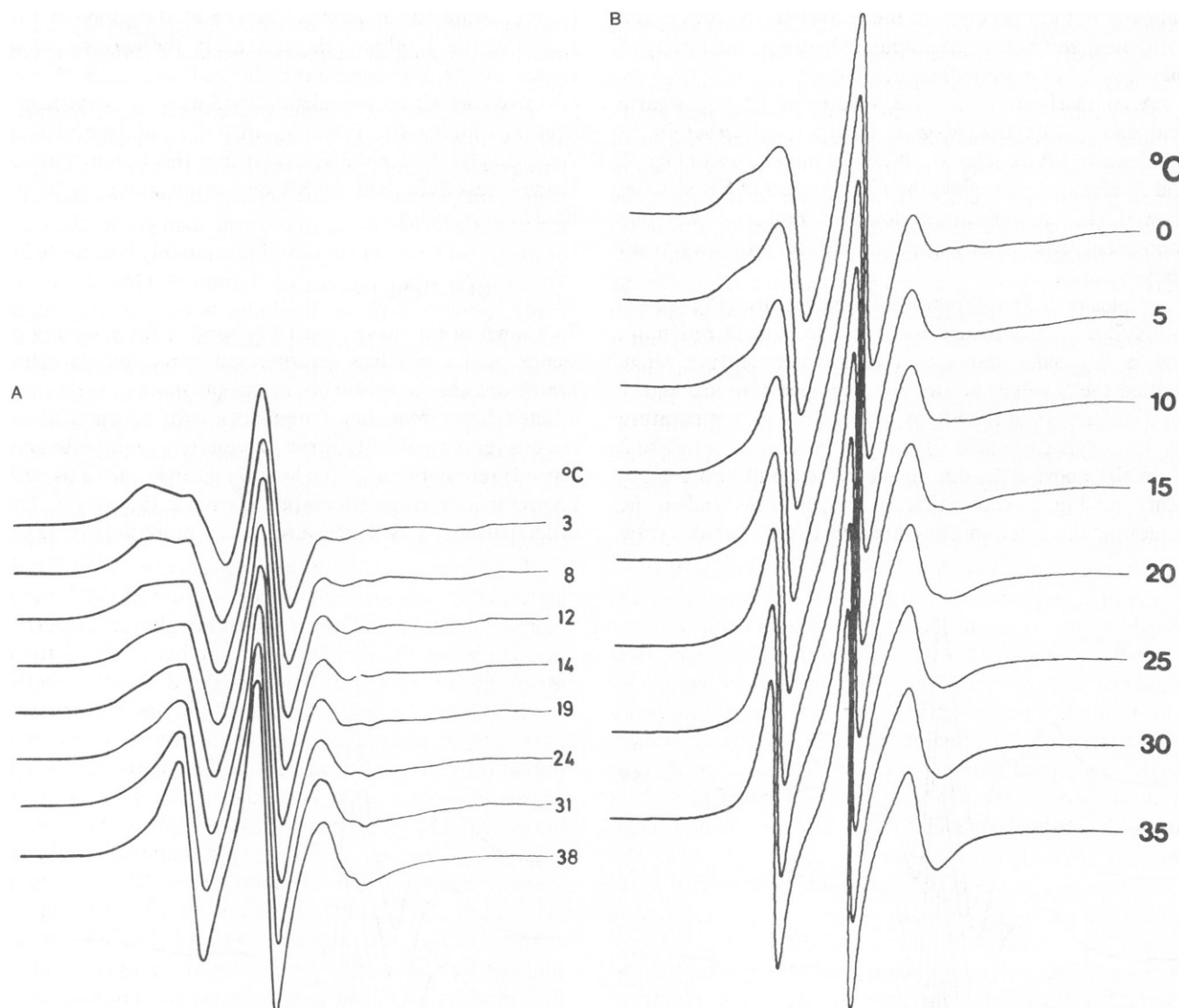


FIGURE 5 EPR spectra of 12- and 14-doxylstearic acid incorporated into sarcoplasmic reticulum membranes. (A) 12-doxylstearic acid; (B) 14-doxylstearic acid.

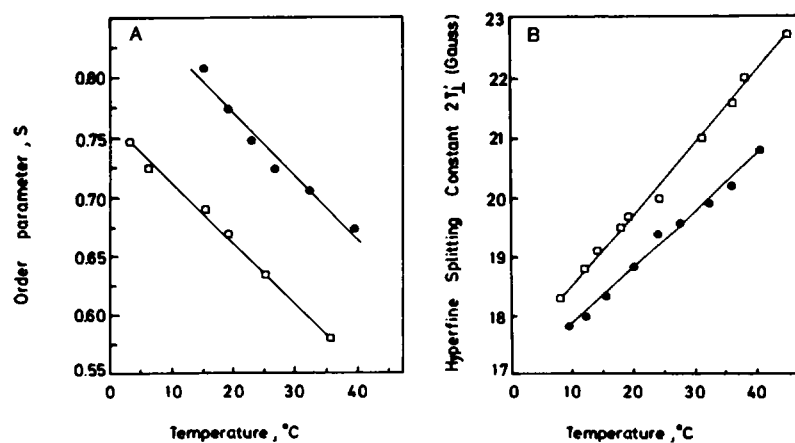


FIGURE 6 (A) Order parameters  $S$  measured at different temperatures for 5-doxylstearic acid incorporated into T-tubule membranes (●) or SR membranes (□). (B) Hyperfine splitting constants as a function of temperature for 12-doxylstearic acid incorporated into T-tubule membranes (●) or SR membranes (□).

rotations (subnanosecond) of the molecular long axis with respect to the average orientation of the fatty acid chains in the bilayer (21, 22). As shown above, the values of the order parameter for the spin labels incorporated in T-tubules are a function of both temperature and position of the doxyl group along the fatty acyl chain. As shown in other membranes studied, at any given temperature the order parameter decreases as the position of the doxyl group approaches the end methyl group. At low temperatures the order parameter has very similar values for the spin labels containing the doxyl group attached to the fifth or seventh carbon atom, and decreases progressively but it is still measurable with the 16-doxylstearic acid spin labels. On increasing temperature, there is a steady decrease of the order parameter obtained with 5-, 7-, 12-, and 14-doxylstearic acid, and the center of the bilayer is characterized by nearly isotropic motion, so that it is no longer possible to measure order parameter with 16-doxylstearic acid. These results show that the T-tubule membrane, in common with many other biological membranes, displays a flexibility gradient from its surface to its center, the flexibility increasing towards the center of the bilayer.

It is noteworthy that the values of the order parameter obtained at 37°C with 5-doxylstearic acid are higher for the T-tubule membrane than for other mammalian membranes studied. This finding might be related to the very high cholesterol content of the T-tubule membrane (10, 11), since cholesterol may increase the packing order of the T-tubule phospholipids (31). However, cholesterol is located to a depth of about carbon 10, so that this presumptive effect of cholesterol would be restricted to the region of the fatty acyl chain closer to the polar head group. Consistent with this view, 12-doxylstearic acid gives, at 37°C, order parameters with values closer to those found in other mammalian membranes, and the spectra of 16-doxylstearic acid at 37°C indicate nearly isotropic motion in the center of the bilayer. However, any detailed model involving cholesterol should be checked experimentally using lipid extracts of T-tubules with or without cholesterol.

In the case of the T-tubule membrane, the EPR spectra apparently show only one component with all the spin labels and at all the temperatures studied. In contrast, for the sarcoplasmic reticulum membrane, the EPR spectra of 12-doxylstearic acid and 14-doxylstearic acid clearly show two components at low temperatures, and even with 16-doxylstearic acid it is possible to detect a two-component spectra at 4°C (32). Although a detailed quantitative description of the two spectral components observed with SR is outside the scope of the present study, it is noteworthy to recall that it has been proposed that the  $\text{Ca}^{2+}$ -ATPase of SR exerts a perturbing effect on 30–35 molecules of lipid per enzyme molecule (32). These lipids, the boundary lipids or annulus, are more immobilized on the EPR time scale than the bulk bilayer lipids and thus yield two-component EPR spectra (29, 32–35). Boundary and

bilayer lipids exchange at rates faster than 10  $\mu\text{s}$ , as indicated by nuclear magnetic resonance (NMR) experiments (36).

In the absence of a quantitative spectral analysis, it is not possible to decide if T-tubules have boundary lipids. However, there is little information as to what the intrinsic membrane proteins of T-tubules are and only one study has reported the existence of an intrinsic membrane, the  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ATPase, which would presumably constitute 20–30% of the total protein of T-tubules (16). Since the lipid-to-protein ratio of T-tubules is considerably higher than that of SR (9, 10), even if the  $\text{Ca}^{2+}$ - or  $\text{Mg}^{2+}$ -ATPase of T-tubules had a similar number of boundary-lipids as the  $\text{Ca}^{2+}$ -ATPase of SR, they would represent <4% of the total lipids, as opposed to 35% in the case of SR. Furthermore, since the T-tubule membrane is less fluid than the SR membrane, it might prove to be difficult to detect a more immobilized boundary lipid component, even if it represented a more significant fraction of the total lipids.

To conclude, it is interesting to speculate what physiological relevance the high degree of order of the T-tubule membrane lipid phase might have. It might serve a purpose in modulating the very low permeability of the T-tubule membrane to ions (37), it might confer different properties to the sodium channels present in T-tubules with regard to those of the external plasma membrane (8) and, more importantly, it might have a role in maintaining a high degree of structural organization of the T-tubule membrane required for successful E-C coupling. Note in this respect that removal of cholesterol of mammalian skinned fibers perturbs excitation-contraction (E-C) coupling (Donaldson, S., personal communication), a finding that supports a role for the lipid phase of T-tubules in this process. Furthermore, a change in the properties of the lipid phase of the T-tubule membranes was proposed as one of several possible explanations for the observed effect of temperature on E-C coupling in frog twitch muscle fibers (38), where a decrease in temperature produces an increase in the latency between depolarization and the onset of the rise in intracellular free  $\text{Ca}^{2+}$ .

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