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Depolarization of dehydroergosterol in phospholipid bilayers

Parkson Lee-Gau Chong and T.E. Thompson

Department of Biochemistry, University of Virginia, Charlottesville, VA 22908 (U.S.A.)

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The behavior in phospholipid bilayers of low concentrations of dehydroergosterol, a fluorescent cholesterol mimic, has been examined by fluorometry and calorimetry. In contrast to many fluorescent membrane probes, dehydroergosterol shows a decrease in fluorescence anisotropy when the matrix phospholipid goes from the liquid-crystalline to the gel state. This was observed in three systems in which the matrix lipid was either dipalmitoyl- or dimyristoylphosphatidylcholine or dilauroylphosphatidylethanolamine. The decrease in anisotropy is the result of a large increase in the fluorescence life time of dehydroergosterol in these bilayer systems which is probably the result of thermal quenching of dehydroergosterol by neighboring molecules. The rotation of dehydroergosterol in these bilayers can be described in terms of the thermal coefficient of frictional resistance offered by the environment (Weber et al. (1984) Biochemistry 23, 6785–6788). The thermal coefficients are observed to change abruptly at the onset and completion temperatures of the gel to liquid-crystalline phase transition temperatures of the three matrix phospholipids. These changes are, however, much smaller than are the corresponding changes in the thermal coefficient observed for the fluorescent probe diphenylhexatriene in dilauroylphosphatidylethanolamine bilayers. The difference in behavior of the two fluorescent probes may be the result of lateral phase separation of dehydroergosterol similar to that reported for cholesterol in similar systems.

Introduction

Cholesterol is an important component of the plasma membranes of most eukaryotic cells. Its dynamic behavior and molecular organization are not well understood, however, in either biological membranes or simple phospholipid bilayers. This situation is due to the fact that it is relatively difficult to observe cholesterol directly by NMR methods and fluorescent or spin-labelled derivatives are generally poor cholesterol mimics in both biological and physical properties. Such is not the case with dehydroergosterol ($\Delta^{5,7,9(11),22}$-ergostatetraene-3-β-ol). This fluorescent sterol occurs naturally in several biological membranes [1,2] and resembles both cholesterol and ergosterol in its physiological properties [3–5]. A number of studies have employed dehydroergosterol as a cholesterol mimic [4,6–8]. Smutzer and Yeagle [7] reported the surprising fact that the steady-state fluorescence anisotropy of dehydroergosterol in the gel state of L-α-dimyristoylphosphatidylcholine (DMPC) is less than that in the liquid-crystalline state. This behavior is surprising since it is in marked contrast to that observed for commonly

Abbreviations: dehydroergosterol (DHE), $\Delta^{5,7,9,(11),22}$-ergostatetraene-3β-ol; DPH, diphenylhexatriene; DMPC, L-α-dimyristoylphosphatidylcholine; DLPE, L-α-dilauroylphosphatidylethanolamine; DPPC, L-α-dipalmitoylphosphatidylcholine.

Correspondence: Dr. T.E. Thompson, Department of Biochemistry, University of Virginia, Charlottesville, VA 22908, U.S.A.

used membrane probes such as diphenylhexatriene (DPH) and perylene which exhibit higher steady-state anisotropies in the gel state than in the liquid-crystalline state in DMPC bilayers (see, for example, Ref. 9); a result that is certainly more reasonable, since diphenylhexatriene anisotropy reflects principally the molecular order in the membrane, and membrane order in the gel state is higher than that in the liquid-crystalline state [10].

The basis for the unusual anisotropy change in dehydroergosterol fluorescence observed at the gel to liquid crystalline phase transition of DMPC and the relation of this change to the alteration in the physical state of the matrix phospholipid have not been elucidated. In this communication, we first examine the origin of the dihydroergosterol depolarization phenomenon described above. In the second part, we use the thermal coefficient of fractional resistance derived from fluorescence measurements as a parameter to describe phenomenologically the rotation of dehydroergosterol in lipid bilayers. Calorimetric studies are used to relate rotation to the physical state of the matrix lipid. A preliminary report of this work has appeared elsewhere [11].

Materials and Methods

Materials. L-α-Dilauroylphosphatidylethanolamine (DLPE), L-α-dipalmitoylphosphatidylcholine (DPPC) and L-α-dimyristoylphosphatidylcholine (DMPC) were purchased from Avanti Biochemicals (Birmingham, AL). Dehydroergosterol was obtained from Frann Scientific Inc. (Columbia, MO) and was purified by high pressure liquid chromatography with a C-18 reverse-phase column (4 mm × 15 cm, Micropak, MCH-5-n-ap, Varian Instrument Incorp, Palo Alto, CA) using methanol/acetonitrile (67 : 33, v/v) as the mobile phase (Schroeder, Barenholz, and Thompson, unpublished results).

Preparation of liposomes. Dehydroergosterol dissolved in methanol was first evaporated to dryness under nitrogen, and then redissolved in chloroform. Either DLPE, DPPC, or DMPC, dissolved in chloroform, was then mixed with dehydroergosterol in chloroform and the mixture was dried under vacuum. Liposomes were prepared under nitrogen as multilamellar vesicles, by the method of Bangham et al. [12], in 22.5 mM phosphate buffer at pH 7.1 or in 50 mM KCl containing 0.02% NaN_3. The liposomes were always prepared at a temperature higher than the main phase transition temperature of the bulk lipids. The phospholipid concentration of the lipid stock solution was determined as inorganic phosphate by the method of Bartlett [13]. The concentration of dehydroergosterol was determined using an extinction coefficient at 326 nm, in dioxane equal to 10 600 $M^{-1} \cdot cm^{-1}$ [6].

DLPE dispersions containing diphenylhexatriene were made by the injection of concentrated diphenylhexatriene in tetrahydrofuran into the phospholipid dispersion with vigorous stirring. The mole ratio of diphenylhexatriene to phospholipid was about 1 : 1900.

Fluorescence measurements. Fluorescence anisotropy and intensity measurements were made with an SLM 4800 fluorometer. A dispersion of multilamellar vesicles with a concentration of phosphate approximately equal to that in the sample was used as the blank solution to correct for scattered light. Similar corrections were made in the determination of emission spectra. Quenching of dehydroergosterol emission by acrylamide was measured by monitoring the total fluorescence intensity from 340 nm to 500 nm using an excitation wavelength of 324 nm. The standard deviation of the polarization values is approximately ± 0.005.

Fluorescence lifetime measurements. Fluorescence lifetimes of dehydroergosterol in liposomes were determined in Dr. E. Gratton's laboratory at the University of Illinois using a continuously variable modulation frequency fluorometer [14]. The light source was an He-Cd laser (Liconix Inc, Sunnyvale, CA). The excitation wavelength used was 325 nm. The excitation polarizer was set at 35° with respect to the vertical plane. No emission polarizer was used. A suspension of glycogen in water was used as the scattering reference signal. For dehydroergosterol emission, a 400 nm interference filter was used. Phase and modulation data were determined with modulation frequencies ranging from 20 MHz to 190 MHz. The data were analyzed using the non-linear least-squares program provided by ISS Inc. (La Spezia, Italy) based on the scheme described in [15,16]. A random

error of 0.2° for phase angle and a random error of 0.004 for modulation values were used in the least-squares fit. Lifetimes of diphenylhexatriene in DLPE were measured using an SLM 4800 fluorometer with a modulation frequency of 6 MHz.

Differential scanning calorimetry. Calorimetric measurements were made with a MicroCal differential scanning calorimeter (Model MC-2, Amherst, MA). The liposome dispersions were first incubated at temperatures above 45°C for at least 1 h and then loaded into the calorimeter at room temperature. The sample was scanned in the ascending temperature mode at an average rate of 15 Cdeg/h.

Results

Dehydroergosterol anisotropy in glycerol

The fundamental anisotropy, A_0, was determined in glycerol at −12°C. Fig. 1 shows the excitation wavelength dependence of A_0. The difference in A_0 between 324 nm and 351 nm is quite small. Fig. 1 also shows the temperature dependence of dehydroergosterol anisotropy in glycerol.

Temperature dependence of dehydroergosterol anisotropy in phospholipid liposomes

Figs. 2A, B show the temperature dependence of the steady-state fluorescence anisotropy of 4.8 mol% dehydroergosterol in DMPC and 4.0 mol% dehydroergosterol in DLPE with excitation at 325 nm. It is apparent that the dehydroergosterol anisotropy decreases in the temperature interval from 35°C to 15°C, the same temperature region in which the matrix phospholipids undergo the liquid-crystalline to gel phase transition. A similar result was obtained for dehydroergosterol in DPPC liposomes (data not shown). These results are in qualitative agreement with that previously reported by Smutzer and Yeagle [7] who used DMPC as the matrix lipid.

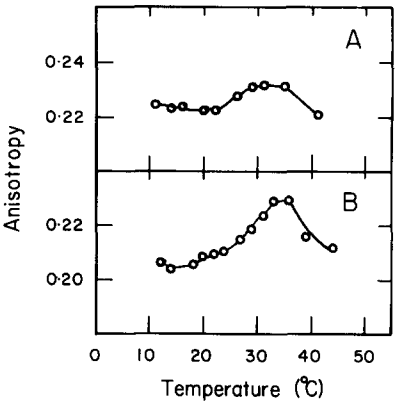

Fig. 2. The steady-state anisotropy of dehydroergosterol emission as a function of temperature (A) in DMPC multilamellar vesicles containing 4.8 mol% dehydroergosterol and (B) in DLPE multilamellar vesicles containing 4.0 mol% dehydroergosterol. λ_{em} 325 nm, average heating rate 4 Cdeg/h, λ_{em} 398 nm.

Red edge effect

Fig. 3 shows the fluorescence intensity of dehy-

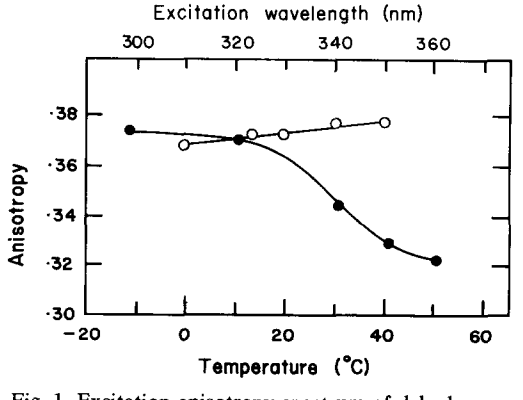

Fig. 1. Excitation anisotropy spectrum of dehydroergosterol in glycerol at −12°C (○), λ_{em} 390 nm; and, dependence of dehydroergosterol anisotropy in glycerol on temperature (●), λ_{ex} 324 nm and λ_{em} 390 nm.

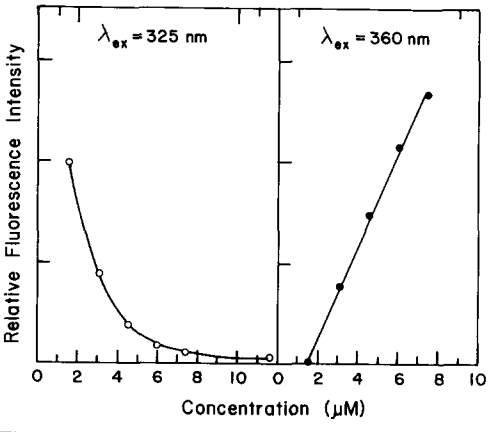

Fig. 3. Fluorescence intensity of dehydroergosterol in ethanol as a function of concentration at two excitation wavelengths. Temperature 12.8°C, λ_{em} 390 nm.

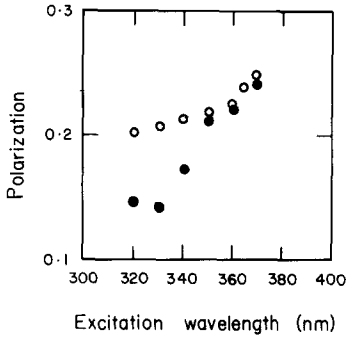

Fig. 4. Excitation polarization spectrum of $3.1 \cdot 10^{-6}$ M (○) and $7.4 \cdot 10^{-6}$ M (●) dehydroergosterol in ethanol. Temperature 12.8°C, λ_{em} 390 nm.

droergosterol in ethanol as a function of concentration at two excitation wavelengths. When using a blue edge wavelength, 325 nm, the intensity decreases with concentration. In contrast, the intensity increases linearly with concentration when a red edge wavelength, 360 nm, was used. Fig. 4 shows the anisotropy of dehydroergosterol in ethanol as a function of excitation wavelength at two concentrations. A depolarization caused by an increase in dehydroergosterol concentration is readily observed when the excitation wavelength is between 320 and 340 nm. This depolarization, however, disappears for excitation wavelengths between 350 nm and 370 nm. Thus, there is a red edge effect which indicates that depolarization resulting from homotransfer occurs [17]. Although homotransfer depolarization usually occurs in the range above 1 mM, it is probable that dehydroergosterol forms aggregates or micelles in the apparent concentration range of 1–10 μM. It is of interest to note that a red edge effect is observed in both depolarization (Fig. 4) and in intensity (Fig. 3). The former is well documented [17], however, the latter is not.

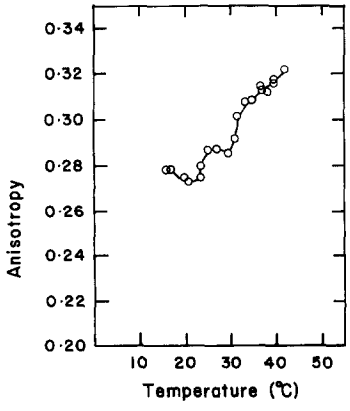

Fig. 5. The steady-state anisotropy of 4.8 mol% dehydroergosterol in DMPC as a function of temperature. λ_{ex} 351 nm. λ_{em} 398 nm.

In order to test whether the depolarization shown in Fig. 2 is caused by homotransfer, 351 nm was used as the excitation wavelength. The results for 4.8 mol% dehydroergosterol in DMPC are reported in Fig. 5. A decrease in anisotropy with decreasing temperature is still seen in the same temperature region where the bulk lipids become more ordered. Thus the depolarization is probably not due to either homotransfer or a change in homotransfer.

Acrylamide quenching

The fluorescence intensity quenching of 4.0 mol% dehydroergosterol in DLPE by acrylamide at 26.2°C and 43.1°C is shown in Fig. 6. Taking the lifetime in the absence of quencher, τ_0, to be 1.24 ns at 26.2°C and 0.52 ns at 43.1°C (see below and Fig. 7B) and assuming a dynamic quenching mechanism, the quenching rate constant, k^*, can be calculated using the equation

$$F^0/F = 1 + k^*\tau_0\,[\text{quencher}]$$

to be $k^* = 0.613\ \text{M}^{-1} \cdot \text{ns}^{-1}$ (at 43.1°C) and $k^* = 0.082\ \text{M}^{-1} \cdot \text{ns}^{-1}$ (at 26.2°C).

Lifetime measurements of dehydroergosterol in DMPC and DLPE

We have examined the temperature dependence

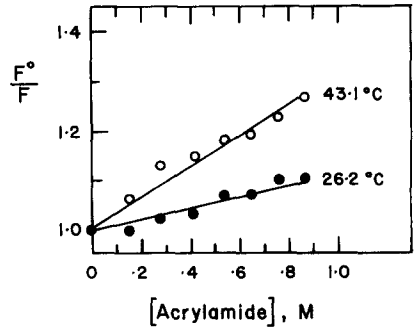

Fig. 6. Relation between F^0/F and acrylamide concentration for dehydroergosterol in DLPE. 5 M acrylamide was used as the stock solution. The intensities were corrected for background signals and for volume changes. λ_{ex} 324 nm.

TABLE I

LIFETIMES, PRE-EXPONENTIAL FACTORS, AND FRACTIONAL INTENSITIES AS ANALYZED BY A TWO-COMPONENT FIT AND A ONE-COMPONENT FIT

DHE, dehydroergosterol.

Temp. (°C)	Two-component fit					One-component fit	
	τ_1 (ns)	τ_2 (ns)	f_1	α_1	χ^2	τ (ns)	χ^2
A. 4.8 mol% DHE in DMPC							
12.0	2.05	1.13	0.882	0.805	2.87	1.89	7.90
18.5	1.44	3.60	0.937	0.974	3.42	1.50	7.53
22.1	1.18	2.01	0.835	0.896	3.09	1.27	6.63
25.4	0.98	13.02	0.999	1.00	4.67	0.99	4.20
28.5	0.82	10000.00	0.993	1.00	4.34	0.82	4.82
32.2	0.69	131.00	0.987	1.00	2.48	0.69	4.04
43.0	0.43	6.18	0.991	1.00	0.92	0.43	2.34
B. 4.0 mol% DHE in DLPE							
18.3	1.28	3.17	0.809	0.913	4.33	1.45	39.6
26.7	0.98	2.83	0.874	0.952	1.51	1.09	28.5
29.4	0.85	5.19	0.964	0.994	3.01	0.87	13.3
31.8	0.75	6.36	0.987	1.00	2.42	0.76	4.27
35.6	0.66	13.50	0.989	1.00	3.12	0.66	5.26
42.0	0.54	9.40	0.990	0.999	2.51	0.54	4.05

of the lifetimes of 4.8 mol% dehydroergosterol in DMPC and of 4.0 mol% dehydroergosterol in DLPE. The measured phase delay and modulation data were fitted to the exponential decay law

$$I(t) = \sum_i \alpha_i \exp(-t/\tau_i)$$

where $I(t)$ is the fluorescence intensity, α_i is the pre-exponential factor, and τ_i is the fluorescence lifetime from the ith component. The results are given in Table I. Note in Table I that

$$f_i = \alpha_i \tau_i / \sum_j \alpha_j \tau_j$$

where f_i is the fraction of the total fluorescence intensity derived from the ith component.

For 4.8 mol% dehydroergosterol in DMPC, at 25.4°C and 28.5°C, no improvement in χ^2 is gained with a two-component compared to a one-component fit. Although at 32.2°C and 43.0°C some improvement in χ^2 is seen with a two-component fit, the pre-exponential factor of the minor component is quite small. In addition, between 25.4°C and 43.0°C, the lifetime of the minor component in the two-component fit varies randomly with temperature. This is most likely the result of random measurement errors. Furthermore, some of the lifetimes of the minor component are unrealistically large (e.g. 10000 ns and 131 ns), apparently the result of overfitting. Thus, dehydroergosterol emission in DMPC within this temperature range is best described by a single exponential decay.

At 12.0, 18.5 and 22.1°C, χ^2 decreases significantly with two-component compared to one-component fits, and the pre-exponential factor of the minor component is substantial. A three-component fit does not give better χ^2 values (data not shown). Thus at temperatures between 12.0°C and 22.1°C, dehydroergosterol emission in DMPC is best described by a double-exponential decay law.

The one-component fit lifetimes at temperatures between 25.4°C and 43.0°C and the average lifetime $\langle\tau\rangle = (\tau_1)f_1 + (\tau_2)f_2$ at temperatures below 25.4°C are plotted in Fig. 7A as a function of temperature. Note that even for best fits χ^2 is not near 1. This is because the estimated errors are smaller than the true errors.

For 4.0 mol% dehydroergosterol in DLPE, similar results were obtained. Between 18.3°C and

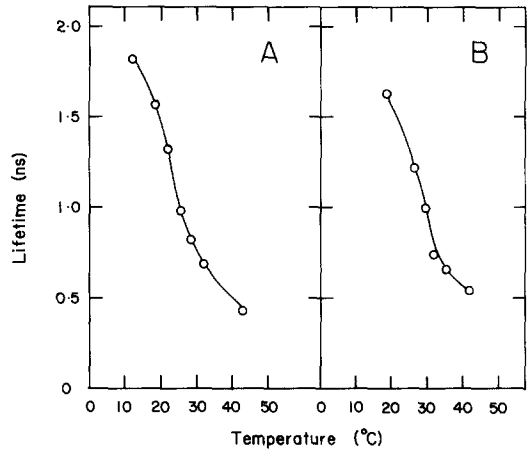

Fig. 7. Dependence of the lifetime of dehydroergosterol emission on temperature for (A) 4.8 mol% dehydroergosterol in DMPC and for (B) 4.0 mol% dehydroergosterol in DLPE.

29.4°C, a large decrease in χ^2 is seen when a two-component fit is compared to a one-component fit, and the pre-exponential factor of the minor component is substantial. In this temperature region, a single-exponential decay law is inadequate to describe the dehydroergosterol emission. Although we have also attempted a three-component fit, no improvement in χ^2 resulted. Thus, dehydroergosterol emission in DLPE at between 18.3°C and 29.4°C can best be described by a double-exponential decay law.

In contrast, at temperatures between 31.8°C and 42.0°C, not much improvement in χ^2 is seen when the two-component fit is compared to a one-component fit. In addition the pre-exponential factor of the minor component is quite small and varies randomly with temperature. In this temperature region, dehydroergosterol emission in DLPE appears to follow a single exponential decay law.

For 4.0 mol% dehydroergosterol in DLPE, lifetimes from the single-component fit at temperatures between 31.8°C and 42.0°C and the average lifetime for temperatures between 18.3°C and 29.4°C are plotted in Fig. 7B as a function of temperature.

Temperature dependence of emission intensity of dehydroergosterol in liposomes

Emission spectra of 4.8 mol% dehydroergosterol in DMPC and 4.0 mol% dehydroergosterol in DLPE were measured as a function of temperature. No shift in emission maxima of dehydroergosterol in lipid bilayers was observed (data not shown). The emission intensity of dehydroergosterol in DMPC and in DLPE as a function of temperature is plotted in Figs. 8A and 8B, respectively. The intensity variation with temperature was substantial in both systems. This result is similar to that previously reported [4,7]. It should be noted that the trend in the intensity variation shown in Fig. 8 is parallel to the trend in the lifetime variation shown in Fig. 7. In addition, the relative intensity change has a magnitude comparable to the relative lifetime change. For example, at 4.8 mol% dehydroergosterol in DMPC, the dehydroergosterol lifetime falls 66% from 20°C to 40°C while the intensity falls 78% over the same temperature range.

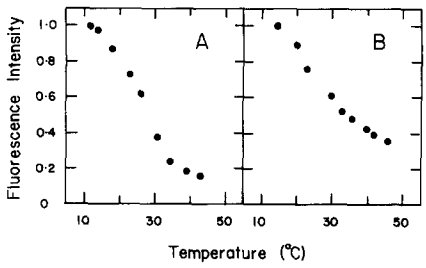

Fig. 8. Dependence of the total intensity of dehydroergosterol emission on temperature for (A) 4.8 mol% dehydroergosterol in DMPC and for (B) 4.0 mol% dehydroergosterol in DLPE.

Frictional resistance to rotation of dehydroergosterol in lipid bilayers

The thermal coefficient of the frictional resistance to the rotation of dehydroergosterol in lipid bilayers can be determined by the method of Weber et al. [18]. For a rotating unit in a medium of viscosity η, the steady-state fluorescence anisotropy A can be related to the fluorescence lifetime, τ, and the effective volume of the rotating unit, V, by the equation

$$(A_0/A) - 1 = RT\tau/\eta V \quad (1)$$

where R is the gas constant, A_0 is the fundamental anisotropy, and T is the absolute temperature. Although the viscosity of the lipid bilayer η is poorly defined, the relationship between the relative viscosity $\eta/\eta(0)$ and the temperature can still

be approximated by

$$\eta/\eta(0) = \exp(b(T(0) - T)) \quad (2)$$

where $\eta(0)$ is the viscosity in the vicinity of the fluorophore at an arbitrarily chosen reference temperature $T(0)$, and b is the thermal coefficient of the viscosity of the lipid bilayer. b is the fractional change in viscosity per degree. Combining Eqns. 1 and 2, one obtains

$$Y = \ln((A_0/A) - 1) - \ln(RT\tau/V) = -\ln(\eta(0)) + b(T - T(0)) \quad (3)$$

Thus, a plot of Y vs. $t = T - T(0)$ should be a straight line if b is independent of temperature.

Using $A_0 = 0.374$ (Fig. 1, 325 nm) and the data shown in Figs. 2A, B, 7A, B, and letting $V = 382$ ml/mol and $T(0) = 0°C$, Y can be calculated for the systems we have examined. The temperature dependence of Y for 4.8 mol% dehydroergosterol in DMPC and for 4.0 mol% dehydroergosterol in DLPE is shown in Figs. 9A, B, respectively.

For 4.8 mol% dehydroergosterol in DMPC (Fig. 9A), all the data can be fitted with three lines with break points at 19.5°C and 27°C. Slopes of these three lines are: b_1(low temperature) = 0.021, b_2(medium temperature) = 0.060, and b_3(high temperature) = 0.042. Since b is deduced from the rotation of dehydroergosterol, the breakpoint temperature will now be referred to as the critical temperature, t_c, for the frictional resistance to the rotation of dehydroergosterol. In the case of 4.8 mol% dehydroergosterol in DMPC, $t_c(1) = 19.5°C$ and $t_c(2) = 27°C$. In the case of 4.0 mol% dehydroergosterol in DLPE (Fig. 9B), the following results are obtained: $t_c(1) = 26.5°C$, $t_c(2) = 32.5°C$, $b_1 = 0.020$, $b_2 = 0.068$, and $b_3 = 0.056$.

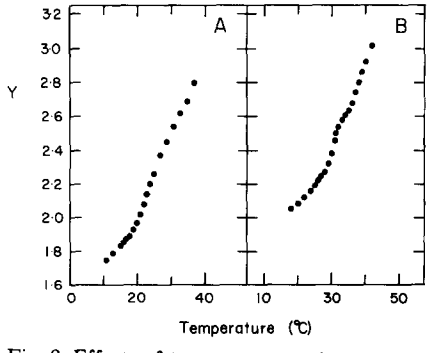

Fig. 9. Effects of temperature on Y for (A) 4.8 mol% dehydroergosterol in DMPC and for (B) 4.0 mol% dehydroergosterol in DLPE.

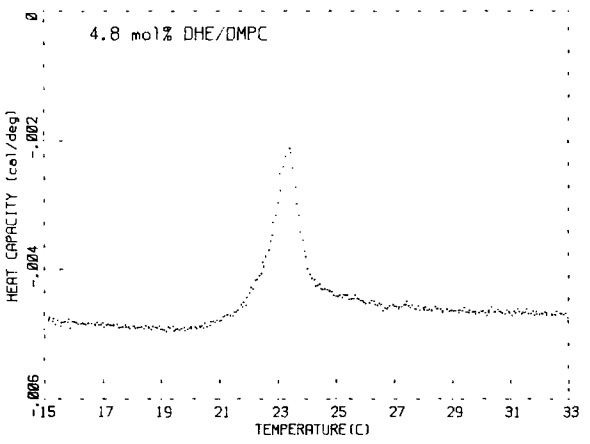

Fig. 10. Differential scanning calorimeter traces of dispersions of 4.8 mol% dehydroergosterol in DMPC.

Differential scanning calorimetry

The thermal behavior of DMPC containing 4.8 mol% dehydroergosterol and of DLPE containing 4.0 mol% dehydroergosterol is shown in Figs. 10 and 11, respectively. The critical calorimetric data together with the Y-plot data are listed in Table II. It is apparent in all cases that the onset temperature of the melting of the matrix phospholipid

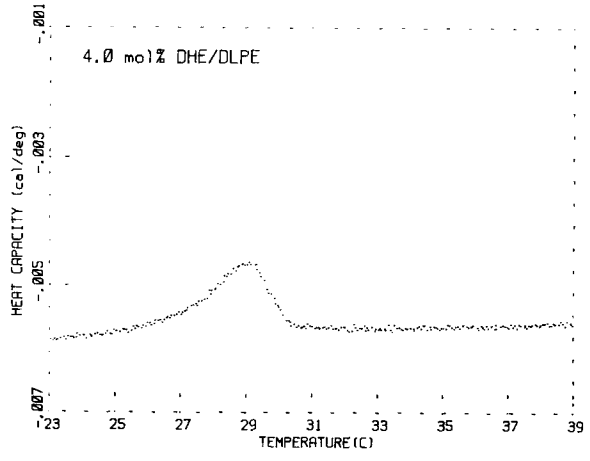

Fig. 11. Differential scanning calorimeter traces of dispersions of 4.0 mol% dehydroergosterol in DLPE.

TABLE II

SUMMARY OF THERMAL COEFFICIENTS, CRITICAL TEMPERATURES, AND THE CALORIMETRIC DATA

DHE, dehydroergosterol.

	Fluorescence					Calorimetry		
	b_1	b_2	b_3	$t_c(1)$ (°C)	$t_c(2)$ (°C)	onset (°C)	maximum (°C)	completion (°C)
4.8 mol% DHE in DMPC	0.021	0.060	0.042	19.5	27	20	23.4	26
4.0 mol% DHE in DLPE	0.020	0.068	0.056	26.5	32.5	26	29.1	31

matches $t_c(1)$ and the completion temperature matches $t_c(2)$.

Comparative data obtained with diphenylhexatriene in DLPE

We have measured the fluorescence anisotropy (Fig. 12A) and lifetime (Fig. 12B) of diphenylhexatriene in DLPE as a function of temperature. The Y plot shown in Fig. 12C gives $b_1 = 0.040$, $b_2 = 0.250$, $b_3 = 0.050$, $t_c(1) = 22°C$, and $t_c(2) = 29°C$. Note that b_2 is much greater than b_1 and b_3. Also b_2 is much larger in the case of diphenylhexatriene than any value of b listed in Table II.

Discussion

There are several possible explanations for the surprising decrease in dehydroergosterol anisotropy in the temperature range where the matrix phospholipid becomes more ordered (Fig. 2). One possibility is that the phase transition causes a reorganization of dehydroergosterol which brings dehydroergosterol molecules in close proximity to each other so that homotransfer increases and a depolarization results [19]. Depolarization caused by homotransfer may be eliminated by excitation at the red edge of the absorption spectrum [17]. However, the data in Fig. 5 show that with excitation at the red edge (351 nm) a depolarization is still observed. Thus an increase in the extent of homotransfer among dehydroergosterol molecules cannot explain the depolarization.

A second possible explanation is based on work by Borochov and Shinitzky [20]. These workers have suggested that a displacement of some membrane components towards the aqueous phase may

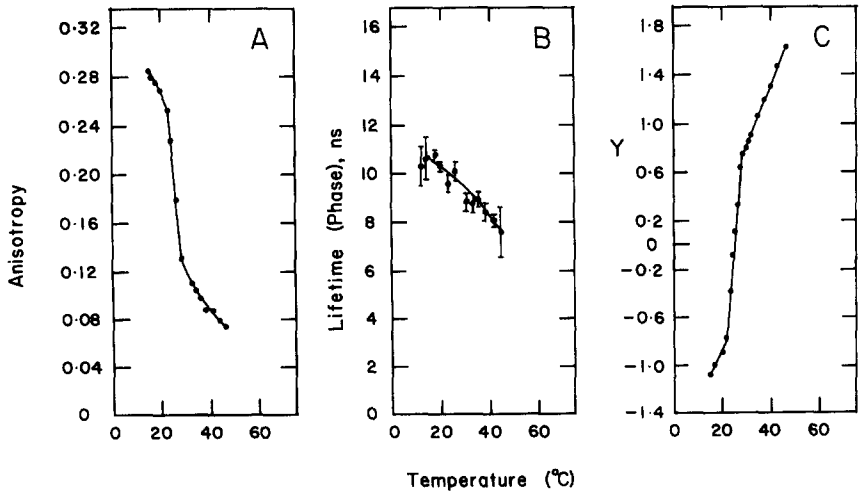

Fig. 12. Dependence of temperature (A) on diphenylhexatriene anisotropy (B) on diphenylhexatriene lifetime and (C) on Y in DLPE $V = 232$ ml/mol and $A_0 = 0.39$ were used for the calculation of Y.

occur when the matrix lipid enters the gel state. Thus when the matrix lipid in our systems undergoes a disorder-to-order phase transition, the dehydroergosterol molecule, most likely the fused ring, may be partially extruded from the membrane. As a result, the chromophore may undergo faster rotation because of less hindrance. This would then lead to increased depolarization.

If this is indeed the case, the dehydroergosterol molecules that are extruded from the membrane should be more accessible to a quencher confined to the aqueous phase. However, the acrylamide quenching constants, $k^*(43.2°C) > k^*(26.1°C)$ derived from the data in Fig. 6, vary just the opposite way to that expected if partial extrusion occurred.

It seems the most likely explanation for the anomalous anisotropy change in dehydroergosterol fluorescence is the large lifetime change with temperature shown in Figs. 7A, B. The temperature dependence of the lifetime change is three times larger for dehydroergosterol in the interval from 7 to 39°C (Figs. 7A, B) than it is for diphenylhexatriene [21]. One possible cause for this large change is thermal quenching of dehydroergosterol emission through collisions with neighboring molecules. This suggestion is strongly supported by the parallel change in both the intensity and the lifetime shown in Figs. 8A, B. If this interpretation is correct, then the frequency and/or the efficiency of the collision between dehydroergosterol and its neighboring molecules increases as the temperature increases.

Once the nature of the anisotropy anomaly is understood, the rotational behavior of dehydroergosterol in bilayers can be analysed. We have undertaken a quantitative description of rotation in terms of the thermal coefficient of frictional resistance offered by the lipid bilayer to dehydroergosterol. The results derived from this spectroscopic approach, combined with the calorimetry data, provide further information about the dynamics of dehydroergosterol in lipid bilayers. The finding that there are critical changes in dehydroergosterol rotation at the onset and completion temperatures of the gel to liquid-crystalline phase transition of the matrix phospholipid is important. The largest value for the thermal coefficient occurs in the temperature range over which gel and liquid-crystalline phase of the matrix phospholipid coexist. It is well known that in this temperature region, the lateral compressibility of the system is anomalously large [22]. Thus, it might be expected that the thermal coefficient of frictional resistance, a viscosity parameter, would reflect the large lateral compressibility. In the case of diphenylhexatriene, b_2 is much greater than b_1 and b_3 (Fig. 12C). But in the case of dehydroergosterol, the difference is not so large (Fig. 9 and Table II). Thus the Y plot of diphenylhexatriene is much more sensitive to the phase transition of the matrix lipid, than is the Y plot of dehydroergosterol. There are at least two possible explanations for this situation. First, it may arise from differences in the location of the chromophores in the bilayer. Diphenylhexatriene is known to be confined to the hydrocarbon region. Since the main lipid phase transition is a result of *gauche-trans* isomerization of the acyl chains, diphenylhexatriene can directly sense the phase transition. In contrast, the chromophore of dehydroergosterol, which presumably resides in the more polar belt region away from the hydrocarbon core [23], does not directly experience the acyl chain isomerization. Thus, the Y plot of dehydroergosterol is not as sensitive to the main phase transition of the phospholipids as is the Y plot of dehydroergosterol. It should be emphasized, however, that even though the chromophore of dehydroergosterol is not in the central region of the bilayer as is diphenylhexatriene, the rotation of diphenylhexatriene still reflects, to a degree, the physical state of the bulk lipid. In this respect, the Y plot method may prove useful in both detecting the phase transition of the bulk lipid and distinguishing between the probes in the hydrocarbon region and those in more polar regions.

An alternative explanation as to why the Y plot of diphenylhexatriene is more sensitive to the phase transition is as follows. As is the case with cholesterol [24], dehydroergosterol molecules may form dehydroergosterol-enriched domains. These domains are laterally separated from the phospholipid domains. In this case, most of the dehydroergosterol molecules do not physically contact the phospholipid molecules and therefore do not sense the phase transition of the bulk lipids directly. This seems to us to be the more likely

explanation. We note that Smutzer and Yeagle [7] have compared the polarization of dehydroergosterol and alpha-parinaric acid in DMPC and they have reached a conclusion similar to this.

The Y plot of DHE in lipid bilayers provides interesting contrasts with published studies on the rotations of peptides and proteins in organic solvents by Weber and co-workers [25,26]. They reported that the thermal coefficient of frictional resistance to the rotation of amino acid residues in peptides or proteins in glycerol/water mixtures is always high below a critical temperature and low above it. In contrast, the thermal coefficient of frictional resistance to the dehydroergosterol rotations in lipid bilayers is just the opposite (Table II and Figs. 9A and 9B).

In the case of peptides or proteins in glycerol/water mixtures, the results have been interpreted as follows [25,26]: Below t_c, the amplitude of peptide rotation increases as the solvent viscosity decreases, as a result of temperature increase; Above t_c, the amplitude still increases as the temperature increases, but the rate is limited by the local environment of peptides; Thus, the thermal coefficient below t_c is greater than the thermal coefficient above t_c.

In the case of dehydroergosterol in lipid bilayers, we interpret our data in a similar fashion. Below $t_c(1)$, the free volume for the dehydroergosterol rotation increases as the temperature increases. Above $t_c(1)$, the increase in the free volume is facilitated by a unique characteristic of lipid bilayers, that is, the order-disorder phase transition. Through this transition, the packing of the lipids becomes loose and a positive volume change is created. As a consequence, the thermal coefficient below $t_c(1)$ is lower than the thermal coefficient above $t_c(1)$. Hence we conclude that the difference in the Y plot between peptide in glycerol/water mixtures and dehydroergosterol in lipid bilayers arises from the order-disorder phase transition of the phospholipids.

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