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A fluorescence anisotropy study on the phase behavior of dimyristoylphosphatidylcholine/cholesterol mixtures

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The phase behavior of L-α-dimyristoylphosphatidylcholine/cholesterol mixtures was studied in multilamellar vesicles by fluorescence polarization of the sterol molecule dehydroergosterol and of the polyene molecule α-parinaric acid. In the absence of cholesterol, dehydroergosterol exhibited an increase in polarization as DMPC vesicles were heated through the phase transition. This rise in polarization anisotropy was observed over a 0.6–1.0°C increase in temperature with the midpoint of the phase transition occurring at 23.6°C. Addition of 5 mol% cholesterol completely obliterated this change in polarization anisotropy through the phase transition of DMPC. α-Parinaric acid underwent a characteristic decrease in polarization anisotropy through the phase transition of DMPC. The change in anisotropy through the phase transition was over 4-fold greater than the values observed with dehydroergosterol. Vesicles containing 5 mol% cholesterol in the presence of α-parinaric acid underwent a decrease in polarization anisotropy that was over 75% of the original decrease in amplitude observed in the absence of any membrane cholesterol. The difference in sensitivity of the two fluorescent probes to the phase transition of DMPC as a function of membrane cholesterol content may be explained by a preferential partitioning of dehydroergosterol (and cholesterol) into a sterol-rich phase at low sterol concentrations. This partitioning allows dehydroergosterol to detect sterol-rich regions in the membrane bilayer.

Introduction

It has long been known that biological membranes vary greatly in their cholesterol content. Bacterial membranes and protein-rich inner mitochondrial membranes contain virtually no cholesterol, while human erythrocyte membranes contain up to 50 mol% cholesterol. Yet the structural and functional role of cholesterol in biological membranes is still largely unknown. One of the principle problems is lack of a suitable method to probe cholesterol behavior in a nonperturbing or minimally perturbing manner.

In 1979, Rogers et al. [1] introduced a derivative of cholesterol that possessed a conjugated triene carbon-carbon double bond system that allowed this molecule to function as a highly sensitive fluorescent probe of membrane cholesterol behavior. Dehydroergosterol is an especially powerful probe of cholesterol behavior since it is similar in structure to cholesterol, and this hydrophobic probe is easy to incorporate into artificial membranes. Furthermore, dehydroergosterol possesses a quantum yield high enough for detection by fluorescence spectroscopy.

Dehydroergosterol has been shown to function in a manner similar to cholesterol in water per-

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Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; PC, phosphatidylcholine; T_m, temperature of the phase transition.

meability studies and cell growth studies [1], glucose permeability studies [2], and in its effect on the order parameters derived from electron spin resonance of spin labels [2]. Circular dichroism studies with dehydroergosterol have been used to measure the behavior of cholesterol in a variety of vesicle systems [3]. Recently, Muczynski and Stahl [4] used a nonspecific phospholipid exchange protein preparation to transfer dehydroergosterol from sonicated lipid vesicles to electroplax membrane fragments without significant loss of membrane $(Na^+ + K^+)$-ATPase activity.

In this paper, we report strong evidence that dehydroergosterol is probing the cholesterol-rich regions of the membrane bilayer and that a phase separation of sterol-rich regions may occur at low cholesterol concentrations in DMPC vesicles.

Materials and Methods

Materials. Dehydroergosterol was synthesized from ergosterol (Sigma Chemical Co.) by dehydration with mercuric acetate/chloroform/acetic acid for 24 h at 35°C in the dark and under a nitrogen atmosphere [5]. The product was recrystallized with ether/ethanol in an acetone/solid CO_2 bath. Infrared spectroscopy of the product indicated that it was in the alcohol form, while melting-point determinations and absorption spectra were similar to published values [1,6]. Dehydroergosterol was stored in the dark in a dessicator at −20°C until use. DMPC (in chloroform) and egg PC were purchased from Avanti Polar Lipids (Birmingham, AL), α-parinaric acid was purchased from Molecular Probes (Junction City, OR) and cholesterol was purchased from Sigma Chemical Co. (St. Louis, MO).

Probe incorporation. For the preparation of multilamellar vesicles containing probe, dehydroergosterol or α-parinaric acid was dissolved in HPLC grade chloroform and added to 4 mg DMPC. The mixture was then dried to a thin film under a stream of argon or nitrogen in an N-evap analytical evaporator apparatus with gentle heating. The resulting thin film of lipid and probe was then dried under high vacuum for a minimum of 2 h to remove residual chloroform. The lipid mixture was suspended in 150 mM NaCl/1 mM EDTA/2.5 mM histidine at pH 7.00 at 30°C by vortexing for 35 s. For heating curves, the vesicles were allowed to equilibrate to room temperature and were then cooled to the starting temperature. For cooling curves, the vortexed suspension was maintained at approx. 30°C and heated to the starting temperature. Final lipid concentration was 0.33 mg/ml.

Fluorescence spectroscopy. Fluorescence spectroscopy was carried out in a Perkin-Elmer Model LS-5 spectrofluorometer equipped with polarizers. For dehydroergosterol, excitation was at 327 nm while emission was measured either at 375 or 395 nm. α-Parinaric acid was excited at 324 nm and emission was measured at 420 nm. The steady-state fluorescence anisotropy, r, was defined as:

$$r = \frac{I_{\parallel} - GI_{\perp}}{I_{\parallel} + 2GI_{\perp}}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities with the analyzer parallel and perpendicular to the vertical polarizer, respectively. G represents the ratio of the sensitivities of the detection system for vertically and horizontally polarized light [7]. Changes in background fluorescence contributed by the membrane sample without probe were measured and subtracted from both the parallel and perpendicular values of the emission [8].

The temperature inside the cuvette was monitored with an Electrotherm digital thermometer. Temperature of the lipid suspension was controlled by use of a water bath connected to the sample chamber of the fluorometer. Heating and cooling rates were approx. 4 deg. C/h.

Fluorescence lifetime measurements of dehydroergosterol in multilamellar DMPC vesicles were carried out under the direction of Dr. Frank Saeva at the Eastman Kodak Corporation, Rochester, NY.

Miscellaneous methods. Quantum yield measurements for dehydroergosterol in cyclohexane were done according to the procedure of Berlman [9] in which solutions of dehydroergosterol and 1-naphthylamine were matched in their absorbance at 300 nm. Total emission energy from a corrected spectrum of dehydroergosterol was then compared to the corrected total emission energy from 1-naphthylamine by plotting fluorescence intensity as a function of wave number. Absorption spectra

were measured on a Cary 14 recording spectrophotometer.

Wavelength correction factors for plotting corrected fluorescence emission spectra were obtained by measuring reflectance from a packed, granular surface of Kodak White Reflectance Standard (Eastman Kodak Co., Rochester, NY) placed at a 45° angle from the incident light beam. Corrected spectra for 1-naphthylamine and dehydroergosterol were then obtained by digitalizing the fluorescence spectra and applying the experimentally determined correction factors as a function of wavelength.

For determinations concerning localization of the probe, multilamellar vesicles with and without dehydroergosterol were centrifuged for 45 min at 35 000 rpm in a Ti-50 rotor at 25°C. The supernatant was removed from the centrifuge tube, and the hard pellet was resuspended in 5 ml histidine buffer by vortexing. Fluorescence emission of the pellet and supernatant was measured at 375 nm.

Results

Characterization of dehydroergosterol fluorescence

Quantum yield measurements of dehydroergosterol fluorescence in cyclohexane yielded a value of 0.032. This value is high enough for the detection of phase changes in lipid bilayers by the technique of fluorescence polarization. The fluorescence emission spectrum of dehydroergosterol in multilamellar vesicles of DMPC containing no cholesterol indicated that maximum fluorescence emission was observed at 375 nm with a side peak at 395 nm. Fig. 1 shows the steady-state fluorescence emission of 0.88 mol% dehydroergosterol in DMPC as a function of temperature (heating curve). As shown in Fig. 1, a sharp drop in fluorescence intensity occurred between 23.30 and 23.95°C, at the T_m of DMPC.

Polarization anisotropy and fluorescence lifetime of dehydroergosterol in pure phospholipid bilayers

The objective of this study was to determine whether or not dehydroergosterol was capable of probing the sterol-rich regions of a membrane bilayer. This will be shown by the differential effects of cholesterol on diminution of the anisotropy change through the T_m of DMPC with

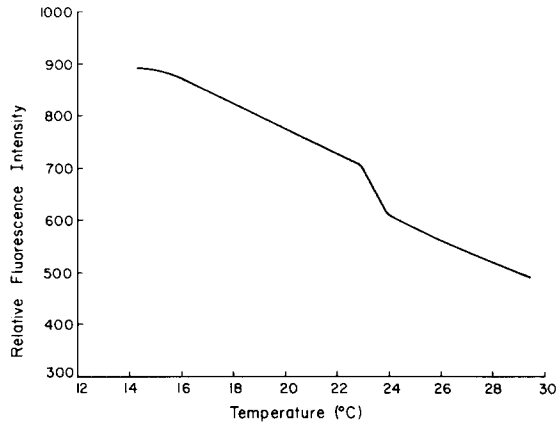

Fig. 1. Steady-state fluorescence emission of 0.88 mol% dehydroergosterol in DMPC vesicles as a function of temperature. Vesicles were heated at a rate of 4 deg. C/h. Emission of dehydroergosterol was monitored at 395 nm in the absence of polarizers.

dehydroergosterol and α-parinaric acid. Implicit in this approach is that the polarization anisotropy change of dehydroergosterol through the T_m of DMPC is sufficiently great to detect the T_m of DMPC.

Fig. 2 shows both heating and cooling curves of the polarization anisotropy changes of dehydroergosterol and a corresponding heating curve of α-parinaric acid as a function of temperature in multilamellar DMPC vesicles that contain no cholesterol. At probe concentrations of 0.88 mol% dehydroergosterol, a change in the slope of the plot of the polarization anisotropy can be observed at 17°C, while the main transition at 23°C can be observed as a sharp increase in polarization anisotropy of the probe. At concentrations of 0.50 and 1.96 mol% dehydroergosterol, a similar rise in anisotropy was also observed. α-Parinaric acid underwent a decrease in anisotropy above the T_m of DMPC, and this decrease was of a much larger magnitude when compared to the anisotropy change with dehydroergosterol.

Table I indicates the observed increase in polarization anisotropy through the phase transition, the temperature range over which the main transition occurred, and the anisotropy value at the midpoint of the main phase transition. The data from Table I indicate that at 1.96 mol% dehydroergosterol, a slight broadening of the phase

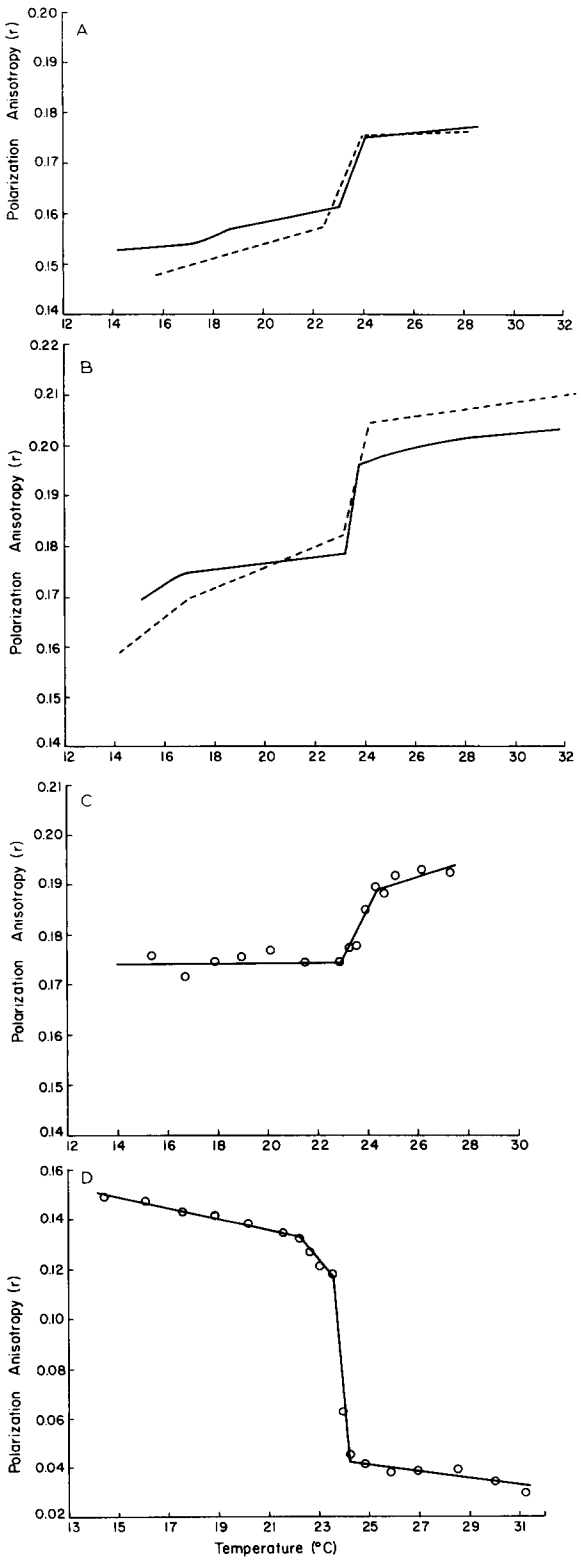

TABLE I

THE EFFECT OF VARIOUS PROBE CONCENTRATIONS OF DEHYDROERGOSTEROL ON THE DETECTION OF PHASE TRANSITIONS IN DMPC VESICLES CONTAINING NO CHOLESTEROL

0.50 and 0.88 mol% dehydroergosterol (DHE) values are the average of three trials, while 1.96 mol% dehydroergosterol is the average of five trials.

Mol% DHE	Range of phase change (°C)	Increase in r	Apparent T_m	r at midpoint of T_m
0.50	0.8	0.143	23.8	0.182
0.88	0.7	0.203	23.6	0.184
1.96	1.1	0.150	23.4	0.171

transition occurred when compared to the values at 0.88 and 0.50 mol%. The amplitudes of the anisotropy changes through the phase transition were relatively independent of the concentration of probe used over the values that were tested. However, we did observe a somewhat larger amplitude change for r at 0.88 mol% dehydroergosterol when compared to the values obtained with 0.50 and 1.96 mol% dehydroergosterol.

As a control, the polarization anisotropy of dehydroergosterol in egg phosphatidylcholine liposomes was measured as a function of temperature. This phospholipid does not exhibit a phase transition in this temperature range. The polarization anisotropy was linear in the temperature region between 14 and 31°C (data not shown).

We observed that r values at the T_m of DMPC increased up to a temperature of 24.15°C and then decreased slightly above 24.15°C. This small decrease in anisotropy above the phase transition of DMPC in heating curves is due to the scattering changes of the lipid itself, since changes in background fluorescence show a small change in ani-

Fig. 2. Fluorescence polarization anisotropy changes of dehydroergosterol and α-parinaric acid in DMPC vesicles as a function of temperature. The vesicles contained no cholesterol. (A) Heating (solid line) and cooling curve (broken line) of 1.96 mol% dehydroergosterol. (B) Heating (solid line) and cooling curves (broken line) of 0.88 mol% dehydroergosterol. (C) Heating curve of 0.50 mol% dehydroergosterol. (D) Heating curve of 0.1 mol% α-parinaric acid.

sotropy at 24.15°C (data not shown). Background fluorescence for the parallel component of dehydroergosterol fluorescence corresponded to 1.56% of the total fluorescence emission, while the perpendicular component corresponded to 0.74% of total fluorescence. These scattering changes can be observed as a slight decrease in fluorescence anisotropy just above the T_m of DMPC.

Fluorescence lifetime measurements of 0.88 mol% dehydroergosterol in DMPC vesicles at 22.5°C yielded a lifetime of 1 ns. Further lifetime measurements were not carried out due to equipment limitations. However, a comparison of the relative quantum yield of dehydroergosterol below and above the T_m of DMPC indicated that dehydroergosterol underwent a decrease in quantum yield through the T_m of DMPC. At 24.35°C, the fluorescence yield was 87.1% of the value measured at 22.85°C.

Polarization anisotropy of dehydroergosterol in cholesterol-phospholipid bilayers

The effect of various concentrations of cholesterol on detection of the DMPC phase transition with 0.88 mol% dehydroergosterol have been examined. 1 mol% cholesterol in the membrane caused an approx. 50% decrease in the anisotropy rise through the phase transition of DMPC without significantly broadening the phase transition. 2 mol% cholesterol caused a further decrease in the anisotropy change and a broadening of the phase transition. At 5 mol% cholesterol, no anisotropy change could be observed at the phase transition of DMPC, indicating that 5 mol% cholesterol totally eliminated the phase transition normally detected with 0.88 mol% dehydroergosterol.

Polarization anisotropy of α-parinaric acid in cholesterol-phospholipid bilayers

α-Parinaric acid exhibited a characteristic decrease in polarization anisotropy as the sample of DMPC multilamellar vesicles was heated through the phase transition [10]. When vesicles were heated at the rate of 4 deg. C/h, this decrease in polarization anisotropy occurred over a temperature range of 2°C (22–24°C). Over 80% of this decrease occurred over a temperature range of 0.7°C with the midpoint temperature of this sharp transition at 23.7°C. When 5 mol% cholesterol was added to DMPC vesicles with α-parinaric acid as the probe, over 75% of the detected phase transition still remained. These results are similar to those reported by Sklar et al. [11].

Fig. 3 is a summary of the fluorescence anisotropy change observed with dehydroergosterol and α-parinaric acid as a function of mol% cholesterol in the membrane. In the absence of membrane cholesterol, α-parinaric acid caused a 4-fold or greater anisotropy change through the T_m of DMPC than did dehydroergosterol. The smaller anisotropy change observed with dehydroergosterol was totally obliterated upon incorporation of 5 mol% cholesterol. Extrapolation indicates that over 17 mol% cholesterol would be required to totally eliminate the anisotropy change of α-parinaric acid at the T_m of DMPC. For comparison of the differential effect of cholesterol on motional parameters of dehydroergosterol and α-parinaric acid, the data in Fig. 3 represent anisotropy changes as a function of membrane cholesterol, and not total membrane sterol. Probe concentrations of dehydroergosterol would increase total membrane sterol content by 0.87–0.88 mol% in dehydroergosterol-cholesterol vesicles.

Location of dehydroergosterol

The distribution of fluorescence between the membrane and aqueous phase was determined by fluorescence emission of the probe after centrifugation of the membranes as described in Materials and Methods. We observed greater than 99% of dehydroergosterol fluorescence in the membrane pellet after centrifugation.

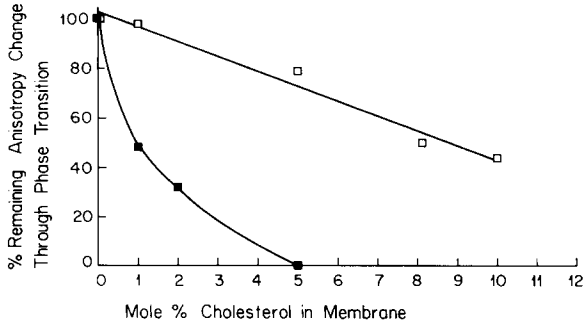

Fig. 3. Effect of membrane cholesterol on dehydroergosterol and α-parinaric acid-detected phase transitions in DMPC multilamellar vesicles. Closed squares represent dehydroergosterol and open squares represent α-parinaric acid.

Discussion

Physical studies have indicated that addition of cholesterol to model membranes increases the order of phospholipid hydrocarbon chains in the liquid crystalline state and decreases the order in the gel state, ultimately causing total elimination of the phospholipid phase transition [12,13]. Cholesterol has also been shown to increase the order parameters characterizing phospholipid hydrocarbon chains [14,15], to cause condensation of phospholipid monolayers [16,17], and to disrupt phospholipid headgroup interactions [18].

These dramatic effects of cholesterol detected by the techniques just mentioned for the most part are observed at relatively high cholesterol concentrations (i.e., greater than 5 mol%). The results of this study indicate that dehydroergosterol fluorescence is highly sensitive to the presence of membrane cholesterol at concentrations less than 5 mol%. Furthermore, this sensitivity is very different from the sensitivity exhibited by the fluorescence depolarization of α-parinaric acid in cholesterol-containing membranes. Dehydroergosterol detects the presence of membrane cholesterol at lower concentrations than is observed with α-parinaric acid.

At a membrane concentration of 5 mol% cholesterol, dehydroergosterol fluorescence no longer detects the phase transitions of DMPC although much of the phospholipid is still undergoing a cooperative phase transition (as detected by parinaric acid depolarization). Therefore, dehydroergosterol is likely to be undergoing a phase separation into regions enriched in membrane sterol at cholesterol concentrations of 5 mol% and more. By so doing, the sterol inhabits a microdomain incapable of a highly cooperative phase transition. In such a domain, it is insulated from the domains of lipid still capable of a highly cooperative phase transition.

An analysis of the partitioning indicates a substantial preference of dehydroergosterol for the phase not undergoing a highly cooperative phase transition. The anisotropy results from α-parinaric acid indicate that over 75% of the DMPC was still undergoing a highly cooperative phase transition at 5 mol% cholesterol. At the same time, less than about 5% of the dehydroergosterol was in a DMPC phase undergoing a cooperative phase transition. While a numerical value for the partition coefficient would be difficult to assign, it clearly indicates a great preference of dehydroergosterol for regions not undergoing a highly cooperative phase transition.

Calorimetry results (in the absence of dehydroergosterol) have indicated that at least two phases are present when cholesterol is in the membrane [19]. Cholesterol has been suggested to remove phospholipids from the highly cooperative phase transition by forming a cholesterol-phospholipid phase rich in cholesterol. The data presented here suggest that at least two phases are present at very low cholesterol concentrations, i.e., at 5 mol% cholesterol. This conclusion is in good agreement with the calorimetric results of Mabrey et al. [13], who noted the presence of a broad transition above 5 mol% cholesterol in DMPC, in addition to the more cooperative DMPC transition. Our data also suggest that at low dehydroergosterol concentrations, the probe partitions into cholesterol-rich regions. This is in agreement with the previous determinations that dehydroergosterol and cholesterol behave in a similar manner in membranes [2]. The indication that dehydroergosterol preferentially partitions into sterol-rich regions of the membrane suggests that this fluorescent probe will provide a powerful means to study cholesterol behavior in membranes. The other observations suggest that one should consider systems containing cholesterol as complex systems even at low cholesterol concentrations.

An interesting additional observation in this study was the increase in anisotropy observed with dehydroergosterol above the T_m of DMPC compared to the anisotropy values observed at lower temperatures. An explanation of the increase in anisotropy above the phase transition could be a decrease in the fluorescence lifetime of dehydroergosterol. Changes in fluorescence lifetime of diphenylhexatriene have been proposed to explain the increase in anisotropy of diphenylhexatriene above the T_m of DMPC in bacteriorhodopsin-DMPC vesicles as the protein to phospholipid ratio is raised [20]. In DMPC vesicles, if one assumes that the emissive rate constant of dehydroergosterol is temperature-independent, then the

quantum yield would be a measure of the fluorescent lifetime. We observed a 13% decrease in quantum yield above the T_m of DMPC, suggesting that a decrease in lifetime may be responsible for the increase in anisotropy above the T_m of DMPC.

A second possible explanation is that depolarization occurs through energy transfer from one probe molecule to another, located at somewhat different orientations relative to each other. Depolarization of dehydroergosterol fluorescence by energy transfer has previously been suggested by Rogers et al. [1]. Depolarization by this mechanism would be more favorable under conditions of increased energy transfer. Such energy transfer is enhanced by shorter intermolecular distances. This would suggest that below the phase transition of the bulk phospholipid, dehydroergosterol displays smaller average intermolecular distances than above the phase-transition temperature.

The energy transfer is a critical function of the intermolecular distance, especially if transfer involves more than two molecules. The bilayer is in a more expanded state above the phase transition than below the T_m, and this could lead to an apparent increase in steady-state anisotropy upon entering the liquid-crystal state due to an increase in the average intermolecular distance between chromophores. A resolution of the problem must await a separate time-resolved fluorescence anisotropy study.

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