Biochimica et Biophysica Acta, 946 (1988) 270–280
Elsevier

BBA 74233

A fluorescent sterol probe study of cholesterol / phospholipid membranes

Greg Smutzer

Department of Biochemistry, School of Medicine, State University of New York at Buffalo, Buffalo, NY (U.S.A.)

(Received 22 January 1988)
(Revised manuscript received 15 August 1988)

Key words: Multilamellar liposome; Cholesterol; Dehydroergosterol; Time correlated fluorescence; Fluorescence

The behavior of dehydroergosterol in L-α-dimyristoylphosphatidylcholine (DMPC) unsonicated multilamellar liposomes was characterized by absorption spectroscopy and fluorescence measurements. Dehydroergosterol exhibited a lowered absorption coefficient in multilamellar liposomes while the steady-state fluorescence anisotropy of dehydroergosterol in these membranes decreased significantly with increasing dehydroergosterol concentration, suggesting membrane sterol–sterol interactions. The comparative steady-state anisotropy of 0.9 mole percent dehydroergosterol in multilamellar liposomes was lower than in small unilamellar vesicles suggesting different sterol environments for dehydroergosterol. Dehydroergosterol fluorescence lifetime was relatively independent of membrane sterol content and yielded similar values in sonicated and unsonicated model membranes. In multilamellar liposomes containing 5 mole percent cholesterol, the gel-to-liquid crystalline phase transition of DMPC detected by 0.9 mole percent dehydroergosterol was significantly broadened when compared to the phase transition detected by dehydroergosterol in the absence of membrane cholesterol (Smutzer, G. et al. (1986) Biochim. Biophys. Acta 862, 361–371). In multilamellar liposomes containing 10 mole percent cholesterol, the major fluorescence lifetime of dehydroergosterol did not detect the gel-to-liquid crystalline phase transition of DMPC. Time-correlated fluorescence anisotropy decays of dehydroergosterol in DMPC multilamellar liposomes in the absence and presence of 5 mole percent cholesterol exhibited a single rotational correlation time near one nanosecond that was relatively independent of temperature and low concentrations of membrane cholesterol. The limiting anisotropy of 0.9 mole percent dehydroergosterol decreased above the gel-to-liquid crystalline phase transition in membranes without cholesterol and was not significantly affected by the phase transition in membranes containing 5 mole percent cholesterol. These results suggested hindered rotational diffusion of dehydroergosterol in multilamellar liposomes. Lifetime and time-correlated fluorescence measurements of 0.9 mole percent dehydroergosterol in multilamellar liposomes further suggested this fluorophore was detecting physical properties of the bulk membrane phospholipids in membranes devoid of cholesterol and was detecting sterol-rich regions in membranes of low sterol concentration.

Abbreviations: dehydroergosterol, ergosta-5,7,9(11),22-tetraen-3β-ol; DMPC, L-α-dimyristoylphosphatidylcholine; EDTA, ethylenediaminetetraacetic acid, sodium salt; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; PC, phosphatidylcholine; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; r, steady-state anisotropy; r_0, zero-time anisotropy; $r_{(0)}$, apparent zero-time anisotropy; r_∞, limiting anisotropy; SUV, small unilamellar vesicles.

Correspondence (present address): G. Smutzer, Department of Biological Sciences, State University of New York at Albany, Albany, NY 12222, U.S.A.

Introduction

It has long been known that cholesterol is an integral component of many biological membranes, and these membranes can exhibit a wide range in cholesterol content. Cholesterol is also an important component of human serum lipoproteins whose function is to transport cholesterol through the blood stream. However, the role of cholesterol in modulating membrane structure and function is still largely unknown.

A major problem for determining cholesterol effects on biological membrane structure and function has been the lack of a suitable method for probing sterol behavior in biological membranes that would minimally perturb the system yet accurately monitor membrane cholesterol behavior. This problem has been alleviated by the recent introduction of fluorescent sterol probes such as dehydroergosterol [1]. Dehydroergosterol is a useful analog of cholesterol that has been shown to function in a manner similar to cholesterol [1–3], and has recently been exploited in a wide variety of biological systems [4–6]. Dehydroergosterol detects membrane sterol phase separations at cholesterol concentrations as low as 5 mole percent [7–9], undergoes an increase in steady-state fluorescence anisotropy above the gel-to-liquid crystalline phase transition of multilamellar liposomes prepared from thin films [7,8], yet does not undergo complex excited state behavior [10]. In this report, the fluorescence of dehydroergosterol in unsonicated multilamellar liposomes is characterized and compared to its fluorescence behavior in sonicated unilamellar vesicles (SUV) under similar experimental conditions. The effects of low sterol concentration on the fluorescence properties of dehydroergosterol in multilamellar liposomes is then analyzed by lifetime and time-correlated anisotropy decays since low membrane cholesterol has been shown to induce unique changes in liposome membrane structure [7,8].

Materials and Methods

Dehydroergosterol was synthesized from ergosterol (Sigma Chemical Co., St. Louis, MO) by oxidation with mercuric acetate and purified by reverse-phase HPLC as previously described [10]. DMPC (in chloroform) was purchased from Avanti Polar Lipids (Birmingham, AL) and cholesterol was obtained from Eastman Kodak (Rochester, NY).

Preparation of liposomes. Multilamellar liposomes of DMPC were prepared from thin films as previously described [7] and suspended in aqueous buffer containing 150 mM NaCl, 5 mM Hepes at pH 7.00, and 1 mM EDTA and shaken for 30 s at approx. 30 °C by vortexing. Multilamellar liposomes were centrifuged for 20 min at 30000 × g in a Beckman J-21 preparatory centrifuge and resuspended in 6 ml of Hepes buffer by vortexing. Final DMPC concentration of all samples was 0.33 mg/ml. Multilamellar liposomes without dehydroergosterol were prepared in an identical manner.

Small unilamellar vesicles were prepared from DMPC and 0.9 mole percent dehydroergosterol by sonication and ultracentrifugation above 25 °C using previously described procedures [3].

Fluorescence spectroscopy. Steady-state fluorescence anisotropy, r, was measured with a PRA fluorescence lifetime spectrofluorometer (Photochemical Research Associates, London, Ontario, Canada) or a Perkin-Elmer LS 5 fluorescence spectrophotometer and was defined as follows:

$$r = \frac{I_{\parallel} - G \cdot I_{\perp}}{I_{\parallel} + 2G \cdot I_{\perp}} \quad (1)$$

$I_{\parallel}$ and $I_{\perp}$ represent the fluorescence intensities with the analyzer parallel and perpendicular to the vertical polarizer, respectively. G represents the ratio of the sensitivity of the detection system for vertically polarized light. Changes in background fluorescence contributed by unlabeled membranes were measured and subtracted from the parallel and perpendicular components of the emission. The temperature of the sample was controlled by a water bath connected to the sample chamber of the fluorometer and was monitored with a digital thermometer.

Fluorescence lifetimes were measured by the single photon counting technique utilizing a PRA fluorescence lifetime spectrophotometer. Excitation by 325 nm light was provided by a hydrogen filled, thyratron-gated lamp operated at 30.9 kHz

and at a potential of 6 kV. Fluorescence emission was measured through a 3 mm KV 370 Schott filter (high pass, 50% transmittance at 370 nm, Schott Optical Co., Duryea, PA) with the emission polarizer at 54.7°. A lamp profile utilizing a scattering solution of glycogen in water was determined for each lifetime measurement, and samples in the absence of probe were examined under identical conditions and subtracted from the labeled data on a channel per channel basis as previously described [10]. Reiterative convolution of the lifetime data was analyzed by least-squares analysis as previously described [10].

Time-correlated fluorescence anisotropy measurements were analyzed by:

$$r(t) = \sum_{j=1}^{m} \beta_j \exp(-t/\phi_j) \tag{2}$$

where β_j is the pre-exponential factor of the $r(t)$ curve and ϕ is the rotational correlation time in nanoseconds. The time-correlated anisotropy decay of dehydroergosterol in multilamellar liposomes was analyzed by setting the rotational correlation time ϕ_2 to represent a constant value:

$$r(t) = (r_0 - r_\infty) \exp(-t/\phi_1) + r_\infty \tag{3}$$

At 0.9 mole percent dehydroergosterol, subtraction of low levels of background fluorescence (2 percent or less in multilamellar liposomes) from the vertical and horizontal components increased the precision of measurement of probe dynamics in these membrane systems. The apparent zero-time anisotropy, $r_{(0)}$, was lowered by approx. 0.01 anisotropy units after background subtraction while r_∞ was decreased by approx. 0.006 anisotropy units since background scatter was less significant at later data acquisition channels where r_∞ was defined. Background correction generally increased the rotational correlation time by 0.3 ns. As an internal control, the fluorescence lifetime of dehydroergosterol obtained from the sum curve of the time-correlated polarized fluorescence emission, $(I_\parallel + 2GI_\perp)$, was compared to the lifetime measured at 54.7° [11].

The r_∞ values were obtained from the data fit using Eqn. 3. The motional order parameter, S, was calculated from the time-correlated anisotropy decays according to Wolber and Hudson [12]:

$$S = \left(\frac{r_\infty}{r_0}\right)^{1/2} \tag{4}$$

with r_0 equal to 0.385 for dehydroergosterol when excited at its major absorption band [13].

Miscellaneous techniques. Absorption spectra of dehydroergosterol in multilamellar liposomes were measured with a Perkin-Elmer Lambda 3B double beam spectrophotometer. Unlabeled liposomes were used in the reference beam of the spectrophotometer.

Results

Spectral properties of dehydroergosterol in liposomes

In order to measure the absorptive properties of dehydroergosterol in multilamellar liposomes, membranes of 10 mole percent fluorophore were prepared. The corresponding absorption maximum of 10 mole percent dehydroergosterol in multilamellar liposomes was 326.5 nm. This value was similar to its absorbance maximum in isotropic solvents [10], and in SUV where 10 mole percent dehydroergosterol exhibited an absorption maximum of 326.0 nm [9]. This observation indicated the most probable electronic transition within the major absorption band of dehydroergosterol occurred at a similar wavelength in unsonicated membranes, in sonicated membranes, and in isotropic solvents. However, 10 mole percent dehydroergosterol in multilamellar liposomes (54.6 mM dehydroergosterol) at 20°C exhibited an absorption coefficient near 3100 ± 100 $M^{-1} \cdot cm^{-1}$ which was significantly lower than its absorption coefficient in non-aqueous isotropic solvents. In these solvents, dehydroergosterol exhibited absorption coefficients near 10000–11000 $M^{-1} \cdot cm^{-1}$ [4,10]. The lowered absorption coefficient in multilamellar liposomes suggested interactions between dehydroergosterol chromophores.

Fluorescence measurements

As a test of the ability of dehydroergosterol to undergo sterol–sterol interactions in multilamellar liposomes, the effect of dehydroergosterol concentration on its steady-state fluorescence ani-

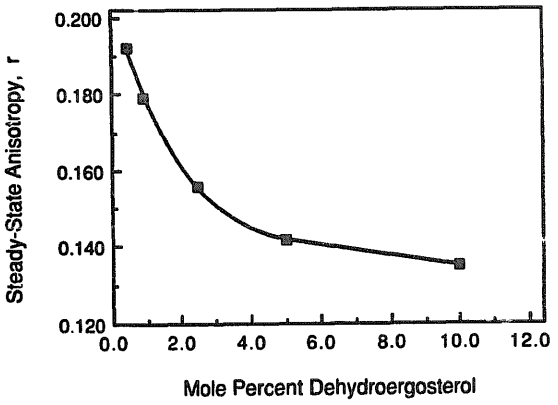

Fig. 1. Steady-state fluorescence anisotropy of dehydroergosterol as a function of its membrane concentration in multilamellar liposomes. Temperature was 19.9 °C, excitation was at 325 nm, and emission was measured through a KV 370 filter.

sotropy was measured. Fig. 1 presents the steady-state fluorescence anisotropy of dehydroergosterol in multilamellar liposomes at 20 °C as a function of dehydroergosterol concentration. Upon excitation at 325 nm, the fluorescence anisotropy of dehydroergosterol was decreased by increasing concentrations of membrane fluorophore. This anisotropy decrease in multilamellar liposomes was most pronounced at concentrations up to 5 mole percent dehydroergosterol while above this concentration, fluorescence anisotropy underwent only a further slight decrease. These results suggested interactions between dehydroergosterol chromophores in multilamellar liposomes as sterol concentration was increased. These steady-state anisotropy results also correspond to previously reported changes in the physical properties of dehydroergosterol observed between 0 and 5 mole percent membrane sterol which indicated the presence of sterol-rich regions in multilamellar liposomes at these concentrations [7,8].

The steady-state anisotropies of low concentrations of dehydroergosterol in multilamellar liposomes presented in Fig. 1 displayed lower values than similar dehydroergosterol concentrations in SUV [9]. These results suggested potentially different membrane environments for dehydroergosterol in these two bilayer systems. In POPC SUV, dehydroergosterol concentrations near 1 mole percent displayed fluorescence anisotropies near 0.26 [9], higher than the anisotropies observed with multilamellar liposomes. The steady-state anisotropy of 0.9 mole percent dehydroergosterol was therefore compared in DMPC SUV and DMPC multilamellar liposomes at an identical temperature above the gel-to-liquid crystalline phase transition where potential fusion of SUV would not occur. Upon excitation at 325 nm and at 26.1 °C, the steady-state anisotropy of dehydroergosterol in DMPC SUV was 0.24 while an identical fluorophore concentration in multilamellar liposomes yielded a value of 0.18. These different steady-state anisotropies under identical conditions suggested that dehydroergosterol may reside within different membrane environments in unsonicated and sonicated model membranes.

To determine if the fluorescence lifetime of 0.9 mole percent dehydroergosterol also differed in SUV and multilamellar liposomes, the fluorescence lifetime of dehydroergosterol in multi-

TABLE I

FLUORESCENCE LIFETIMES OF DEHYDROERGOSTEROL IN DMPC MODEL MEMBRANES

MLV, multilamellar liposomes.

Vesicle type	Mole% dehydroergosterol	Temperature (°C)	τ_1	A_1 [a]	τ_2	A_2
SUV	0.9	26.1	0.90±0.01	100.0	0.0	0.0
MLV	0.9	26.1	0.87±0.01	100.0	0.0	0.0
MLV	0.9	19.9	1.43±0.01	98.7	4.54±0.36	1.3
MLV	2.5	19.9	1.38±0.01	99.7	5.72±0.35	0.3
MLV	5.0	19.9	1.39±0.01	99.6	8.92±0.66	0.4
MLV	10.0	19.9	1.36±0.01	99.5	9.12±0.44	0.5

[a] Relative amplitudes A_1 and A_2 were normalized to 100.0%.

lamellar liposomes was compared to its fluorescence lifetime in DMPC SUV at a comparable temperature above the gel-to-liquid crystalline phase transition of DMPC. Table I presents these results and indicates that in DMPC SUV containing no cholesterol, dehydroergosterol exhibited a single fluorescence lifetime whose decay was similar in duration to its single lifetime in unsonicated DMPC multilamellar liposomes devoid of cholesterol. The fluorescence lifetime of dehydroergosterol in SUV was very similar to that reported by Schroeder et al. in POPC SUV [9]. Thus, the fluorescence lifetime of dehydroergosterol did not appear sensitive to vesicle configuration when compared to its steady-state fluorescence anisotropy.

Table I also lists the fluorescence lifetime data of dehydroergosterol in multilamellar liposomes as a function of fluorophore concentration. The major fluorescence lifetime of dehydroergosterol decreased slightly as membrane concentrations of dehydroergosterol increased from 0.9 to 10.0 mole percent. A similar observation has been reported for dehydroergosterol in SUV [9]. The fluorescence lifetime of dehydroergosterol in membranes was not significantly affected by potential sterol–sterol interactions in membrane bilayers as fluorophore concentration was increased. In summary, fluorescence lifetimes of dehydroergosterol appeared relatively insensitive to its membrane concentration and to its membrane configuration. These results are consistent with lifetime decays of dehydroergosterol in other lipid systems [6].

Effect of low membrane cholesterol on the fluorescence lifetime of probe levels of dehydroergosterol in liposomes as a function of temperature

Fluorescence lifetime studies of dehydroergosterol in multilamellar liposomes were measured to quantitate the effects of low cholesterol concentrations on membrane structure. Previous reports have indicated the unpolarized fluorescence emission and major fluorescence lifetime of dehydroergosterol both underwent an overall decrease in multilamellar liposomes as the membranes were heated [7,8,10]. Probe concentrations of dehydroergosterol also undergo a sharp decrease in fluorescence lifetime and relative quantum yield of approx. 15 percent at the gel-to-liquid crystalline phase transition of multilamellar liposomes, consistent with an increase in steady-state anisotropy above this phase transition [7,10]. Cholesterol concentrations as low as 5 mole percent eliminated the steady-state anisotropy increase in DMPC multilamellar liposomes at 23°C, suggesting sterol-rich regions in these membranes. Detection of the gel-to-liquid crystalline phase transition in DMPC SUV near 24°C has also been observed by fluorescence intensity measurements [14].

Fig. 2 presents the corresponding effect of low membrane cholesterol on the fluorescence lifetime of 0.9 mole percent dehydroergosterol in multi-

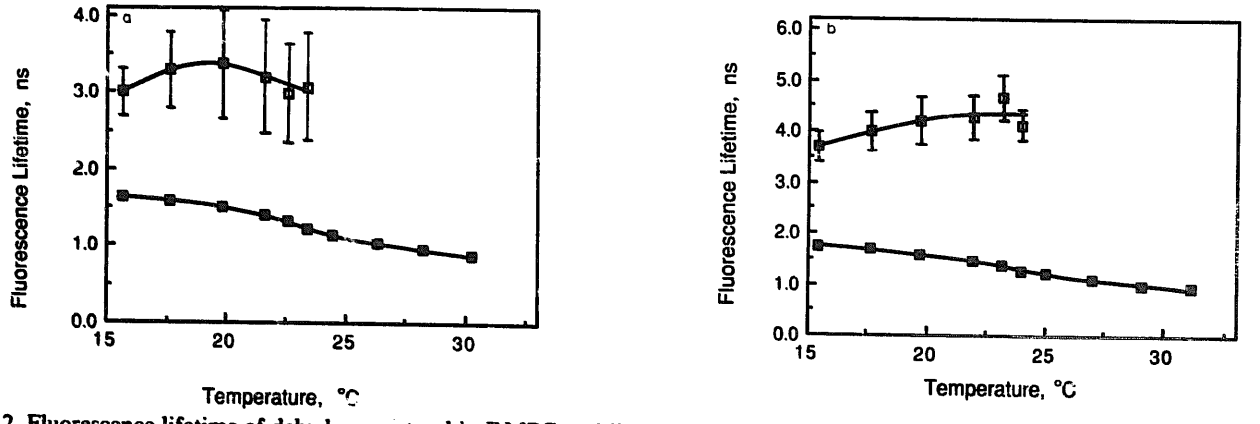

Fig. 2. Fluorescence lifetime of dehydroergosterol in DMPC multilamellar liposomes as a function of temperature. (a) 5 mole percent cholesterol, and (b) 10 mole percent cholesterol (heating curves). Closed squares represent shorter lifetime component while open squares represent the longer lifetime component of dehydroergosterol. Standard deviations for the shorter lifetime component were ±0.04 ns or less.

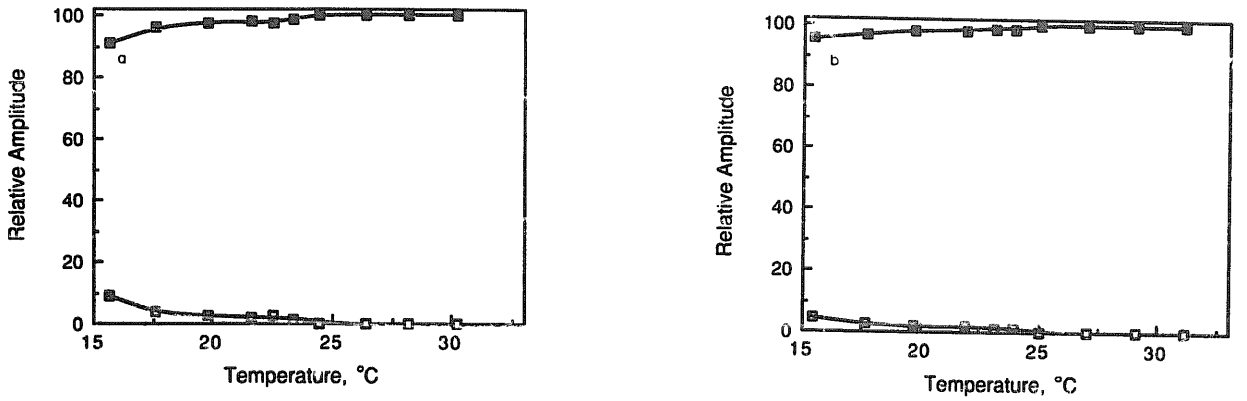

Fig. 3. Relative amplitudes of dehydroergosterol fluorescence lifetime components in DMPC multilamellar liposomes as a function of temperature. (a) 5 mole percent cholesterol, and (b) 10 mole percent cholesterol. Closed squares represent relative amplitudes of the shorter lifetime component.

lamellar liposomes as a function of temperature. At five mole percent cholesterol, the major fluorescence lifetime of dehydroergosterol again decreased with increasing temperature and the previously reported decrease in fluorescence lifetime at the gel-to-liquid crystalline phase transition of DMPC was essentially eliminated [10]. A similar fluorescence lifetime pattern for dehydroergosterol was observed with multilamellar liposomes that contained 10 mole percent cholesterol (see Fig. 2b). Membrane cholesterol concentrations of 5 and 10 mole percent did not significantly affect the duration of the second, minor fluorescence lifetime component of dehydroergosterol whose decay was essentially unchanged by membrane cholesterol addition.

Fig. 3 presents the relative amplitudes of the two fluorescence lifetime components of dehydroergosterol in membranes containing 5 and 10 mole percent cholesterol. Cholesterol did not significantly affect the relative amplitudes of these lifetime components in multilamellar liposomes when compared to membranes without cholesterol. This second lifetime component was still only detected below 24°C, or below the gel-to-liquid crystalline phase transition of DMPC. In membranes containing 10 mole percent cholesterol, the relative amplitude of the major fluorescence lifetime of 0.9 mole percent dehydroergosterol was increased slightly at temperatures below the gel-to-liquid crystalline phase transition of DMPC when compared to membranes with 5 mole percent cholesterol.

The major fluorescence lifetime component of dehydroergosterol in multilamellar liposomes is present both below and above the phospholipid phase transition, and is thought to represent dehydroergosterol whose long molecular axis is parallel to the hydrocarbon chains of the membrane phospholipids [10]. The minor lifetime component has only been observed below the gel-to-liquid crystalline phase transition of DMPC when the membrane bilayer is more ordered, and may represent a distinct environment for dehydroergosterol in multilamellar liposomes [10]. The minor lifetime component of dehydroergosterol exhibits a similar decay pattern in model membranes that contain no cholesterol [8,10], and in membranes that contain 5 and 10 mole percent cholesterol. The presence or absence of the minor lifetime component of dehydroergosterol may be dependent on the phase of the bulk phospholipids in these mem-

TABLE II

EFFECT OF MEMBRANE CHOLESTEROL ON THE MAJOR FLUORESCENCE LIFETIME OF DEHYDROERGOSTEROL IN DMPC MULTILAMELLAR LIPOSOMES AT A TEMPERATURE BELOW AND ABOVE THE GEL-TO-LIQUID CRYSTALLINE PHASE TRANSITION

Mole% cholesterol	τ_1 [a]	
	at 20°C	at 26°C
0	1.32	0.87
5	1.45	1.03
10	1.57	1.15

[a] Standard deviations for lifetime measurements were ±0.02 ns or less.

brane systems. Therefore, the minor lifetime component of dehydroergosterol can be used to report the phase transition of the bulk phospholipids both in the absence and presence of membrane cholesterol.

Table 1 summarizes the effect of temperature on the major fluorescence lifetime of 0.9 mole percent dehydroergosterol at a temperature below and above the gel-to-liquid crystalline phase transition of DMPC as a function of membrane cholesterol. At identical temperatures, 5 and 10 mole percent cholesterol slightly increased the major fluorescence lifetime of dehydroergosterol when compared to membranes without cholesterol, and this increase occurred at temperatures both below and above the phase transition temperature of DMPC. Neither membrane cholesterol or dehydroergosterol significantly altered the fluorescence lifetime of dehydroergosterol in multilamellar liposomes at concentrations up to 10 mole percent sterol, consistent with previous results [6].

Time-correlated fluorescence anisotropy decays of dehydroergosterol in multilamellar liposomes in the absence and presence of 5 mole percent cholesterol

Time-correlated fluorescence anisotropy measurements can yield information on the dynamic properties of fluorescent membrane probes since these studies detect decay of fluorescence anisotropy on the nanosecond time scale. The time-correlated decay of fluorescence anisotropy can reveal physical characteristics of the local membrane environment. Membrane effects on the rotational correlation time (ϕ), and limiting anisotropy (r_∞) observed at times much longer than the fluorescence lifetime, can yield information on the rotational motion and local environment of a membrane fluorophore, respectively.

Time-correlated fluorescence anisotropies of 0.9 mole percent dehydroergosterol in unsonicated DMPC multilamellar liposomes were initially analyzed with membranes that contained no membrane cholesterol. Fig. 4a presents the effect of temperature on the rotational correlation time of 0.9 mole percent dehydroergosterol in multilamellar liposomes at temperatures that included the gel-to-liquid crystalline phase transition of DMPC. Dehydroergosterol exhibited a single rotational correlation time near 1 ns both below and above the phospholipid phase transition of DMPC. Within the temperature range of 15°C to 30°C, the rotational correlation time of dehydroergosterol in multilamellar liposomes without cholesterol was essentially independent of temperature. The rotational correlation time decayed to a finite, positive anisotropy over the measured time span of 35 ns indicating the fluorescence decay in multilamellar liposomes was best described as a single rotational correlation time plus a constant.

Fig. 4b presents the time-correlated anisotropy decay of dehydroergosterol as a function of temperature in multilamellar liposomes that contained 5 mole percent cholesterol. The time-correlated depolarization of dehydroergosterol exhibited a pattern similar to that of membranes without cholesterol. However, a slightly longer rotational correlation time was measured below approximately 21°C in membranes containing 5 mole percent cholesterol.

Fig. 5a summarizes the effect of temperature on the limiting anisotropy of dehydroergosterol in multilamellar liposomes without cholesterol. Al-

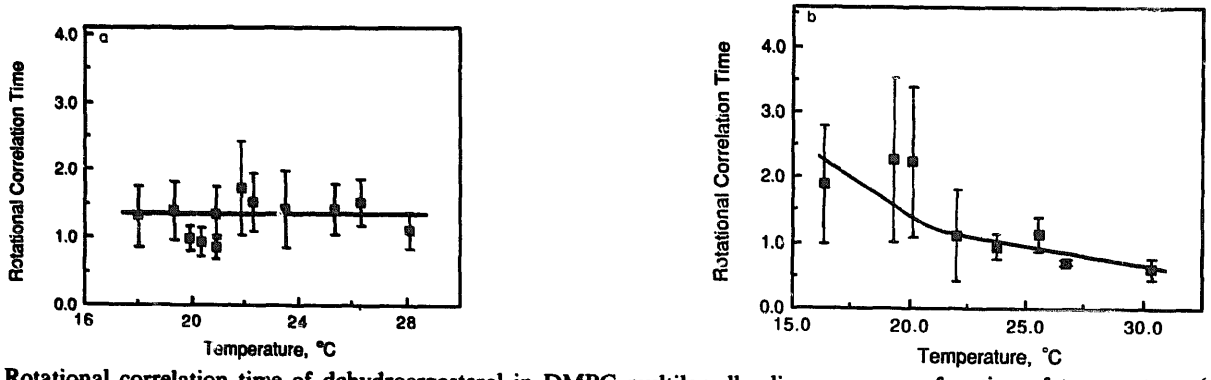

Fig. 4. Rotational correlation time of dehydroergosterol in DMPC multilamellar liposomes as a function of temperature. (a) No membrane cholesterol, and (b) 5 mole percent cholesterol.

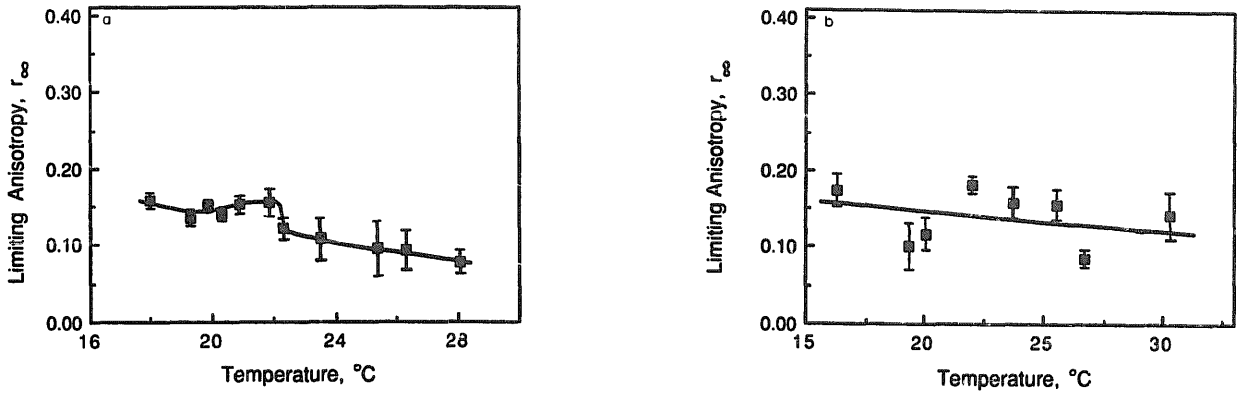

Fig. 5. Limiting anisotropy (r_∞) of the single rotational correlation time of dehydroergosterol in multilamellar liposomes. (a) No membrane cholesterol, and (b) 5 mole percent cholesterol.

though the short fluorescence lifetime and rotational correlation time of dehydroergosterol in multilamellar liposomes did not afford a precise analysis of r_∞ values, the r_∞ of dehydroergosterol decayed to a positive, finite value which decreased with increasing temperature. A sharp decrease in r_∞ was observed near the gel-to-liquid crystalline phase transition of DMPC. The r_∞ of dehydroergosterol in multilamellar liposomes was significantly different from its decay in solvents such as mineral oil or glycerol where complete randomization of polarized fluorescence was detected over a similar time span [8,10]. Decay of anisotropy to a finite, positive value suggested hindered rotational diffusion of dehydroergosterol, and in turn, ordering of dehydroergosterol in multilamellar liposomes. At temperatures above the DMPC phase transition, rotational motion of 0.9 mole percent dehydroergosterol appeared relatively less hindered as the phospholipid membrane bilayer entered the less ordered liquid crystalline state. These results suggested the r_∞ of 0.9 mole percent dehydroergosterol was monitoring the phase behavior of the bulk phospholipids in membranes devoid of cholesterol.

Fig. 5b presents corresponding r_∞ values of 0.9 mole percent dehydroergosterol in unsonicated membranes of 5 mole percent cholesterol as a function of temperature. The r_∞ again decayed to a constant value. However, r_∞ was not significantly affected by temperature suggesting that five mole percent membrane cholesterol essentially eliminated the ability of r_∞ to detect the gel-to-liquid crystalline phase transition of DMPC. Cholesterol also increased the r_∞ of dehydroergosterol above the phase transition of DMPC. This observation suggested 5 mole percent cholesterol increased the motional order of membranes whose phospholipids were above the phase transition temperature. A similar effect of cholesterol on the r_∞ of 1,6-diphenyl-1,3,5-hexatriene has been reported [15].

The $r_{(0)}$ of 0.9 mole percent dehydroergosterol in multilamellar liposomes was largely unaffected by temperature (data not shown). However, the $r_{(0)}$ of dehydroergosterol in multilamellar liposomes appeared lower than in solvents under similar instrumental conditions [10], in glycerol [8], and in sonicated POPC vesicles where reported values approached 0.385 [9]. The results with multilamellar liposomes suggested some fluorescence depolarization may have occurred in membranes that was not observable on the nanosecond time scale. A similar observation has been reported in human serum LDL labeled with dehydroergosterol [6] and in macrophage membranes labeled with 1,6-diphenyl-1,3,5-hexatriene [16].

Calculations of dehydroergosterol motional order parameters utilizing r_∞ estimated from time-correlated fluorescence data and r_0 equal to 0.385 [13] indicated S values near 0.6 below the gel-to-liquid crystalline phase transition, and S values near 0.5–0.4 above the phase transition of nsonicated multilamellar liposomes without cholesterol. S values near 0.6 were also calculated for membranes containing 5 mole percent cholesterol. These motional order parameters qualitatively agree with those of membranes con-

taining low membrane cholesterol whose probe reporter groups are located near the polar region of phospholipid bilayers [17].

In summary, the time-correlated data indicated that dehydroergosterol exhibited a rapid rotational rate within the restricted phospholipid environment of DMPC. The time-correlated results are also consistent with steady-state anisotropies of dehydroergosterol in DMPC multilamellar liposomes [7] since a time-averaged steady-state anisotropy slightly above r_∞ would be predicted for a fluorescent probe that essentially exhibited a single exponential lifetime decay [18].

Discussion

The objectives of this study were to quantitate the effects of low membrane cholesterol on membrane structure in unsonicated membranes by steady-state, lifetime, and time-correlated fluorescence anisotropy studies and to determine potential differences of sterol environments in multilamellar liposomes and SUV at low membrane sterol concentrations. The decreased absorption coefficient of dehydroergosterol suggested probe–probe interactions in multilamellar liposomes where the rigid chromophore of dehydroergosterol was ordered by its phospholipid environment [7,8], and formed sterol-rich regions in membranes at concentrations near 5 mole percent or greater [7–9]. In contrast, low concentrations of dehydroergosterol in solution are not ordered by phospholipid bilayers and may randomly orient in solvent. Since intensity of the absorption band of a chromophore is proportional to the size of its corresponding transition dipole [19], dehydroergosterol may exhibit a smaller transition dipole in multilamellar liposomes than in solvents since membrane-induced sterol-sterol interactions may decrease the transition dipole due to phospholipid ordering of sterol and subsequent sterol segregation from bulk membrane phospholipids at low membrane sterol concentration [7]. This membrane orientation would favor interactions between dehydroergosterol chromophores.

Transition dipole moments induced by absorption of light in neighboring dehydroergosterol rings may then induce transition dipole moments in the originally excited sterol that may oppose the transition dipole moment directly induced by light, similar to the effect observed in stacked nucleotide bases of DNA [20]. While the immediate membrane environment that solvates dehydroergosterol may also affect its absorptive properties in bilayers, the lowered absorption coefficient of dehydroergosterol in DMPC multilamellar liposomes is consistent with sterol–sterol interactions that may either not occur in less ordered solvents or may occur to a lesser extent.

The effect of dehydroergosterol concentration on its steady-state anisotropy further suggested sterol–sterol interactions occurred in multilamellar liposomes at low membrane sterol concentrations. The physical effects of low dehydroergosterol concentrations in multilamellar liposomes were analogous to the anisotropy changes induced by membrane cholesterol in multilamellar liposomes at similar concentrations [7]. Thus, the sterols dehydroergosterol and cholesterol both exerted measurable physical changes in multilamellar liposomes at concentrations near 5 mole percent.

The steady-state anisotropy of dehydroergosterol in unsonicated multilamellar liposomes contrasts with the concentration-dependent depolarization of dehydroergosterol in SUV where Schroeder et al. [9] reported dehydroergosterol steady-state fluorescence anisotropy was not significantly altered below approx. 5 mole percent dehydroergosterol. Above this fluorophore concentration, depolarization of dehydroergosterol readily occurred [9]. Based on the ability of dehydroergosterol to decrease its steady-state anisotropy in membranes, dehydroergosterol fluorescence anisotropy may reflect sterol-sterol interactions in multilamellar liposomes, but at lower membrane fluorophore concentrations than in SUV.

The differential effect that increased dehydroergosterol concentrations exerted on its steady-state fluorescence anisotropy in multilamellar liposomes and SUV may have several possible explanations. One interpretation is based on differences in surface curvature between multilamellar liposomes and SUV. Multilamellar liposomes possess a significantly less curved surface than the much smaller, one bilayer thick membranes of SUV. The lower steady-state fluorescence ani-

sotropy in multilamellar liposomes could represent differences in packing of sterols with phospholipids in SUV and multilamellar liposomes based on the larger radius of curvature in multilamellar liposomes. Comparative circular dichroism spectra of dehydroergosterol in multilamellar liposomes and SUV indicated that dehydroergosterol yielded significant differences in circular dichroic spectra due to asymmetry of sterol packing with phospholipids across SUV and multilamellar liposomes of PC [21]. Yeagle et al. concluded that packing of sterol with phospholipids across bilayer leaflets of multilamellar liposomes was less asymmetric than across corresponding SUV bilayer leaflets [21].

A second interpretation of the lowered steady-state fluorescence anisotropies in multilamellar liposomes is based on differences in formation of multilamellar liposomes and SUV. Multilamellar liposomes are thought to undergo osmotic compression due to solute exclusion upon formation by hydration of dried films [22]. In contrast, SUV formation by sonication will likely insure uniform mixing of membrane components and solute as suggested by fluorescence quenching studies [6] and by proton nuclear magnetic resonance studies [23] of membranes containing low membrane sterol. In contrast, the solute exclusion unique to multilamellar liposomes may expand or shrink these concentric bilayers to accommodate the stress [22] potentially causing uneven membrane distribution of dehydroergosterol upon hydration. This osmotically-induced distribution of dehydroergosterol in multilamellar liposomes could result in localized dehydroergosterol concentrations able to decrease steady-state anisotropies at lower dehydroergosterol concentrations in multilamellar liposomes than in SUV. This uneven distribution could affect relative membrane order as suggested by the lowered anisotropy of dehydroergosterol in liposomes.

Although the absorption and emission spectra of dehydroergosterol in multilamellar liposomes overlap, the concentration-dependent steady-state depolarization of dehydroergosterol in multilamellar liposomes may not be caused by homotransfer since neither unsonicated or sonicated membranes containing dehydroergosterol undergo a red edge effect [8,9]. Lifetime changes may not fully explain the depolarization since increased concentrations of dehydroergosterol caused a very small change in its fluorescence lifetime in multilamellar liposomes while significantly decreasing its steady-state anisotropy. A possible mechanism for the depolarization of low dehydroergosterol concentrations in multilamellar liposomes consists of a concentration-dependent quenching of dehydroergosterol fluorescence in membranes that is based on its ability to self-aggregate within the ordered phospholipid bilayer environment. A self-quenching mechanism for dehydroergosterol fluorescence has been proposed in SUV at membrane fluorophore concentrations above 5 mole percent [9].

Fluorescence lifetimes of dehydroergosterol in unsonicated membranes indicated low levels of cholesterol essentially obliterated the sharp drop in fluorescence lifetime previously measured at the gel-to-liquid crystalline phase transition of DMPC. These results further indicated dehydroergosterol was laterally phase separated in multilamellar liposomes at 5 mole percent cholesterol or higher and unable to detect the bulk phospholipid phase transition near 23° C.

The duration of the single rotational correlation time of dehydroergosterol in multilamellar liposomes at temperatures near the phospholipid phase transition was not significantly altered by the gel-to-liquid crystalline phase transition in membranes devoid of cholesterol or by low concentrations of membrane cholesterol. However, a slightly shorter rotational correlation time in liquid-crystalline DMPC bilayers containing 5 mole percent cholesterol is consistent with cholesterol effects on depolarization of 1,6-diphenyl-1,3,5-hexatriene in large unilamellar PC vesicles [24], and in sonicated vesicles [25]. Furthermore, the loss of sensitivity of r_∞ of dehydroergosterol to bulk phase changes of the phospholipids in multilamellar liposomes containing 5 mole percent cholesterol further suggested that cholesterol formed sterol-rich regions in multilamellar liposomes.

The decreased r_∞ of dehydroergosterol in multilamellar liposomes without cholesterol at temperatures above 23° C did not result in a shorter rotational correlation time. Thus, the decreased motional order of the bulk phospholipids of multilamellar liposomes did not significantly alter the rate of dehydroergosterol rotation in these membranes.

The rotational correlation time of dehydro-

ergosterol in multilamellar liposomes was slightly longer in duration (lower rotational rate for dehydroergosterol in multilamellar liposomes) when compared to POPC SUV [9], and could be explained by inherent differences in the two types of membrane systems studied. The possibilities include radius of curvature effects on cholesterol packing in multilamellar liposomes and SUV, potential osmotic effects in multilamellar liposomes, or temperature effects on the lipid matrix states of the two membrane systems.

In summary, differences in steady-state fluorescence properties of dehydroergosterol in SUV and multilamellar liposomes were observed that suggested potential sterol–sterol interactions in multilamellar liposomes at lower concentrations than in SUV. Fluorescence lifetimes and rotational correlation times in multilamellar liposomes were qualitatively similar to those observed with SUV [9]. Time-correlated fluorescence studies further suggested that low membrane concentrations of dehydroergosterol exhibited a rapid rotational rate within the ordering environment of multilamellar liposomes. This study indicates dehydroergosterol is a promising molecule for the study of sterol dynamics in model membranes which can yield important information on sterol interactions with other membrane sterols, membrane phospholipids, and membrane proteins.

Acknowledgements

The author wishes to thank Dr. Harvey A. Berman for extended use of the fluorescence lifetime spectrophotometer, for use of his laboratory facilities, and for his interest in this project. He wishes to thank Dr. L. Chamberlin, Dr. E. Dahmer, Dr. S. Ohki, D. Badgett, B. Crawford, M. Decker, J. Kaars, M. Nowak, W. Pudlak, and J. Young for their valuable assistance. The author also wishes to thank Dr. V. Chen, Dr. G. Holtom, and M. Rusiniak for valuable discussions. The fluorescence lifetime apparatus was obtained from a Regional Resource Grant from the National Institutes of Health (RR-01705-01).

References

1 Rogers, J., Lee, A.G. and Wilton, D.C. (1979) Biochim. Biophys. Acta 552, 23–37.
2 Archer, D.B. (1975) Biochem. Biophys. Res. Commun. 66, 195–201.
3 Yeagle, P.L., Bensen, J., Greco, M. and Arena, C. (1982) Biochemistry 21, 1249–1254.
4 Muczynski, K.A. and Stahl, W.L. (1983) Biochemistry 22, 6037–6048.
5 Schroeder, F. (1981) FEBS Lett. 135, 127–130.
6 Smutzer, G. (1988) Biochim. Biophys. Acta 958, 323–333.
7 Smutzer, G. and Yeagle, P.L. (1985) Biochim. Biophys. Acta 814, 274–280.
8 Chong, P.L.-G. and Thompson, T.E. (1986) Biochim. Biophys. Acta 863, 53–62.
9 Schroeder, F., Barenholz, Y., Gratton, E. and Thompson, T.E. (1987) Biochemistry 26, 2441–2448.
10 Smutzer, G., Crawford, B.F. and Yeagle, P.L. (1986) Biochim. Biophys. Acta 862, 361–371.
11 Weber, G. (1953) Adv. Protein Chem. 8, 415–459.
12 Wolber, P.K. and Hudson, B.S. (1981) Biochemistry 20, 2800–2810.
13 Fischer, R.T., Stephenson, F.A., Shafiee, A. and Schroeder, F. (1985) J. Biol. Phys. 13, 13–24.
14 Kier, A.B., Sweet, W.D., Cowlen, M.S. and Schroeder, F. (1986) Biochim. Biophys. Acta 861, 287–301.
15 Heyn, M. (1979) FEBS Lett. 108, 359–364.
16 Petty, H.R., Niebylski, C.D. and Francis, J.W. (1987) Biochemistry 26, 6340–6348.
17 Stockton, G.W. and Smith, I.C.P. (1976) Chem. Phys. Lipids 17, 251–263.
18 Van der Meer, B.W., Van Hoeven, R.P. and Van Blitterswijk, W.J. (1986) Biochim. Biophys. Acta 854, 38–44.
19 Eisenberg, D. and Crothers, D. (1979) Physical Chemistry with Applications to the Life Sciences, pp. 562–645, Benjamin/Cummings Publishing Company, Menlo Park, CA.
20 Bloomfield, V.A., Crothers, D.M. and Tinoco, I., Jr. (1974) Physical Chemistry of Nucleic Acids, Harper & Row, New York.
21 Yeagle, P.L., Bensen, J., Boni, L. and Hui, S.W. (1982) Biochim. Biophys. Acta 692, 139–146.
22 Gruner, S.M., Lenk, R.P., Janoff, A.S. and Ostro, M.J. (1985) Biochemistry 24, 2833–2842.
23 Huang, C., Sipe, J.P., Chow, S.T. and Martin, R.B. (1974) Proc. Natl. Acad. Sci. USA 71, 359–362.
24 Straume, M. and Litman, B.J. (1987) Biochemistry 26, 5121–5126.
25 Kawato, S., Kinosita, K., Jr. and Ikegami, A. (1978) Biochemistry 17, 5026–5031.