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A Fluorescence Study of Dehydroergosterol in Phosphatidylcholine Bilayer Vesicles†

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ABSTRACT: The fluorescent sterol $\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol (dehydroergosterol) was incorporated into 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) small unilamellar vesicles (SUV) with and without cholesterol in order to monitor sterol–sterol interactions in model membranes. In the range 0–5 mol % fluorescent sterol, dehydroergosterol underwent a concentration-dependent relaxation characterized by red-shifted wavelengths of maximum absorption as well as altered ratios of absorbance maxima and fluorescence excitation maxima at 338 nm/324 nm. Fluorescence intensity per mole of dehydroergosterol increased up to 5 mol % in POPC vesicles. In contrast, quantum yield, steady-state anisotropy, limiting anisotropy, lifetime, and rotational rate remained relatively constant in this concentration range. Similarly, addition of increasing cholesterol in the range 0–5 mol % in the presence of 3 mol % dehydroergosterol also increased the fluorescence intensity per mole of dehydroergosterol, red-shifted wavelengths of maximum absorption, and altered ratios of absorbance maxima. In POPC vesicles containing between 5 and 33 mol % dehydroergosterol, the fluorescent dehydroergosterol interacted to self-quench, thereby decreasing the fluorescence intensity, quantum yield, steady-state anisotropy, and limiting anisotropy and increasing the rotational rate (decreased rotational relaxation time) of the fluorescent sterol. The fluorescence lifetime of dehydroergosterol remained unchanged. The results were in accord with the interpretation that below 5 mol % sterol, the sterols behaved as monomers exposed to some degree to the aqueous solvent in POPC bilayers. At higher concentrations, the sterol laterally segregated. Above 33 mol % sterol, only a single type of sterol phase existed within the POPC bilayer. At low mole percent fluorescent sterol, dehydroergosterol was an excellent probe molecule for determination of the motional properties (dynamic and static) of sterols in POPC membranes. Differential polarized phase fluorescence spectroscopy indicated that the dehydroergosterol had a rapid rotational rate and a high degree of order at 24 °C in POPC vesicles. Between 7 and 50 mol % cholesterol, the limiting anisotropy, quantum yield, and lifetime increased while the rotational rate decreased. In summary, in POPC vesicles containing mixtures of dehydroergosterol and cholesterol, the two sterols appear similar in motional as well as dielectric properties.

Understanding the central role of cholesterol in modulating the structure and properties of many biological membranes requires knowledge of cholesterol–cholesterol interactions as well as the interactions of cholesterol with other membrane components. Many aspects of this problem have been investigated including cholesterol lateral phase separation, cholesterol–phospholipid interaction, and cholesterol–protein interactions. The information obtained by these various approaches has been reviewed by Schroeder (1984).

A major difficulty in this field has been the identification of suitable probe molecules which, when inserted into membranes, accurately mimic the behavior of cholesterol. Ideally, proton or carbon-13 NMR methods would be the best, but the signals from these atoms in the cholesterol ring systems are generally poorly resolved (Darke et al., 1972; de Kruijff, 1978). However, deuterium NMF of deuteriated cholesterol probes has proven useful to detect motional properties of sterols in model and red blood cell membranes despite some difficulties in practical application (Taylor et al., 1981, 1982; Kelusky et al., 1983). Although spin-labeled derivatives have been used, there is some uncertainty of interpretation because of possible perturbations caused by the bulky nitroxide moiety (Butler et al., 1970; Hubbell & McConnell, 1971; Träuble & Sackman, 1972; Mailer et al., 1974; Presti et al., 1982; Presti & Chan, 1982). More recently, fluorescent analogues of cholesterol have been utilized [reviewed by Schroeder (1984)]. In this paper, we report the use of dehydroergosterol to investigate sterol–sterol and sterol–phospholipid interactions in 1-palmitoyl-2-oleoylphosphatidylcholine (POPC)[1] small unilamellar vesicles (SUV).

Materials and Methods

Reagents. 1-Palmitoyl-2-oleoylphosphatidylcholine was purchased from Avanti Biochemicals, Inc., Birmingham, AL. Cholesterol was obtained from Mann Research Labs, New York, NY. These lipids were checked for purity by thin-layer

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SUV, small unilamellar vesicle(s); dehydroergosterol, $\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol; PIPES, piperazine-*N*,*N*′-bis(2-ethanesulfonic acid); PPO, 2,5-diphenyloxazole; HPLC, high-performance liquid chromatography.

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chromatography and stored in sealed ampules under N_2 at −70 °C. Dehydroergosterol was synthesized and purified as described previously (Fischer et al., 1985). Purity was confirmed by high-performance liquid chromatography, absorbance peak ratios, and comparison with dehydroergosterol standards obtained from Frann Scientific Inc., Columbia, MO.

Preparation of Liposomes. Small unilamellar vesicles were prepared as follows: POPC, cholesterol, and dehydroergosterol were dissolved in chloroform or ethanol in proportions indicated in the legends to figures and tables. Samples were placed in solvent-cleaned glass tubes and evaporated under N_2, and trace amounts of solvents were removed in vacuo for 24–48 h. Sufficient buffer (10 mM PIPES/0.02% NaN_3, pH 7.4) was added at 24 °C to provide a final lipid concentration of 4 mM. The samples were vortexed and sonicated for 3 min with a 125-W bath sonicator (Heat Systems Inc., Plainview, NJ) or with a Sonogen bath sonicator (Branson Instruments, Stamford, CT) to remove the lipid from the sides of the tube and yield a milky suspension of multilamellar vesicles. This suspension was then further sonicated either in five 3-min bursts using a titanium microtip probe and a W-350 sonifier cell disruptor (Branson Sonic Power Co., SKF, Philadelphia, PA) set at 200-W output or in three 10-min bursts with a Sonic Dismembrator, Model 300 (Fisher Scientific, Inc., Pittsburgh, PA), set at 33% maximum output. Sonication was always performed under N_2 and above the phase transition temperature of the matrix phospholipid. The resulting SUV were separated from large vesicles and multilamellar liposomes by differential ultracentrifugation for 2 h with a 40 Ti rotor and a L3-40 or L5-65 ultracentrifuge (Beckman Instruments, Fullerton, CA) as described earlier (Barenholz et al., 1977). The yield of POPC SUV in the supernatant in the presence of dehydroergosterol decreased from approximately 60% at 0.5 mol % fluorescent sterol to 20% at 60 mol % sterol. This decrease was similar to that obtained in the formation of POPC SUV with increasing mole percent cholesterol (data not shown).

Lipid Composition. The lipid composition of the SUV was examined in order to determine if the fluorescent sterol and cholesterol were incorporated into SUV in the same proportion (sterol:POPC ratio) as was present in the starting mixture prior to sonication. The lipid composition of the of the SUV was determined as follows: Total phospholipid concentration was estimated by determination of phosphorus (Bartlett, 1959). The relative mole percent of POPC was determined after lipid extraction (Bligh & Dyer, 1959), two-dimensional thin-layer chromatography, visualization with I_2 vapor, elution, and phosphate assay (Schroeder et al., 1976). The mole percent cholesterol and dehydroergosterol was determined by using stigmasterol or ergosterol, added as an internal standard prior to lipid extraction, and separation of neutral lipids from phospholipids by silicic acid chromatography (Schroeder et al., 1976). Sterols were resolved both by analytical high-performance liquid chromatography (Fischer et al., 1985) and by fluorescence analysis (Schroeder, 1984). In the latter method, the fluorescent dehydroergosterol was quantitated by dissolving the neutral lipid fraction in ethanol, determining the fluorescence or absorbance at 324-nm excitation (376-nm emission) as described below, and comparing fluorescence intensities to those of a standard curve for dehydroergosterol, also in ethanol. Dehydroergosterol was incorporated into POPC SUV vesicles essentially in the same molar proportion as initially present prior to sonication. Plots of measured mole percent fluorescent sterol vs. starting mole percent fluorescent sterol were linear with a slope of 0.8 for dehydroergosterol.

Fluorescence Spectroscopy. Fluorescence parameters were measured with four instruments. Absorbance, absorption-corrected fluorescence, relative fluorescence efficiency, and corrected fluorescence emission were determined simultaneously with a computer-based spectrofluorometer previously described (Christman et al., 1980; Schroeder, 1980). Corrected fluorescence excitation spectra, emission spectra, and fluorescence intensity were obtained with a photon-counting Fluorolog spectrofluorometer (Spex Instruments, Metuchen, NJ). Light scattering was reduced by using dilute samples and by using narrow band-passes in the excitation and emission monochromators. Where light scattering was detectable, fluorescence intensities were corrected by subtracting the signal of an analogous vesicle preparation containing cholesterol instead of fluorescent sterol. The scattering correction was always less than 2%.

Steady-state fluorescence polarization, P, was determined at the University of Missouri and at the University of Virginia using an SLM 4800 subnanosecond spectrofluorometer (SLM Instruments, Inc., Urbana IL) in the L format. In determination of polarization, light scattering was reduced by using narrow band-passes in the excitation monochromator and emission monochromator. In addition, samples were serially diluted, and polarization was measured and then extrapolated to zero absorbance (Lentz et al., 1979; Chong & Colbow, 1976). Steady-state anisotropy, r, was measured as

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

The steady-state polarization, P, was corrected for grating induced anisotropies (Chen & Bowman, 1965).

Fluorescence lifetimes were measured by phase and modulation with two types of instruments: (1) SLM 4800 subnanosecond spectrofluorometers equipped with an excitation monochromator, either an emission monochromator or an appropriate cutoff filter, and a xenon arc light source. These instruments were limited to operation at three fixed modulation frequencies (6, 18, and 30 MHz). All fluorescence lifetimes were measured with the excitation polarizer set at 0° and the emission polarizer set at the magic angle, 55°, in order to eliminate Brownian motion as a determinant of apparent lifetime. Fluorescence lifetimes were simultaneously measured relative to a reference solution of 2,5-diphenyloxazole (PPO) (Chemalog Chemical Dynamics Corp., South Plainfield, NJ) in absolute ethanol as described previously (Lakowicz et al., 1981). The excitation wavelength for dehydroergosterol in SUV was 324 nm; fluorescence emission was monitored at 376 nm. (2) Alternately, a multifrequency phase and modulation fluorometer (1-300 MHz), described elsewhere (Gratton & Linkeman, 1983), was utilized. The latter instrument is based on the cross-correlation principle introduced by Spencer and Weber (1969). A He/Cd laser (Model 4240NB, Liconix, Sunnyvale, CA), whose emission intensity at 325 nm was modulated sinusoidally with a Pockels cell, was the light source. The excitation polarizer was set at 35°. Usually, 14 modulation frequencies were utilized; at each frequency, both phase and modulation of the fluorescence were determined with respect to a glycogen scatter solution. Scattered light was observed after it passed through an interference filter. Emission was observed through a Corning (0-52) or Schott KV-370 sharp-cutoff filter. Data were collected by an Apple II or IBM PC computer using an ISS01 interface (ISS Inc., Champaign, IL). The set of phase and modulation data was statistically analyzed by a nonlinear least-squares routine using software for an IBM PC (IBM Inc.) obtained from ISS Inc., Urbana, IL. The method of data analysis is described elsewhere (Jameson & Gratton, 1983; Lakowicz et al., 1984a).

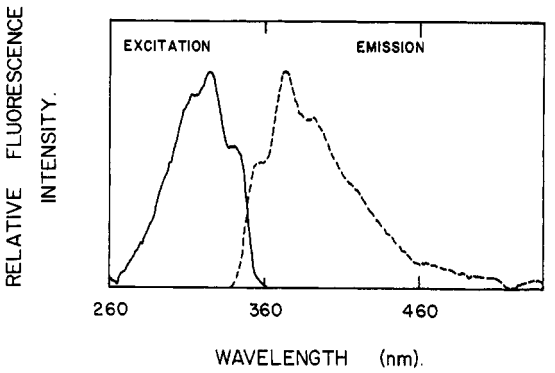

FIGURE 1: Fluorescence excitation and emission spectra of dehydroergosterol in POPC vesicles. Spectra were obtained with dehydroergosterol (0.5 mol %) in POPC single unilamellar vesicles using the Spex Fluorolog spectrofluorometer. Excitation and emission were at 324 and 376 nm, respectively. Emission spectra were corrected for monochromator and source-dependent variation in intensity.

The data were fitted to one- or two-exponential terms. In the latter case, each term was characterized by a lifetime (τ) and a fractional intensity (f). The reduced χ^2 parameter was utilized as described by Lakowicz et al. (1984a) to judge the quality of fit. Usually, χ^2 values near 3 were considered acceptable while values in excess of 10 were indicative of large discrepancies between calculated theoretical curves and experimentally determined ones. The error in each parameter was determined by using a covariance matrix of errors (Lakowicz et al., 1984a). The statistical analysis does not attribute physical significance to the parameters but only that the data fit the model used.

Differential polarized phase fluorometry was used to obtain R, the rotational rate (radians per second) and r_∞, the limiting anisotropy, of dehydroergosterol fluorescence in membranes according to the theory developed by Weber (1978) and extended by Lakowicz et al. (1984b). In the curve-fitting procedure, the values of r_0 (the anisotropy in the absence of rotational motion), r_∞, and Φ (the rotational correlation time in nanoseconds) were floating parameters. The rotational rate (radians per second) equals $6\Phi^{-1}$. This procedure utilized differential polarized phase and modulation data obtained at 14 frequencies between 10 and 300 MHz. Values of limiting anisotropy and rotational rate obtained thereby differ from those obtained by an earlier method (Weber, 1978; Lakowicz et al., 1979). In the latter method, only a single fixed frequency and a fixed $r_0 = 0.385$ for dehydroergosterol [determined at 324 nm excitation according to the conditions described by Shinitzky and Barenholz (1974)] were utilized. The He/Cd laser line at 325 nm was used as the dehydroergosterol excitation wavelength.

Photobleaching is a potential problem associated with fluorescence measurement of many fluorophores. Over a 2-h time period, during which dehydroergosterol in POPC SUV was exposed to strong ultraviolet light (450-W xenon lamp), the relative fluorescence intensity of the dehydroergosterol (2 or 6 mol %) did not diminish significantly (100 ± 3 vs. 96 ± 3 and 100 ± 2 vs. 96 ± 2, respectively).

Results

Spectroscopic Characterization of Dehydroergosterol. The maxima in the corrected excitation spectrum of dehydroergosterol in POPC vesicles were at 310, 324, and 340 nm while emission maxima were at 356, 376, and 390 nm (Figure 1). The maxima were similar to those obtained for the dehydroergosterol in ethanol. The similarity in spectral shape of corrected fluorescence excitation spectra with absorption spectra is indicative of the purity of the dehydroergosterol

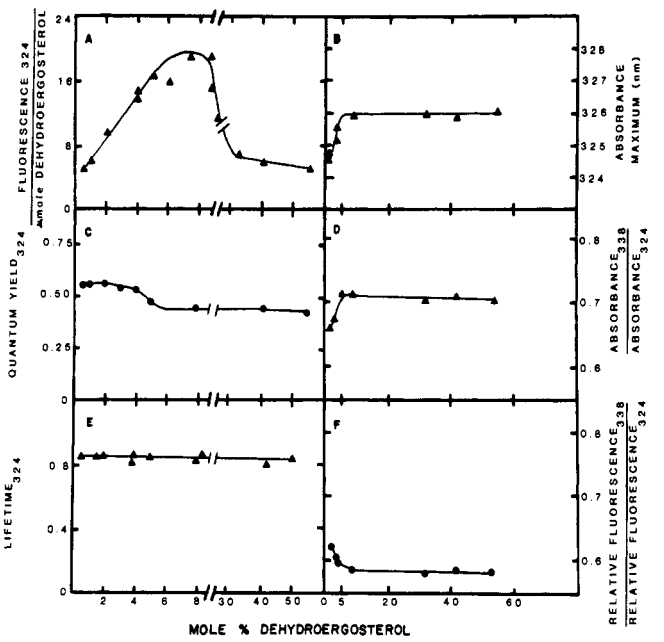

FIGURE 2: Effect of increasing mole percent dehydroergosterol on fluorescence properties in POPC vesicles. All determinations were performed on intact SUV. The mole percent dehydroergosterol refers to the actual quantity of fluorescent sterol in the SUV (measured by HPLC and by fluoresecence of the extracted neutral lipid fraction suspended in ethanol). (A) The Spex Fluorolog spectrofluorometer was used to determine the relative fluorescence excitation intensity at the fluorescence excitation maximum near 324 nm. (B) Absorbance spectra were determined with an Aminco-Bowman DW-2 UV–vis spectrometer. (C) Quantum yields were calculated at 24 °C by comparison of fluorescence and absorbance of the fluorescent sterol relative to a standard, 1,6-diphenyl-1,3,5-hexatriene in benzene (quantum yield = 0.80). (D) Absorbance spectra were obtained as for (B). The ratio of the absorbance maximum near 338 nm to the absorbance maximum near 324 nm was taken from these spectral scans. (E) Fluorescence lifetime was determined as described under Materials and Methods. (F) Fluorescence excitation spectra were obtained with the Spex Instruments photon-counting Fluorolog spectrofluorometer. The ratio of the relative fluorescence intensity excitation maximum near 338 nm to the relative fluorescence excitation maximum near 324 nm was taken from these spectral scans.

(HPLC grade) utilized herein.

The fluorescence dehydroergosterol exhibited a significant Stokes shift. The fluorescence emission maxima of dehydroergosterol were near 402, 426, and 440 nm when dehydroergosterol in a small amount of ethanol was dispersed in phosphate-buffered saline. When dehydroergosterol was dissolved in a series of dioxane/water mixtures, the ratio of absorbance-corrected fluorescence at 338 nm/absorbance-corrected fluorescence at 324 nm decreased with increasing hydrophobicity, from 0.75 (pure water) to 0.60 (dioxane). In POPC vesicles, this ratio was 0.60. Interaction with the more polar aqueous solvent, as compared to POPC hydrophobic alkyl chains, apparently stabilized the ground state to a greater extent. Taken together, these results indicate that in POPC vesicles, the fluorescent sterol resides in a nonpolar environment.

Fluorescence Properties of Dehydroergosterol in POPC Vesicles. The fluorescence emission at 376 nm when excited at 324 nm was determined for different concentrations of dehydroergosterol in POPC vesicles. The data plotted as fluorescence per mole of dehydroergosterol vs. mole percent dehydroergosterol in the vesicles are shown in Figure 2A. Relative fluorescence intensity increased with increasing mole percent dehydroergosterol until about 6 mol %. Above this concentration, this parameter decreased approximately 50% for dehydroergosterol. At mole percents greater than 30 mol

Table I: Lifetime Analysis of Dehydroergosterol in POPC SUV Using a Multifrequency Phase and Modulation Fluorometer[a]

mol % dehydroergosterol	one-lifetime analysis, τ (ns)	statistical χ^2 value
2	0.860 ± 0.004	2.76
5	0.848 ± 0.005	1.52
50	0.842 ± 0.013	2.31

[a] Lifetime of dehydroergosterol in POPC SUV was measured at 24 °C by phase and modulation techniques at 10, 20, 30, 40, 60, 70, 80, 90, 100, 115, 120, 130, 140, and 150 MHz. Values represent the mean ± SEM (n = 14).

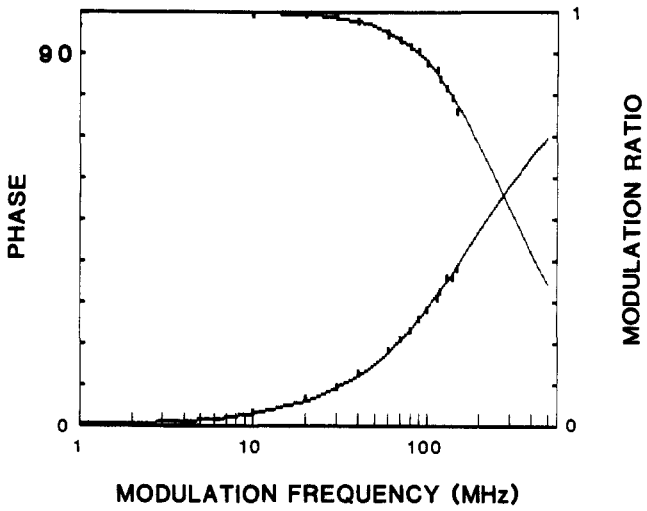

FIGURE 3: Phase and modulation data for dehydroergosterol in POPC SUV. Dehydroergosterol (2 mol %) was incorporated into POPC SUV, and phase angles (degrees, bottom curve) or modulation ratios (top curve) were obtained at 24 °C as a function of modulation frequency (1–160 MHz). The solid lines indicate the best one-component fit. The data points are the experimental values.

%, the fluorescence of dehydroergosterol decreased more slowly, apparently to a limiting value.

Three changes in absorbance at low mole percent fluorescent sterol are also noted. First, for 0.5 mol % dehydroergosterol, the major absorption maximum in POPC SUV occurred at 324.6 nm; with increasing mole percent fluorescent sterol, the absorption maximum was red-shifted. This shift was 1.4 nm near 5 mol % dehydroergosterol. At higher sterol concentrations, no further shift was observed. This is shown in Figure 2B. Second, there was a change in the ratio of the absorbance at 338 nm to the absorbance at 324 nm (Figure 2D) and the fluorescence excitation at 338 and 324 nm (Figure 2F). The ratio of absorbance at 338 nm/324 nm increased for dehydroergosterol until 5 mol % fluorescent sterol. The ratio of fluorescence excitation peak intensities, in contrast, decreased (due to the lower fluorescence emission of this probe molecular at 338 nm than at 324 nm) until 5 mol % fluorescent sterol. Third, the absorbance at 324 nm per micromole of fluorescent sterol increased linearly with increasing mole percent dehydroergosterol until about 5 mol % (data not shown).

Dependence of Lifetime, Quantum Yield, and Anisotropy on Sterol Concentration. A multifrequency (1–300 MHz) phase and modulation spectrometer was utilized to determine if dehydroergosterol has one or multiple lifetime components in POPC vesicles (Table I). Statistical analysis for a one-lifetime fit showed χ^2 values below 3 for 2–50 mol % dehydroergosterol in POPC vesicles. The data for 2 mol % dehydroergosterol fit a one-component lifetime analysis near 0.860 ns (Figure 3). Attempts at two-component fitting yielded two lifetimes at 0.846 ± 0.038 ns (fractional intensity, 0.98) and 2.19 ± 4.26 ns (fractional intensity, 0.02), respectively. The

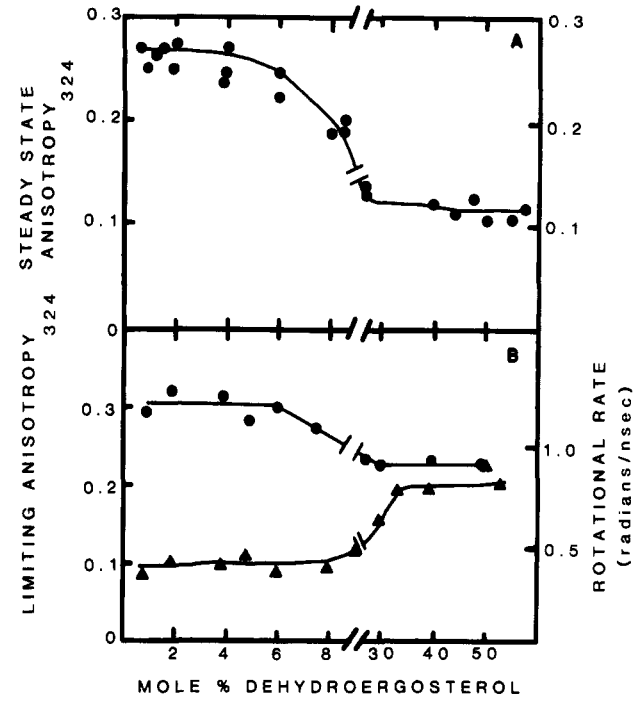

FIGURE 4: Effect of mole percent dehydroergosterol on anisotropy and rotational rate of dehydroergosterol in POPC vesicles. (A) Steady-state anisotropy was measured by using the T-format SLM fluorometer as described under Materials and Methods. (B) All parameters were determined by multifrequency phase and modulation techniques at 14 different frequencies as described under Materials and Methods. Limiting anisotropy (●); rotational rate (▲).

Table II: Red-Edge Effect for Dehydroergosterol Self-Quenching in POPC SUV[a]

mol % dehydroergosterol	mol % POPC	r_{324}	r_{356}
2	98	0.265 ± 0.005	0.270 ● 0.006
48	52	0.140 ± 0.003	0.152 ± 0.003
59	41	0.114 ± 0.004	0.127 ± 0.005

[a] Fluorescence emission was measured at 376 nm. Values represent the mean ± SEM (n = 3).

smaller component with fractional intensity 0.02 may be due to background fluorescence. The χ^2 value was not decreased by using two components. For a two-component analysis, χ^2 values were near 5. Similar results were obtained at 5 and 50 mol % dehydroergosterol (not shown). Therefore, we conclude that dehydroergosterol in the range 2–50 mol % in POPC SUV has a single lifetime at 24 °C near 0.860 ± 0.038 ns.

Both the relative quantum yield (Figure 2C), measured relative to diphenylhexatriene in benzene at 24 °C (quantum yield of 0.80), and the fluorescence lifetime of dehydroergosterol in POPC vesicles were constant near 0.56 and 0.86 ns, respectively, up to 4–5 mol % fluorescent sterol (Figure 2E). At concentrations higher than 4 mol % fluorescent sterol, the relative quantum yield, but not lifetime, decreased to limiting values near 33 mol % sterol.

The steady-state fluorescence anisotropy of dehydroergosterol was constant up to 4–5 mol % fluorescent sterol in POPC SUV (Figure 4A). This result is not consistent with sterol–sterol aggregation. Then r value decreased from 0.270 to 0.115 for dehydroergosterol at 33 mol %. At higher (above 33 mol %) concentrations of dehydroergosterol, the steady-state anisotropy reached a limiting value near 0.110. The data in Table II indicate that dehydroergosterol concentration quenching resulting in decreased anisotropy was noted both

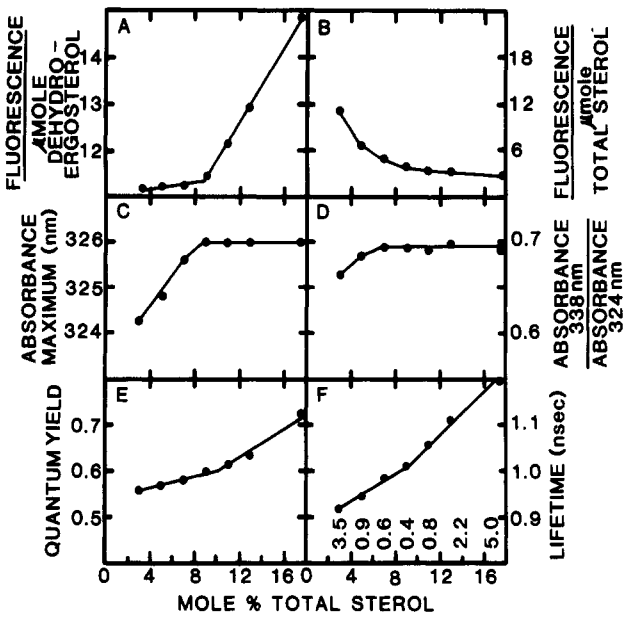

FIGURE 5: Effect of cholesterol on fluorescence properties of dehydroergosterol in POPC vesicles. POPC SUV contained 3 mol % dehydroergosterol and 0–15 mol % cholesterol. Lifetime data were obtained by using phase and modulation data at three fixed frequencies. Absorbance spectra were recorded with a Cary 210 spectrophotometer. Other parameters were determined as described under Materials and Methods. The numbers in (F) refer to χ^2 values for a one-component analysis for 3 mol % dehydroergosterol at the indicated total sterol content.

at the red edge of the absorption spectrum (356 nm) and at the major absorption maximum (324 nm), indicating the absence of a red edge effect.

The limiting anisotropy of dehydroergosterol (0.5 mol %) in POPC SUV was determined with the multifrequency (1–300 MHz) phase and modulation instrument using 14 different frequencies (10, 20, 30, 40, 60, 70, 80, 90, 100, 115, 120, 130, 140, and 150 MHz). The limiting anisotropy thereby obtained and the rotational rate calculated according to the equations developed by Lakowicz et al. (1984b) were 0.298 and 0.411 rad/ns, respectively, at 24 °C (Figure 4B). Thus, dehydroergosterol appears highly ordered with a restricted range of rotation but rapid rate of motion in POPC membrane bilayers. The limiting anisotropy parameter was constant up to 4–5 mol % sterol and then decreased with increasing mole percent fluorescent sterol to nonzero limiting values near 0.090 for dehydroergosterol at 33 mol % sterol (Figure 4B). Concomitant to the decrease in limiting anisotropy was a faster rotational rate (shorter rotational relaxation time).

Effect of Cholesterol on Fluorescence Properties of Dehydroergosterol in POPC Vesicles. The similarity of cholesterol and dehydroergosterol was examined by using a low concentration of dehydroergosterol (3 mol %) and increasing cholesterol (0–14 mol %) in POPC SUV. The specific fluorescence of dehydroergosterol (fluorescence per micromole of dehydroergosterol) increased with increasing mole percent cholesterol (Figure 5A) both in the range 0–7 mol % and even more above 8 mol % cholesterol. As expected, the fluorescence per micromole of total sterol decreased (Figure 5B). Betwen 0 and 7 mol % total sterol, the major absorbance maximum near 324.3 nm was red-shifted (Figure 5C), and the ratio of absorbance at 338 nm/324 nm increased (Figure 5D). These observations are very similar to those obtained above the POPC SUV containing only increasing concentrations of dehydroergosterol between 0 and 5 mol % (Figure 2A, B, D, and F).

Increasing the cholesterol content of the POPC SUV (up to 7 mol % total sterol) increased the quantum yield (Figure 5E) and fluorescence lifetime (Figure 5F) of dehydroergosterol. Analysis of lifetime data for a single-lifetime fit resulted in χ^2 values between 0.4 and 3.5 (Figure 5F). Two-component analysis did not improve the χ^2 values. The steady-state anisotropy, limiting anisotropy, and rotational rate of dehydroergosterol were essentially unaltered between 0 and 7 mol % total sterol (Figure 6A–C). At sterol concentrations above 7 mol % total sterol, the fluorescence per micromole of dehydroergosterol (Figure 5A), quantum yield (Figure 5E), lifetime (Figure 5F), steady-state anisotropy (Figure 6A), limiting anisotropy (Figure 6B), and rotational relaxation time [equivalent to (6 × rotational rate)$^{-1}$ in Figure 6C] all increased. In POPC vesicles containing 50 mol % cholesterol, restriction to motion of dehydroergosterol was even greater (data not shown). The steady-state anisotropy and limiting anisotropy of low concentrations of dehydroergosterol (0.5 or 3 mol %) in POPC SUV increased 1.18- and 1.45-fold, respectively, when the total sterol content was increased from 0.5 to 50 mol %. Concomitantly, the rotational rate decreased from 0.4 to less than 0.1 rad/ns. These results indicate that the addition of cholesterol restricts both the range and rate of motion of the fluorescent sterol in the POPC SUV.

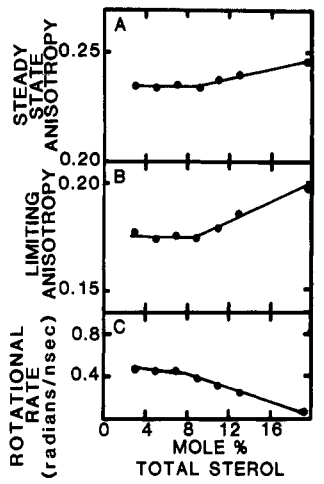

FIGURE 6: Effect of cholesterol on dynamic and static properties of dehydroergosterol in POPC vesicles. All conditions were as described in the legend to Figure 5. Steady-state anisotropy (A), limiting anisotropy (B), and rotational rate (C) were determined as described under Materials and Methods using phase phase and modulation data at three fixed frequencies. The limiting anisotropy and rotational rate were calculated, by using a fixed instead of a floating value for r_0, also as described under Materials and Methods.

In summary, dehydroergosterol and cholesterol appear to have a similar effect on dehydroergosterol fluorescence parameters explained by dielectric changes at low mole percent total sterol (see Discussion). At higher mole percent total sterol, the added cholesterol rigidifies the POPC (Figures 5 and 6) while added dehydroergosterol results in self-quenching.

Discussion

Several observations obtained with the fluorescent dehydroergosterol indicate that at low mole percent in POPC SUV this sterol is sensitive to dielectric effects. At concentrations up to about 5 mol %, the specific fluorescence per micromole of fluorescent sterol increased with concentration in the POPC bilayer. At the same time, the absorbance maximum near 324 nm was red-shifted 1.4 nm. In addition, the ratio of the relative fluorescence excitation near 338 nm to the relative fluorescence excitation near 324 nm also increased in the concentration range from 0 to 5 mol %. The quantum yield, however, remained constant between 0 and 5 mol % fluorescent sterol because the specific absorbance per micromole of fluorescent

sterol increased in parallel. In the presence of 3 mol % dehydroergosterol, addition of cholesterol from 0 to 5 mol % cholesterol also increased the specific fluorescence, red-shifted the absorbance maximum, and altered absorbance peak ratios. These data indicate that dehyderoergosterol behaved similarly to cholesterol at low mole percent sterol in POPC vesicles. If dehydroergosterol were not a good analogue for cholesterol, then self-quenching of laterally segregated dehydroergosterol might be expected with increasing concentration of cholesterol. The above observations may be explained by dielectric constant effects. The addition of dehyderoergosterol (and cholesterol) can cause a change of the average dielectric properties of the medium. Medium here refers to the POPC membrane microenvironment wherein the dehydroergosterol fluorophore is located. Space-filling models of cholesterol (and dehydroergosterol) indicate that the conjugated triene double-bond series of dehydroergosterol is located within a few angstroms of the OH group. The fluorescence intensity and lifetime are strongly dependent on the dielectric constant of the medium. The data reported here indicate that the polarity (dielectric constant) of the medium alters the fluorescence properties of dehydroergosterol. The data are consistent with the interpretation that with increasing concentration up to 5 mol % dehydroergosterol in the POPC vesicles, there is a linear change of the medium dielectric constant. That the medium has some influence on the decay is demonstrated by the values of τ phase and τ modulation. At high frequencies, τ phase is greater than τ modulation. This situation can occur only in the presence of an excited-state reaction. We propose that in this case the reaction is the relaxation (reorientation) of dipoles or charges close to the excited dehydroergosterol. A prediction can be made that if the red part of the emission spectrum is used for the lifetime measurements (using a longer wavelength cutoff filter) the value of τ phase will be even larger than τ modulation. This effect was observed. The absence of anisotropy changes rules out aggregation of dehydroergosterol below 5 mol % dehydroergosterol. This interpretation is also consistent with data reported by others (Kao et al., 1978) using another fluorescent sterol analogue, *N*-(2-naphthyl)-23,24-dinor-5-cholen-22-amin-3β-ol. The fluorescent properties of this fluorescent sterol were also solvent polarity sensitive. The hydrocarbon region of the bilayer sensed by this fluorophore had a polarity comparable to that of acetonitrile. This polarity was reduced by addition of increasing amounts of cholesterol. Therefore, the first conclusion to be made from the data is that below 5 mol % dehydroergosterol this molecule is more sensitive to polar effects than at greater mole percent sterol, possibly due to being inserted into the bilayer less deeply or the lipid polar head groups occupying more area and allowing greater penetration of H_2O. Either effect would result in a greater interaction of the fluorophore with the aqueous interface (higher dielectric constant) below 5 mol % sterol. The molecular origins of this phenomenon and why it disappears near 5 mol % sterol are not known. One simple explanation might be that cholesterol and dehydroergosterol insert themselves into the liquid-crystalline (fluid) POPC bilayer in a highly specific fashion which leads to a condensing or ordering effect. This would be expected to reduce the number of vacancies occurring in the bilayer and consequently to reduce the permeability of the bilayer to water and small molecules.

A second conclusion, derived from data at higher mole percent dehydroergosterol, is that putative complexes interact with each other to self-quench fluorescence without further change in absorption parameters. This conclusion is consistent with that of others (Rogers et al., 1979) and is supported by the following observations: Above 5 mol % fluorescent sterol, the quantum yield, steady-state anisotropy, and limiting anisotropy of dehyderoergosterol in POPC SUV continuously decreased to limiting values near 30–35 mol % sterol. However, the lifetime was relatively constant. A similar finding of a decrease in polarization and quantum yield, but not in lifetime, was reported for chlorophyll *b* self-quenching (Kelly & Patterson, 1971). Fluorescence self-quenching at high concentration due to formation of aggregates could result in the observed quenching of fluorescence, since such aggregates are often not fluorescent (Parker, 1968). Planarity is a feature common to many molecules that undergo concentration quenching (Berlman, 1971). Cholesterol also considerably reduces the rate of cholesterol self-diffusion in fluid bilayers (Golan et al., 1984). It is interesting to note that the lateral diffusion coefficient of NBD-cholesterol in egg phosphatidylcholine at 37 °C and in dimyristoylphosphatidylcholine at 26 °C decreases linearly with increasing cholesterol concentration between 5 and 20 mol %, while at 15 and 19 °C, respectively, the lateral diffusion coefficient of NBD-cholesterol is constant between 0 and 10 mol % cholesterol (Golan et al., 1984; Alecio et al., 1982). When POPC (the major component of egg PC), egg PC, and dimyristoylphosphatidylcholine are all in the fluid state, it may be postulated that above 5 mol % cholesterol coexisting, immiscible fluid domains may be present under certain conditions. The existence for such coexisting fluid phase lipids was suggested earlier from model membranes (Rechtenwald & McConnell, 1981). The present observations would imply that in the fluid phase bilayer, cholesterol may be present as monomers below 5 mol % and that above 5 mol % separate cholesterol-rich domains may coexist with the fluid phase POPC. The composition or nature of these cholesterol-rich domains is not known. However, the above interpretation is consistent with one suggested by other investigators for cholesterol (Smutzer & Yeagle, 1985). The existence of a long-lived cholesterol–lipid complex or cholesterol dimer that may form near 4 mol % cholesterol has been postulated (Phillips & Finer, 1974; Presti et al., 1982). Several models for phosphatidylcholine–cholesterol interaction have been proposed, one of which invokes the presence of cholesterol dimers (Martin & Yeagle, 1978). Cholesterol dimer formation has been observed in chloroform solution (Feher et al., 1974). There is also some biological evidence which suggests clustering of cholesterol within the plane of mouse and frog rod outer segment plasma membranes and basal disks (Andrews & Cohen, 1979). Vertebrate rod outer segment membranes contain very small amounts of sterols, approximately 5–7 mol % in bovine retinal rod outer segment membranes (Fliesler & Schroepfer, 1982).

The present observations may be further extended to high mole percent cholesterol in fluid phase POPC. Above 30 mol % cholesterol, the putative dimers or other complexes may laterally segregate or coalesce in a pure cholesterol–POPC domain. In the fluid state, the maximum amount of cholesterol accommodated in phospholipids before a pure cholesterol phase separates out is 50–67 mol %, depending on the phospholipid polar head group (Houslay & Stanley, 1982). When a pure cholesterol phase separates out, the permeability of dipalmitoylphosphatidylcholine vesicles is greatly increased (Blok et al., 1977). These and other interpretations were reviewed recently by Presti et al. (1982). It is significant to note that there was no apparent change at about 20 mol % dehydroergosterol in fluid phase POPC. This is important since various models for cholesterol–lipid interactions have included sug-

gestions of complex formation at 20 (Copeland & McConnell, 1980), at $33^1/_3$ (Engelman & Rothman, 1972), or at 50 (Phillips & Finer, 1974) mol % sterol. Different experimental techniques appear to offer contradictory determinations of the associated stoichiometry. It is possible that the fluorescent dehydroergosterol is insensitive to effects at 20 mol %. However, direct observation of the properties of deuteriated cholesterol in egg PC bilayers with increasing cholesterol by NMR techniques indicates the presence of a breakpoint near 30–40 mol %, but not near 20 mol %, cholesterol for quadruple splittings of cholesterol-3α-d_1, and cholesterol-7,7-d_2 probes (Taylor et al., 1982). The major phospholipid species in egg PC is POPC. Thus, the data presented with the fluorescent dehydroergosterol appear more consistent with appearance of separate cholesterol–lipid domains in fluid phase POPC near 30 mol % cholesterol. Earlier data from several laboratories suggested the existence of lateral phase separation of cholesterol resulting in cholesterol–cholesterol association in liposomes. Cholesterol association with the more fluid phase has been predicted in phase-separated disaturated/disaturated and disaturated/monounsaturated phosphatidylcholine mixtures (Verkleij et al., 1974; de Kruyff et al., 1974). Above 25 mol % cholesterol, spin-labeled steroids were no longer completely miscible with dipalmitoylphosphatidylcholine membranes (Trauble & Sackman, 1972). X-ray investigations indicated that below 50 mol % cholesterol, a ribbonlike structure was noted below the phase transition of dipalmitoylphosphatidylcholine corresponding to cholesterol-poor and cholesterol-rich areas (Hui & Parsons, 1975). However, these structures still allowed phospholipid–sterol interactions. Changes of slope in phase diagrams occurring near 22 and 30 mol % sterol have been interpreted either as representing the appearance of nonideal mixing of cholesterol–phospholipid complexes or as representing the appearance of cholesterol–cholesterol dimers (Houslay & Stanley, 1982). Future investigations with dehydroergosterol in phase-separated or gel phase phospholipids are needed to resolve whether a breakpoint may be obtained near 20 mol % cholesterol in such membranes or whether the data obtained here indicate a different behavior for the fluorescent sterol.

A third conclusion that may be drawn from the measurements concerns the dynamic (motional) and static (structural) properties of dehydroergosterol. The data indicate that a low mole percent in liquid-crystalline POPC SUV at 24 °C this sterol has a fast rotational relaxation time (0.42 ns) and a high degree of order. These findings are consistent with those of other investigators obtained by deuterium NMR of deuteriated cholesterol in multilayers of egg phosphatidylcholine: order parameters and rotational relaxation times were near 0.67 and 0.5 ns, respectively (Taylor et al., 1981, 1982; Kelusky et al., 1983). In addition, increasing the cholesterol content from 0 to 50 mol % increased the order parameters of the deuteriated cholesterol to 0.78 in egg phosphatidylcholine multilayers (Taylor et al., 1982), 0.80 in dimyristoylphosphatidylcholine (Kelusky et al., 1983), and 0.76 in human erythrocyte ghosts (Kelusky et al., 1983). Similarly, the results presented here showed that the limiting anisotropy which reflects the order of dehydroergosterol in POPC SUV increased with increasing cholesterol. Although the amplitude determined from the limiting anisotropy and the rate of anisotropic motion are not necessarily correlated, one may expect that an increase in order would be accompanied by a decrease in rotational rate (an increase in rotational relaxation time). Investigations of Veatch and Stryer (1977) demonstrated that in bis(dihydrosterculoyl)phosphatidylcholine liposomes containing up to 33 mol % cholesterol, there was no change in fluorescence lifetime or rotational rate of diphenylhexatriene. In contrast, the results obtained with dehydroergosterol are not consistent with this prediction: cholesterol increased both the lifetime and the rotational relaxation time of the fluorescent sterol in POPC SUV. Thus, the motion of sterols is not affected by cholesterol in the same way as the motion of diphenylhexatriene in liposomes. Deuterium NMR measurements of deuteriated cholesterol were quite insensitive to variation in motional rates, indicating that any changes in the dynamics of cholesterol caused by variation of the cholesterol content of the membrane would not be easily detectable by using deuterium NMR relaxation time measurements (Taylor et al., 1982).

In conclusion, the fluorescent sterol dehydroergosterol is a useful probe molecule analogue for cholesterol. In POPC SUV, it appears quite sensitive to dielectric effects below 5 mol % and to sterol interactions with other sterols and/or olipids above 5 mol % sterol. In addition, the data presented here indicate that at low concentrations the fluorescent sterol is an excellent probe molecule for examining the motional properties (dynamic and static) of cholesterol in model phospholipid membranes. The motional properties of the fluorescent sterol indicate rapid motion (dynamics) with a restricted cone angle or rotation (order). Both parameters are affected by increasing mole percent cholesterol in the POPC vesicles. Fluorescence self-quenching of this probe molecule is greater than 5 mol % was consistent with a lateral phase separation of sterol in POPC SUV.

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