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Physical properties of the fluorescent sterol probe dehydroergosterol

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Spectroscopic studies were performed on the fluorescent sterol probes ergosta-5,7,9(11),22-tetraen-3β-ol (dehydroergosterol) and cholesta-5,7,9(11)-trien-3β-ol (cholestatrienol). In most isotropic solvents, these molecules exhibited a single lifetime near 300 ps. Fluorescence lifetimes in 2-propanol were independent of emission wavelength and independent of excitation wavelength. Excited state behavior of these probes appears relatively simple. In isotropic solvents, dehydroergosterol fluorescence emission underwent at most a small Stokes shift as solvent polarity was modified. Time-resolved anisotropy decays indicated that dehydroergosterol decay was monoexponential, with rotational correlation times dependent on solvent viscosity. When incorporated into L-α-dimyristoylphosphatidylcholine liposomes at a concentration of 0.9 mol%, dehydroergosterol fluorescence lifetime decreased at the phase transition of this phospholipid indicating that the sterol probe was detecting physical changes of the bulk phospholipids. Furthermore, total fluorescence decays and anisotropy decays were sensitive to the environment of the sterol. Dehydroergosterol and cholestatrienol are thus useful probes for monitoring sterol behavior in biological systems.

Introduction

A serious problem impeding progress towards understanding cholesterol behavior in biological systems has been the lack of adequate techniques for monitoring cholesterol. The native sterol molecule offers no physical or structural attributes which can readily be exploited to directly observe cholesterol behavior. Recently, two fluorescent sterol derivatives have been introduced that alleviate this deficiency. Ergosta-5,7,9(11),22-tetraen-3β-ol (dehydroergosterol) and cholesta-5,7,9(11)-trien-3β-ol (cholestatrienol) are sterols into which a conjugated triene system has been introduced synthetically to provide a valuable fluorophore. The structural modification is minimally perturbing, leaving unmodified the 3β-hydroxyl and the tails of the molecules. Modifications of these latter two structures can lead to non-cholesterol behavior [1].

As expected from their structural similarity to cholesterol, these sterol probes exhibit considerable similarity to cholesterol in water permeability and cell growth studies [2], glucose permeability studies, and in membrane ordering [3]. These molecules have already proven useful in monitoring cholesterol behavior in membrane and serum lipoproteins by fluorescence techniques [3–8].

Because these probes are useful and unique for studying sterol behavior, it is necessary to have an adequate understanding of their absorptive and

Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DMF, dimethylformamide; EDTA, ethylenediaminetetraacetic acid, sodium salt; PC, phosphatidylcholine; SUV, small unilamellar vesicles; T_m, temperature of the phase transition.

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fluorescent emission properties as well as how those properties may reflect the molecular environment of the sterol. This report provides that information.

Materials and Methods

Dehydroergosterol was directly synthesized from ergosterol (Sigma Chemical Co., St. Louis, MO) by dehydration with mercuric acetate using a modification of the procedure of Windhaus [9]. 2 g of ergosterol were reacted with 3 g of mercuric acetate in 20 ml of chloroform and 17 ml of glacial acetic acid at 37–40 °C in the dark under a nitrogen atmosphere. After 24 h, the material was twice filtered to remove mercurous acetate and concentrated to less than 5 ml in a rotary evaporator. The product was then recrystallized in warm/cold ethanol, filtered, washed with ice-cold ethanol, and then dissolved in ether. The dehydroergosterol solution was refiltered through Whatman No. 1 filter paper and the filtrate was placed under a stream of nitrogen in the dark to remove the ether. The product was then recrystallized in warm/cold ethanol for several hours and dried overnight under vacuum and directly used or passed through a Beckman HPLC chromatography system equipped with a reverse-phase C-18 preparatory HPLC column (Ultrasphere-ODS, 10 mm × 25 cm). The column separated any unreacted ergosterol from dehydroergosterol and was equilibrated with methanol/acetonitrile (1 : 19, v/v). Flow rates were 3 ml/min and dehydroergosterol eluted from the column 24 min after injection as a single peak. Melting point determinations and absorption spectra for dehydroergosterol were similar to published values [10].

Cholestatrienol was synthesized from 7-dehydrocholesterol (Sigma Chemical Co.) using the procedure of Antonucci et al. [11] and was also purified by passage through a C-18 reverse phase HPLC column [12]. Rechromatography of the isolated fraction produced a single peak, with only a hint of a second peak at less than the 1% level. Infrared spectroscopy indicated that the product was a free alcohol, and the ^{13}C-NMR spectrum at 67 MHz was identical with the spectrum of cholestatrienol previously published [12].

Extinction coefficients

For the determination of extinction coefficients and wavelength maxima of dehydroergosterol in organic solvents, absorption spectra were measured on a DW-2c double-beam spectrophotometer (SLM-Aminco, Urbana, IL). Baseline corrections due to lamp intensity output were corrected prior to sample measurement, and the monochromator was calibrated to within 0.1 nm using the hydrogen line of the deuterium lamp at 486.0 nm. Extinction coefficients were measured at three different concentrations (12.5 μM or less) for dehydroergosterol in each solvent.

Quantum yield

The quantum yield of dehydroergosterol relative to 1-naphthylamine was measured by comparing the total emission energy from a corrected fluorescence emission spectrum of dehydroergosterol to that of a corrected emission spectrum of 1-naphthylamine in cyclohexane at 20 °C [13]. All steady-state fluorescence spectra were measured on a Perkin-Elmer model LS-5 spectrofluorometer and correction factors were calculated according to the procedure of Chen [14].

Fluorescence lifetime analysis

Fluorescence lifetimes of dehydroergosterol and cholestatrienol were measured in either of two ways. Some fluorescence lifetime measurements were carried out at the Regional Laser and Biotechnology Laboratories of the University of Pennsylvania. This lifetime apparatus was composed of a time-correlated single-photon counting system incorporating a mode-locked argon-ion laser, pumping a cavity-dumped dye laser whose frequency was doubled to excite the sample at 325 nm. Detection of fluorescence was at 375 or 395 nm and was measured through a magic angle polarizer. The detection system was composed of a monochromator on the emission side and a microchannel plate detector. A red filter was used to eliminate the fundamental frequency, and a WG345 cutoff filter was used to block the excitation wavelength when collecting fluorescence. Fluorescence lifetimes were obtained from the deconvolution of the stored data in 512 channels from a stable instrument function of approximately 150 ps. A water bath connected to the

sample chamber was used to regulate the temperature of the sample.

Fluorescence lifetimes were also measured with a flashlamp system using hydrogen as the gas (PRA, London, Ontario), and excitation was accomplished with the output of a thyraton-gated flashlamp at an output of 6 kV. For the PRA system, fluorescence emission was detected using a monochromator on the excitation side, and a 3 mm KV 370 Schott filter on the emission side (high pass, 50% transmittance at 370 nm; Schott Optical Glass Co, Duryea, PA). All fluorescence lifetime data were collected with the polarizers oriented at the magic angle. A lamp profile was measured after each lifetime determination with a glycogen solution in water. In isotropic solvents, dehydroergosterol concentration was 50 μM unless stated otherwise. A minimum of 12000 counts were normally recorded in the maximum emission channel. Temperature was regulated by means of a water bath. The decay of the total emission was analyzed according to a decay law of the following form

$$F(t) = \sum_i F_i \, e^{-t/\tau_i} \qquad (1)$$

where $F(t)$ is equivalent to the observed decay without the contributions of the lamp pulse. The lifetimes (τ_i) and the pre-exponential factors were fit by iterative convolutions according to the least-squares analysis of Grinvald and Steinberg [15]. A chi-squared value, a display of residuals, and an autocorrelation function were employed as a measure of the quality of the fit. The importance of more than one exponential in the decay was detected by a significant improvement in the chi-squared value (decrease) and in the residual display (less coherent oscillation). All chi-squared values were less than 3.0.

For time-resolved fluorescence polarization measurements, the polarized decay curves $I_{vv}(t)$ and $I_{hv}(t)$ were collected along with the excitation profiles for each sample. The experimental emission anisotropy curve $r(t)$ was calculated by

$$r(t) = \frac{I_{vv}(t) - QI_{hv}(t)}{I_{vv}(t) + 2QI_{hv}(t)} \qquad (2)$$

where I is the intensity; v represents the excitation or emission polarizer in the vertical position, respectively; h represents the excitation or emission polarizer in the horizontal position, respectively. Q is the total scaling factor used in data manipulation.

DMPC vesicles

For the preparation of multilamellar liposomes of DMPC that contained dehydroergosterol, the probe was dissolved in HPLC grade chloroform and added to 2 mg of DMPC in chloroform (Avanti Polar Lipids, Birmingham, AL). The solution was then dried to a thin film under a stream of argon in an N-evap analytical evaporator apparatus with gentle heating. The resulting thin film of lipid and dehydroergosterol was subsequently dried under high vacuum for 2 h to remove residual chloroform. The lipid was suspended in 150 mM NaCl/1 mM EDTA/2.5 mM histidine at pH 7.00 at approx. 25°C by vortexing to yield a phospholipid concentration of 0.33 mg/ml. Unlabeled liposomes that did not contain dehydroergosterol were prepared in an identical manner. The liposomes were either directly used for measurement or were centrifuged for 20 min at 25000 rpm in a Beckman Ti-50 rotor at 20°C. The centrifuged samples were carefully resuspended in 6 ml of the same histidine buffer. For fluorescence lifetime measurements, the vesicles were allowed to equilibrate to the starting temperature for approximately 1 h. Overall heating rates were approx. 1.25 C deg/h.

Small unilamellar vesicles (SUV) were prepared from egg PC (Avanti Polar Lipids) as described previously [16]; for fluorescence lifetime measurements the final lipid concentration was 0.33 mg/ml.

Results

Fig. 1 shows a corrected fluorescence emission spectrum of HPLC-purified dehydroergosterol in 2-propanol. Maximum fluorescence emission occurred at 372.5 nm with shoulders at 395 and 355 nm. The maximum absorption and emission wavelengths of dehydroergosterol as a function of solvent are listed in Table I. The data indicate the absence of a Stokes shift as solvent polarity is increased. A plot of the Stokes shift (absorption

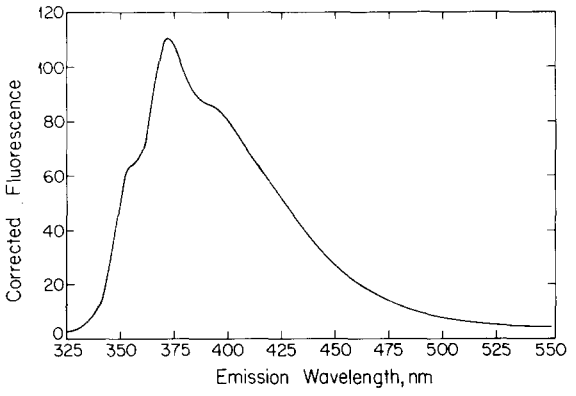

Fig. 1. Corrected fluorescence emission spectrum of 50 μM dehydroergosterol in 2-propanol at 20°C. Excitation was at 310 nm.

maximum – emission maximum) versus the orientational polarizability of the solvent, Δf [17] can be used to determine the increase in dipole moment of dehydroergosterol upon excitation [17]. The polarizability of the solvent is defined by

$$\Delta f = \frac{D-1}{2D+1} - \frac{n^2-1}{2n^2+1} \tag{3}$$

where n is the refractive index of the solvent and D is its dielectric constant (see Table I). The increase in dipole moment upon excitation of dehydroergoesterol can then be calculated according to Lippert [17]

$$\mu^* - \mu = \left[\frac{2a^3}{\Delta f} hc\Delta\bar{\nu}\right]^{1/2} \tag{4}$$

where μ^* and μ are the dipole moments in the excited and ground state, a is the radius of the cavity in the solvent required for the fluorophore, and $\Delta\bar{\nu}$ is the Stokes shift caused by a change in polarizability, Δf. Using 10 Å as an approximate radius of dehydroergosterol (based on X-ray crystallography data of cholesterol), a value of less than 1 Debye unit increase in dipole moment can be calculated from these equations when dehydroergosterol is excited to its lowest singlet state. This calculation clearly shows that dehydroergosterol is virtually insensitive to solvent polarity and does not undergo charge separation upon excitation in any of the solvents tested. Even in water, a Stokes shift of only 4000 cm^{-1} has been observed for dehydroergosterol [7]. These data, along with substantial overlap of emission and absorption spectra, indicate that energy transfer must be considered as a possible mechanism for energy dissipation at high fluorophore concentrations.

Table II lists the extinction coefficients for dehydroergosterol in a series of organic solvents. Dehydroergosterol absorbance is relatively insensitive to the nature of the solvent, with most extinction coefficients near 10000 $M^{-1} \cdot cm^{-1}$.

TABLE I

SPECTRAL PROPERTIES OF DEHYDROERGOSTEROL SOLUTIONS

Solvent	Absorption λ_{max} (nm)	Emission λ_{max} (nm)	Stokes shift (cm^{-1})	Δf [a]
Cyclohexane	325.6	372.5	3867	0.001
Benzene	327.5	375.0	3868	0.004
Chloroform	327.8	378.0	4051	0.016
Ether	324.3	373.0	4026	0.167
Ethyl acetate	325.2	373.0	3941	0.201
Pyridine	329.6	–	–	–
DMSO	328.6	378.0	3977	0.262
1-Butanol	325.2	373.0	3941	0.265
2-Propanol	324.8	372.5	3943	0.274
Methanol	324.4	372.5	3981	0.310
DMF	327.3	–	–	–
DMPC	327.5	375.5	3956	–

[a] Δf, orientational polarizability defined by Eqn. 3.

TABLE II

EXTINCTION COEFFICIENTS OF DEHYDROERGOSTEROL SOLUTIONS

Solvent	Extinction coefficient ($M^{-1} \cdot cm^{-1}$)
Cyclohexane	10100
Benzene	11500
Chloroform	11200
Ether	11700
Ethyl Acetate	10200
DMSO	10400
1-Butanol	10600
2-Propanol	10600
Methanol	10500
DMF	11000

TABLE III

QUANTUM YIELD AND FLUORESCENCE LIFETIME OF DEHYDROERGOSTEROL IN ORGANIC SOLVENTS

Solvent	Quantum yield	τ_1 (ns)	Rel. ampl. [a]	τ_2 (ns)	Rel. ampl.	Chi square
Acetone	< 0.001	0.33	98.8	4.68	1.2	2.54
Chloroform	0.003	0.33	98.9	5.00	1.1	2.58
Methanol	0.024	0.18	100.0	–	1.2	1.21
Cyclohexane	0.032 [b]	0.20	100.0	–	–	1.21
Ethanol	0.040	0.18	100.0	–	–	2.24
Ether	0.030	0.20	99.7	4.37	0.3	1.12
2-Propanol	0.051	0.27	100.0	–	–	1.24
		0.25 [c]				1.91
Benzene	0.058	0.32	100.0	–	–	1.00
DMF	0.058	0.39	99.5	6.12	–	1.31

[a] Normalized to 100%.

[b] From Smutzer and Yeagle [5].

[c] Data from picosecond laser; dehydroergosterol concentration 10 μM.

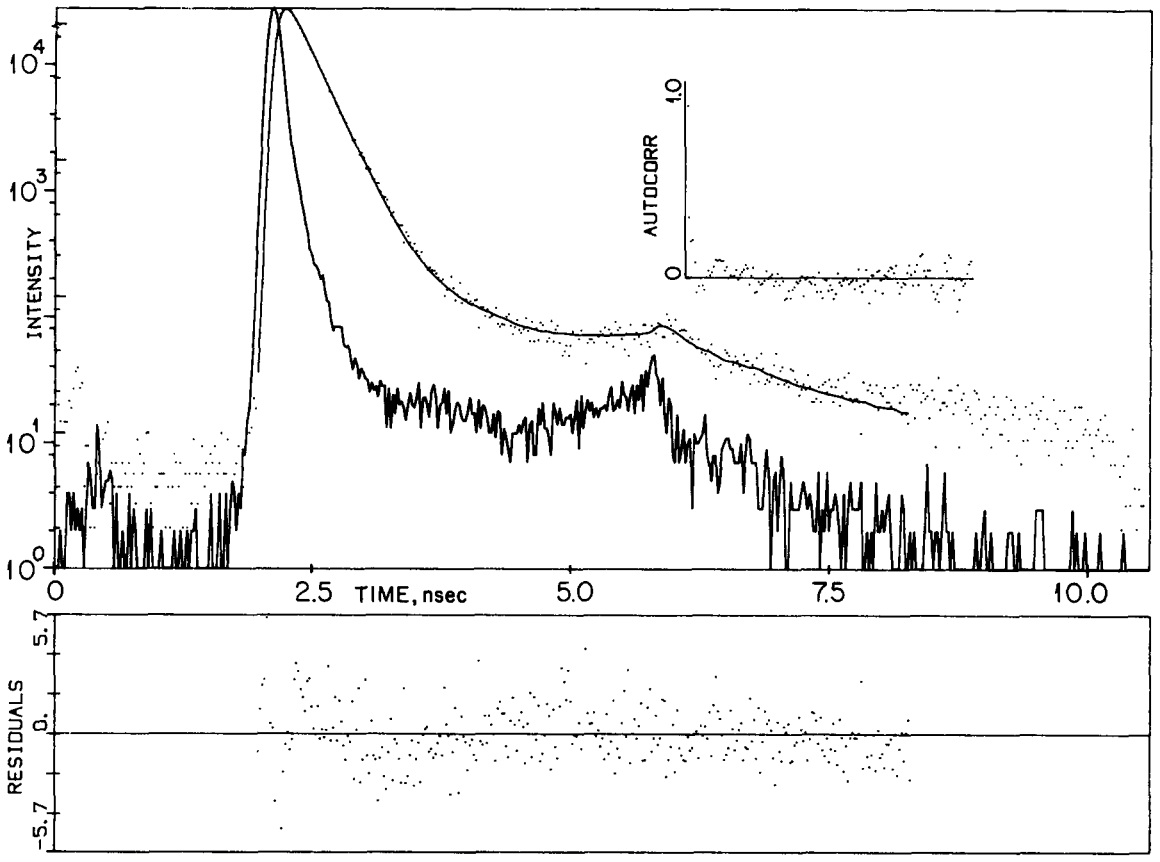

Fig. 2. Fluorescence decay of 10 μM dehydroergosterol in 2-propanol measured with a laser-based system. Dots (may not reproduce legibly) represent the fluorescence emission in each channel, and the smooth solid line through these dots is the exponential fit. The jagged, heavy solid line represents the instrument function. Each channel represents 0.0207 ns. The temperature was 20°C.

Table III lists the relative quantum yield of dehydroergosterol as a function of solvent and the fluorescence lifetime and relative amplitudes in these solvents. Quantum yield is dependent on solvent but does not show a strong dependence. When compared to the majority of solvents, a noticeable decrease in quantum yield was observed in acetone, and to a lesser degree in chloroform. Acetone absorbs strongly in the region that dehydroergosterol absorbs, and with solvent concentration high relative to dehydroergosterol, absorbance of light energy by acetone may directly block absorbance by the fluorescent molecule.

Fluorescence lifetime measurements of dehydroergosterol in isotropic media as a function of solvent from both the laser system and the flashlamp system are presented in Table III while Fig. 2 shows the time-dependent fluorescence emission of dehydroergosterol in 2-propanol. The fluorescence lifetime of dehydroergosterol in organic solvent showed little change with respect to solvent. Except for acetone and chloroform, lifetime changes paralleled changes in quantum yield. The chromophores of cholestatrienol and dehydroergosterol are identical, and cholestatrienol exhibited a fluorescence lifetime similar to dehydroergosterol in the three solvents studied ($\tau_{2\text{-propanol}} = 0.28$ ns).

To determine if excited state isomerization occurred, fluorescence lifetime measurements were recorded as a function of excitation wavelength as well as a function of emission wavelength (Figs. 3A and 3B). A variation in lifetime with emission wavelength would be indicative of photoisomerization. Virtually no change in fluorescence lifetime was observed in either case, and no new lifetime components were measured over the range of wavelengths of probe absorption and emission.

The effect of sample illumination time (at 325 nm) on lifetime was determined. No significant change in the dominant lifetime was observed as a function of time of laser illumination between 45 and 600 s (data not shown).

The effect of dehydroergosterol concentration on lifetime in 2-propanol was also measured (see Table IV). No significant change in lifetime occurred as a function of probe concentration, although fluorescence emission significantly decreased at concentrations above 100 μM dehydroergosterol as detected by a longer data collection time. These results suggested that above 100 μM, dehydroergosterol underwent self-quenching.

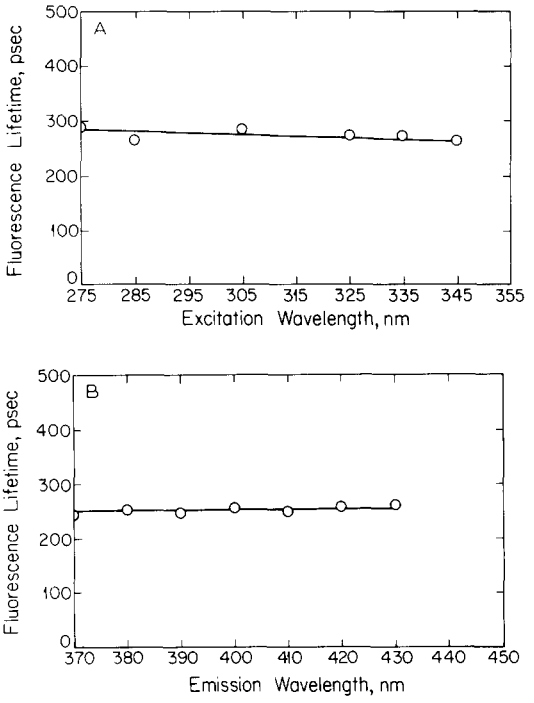

Fig. 3. (A). Fluorescence lifetime of dehydroergosterol in 2-propanol measured as a function of excitation wavelength. Excitation pulse was generated by a flash lamp, and dehydroergosterol concentration was 25 μM. Fluorescence emission was measured through a KV370 filter. (B). Fluorescence lifetime as a function of emission wavelength. Lifetime was measured with a laser-based system at 20°C.

In mineral oil, two lifetime components for dehydroergosterol were observed. The major lifetime component, whose amplitude was 95% or more of the total fluorescence decay, was temperature dependent, with lower temperatures leading to a longer major lifetime component. The major lifetime component for mineral oil is presented in Table V as a function of temperature.

After a characterization of the physical properties of dehydroergosterol in isotropic solvents, the probe was incorporated into egg PC SUV and DMPC multilamellar liposomes at a concentration of 0.9 mol%. In SUV at 20°C, dehydroergosterol fluorescence possessed two lifetime components. The major component was 0.97 ns and the minor lifetime component was 4.1 ns.

TABLE IV

EFFECT OF DEHYDROERGOSTEROL CONCENTRATION ON FLUORESCENCE LIFETIME

Concentration (μM)	Lifetime (ns)
10	0.30
25	0.29
100	0.31
250	0.33
500	0.37

In DMPC liposomes, the temperature range examined included the temperature at which DMPC undergoes a gel-to-liquid-crystalline phase transition at 23.6°C. Background scatter from unlabeled liposomes, from the optical system itself, or from phospholipid, was removed from the final data by time-resolved measurements on suitable controls. The background contribution, which totaled less than 5% of total counts in the presence of dehydroergosterol, was subtracted from the total emission decay data. This procedure made it possible to measure dehydroergosterol emission exclusively. Two fluorescent lifetime components were observed in DMPC liposomes below the phase transition with the PRA system (see Fig. 4). The major lifetime component was near 1 ns. The same value was obtained from the laser-based instrument. A second minor lifetime component of approximately 3.5 ns was also observed. As shown in Fig. 5, this minor component decreased

TABLE V

FLUORESCENCE LIFETIMES AND FLUORESCENCE ANISOTROPY DECAYS OF DEHYDROERGOSTEROL IN MINERAL OIL

Temp. (°C)	Lifetime [a] (ns)	Chi square [b]	Rotational corr. time (ns)
6.0	1.04	2.16	n.m. [c]
10.2	0.85	1.56	8.20
19.8	0.65	1.01	5.54
24.0	0.58	1.35	3.89
30.1	0.46	1.31	2.31

[a] Measured at 54.7°C.
[b] Derived from lifetime analysis.
[c] n.m., not measured.

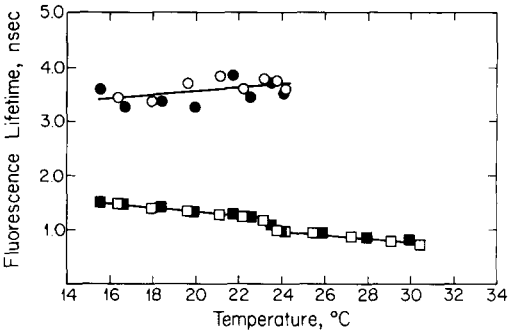

Fig. 4. Fluorescence lifetime of dehydroergosterol in DMPC liposomes as a function of temperature (heating curve). Final lipid concentration was 0.33 mg/ml, and dehydroergosterol concentration was 0.9 mol%. The open and closed symbols represent results from two different experiments. Squares and circles represent the two lifetime components of dehydroergosterol observed in liposomes.

in amplitude with an increase in sample temperature, and was less than 0.1% of the total decay amplitude at 24°C. The major lifetime component near 1 ns underwent approximately an 18% decrease in lifetime at 23°C. Concurrently the total photon counts emitted, over a time span of 35 ns, also decreased 18%. The quantum yield has been shown to decrease 13% in DMPC liposomes in the region of the phase transition when the phase change was detected by steady-state emission [5]. The phase transition detected by lifetime analysis occurred over a temperature range of 0.9°C. This temperature-dependent change appears to repre-

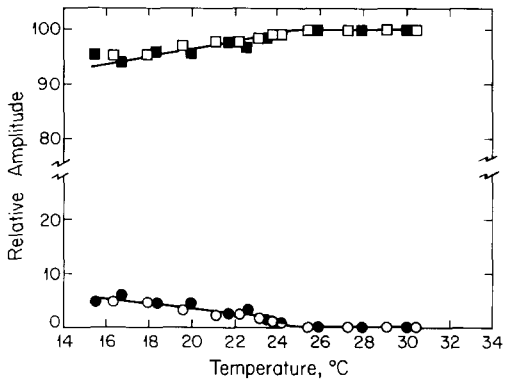

Fig. 5. Relative amplitudes of the lifetime components of dehydroergosterol in DMPC liposomes. Conditions were the same as in Fig. 4. Open and closed symbols represent the results from two different experiments. Squares represent relative amplitudes of the short-lifetime component while circles represent relative amplitudes of the long-lifetime component.

sent the gel-to-liquid-crystalline phase transition of DMPC because it occurs at the DMPC phase transition temperature. The same results were observed with two different batches of DMPC. Higher concentrations of probe (4.8 mol%) in DMPC liposomes yielded lifetime results consistent with the results obtained at probe levels of 0.9% (data not shown).

The time-dependent depolarization of dehydroergosterol fluorescence as a function of temperature in mineral oil was also measured. At increasing temperatures, the mineral oil became less viscous, and a decrease in lifetime as well as a decrease in the rotational correlation time of dehydroergosterol was observed. The rotational correlation times are shown in Table V as a function of temperature in mineral oil.

Discussion

Dehydroergosterol in isotropic solvents

The extinction coefficient of dehydroergosterol changed very little with solvent, with an extinction coefficient near 10000 units $M^{-1} \cdot cm^{-1}$ for the major absorption band of dehydroergosterol. Likewise the emission maxima did not show strong sensitivity to solvent. Furthermore, there is little temperature effect on the wavelength of emission maxima down to 77 K [19]. The lack of a substantial blue shift at low temperature [19] may indicate only a small role for solvent relaxation [18].

In isotropic environments, dehydroergosterol possesses a single, short lifetime of approximately 300 ps. To determine if photoisomerization of dehydroergosterol occurred during the time of measurement by actinic light, the fluorescence lifetime of dehydroergosterol was measured as a function of emission wavelength. If there were photochemical processes occurring such as photoisomerization, a change in fluorescence lifetime as wavelength was varied or more than one decay component upon excitation would be expected. In 2-propanol, no change in dehydroergosterol fluorescence lifetime was observed as emission wavelength was varied in contrast to fluorescent molecules such as 2-naphthol [20]. Likewise, no lifetime changes were observed when the excitation wavelength was varied and the emission wavelength was held constant. Furthermore, only a single decay component was observed. Thus no photochemical reactions or intermediates due to photoisomerization upon excitation of dehydroergosterol could be detected by lifetime analysis.

In some solvents, a second minor lifetime component was observed with a value near 4 ns. In solvents such as acetone or chloroform where the quantum yield was greatly reduced, this second longer component was most pronounced, with relative amplitudes approaching 2 percent of total fluorescence emission. The second lifetime component in acetone as well as in chloroform could be due to a minor amount of photodecomposition. Because of the low relative quantum yield, dehydroergosterol in these solvents required the longest exposure to light in the time-resolved fluorescence measurements. Photodecomposition has been observed previously with dehydroergosterol under intense ultraviolet light illumination [21]. We have observed the introduction of a second longer lifetime component with extended laser illumination in 2-propanol. With a 10-fold increase in total illumination time (over that normally used), the magnitude of the second component increased from a negligible value to about 1.4% of the total amplitude of the decay (data not shown). Thus under normal conditions in organic solvent, photodecomposition is negligible, with the possible exception of chloroform and acetone where extended illumination times were required for lifetime analysis.

In mineral oil, the decay of the total fluorescence emission could be altered by changing the temperature, and thus the viscosity of the solvent. Magic angle fluorescence emission yielded temperature-dependent lifetimes intermediate in length between those seen in isotropic organic solvents and those seen in more restricted, anisotropic membrane environments. A decrease in motion of the probe affects the non-radiative emission pathway. Decreased motion inhibits the transfer of excited state energy to the lattice through mechanisms involving vibrational modes. Thus longer lifetimes are expected in motionally restricted circumstances, such as in mineral oil at low temperatures.

Overall, the major lifetime component reflected the relative motional restriction of the environment. The shortest lifetimes were measured in low

viscosity isotropic solvents and were near 300 ps. In mineral oil, a high viscosity media, fluorescence lifetime increased from a value near that in the isotropic solvents to about 1 ns at 6°C. The restricted environment that is found in the DMPC bilayers produced a lifetime of about 1 ns. Finally in isopentane at 77 K, where the probe is immobilized, cholestatrienol (which has the same fluorophore as dehydroergosterol) exhibits a lifetime near 3 ns [19].

Dehydroergosterol in phospholipid bilayers

Since dehydroergosterol can be used as a fluorescent probe of sterol behavior [5], the lifetime of dehydroergosterol was measured as a function of temperature in DMPC liposomes. When incorporated into multilamellar liposomes at probe concentrations, the major lifetime component of dehydroergosterol decreased at 23°C, indicating that the majority of dehydroergosterol molecules were detecting the phase change of the DMPC. This decrease in lifetime agrees well with the decrease in quantum yield observed with dehydroergosterol at the T_m of DMPC [5]. In addition to this major lifetime component, a second minor lifetime was observed below the T_m of DMPC. The relative and absolute amplitudes of the minor component gradually decreased to zero as the temperature was increased towards the T_m of DMPC. Centrifugation of the labeled liposomes before measurement precluded the presence of probe in the aqueous environment while the ability to perform background subtraction on the liposomes removed from the data set potential artifacts due to scattering, potential fluorescence from phospholipids and buffer, or from the optical system itself.

Two lifetime components have also been measured with 1,6-diphenyl-1,3,5-hexatriene (DPH) in DMPC liposomes below the T_m where the second component is a very minor component; a single lifetime component was observed above the T_m [22]. Some controversy exists concerning the reality of this minor second lifetime component for DPH below the T_m [22], because DPH exposed to room light for 3 to 6 h is more likely to exhibit a second component in liposomes.

With dehydroergosterol in DMPC liposomes, the second minor component does not appear to be due to photodecomposition for at least two reasons. First, the amplitude of the second component was temperature-dependent and slowly decreased to zero amplitude at elevated temperature. Second, the time of illumination did not of itself give rise to a second component. These experiments were performed stating from the lowest temperature and ending with the highest temperature. The temperature at which the samples had experienced the greatest illumination times (highest temperatures) showed no evidence of the second component. These results suggest that the second minor component does not represent a photodecomposition product. Furthermore, the ability to correct for background fluorescence reduces the chance of scattering artifacts in this analysis. Therefore, this second minor component, whose fractional intensity at most is less than 0.05 below the T_m of DMPC, may represent a distinct environment for dehydroergosterol in the bilayer. An examination of these phenomena is the subject of a separate study.

Dehydroergoesterol fluorescence anisotropy decays

Experiments on the time-dependent depolarization of dehydroergosterol in mineral oil have been used as a method to quantitate rotational motion of fluorescent probes during the time that emission occurs [23]. This fluorescence emission is dependent on the temperature, viscosity, shape, and size of the rotating unit [24,25]. Weber [26] found that the extent of fluorescence depolarization depended on the fluorescence lifetime and on the rotational motion of the probe.

The decay of the anisotropy, $r(t)$, can be related in the case of simple isotropic motion to the rotational correlation time of the fluorophore in terms of a single exponential decay:

$$r(t) = r_0 \, e^{-t/\phi} \tag{5}$$

Since this molecule is not spherical, however, rotation about the long axis of the molecule may be more rapid than other tumbling modes.

In mineral oil the rotational correlation times, as derived from anisotropy decay, are temperature-dependent since temperature of this viscous solvent affects molecular motion of dehydroergosterol. The anisotropy decayed to about $r = 0.01$, suggesting complete randomization of the initially polarized fluorescence during the excited-

state lifetime. As the temperature was increased, $r(t)$ decayed more rapidly. Energy transfer is not expected to be an important mechanism for decay of the anisotropy because of the low concentration of the sterol. Thus the rotation of the fluorophore in mineral oil was temperature (i.e., viscosity) dependent (as was the fluorescence lifetime).

The single exponential for the anisotropy decay for dehydroergosterol in mineral oil may have a simple explanation. It has been calculated that the emission transition dipole is likely to lie nearly colinear with the long axis of the sterol (Hudson, B., personal communication). This is in agreement with theory and experimental data from other polyenes [27]. The best value for r_0 at present is obtained from the anisotropy decay in mineral oil, extrapolating back to time zero. From this lower estimate, r_0 is at least 0.36. Fischer, et al. [31] determined r_0 to be 0.385.

Due to the orientation of the emission transition dipole, rotation about the long axis will not cause a decay of the anisotropy. Rotational modes other than rotation about the long molecular axis must be responsible for the anisotropy decay. These rotational modes are not likely to be distinguishable as a multiexponential decay because the rates of these non-axial rotations are too similar. Therfore, single exponential decay is observed in this temperature region.

This should make the rate of anisotropy decay very sensitive to behavior of a sterol in a phospholipid bilayer. Rotation about the long molecular axis is little affected by insertion into a membrane, but the time-dependent reorientation of that axis (off-axis motions) is strongly affected by the membrane [28].

In summary, we have described a one-step synthesis of dehydroergosterol, and have characterized its physical characteristics. The molecule is a suitable probe for studying the sterol behavior of cholesterol in bilayer membrane systems and possesses a fluorescence lifetime long enough to detect lifetime changes as well as anisotropy decays in biological systems. Excited-state behavior of these probes appears simpler than for parinaric acid [29]. Furthermore, the location of these β-hydroxyl fluorescent sterols in the membrane would appear to be better defined than for DPH. The location of cholesterol in phospholipid bilayers has been determined using neutron diffraction [30]. Based on the similarity in structure and function between these probes and cholesterol, dehydroergosterol and cholestatrienol are likely found in the same membrane location. Therefore, the position of the fluorophore in the membrane can be accurately deduced.

This work demonstrates that information concerning sterol environments and behavior in biological systems can be obtained through the use of sterol fluorescent probes such as dehydroergosterol and cholestatrienol that possess the triene chromophore.

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