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Influence of cholesterol and ergosterol on membrane dynamics: a fluorescence approach☆

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Abstract

Sterols are essential membrane components of eukaryotic cells and are important for membrane organization and function. Cholesterol is the most representative sterol present in higher eukaryotes. It is often found distributed non-randomly in domains or pools in biological and model membranes. Cholesterol-rich functional microdomains (lipid rafts) are often implicated in cell signaling and membrane traffic. Interestingly, lipid rafts have also recently been isolated from organisms such as yeast and *Drosophila*, which have ergosterol as their major sterol component. Although detailed biophysical characterization of the effect of cholesterol on membranes is well documented, the effect of ergosterol on the organization and dynamics of membranes is not very clear. We have monitored the effect of cholesterol and ergosterol on the dynamic properties of both fluid (POPC) and gel (DPPC) phase membranes utilizing the environment-sensitive fluorescent membrane probe DPH. Our results from steady state and time-resolved fluorescence measurements show, for the first time, differential effects of ergosterol and cholesterol toward membrane organization. These novel results are relevant in the context of lipid rafts in ergosterol-containing organisms such as *Drosophila* which maintain a low level of sterol compared to higher eukaryotes.

Keywords: Cholesterol; Ergosterol; Fluorescence polarization; Fluorescence lifetime; Apparent rotational correlation time

Sterols present in the plasma membranes of eukaryotic cells are essential for the organization and function of membranes, and are the products of a long biochemical evolution [1]. The end product of the sterol pathway in the course of evolution is cholesterol which is the most representative sterol present in vertebrate membranes. Cholesterol plays a crucial role in membrane organization, dynamics, function, and sorting [2–4]. It is often found distributed non-randomly in domains or pools in biological and model membranes [2–9]. Many of these domains are believed to be important for the maintenance of membrane structure and function. Recent observations suggest that cholesterol exerts many of its actions by maintaining a specialized type of membrane domain, termed "lipid raft," in a functional state [7,10–12]. Although the existence of lipid rafts in membranes has not been unequivocally shown [13], they are thought of as lateral organizations on the plane of the membrane that are enriched in cholesterol and sphingolipids and specific proteins that are implicated in cell signaling and traffic. The integrity of the raft regions of the membrane is thought to be crucial to regulate signal transduction events [14] and entry of pathogens into the cell [15,16].

While cholesterol is the major sterol present in plasma membranes of higher eukaryotes, ergosterol is the major sterol component present in lower eukaryotes such as certain protozoa, yeast, and other fungi, and in insects such as *Drosophila* [1]. The chemical structure of ergosterol differs from that of cholesterol in having two additional double bonds (at positions C_7 and C_{22}) and a methyl group at C_{24} of the side chain (see Fig. 1). Both structural features appear relatively late during

☆ *Abbreviations:* DMPC, dimyristoyl-*sn*-glycero-3-phosphocholine; DPH, 1,6-diphenyl-1,3,5-hexatriene; DPPC, dipalmitoyl-*sn*-glycero-3-phosphocholine; MLV, multilamellar vesicle; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine.

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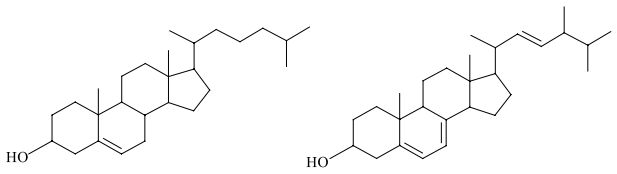

Cholesterol Ergosterol

Fig. 1. Chemical structures of the sterols used.

ergosterol biosynthesis in response to some specialized requirements related to the physiology of organisms containing ergosterol as the major sterol [1].

Interestingly, lipid rafts have recently been isolated from organisms such as yeast [17] and *Drosophila* [18] which have ergosterol as their major sterol component. Although detailed biophysical characterization of the effect of cholesterol on membranes is well documented [2,5], the effect of ergosterol on the organization and dynamics of membranes has not been studied in detail, especially using fluorescence spectroscopy. In this paper, we have monitored the effect of cholesterol and ergosterol on the dynamic properties of both fluid (POPC) and gel (DPPC) phase membranes utilizing the environment-sensitive fluorescent membrane probe DPH. DPH is extensively used to monitor the organization and dynamics of the interior regions in membranes [19]. Our results, using fluorescence polarization and time-resolved fluorescence measurements of DPH, show differential effects of ergosterol and cholesterol toward membrane dynamics.

Materials and methods

Materials. DPPC and POPC were obtained from Avanti Polar Lipids (Alabaster, AL, USA). DMPC, cholesterol, ergosterol, and DPH were purchased from Sigma Chemical (St. Louis, MO, USA). All lipid stock solutions were made in methanol except ergosterol which was dissolved in ethanol. Phospholipids were checked for purity by thin layer chromatography on pre-coated silica gel plates (Sigma) in chloroform/methanol/water (65:35:5, v/v/v) and were found to give only one spot in all cases with a phosphate-sensitive spray and on subsequent charring [20]. The concentrations of the phospholipids were determined by phosphate assay subsequent to total digestion by perchloric acid [21]. DMPC was used as an internal standard to assess lipid digestion. Concentration of stock solution of DPH in methanol was estimated from its molar absorption coefficient (ε) of 88,000 M^{-1} cm^{-1} at 350 nm [22]. All other chemicals used were of the highest purity available. Solvents used were of spectroscopic grade. Water was purified through a Millipore (Bedford, MA, USA) Milli-Q system and used throughout.

Sample preparation. Multilamellar vesicles (MLVs) of either POPC or DPPC containing increasing concentrations (0–50 mol%) of sterol (cholesterol or ergosterol) and 1 mol% DPH were prepared. For this, 80 nmol of total lipid (phospholipid and sterol) and 0.8 nmol of DPH were mixed well and dried under a stream of nitrogen while being warmed gently (~35 °C). After the lipids were dried further under a high vacuum for at least 3 h, they were hydrated (swelled) by adding 1.5 ml of 10 mM sodium phosphate, 150 mM sodium chloride, pH 7.4 buffer, and each sample was vortexed for 2 min to uniformly disperse the lipids and form homogeneous MLVs. The buffer was always maintained at a temperature well above the phase transition temperature of the phospholipid used as the vesicles were made. Thus, the lipids were swelled at a temperature of 40 °C for POPC and 60 °C for DPPC samples. The samples were freeze–thawed five times by cycling the samples in liquid nitrogen and water bath maintained at 60 °C to ensure solute equilibration between trapped and bulk solutions. Background samples were prepared in the same way except that DPH was not added to them. The optical density of the samples measured at 358 nm was less than 0.1 in all cases which rules out any possibility of scattering artifacts in the polarization measurements [23]. Samples were kept in dark for 45 min before measuring fluorescence. All the experiments were carried out with multiple sets of samples at room temperature (23 °C).

Fluorescence polarization measurements. Steady state fluorescence polarization measurements were performed with a Hitachi F-4010 spectrofluorimeter using a Hitachi polarization accessory. Quartz cuvettes with a path length of 1 cm were used. The excitation wavelength was set at 358 nm and emission was monitored at 430 nm. Excitation and emission slits with bandpass of 1.5 and 10 nm were used for all measurements. The excitation slit used was the minimum possible to minimize any photoisomerization of DPH during irradiation. Fluorescence was measured with a 30 s interval between successive openings of the excitation shutter to reverse any photoisomerization of DPH [24]. Polarization values were calculated from the equation [25]:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}, \tag{1}$$

where I_{VV} and I_{VH} are the measured fluorescence intensities with the excitation polarizer oriented vertically and the emission polarizer vertically and horizontally oriented, respectively. G is the grating correction factor and is the ratio of the efficiencies of the detection system for vertically and horizontally polarized light, and is equal to I_{VV}/I_{VH}. All experiments were done with multiple sets of samples and average values of polarization are shown in Fig. 2. The contribution from the backgrounds was found to be negligible.

Time-resolved fluorescence measurements. Fluorescence lifetimes were calculated from time-resolved fluorescence intensity decays using a Photon Technology International (London, Western Ontario, Canada) LS-100 luminescence spectrophotometer in the time-correlated single photon counting mode. This machine uses a thyratron gated nanosecond flash lamp filled with nitrogen as the plasma gas (16 ± 1 in. of mercury vacuum) and is run at 22–25 kHz. Lamp profiles were measured at the excitation wavelength using Ludox (colloidal silica) as the scatterer. To optimize the signal to noise ratio, 5000 photon counts were collected in the peak channel. The excitation and emission wavelengths used were 358 (which corresponds to a peak in the spectral output of the nitrogen lamp) and 430 nm, respectively. All experiments were performed using excitation slit with a nominal bandpass of 2 nm, and emission slit with a bandpass of 5 nm or less. The sample and the scatterer were alternated after every 10% acquisition to ensure compensation for shape and timing drifts occurring during the period of data collection. This arrangement also prevents any prolonged exposure of the sample to the excitation beam thereby avoiding any possible photodamage to the fluorophore. The data stored in a multichannel analyzer were routinely transferred to an IBM PC for analysis. Fluorescence intensity decay curves so obtained were deconvoluted with the instrument response function and analyzed as a sum of exponential terms:

$$F(t) = \Sigma_i \alpha_i \exp(-t/\tau_i), \tag{2}$$

where $F(t)$ is the fluorescence intensity at time t and α_i is a pre-exponential factor representing the fractional contribution to the time-resolved decay of the component with a lifetime τ_i such that $\Sigma_i \alpha_i = 1$. The decay parameters were recovered using a non-linear least square

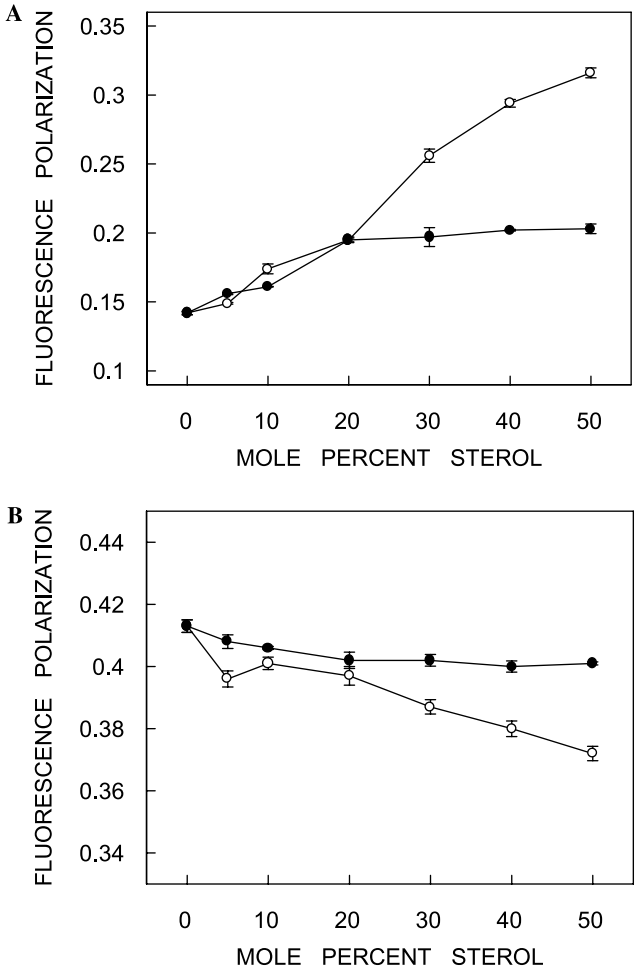

Fig. 2. Fluorescence polarization of DPH in (A) POPC and (B) DPPC membranes as a function of increasing concentration of cholesterol (○) and ergosterol (●). The ratio of DPH to total lipid was 1:100 (mol/mol) and total lipid concentration was 53 μM in all cases. The excitation wavelength used was 358 nm and emission was monitored at 430 nm. Measurements were carried out at room temperature (23 °C). The data points shown are means ± SE of at least three independent measurements. See Materials and methods for other details.

iterative fitting procedure based on the Marquardt algorithm [26]. The program also includes statistical and plotting subroutine packages [27]. The goodness of fit of a given set of observed data and the chosen function was evaluated by the reduced χ^2 ratio, the weighted residuals [28], and the autocorrelation function of the weighted residuals [29]. A fit was considered acceptable when plots of the weighted residuals and the autocorrelation function showed random deviation about zero with a minimum χ^2 value not more than 1.3. Mean (average) lifetimes $\langle\tau\rangle$ for biexponential decays of fluorescence were calculated from the decay times and pre-exponential factors using the following equation [25]:

$$\langle\tau\rangle = \frac{\alpha_1\tau_1^2 + \alpha_2\tau_2^2}{\alpha_1\tau_1 + \alpha_2\tau_2}. \quad (3)$$

Results

The change in fluorescence polarization of DPH with increasing sterol concentration is shown in Fig. 2. Fluorescence polarization is correlated to the rotational diffusion rate [25] of membrane embedded probes which is sensitive to the packing of fatty acyl chains and sterols. Fig. 2A shows that with increase in cholesterol concentration, the DPH polarization in fluid POPC membranes shows a continuous increase up to the highest concentration of cholesterol used. Thus, there is large (123%) increase in polarization when 50 mol% cholesterol was incorporated in POPC membranes. This could possibly indicate that the membrane becomes more ordered (rigid) with increasing concentration of cholesterol when added to membranes in the fluid phase. This is in agreement with previous studies in which DPH polarization values were reported for POPC [30] and egg PC [31] membranes containing cholesterol.

As mentioned above, although the biophysical characterization of the effect of cholesterol on membranes is well documented by use of DPH fluorescence [30–32], the effect of ergosterol on the organization and dynamics of membranes has not been studied in detail, especially using DPH fluorescence. In contrast to what was observed in case of cholesterol containing POPC membranes, the fluorescence polarization of DPH in POPC membranes shows an increase (37%) only up to ~20 mol% when ergosterol was used as the sterol component (Fig. 2A). At higher concentrations of ergosterol, the polarization values stabilize and display a marginal increase with increasing ergosterol concentration up to 50 mol%. This suggests that ergosterol does not influence the membrane order and dynamics in fluid membranes beyond a certain concentration. The effect of ergosterol therefore appears to be markedly different than that of cholesterol on the dynamics (order) of fluid phase POPC membranes. To the best of our knowledge, this is the first report describing this unique effect of ergosterol using DPH fluorescence. It is important to note here that the polarization values determined remained identical even after dilution of membrane samples indicating the absence of any scattering artifacts [23].

Fig. 2B shows the corresponding changes in DPH polarization when the sterols were incorporated in the gel phase DPPC membranes at room temperature (23 °C). In DPPC membranes containing increasing amounts of cholesterol, the polarization values show a small (~10%) decrease up to 50 mol% cholesterol. This is in agreement with previous results in which it was shown that incorporation of cholesterol makes the gel phase DPPC membrane somewhat less ordered [32]. The effect of ergosterol incorporation on DPPC membranes is considerably less than that of cholesterol. DPH polarization shows a very small (3%) decrease in DPPC membranes even in the presence of 50 mol% ergosterol (shown in Fig. 2B). Therefore even in this case, the effect of ergosterol was found to be much smaller when compared to the effect induced by cholesterol.

Fluorescence lifetime serves as a faithful indicator of the local environment in which a given fluorophore is placed [33]. In addition, it is well known that the fluorescence lifetime of DPH is sensitive to polarity changes in its environment [34]. All fluorescence decays could be fitted well with a biexponential function. The mean fluorescence lifetimes were calculated from the decay curves according to Eq. (2) and using Eq. (3) and are shown in Fig. 3. Fig. 3A shows an increase (~20%) in the mean lifetime of DPH with increasing cholesterol concentration in the fluid phase POPC membranes. Since DPH lifetime is known to be reduced by the presence of water in its immediate environment [34,35], we interpret the increase in DPH lifetime with increasing cholesterol due to a decrease in water penetration in the bilayer [36] because of the rigidification of the fluid membrane upon cholesterol incorporation (Fig. 2A). The corresponding change in DPH lifetime due to incorporation of increasing concentrations of ergosterol in the membrane is considerably less and shows a modest reduction in lifetime beyond 30 mol% of ergosterol (Fig. 3A). This may indicate that water penetration is more in case of ergosterol perhaps due to a relatively less rigidifying effect of ergosterol in POPC membranes as previously shown by change in fluorescence polarization (see Fig. 2A).

The effect of sterols on the mean fluorescence lifetime of DPH in gel phase DPPC membranes is less and is shown in Fig. 3B. Interestingly, DPH lifetime shows an initial decrease (up to 10 mol%) for both cholesterol and ergosterol possibly due to increased water penetration in the bilayer due to the fluidization of the gel membrane. However, the mean lifetime values show an increase beyond this point. The initial decrease in DPH lifetime is only observed with the gel phase DPPC membranes at low concentrations. These conditions are similar to

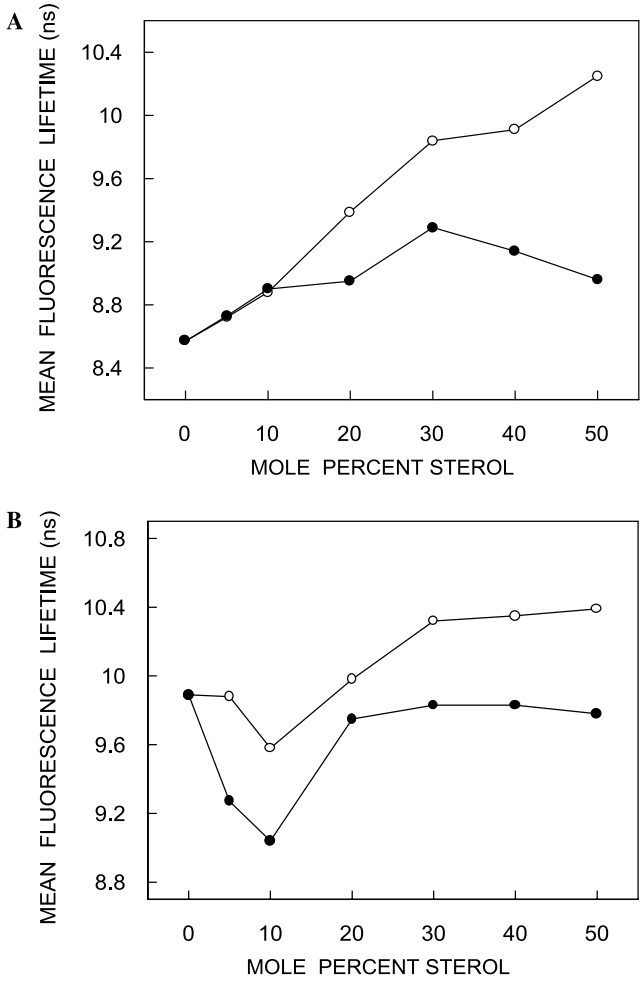

Fig. 3. Mean fluorescence lifetimes of DPH in (A) POPC and (B) DPPC membranes as a function of increasing concentration of cholesterol (○) and ergosterol (●). Mean fluorescence lifetimes were calculated from the decay parameters using Eq. (3) as described in Materials and methods. All other conditions are as in Fig. 2. See Materials and methods for other details.

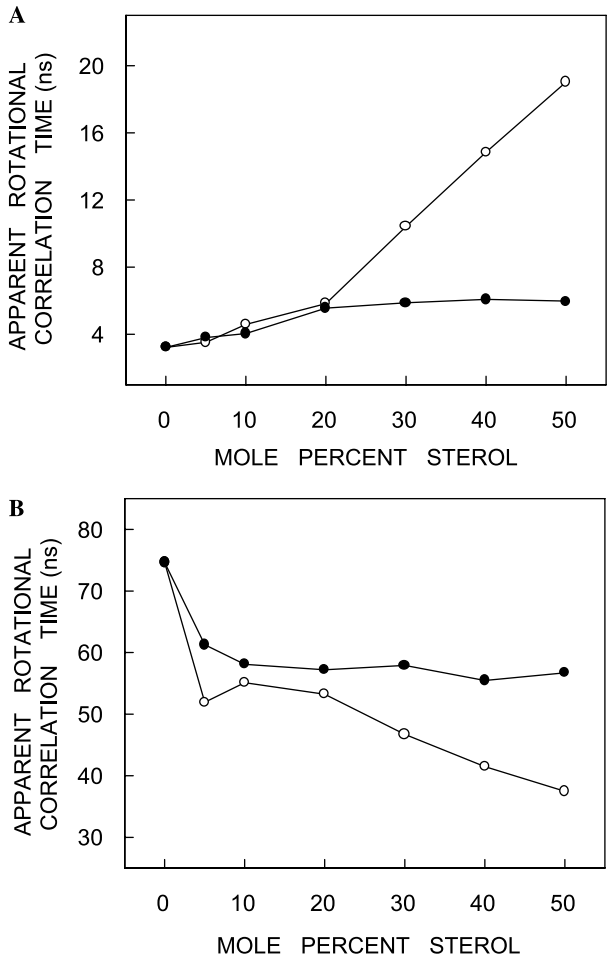

Fig. 4. Apparent rotational correlation times of DPH in (A) POPC and (B) DPPC membranes as a function of increasing concentration of cholesterol (○) and ergosterol (●). Apparent rotational correlation times were calculated from fluorescence polarization values from Fig. 2 and mean fluorescence lifetimes from Fig. 3 using Eq. (4). All other conditions are as in Fig. 2. See text for other details.

those in which we have earlier reported that cholesterol [6] and dehydroergosterol [6,9] may exist as transleaflet tail-to-tail dimers. Whether the initial decrease in DPH lifetime is due to the transleaflet sterol dimer formation is not clear but represents an interesting possibility.

In order to ensure that the polarization values measured for DPH (Fig. 2) are not influenced by lifetime-induced artifacts, the apparent (average) rotational correlation times were calculated using Perrin's equation [25]:

$$\tau_c = \frac{\langle\tau\rangle r}{r_0 - r}, \tag{4}$$

where r_0 is the limiting anisotropy of DPH, r is the steady state anisotropy (derived from the polarization values using $r = 2P/(3-P)$), and $\langle\tau\rangle$ is the mean fluorescence lifetime taken from Fig. 3. The values of the apparent rotational correlation times, calculated this way using a value of r_0 of 0.36 [37], are shown in Fig. 4. As is evident from the figure, the apparent rotational correlation times show similar trends with increasing sterol concentration as seen in Fig. 2 when DPH polarization was plotted. This clearly shows that the observed changes in polarization values are free from lifetime-induced artifacts.

Discussion

Cholesterol is the major sterol molecule ubiquitously present in mammalian cells. It is hypothesized that cholesterol is selected in the long natural evolution process for its ability to optimize certain physical requirements of cell membranes (such as packing) in relation to functions performed by the cell [1,38,39]. Cholesterol appears to be the appropriate molecule to maintain a delicate balance between membrane rigidity to allow for large cell volumes (as found in higher eukaryotes) and membrane softness and fluidity necessary to allow membrane embedded proteins to function properly. The organization and dynamics of cholesterol in cellular membranes have been extensively studied at the biophysical, biochemical, and cell biological levels [2–9]. In the last few years, there has been an ever increasing number of studies linking organization of membrane cholesterol with lipid rafts [4,7,8].

Ergosterol, on the other hand, is found in lower eukaryotes such as fungi [40], and protozoan parasites such as *Leishmania* and *Trypanosoma* [41]. It is alkylated at its side chain and it has been postulated that this specialized structural feature has been chosen by yeast during sterol evolution for enhancing membrane disorder as an alternative to synthesis of unsaturated fatty acids [1]. Although insects such as *Drosophila* cannot synthesize sterols and require a dietary source [42], the predominant membrane sterol in *Drosophila* is ergosterol [18].

The effect of cholesterol on the physical properties of membranes is well documented [2,5]. However, the effect of ergosterol on the organization and dynamics of membranes has not been studied in detail, especially using fluorescence spectroscopy. The effect of ergosterol on membrane organization and dynamics assumes significance in view of the recent reports about isolation of lipid rafts from organisms such as yeast [17], *Drosophila* [18], and protozoan parasites such as *Leishmania* and *Trypanosoma* [43] which have ergosterol as their major sterol component. Ergosterol has also been recently shown to be crucial for targeting of plasma membrane proteins in yeast [44]. In addition, one of us has recently shown that ergosterol mutants of the pathogenic yeast *Candida albicans* show drug resistance which could be related to the organization, dynamics, and specific interaction of membrane ergosterol with sphingolipids in the plasma membrane [45]. Likewise, drug resistance in *Leishmania donovani* has been correlated to ergosterol biosynthesis and resulting changes in membrane dynamics [41]. The understanding of ergosterol organization in membranes is therefore crucial.

Although the effects of ergosterol in membrane dynamics have been previously addressed using magnetic resonance spectroscopy and neutron scattering [46–49], there have been fewer studies employing fluorescence spectroscopic approaches. Fluorescence spectroscopy is widely used in the analysis of membrane structure and dynamics. The advantages of using fluorescence techniques are intrinsic sensitivity, suitable time resolution, non-invasive nature, and minimum perturbation. Application of fluorescent probes such as DPH therefore offers a powerful approach for monitoring membrane organization and dynamics due to their high sensitivity and multiplicity of measurable parameters.

In this paper, we have monitored the effect of cholesterol and ergosterol on the dynamic properties of both fluid and gel phase membranes utilizing environment-sensitive fluorescent membrane probe DPH. DPH is a rod-like molecule and partitions into the interior (hydrophobic core) of the bilayer. The average location of DPH has been shown to be ~ 8 Å from the center of the bilayer [50]. However, its precise orientation in the membrane interior is not known. Fluorescence quenching experiments in model membranes containing co-existing gel and fluid phases have earlier shown that DPH partitions homogeneously between these phases [51]. The partitioning property of DPH is particularly advantageous since it reports the phase-averaged rotational properties of the membrane lipid components without bias toward any particular phase of the membrane.

Our results, using fluorescence polarization and time-resolved fluorescence measurements of DPH, show differential effects of ergosterol and cholesterol on membrane dynamics for both fluid and gel phase

membranes. The rotational mobility of DPH, derived from steady state and time-resolved fluorescence measurements, decreases as a function of increasing sterol concentration in fluid POPC membranes. This could be due to the rigidifying effect of sterol in fluid membranes. Interestingly, this effect is more pronounced in membranes containing high concentrations of cholesterol than ergosterol. At concentrations above 15–20 mol%, ergosterol does not appear to influence the membrane order and dynamics in fluid POPC membranes further. This is an interesting observation since the ergosterol content of the plasma membrane of ergosterol-containing organisms such as *Drosophila* has previously been shown to be in this range [18].

In addition, the rotational mobility of DPH in DPPC membranes was found to increase as a function of cholesterol concentration indicating the fluidizing effect of cholesterol in gel phase membranes. The corresponding change with ergosterol is less pronounced and stabilizes after a certain concentration (~10 mol%) of ergosterol in the membrane. Further, mean fluorescence lifetimes of DPH in POPC and DPPC membranes with increasing sterol concentration indicate changes in environment polarity. In summary, our results show that the effect of ergosterol on membrane order and dynamics is markedly different than that of cholesterol. These results are relevant in the context of membrane domains (lipid rafts) in ergosterol-containing organisms such as *Drosophila* which maintain a low level of sterol compared to higher eukaryotes.

Acknowledgments

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