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Time-Resolved Fluorescence Investigation of Membrane Cholesterol Heterogeneity and Exchange†

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ABSTRACT: The fluorescent sterol $\Delta^{5,7,9(11),22}$-ergostatetraen-3β-ol (dehydroergosterol) was investigated as a cholesterol analogue to examine sterol domains in and spontaneous exchange of sterol between 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) small unilamellar vesicles (SUV). Fluorescence lifetime, acrylamide quenching analyses, and intermembrane exchange kinetics were consistent with the presence of at least two sterol domains in POPC. Fluorescence lifetime was determined by phase and modulation fluorescence spectroscopy and analyzed by nonlinear least-squares as well as continuous distributional analyses. Both methods demonstrated that pure dehydroergosterol in POPC SUV had two lifetime components (C) and fractional intensities (F) near C_1 = 0.851 ns (F_1 0.96) and C_2 = 2.668 ns (F_2 0.04). In contrast to component C_1, the center of lifetime distribution, fractional intensity, and peak width of dehydroergosterol lifetime component C_2 was dependent on the polarity of the medium and vesicle curvature. The sterol domain corresponding to dehydroergosterol component C_2 was preferentially quenched by acrylamide. Acrylamide quenching of dehydroergosterol fluorescence demonstrated that the two lifetime components of dehydroergosterol were not due to transbilayer sterol domains with different lifetimes. In a spontaneous exchange assay not requiring separation of donor and acceptor SUV, the lifetime component C_2, but not C_1, shifted to a shorter lifetime with altered distributional width. The kinetics of these lifetime and distributional width changes best fitted a two-exponential function, with a fast exchange rate constant k_1 = 0.0325 min^{-1}, $t_{1/2}$ = 21.3 min, and a slow rate constant k_2 = 0.00275 min^{-1}, $t_{1/2}$ = 261 min. The fast exchanging pool correlates with the longer lifetime component C_2. These kinetics were confirmed both by dehydroergosterol exchange measured with fluorescence intensity and by [^{3}H]cholesterol exchange. In summary, lifetime, distributional width, acrylamide quenching, and classical exchange assay data are consistent with the presence of at least two pools of sterol in POPC SUV.

Recently the fluorescent sterol dehydroergosterol[1] gained popularity as a probe molecule for monitoring the structural and rotational dynamic properties of cholesterol in model membranes (Rogers et al., 1979; Hale & Schroeder, 1982; Yeagle et al., 1982; Schroeder, 1984; Fischer et al., 1985a; Smutzer et al., 1986; Chong & Thompson, 1986; Schroeder et al., 1987), biological membranes (Schroeder, 1981; Hale & Schroeder, 1982; Muczynski & Stahl, 1983; Kier et al., 1986), and lipoproteins (Smith & Green, 1974; Schroeder et al., 1979a,b; Yeagle et al., 1982). In addition, dehydroergosterol was used to examine sterol–protein interactions with cytosolic sterol carrier protein (Fischer et al., 1985b), membrane glycophorin (Yeagle et al., 1982), and plasma lipoprotein apoproteins (Smith & Green, 1974b).

Despite these observations, only one report utilized dehydroergosterol to examine the presence of sterol domains in membranes (Schroeder et al., 1987). Neither has dehydroergosterol heretofore been reported as a probe molecule for

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SUV, small unilamellar vesicles; dehydroergosterol, $\Delta^{5,7,9(11),22}$-ergostateraen-3β-ol.

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determination of sterol exchange dynamics between model or biological membranes. To measure cholesterol exchange, previous investigators utilized radiolabeled sterols and separation of donor and acceptor biomembranes, lipoproteins (Bell, 1978; Smith & Scow, 1979; Bruckdorfer & Sherry, 1984; Johnson et al., 1986), or model membranes (McLean & Phillips, 1981, 1982; Backer & Dawidowicz, 1981; Bar et al., 1986). The observation that exchange measured with fluorescent sterols such as pyrene-labeled cholesterol was much faster than that of radiolabeled cholesterol (McLean & Phillips, 1981) indicated that such sterols may not be suitable probe molecules for exchange assays. In contrast, dehydroergosterol much more closely resembles cholesterol in structure and membrane properties [see Schroeder (1984) for a review; Schroeder et al., 1987]. Herein is reported the use of dehydroergosterol in combination with distributional analysis of time-resolved fluorescence lifetime measurements to examine membrane sterol domains and intermembrane sterol exchange. The results (1) examine the molecular domains of sterols, (2) provide new insights into the mechanism of sterol exchange, and (3) show a new method for monitoring sterol exchange without separation of donor and acceptor membranes.

Materials and Methods

Reagents. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) was obtained from Avanti Polar Lipids Inc. (Birmingham, AL). Cholesterol was purchased from Applied Science Laboratories Inc. (State College, PA). Dehydroergosterol was synthesized and purified as described previously (Fischer et al., 1985). The purity of recrystallized dehydroergosterol (98%) was confirmed by high-performance liquid chromatography, absorbance peak ratios, and comparisons with dehydroergosterol standards obtained from Frann Scientific Inc. (Columbia, MO). Cholesterol was purchased from Applied Science Laboratories Inc. (State College, PA). These lipids were checked for purity by thin-layer chromatography on silica gel G (250 μm, Analtech Inc., Neward, DE) with chloroform/methanol (100:2 v/v) as the eluting solvent for cholesterol and chloroform/methanol/water (65:25:4) for phospholipid. Standard lipids in one lane of the thin-layer plate were visualized with Rhodamine 6G (0.01% in 0.1 M NaOH) as single spots. The corresponding sample areas in parallel lanes on the same thin-layer plate were then eluted, dried and flushed with N_2, and stored at −70 °C. [1,2-^{3}H(N)]cholesterol and [*oleate*-1-^{14}C]cholesterol oleate (sp act. 57 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Their purity was also checked as described above. The purity of the [^{14}C]cholesterol oleate (by radioactivity) was better than 99%. In contrast, the [1,2-^{3}H(N)]cholesterol had to be repurified by thin-layer chromatography. All other procedures were as described (Bar et al., 1986). DEAE-Sepharose CL-6B was obtained from Pharmacia Inc. (Piscataway, NJ). Prior to use, 100 mL of the gel was washed three times with 200 mL of SUV sonication buffer. Dicetyl phosphate was purchased from Sigma Chemical Co. (St. Louis, MO).

Preparation of Liposomes. Small unilamellar vesicles (SUV) were prepared as described earlier (Schroeder et al., 1987), except that the sonication buffer, pH 7.4, was prefiltered through a 0.22-μm Millipore filter prior to preparation of liposomes. The final lipid concentration of the investigated SUV varied between 2.5 and 5 μmol/mL. Multilamellar liposomes were formed by drying the lipids in chloroform under N_2 and vortexing for about 2 min in a buffer (10 mM PIPES/0.02% NaN_3, pH 7.4) at 24 °C. The lipid suspension was sonicated for 3 min with a Sonogen bath sonicator (Branson Instruments, Stanford, CT) to disperse the lipids. The largest particles were removed by centrifugation at 250*g* for 10 min.

[^{3}H]Cholesterol Exchange. In all radiolabeled cholesterol transfer experiments two populations of POPC vesicles were used: negatively charged donor vesicles and neutral acceptor vesicles. The vesicles were separated on DEAE-Sepharose CL-6B ion-exchange columns as described (Bar et al., 1986). It is important to note that this procedure utilized an elution buffer at pH 7.2 while an earlier method (McLean & Phillips, 1982) used pH 6.0. The pH of the eluting buffer can have a significant effect on $t_{1/2}$ for exchange. In the ion exchange column experiments the donor vesicles contained 3 mol % dehydroergosterol, trace amounts of [^{3}H]cholesterol, 15 mol % dicetyl phosphate, and varying proportions of cholesterol while the acceptor vesicle had trace amounts of [^{14}C]cholesterol oleate (as a nonexchangeable marker to monitor vesicle recovery) and the same sterol/phospholipid molar ratio as the donor vesicles. In all experiments, acceptor vesicles (1 mg/mL) were present in at least 10-fold excess over donor vesicles (0.1 mg/mL) in order to minimize back-exchange of [^{3}H]-cholesterol or dehydroergosterol. In the 300-min time course of the experiments, less than 1% of charged vesicles and 80–85% of the neutral vesicles were recovered in the eluate from the ion-exchange columns. At varying time points during the exchange, aliquots were removed and resolved by the ion-exchange column. The acceptor vesicles were eluted. An aliquot of the eluant was counted for radioactivity while another aliquot was assayed for fluorescent dehydroergosterol content. Fluorescence intensity of dehydroergosterol in acceptor vesicles eluted from the ion-exchange columns above was determined as described earlier (Schroeder et al., 1987).

Dehydroergosterol Exchange: Lifetime Distribution. Five types of SUV were prepared with composition of the following mol percent ratios: (I) POPC/cholesterol, 65:35; (II) POPC/dehydroergosterol, 65:35; (III) POPC/dehydroergosterol, 97:3; (IV) POPC/cholesterol, 97:3; (V) POPC/cholesterol/dehydroergosterol, 65:32:3. Exchange was monitored with lifetime distributional analysis (see below) without separation of donor and acceptor vesicles. The donor/acceptor vesicle lipid ratio was 1:10.

Lipid Composition. Lipid composition of liposomes was determined as described previously (Schroeder et al., 1987) in order to assume that the final lipid composition of the SUV was the same as that in the starting mixture.

Fluorescence Spectroscopy. Fluorescence parameters were measured exactly as described earlier (Schroeder et al., 1987). The inner-filter effect was avoided by diluting all samples such that the absorbance at 325 nm, the excitation wavelength, was less than 0.2. Light scattering was reduced by use of dilute samples and by appropriate cutoff filters in the emission system. Light scattering was not detectable with the SUV used herein. Except where indicated, all fluorescence measurements were made with an SLM 4800 subnanosecond spectrofluorometer (SLM Instruments, Inc., Urbana, IL) modified to 1–250-MHz multifrequency capability (ISS Inc., Urbana, IL). The excitation source was a He/Cd laser (Model 4240NB, Liconix, Sunnyvale, CA) whose emission intensity at 325 nm was modulated sinusoidally with a Pockels cell. Emission was observed through a Janos GG-375 sharp cutoff filter. The light intensity detection was in L format. Data were collected by an IBM PC computer with math coprocessor and 20-MByte Seagate hard disk using an ISS-ADC interface (ISS Instruments Inc., Urbana, IL). The precise description of this instrument construction and theory was provided earlier (Gratton & Limkeman, 1983).

Lifetime Determination. Fluorescence lifetime was measured by the phase and modulation instrument described above. Light scattering was reduced by a Janos GG-375 cutoff filter. Fluorescence lifetime is sensitive to changes in light scattering, especially due to aggregation or fusion of vesicles (Schullery et al., 1980). However, the turbidity of the POPC/sterol-containing vesicles described herein was constant over the time of the experiments. In fact, light scattering was insignificant even for SUV preparations that were over 2 weeks old. The excitation polarizer was 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. Data were usually obtained at 8–12 modulation frequencies (20–140 MHz). At each frequency both phase and modulation of the fluorescence were determined with respect to dimethyl-POPOP in ethyl alcohol in the reference cell. The lifetime of dimethyl-POPOP in ethanol at 24 °C was 1.45 ns (Lakowicz et al., 1981). The sample temperature was controlled and thermostated for 24 °C as described elsewhere (Schroeder et al., 1987). Data were collected and analyzed with an ISS-01 or ISS-187 interface/program (ISS Instruments Inc., Urbana, IL) as described above.

Lifetime Nonlinear Least-Squares Analysis. Nonlinear least-squares data analysis was performed by a multiexponential fit routine (Lakowicz et al., 1984) with ISS-01 software (obtained from ISS Instruments Inc., Urbana, IL) for the above IBM PC computer (IBM Inc.). In the nonlinear least-squares method, the data were fitted to one or two exponential terms. 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. (1984) to judge quality of fit. The error in each parameter was determined from a covariance matrix of errors (Lakowicz et al., 1984). The statistical analysis does not attribute physical significance to the parameters but only that the data fit the model used.

Lifetime Distributional Analysis. Lifetime distributional analysis (Caceri & Cacheris, 1980) was performed on ISS-187 software. The data were fitted to uniform, Gaussian, and Lorentzian distributions. The Lorentzian distribution provided the best fits. The derivation of continuous distribution of lifetime values in frequency domain fluorometry is reported elsewhere (Fiorini et al., 1987). Herein, each component distribution is characterized by a Lorentzian shape according to the following functional relationship:

$$f(\tau) = A/\{1 + [(\tau - C)/(W/2)]^2\} \quad (1)$$

where C is the center position, W is the width of the distribution at half-height, τ is the lifetime, and $f(\tau)$ represents the function that minimizes the reduced χ^2. The constant A can be obtained from the normalization condition.

For both nonlinear least-squares and distributional analysis the ISS-187 program minimized the reduced χ^2 defined by

$$\chi^2 = \sum\{[(P_m - P_c)/S^P]^2 + [(M_m - M_c)/S^M]^2\}/[(2n - f - 1)] \quad (2)$$

where suffixes c and m indicate the calculated and measured values, respectively, of phase (P) and modulation (M), n is the number of frequencies employed, f is the number of free parameters, and S^P and S^M are the standard deviations of each phase and modulation determination, respectively. The standard deviations do not depend on the modulation frequency and are constant for each determination in phase fluorometry (Gratton et al., 1984). Therefore, they factor out in the χ^2 expression. The minimum value of χ^2 is not dependent on a common multiplicative factor. In order to increase the speed of calculation, a fixed value for the standard deviation is used.

Photobleaching is a potential problem associated with fluorescence determinations of many fluorophores. Over a 3-h time period, during which dehydroergosterol in POPC was exposed to continuously strong ultraviolet light (He/Cd laser), the relative fluorescence intensity of dehydroergosterol did not decrease significantly. However, fluorescence decreased thereafter such that by 5–6 h continuous exposure the intensity was reduced 30–40%. All experiments performed herein were completed within 3 h, or the excitation shutter was closed between measurements.

Kinetic Analysis. The kinetics of exchange were calculated in three ways. First, for radioisotope-containing vesicles separated by ion-exchange chromatography, the fraction of label remaining in the donor vesicle at time t for negatively charged donor vesicles was determined as

$$X(t) = \frac{(^3H/^{14}C)_0 - (^3H/^{14}C)_t}{(^3H/^{14}C)_0} \quad (3)$$

where $(^3H/^{14}C)_t$ and $(^3H/^{14}C)_0$ represent the ratio of [^{3}H]-cholesterol to [^{14}C]cholesterol oleate in the eluate at time t and in the starting incubation mixture, respectively. Second, for the vesicles containing dehydroergosterol and separated by ion-exchange chromatography, the fraction of label remaining in the donor vesicle at time t for negatively charged donor vesicles was determined as

$$X(t) = \frac{(I/^{14}C)_0 - (I/^{14}C)_t}{(I/^{14}C)_0} \quad (4)$$

where $(I/^{14}C)_t$ and $(I/^{14}C)_0$ represent the ratio of dehydroergosterol fluorescence intensity to [^{14}C]cholesterol oleate in the eluate at time t and in the starting incubation mixture, respectively. Third, for lifetime kinetic data, the curves were fitted to one-exponential or multiexponential functions by PC NONLIN or JANA iterative nonlinear least-squares analysis (Statistical Consultant Inc., Lexington, KY) on an NCR PC6 (NCR G.m.b.h., Augsburg, Germany) personal computer.

Acrylamide Fluorescence Quenching. The classical Stern–Volmer equation describes the collisional quenching of fluorescence (Stern & Volmer, 1919):

$$F_0/F = 1 + K_{sv}[Q] \quad (5)$$

where F_0 and F are the fluorescence in the absence and presence of the quencher, respectively. [Q] is the concentration of quencher; K_{sv} is the Stern–Volmer constant for the collisional quenching process. K_{sv} is equal to $k_q\tau_0$, where k_q is the apparent bimolecular rate constant for the quenching process. The Stern–Volmer equation predicts a linear dependence of F_0/F on [Q] for a homogeneously emitting solution.

RESULTS

Fluorescence Lifetime of Dehydroergosterol in Palmitoyloleoylphosphatidylcholine Small Unilamellar Vesicles. In order to accurately determine sterol exchange between membranes from fluorescent lifetime experiments, it was first necessary to determine factors that affect fluorescence lifetime parameters of dehydroergosterol in POPC SUV. The first of these variables is fluorescent sterol purity. Small quantities of oxidized sterols are known to enhance half-times for cholesterol exchange by nearly an order of magnitude (Bar et al., 1986). In addition, the fluorescence lifetime of dehydroergosterol in POPC SUV is very sensitive to sterol oxidation. Partially oxidized dehydroergosterol (e.g., 89% pure) can give rise to four or more lifetimes in POPC SUV (data not shown). In contrast, HPLC-purified dehy-

Table I: Analysis of Fluorescence Emission Decay of Dehydroergosterol in POPC SUV Analyzed as Discrete Exponential Components or as a Lorentzian Distribution[a]

Exponential Analysis				
τ	F_1	τ_2	F_2	χ^2
0.852	0.947	3.780	0.053	2.00

Lorentzian Distributional Analysis						
C_1	W_1	F_1	C_2	W_2	F_2	χ^2
0.851	0.050	0.952	2.668	0.127	0.048	2.47

[a] Dehydroergosterol (98+% pure) was incorporated into POPC SUV. The SUV were composed of POPC/dehydroergosterol, 97:3. τ is the lifetime in nanoseconds; F is the fractional intensity; C is the center of distribution in nanoseconds; W is the full width at half-maximum of the distribution in nanoseconds; χ^2 is the reduced χ^2.

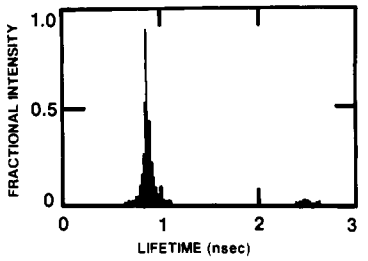

FIGURE 1: Distributional analysis using two Lorentzian functions for dehydroergosterol in small unilamellar vesicles (POPC/DHE, 97:3 mol %) at 24 °C.

droergosterol displayed only two lifetime components in POPC SUV. For 3 mol % HPLC-purified dehydroergosterol in POPC SUV, nonlinear least-squares statistical analysis for two-component fit yielded two lifetime components near 0.852 ± 0.028 ns, fractional intensity 0.95 ± 0.03, and 3.78 ± 1.49 ns, fractional intensity 0.05 ± 0.03 (Table I). A three-component fit increased the χ^2 values to over 30. Adding an additional fitting parameter at the same time as increasing the number of components from two to three does not lead to a decreased χ^2 since the data do not actually fit a three-component analysis very well. In ethanol below the critical micelle concentration HPLC-purified dehydroergosterol displayed only a single lifetime component near 0.4 ns, fractional fluorescence 1.00.

The above data were also analyzed by using a sum of two continuous distributions of lifetime values characterized by a Lorentzian shape centered at decay times C_1 and C_2 and having widths W_1 and W_2. The dehydroergosterol showed a biphasic lifetime distribution in POPC SUV (Figure 1 and Table I) with two components centered near 0.851 ± 0.007 and 2.668 ± 0.762 ns (n = 5 experiments), respectively (χ^2 = 2.47 ± 0.81). The fractional fluorescence contributed by the two components was 0.96 and 0.04, respectively. The major component C_1 at 0.851 ns had a narrow lifetime distribution with a width at half-height of 0.05 ns. The component centered near C_2 had a much broader lifetime distribution with a peak width at half-height of 0.127 ns. The mean reduced χ^2 for dehydroergosterol lifetimes with the Lorentzian distributional analysis (2.47) was about the same as that obtained with the dual exponential analysis (2.00). The major lifetime component of the Lorentzian distribution, mean lifetime 0.851 ± 0.007 ns (mean of five experiments), was nearly indistinguishable from the short-lifetime component (0.852 ± 0.028 ns) obtained by using a double-exponential analysis. The Lorentzian distributional analysis of oxidized dehydroergosterol in POPC SUV also indicated the appearance of additional lifetime components (not shown). From the above lifetime analyses it may be concluded that the two methods of analysis (double exponential and Lorentzian distribution) may be used interchangeably. However, the Lorentzian distributional analysis provides additional parameters (e.g., width of the distribution) that, as shown below, may be sensitive to the environmental heterogeneity of each lifetime component.

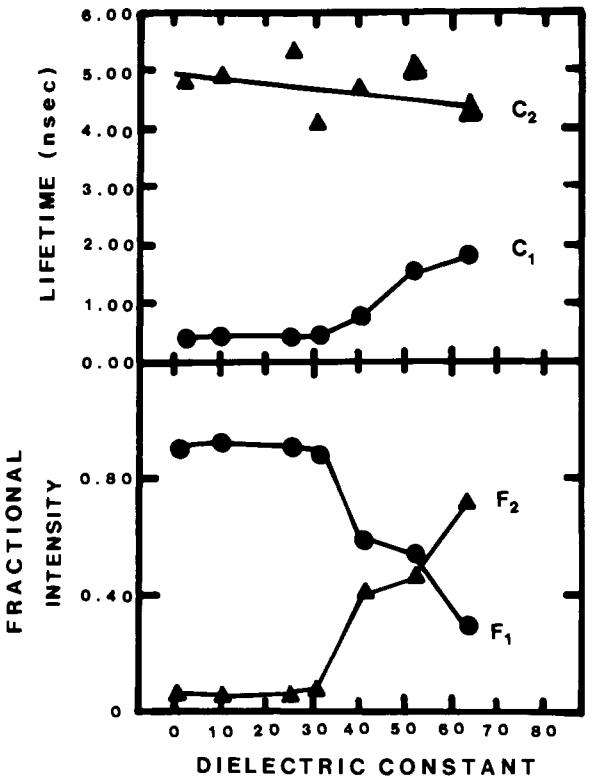

FIGURE 2: Effect of solvent dielectric properties on fluorescent lifetime components of dehydroergosterol (0.5 μg/mL) in dioxane/water mixtures. Lifetime values are derived from the centers of Lorentzian distribution. (●) Main lifetime component; (▲) second lifetime component with fractional intensity increased by the elevated polarity of the medium.

Dehydroergosterol Lifetime Dielectric Sensitivity. Below the critical micelle concentration dehydroergosterol has only one lifetime component near 0.4 ns in organic solvents such as ethanol. However, in solvents such as dioxane and above the critical micelle concentration, dehydroergosterol has essentially two lifetime components near 0.4 ns (fractional intensity >0.96) and 5 ns (fractional intensity <0.05) (Figure 2). The fractional fluorescence of the longer lifetime component, due to micellar dehydroergosterol, increased with increasing micellar content.

The dehydroergosterol lifetime is sensitive to the dielectric constant of the medium (Figure 2). The fluorescence lifetime of dehydroergosterol was examined as a function of dielectric constant in a series of dioxane/water mixtures. The data were analyzed as a Lorentzian distribution for two lifetime components. A significant change in lifetime occurred when the medium dielectric constant exceeded 30. The lifetime of the 0.4-ns component increased, the lifetime of the 5-ns component decreased, and the fractional fluorescence due to the 5-ns component increased. Thus, increased medium polarity resulted in formation of more dehydroergosterol micelles as well as a shifting of the lifetime of monomeric dehydroergosterol to longer values.

The dielectric sensitivity of dehydroergosterol was also examined in POPC SUV where two lifetime components C_1 = 0.851 ns and C_2 = 2.668 ns were obtained from Lorentzian distributional analysis. From the observation that higher medium polarity increased the lifetime of dehydroergosterol

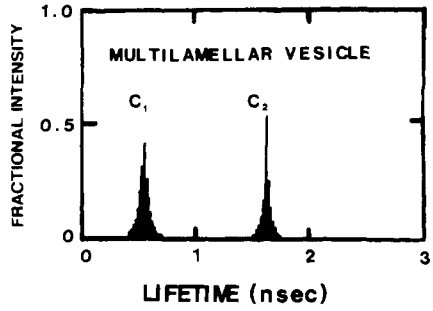

FIGURE 3: Distributional analysis using two Lorentzian functions of dehydroergosterol in multilamellar vesicles (POPC/DHE, 97:3 mol %) at 24 °C.

in organic solvents, one may predict that in POPC the longer lifetime component C_2 may be located in a more polar environment in the bilayer than component C_1. This possibility was tested by using acrylamide, a water-soluble quencher that does not permeate the membrane bilayer. At low concentrations acrylamide (0–1.2 mM) is expected to partially quench dehydroergosterol fluorescence. At 3 mol % dehydroergosterol in POPC, these low concentrations of acrylamide preferentially quenched the C_2 component 25–30% without quenching the C_1 component lifetime. Likewise, at 35 mol % dehydroergosterol low acrylamide concentrations quenched the C_2 component 46–50% without affecting component C_1. Thus, dehydroergosterol lifetime component 2 may arise from a dehydroergosterol pool more sensitive to the aqueous phase than component C_1.

Effect of POPC Vesicle Curvature on Dehydroergosterol Lifetime. The effect of vesicle curvature on dehydroergosterol lifetime distributions was examined. Small unilamellar POPC vesicles have a limiting radius of curvature while large multilamellar vesicles do not. The lifetime of dehydroergosterol (3 mol %) in multilamellar vesicles of POPC (Figure 3) fits a two-component Lorentzian distribution centered near 0.5 and 1.6 ns with two nearly equal fractional fluorescence intensities of dehydroergosterol, 0.43 (component 1) and 0.57 (component 2) ($\chi^2 = 1.9$). In contrast, in POPC SUV containing 3 mol % dehydroergosterol (Table I) the Lorentzian function fits two components centered near 0.851 (fractional fluorescence 0.95) and 2.668 (fractional fluorescence 0.05). The widths of these components in the POPC SUV were smaller (0.05 and 0.127 ns, respectively) than those in the multilamellar vesicles (0.054 and 0.174 ns, respectively).

Sensitivity of Dehydroergosterol Lifetime to Total Sterol Content of POPC SUV. When POPC SUV liposomes contained cholesterol in addition to dehydroergosterol (POPC/cholesterol/dehydroergosterol, 65:32:3), the width of component C_2 increased from 0.127 to 0.35 ns (Figure 4, top panel, vs Table I). In these sterol-rich POPC SUV the short-lifetime component of dehydroergosterol (Figure 4, top panel) shifted to slightly longer lifetime (from 0.851 ± 0.028 to 1.127 ± 0.045 ns, fractional intensity 0.906 ± 0.052). If POPC SUV contained 35 mol % dehydroergosterol without cholesterol (Figure 4, lower panel), the lifetime of component 1 also increased (1.198 ns). The Lorentzian distributional analysis of 35 mol % dehydroergosterol in POPC SUV showed similar distributional widths near 0.05 and 0.35 ns and fractional fluorescence $F_1 = 0.903$ and, $F_2 = 0.097$ (Figure 4, lower panel). In summary, high cholesterol or high dehydroergosterol content of POPC SUV shifted the lifetime of C_1 to slightly longer values and increased the width W_2 of component C_2 as compared to those of dehydroergosterol in POPC SUV of low mole percent sterol.

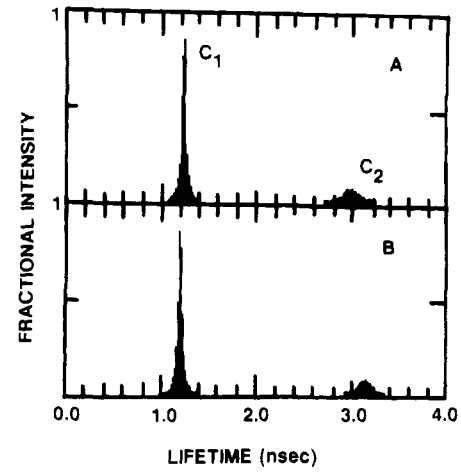

FIGURE 4: Distributional analysis using two Lorentzian functions for dehydroergosterol (A) in POPC/Chol/DHE (65:32:3 mol %) and (b) in POPC/DHE (65:35 mol %) SUV.

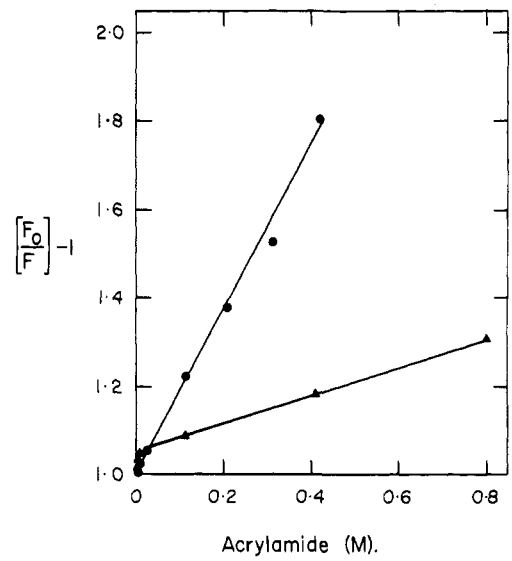

FIGURE 5: Acrylamide quenching of dehydroergosterol in homogeneous and heterogeneous systems. Fluorescence of dehydroergosterol (3.9 mol %) in POPC vesicles was determined in the presence of increasing concentrations of acrylamide (▲). Fluorescence of dehydroergosterol (1 μg/mL) in ethanol was determined in the presence of increasing concentrations of acrylamide (●).

Location of Dehydroergosterol in POPC SUV. The molecular origin of the two lifetime components of dehydroergosterol in POPC SUV reported above is not known. One possibility may be that the two lifetime values of dehydroergosterol in POPC SUV may reflect two different populations of dehydroergosterol in the outer and inner leaflets of the liposomal membranes, respectively. In multilamellar vesicles where there is little geometric constraint, a more equal transbilayer distribution would be expected. In contrast, in SUV with their limiting radius of curvature and associated high geometric constraint the transmembrane distribution would be unequal, consistent with the observations in Table I. In summary, these lifetime components could be interpreted as being due to the presence of sterol in two transbilayer domains. This possibility was further examined by determining the effect of total sterol content on the transbilayer distribution of dehydroergosterol in SUV. For this purpose high concentrations of acrylamide, a nonpenetrating quencher (Eftink & Ghiron, 1981), were used in a similar manner as described previously (Chong & Thompson, 1986). The quenching of dehydroergosterol by acrylamide in a homogeneous system

Table II: Accessibility of Dehydroergosterol to Acrylamide Quenching in Palmitoyloleoylphosphatidylcholine Vesicles

mol %			limiting quenching (%)[a]
dehydroergosterol	cholesterol	POPC	
0.48	0	99.5	64 ± 5
0.19	48.9	50.9	30 ± 6
4.83	0	95.2	52 ± 5
3.98	40.3	55.7	26 ± 3

[a] Limiting quenching is defined as (maximal percent decrease in fluorescence of dehydroergosterol in vesicles upon addition of 800 mM acrylamide)/(maximal percent decrease in fluorescence of dehydroergosterol in ethanol upon addition of 800 mM acrylamide). The concentration of vesicles was 4 mM lipid in 10 mM PIPES/0.02% NaN_3, pH 7.4, buffer. Values represent the mean ± SEM (n = 4).

such as ethanol results in a linear Stern–Volmer plot (upper curve of Figure 5). The Stern–Volmer apparent quenching constant for dehydroergosterol obtained in ethanol is 1.9 M^{-1}. In contrast, in POPC SUV the dehydroergosterol Stern–Volmer plot of dehydroergosterol fluorescence (lower curve in Figure 5) shows two essentially linear segments, one quenched with high efficiency at low acrylamide concentration (0–100 mM) and another required much more acrylamide (0.1–0.8 M). The data may result from two different effects: (1) Low concentrations of acrylamide preferentially quench the smaller dehydroergosterol pool that may be in closer proximity to the aqueous phase while at high acrylamide concentration the second larger dehydroergosterol pool is also quenched. (2) Alternately, dehydroergosterol in POPC SUV may be described as a two-component system comprised of fluorophores located in the outer and the inner monolayers of the bilayer, respectively. In this case the dehydroergosterol in the inner leaflet would be quenched only at high acrylamide concentrations where penetration of the bilayer by acrylamide may occur. These two possibilities were further resolved. Due to the low permeability of the bilayer to acrylamide, the inner surface of the bilayer is only poorly accessible to this quencher. If added to a preformed vesicle dispersion, acrylamide would therefore be expected to quench the fluorophores in the external monolayer. When acrylamide was present at high concentration both inside and outside the POPC SUV, both pools of dehydroergosterol in both leaflets are quenched efficiently (data not shown). This quenching of dehydroergosterol fluorescence intensity in POPC vesicles by acrylamide added to preformed vesicles was dependent on the total sterol content of the POPC SUV bilayers. In the absence of cholesterol and at low mol percent fluorescent sterol, the limiting quenching was 52–64% (Table II). Addition of cholesterol in the range of 40–48 mol % decreased the acrylamide limiting quenching of dehydroergosterol to 25–30%. Again, if the acrylamide was present both inside and outside the vesicle, nearly complete quenching of dehydroergosterol fluorescence was observed. Thus, the distribution of sterol between the transbilayer leaflets of POPC SUV is highly dependent on the mol percent sterol in the vesicle bilayer. However, dehydroergosterol both at 3 mol % and at 35 mol % in POPC SUV displayed two components with very similar fractional fluorescence, F_1 = 0.955 ± 0.012 and F_1 = 0.914 ± 0.013 (n = 5), respectively. Therefore, the two lifetime components of dehydroergosterol in POPC SUV do not appear due to one lifetime component being associated with the outer monolayer while the other component is associated with the inner monolayer of the membrane.

Dehydroergosterol Exchange between POPC SUV: A Fluorescence Lifetime Study. The exchange of dehydroergosterol between POPC/dehydroergosterol (65:35) donor and POPC/cholesterol (65:35) acceptor SUV was examined by the alteration in lifetime distributional parameters during the exchange process (Figure 6). The lifetime (ns) C_2, but not C_1, decreased with time of exchange. These exchange data best fit two exponential components: k_1 = 0.0325 ± 0.0033, $^1t_{1/2}$ = 21.3 ± 2 min; k_2 = 0.00275 ± 0.00058, $^2t_{1/2}$ = 261 ± 54 min. The peak width at half-height (ns) W_2 of component C_2 in the POPC/sterol (65/35) SUV was not dependent on the exchange process. However, the fractional intensity F_2 of C_2 increased exponentially (Figure 7A). This process was maximal in about 4 h. These data also best fit two exponential components: k_1 = 0.0204 ± 0.0075, $^1t_{1/2}$ = 34 ± 12 min; k_2 = 1.84 × 10^{-3} ± 4.6 × 10^{-4}, $^2t_{1/2}$ = 377 ± 94 min. The exchange of dehydroergosterol between POPC SUV containing 3 mol % sterol (donor, POPC/dehydroergosterol, 97:3; acceptor, POPC/cholesterol, 97:3) showed similar kinetics as

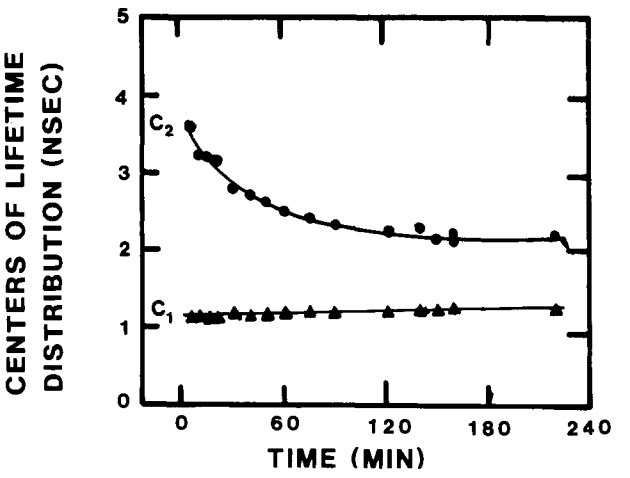

FIGURE 6: Changes of the Lorentzian distributional parameter: lifetime center alterations during exchange of dehydroergosterol between POPC SUV. POPC/Chol (65:35 mol %) and POPC/DHE (65:35 mol %). (▲) Main lifetime component C_1; (●) second lifetime component C_2.

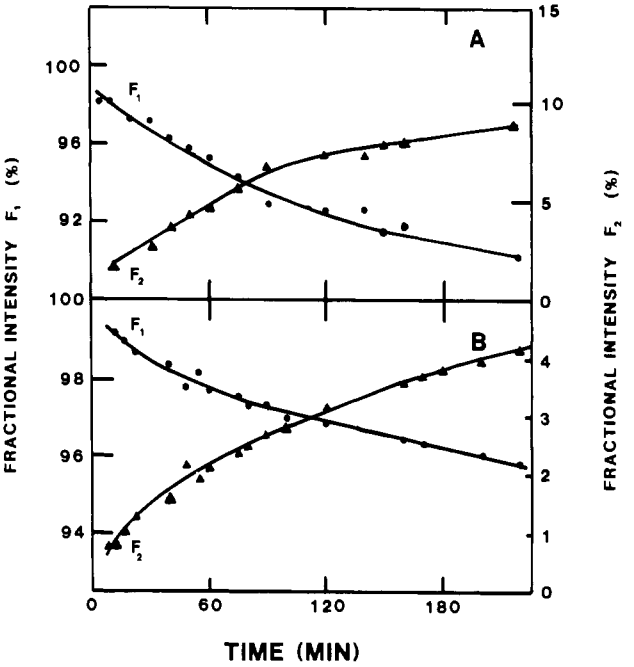

FIGURE 7: Changes of the Lorentzian distributional parameters: fractional intensity of lifetime during exchange of dehydroergosterol between POPC SUV. (A) POPC/DHE (65:35 mol %) and POPC/Chol (65:35 mol %); (B) POPC/DHE (97:3 mol %) and POPC/Chol (97:3 mol %). (●) Main lifetime component intensity F_1; (▲) second lifetime computer intensity F_2.

Table III: Sterol Exchange Kinetics between POPC SUV[a]

label	rate constant ($\times 10^{-3}$ min^{-1}) k_1	k_2	half-time (min) $^1t_{1/2}$	$^2t_{1/2}$	distribution of exchangeable sterol X_1	X_2	% nonexchangeable sterol
			Ion-Exchange Separation				
[^{3}H]cholesterol	27	2.4	26	291	5	95	47
dehydroergosterol[b]	30	2.6	23	269	11	89	38
			Lifetime Analysis without Separation				
dehydroergosterol	32	2.7	21	261			

[a] In these equilibrium exchange experiments, the donor and the acceptor vesicles had the same dehydroergosterol (which contained [^{3}H]cholesterol) or cholesterol concentration (35 mol %). Exchange was determined at 24 °C (Nemecz et al., 1988). [b] The fluorescence intensity of dehydroergosterol which was transferred from donor vesicles was measured in the acceptor vesicles.

noted above for POPC SUV containing 35 mol % sterol (both donor and acceptor): $^1k_1 = 0.033 \pm 0.004$, $^1t_{1/2} = 21 \pm 3$ min; $k_2 = 3.54 \pm 10^{-3}$, $^2t_{1/2} = 296 \pm 45$ min (Figure 7B).

Dehydroergosterol and [^{3}H]Cholesterol Exchange: An Ion-Exchange Column Investigation. The above results for lifetime alteration kinetics during sterol exchange obtained without separation of donor and acceptor membranes were confirmed with classical exchange assays requiring separation of donor (charged) and acceptor (uncharged) POPC SUV (Table III). [^{3}H]Cholesterol (trace amounts) and dehydroergosterol (35 mol %) were incorporated into donor vesicles along with dicetyl phosphate. Acceptor vesicles, in 10-fold excess, contained 35 mol % cholesterol with trace amounts of [^{14}C]cholesterol oleate as a nonexchangeable marker to monitor vesicle recovery. The spontaneous exchange curve for dehydroergosterol, as determined by measuring dehydroergosterol fluorescence intensity in the acceptor SUV, displayed similar kinetics as the spontaneous exchange of [^{3}H]cholesterol, measured by radioactivity in the acceptor SUV ($^1t_{1/2} = 26$ vs 23 min and $^2t_{1/2} = 291$ vs 269 min). More important, the [^{3}H]cholesterol spontaneous exchange kinetics were also like those obtained from kinetics of lifetime changes ($^1t_{1/2} = 23$ vs 21 min and $^2t_{1/2} \simeq 269$ vs 261 min). The classical exchange assay also confirmed that the size of the nonexchangeable sterol for [^{3}H]cholesterol and dehydroergosterol was 47% and 38%, respectively. In addition, the distributions of the exchangeable pools of [^{3}H]cholesterol and dehydroergosterol ($X_1 = 5$ vs 11 and $X_2 = 95$ vs 89) were also similar.

Mass Transfer of Sterol. The mass transfer rate of cholesterol between POPC/cholesterol/dehydroergosterol (65:32:3) donors and POPC/dehydroergosterol (97:3) acceptor SUV was determined (Figure 8). In contrast to the above experiments, in this instance the donor SUV was present in 10-fold excess. The center of lifetime distribution of the C_2 lifetime component, but not the C_1 component, decreased with time in a biexponential manner ($^1t_{1/2} = 26$ min; $^2t_{1/2} = 9.1$ h) (Figure 8A). At the same time, the width W_2 of lifetime component C_2 also decreased in a biexponential manner with a $^1t_{1/2} = 21$ min and $^2t_{1/2} = 9.7$ h (Figure 8B). The width, W_1, of lifetime component C_1 remained constant. The fractional intensity, F_1 (0.94) and F_2 (0.06), of lifetime components C_1 and C_2 did not change significantly during the exchange process (data not shown). It should be noted that in the initial phase (0–30 min), but not in the second phase (30–300 min), of mass transfer the sterol/phospholipid ratio was not significantly altered.

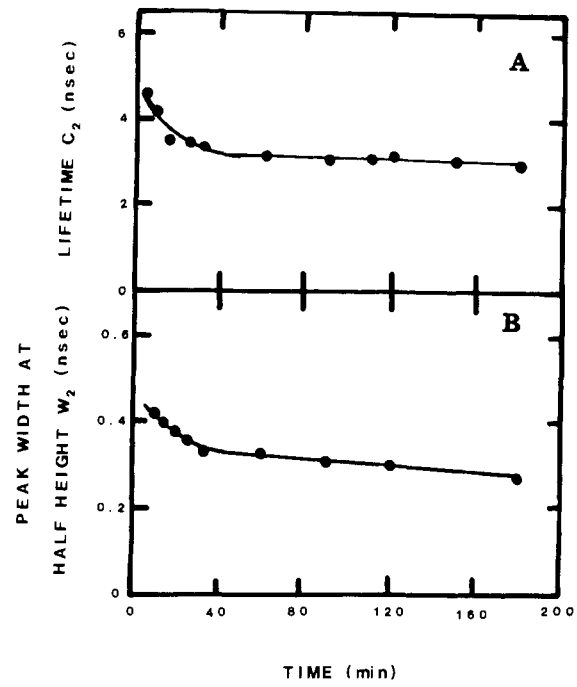

FIGURE 8: Changes of the Lorentzian distributional parameters: (a) center and (b) width at peak half-height during transfer of cholesterol between POPC SUV. Donor POPC/cholesterol/dehydroergosterol (65:32:3 mol %) and acceptor POPC/dehydroergosterol (97:3 mol %).

Discussion

The evidence presented herein indicates that (1) sterols may exist in more than one pool within POPC SUV membranes, (2) the size and properties of these pools are dependent on sterol content and membrane curvature, and (3) spontaneous exchange of sterol between membranes may occur preferentially from one of these pools. Consequently, all sterols in a membrane may not be alike. Previously, this laboratory provided spectroscopic evidence derived from dehydroergosterol concentration dependent quenching that two pools of dehydroergosterol may exist in POPC SUV (Schroeder et al., 1987). Other investigators have also suggested the presence of a phase-separated cholesterol domain comprised of 1:1 cholesterol–phospholipid complexes (McLean & Phillips, 1982) or the presence of two kinetically defined pools of cholesterol (Bar et al., 1986). There is, however, considerable disagreement about interpretations of the complexity of such bilayer pools (Klausner & Kleinfeld, 1984; Yeagle, 1985). Herein, multifrequency phase fluorometry in conjunction with continuous lifetime distributional analysis were used to characterize further the existence of sterol domains in POPC vesicles. Several sets of evidence support the existence for at least two dehydroergosterol domains in POPC SUV membranes:

First, in a homogeneous POPC SUV environment the pure dehydroergosterol can be characterized by one main fluorescence lifetime component having a normalized fractional intensity near 0.95 and a second longer lifetime component

having a fractional intensity <0.05. In a previous investigation from this laboratory the existence of two lifetime components for dehydroergosterol in POPC vesicles was observed (Schroeder et al., 1987). However, because one of the two components represented such a small proportion of the total fluorescence and since it was not possible at that time to accurately resolve this component, the lifetime data were treated as essentially one component in the earlier report. In the present investigation the lifetime data acquisition and analysis was improved to the extent that two components could be accurately resolved on the basis of the fact that the χ^2 value was significantly lower in this case as compared to the assumption of a single lifetime value. The data were subjected to both nonlinear least-squares and to continuous distributional fits. In both cases the χ^2 values were significantly lower for two-component fits. When the two models were compared, the distributional and exponential analyses yielded similar χ^2 values. Since a two-component distributional analysis contains two more fitting parameters than a two-component exponential model, a significantly decreased χ^2 might be expected for the former. However, the results in Table I do not show such a major difference. Likewise, other investigators have also not consistently observed major differences in χ^2 between the two models (Fiorini et al., 1981). All of the χ^2 values were near 1. In fact, these workers concluded that, due to the inherent limitations of present-day instrumentation, it may be impossible to decide if a decay is multiexponential or distributed, on the basis of χ^2 alone. The real motivation claimed for using lifetime distributional analysis arises from physical intuition rather than from statistical demonstration. In some cases, it is desirable to describe decay by use of continuous distributions rather than discrete exponentials which may require more parameters and have no direct physical interpretation (Fiorini et al., 1987). Moreover, the distributional model may describe reality more adequately than the superposition of a finite number of exponentials, particularly if the fluorophore is not in a homogeneous isotropic solution. In a membrane, molecules can be phase separated or partially separated, and thus not every fluorophore may experience the same microenvironment. Thus, we can tentatively assume that the distribution width reflects the homogeneity of the environment in which the fluorophore is embedded. If this reasoning is valid, the exponential model could be considered a special case of the general distributional model, namely, the case when the fluorophore is distributed among domains with defined, sharp boundaries. In short, the major rationale for using the distributional rather than exponential fitting in the present investigation is that the former provides us with an additional parameter, distributional width, that may reflect homogeneity or polarity changes (Fiorini et al., 1987). The kinetics of dehydroergosterol width changes thus provide an additional set of parameters that not only yield half-times for exchange confirming those obtained from kinetics of lifetime, C_2, shifts but may also provide additional information on the molecular basis for these shifts. In addition, the lifetime data are consistent with the presence of at least two lifetime components. The two lifetime components may arise from differences in the rotational motions of the probe in the bilayer microenvironments rather than polarity differences of these environments. However, we have shown earlier that the lifetime of dehydroergosterol in POPC SUV is relatively insensitive (increases from 0.9 to 1.1 ns) with increasing cholesterol content (from 0 to 16 mol %) of SUV while over the same concentration range the rotational rate decreased from 0.5 to 0.04 rad/ns (Schroeder et al., 1987). In addition, even if the two lifetimes were due to rotational motion differences, such differences alone would imply the existence of different sterol environments or pools within the bilayer.

Second, the fluorescence properties of the two dehydroergosterol lifetime components appear to be differentially susceptible to medium polarity. The greater peak width, longer lifetime, and preferential quenching by acrylamide of dehydroergosterol lifetime component C_2 in POPC SUV were consistent with the interpretation that dehydroergosterol in this domain appeared to be located in an environment more polar than that of dehydroergosterol associated with component C_1.

Third, the dehydroergosterol lifetimes in multilamellar vs SUV membranes as well as the acrylamide quench (SUV) data presented herein are consistent with the interpretation that dehydroergosterol exists in both outer and inner monolayers of the SUV membrane in two pools with a short lifetime and a long lifetime component, respectively. The two lifetime components of dehydroergosterol do not represent sterol in the outer and inner face of the POPC membrane. However, the results presented herein are in agreement with data from a number of laboratories indicating the existence of an asymmetric transbilayer sterol distribution in SUV that is highly dependent on sterol/phospholipid ratio. For example, exchange experiments using [^{3}H]cholesterol (de Kruijff et al., 1976; Smith & Green, 1974a) and 3-thiocholesterol (Huang et al., 1974) demonstrated that, above 33 mol % sterol, the sterol was enriched in the inner leaflet of SUV, consistent with results presented herein. At low mol percent sterol the opposite behavior may be predicted. As shown in Table II and by other investigators (Poznansky & Lange, 1976a; Huang et al., 1970), at low mol percent sterol more than 70% of sterol is located in the outer monolayer of SUV. Thus, if the two lifetime components of dehydroergosterol in POPC SUV indeed arose from a differential transbilayer location of dehydroergosterol, then at high vs low mol percent sterol in POPC SUV the fractional fluorescence of each component should differ markedly. However, the data indicate that this was not the case. At 3 mol % dehydroergosterol in POPC SUV the major and minor lifetime component fractional fluorescences (0.96 and 0.04, respectively) were similar to those obtained from 35 mol % dehydroergosterol in POPC SUV (0.92 and 0.08), respectively. Thus, although the transbilayer distribution of the dehydroergosterol is asymmetric in POPC SUV with high mol percent sterol, the two transbilayer pools do not correspond to the two lifetime components C_1 and C_2, respectively.

In conclusion, the most likely possibility explaining the presence of two dehydroergosterol lifetime components in POPC SUV is that they represent two different environments or domains of different polarity. Medium polarity can affect lifetime of fluorophores such as dehydroergosterol (Schroeder et al., 1987). The dielectric constant gradient along the membrane normal can, in part, determine the distribution of diphenylhexatriene decay rates (Fiorini et al., 1987). Increased polarity increased the lifetime of the short lifetime component of dehydroergosterol in dioxane/water mixtures. The lifetime component near 5 ns in such mixtures was most likely due to the presence of micelles and has also been noted previously by others (Smutzer et al., 1986; Fischer et al., 1985b). In addition, investigators using another fluorescent sterol analogue, *N*-(2-naphthyl)-22-amino-23,24-dinor-5-cholen-3β-ol, showed that this fluorescent sterol is also solvent polarity sensitive (Kao et al., 1978). However, in this early work individual pools or domains of fluorescent sterol were not resolved.

The presence of two lifetime components for dehydroergosterol in POPC SUV indicating that a small portion of the dehydroergosterol is in a pool of considerably different polarity from the majority of sterol appears very significant to sterol exchange between membranes. As shown by the exchange data, the fast spontaneous exchange of sterol between membranes appears to occur from the medium polarity sensitive pool. At any one time only a small portion of the dehydroergosterol molecules in POPC SUV appear located in the membrane surface such that they are sensitive to the polarity of the medium and may undergo spontaneous desorption into the aqueous phase. This unique observation was made possible by time-resolved multifrequency phase and modulation fluorometry and distributional lifetime analysis. The presence of such a small, very active, and spontaneously exchanging sterol pool was not previously reported from radioisotope exchange experiments. That the behavior of dehydroergosterol reflects that of cholesterol is supported by the observation that the dehydroergosterol exchange kinetics appear to be similar to those observed for [^{3}H]cholesterol.

It has been shown that cholesterol exchange between cholesterol/phosphatidylcholine vesicles proceeds through the aqueous phase and follows single-exponential kinetics (McLean & Phillips, 1981, 1982; Backer & Dawidowicz, 1981; Bar et al., 1986). Although some investigators have found only a single pool of cholesterol (Bloj & Zilversmit, 1977; McLean & Phillips, 1981; Backer & Dawidowicz, 1979), a number of investigators have shown that a major portion of the cholesterol pool is exchangeable and a minor portion is nonexchangeable (Pozansky & Lange, 1978; Bar et al., 1986). Our observations support that the transfer of cholesterol occurs by diffusion through the aqueous phase and follows exponential kinetics. The exchange of sterol between bilayers may be measured by following the change in lifetime distribution of the sterol in lipid bilayers of similar composition, but containing cholesterol rather than dehydroergosterol. Earlier we demonstrated that dehydroergosterol self-quenches at 35 mol % resulting in decreased fluorescence polarization (Schroeder et al., 1987). However, it was also shown therein that fluorescence lifetime was insensitive to the self-quenching phenomenon (Schroeder et al., 1987). Analysis of the exchange curves monitored by lifetime changes showed a better fit for a two-exponential function. A rapid exchange or desorption occurs in the first 30 min, and a slower component occurs with a half-time of 3.5–5 h, similar to that observed by other investigators using radiolabeled cholesterol and techniques that separate donor and acceptor vesicles (Bar et al., 1986; McLean & Phillips, 1982). The lifetime distribution changes per se are consistent with but do not prove a quantitative relationship between the mass of dehydroergosterol exchanged and cholesterol exchange. Therefore, the mass of dehydroergosterol and radiolabeled cholesterol exchanged was followed by measuring the quantity of dehydroergosterol and radiolabeled cholesterol in acceptor vesicles separated from donor vesicles by ion-exchange column chromatography as described under Materials and Methods and Results. The fluorescence intensity of dehydroergosterol appearing in the acceptor vesicles (present in 10-fold excess) increased linearly with increasing dehydroergosterol content in the acceptor vesicle. In addition, this intensity correlated directly with dehydroergosterol content quantitated by HPLC or fluorescence assay of sterols extracted from the acceptor vesicles (Fisher et al., 1985a). In either case, the mass of dehydroergosterol in the acceptor vesicle increased in a biexponential manner with half-times of 26 and 291 min, respectively. Therefore, the close similarity of the exchange kinetics in these experiments (compare the above half-times with those observed from the lifetime distribution experiments, 21 ± 2 and 261 ± 54 min) reflects the mass of dehydroergosterol exchanged. The above experiments have also been repeated with another fluorescent sterol analogue, cholestatrienol, and essentially the same exchange kinetics were observed with half-times of 33 ± 4 and 316 ± 84 min, respectively (Nemecz, Fontaine, and Schroeder, unpublished observation). The close correlation of the exchange kinetics from lifetime and ion-exchange experiments provides a reasonable basis for ascribing a physical meaning to the lifetime distributional changes. It should be pointed out that, in contrast to the results presented here with lifetime investigations, the ion exchange column procedures require that the donor and acceptor differ in having a charged component added to either donor acceptor vesicles. The ion-exchange procedures are also limited in time resolution such that fewer data points are obtainable in less than 15 min. The advantage of the fluorescent sterol exchange method is that the vesicles do not differ in charge properties, size, or density and that exchange can be continuously monitored every few seconds from time zero. It should be noted that the exchange $t_{1/2}$ observed herein, 3.5–5 h at 24 °C and pH 7.2, agrees closely with that of Bar et al. (1986), who examined [^{3}H]cholesterol exchange at pH 7.0. Both observations, however, differ from that of McLean and Phillips (1982), who determined [^{3}H]cholesterol exchange at pH 6. The differences appear due to slower exchange at lower pH (Nemecz, Fontaine, and Schroeder, unpublished observation).

The above observations regarding sterol exchange conditions were made under equilibrium conditions; i.e., the sterol/phospholipid molar ratios in the donor and acceptor were the same. Two kinetic pools were identified, $^1t_{1/2}$ = 18–30 min and $^2t_{1/2}$ = 190–260 min. In contrast, for mass transfer the donor and acceptor SUV sterol/phospholipid ratios were 65:32:3 (POPC/cholesterol/dehydroergosterol) and 97:3 (POPC/dehydroergosterol) with the donor in 10-fold excess. Two kinetic pools were also identified for mass exchange, $^1t_{1/2}$ = 20–30 min and $^2t_{1/2}$ = 9.7 h. The latter observation seems at variance with that of other groups reporting that the half-times of equilibrium exchange and mass transfer were the same (Bar et al., 1986). However, these investigators did not resolve the short $t_{1/2}$ near 30 min, due perhaps to a paucity of data points between 0 and 30 min. In addition, net transfer was measured from 1 mol % cholesterol containing donor vesicles. In contrast, the present investigation POPC vesicles containing 35 mol % sterol were used in the donor SUV. The initial cholesterol concentration dramatically affects transfer rates (Bar et al., 1986; McLean & Phillips, 1982). Between 1 and 20 mol % cholesterol the $t_{1/2}$ of exchange doubled (Bar et al., 1986) while between 1 and 40 mol % cholesterol the $t_{1/2}$ increased 2.6-fold (McLean & Phillips, 1982).

The data provided here provide evidence consistent with the presence of two pools of sterol in SUV membranes. Only one of these pools appears to participate in cholesterol exchange between membranes. The physiological significance of these pools is not known. Certainly, one factor that may determine the proportion of the longer lifetime rapidly exchangeable pool includes the vesicle curvature, or geometric constraint. However, other factors such as lipid composition, pH, ions, proteins, etc. that may influence this distribution are completely unknown at this time. Sterol carrier protein has been shown to interact in a 1:1 molar complex with sterols such as dehydroergosterol (Fischer et al., 1985b). It has been suggested that since spontaneous sterol exchange between mem-

branes operates by an aqueous diffusion mechanism that is about 6-fold more rapid than spontaneous exchange of phospholipid, an exchange protein is required in vivo for sparingly soluble phospholipids but not for cholesterol (McLean & Phillips, 1981). Such a suggestion, however, assumes that all cholesterol molecules in membranes exist as a single pool. Data presented both herein and elsewhere (Bar et al., 1986) are consistent with the presence of more than one pool. In addition, the present work demonstrates that these pools are not equivalent in their spontaneous exchangeability. Whether sterol carrier proteins affect one, both, or neither pool thus remains an interesting question.

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