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Spontaneous transfer between phospholipid bilayers of dehydroergosterol, a fluorescent cholesterol analog

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The spontaneous interbilayer transfer of dehydroergosterol, a fluorescent cholesterol analog, was examined using small unilamellar phospholipid vesicles. The kinetic data were best fit by an equation of the form $A\exp(-kt)+B$. Qualitatively, the general trend of the half-time for transfer and the base values (B) obtained for dehydroergosterol resemble the corresponding values obtained in the earlier studies of cholesterol transfer. However, quantitative differences, which reflect the molecular structure of the sterol, were observed. Acrylamide quenching performed on the donor vesicles at different stages of the transfer indicated that a time-dependent organization of DHE within the vesicles occurs.

We have been studying the organization of cholesterol in bilayers by following the spontaneous exchange and net transfer of this sterol between bilayer membranes [1,2]. Previous kinetic studies of the spontaneous exchange and net transfer of [^{3}H] cholesterol between small unilamellar vesicles comprised of 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) demonstrated that about 20% of the total cholesterol is neither exchangeable nor transferable in the 8-h time frame of the experiment [1]. The size of the nonexchangeable pool varies with temperature and with the type of phospholipid [1,2]. The physical nature of this pool is, however, not clear due to the difficulty in observing cholesterol directly by spectroscopic methods. This problem can be overcome in part by the use of a suitable fluorescent cholesterol analog which, when inserted into membranes, mimics the physical and biological behavior of cholesterol. Several reports suggest that DHE is an excellent cholesterol mimic [3,4].

In this study we examine net transfer of DHE, from small unilamellar vesicles composed of either POPC or bovine brain sphingomyelin SPM. We demonstrate that not all the DHE in the donor vesicles is transferable in the 8-h time frame of the experiment and that the size of this nontransferable DHE pool varies with temperature and the type of phospholipid matrix. These results are comparable to those obtained for cholesterol and thus strengthen the previous assertion that DHE resembles cholesterol in its physical chemical properties [4–6]. Based on the idea that DHE is a good cholesterol mimic, we use the fluorescent properties of this molecule to examine certain characteristics of the nontransferable pool.

Transfer experiments. DHE transfer was followed by monitoring the decrease in fluorescence intensity at 398 nm as a function of time in small unilamellar donor vesicles. Fluorescence intensity measurements were made with a SLM 4800 fluorometer. The excitation and emission wavelengths were 324 nm and 398 nm, respectively. No excitation or emission polarizers were used. Light scattering and photobleaching were minimized by using dilute samples and narrow band widths in the excitation and emission monochromators. The small unilamellar vesicles were made by sonication as described elsewhere [1]. Except in special experiments discussed later, two populations of vesicles were used: neutral donors and negatively charged acceptors. The donor vesicles contained DHE, phospholipid and also traces of [^{14}C]cholesterol oleate which served as a nonexchangeable marker used to monitor vesicle recovery and to normalize fluorescence intensity. Acceptor

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Abbreviations: DHE, dehydroergosterol, Δ5,7,9(11),22-ergostatetraene-3β-ol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SUV, small unilamellar vesicles; SPM, bovine brain sphingomyelin.

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vesicles were present in 10-fold excess over donors. They contained the same phospholipid as the donor vesicles plus 15 mol% phosphatidylglycerol but no DHE. After the incubation period, the charged acceptor vesicles were separated from the neutral donors on a pre-equilibrated, DEAE-Sephacel ion exchange column (Pharmacia Fine Chemicals) by a procedure similar to that used in previous work [1,2]. Typically, less than 1% of the charged vesicles and 90–95% of the neutral vesicles were recovered in the eluate at all time intervals examined.

The fraction of DHE remaining in the uncharged donor vesicles at time t, is given by:

$$X_t = (F/^{14}C)_t/(F/^{14}C)_{donors}$$

where $(F/^{14}C)_t$ is the ratio of the fluorescence intensity to [^{14}C]cholesterol oleate in the eluate at time t, and $(F/^{14}C)_{donors}$ is the ratio of the fluorescence intensity to [^{14}C]cholesterol oleate in the donors after column elution at $t = 0$. Kinetic data were fitted to a single- or a two-exponential function and one value for $t = \infty$ using an iterative, non-linear least-squares analysis program on a Control Data Corp. Cyber 730 computer [7].

Typical DHE transfer data between POPC vesicles are shown in Fig. 1. The kinetic data for DHE net transfer from both POPC and SPM vesicles are best fit by a single-exponential function and a base value for the percent of fluorescence intensity remaining in the donor vesicles. The kinetic parameters obtained for transfer to a 10-fold excess of negatively charged acceptor vesicles as a function of DHE concentration and temperature are summarized in Table I. Also in Table I, for comparison, are value in parenthesis obtained for cholesterol net transfer under the same conditions [1]. In all cases it was assumed in calculating these parameters that at equilibrium DHE is homogeneously distributed between donor and acceptor vesicles. The expected

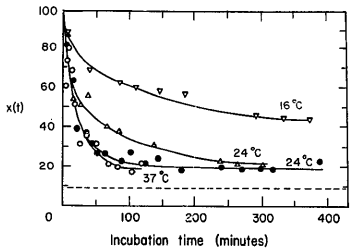

Fig. 1. Typical curves for dehydroergosterol transfer at three temperatures from neutral POPC donor vesicles at two different concentrations of DHE; open symbols, 10 mol% DHE; filled circles, 1 mol% DHE. The dashed line represents the expected value for the percent of label remaining in the donors when DHE equilibrium is established between the two vesicle populations. (For details see text).

base value is then 9.09%. It can be seen in Table I that the base values calculated using the fitting procedure are substantially larger in all experiments. It is also apparent in both POPC and SPM vesicles that the nontransferable base values decrease with increasing temperature as do the half-times for transfer. It is clear from the data in Table I and Fig. 1 that the general trends of DHE transfer rather closely resemble those of cholesterol transfer. Even though the half-times for DHE transfer are substantially smaller than the values obtained for cholesterol transfer, the nontransferable pools are of comparable size. Moreover, as previously reported for cholesterol transfer, DHE transfer from POPC is faster and the base values smaller than for transfer from SPM vesicles at equivalent temperatures. It also is apparent that increasing the DHE concentration from 1 to 10 mol% increases the half-time for

TABLE I

Temperature and concentration effects on the kinetic parameters of DHE transfer from SUV

Phospholipid [b] matrix	mol % DHE [a]	Incubation temperature	$t_{1/2}$ (min)	Base value (%)
POPC	10	16 °C	61 ± 14	45.4 ± 3.6
POPC	1	25 °C	15 ± 3	20.8 ± 2.0
POPC	10	25 °C	27 ± 1 (213 ± 55) [c]	21.1 ± 5.0 (32.4 ± 8) [c]
POPC	10	37 °C	14 ± 4 (65 ± 5) [c]	21.3 ± 5.0 (19.7 ± 2) [c]
Bovine brain SPM	10	37 °C	177 ± 21	76.8 ± 4.0
Bovine brain SPM	10	45 °C	102 ± 19	60.8 ± 6.5
Bovine brain SPM	10	50 °C	88 ± 21	47.0 ± 10.0

[a] Δ5,7,9,(11),22-ergostatetraen-3β-ol (dehydroergosterol or DHE) was obtained from Fran Scientific Inc. (Cincinnati, OH), purified by HPLC [10] and stored under nitrogen at −70 °C. DHE concentration was determined using an absorption coefficient equal to 10600 $M^{-1} \cdot cm^{-1}$ at 326 nm in dioxane [11].

[b] The phospholipids POPC, POPG and SPM were supplied by Avanti Polar Lipid, Inc. (Birmingham, AL) and were used without further purification. For more details, see text.

The numbers in parenthesis are the kinetic parameters for cholesterol net transfer under the same experimental conditions [1].

transfer, but does not affect the base value of nontransferable DHE as was the case for cholesterol exchange [1].

Previous studies of lipid transfer and exchange [1] as well as this study of DHE transfer suggest that the rate-limiting step in transfer is desorption of DHE from the donor vesicle. Since desorption involves partition from the lipid/water interface into water, the water solubility of the transferring amphiphilic molecule is reflected in the rate of desorption so that the rate of transfer increases with increasing water solubility of the lipid. Because of the additional double bonds in DHE, this sterol is expected to be more polar and therefore more soluble in water than is cholesterol. As the water solubility increases, the activation energy barrier for desorption decreases and therefore the rate of exchange of DHE is less temperature dependent than the cholesterol rate. This is seen in the activation energy of 12.8 kcal/mol calculated for DHE desorption from POPC vesicles, compared with the cholesterol activation energy of 22 kcal/mol in POPC vesicles [2].

An experiment was carried out to see if a nontransferable pool of DHE is present in the acceptor population after the transfer of DHE. SPM was chosen as the phospholipid matrix since it has the largest nontransferable DHE pool. Neutral SPM acceptor vesicles containing DHE were prepared by transferring DHE from negatively charged SPM donors containing DHE. The negatively charged donors were separated from the acceptors after 3 h of transfer using our standard ion exchange column [1,2]. These acceptors were then used as donors to a new population of negatively charged acceptors in a second transfer experiment and transfer as a function of time was followed in the usual manner, but under two sets of conditions. (1) An aliquot of the new donors was incubated with acceptor vesicles immediately after separation. (2) An aliquot was used in a transfer experiment after 24 h storage at 50°C. The results are shown in Fig. 2 where the filled circles are data obtained in the immediate second transfer experiment, and the open circles are transfer data obtained following storage at 50°C. It is quite clear that immediately after preparation by transfer, the new donors are able to transfer out essentially all the DHE. However, after 24 h storage at 50°C, the 50% non-exchangeable pool is regenerated in these vesicles and they become indistinguishable from the original donor vesicles prepared by sonication (Table I).

Fluorescence quenching experiments. The quenching of DHE fluorescence by acrylamide [8] was used to examine the characteristics of the non-exchangeable pool in donor vesicles. Quenching measurements were carried out on donor vesicle dispersions at the beginning and the end of a transfer experiment. To do this, 20 μl portions of a 5 M acrylamide stock solution were added serially to a 1.5 ml sample of donor vesicles in

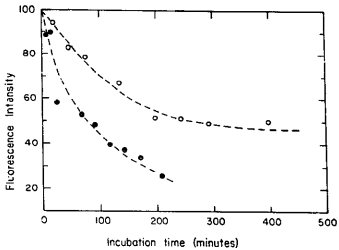

Fig. 2. Net transfer of DHE at 50°C from SPM vesicles. SPM vesicles containing DHE were made by exchange for 3 h between negatively charged donor vesicles containing DHE to vesicles containing SPM only. Filled circles show the transfer experiment using the acceptor vesicles as donors immediately after the first transfer was interrupted and the two populations separated. The open circles show the transfer experiment using another aliquot of the same acceptor vesicles but after 24 h storage at 50°C. (For details see text).

final concentration range of 0–0.5 M. DHE fluorescence lifetimes were also determined at the beginning and end of the transfer experiments, as described in Table II. Fluorescence intensity was determined at the same temperature as the transfer experiments and were corrected for volume changes and background so that apparent Stern-Volmer constants, K_{SV}, were calculated [9].

Table II summarizes the results for acrylamide quenching of DHE fluorescence in SPM donor vesicles at 37°C. Quenching was examined in donor vesicles containing initially 10 mol% DHE at zero time (D_0), immediately at the end of the transfer experiment (D_∞), then after 48 h (D_∞, 48) and 72 h (D_∞, 72) of storage at 37°C. The value of (k^*) the biomolecular quenching rate constant obtained immediately at the end of the transfer is significantly smaller than the initial value of k^* and the values of k^* obtained after 48 and 72 h storage. This result shows that the accessibility of dehydroergosterol to acrylamide (which presumably resides in the aqueous media) is less in the non-exchangeable pool than it is in the exchangeable pool. However, after incubation the increase in k^* suggests that dehydroergosterol spontaneously rearranges in the vesicle bilayer to an organization similar to that at zero time. A plot of the ln k^* versus time for SPM donor vesicles (Table II) is a straight line indicating that reorganization is a first order process with a half-time of roughly 24 h.

In order to determine whether this reorganization produces a fresh population of transferable DHE, a second transfer at 50°C was carried out with fresh acceptors using SPM donor vesicles collected at the end

TABLE II

Lifetimes and acrylamide quenching constants for DHE in SPM donor vesicles at 37°C

	D_0 [a]	D_∞ [a]	D'_∞,48 [b]	D''_∞,72 [b]
τ_0 (ns) [c]	0.887	0.937	0.922	0.922
k^* ($M^{-1} \cdot ns^{-1}$) [d]	0.28	0.06	0.12	0.21

[a] D_0 and D_∞ designate data obtained with donor vesicles separated from acceptor vesicles by ion exchange chromatography at the beginning and immediately at the end of the transfer experiment, respectively.

[b] D'_∞,48 and D''_∞,72 designate data obtained with donor vesicles separated from acceptor vesicles by ion exchange chromatography at the end of the incubation period, followed by 48 h and 72 h storage, respectively. For details, see text.

[c] Fluorescence lifetime of dehydroergosterol, τ_0, in vesicles was determined by means of multifrequency phase-modulation fluorometry [12]. The instrument was a SLM 4800 fluorometer (Urbana, IL). The light source was a He-Cd laser (Liconix, Inc., Sunnyvale, CA) with a 325 nm excitation wavelength. The excitation polarizer was set at 35° with respect to the vertical plane. A suspension of glycogen in water was used as the scattering reference signal. Phase and modulation data were determined using modulation frequencies ranging from 5 MHz to 200 MHz and the data were fitted either with the conventional multi-exponential decay law or with the Lorentzian distribution function [13], using a non-linear least square procedure developed by ISS, Inc. Both fitting procedures fit equally well to a single lifetime made with X^2 near 1.

[d] The dynamic quenching rate constant, k^*, can be calculated assuming that only a dynamic quenching mechanism operates, by the following equation: $F^0/F = 1 + k^* \cdot \tau_0[Q]$ where F^0/F is the ratio between the fluorescence intensities in the absence of quencher and each quencher addition, corrected for volume changes in background, τ_0 is the lifetime of DHE in the absence of quencher and [Q] is the acylamide concentration. At least seven different concentrations were used for each Stern-Volmer plot and the correlation coefficient of the linear regression was better than 0.95.

of the initial transfer, but then stored for 24 h. As a result of the 50-fold dilution occurring during separation of donors and acceptors on the ion exchange column, it was difficult to obtain accurate intensity measurements during the second transfer. However, a significant amount of transfer was detected (data not shown). Thus a transferable population of DHE was regenerated concomitant with the return of normal quenching characteristics. The same behavior, that is, the decrease in k^* in donor vesicles at the end of the transfer and then the regeneration of the original quenching characteristics of the donors, is also seen in POPC donor vesicles but at a much more rapid rate (data not shown). It thus is clear that the rate of reorganization of DHE in the non-exchangeable pool depends on the type of matrix phospholipid. This is also the case with the DHE transfer rate constants and the size of the exchangeable pools. For SPM vesicles compared to POPC vesicles under similar conditions, the transfer rate as well as the reorganization rate of the non-exchangeable pool are smaller, but the size of this pool is larger. The differences in the transfer rate between the two phospholipids can be explained, as in the case of cholesterol, as a consequence of the differences in the lateral packing density and therefore in the Van der Waals attractive interactions between the sterol and the phospholipids matrix [14,15], while the differences in the nonexchangeable pool are a result of the co-existence of a sterol-rich and a sterol-poor domains as explained elsewhere [2].

The data presented in this paper establish the fact that in phospholipid vesicles containing DHE, this sterol is organized into a readily transferable pool and a second pool that in the time frame of the experiments is non-transferable suggesting a general pattern of behavior of sterol in bilayers. In SPM vesicles in particular the intravesicular generation of one pool from the other occurs at quite different rates. The generation of the non-transferable pool from the transferable pool appears to be the faster of the two processes. The experimental designs developed in this study will permit us to generate SPM/DHE and SPM/cholesterol vesicles which have only a non-exchangeable or only an exchangeable pool of sterol. Studies are also under way to evaluate the interactions between DHE and cholesterol when both are present in the same bilayer.

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