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A fluorescence and radiolabel study of sterol exchange between membranes

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The fluorescent sterols $\Delta^{5,7,9,(11),22}$-ergostatetraen-3β-ol (dehydroergosterol) and $\Delta^{5,7,9,(11)}$-cholestatrien-3β-ol (cholestatrienol) as well as [1,2-^{3}H]cholesterol were utilized as cholesterol analogues to examine spontaneous exchange of sterol between 1-palmitoyl-2-oleoylphosphatidylcholine (POPC) small unilamellar vesicles (SUV). Exchange of fluorescent sterols was monitored at 24°C by release from self-quenching of polarization from the time of mixing without separation of donor and acceptor vesicles. The polarization curve for 35 mol% sterol in POPC best fitted a two-exponential function, with a fast-exchange rate constant $k_1 = 0.0217$ min^{-1}, $^1t_{1/2} = 32$ min, size pool 1 = 12%, and a slow rate constant $k_2 = 2.91 \cdot 10^{-3}$ min^{-1}, $^2t_{1/2} = 238$ min, size pool 2 = 88%. In addition to the above two exchangeable pools of sterol, the data were consistent with the presence of a slowly or nonexchangeable pool, 42% of total sterol, that was highly dependent on sterol content. These results were confirmed by simultaneous monitoring of [1,2-^{3}H]cholesterol radioactivity and dehydroergosterol fluorescence intensity after separation of donor and acceptor vesicles by ion-exchange column chromatography. Thus, dehydroergosterol or cholestatrienol exchange as measured by fluorescence parameters (polarization and/or intensity) provides two new methods to follow cholesterol spontaneous exchange. These methods allow resolution and quantitation of a shorter exchange $t_{1/2}$ near 30 min previously not reported. Thus, the cholesterol desorption rate from membranes may be faster than previously believed. In addition, the presence of a slowly non-exchangeable pool was confirmed.

Introduction

Cholesterol is a major component of mammalian cell plasma membranes. Cholesterol influences membrane passive permeability to ions, glucose, nonelectrolytes, and water [1–5]. It may stimulate or inhibit activities of membrane bound enzymes and receptor-effector systems. Cholesterol is required for protein and membrane biosynthesis (for review, see Ref. 5). Cells preferentially incorporate cholesterol from the extracellular medium by two mechanisms: receptor-mediated endocytosis of cholesterol-rich lipoproteins or by collisional/diffusional net transfer from lipoproteins or blood cells [6,7]. The latter passive transfer of cholesterol has been investigated between biological or artificial membranes using radiolabeled sterols. However, separation of donor from acceptor biomembranes, lipoproteins [8–11], or model

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

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membranes [6,12–16] requires ion-exchange chromatography, gel permeation chromatography, or ultracentrifugation. For these reasons, most investigations have included little data at incubation time points less than 30 min, where important interactions may occur.

In the past few years the fluorescent sterol, dehydroergosterol, has been used as a probe molecule for monitoring the structural and rotational dynamic properties of cholesterol in model membranes [17–27] biological membranes [18,19,28,29] and lipoproteins [30–34]. Dehydroergosterol has also been used to examine sterol–protein interactions such as with rat liver sterol-carrier-protein [23], glycophorin [34], and LDL and HDL apoproteins [34]. Despite these observations, dehydroergosterol has heretofore not been reported as a probe molecule for determination of sterol exchange dynamics between model or biological membranes.

To measure exchange, previous investigations utilized radiolabeled sterols and separation of donor from acceptor biomembrames, lipoproteins [19–22], or model membranes [23–28]. Herein we report the use of dehydroergosterol in combination with steady-state polarization to monitor 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). These lipids were checked for purity by thin-layer chromatography on Silica Gel G (250 μm, Analtech Inc., Newark, DE) with chloroform/methanol (100 : 2, v/v) as the eluting solvent for cholesterol and chloroform/methanol/water (65 : 25 : 4, v/v) for phospholipid. Standard lipids were visualized with Rhodamine 6G (0.01% in 0.1 M NaOH). The distribution of lipid components was determined by scraping plates at positions conforming to standards, eluting and storing under N_2 at −70 °C. Dehydroergosterol was synthesized and purified by HPLC as described previously [22,23]. Cholestatrienol was synthesized and purified also as described previously [35,36]. The purity of recrystallized dehydroergosterol and cholestatrienol were confirmed by high-performance liquid chromatography (98 + %), absorbance peak ratios, and comparisons with standards obtained from Frann Scientific Inc. (Columbia, MO). [1,2(n)-^{3}H]cholesterol (spec. act. 58 Ci/mmol) and cholesteryl [1-^{14}C]oleate (spec. act. 57 mCi/mmol) were purchased from New England Nuclear (Boston, MA). Their purity was also checked as described above. The purity of the cholesteryl [1-^{14}C]oleate (by radioactivity) was better than 99%. In contrast, the [1,2-^{3}H]cholesterol was only 86% pure. The 14% impurity was due to the presence of cholesterol oxidation products. These impurities transferred much faster than cholesterol with a half-time similar to that reported by Bar et al. [16]. The oxidized sterols were removed by thin-layer chromatography. Within the time frame of our exchange assay at 24 °C, we cannot detect cholesterol, dehydroergosterol, or cholestatrienol oxidation products. DEAE-Sepharose CL6B 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 Company, St. Louis, MO.

Preparation of liposomes. Small unilamellar vesicles (SUV) were prepared as described earlier [27] except that the sonication buffer (pH 7.2) 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 μM/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 7.2) at 24 °C. The lipid suspension was sonicated for 3 min with a Sonogen bath sonicator (Branson Instr., Stanford, CT) to disperse the lipids. The largest particles were removed by centrifugation at 250 × g for 10 min.

Lipid composition. Lipid composition of liposomes was determined as described previously [27]. In fluorescence polarization experiments four types of SUV were prepared with composition of the following mol% ratios: (I) POPC/cholesterol (65 : 35), (II) POPC/dehydroergosterol or cholestatrienol (65 : 35), (III) POPC/dehydroergosterol (97 : 3), and (IV) POPC/cholesterol/dehydroergo-

sterol (65 : 32 : 3). In ion-exchange column experiments the SUV were prepared with a composition of the following mole ratios: (I) POPC/dicetyl phosphate/dehydroergosterol (82 : 15 : 3), (II) POPC/cholesterol (97 : 3), (III) POPC/dicetyl phosphate/dehydroergosterol (50 : 15 : 35), (IV) POPC/cholesterol (65 : 35), (V) POPC/dicetyl phosphate/dehydroergosterol (75 : 15 : 10), (VI) POPC/cholesterol (90 : 10).

Cholesterol transfer. Only neutral donor and acceptor vesicles were utilized for the polarization experiments. However, for the experiments utilizing ion-exchange columns, negatively charged donor vesicles were separated from neutral acceptors on DEAE-Sepharose CL-6B ion-exchange columns as described by Bar et al. [16]. In the ion-exchange experiments the donor vesicles contained [^{3}H]cholesterol while the acceptor vesicles had cholesteryl [^{14}C]oleate as a nonexchangeable marker to monitor vesicle recovery. 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.

Fluorescence spectroscopy. Fluorescence polarization was measured exactly as described earlier [27]. 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 using dilute samples and by using appropriate cutoff filters in the emission system. Light scattering was not detectable with the SUV at the concentrations 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 cut-off filter. The light intensity detection was in L or T format for steady-state polarization. Data were collected by an IBM PC computer using an ISS-ADC interface. The precise description of this instrument construction and theory was described earlier [37]. Steady-state fluorescence polarization, P, was measured every three minutes in the L format as

$$P = \frac{I_{vv} - GI_{vh}}{I_{vv} + GI_{vh}} \tag{1}$$

where G, the ratio of the detection system sensitivity for vertically and horizontally polarized light, is equal to I_{hv}/I_{hh}. I_{vv} corresponds to vertically polarized excitation and vertically polarized emission, I_{vh} corresponds to vertically polarized excitation and horizontally polarized emission. In the T format the ratio of I_{vv}/I_{vh} was monitored continuously with data collected by the computer every 20 seconds. In determination of polarization, light scattering was reduced by a Janos GG-375 cutoff filter. Fluorescence polarization is very sensitive to changes in light scattering, especially due to aggregation or fusion of vesicles [38]. 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.

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 is determined as follows:

$$X(t) = \frac{(^3H/^{14}C)_0 - (^3H/^{14}C)_t}{(^3H/^{14}C)_0} \tag{2}$$

where $(^3H/^{14}C)_t$ and $(^3H/^{14}C)_0$ represent the ratio of [^{3}H]cholesterol to cholesteryl [^{14}C]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 vesicles at time t for negatively charged donor vesicles was determined as follows:

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

where $(I/^{14}C)_t$ and $(I/^{14}C)_0$ represent the ratio of dehydroergosterol fluorescence intensity to cholesterol [^{14}C]oleate in the eluate at time t and in the starting incubation mixture, respectively. Third, for the polarization studies, the data were fitted to one- or multiexponential functions using PC NONLIN or JANA iterative nonlinear least-squares analysis (Statistical Consulant Inc., Lexington, KY) with an NC PC6 (NCR, Augsburg, F.R.G.) personal computer.

Results

Dehydroergosterol exchange between POPC SUV: a polarization study

Concentration-dependent self-quenching of fluorescence was used to develop a fluorescent sterol exchange assay that does not require separation of donor and acceptor membrane vesicles. Above 6 mol% dehydroergosterol in POPC SUV, the polarization of dehydroergosterol is quenched (Fig. 1). The fluorescence polarization in 3% and 35 mol% dehydroergosterol POPC SUV is 0.348 and 0.163, respectively; a difference of 0.185. It seems reasonable to predict that if the donor vesicle contains 35 mol% dehydroergosterol, and if the quenched dehydroergosterol in the donor SUV spontaneously exchanges into acceptor SUV with the same mol% cholesterol then release from quenching and increase in polarization should occur. To follow the spontaneous exchange of cholesterol and dehydroergosterol between membranes, two populations of POPC vesicles were used: acceptor POPC/cholesterol (65 : 35 mol%) and donor POPC/dehydroergosterol (65 : 35 mol%). The exchange was followed by measuring fluorescence polarization. Polarization was monitored as a function of time at 24°C immediately after mixing the above vesicles in a 1 : 10 donor/acceptor ratio to minimize back exchange (Fig. 2, lower portion of curve). With the spectrofluorimeter in the L format, data were obtained every three minutes while in the T format, data were obtained continuously every 20 seconds. Similar kinetics were obtained for both formats. The polarization data in Fig. 2 best fit a two-exponential curve. The initial first component had a rate constant $k_1 = 0.0217 \pm 0.0046$ min^{-1} and half-time for exhange $t_{1/2} = 32 \pm 7$ min ($n = 3$), respectively (Table I). The second component parameters were $k_2 = 0.00291 \pm 0.00030$ min^{-1} and 238 ± 24 min, respectively. The distribution of exchangeable dehydroergosterol was 12.5% and 87.5%, respectively. However, 42% of the dehydroergosterol was not exchangeable (Table I). The weighted average of the two half-times for exchange was 208 ± 38 min. Similar data were obtained when cholestastrienol was used to monitor exchange. The rate constants and half-times for exchange were $k_1 = 0.021 \pm$

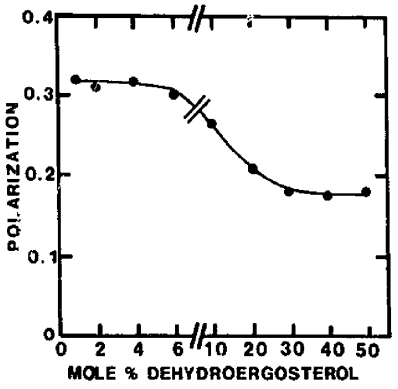

Fig. 1. Effect of increasing mole% dehydroergosterol on polarization of dehydroergosterol in POPC SUV. Steady-state polarization was measured in POPC vesicles containing increasing dehydroergosterol content as described in Materials and Methods.

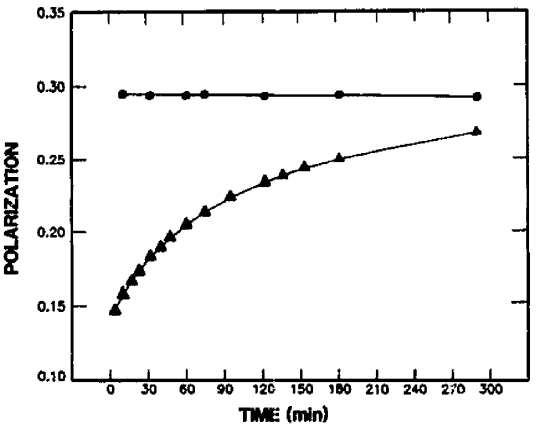

Fig. 2. Changes in polarization of dehydroergosterol (DHE) during exchange between POPC SUV. SUV consist of (■) POPC/Chol/DHE (65:32:3 mol%) and POPC/DHE (97:3 mol%), or (▲) POPC/Chol (65:35 mol%) and POPC/DHE (65:35 mol%).

TABLE I

STEROL EXCHANGE KINETICS BETWEEN POPC SUV DETERMINED FROM FLUORESCENCE POLARIZATION WITHOUT SEPARATION

In these equilibrium exchange experiments, the donor and acceptor vesicles contained 35 mol% dehydroergosterol (or cholestatrienol) and 35 mol% cholesterol, respectively. The donor vesicles also contained [^{3}H]cholesterol in trace amounts.

	Rate constant (10^{-3} min^{-1})		Half-time (min)		Distribution of exchangeable sterol %		% nonexchangeable sterol
	k_1	k_2	$^1t_{1/2}$	$^2t_{1/2}$	Pool 1	Pool 2	
Dehydroergosterol							
Mean ± S.D.	21.7 ± 4.6	2.91 ± 0.3	32 ± 7	238 ± 24	12.5 ± 0.5	87.5 ± 0.5	42.0 ± 4.2
Cholestatrienol							
Mean ± S.D.	21.0 ± 2.8	2.19 ± 0.5	33 ± 4	316 ± 84	10.6 ± 0.6	89.4 ± 2.6	38.5 ± 4.8

0.003 min, $k_2 = 0.00219$ min^{-1}, $^1t_{1/2} = 33 \pm 4$ min, $^2t_{1/2} = 316 \pm 84$ min, respectively.

The spontaneous exchange of dehydroergosterol between two other populations of vesicles, POPC/dehydroergosterol (97 : 3) and POPC/cholesterol/dehydroergosterol (65 : 32 : 3), in a 1 : 10 ratio did not alter the polarization of dehydroergosterol (Fig. 2, top curve). This observation indicated that the exchange of dehydroergosterol, from the dehydroergosterol-rich vesicles, and not the back exchange of cholesterol from the cholesterol-rich vesicles was responsible for the observed release from polarization quenching.

Dehydroergosterol vs. [^{3}H]cholesterol exchange between POPC SUV: an ion-exchange column study

The above results were confirmed with a classical exchange assay in order to test whether cholesterol and dehydroergosterol spontaneous exchange between vesicles displayed similar kinetics. [^{3}H]Cholesterol and dehydroergosterol (3 mol%) were incorporated into negatively charged donor POPC vesicles. At 3 mol% dehydroergosterol in POPC SUV the dehydroergosterol does not self-quench (Fig. 3). Thus, its fluorescence intensity is directly proportional to dehydroergosterol content in the POPC SUV. Acceptor vesicles contained POPC/cholesterol (97 : 3) with trace amounts of cholesteryl [^{14}C]oleate as a nonexchangeable marker to monitor vesicle recovery.

After mixing the vesicles, at various time points aliquots were placed on ion exchange columns. Acceptor vesicles were completely eluted within 2 min. The fluorescence intensity of the eluant acceptor vesicles was determined. Thereafter the eluant sample was transferred to scintillation vials and [^{3}H]cholesterol and cholesteryl [^{14}C]oleate were quantitated by scintillation counting. The spontaneous exchange curves for dehydroergosterol, as measured by fluorescence intensity in the acceptor, and cholesterol, as measured by [^{3}H]cholesterol in the acceptor displayed similar kinetics (Fig. 4). Both curves best fit-exponential components. The rate constants, $t_{1/2}$, and pool size of exchangeable [^{3}H]cholesterol were $k_1 = 56.4 \cdot 10^{-3}$ min^{-1}, $k_2 = 3.08 \cdot 10^{-3}$ min^{-1}, $^1t_{1/2} = 12$ min, $^2t_{1/2} = 225$ min and pool 1 = 8%, pool

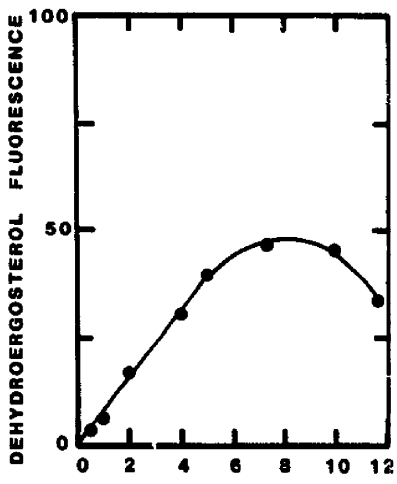

Fig. 3. Linearity of dehydroergosterol fluorescence at low mol% dehydroergosterol in POPC SUV. All conditions were as described in legend to Fig. 1.

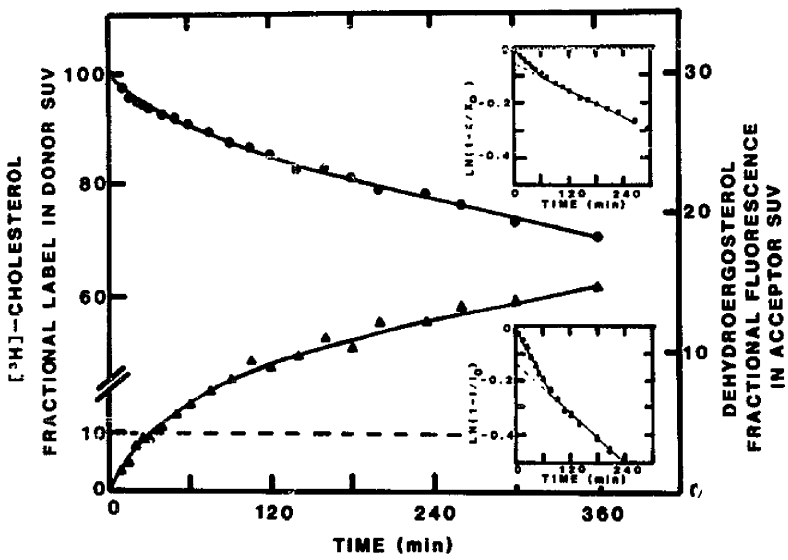

Fig. 4. Simultaneous exchange of [^{3}H]cholesterol and dehydroergosterol. Donor vesicles contained POPC/dicetyl phosphate/dehydroergosterol (50:15:35) as well as trace amounts of [^{3}H]cholesterol. The acceptor vesicles contained POPC/cholesterol (65:35) as well as trace amounts of cholesteryl [^{14}C]oleate. X is the fraction of labeled molecules transferred to acceptor at time t. X_0 represents the fraction at equilibrium. I is the fluorescent intensity at time t. I_0 at equilibrium. The data were corrected for back exchange of sterol as described in Materials and Methods. ●, [^{3}H]cholesterol; ▲, dehydroergosterol fluorescence; Upper inset, [^{3}H]cholesterol; lower inset, dehydroergosterol fluorescence.

$2 = 92\%$ (Table II). The same parameters for dehydroergosterol exchange were $k_1 = 71.7 \cdot 10^{-3}$ min^{-1}, $k_2 = 3.94 \cdot 10^{-3}$ min^{-1}, $^1t_{1/2} = 9.7$ min, $^2t_{1/2} = 176$ min, and pool 1 = 13.5%, pool 2 = 86.5%. It should be noted that 25.9% of the [^{3}H]cholesterol and 21.7% of the dehydroergosterol in these vesicles containing only 3 mol% sterol were in a nonexchangeable pool (Table II).

The effect of sterol content on the rate constants, half-time for exchange, and pool size were

TABLE II

STEROL EXCHANGE KINETICS BETWEEN POPC SUV FROM ION EXCHANGE SEPARATION

In these equilibrium exchange experiments, the donor and the acceptor vesicles had 35 mol% dehydroergosterol (which contained [^{3}H]cholesterol) and 35 mol% cholesterol, respectively. The fluorescence intensity of dehydroergosterol which was transferred from donor vesicles was measured in the acceptor vesicles.

Mol% sterol	Rate constant (10^{-3} min^{-1})		Half-time (min)		Distribution of exchangeable sterol %		% nonexchangeable sterol
	k_1	k_2	$^1t_{1/2}$	$^2t_{1/2}$	Pool 1	Pool 2	
[^{3}H]Cholesterol							
3	56.4± 5.0	3.08±0.19	12.0±1.0	225±24	8.0±0.5	92.0±0.5	25.9±4.2
10	33.6± 6.4	2.66±0.24	21.0±4.0	260±23	7.2±0.6	92.8±0.6	36.0±5.2
35	27.0± 3.1	2.38±0.15	26.0±7.0	291± 4.7	5.0±0.4	95.0±0.4	47.0±6.5
Dehydroergosterol							
3	71.7±18.5	3.94±0.74	9.7±2.4	176±33	13.5±0.5	86.5±0.5	21.7±3.2
10	51.1±16.0	2.68±0.20	14.0±4.3	258±19	14.0±1.5	86 ±1.5	30.6±4.0
35	30.1± 9.6	2.58±0.18	23.0±8.0	269±38	11.4±1.2	88.6±1.2	38.0±4.8

dramatic. With increasing sterol content (either cholesterol or dehydroergosterol) the k_1 and k_2 decreased, $^1t_{1/2}$ and $^2t_{1/2}$ increased (Table II). The proportion of exchangeable sterol decreased, concomitantly. In addition, the % nonexchangeable sterol increased from 22–26% in 3 mol% sterol in POPC SUV to 38–47% in 35 mol% sterol in POPC SUV.

Discussion

Cholesterol homeostasis in living cells is a multifactorial process. The various subcellular membranes such as plasma membranes, microsomes, and mitochondria vary widely in cholesterol content and cholesterol/phospholipid ratio. Although cholesterol can spontaneously desorb from membranes and transfer between membranes, these processes are still poorly understood. Likewise, mechanisms controlling the intracellular distribution of cholesterol between membranes are not fully known. Part of the difficulty lies in that previous investigations utilized exchange assays that require separation of donor and acceptor membranes. For separations of donor and acceptor membranes of equal charge, size, etc., only sedimentation methods have been useful. Sedimentation methods may diminish temporal resolution of rapid exchange components and also may allow for cross contamination of donor and acceptor membranes. Herein is described a new assay not requiring separation of donor and acceptor membranes. This assay circumvents the above difficulties and resolves the presence of a hitherto unreported rapidly exchangeable pool of cholesterol between membranes. It should be noted that determination of exchange kinetics by measuring changes in fluorescence polarization does not directly measure the number of sterol transferred. However, since these kinetic parameters were very similar to those obtained by [^{3}H]cholesterol exchange and fluorescence intensity of dehydroergosterol appearance in acceptor POPC SUV (ion-exchange columns), the polarization change kinetics do reflect the number of sterol molecules transferred.

The measurement of spontaneous cholesterol exchange in the present investigation reports two significant advances. First, as shown herein, replacement of radiolabeled cholesterol by dehydroergosterol and measurement of dehydroergosterol fluorescence in the acceptor vesicles separated from the donor obviates the need for radioisotopes. However, the kinetics of dehydroergosterol exchange and [^{3}H]cholesterol exchange measured even in a system that requires separation of donor and acceptor vesicles were essentially the same. In contrast, the rate of exchange of another fluorescent pyrene-labeled sterol analog (3-pyrene methyl ester of 3β-hydroxy-22,23-dinor-5-cholenic acid) was 4.4-fold faster than the $t_{1/2}$ of radiolabeled cholesterol exchange under identical conditions [6]. Thus, dehydroergosterol appears to be a good fluorescent analogue to monitor cholesterol exchange. A second major finding from this investigation is the observation that fluorescence polarization of dehydroergosterol can be used to monitor the exchange of cholesterol without separation of donor and acceptor vesicles. This obviates the need for incorporating charged lipid, or glycolipid into the donor or acceptor. Likewise it also eliminates the need to separate vesicles when donor and acceptor are not the same size. These factors may all have some affect on exchange kinetics of cholesterol in vesicles. At the very least such assays are all more time consuming and need correction for cross contamination of donor and acceptor vesicles in the eluants.

It has been shown that cholesterol exchange between cholesterol/phosphatidylcholine (PC) vesicles proceeds through the aqueous phase and follows single-exponential kinetics [6,12,15,16]. Although some investigators have found only a single pool of cholesterol [6,14,39], a number of other workers have shown two pools: a major portion of the cholesterol pool is exchangeable and a minor portion is nonexchangeable [16,40]. Our observations support the model where the transfer of cholesterol occurs by diffusion through the aqueous phase and follows exponential kinetics. In addition, analysis of the exchange curves showed better fits for a two-exponential function. The r values for best fit were 0.939 ± 0.006 ($n = 7$) for one-exponential fit and 0.993 ± 0.002 ($n = 7$) for two-exponential fit. In all cases the r value for a two-exponential fit was higher than for a one-exponential fit. The existence of two sterol domains

in POPC SUV is also supported by fluorescence lifetime distributional analysis [41,43] of dehydroergosterol and cholestatrienol. In this work the range of fractional fluorescence intensities was $F_1 = 0.92$ to 0.95 and $F_2 = 0.05$ to 0.08. The occurrence of two exchangeable cholesterol pools may be explained as follows. A rapid exchange or desorption occurs in the first 30 min and a slower component occurs with a half-time of 3–5 hours, similar to that observed by other investigators using radiolabeled cholesterol and techniques that separate donor and acceptor vesicles. The latter procedures require that the donor and acceptor not be identical in lipid composition and are also limited in time resolution such that few data points are obtainable in less than 15 minutes. The advantage of the fluorescent sterol exchange method is that the vesicles do not differ in charge properties or size and that exchange can be continuously monitored every few seconds from time zero. Our observed exchange rates at 24°C for the longer $t_{1/2}$ compare favorably with that reported by Bar et al. [16] who determined an exchange $t_{1/2}$ of 3.5 h at 24°C and pH 7.2. In contrast, other investigators [6,12,13] observed an exchange rate of 7.4 h at pH 6.0 and 24°C. When the experiments in the present study were performed at lower pH (e.g., pH 6.8) the exchange was also slower (longer $t_{1/2}$).

The last important point to be made was that previous investigators observed a single $t_{1/2}$ near 3–4 h [6,12,13,16] with a second very slowly or nonexchanging pool [16,40] with a $t_{1/2}$ of 9 h or more. However, in these earlier reports very few data points were obtained between 0 and 30 min exchange. Thus, the presence of a $t_{1/2}$ near 30 min at physiological concentrations of sterol (35 mol%) was not observed or resolved. The data presented here (Table I) are consistent with the presence of a nonexchangeable sterol pool; the size of which depends on the sterol content of the vesicles.

Arguably, it is possible that a rapid exchange component may arise from the presence of oxidized sterol. In fact one report [16] indicated that oxidized cholesterol exchanges with a very short $t_{1/2}$ near 4 min. We have confirmed this observation with 86% pure [^{3}H]cholesterol obtained from the radioisotope supplier. However, when the [^{3}H]cholesterol was purified by thin-layer chromatography this very short $t_{1/2}$ was not observed. In addition, unlike the impure [^{3}H]cholesterol the dehydroergosterol showed only a single spot on thin-layer chromatography. Both pure dehydroergosterol exchange (fluorescence intensity as well as polarization) and pure [^{3}H]cholesterol exchange indicated very similar kinetics. However, some data indicate that cholesterol has a unique structure (Fig. 5) and that interactions with phospholipids [6,19,30,41–43] or proteins [35] may not be identical for some fluorescent sterols. Therefore, cholestatrienol, a closer structural analogue (Fig. 5) was utilized to examine the spontaneous exchange kinetics of sterol in POPC SUV. The comparative exchange kinetic study presented herein with cholestatrienol showed almost identical exchange kinetics as dehydroergosterol and [^{3}H]cholesterol. These data are also consistent with the similar interactions of dehydroergosterol and cholestatrienol with phospholipids [27,41,43] and with proteins [23,35]. In addition, the data presented here are consistent with those shown earlier indicating that the presence of multiple sterol pools was not due to vesicle size heterogeneity or fusion of vesicles [16]. In conclusion, both fluo-

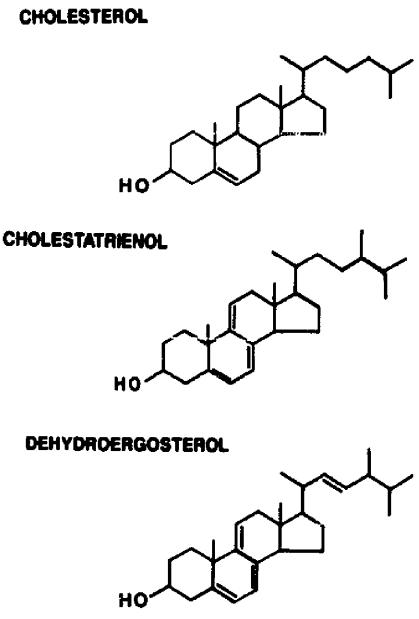

Fig. 5. Structures of cholesterol, dehydroergosterol and cholestatrienol.

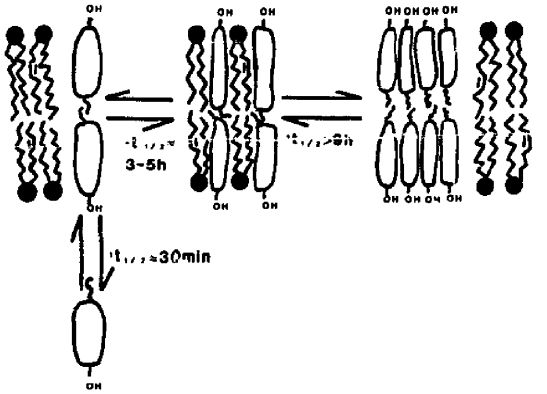

Fig. 6. Model for spontaneous cholesterol desorption from POPC SUV. The half-times for exchange are at 24°C.

rescent sterols and radiolabeled cholesterol showed very similar exchange kinetics by three very different exchange assays. These data indicate the usefulness of utilizing fluorescent dehydroergosterol or cholestatrienol to monitor cholesterol exchange.

The observations regarding the above three different $t_{1/2}$ for exchange near 30 min, 3–5 h, and 9 h may be rationalized by the model shown in Fig. 6. Basically, the model proposes that the desorption rate of cholesterol at 24°C (pH 7.2) from POPC vesicles has a $t_{1/2}$ near 30 min. The cholesterol is desorbed as a monomer. Considerable evidence summarized elsewhere [6,12,13] indicates that the half-times for cholesterol and phospholipid transfer differ by 6–10-fold. In addition, the rate of cholesterol desorption from phospholipid vesicles appears to be independent over the cholesterol concentration range 1–40 mol% cholesterol [12,27]. The transbilayer migration rate has been determined in a variety of model membrane systems to be as short as seconds to as long as days (reviewed in Ref. 44). Likewise, in biological membranes the transbilayer migration rate of cholesterol has also been reported to range between seconds to days (reviewed in Ref. 44). Thus, although some studies indicate that the transbilayer migration rate is not rate limiting in cholesterol exchange [6,12,13], a number of other factors including lipid and protein composition as well as vesicle curvature may be relevant. The high rates observed herein may be due to the high curvature of the SUV vesicle membranes. Extremely low rates have been reported for the transfer of cholesterol from monolayers to vesicles [45]. In any case, no evidence has to our knowledge been reported indicating that the rate-limiting step in cholesterol exchange is the transbilayer migration rate. These observations are consistent with the above proposal. We have shown elsewhere [27,41,43] that at mol% sterol above about 5 mol% in POPC vesicles the sterol interacts with other sterols and/or phospholipid. Thus, the present model proposes the existence of cholesterol-phospholipid (1 : 1) complexes in POPC vesicles. Other evidence for the existence of such 1 : 1 complexes has been provided [12]. Neither the data described herein or elsewhere are consistent with cholesterol : cholesterol clustering below 35 mol% cholesterol. Cholesterol dimer formation has been observed in chloroform solution [46] and pure cholesterol phases have been reported to separate out from phospholipids at high mol% cholesterol [47]. Both the data presented here and earlier [11,24] are inconsistent with desorption of cholesterol-phospholipid complexes. The data suggest that the second $t_{1/2}$ near 3–5 h may reflect dissociation of cholesterol-phospholipid complexes to form cholesterol monomers that may subsequently desorb. The third $t_{1/2}$ near 9 h may be interpreted as being due to the existence of cholesterol-cholesterol complexes dissociating to form cholesterol-phospholipid complexes.

The relevance of the above exchange rates as discussed in the proposed model (Fig. 6) to biological membranes must be considered. Several investigators using [^{14}C]cholesterol exchange from *Mycoplasma gallisepticum* and *Acholeplasma laidlawii* membrane have also reported biphasic kinetics [48,49]. In contrast, cholesterol exchange kinetics were monoexponential for erythrocyte membranes [50–52]. The rate of efflux of cholesterol from membranes is highly dependent on cell type, e.g. GM 3468 fibroblasts and Fu 5AH hepatoma cells differ by an order of magnitude in efflux rate [53]. Obviously, a number of factors must be regulating such a diversity of cholesterol exchange rates including donor and acceptor membrane curvature, acceptor protein, donor and acceptor phospholipid polar head group and acyl chain length, surface hydration, etc. [45,54,55].

In conclusion, much more remains to be done to elucidate factors regulating the distribution of

cholesterol between the proposed three pools. The development of the rapid fluorescent sterol polarization assay described herein should help provide impetus to these investigations. The assay does not require separation of donor and acceptor membranes and should therefore be particularly useful for determination of sterol exchange between biological membranes or lipoproteins where such particles do not differ in charge, size, density, or other parameters. In addition, this technique may eliminate the need for radioisotope labelled sterol in the more classical exchange assays.

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