Erythrocyte Membrane Lateral Sterol Domains: A Dehydroergosterol Fluorescence Polarization Study†

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Received September 10, 1993; Revised Manuscript Received December 28, 1993®

ABSTRACT: Structural domains of cholesterol and their regulation in the erythrocyte membrane are poorly understood. Dehydroergosterol fluorescence polarization change was used to continuously monitor the kinetics of sterol exchange and sterol domain size in erythrocyte ghost membranes. Direct correlation between molecular sterol exchange and steady-state dehydroergosterol fluorescence polarization measurements was obtained without separation of donor and acceptor membranes. Three important observations were made. First, sterol exchange between small unilamellar vesicles (SUV) with the same cholesterol/phospholipid ratio as the erythrocyte membrane (1-palmitoyl-2-oleoylphosphatidylcholine/cholesterol = 1:1) was resolved into three kinetic cholesterol domains: 23 ± 9% of total sterol was rapidly exchangeable, with $t_{1/2}$ = 23 ± 6 min; 59 ± 9% of total sterol was slowly exchangeable, with $t_{1/2}$ = 135 ± 3 min; and 19 ± 9% of total sterol was essentially nonexchangeable, with a $t_{1/2}$ of days. Second, the substitution of erythrocyte ghosts for SUV as an acceptor significantly altered the kinetic parameters of sterol exchange from donor SUV, graphically showing that both the properties of the acceptor and spontaneous desorption of cholesterol from the donor SUV influenced spontaneous cholesterol transfer. Third, studies of exchange between erythrocyte ghosts revealed multiple kinetic pools of sterol differing from those in the SUV: 4 ± 2% of total sterol was rapidly exchangeable, with $t_{1/2}$ = 32 ± 9 min; 29 ± 3% of total sterol was very slowly exchangeable, with $t_{1/2}$ = 23 ± 7 h; and a surprisingly large 67 ± 2% of total sterol was nonexchangeable, with a $t_{1/2}$ of days.

The first evidence for an asymmetric distribution of lipids across a biological membrane was reported in the erythrocyte plasma membrane by Bretscher (1972). Since that exciting discovery of phospholipid asymmetry, considerable data substantiating both phospholipid [reviewed in Op den Kamp (1979), Bergelson and Barsukov (1977), Devaux (1988), Sweet and Schroeder (1988), and Jain (1984, 1985, 1986)] and cholesterol (Schroeder & Nemecz, 1990; Schroeder et al., 1991b) transbilayer asymmetry across plasma membranes has been accumulated. More recently, and again in the erythrocyte membrane, studies consistent with the presence of lateral cholesterol domains appeared (Schroeder et al., 1991a; Gordon & Mobley, 1984; Lange et al., 1984). Although significant literature exists regarding the exchange of cholesterol between erythrocytes and plasma lipoproteins [reviewed in Phillips et al. (1987)], general agreement on the presence of multiple domains and their size in erythrocyte membranes has been elusive. Instead, a wide range of exchange kinetics of cholesterol transfer between erythrocyte and plasma has been reported. Some authors, for example, found that only part of the erythrocyte cholesterol is available for exchange (Bell & Schwartz, 1971; Bruckdorfer & Sherry, 1984; Bjornson et al., 1975; Lange & D'Alessandro, 1977), while others showed that all erythrocyte cholesterol was exchangeable (d'Hollander & Chevalier, 1972; Gold and Phillips, 1990; Hagerman & Gould, 1951; Murphy, 1962; Bruckdorfer & Green, 1967; Quarfordt & Hilderman, 1970; Giraud & Claret, 1979). The reported half-times of exchange range from 2 h for cholesterol exchange between dog erythrocyte and dog plasma (Hagerman & Gould, 1951) to 9 h for cholesterol exchange from human erythrocyte to human plasma (Schick & Schick, 1985). These studies assumed that the rate-limiting step was the desorption of cholesterol from the donor membrane and that the properties of the acceptor were insignificant. Consequently, there is a paucity of information regarding cholesterol exchange between erythrocyte membranes themselves. Only two studies examined cholesterol transfer between erythrocytes and showed only negligible (Lange, 1982) or 10% (Steck et al., 1988) transfer of cholesterol in 24 h.

The presence of rapidly exchangeable sterol domains in erythrocyte and other membranes has been difficult to prove. Most studies of cholesterol exchange used [^{3}H]cholesterol or [^{14}C]cholesterol in vesicles. The time resolution of such methods is limited because of the need to separate the donor and acceptor membranes. Moreover, such methodology requires that the donor and acceptor membranes differ in a size/density/affinity label, which renders its application to the study of cholesterol exchange among ghosts or intact erythrocytes impractical. The present paper reports a method to study the molecular exchange of a fluorescent cholesterol analogue, dehydroergosterol, between erythrocyte ghosts that does not require separation of donor and acceptor. The assay was based on the continuous monitoring of dehydroergosterol fluorescence polarization as first described for cholesterol exchange between small unilamellar vesicles (SUV)[1] (Schroeder et al., 1987; Nemecz et al., 1988) and subsequently refined by Butko et al. (1992). Dehydroergosterol, a naturally occurring sterol, was shown to be an excellent fluorescent cholesterol analogue, whose exchange in SUV was very similar to that of radiolabeled cholesterol and whose organization

† This work was supported in part by the USPHS National Institute of Health [HL 37515 to C.H.J.; GM 31651 to F.S.].

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® Abstract published in *Advance ACS Abstracts,* February 15, 1994.

0006-2960/94/0433-2880$04.50/0

into sterol domains in SUV was similar to that of cholesterol (Schroeder et al., 1987). The goal of this study was to measure the kinetics of cholesterol exchange in erythrocyte membranes and to clarify the issue of multiple sterol domains.

MATERIALS AND METHODS

Materials. 1-Palmitoyl-2-oleoylphosphatidylcholine (POPC) was purchased from Avanti Polar Lipids, Inc. (Birmingham, AL). Cholesterol was from Applied Science Laboratories, Inc. (State College, PA). [^{3}H]cholesterol (46 mCi/mmol) was obtained from Amersham (Arlington Heights, IL). ATP (magnesium salt) and ergosterol were purchased from Sigma Chemical Company (St. Louis, MO). Dehydroergosterol, synthesized and purified according to Fischer et al. (1984, 1985), was 96% pure as determined by reverse-phase HPLC. Sterol carrier protein 2 (SCP-2) was isolated and characterized as in Noland et al. (1980) and Nemecz et al. (1991). HPLC grade acetonitrile and methanol were from Fisher Scientific (Pittsburgh, PA). All other chemicals were of reagent grade or better.

Small Unilamellar Vesicles (SUV) Preparation. SUV composed of POPC/dehydroergosterol, POPC/cholesterol, POPC/cholesterol/[^{3}H]cholesterol (trace), or POPC/cholesterol/[^{3}H]cholesterol/[^{14}C]triacylglycerol (trace) (PC/sterol molar ratios = 65:35 or 50:50) were prepared as described earlier (Schroeder et al., 1987; Nemecz et al., 1988) with 50 mM Tris buffer, pH 7.4, and 0.1 mM PMSF.

Erythrocyte Ghosts Preparation. The method of ghost preparation was a modification of that of Shoemaker and Lauf (1983). Unsealed ghosts were prepared for sterol-exchange assays, but the procedures employed were designed to be comparable to those required for preparation of sealed ghosts to simplify comparisons to other studies. The following solutions were used. Tris-buffered saline (TBS): 165 mM NaCl and 2 mM Tris·HCl, pH 7.2, at 0 °C. Hemolyzing solution I: 4 mM $MgCl_2$, pH 3.6–4.0 at 0 °C. Hemolyzing solution II: 4 mM $MgCl_2$ and 0.1 mM EGTA, pH 6.0, at 0 °C. Restoring solution: 2 M NaCl. Sucrose cushion: 43%, w/v, sucrose, 50 mM NaCl, and 25 mM Tris·HCl, pH 7.2, at 0 °C.

Fresh human blood was collected from healthy volunteers by venipuncture using a heparin solution as an anticoagulant. Blood was washed three times in TBS, pH 7.2, at 4 °C by centrifugation at 3750 rpm in a Beckman GPR centrifuge (Model GH 3.7 rotor; Beckman Instruments, Inc., Palo Alto, CA). Plasma and buffy coat were removed, and erythrocytes were sedimented for 10 min at 3750 rpm at 4 °C. The supernatant was removed.

The pellet of packed erythrocytes was diluted into a 100× volume of hemolyzing solution I at 0 °C, and the pH was adjusted to 6.0. The suspension was stirred for 5 min, and then the ghosts were sedimented at 30000*g* in a Model J2-21M centrifuge, rotor JA-17 (15 000 rpm) (Beckman Instruments, Inc., Palo Alto, CA), for 6 min at −1 °C). All but 30 mL of the supernatant was removed, and the pellet was returned to an ice bath. Three milliliters of 10 mM ATP and 3 mL of restoring solution were added to give a final volume of 36 mL of erythrocyte ghosts suspension. The pH was adjusted to 7.2 at 0 °C, and the suspension was stirred for 15 min. The ghost suspension was then centrifuged again at 30000*g* for 6 min at −1 °C, and all but 3 mL of the supernatant was removed. Although resealing was not expected with 0 °C incubation, the presence of ATP was required for the preparation of white ghosts.

The 3 mL of ghosts suspension from above was diluted into 300 mL of hemolyzing solution II at 0 °C, pH 6.0, and stirred for 5 min. The suspension was then centrifuged at 30000*g* at −1 °C for 6 min. All but 30 mL of the supernatant was removed. The pellet was resuspended and returned to an ice bath. In several preparations, centrifugation on a sucrose cushion (27000*g* for 40 min) confirmed that such ghosts were unsealed. Sodium azide was added to a final concentration of 0.1 mM. The ghosts, stored refrigerated (4 °C), were stable over 6 days.

Membrane protein was measured by the method of Lowry et al. (1951) with the addition of 1% sodium dodecyl sulfate to all samples and standards.

Loading of Dehydroergosterol into Erythrocyte Membranes. Dehydroergosterol was loaded into ghosts by incubation with SUV (POPC/dehydroergosterol, 1:1). The SUV/ghost lipid ratio was 30:1 in 50 mM Tris buffer, pH 7.4 (1 mL total), in the presence of 0.1 mM PMSF, unless otherwise stated. The samples were typically incubated for 24 h at 37 °C in a shaking water bath. After incubation, the SUV and ghosts were separated by centrifugation at 15 000 rpm in an Eppendorf microcentrifuge, Model 5412 (Brinkmann Instruments, Inc., Westbury, NY), for 5 min. The pellet was washed twice to remove any remaining SUV associated with ghosts. Control experiments using SUV cosonicated with a trace amount of [^{14}C]triacylglycerol, a very slowly exchangeable marker, showed less than 3% of the radioactivity in the pellet being due to fusion and/or sticking of SUV to ghosts. The pellet was resuspended in 50 mM Tris buffer, pH 7.4.

Determination of Sterol Content and Extent of Dehydroergosterol Loading in Erythrocyte Ghost Membrane. Ergosterol, used as an internal standard, was added before lipid extraction to the ghost suspension. The plasma membrane lipids were then extracted by the Bligh–Dyer method and fractionated into neutral lipids and phospholipids (Schroeder et al., 1976). The amount of dehydroergosterol loaded into the membrane was determined by reverse-phase HPLC (Adsorbosphere C18 3U column, Alltech Assoc., Inc., Deerfield, IL) of the neutral lipid fraction using a Series 4 liquid chromatograph equipped with an LC-55 UV/vis spectrophotometer detector (Perkin-Elmer Inc., Norwalk, CT).

Steady-State Photon Counting Fluorescence Measurement. Steady-state dehydroergosterol fluorescence polarization was measured with a PC-1 photon counting spectrophotometer (ISS Instruments, Inc., Champaign, IL) in the T-format. Fluorescence polarization in samples with increasing dehydroergosterol content, for establishment of standard curves, was measured in L-format. The light source was a 300-W xenon arc lamp. The excitation wavelength was 325 nm. The inner-filter effect was precluded by using dilute sample suspensions (absorbance at 324 nm was maintained below 0.2) and GG-375 emission cutoff filters (Janos Technology, Inc., Townshend, VT).

Sterol-Exchange Assay. Details of the theoretical derivation and basis of the assay are provided in Appendix 1. Briefly, in the donor membranes the dehydroergosterol concentration is sufficiently high to exhibit self-quenching resulting in low polarization values. Because the dehydroergosterol quantum yield is inversely proportional to the solvent dielectric (Nemecz & Schroeder, 1988), dehydroergosterol

[1] Abbreviations: SUV, small unilamellar vesicles; DHE, dehydroergosterol; POPC, 1-palmitoyl-2-oleoylphosphatidylcholine; SCP-2, sterol carrier protein 2; TBS, Tris-buffered saline; HPLC, high-performance liquid chromatography; Tris, tris(hydroxymethyl)aminomethane; RBC, red blood cell; ATP, adenosine triphosphate; PMSF, phenylmethanesulfonyl fluoride.

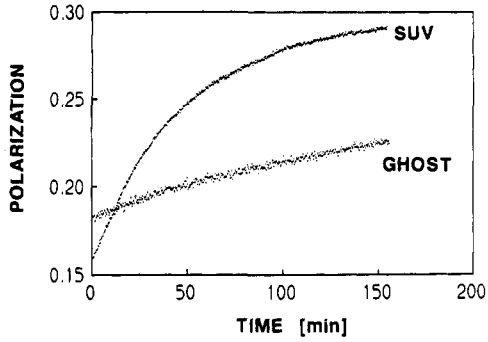

FIGURE 1: Dehydroergosterol fluorescence polarization change as a result of sterol exchange. To 20 μL of donor membranes was added 3 μg sterol acceptor to produce a final amount of 33 μg of sterol at 37 °C. Polarization of the donor + acceptor mixture was recorded every 20 s for 3 h. The change of polarization for exchange of dehydroergosterol from SUV to SUV (SUV) as well as that from ghosts to ghosts (GHOST) is depicted.

desorbed from the donor membrane into the aqueous buffer is nonfluorescent. Dehydroergosterol entering the acceptor membrane is released from self-quenching, and the polarization increases. Dehydroergosterol in the donor and cholesterol in the acceptor exchange one for one. This exchange of dehydroergosterol for cholesterol between donor and acceptor membranes was monitored continuously at 37 °C for 3 h by measuring the fluorescence polarization of the donor and acceptor mixture. Aliquots of a donor (either SUV or ghosts) were diluted in 50 mM Tris, pH 7.4, to a final concentration of 1.5 μg of sterol/mL. After 10 min acceptor, 30 μg total sterol concentration into 2 mL total volume, was then added to start the exchange. Data points were averaged over 20 s. The sample was continuously stirred with a resident stirring bar during data collection.

Before data analysis, polarization values were converted to anisotropy [$r = 2P/(3 - P)$]. Residual light scatter contribution from both donor and acceptor membranes was subtracted from all data. This allowed comparison of the polarization (anisotropy) value at the beginning of the exchange with the standard curve; see Appendix 2. The corrected data were computer fit to a one- or two-exponential equation to provide the kinetic parameters of sterol exchange as described in Appendix 1.

RESULTS

Sterol Exchange between SUV. In the present investigation SUV containing dehydroergosterol were used to load dehydroergosterol into erythrocyte ghost membranes. To prevent disruption of the erythrocyte sterol/phospholipid molar ratio, SUV were used with the same sterol/phospholipid ratio as erythrocytes (1:1; Lubin et al., 1988). Although considerable work has been reported regarding sterol exchange and sterol domains between donor and acceptor SUV containing 35 mol % of total lipid as sterol (Nemecz et al., 1988; Butko et al., 1992), the effect of increased sterol content to 50 mol % is not known. Therefore, the exchange of sterol between SUV with total sterol content of 50 mol % total lipid was determined. Figure 1 (SUV) illustrates the dehydroergosterol fluorescence polarization change during sterol exchange between SUV containing 50 mol % sterol. As dehydroergosterol from donor SUV was exchanged into acceptor SUV, fluorescence quenching decreased and polarization increased (Figure 1, SUV). After curve fitting for one or two exponentials, the deviation of the data from the fitted curves was calculated. These deviations, or residuals, are plotted in Figure 2 for one- (Figure 2A) and two-exponential (Figure 2B) fits. For the two-exponential fit, the residuals cluster about 0 throughout the time course of the experiments, showing a superior fit of this curve to the data compared to the one-exponential fit. Using three exponents (three exchangeable plus one nonexchangeable domain) did not improve the fit of the data. Thus, the sterol exchanges in three kinetically resolved domains: the two exponential components corresponding to exchangeable domains plus one nonexchangeable domain. The data were resolved into a rapidly exchangeable domain, f_1, a slowly exchangeable domain, f_2, and an essentially nonexchangeable domain, f_3, representing 23 ± 9%, 59 ± 9%, and 19 ± 9% of total sterol, respectively (Table 1). The half-times of exchange of these domains were 23 ± 6 min, 145 ± 19 min, and days, respectively. A similar result was previously reported for sterol exchange between SUV of 35 mol % total sterol content (Butko et al., 1992). The increase of sterol content in SUV from 35 to 50 mol % did not significantly affect sterol kinetic exchange parameters between SUV (Table 1).

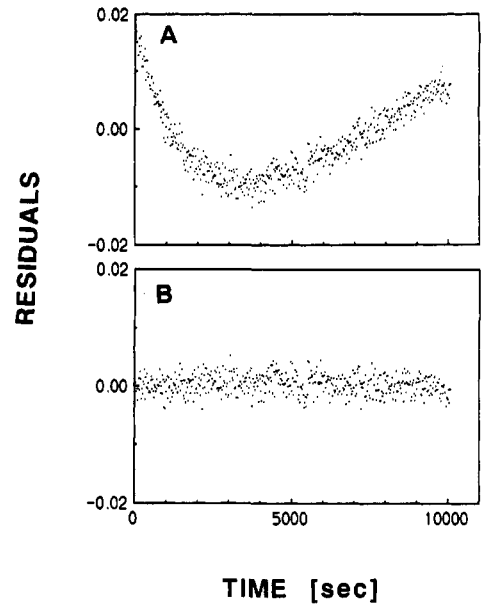

FIGURE 2: Comparison of residuals from one-exponential (A) and two-exponential fits (B) applied to the same experimental data for sterol exchange between SUV.

Table 1: Kinetic Parameters of Sterol Exchange from SUV to SUV and from SUV to Erythrocyte Ghosts[a]

type of exchange	sterol domain distribution (%)				
	f_1	f_2	f_3	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (min)
SUV–SUV (35 mol %)	11 ± 4	70 ± 1	19 ± 2	27 ± 6	145 ± 19
SUV–SUV (50 mol %)	23 ± 9	59 ± 9	19 ± 9	23 ± 6	135 ± 3
SUV–RBC	1 ± 0.4	57 ± 4	42 ± 4	0.48 ± 0.18	120 ± 6

[a] Exchange assays were performed as described in Materials and Methods. SUV (50 mol % total sterol) were used as both donor and acceptor, or SUV were used as donors and erythrocyte ghosts as acceptors (donor/acceptor ratio = 1:10). Polarization changes were fit by a two-exponential equation. The use of a three-exponential equation did not lead to further improvement of the fit. Average values ± SEM of seven experiments are given.

Sterol Exchange between SUV and Erythrocyte Ghost Membranes. Since erythrocyte ghosts must first be loaded with dehydroergosterol from SUV before they can be used to study ghost to ghost sterol exchange, it is necessary to understand the dynamics of the SUV to ghost exchange. It

is commonly assumed in the literature [reviewed in Phillips et al. (1987)] that the rate-limiting step in the spontaneous cholesterol exchange process (e.g., from a SUV donor to a SUV acceptor) is the rate of desorption of sterol from the donor membrane and that the acceptor is without effect. The validity of this assumption for SUV to ghost exchange (used to load erythrocyte ghosts) was tested as follows: A new standard curve for the dehydroergosterol exchange between dissimilar donor and acceptor pairs (from SUV to erythrocyte ghosts) was constructed (Appendix 2) and used for analysis of the kinetics of exchange. Similarly to SUV–SUV exchange, in the SUV–ghost exchange a significantly better fit, as determined by residuals, is provided by a two-exponential equation, which shows the existence of three kinetically resolved sterol domains. The expected independence of sterol exchange kinetic parameters from the type of acceptor, however, was not observed. The parameters are summarized in Table 1. Although the donor SUV composition and the donor/acceptor ratio (1:10) was kept the same as for the SUV to SUV exchange, there were dramatic differences in the SUV to ghost exchange kinetics. The f_1 decreased from 23 ± 9% to almost zero, 2 ± 1%, $P < 0.001$, while the half-time of 23 ± 6 min dramatically decreased to 0.71 ± 0.41 min. The size of the f_2 was unchanged, 59 ± 9% and 57 ± 4%, and its half-time decrease was not statistically significant: from 135 ± 3 to 114 ± 8 min, $P < 0.1$. The size of the f_3 doubled from 19 ± 9% to 43 ± 4%, $P < 0.01$. Thus, not only the properties of the donor membrane but also those of the acceptor membrane, in the case of erythrocyte ghosts, are important determinants of sterol-exchange kinetics and domain sizes in the donor. Consequently, to obtain a correct kinetic description for erythrocyte ghost sterol domains, the exchange of sterol from ghosts to ghosts must be determined.

Loading of Dehydroergosterol into the Erythrocyte Membrane. Most previous studies with radiolabeled cholesterol assumed that loading the radiolabeled cholesterol into erythrocyte membranes uniformly labeled all cholesterol domains in the membrane. The results of the SUV to erythrocyte ghost exchanges described above suggest that this assumption may not be correct. To test this directly, dehydroergosterol was loaded into the erythrocyte membrane by incubation of ghost membranes with SUV containing 50 mol % sterol as dehydroergosterol for 24 h as described in Materials and Methods. The amount of dehydroergosterol loaded into the acceptor erythrocyte ghost membrane was determined after lipid extraction of the loaded erythrocyte ghost membranes and subsequent sterol analysis of the neutral lipid fraction by reverse-phase HPLC (Figure 3A). The peak at low retention time is solvent (ethanol). Peaks 1, 2, and 3 are due to dehydroergosterol, ergosterol (internal standard), and cholesterol, respectively. The dependence of peak area on the amount of sterol is shown in Figure 3B. The standard curves in Figure 3B were used to determine response factors of dehydroergosterol and cholesterol to calculate dehydroergosterol content from HPLC scans of lipids extracted from dehydroergosterol-loaded erythrocytes.

Figure 4 illustrates the dependence of the dehydroergosterol amount in ghost membranes on time of incubation with an SUV/ghost ratio = 30:1. Two important pieces of information are obvious from Figure 4: (a) the maximum dehydroergosterol loaded in 72 h was only 16 mol % total lipid; (b) sterol exchange reached its maximum in 24 h, and further incubation did not lead to increased dehydroergosterol content in ghosts.

The sterol content in RBC membrane is 50 mol % total lipid (Lubin et al., 1988). If the sterol in RBC ghosts were

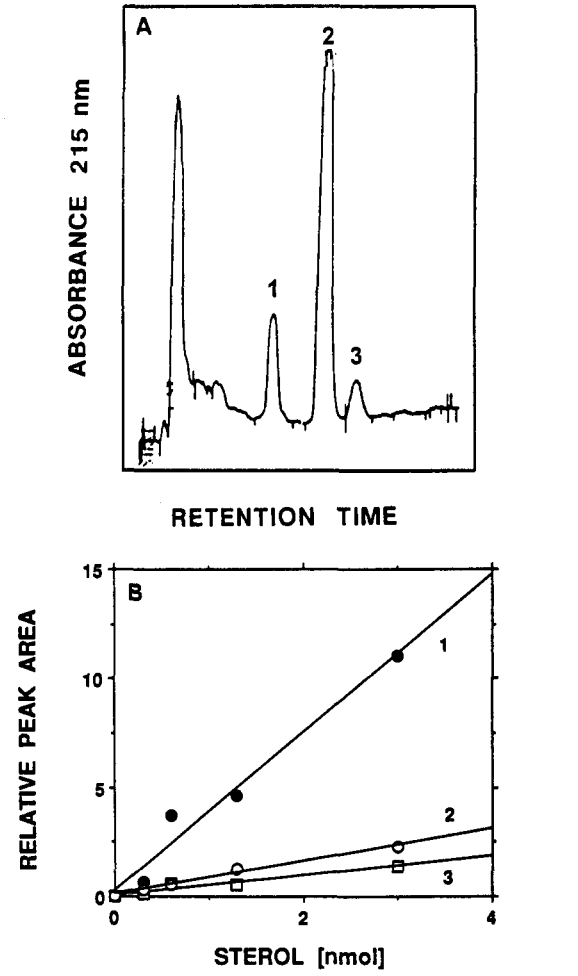

FIGURE 3: Determination of sterol content of erythrocyte membrane. Erythrocyte ghosts were incubated with SUV at 37 °C as described in Materials and Methods. After separation of ghosts from SUV by centrifugation, lipid was extracted by the Bligh–Dyer method and the neutral lipid fraction was collected on silica gel columns. This fraction was then analyzed by HPLC. Panel A illustrates a record of an HPLC analysis. Peaks 1, 2, and 3 refer to dehydroergosterol, ergosterol (internal standard), and cholesterol, respectively. The high peak at the beginning of the record is from the ethanol solvent. Areas of the peaks were calculated using an integrator, and the relationship between each area peak and the amount of compound was determined by means of the calibration curves in panel B.

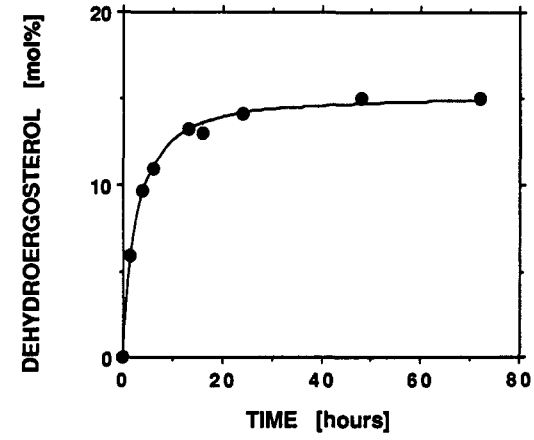

FIGURE 4: Dependence of dehydroergosterol content of loaded ghosts on incubation time. Ghosts were incubated with SUV (in a 1:10 ratio). After separation from SUV, the lipid was extracted and the dehydroergosterol content was determined using HPLC analysis.

100% exchangeable, the maximum content of dehydroergosterol should rise to approx. 48 mol % during incubation of

Table 2: Loading of Dehydroergosterol into Erythrocyte Membrane[a]

SUV:RBC ratio	SCP-2	DHE loaded (mol % of total)	[^{3}H]cholesterol loaded (mol % of total lipid)
2:1	–	14 ± 2	n.a.
30:1	–	16 ± 1	n.a.
30:1	+	19 ± 2	n.a.
1:1 + ^{3}H	–	n.a.	14 ± 3

[a] Amount of dehydroergosterol or [^{3}H]cholesterol loaded into erythrocyte ghosts under different SUV/ghosts ratios and in the absence or presence of SCP-2 during the loading process. Average values ± SEM of three measurements are depicted; n.a., not applicable.

ghosts with SUV in an SUV/ghosts ratio = 30:1. That maximal DHE loading was only 16 mol % total lipid means that only 33% (16/48 × 100%) of the total sterol was loaded. Thus, 67% of RBC membrane sterol was nonexchangeable. To confirm this finding of the existence of a large nonexchangeable domain in erythrocyte ghost membrane, several other loading experiments were performed.

First, the effect of a different ratio of donor to acceptor ghosts on the amount of dehydroergosterol loaded was determined. The ratio of loading SUV to acceptor ghosts was changed from 30:1 to 2:1. This change, however, did not lead to a significant change in the amount of dehydroergosterol loaded into the ghost membranes—16 ± 1 vs 14 ± 2 mol % total lipid (Table 2).

Second, the possibility that the large nonexchangeable sterol domain in erythrocyte membranes might be due to unique properties of dehydroergosterol, rather than cholesterol, was examined. SUV containing [^{3}H]cholesterol rather than dehydroergosterol were used for loading erythrocyte ghosts (in an SUV/ghosts ratio = 1:1) for 24 h at 37 °C. From the specific activity of [^{3}H]cholesterol in the cholesterol fraction of HPLC eluants of the total lipid extract from the acceptor ghosts, the amount of radiolabeled cholesterol loaded in the ghosts was determined to be 14 ± 3 mol % total lipid.

Third, sterol carrier protein 2 (SCP-2) is a cytosolic cholesterol binding protein that enhances the exchange of dehydroergosterol between SUV and between L-cell fibroblast plasma membranes (Schroeder et al., 1991a; Woodford et al., 1994) and reduces the proportion of the nonexchangeable sterol domain. Therefore, SCP-2 was used in the dehydroergosterol loading experiment to determine if SCP-2 could also decrease the large nonexchangeable pool in erythrocyte ghost membrane and increase the dehydroergosterol loading. However, the presence of SCP-2 at a concentration known to increase sterol exchange in the above systems increased the amount of dehydroergosterol loaded into acceptor erythrocyte membrane only very slightly: 19 ± 2 vs 16 ± 1 mol % total lipid was loaded (Table 2), which corresponds with a slight decrease of the nonexchangeable domain, 61 ± 4% vs 67 ± 2%.

These experiments indicate that the slowing down of the sterol exchange determined in SUV to erythrocyte ghosts vs SUV to SUV exchange is due to the inability of some sterol desorbed from donor SUV to enter the acceptor erythrocyte membrane. It is also consistent with the presence of a large (about 67% of all sterol) nonexchangeable domain of cholesterol in the erythrocyte membrane. Thus, only 33% of total erythrocyte ghost sterol is accessible to loading from SUV and can subsequently participate as a donor for sterol exchange between erythrocyte ghost membranes.

Kinetic Parameters for Sterol Exchange from Ghosts to Ghosts. Sterol exchange between erythrocyte ghosts is shown in Figure 1 (GHOST). Note that the maximal polarization change for the SUV to SUV exchange was about twice the maximal polarization change observed for ghost to ghost exchange. This was because the SUV donor could be prepared with much higher dehydroergosterol content in SUV (100 mol % of total sterol) compared to erythrocyte ghosts, in which maximal dehydroergosterol incorporation was 38 mol % of total sterol (see above).

Table 3: Kinetic Parameters of Sterol Exchange from Erythrocyte Ghosts to Erythrocyte Ghosts[a]

	sterol domain distribution (%)					
	f_1	f_2	f_3	$^1t_{1/2}$ (min)	$^2t_{1/2}$ (h)	n
control	4 ± 2	29 ± 3	67 ± 2	32 ± 9	23 ± 7	7
+SCP-2	2 ± 0.5	31 ± 1	67 ± 2	22 ± 2	17 ± 1	2

[a] Erythrocyte ghosts were dehydroergosterol loaded by incubation of ghosts with SUV (1:30) for 24 h at 37 °C as described in Materials and Methods. The dehydroergosterol-loaded ghosts were used as donors for sterol exchange from erythrocyte ghosts to nonloaded acceptor (donor/acceptor ratio = 1:10) in the absence (control) or the presence of 2 μM SCP-2. A two-exponential equation was used to fit polarization data. The fraction of f_3 was calculated from chemical determination by HPLC analysis of the dehydroergosterol to cholesterol ratio in erythrocyte ghosts at the end of the 24-h loading period.

The data in Figure 1 (GHOST) were best fit by a two-exponential equation, as determined by residuals. The use of a three-exponential equation did not improve the fit. These data show that there are three kinetic sterol domains in erythrocyte ghost membranes: two exchangeable domains corresponding to the exponential components of the polarization assay and a third nonexchangeable domain not labeled by dehydroergosterol (not loaded with dehydroergosterol). This nonexchangeable domain, f_3 = 67% of total sterol, was calculated from the chemical determination of dehydroergosterol content of donor ghosts by HPLC as described above. The results of several exchange experiments are summarized in Table 3. The rapidly exchangeable domain accounted for a size of 4 ± 2% of total sterol, with an exchange half-time of 32 ± 9 min. The f_2 domain, representing 29 ± 3% of total sterol, was very slowly exchangeable with a half-time of 23 ± 7 h.

Effect of SCP-2 on Sterol Exchange between Ghosts. Previous results using model membranes (Butko et al., 1990, 1992; Schroeder et al., 1990a, 1991a) and L-cell fibroblast plasma membranes for the exchanges (Schroeder et al., 1991a; Woodford et al., 1994) showed a pronounced effect of the presence of SCP-2 on sterol-exchange kinetics. What is most important, SCP-2 altered not only the half-times but also the size of the nonexchangeable sterol domain in those studies. Therefore, two experiments were performed to test whether SCP-2 also modulated erythrocyte ghost sterol domain dynamics and the nonexchangeable domain.

First, SCP-2 had little effect on dehydroergosterol loading of erythrocyte ghosts, as mentioned above. When 2 μM SCP-2 was present during the erythrocyte ghosts dehydroergosterol loading process, there was only a slight increase (from 16 ± 1 to 19 ± 2 mol % of total sterol) in the amount of dehydroergosterol loaded (Table 2).

Second, erythrocyte ghosts were loaded in the absence of SCP-2 and then used as donors in a ghost to ghost exchange assay. The presence of 2 μM SCP-2 in the assay mixture did not alter sterol-exchange parameters (Table 3). These results are in contrast to the effect of SCP-2 on sterol-exchange parameters between L-cell plasma membranes, where the presence of 1.5 μM SCP-2 decreased the $^1t_{1/2}$ of the exchange 2.5-fold, the $^2t_{1/2}$ 1.5-fold, and the nonexchangeable domain from 65% to 56% (Woodford et al., 1994).

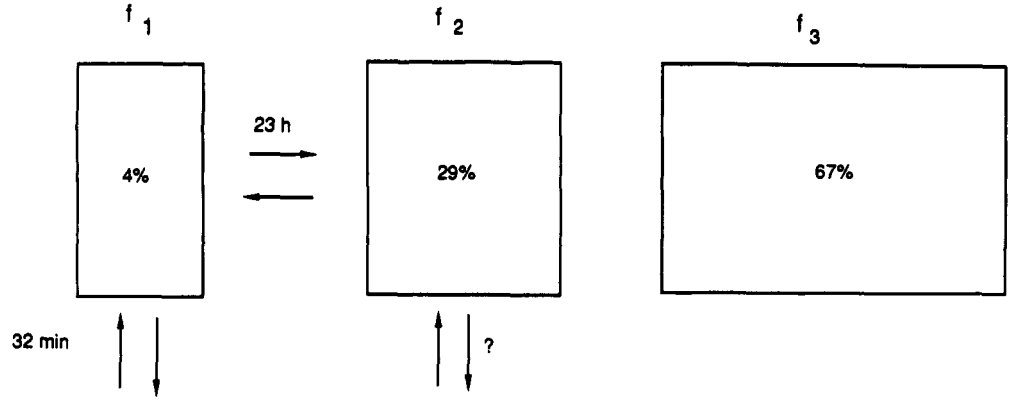

FIGURE 5: Sterol kinetic domain model for erythrocyte membrane. Three domains are shown: one containing 4% of total sterol, fast exchangeable, with a half-time of 32 min; one containing 29% of total sterol, slowly exchangeable, with a half-time of 26 h; and one containing 67% of total sterol, nonexchangeable.

DISCUSSION

The results described in this paper extend the methodology, developed earlier for SUV and nucleated cell membranes by this and other laboratories, to examine erythrocyte sterol domains. The theoretical basis for using fluorescent sterol polarization change to determine molecular sterol transfer has been established in a detailed theoretical paper (Butko et al., 1992). Therein use of the dehydroergosterol standard curve, correction factors, etc., for determining molecular sterol exchange was validated.

The findings presented here as well as those from other laboratories provide strong evidence for the existence of multiple cholesterol domains in biological and model membranes:

(1) Extent of fluorescent sterol and radiolabeled cholesterol loading of erythrocyte ghost membrane (present work). There is a large part, about 67% of the total, of nonexchangeable sterol in the erythrocyte membrane.

(2) Kinetic exchange evidence based on sterol fluorescence polarization change in erythrocyte ghost membrane (present work), in L-cell plasma membrane (Schroeder et al., 1991a; Woodford et al., 1993), and in model membranes (Schroeder et al., 1990a; Hapala et al., 1990; Butko et al., 1990, 1992; Schroeder et al., 1990b). Thus, the membrane sterol is distributed in multiple kinetic domains.

(3) Kinetics of radiolabeled cholesterol exchange in SUV. The kinetics and multiple domains resolved by use of radiolabeled cholesterol are very similar to those observed with fluorescent sterol in SUV (Nemecz et al., 1988). The comparison between dehydroergosterol and radiolabeled cholesterol exchanges between SUV was performed previously by this laboratory (Nemecz et al., 1988). In these experiments the SUV dehydroergosterol was exchanged for SUV cholesterol and radiolabeled cholesterol for unlabeled SUV cholesterol. There was no significant difference between kinetic parameters of sterol exchange obtained be these two methods. This indicates the 1:1 exchange between SUV dehydroergosterol and SUV cholesterol. In our experiments with erythrocyte membranes 14 ± 2% of sterol was exchanged (at a SUV/RBC ratio = 2:1). Using radiolabeled cholesterol revealed 14 ± 3% of exchanged sterol (SUV/RBC ratio = 1:1). Thus, there is no significant difference in amounts of dehydroergosterol vs radiolabeled cholesterol exchanged for unlabeled cholesterol in erythrocyte ghost membranes. These two studies document the 1:1 exchange between SUV dehydroergosterol and erythrocyte cholesterol.

(4) Static experiments with the sterol (dehydroergosterol and cholestatrienol) fluorescent polarization and limiting anisotropy in SUV membranes (Nemecz et al., 1988; Schroeder et al., 1988; Chong & Thompson, 1986; Hyslop et al., 1990; Bar et al., 1989; Schroeder & Nemecz, 1989). The size and number of cholesterol domains in membranes determined with sterol fluorescence polarization are similar to those observed in experiments studying membrane phase diagrams using other methods [reviewed in Schroeder et al. (1991a)].

(5) Dynamic lifetime evidence with fluorescent sterols dehydroergosterol and cholestatrienol in SUV and L-cell plasma membranes (Schroeder & Nemecz, 1989; Schroeder et al., 1988, 1991a). Multiple fluorescent sterol lifetimes in the membranes, as opposed to single lifetimes in solvents, are consistent with multiple sterol domains.

A model of sterol kinetic distribution in the erythrocyte membrane, based on the findings detailed herein as well as those in the literature, is proposed (Figure 5). On the basis of dehydroergosterol quenching by acrylamide in experiments with model membranes (Nemecz & Schroeder, 1988) sterol may enter the erythrocyte membrane by exchange through the fast-exchangeable domain. However, the erythrocyte membrane is much more complex than SUV, so the possibility of sterol entering the membrane via parallel pathway(s) to other sterol domains cannot be excluded. Sterol then may equilibrate laterally either in parallel or in series with the slowly exchangeable domain.

From studies of model membranes it is known that numerous factors affect the kinetic parameters of sterol exchange. These include lipid composition and topology, sterol content, the presence of protein in biological membranes, and lipid packing, which may also be affected by the radius of curvature of vesicles or ghosts (Fugler et al., 1985). Thus it is not surprising that the kinetic parameters of sterol exchange from SUV to SUV differ from those of exchange from SUV to ghost and from ghost to ghost. Because of these complexities and the fact that exchange kinetics are determined by both donor and acceptor membrane composition, inferences about the domain structure of membranes can only be made from experiments in which homologous exchange—SUV to SUV or ghost to ghost—is examined.

The results presented herein with erythrocyte sterol domains help to resolve the considerable disagreement regarding the existence of a nonexchangeable cholesterol domain(s) in the erythrocyte. Some authors report the occurrence of the nonexchangeable sterol pool (Bell & Schwartz, 1971; Bruckdorfer & Sherry, 1984; Lange, 1982; Steck et al., 1988), while others report no nonexchangeable pool (d'Hollander & Chevalier, 1972; Gold & Phillips, 1990). Two sets of evidence

are consistent with the existence of a large nonexchangeable sterol domain in human erythrocytes.

First, the amount of dehydroergosterol exchanged to red blood cell ghosts was measured directly using HPLC of lipid extracts. This provided direct evidence for the existence of a large nonexchangeable sterol domain representing about 67% of total sterol in the erythrocyte membrane. The existence of a nonexchangeable domain in SUV (about 20% of total sterol) raises the possibility that the erythrocyte nonexchangeable sterol may be due to inability of some dehydroergosterol in SUV to exchange and/or nonspecific sticking of SUV to the ghosts during loading. As indicated in Materials and Methods, use of the nonexchangeable marker [^{14}C]-triacylglycerol showed that less than 3% of the dehydroergosterol loaded into erythrocyte ghosts was due to nonspecific sticking/fusion of SUV to ghosts during loading.

Second, these observations of a large nonexchangeable sterol domain in the erythrocyte membrane were not due to a peculiar property of dehydroergosterol. Radiolabeled cholesterol loading also showed a large (66% of total sterol) nonexchangeable sterol domain in the human erythrocyte.

Although spontaneous cholesterol exchange has been proposed to proceed by several other mechanisms, e.g., by a collision-induced (Mutsch et al., 1986) or an activation–collision (Steck et al., 1988) mechanism, a simple aqueous diffusion mechanism (Gold & Phillips, 1990; Johnson et al., 1991) is the most commonly accepted. According to the aqueous diffusion mechanism the transfer of sterol between donor and acceptor membranes involves three steps: desorption of the sterol molecule from donor membrane, its diffusion through the aqueous phase, and incorporation into the acceptor membrane. On the basis of model membrane exchange studies requiring separation of donor and acceptor membranes, it was concluded that the rate-limiting step in intermembrane sterol transfer was the desorption of sterol from the donor membrane [reviewed in Phillips et al. (1987)]. In contrast, a recent observation with the SUV–SUV exchange using the fluorescence polarization sterol exchange assay showed that the properties of the acceptor SUV might also influence the rate of intermembrane exchange (Schroeder & Nemecz, 1989). In the latter studies, if the donor SUV were composed of very viscous distearoylphosphatidylcholine/dehydroergosterol and the acceptor SUV were composed of fluid dimyristoylphosphatidylcholine/cholesterol, intermembrane sterol exchange was relatively fast. More important, in the reverse case where the donor SUV consisted of fluid SUV and the acceptor SUV consisted of very viscous SUV, then intermembrane sterol exchange was extremely slow. Similar dependence of spontaneous cholesterol transfer (from SUV to synaptosomal membranes) on the acceptor membrane properties has also been reported (Rao et al., 1994). In addition, Geldwerth et al. (1991) showed that spontaneous phospholipid transfer from maize seedlings also depends on the type of acceptor.

The present work provides further evidence of the importance of the type of acceptor for sterol-exchange kinetics. The kinetic parameters of the sterol exchange between SUV and those between erythrocyte ghosts are very different (Tables 1 and 2). In SUV–SUV exchange there is 11% of sterol exchanging with a half-time of 27 min. This is comparable to the f_1 domain in the erythrocyte membrane consisting of 4% of sterol with a half-time of 32 min. However, the f_2 domain in SUV–SUV exchange containing 70% of total sterol with a half-time of 145 min has no equivalent domain in the erythrocyte membrane. The f_2 in erythrocyte membrane contains 29% of total sterol, but its half-time is 23 h. Thus, in sterol exchange between SUV and ghosts dehydroergosterol was desorbed from SUV donor much more rapidly than it was absorbed by erythrocyte ghost acceptor membrane and was reabsorbed by other SUV rather than by acceptor ghost membranes. These data provide direct evidence that the rate of desorption of sterol from the donor membrane may not be the only regulatory mechanism of sterol exchange. Furthermore, the increase of the nonexchangeable domain in SUV from 19% in SUV–SUV to 42% in SUV–ghost exchange even in a 10-fold excess of acceptor erythrocyte ghosts further underlines the importance of acceptor membranes in sterol exchange. The increase might be caused by an interaction between donor and acceptor membranes, which, however, did not necessarily affect the mechanism of sterol transfer.

If biological membranes such as erythrocyte ghosts fit with the earlier model where the rate-limiting step is sterol desorption from the donor, then the substitution of erythrocyte ghosts for SUV in the SUV to SUV sterol exchange assay should not greatly affect the kinetic exchange parameters. However, the experiments shown herein show that there was a significant change of the sterol exchange kinetic parameters. Two explanations may be possible: (A) The acceptor as well as the donor affects the sterol-exchange process. (B) A collisional mechanism could account for the observations. Some investigators have linked the collisional mechanism of sterol transfer with the presence of a glycocalyx surrounding the erythrocyte membrane (Bittman, 1993). However, several considerations argue against the collisional mechanism being responsible for the above observations. First, the limiting criterion for significant contribution by the collisional mechanism of exchange is that there be a sufficiently high concentration of total lipid. In experiments that were interpreted to reflect sterol exchange via the aqueous diffusion mechanism, Gold and Phillips (1990) used total lipid concentrations (180 μg/mL), which were 4 times higher than those used in the present study (45 μg/mL). Second, the erythrocyte ghosts used herein were unsealed in the exchange process. Because the majority (75%) of cholesterol is in the cytofacial leaflet and there is no glycocalyx on the cytofacial leaflet, it is unlikely that a glycocalyx significantly altered the sterol exchange. Third, other investigators using sterol exchange between SUV and synaptosomal plasma membranes also observed dependence on the acceptor membrane (Rao et al., 1994; Wood et al., 1994).

The experimental detection of a large nonexchangeable sterol domain in the erythrocyte membrane is crucially dependent on the method used. The method most often used for labeling the donor erythrocyte membranes is by exchange of radiolabeled cholesterol from donor plasma, serum, or SUV to the acceptor erythrocyte. The radiolabeled cholesterol then exchanges for unlabeled cholesterol in the membrane. As shown herein, the radiolabeled cholesterol can enter only the exchangeable sterol domain(s). Consequently, in such studies all radiolabeled cholesterol loaded into the erythrocyte could be exchanged out again in the presence of acceptor, giving the impression of full exchangeability of sterol and a lack of a nonexchangeable domain. In most preceding studies it was not determined whether the radiolabeled sterol uniformly labeled all of the erythrocyte cholesterol. This requires measurement of the specific activity of [^{3}H]cholesterol in the loaded ghost membranes, as was done in this study.

The sterol kinetic domains defined by sterol exchange are likely to reflect structural domains of cholesterol in the membrane. It has been suggested in model membranes that

the fast-exchangeable domain involves sterol monomers (Schroeder et al., 1991a). With increasing cholesterol content, cholesterol undergoes phase separation in model membranes [reviewed in Schroeder et al. (1991a) and Schroeder and Wood (1994)]. However, SUV containing 35 or 50 mol % sterol have 11% and 23% rapidly exchanging sterol, respectively, while the erythrocyte membrane with 50 mol % sterol has only about 4% rapidly exchanging sterol. This suggests that other factors (e.g., protein or other lipids) constrain most of the erythrocyte sterol to be in a phase-separated form. The large nonexchangeable sterol domain may be a pure sterol domain in a nearly crystalline or other inaccessible form (Nemecz et al., 1988; Schroeder et al., 1991a; Schroeder & Wood, 1994). The slowly exchangeable domain may represent an organized array of sterol and phospholipid (Schroeder et al., 1991a; Nemecz et al., 1988), or it may consist of sterol interacting with protein molecules, e.g., the spectrin–actin system, which may have a strong stabilizing effect (Wood et al., 1994; Gordon & Mobley, 1984; Schroeder et al., 1991a; Yeagle, 1987; Thurnhofer et al., 1991).

The structure of membrane sterol domains may have significant functional importance. Cholesterol content is a primary determinant of membrane microviscosity (Yeagle, 1987), and phospholipid content and composition, in turn, dramatically affect cholesterol domain structure (Schroeder et al., 1991a; I. Hapala et al., unpublished data). In two closely related murine fibroblast cell lines, LM and L929, changes in domain structure (Schroeder et al., 1991a) are associated with differences in metastatic potential (Kier & Franklin, 1991; Kier & Schroeder, 1982). Transbilayer sterol distribution in synaptic plasma membrane is altered by chronic ethanol exposure (Wood et al., 1990). Cholesterol enrichment or depletion of red cell membranes has been shown to alter several membrane transport processes (Jemuri & Philipson, 1989; Yeagle, 1983; Grey & Lauf, 1980). In the sickle red cell, cholesterol uptake from plasma has been reported to be abnormal (Ngogang et al., 1989; Jain & Shohet, 1982), given the abnormalities in membrane skeletal proteins (Hebbel, 1991; Rank et al., 1985; Platt et al., 1985) and phospholipid asymmetry (Choe et al., 1986; Franck et al., 1985) in these cells. It is possible that deranged sterol domain structure may also occur. Explaining the relationship of such abnormalities to the other structural and functional abnormalities in sickle cells (Hebbel, 1991; Joiner, 1993) may shed light on the mechanisms regulating cholesterol domains in erythrocytes, as well as the functional consequences of lipid microstructure in biological membranes.

ACKNOWLEDGMENT

The authors wish to thank Ms. Eileen Cooper for her help with erythrocyte ghosts preparation.

APPENDIX 1: THEORETICAL BACKGROUND FOR THE EXCHANGE ASSAY—DERIVATION OF THE STANDARD CURVE FOR STEROL EXCHANGE IN ERYTHROCYTE MEMBRANE

The erythrocyte dehydroergosterol exchange assay is based on that originally developed for model membrane SUV (Nemecz et al., 1988; Nemecz & Schroeder, 1988; Butko et al., 1990, 1992; Schroeder et al., 1990). In the erythrocyte membrane the dehydroergosterol polarization is a nonlinear function of dehydroergosterol concentration and is described by the equation

$$P = p_0/(1 + Kc) \tag{A1}$$

where p_0 is a constant defined as the polarization at infinite dilution, K = constant $\times$ p_0 $\times$ τ, c is the dehydroergosterol concentration, and τ is the fluorescence lifetime. It was shown in earlier work from this laboratory that the fluorescence lifetime of dehydroergosterol is independent of the dehydroergosterol concentration in the range 0–50 mol % (Schroeder et al., 1987).

Polarization measured in the exchange assays resulted from contributions by both donor and acceptor membranes. Polarization itself, however, is not an additive parameter. Using the relation between polarization, P, and anisotropy, r, $P = 3r/(2 + r)$, eq A1 can be rewritten as

$$r = r_0/(1 + K'c) \tag{A2}$$

where r_0, analogous to p_0, is the anisotropy at infinite dilution and the constant $K' = K(1 + r_0/2)$.

The resulting anisotropy r of a mixture of donor and acceptor erythrocyte membranes with anisotropies r_D and r_A, respectively, is the linear combination $r = f_D r_D + f_A r_A$, where f_i values are fractional fluorescence intensities (Butko et al., 1992; Lakowicz, 1983). The concentrations of dehydroergosterol in donor and acceptor membranes fulfill the condition

$$c_I = c_D + kc_A \tag{A3}$$

where c_I is initial dehydroergosterol concentration in donor (e.g., c_I = 35 or 50 mol % of total lipid for SUV, and c_I = 16 mol % for erythrocyte membrane loaded by SUV with an SUV/ghosts ratio = 30:1), c_D and c_A are the concentrations of dehydroergosterol in donor and acceptor membranes, respectively, and k is the acceptor/donor ratio (in our case, k = 10). Note that the meaning of this equation is that dehydroergosterol exchanges to acceptor membranes homogeneously, i.e., it assumes that the amount of dehydroergosterol (not its distribution) in all acceptor membranes is the same.

The relative dehydroergosterol concentration in donor membranes is defined as x_D:

$$x_D = c_D/c_I \tag{A4}$$

At the beginning of the exchange, $c_D = c_I$ and so $x_D = 1$. As the exchange proceeds, the x_D decreases, fluorescence quenching also decreases, and polarization of dehydroergosterol in donor membranes increases. Because of an excess of acceptor, the dehydroergosterol concentration in acceptor membranes is too low to cause the quenching of fluorescence. The resulting anisotropy of a donor and acceptor mixture as a function of donor concentration, $r(x_D)$, is given by eq A5 (Butko et al., 1992):

$$r(x_D) = r_0\{x_D/(1 + Dx_D) + (1 - x_D)/[1 + D(1 - x_D)/k]\} \tag{A5}$$

where $D = K'c_I$. Here, the fractional intensity of donor, f_D, was substituted by dehydroergosterol concentration in donor, x_D, by neglecting the self-quenching of the donor fluorescence intensity. This assumption has very little if any effect on sterol exchange kinetic parameters (Butko et al., 1992). The anisotropy, $r(x_D)$, can be converted to polarization, $P(x_D)$, using the equation $P = 3r/(2 + r)$. So calculated polarization of a donor and acceptor mixture as a function of dehydroergosterol concentration in donor only, $P(x_D)$, can be fit by a quadratic equation (eq A6), the standard curve for sterol

exchange:

$$P(x_D) = -mx_D^2 + n \qquad \text{(A6)}$$

the parameters a and c from Butko et al. (1992) were renamed m and n to prevent confusion by mistaken exchange of a and c for A and C in eq A7 below.

For a two-exponential process (Nemecz et al., 1988; Bruckdorfer & Green, 1967; Butko et al., 1992), the time dependence of dehydroergosterol concentration in donor membranes, x_D, is given by the equation

$$x_D(t) = A\exp(-k_1t) + B\exp(-k_2t) + C \qquad \text{(A7)}$$

where k_1 and k_2 are rate constants of the exchange corresponding to the domains of size A and B, respectively, and C is the size of the nonexchangeable domain. Similar equations may be written using more or fewer exponential terms.

For time $t = 0$, $x_D(0) = A + B + C$, but also $x_D = 1$ (because all dehydroergosterol is in donor), and so parameters A, B, and C must fulfill the condition $A + B + C = 1$.

After substitution of eq A7 into the eq A6, the time dependence of polarization, $P(t)$, during exchange will be

$$P(t) = -m\{A^2\exp(-2k_1t) + B^2\exp(-2k_2t) + 2AB\exp[-(k_1+k_2)t] + 2AC\exp(-k_1t) + 2BC\exp(-k_2t) + C^2\} + n \qquad \text{(A8)}$$

On the basis of residuals obtained for one-, two-, and three-exponential fits, it was determined that the two-exponential equation best fit the data and eq A8 was used in curve fitting the data points. Note that for limited case $t = 0$

$$P(0) = -m(A + B + C) + n = n - m \qquad \text{(A9)}$$

For another limited case of eq A8, $t \rightarrow \infty$, the polarization $P(\infty)$ would be

$$P(\infty) = -mC^2 + n \qquad \text{(A10)}$$

Comparing this result with eq A6, we get $x_D = C$ (for $t \rightarrow \infty$); i.e., C is the size of the nonexchangeable pool, as stated above. Also, eq A10 provides an expression for C:

$$C = \{[n - P(\infty)]/m\}^{1/2} \qquad \text{(A11)}$$

and again from eq A11 the importance of the standard curve for determining the kinetic parameters of the exchange, in this case the nonexchangeable domain, is apparent. For the same value of polarization at infinite time, $P(\infty)$, the calculated size of the nonexchangeable domain depends strongly on parameters m and n.

Parameters of the Standard Curve for Heterogeneous Exchange. Calculation of the parameters of the standard curve for heterogeneous exchange is derived as follows: At the beginning of the exchange, i.e., from SUV to ghosts, all of the dehydroergosterol is in donor membranes (SUV). The initial polarization, $P(0)_H = n_H - m_H$, where the subscript H indicates "heterogeneous", fulfills the condition of the standard curve for donor membranes (SUV):

$$n_H - m_H = n_D - m_D \qquad (t = 0) \qquad \text{(A12)}$$

where the subscript D is for "donor". The parameter m_H of the heterogeneous standard curve is calculated as follows: At equilibrium the polarization will be the sum of contributions from donor as well as acceptor membranes. In a 10× excess of acceptor over donor, the equilibrium amount of fully exchangeable dehydroergosterol present in donor will be 1/11 of its initial concentration. It was determined that direct calculation using n_D and n_A, as opposed to conversion of the values to anisotropies for the calculation, did not introduce any significant error. Therefore, the final value of the parameter n_H is given by the equation

$$n_H = n_D/11 + 10n_A/11 \qquad (t \rightarrow \infty) \qquad \text{(A13)}$$

The resulting standard curve for heterogeneous exchange, $P_H(x_D)$, has the form

$$P_H(x_D) = [-m_D + (12n_D + 10n_A)/11]x_D^2 + (n_D + 10n_A)/11 \qquad \text{(A14)}$$

where m_D and n_D are the standard curve parameters for the donor membranes and n_A is that from the standard curve of acceptor. It should be noted that the standard curve has to be calculated with the same initial dehydroergosterol concentration in the donor, c_I.

The meaning of the standard curve is schematically shown in Figure A1. Both the standard curve and the polarization data from the experiment are presented. The initial polarization value of the experiment, $P(0)$, is equal to the value $n - m$ from the standard curve. The standard curve provides the theoretical polarization. The concept of exchangeable and nonexchangeable pools of sterol, from the polarization change measurement approach, is also apparent from Figure A1. Fitting to exchange data provides the polarization value at the infinite time of the exchange as $P(\infty)$. This value, $P(\infty)$, determines the value of the relative donor concentration at infinite time, $x_D(\infty)$, according to the standard curve. The concentration $x_D(\infty)$ divides the total donor dehydroergosterol into two parts: the exchangeable portion, determined by the interval $\langle x_D(\infty), 1\rangle$, and the nonexchangeable portion, given by the interval $\langle 0, x_D(\infty)\rangle$.

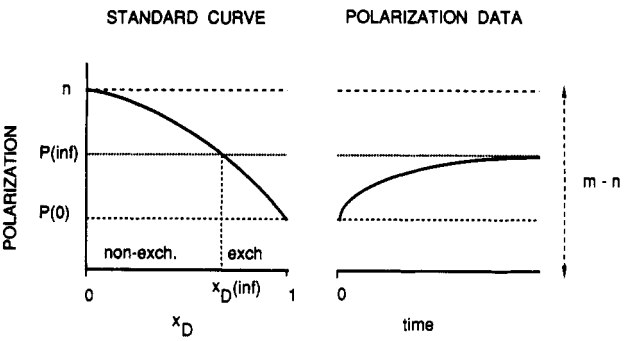

FIGURE A1: Scheme of analyzing the polarization data by means of a standard curve. At the beginning of the exchange all dehydroergosterol is in donor: $x_D = 1$ and $P_0 = n - m$. The maximum possible change of polarization is m (according to the standard curve). The nonexchangeable pool of sterol is determined by the value of polarization at equilibration, $P(\infty)$. It has to be mentioned, however, that the nonexchangeable domain determined by the polarization measurement reflects only the portion of fluorophore (in our case dehydroergosterol) that was not exchanged during the exchange assay. It does not reflect the nonexchangeable portion of the sterol, where the fluorophore was not incorporated. This part can be determined by other methods, e.g., HPLC analysis.

APPENDIX 2: CONSTRUCTION OF THE STANDARD CURVE FOR STEROL EXCHANGES

The standard curve is different for homogeneous and heterogeneous exchange, so eq A6 needs to be constructed for

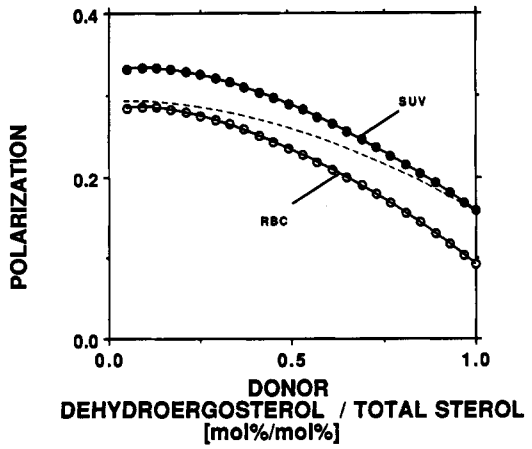

FIGURE A2: Construction of the standard curve for heterogeneous exchange. First, the standard curves of membrane systems involved in the exchange, i.e., SUV and ghosts, were constructed (c_I for both curves was 50 mol %). Then the standard curve for the exchange from SUV to ghosts was constructed (dashed curve) (see text for explanation).

each system involved in the exchange separately. However, the principles of developing the standard curve are same.

First, the dependence of polarization on dehydroergosterol concentration in the system was experimentally determined and fit to eq A1. The SUV with different dehydroergosterol content were prepared, and the polarization of each was measured. Then, the dependence of polarization as a function of the reciprocal of the dehydroergosterol concentration ($1/c_{DHE}$) was determined.

For biological membranes, e.g., erythrocyte ghosts, the construction of eq A1 is more complicated. First, various amounts of dehydroergosterol need to be incorporated into the membranes. This may be achieved by different times of incubation of ghosts with SUV. Then, each sample corresponding to each incubation time is measured for polarization and analyzed by HPLC for sterol content as described above. The dependence of polarization on the reciprocal value of the dehydroergosterol concentration $[P(1/c_{DHE})]$ is then constructed and is fit by a rectangular hyperbola, $P = (p_0/c_{DHE})/[K + (1/c_{DHE})]$, which provide parameters $p_0 = 0.3627$ and $K = 0.0588$ for eq A1. Second, using parameters p_0 and K, the constants r_0 and D are calculated: $r_0 = 2p_0/(3 - p_0)$ and $D = Kc_I(1 + r_0/2)$, where c_I is the initial concentration of dehydroergosterol in donor membranes. Third, based on parameters r_0 and D, the dependence of anisotropy of the mixture of donor and acceptor, $r(x_D)$, on the concentration of dehydroergosterol in donor, x_D, eq A5 is constructed for many values of x_D. After anisotropy $r(x_D)$ is transformed to polarization $P(x_D)$, the dependence of $P(x_D)$ on x_D, i.e., the standard curve, is constructed.

The construction of the standard curve for the exchange between SUV and ghosts (c_I = 50 mol %) (heterogenous exchange) is illustrated in Figure A2 (dashed curve). At the beginning of the exchange all dehydroergosterol is in donor SUV, so the beginning value of polarization equals that from the standard curve for SUV (concentration in donor = 1). At equilibrium the dehydroergosterol would be evenly distributed among all membranes involved. In the case of a donor/acceptor ratio = 1:10 and full exchangeability of sterol between SUV and ghosts, the polarization would be between the equilibrium polarization values from standard curves for SUV and ghosts at 1/11th of their difference from the acceptor value (Figure A2). Note that the standard curve has to be constructed by assuming that all sterol is exchangeable (up to 50 mol %), although in the case of erythrocyte ghosts only about 35% of the sterol is exchangeable.

Table A1: Standard Curve Parameters for Different Types of Exchanges[a]

type of exchange	m	n
SUV–SUV (35 mol %)	0.1673	0.3235
SUV–SUV (50 mol %)	0.1749	0.3129
SUV–ghosts (50 mol %)	0.1316	0.2896
ghosts–ghosts (16 mol %)	0.1473	0.3342

[a] The parameters were either read directly from the standard curve (homogeneous exchange) or calculated as described in the text (heterogeneous exchange). Beginning dehydroergosterol concentration in donor is given in parentheses.

Table A1 summarizes the standard curve parameters for different types of exchanges used in this paper.

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