Spontaneous Rates of Sitosterol and Cholesterol Exchange between Phospholipid Vesicles and between Lysophospholipid Dispersions: Evidence That Desorption Rate Is Impeded by the 24α-Ethyl Group of Sitosterol

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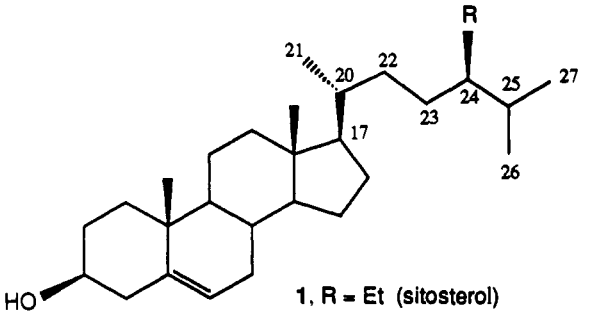
Abstract: The rates of spontaneous movement of [4-^{14}C]sitosterol and [4-^{14}C]cholesterol between unilamellar vesicles prepared with different phospholipid–sterol molar ratios and with different classes of phospholipids and between lysophospholipid-enriched dispersions have been measured. The rates of desorption of sitosterol from dipalmitoylphosphatidylcholine, egg phosphatidylcholine, and sphingomyelin bilayers are lower than those of cholesterol by factors of 3–9 over the range of 1–50 mol % sterol at 50 °C. The rate of movement of sitosterol from aqueous dispersions containing 1.5 mol % sterol and 50 mol % lysophosphatidylcholine is lower than that of cholesterol by a factor of 4 at 37 °C. The additional van der Waals forces made possible by the 24-ethyl group of sitosterol and its nearest neighbors, together with a lower aqueous phase solubility because of the presence of the 24-alkyl group, are considered to contribute to the slower desorption of sitosterol from the lipid–water interfaces relative to cholesterol. Since cholesterol is carried by mixed micelles and phospholipid vesicles in vivo, our results suggest that the 24-alkyl group of the important plant sterols sitosterol and campesterol may play an important role in the phenomenon of selective uptake of exogenous cholesterol by intestinal cells from lipid surfaces. We also discuss the extent of spontaneous cholesterol exchange between vesicles at equilibrium in relation to results obtained in earlier studies.

Intestinal absorption of β-sitosterol (**1**, referred to below as sitosterol) is much less efficient than that of cholesterol. It has been estimated that about 50% of the daily dose of ingested cholesterol is absorbed, whereas less than 5% of dietary sitosterol is absorbed; campesterol (**2**) and stigmasterol (**3**), which are the other characteristic sterols of plants, are also absorbed less efficiently than cholesterol.[1] Furthermore, plant sterols (phytosterols) inhibit the intestinal absorption of cholesterol.[1a,2,3] The mechanisms that govern cell discrimination between cholesterol and the structurally related phytosterols are not clear, nor is it understood how phytosterols lower plasma cholesterol. Among the factors that have been considered in the absorptive recognition process of exogenous sterols are their solubilities in bile salt micelles,[3b,4] affinities for components of bile salt micelles and of intestinal epithelial plasma membranes, intracellular reactions (especially with intestinal esterifying enzymes), and uptake into chylomicrons (triglyceride-rich plasma lipoproteins that play a major role in the transport of intestinally absorbed lipids).[1b,5] Interactions between proteins in the plasma membranes of intestinal cells and ingested sterols may also contribute to the preferential uptake in vivo of exogenous cholesterol by small-intestinal cells.[2a,6]

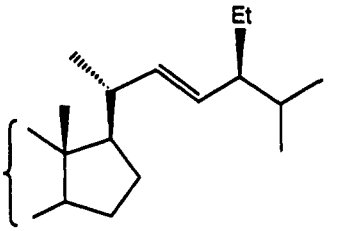
Sitosterol (**1**) differs from cholesterol (**4**) only by the presence of the ethyl group at C_{24} of the sterol side chain. We sought to determine whether the 24-alkyl group in the sterol side chain influences the rate of sterol transfer between unilamellar vesicles and between micelles. Although the physical form of sterols in the aqueous intestinal contents has not been fully established, it appears that sterols are present as a mixture of mixed bile salt micelles and unilamellar vesicles.[7] Since phosphatidylcholine (PC) and sphingomyelin are present in high amounts in many intestinal cell membranes,[8] we have chosen these phospholipids to form our



1, R = Et (sitosterol)

2, R = Me (campesterol)

4, R = H (cholesterol)



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vesicles. In the present work, we report the first use of mixed stearoyllysophosphatidylcholine (C18-lyso-PC) dispersions as the donor and acceptor species for the study of sterol exchange kinetics. We report herein the rates of exchange of [4-^{14}C]sitosterol and [4-^{14}C]cholesterol between unilamellar vesicles prepared with different phospholipids and different sterol-to-phospholipid molar ratios and between dispersions prepared with 50 mol % C18-lyso-PC.[9] The data show that the presence of the 24α-ethyl group impedes the rate of movement of sterol but not the extent of exchange at equilibrium between bilayers prepared with egg PC and dipalmitoyl-PC over a wide range of sterol-to-phospholipid ratios. The exchange rate of sitosterol between egg sphingomyelin bilayers is also lower than that of cholesterol. Our results suggest that the C_{24}-alkyl substituent of sitosterol may have a significant effect on the distribution of the sterol between micelles and plasma membranes of intestinal cells, implying that simple competition between micelles and plasma membranes may contribute significantly to the early events in the absorptive discrimination of sterols observed in vivo during the interaction of digested sterols with the surface of small intestinal enterocytes.

Experimental Section

Materials. [4-^{14}C]Cholesterol (specific activity 57.5 mCi/mmol) and [9,10-^{3}H(N)]glycerol trioleate (specific activity 15.4 Ci/mmol) were purchased from DuPont New England Nuclear (Boston, MA), and [4-^{14}C]sitosterol (specific activity 56 mCi/mmol) was obtained from Amersham Corp. (Arlington Heights, IL). Egg PC, egg PG, dipalmitoyl-PC, egg sphingomyelin, dicetyl phosphoric acid, cholesterol, sitosterol, bovine serum albumin (Fraction V, fatty acid poor), diethylaminoethyl (DEAE)-Sepharose CL-6B, and DEAE-Sephacel were purchased from Sigma Chemical Co. (St. Louis, MO). 1-Stearoyl-*sn*-glycero-3-phosphocholine (C18-lyso-PC) was obtained from Avanti Polar Lipids (Pelham, AL) and from Sigma. Cholesterol and sitosterol were recrystallized twice before use from ethanol–acetone (95:5 v/v). Lipid purities were assayed by thin-layer chromatography (TLC) on silica gel G plates (Analtech, Newark, DE) with solvent systems described previously.[10] The solvent system for C18-lyso-PC was chloroform–methanol–water, 65:35:8. The sterols, PCs, and egg sphingomyelin migrated as single spots when visualized by spraying with H_2SO_4 solution and charring.[11] The sphingomyelin used in this study was purified from egg yolk and was highly enriched in saturated fatty acids (24:0, 22:0, and 16:0).[12] The purities of the radiolabeled sterols were determined by TLC (elution with ethyl acetate–benzene, 3:2 v/v); zones were cut at positions corresponding to standards and counted in 5 mL of scintillation cocktail (Ecoscint, National Diagnostics, Highland Park, NJ). It was determined that the purities of [4-^{14}C]cholesterol and [4-^{14}C]sitosterol were ≥99%, with ~0.3% of each 7-ketosterol present. Since the TLC system did not separate sitosterol from campesterol (24α-methylcholesterol), we used reverse-phase HPLC (C_{18} Carbosphere, 4.6 × 250 mm column, elution with acetonitrile–2-propanol, 4:1 v/v, flow rate of 2.0 mL/min) to analyze the purity of labeled sitosterol. We found the labeled sitosterol to be more than >98% pure. Thus, TLC and HPLC indicated that the radioactive and chemical purity of the labeled sitosterol was >98%. The purity of nonlabeled sitosterol was checked by using the same reverse-phase HPLC conditions (detection at 210 nm) and by GC/MS (30 m × 0.25 mm i.d., 0.25 μm DB-5 bonded phase fused silica capillary column, J & W Scientific, Folsom, CA). Most preparations were found to consist of about 55% sitosterol (HPLC, R_t 24.2 min, flow rate 2.0 mL/min; GC, R_t 24.8 min, flow rate 1.0 mL/min; MS, m/z 414.45) and 45% campesterol (HPLC, R_t 21.2 min, flow rate 2.0 mL/min; GC, R_t 20.8 min, flow rate 1.0 mL/min; MS, m/z 400.30). Sitosterol was purified by semipreparative HPLC (C_{18} Carbosphere, 10 × 250 mm column, elution as described above); analysis on the analytical HPLC column indicated that the purified sitosterol contained only 8% of campesterol as the sole contaminant. The HPLC-purified sitosterol was used where indicated.

Preparation and Characterization of Vesicles. The vesicles were prepared by sonication in 20 mM sodium phosphate buffer containing 1 mM EDTA and 0.05% w/v sodium azide, pH 6.0, as described previously[13] with a slight modification. Donor (1.0 mM total lipid) and acceptor (10.9 mM total lipid) vesicles contained the same phospholipid (i.e., egg PC, dipalmitoyl-PC, or egg sphingomyelin) in order to minimize phospholipid exchange during the prolonged incubation periods required to approach equilibrium. The molar ratio of sterol to phospholipid was the same in the donor and acceptor. Donor vesicles contained 15 mol % dicetyl phosphate to confer negative charge (to permit chromatographic separation) and a trace (0.08 μCi) of radiolabeled sterol as the exchangeable lipid. The neutral acceptor vesicles contained a trace (0.04 μCi) of [^{3}H]triolein as a nonexchangeable marker to monitor their recovery. Aqueous dispersions of the lipids were incubated for 10 min in a water bath maintained at ~10 °C above the phospholipid phase transition temperature and were then sonicated at room temperature in a Heat Systems Ultrasonics (Farmingdale, NY) Model W375 sonicator. The donor vesicles were sonicated with a cup-horn for a continuous period of 1 h, and the acceptor vesicles were sonicated with a microtip at 50% duty cycle for 1 h. The sonicated lipid dispersions were centrifuged for 10 min at 10 000 rpm in a DuPont Sorvall centrifuge with a SS-34 rotor. Pellets containing undispersed lipids, multilamellar liposomes, and titanium fragments were discarded.

The sizes and homogeneities of the donor vesicles were estimated by photon correlation spectroscopy at 50 °C and 90° scattering angle with a 15 mW He-Ne laser (632.8 nm) equipped with a Brookhaven Instruments Corp. (Holtsville, NY) Model Bi-2030AT 128-channel digital correlator. (We did not measure the sizes of the acceptor vesicles because their curvature does not influence the rate of sterol exchange between vesicles.[13]) Polystyrene spheres were used as size standards. Vesicles were prepared as described above except that the buffer was prefiltered five times through Millipore GS 0.2-μm filters. The total lipid concentration was usually 1 mM. Some preparations were diluted to 0.1 mM, without significant change in size, indicating that intervesicular interactions did not influence the measurements. The diameters of the donor vesicles (index of polydispersity of parentheses) prepared with dipalmitoyl-PC, 15 mol % dicetyl phosphate, and 1, 10, and 50 mol % cholesterol were in the range of 188–264 nm (0.19–0.29); dipalmitoyl-PC with the corresponding mol % sitosterol had diameters in the range of 177–221 nm (0.17–0.28). Vesicles prepared from egg PC and sterols had a similar diameter, 156–175 nm (0.17–0.19). Sphingomyelin-containing vesicles with cholesterol or sitosterol and egg PC had diameters in the range of 200–240 nm. It should be noted that the diameters of the sonicated vesicles appear larger than expected, probably because ultrasonic radiation generated by using the cup horn is of significantly lower energy than that from a standard microtip. Negative-staining electron microscopy showed that the donor vesicles were entirely unilamellar. The sizes of the vesicles employed in this study (range of ~150–265-nm diameter) are larger than the sizes of vesicles found to display a dependence between the rate of cholesterol efflux and vesicle curvature (e.g., ref 14). Thus, the differences we found in sterol exchange rates between vesicles of different composition do not arise from a marked difference in vesicle size.

Preparation of a Biosurfactant Model System. Both the donor and acceptor particles contained 50 mol % C18-lyso-PC. This model system resembles the egg lyso-PC/egg PC/cholesterol mixed lipid "micellar" system used previously as *donors* of cholesterol to brush border membrane vesicles as acceptors.[15,16] Our donor dispersions contained 15 mol % of negatively charged lipids [egg phosphatidylglycerol (PG)] to impart a charge to allow separation from neutral acceptors on ion-exchange columns, whereas the acceptor dispersions were neutral. Sterol, egg PC, and egg PG (when present) in chloroform and C18-lyso-PC in chloroform/methanol 2:1 were mixed and dried under a stream of nitrogen and then under vacuum overnight. The donor preparation (total lipid concentration, 2 mM) consisted of 1.5 mol % sterol (with a trace of [^{14}C]-sterol), 15 mol % egg PG, 33.5 mol % egg PC, and 50 mol % C18-lyso-PC. The acceptor preparation (total lipid concentration, 20 mM) contained 1.5 mol % sterol, 48.5 mol % egg PC, 50 mol % C18-lyso-PC, and a trace of [^{3}H]triolein as a nonexchangeable marker. The lipid film was dispersed in 20 mM sodium phosphate buffer, pH 7.4, containing 0.05% w/v sodium azide, without sonication. After incubation at 37 °C for 30

(9) We used C18-lyso-PC instead of a bile salt to form a micellar-type dispersion because we were unable to obtain high recovery of acceptor micelles in the eluate of DEAE-Sepharose and DEAE-Sephacel columns when we attempted to measure sterol transfer rates between two populations (neutral and charged) of bile salt micelles. Lyso-PC dispersions (containing 50 mol % C18-lyso-PC) are used here as a biosurfactant model system; freeze-fracture electron microscopy of these preparations indicated that they consist of mixtures of micelles and small vesicles.

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(16) Although Thurnhofer and Hauser (ref 15) referred to the donor mixture as mixed micelles, we use the more general term dispersions to refer to the surfactant-containing mixtures because freeze-fracture electron microscopy indicated that they consist of mixtures of micelles and small vesicles.

min, donors and acceptors were shaken vigorously by vortexing until the dispersions were clear. Quasi-elastic light scattering was used to assess the size and polydispersity of the donor particles. The average mean diameters of the cholesterol-containing donors (0.7–2.0 mM total lipid concentration) were 165–180 nm, with a polydispersity of 0.15–0.22; sitosterol-containing preparations had average mean diameters of 210–220 nm and a polydispersity of 0.20. Freeze-fracture electron microscopy indicated that the dispersions consisted of a mixture of micelles and vesicles. The mean diameter of the latter, as estimated by electron microscopy, was ~95–110 nm; similar discrepancies in vesicle sizes estimated by light scattering and electron microscopy have been found.

Efflux of Trapped [^{14}C]Glucose. A trace of D-[U-^{14}C]glucose (1.5×10^6 dpm), specific activity 290 Ci/mol (Research Products International), was added to 6 mL of a 5 mM glucose/50 mM NaCl solution. A portion (1.5 mL) of this solution was added to a dry film of lipids (1.5 mol % sterol, 15 mol % egg PG, 33.5 mol % egg PC, 50 mol % C18-lyso-PC; 10 mM total lipid). After the lipid film was dispersed as described above, the dispersions were placed in dialysis sacs, and untrapped glucose was removed by extensive dialysis at 4 °C against large volumes of 50 mM NaCl solution, with frequent changes of the dialysate. We found that <1% of the glucose was trapped; under similar conditions, liposomes prepared from dipalmitoyl-PC and sterol trapped >4% of the total [^{14}C]glucose at 4 °C. Thus, the mixed C18-lyso-PC dispersions have a very small trapped aqueous volume.

Measurement of [^{14}C]Sterol Movement between Vesicles. Acceptor vesicles with 4% albumin (w/v) and donor vesicles without albumin were preincubated separately at 50 °C in a Labline shaking water bath. Exchange was initiated by mixing and vortexing equal volumes of donor and acceptor vesicles. Incubations were carried out at 50 °C, which is above the transition temperatures of the phospholipids we used. Aliquots (200 μL) of the incubation mixture were applied to DEAE-Sepharose CL-6B columns (0.6 × 3 cm) at room temperature; the columns were prewashed with the buffer. To begin the collection of the eluate, the column was washed with 300 μL of buffer as soon as the aliquot had entered completely into the column. An additional 1 mL of buffer was applied to the column, and the eluate was collected in the same liquid scintillation vial. To the eluate was added 8 mL of ScintiVerse II (Fisher Scientific Co.) or 5 mL of Ecoscint scintillation cocktail. After the mixture was shaken by vortex for about 30 s, the vials were counted in a Packard 2000CA liquid scintillation counter for 10 min. Data were corrected for spillover (typically, 2.5% spillover of ^{3}H dpm into the ^{14}C channel, 0% spillover of ^{14}C dpm into the ^{3}H channel). The recovery of the neutral acceptor vesicles in the eluate was approximately 85–90%, and <0.5% of the negatively charged donor vesicles were eluted.[13]

Measurement of [^{14}C]Sterol Movement between C18-lyso-PC-Containing Dispersions. Donor and acceptor dispersions were incubated at 37 °C, and the exchange was initiated by mixing equal volumes of donors and acceptors. Aliquots (200 μL) were withdrawn at the desired time intervals, placed on Pasteur pipets containing DEAE-Sepharose CL-6B or DEAE-Sephacel, and eluted by washing the column first with 100 μL of buffer (after the incubation mixture had entered into the column completely) and then with an additional 1 mL of buffer. The retention of ^{3}H dpm in the eluate (which consists of the neutral C18-lyso-PC dispersions) as a function of time of incubation indicated that the recovery of acceptor micelles was efficient (>80%) and that the percent transfer of [^{3}H]triolein to the donor particles was not extensive.

Analysis of Sterol Oxidation. The extent of cholesterol oxidation in vesicles subjected to 4-day incubation periods at 50 °C was examined by using two methods. The total lipids were extracted with chloroform–methanol (1:1, v/v). In a gas chromatographic method of analysis, an aliquot of the extracted lipids was injected onto a 30 m × 0.25 mm DB-5 fused silica capillary column (J & W Scientific) in a Hewlett-Packard Model 5988A GC/MS equipped with an H-P 1000 data system. Under the conditions used (1.0 mL/min flow rate), the R_t values for cholesterol, 7-ketocholesterol, 7α-hydroxycholesterol, and 7β-hydroxycholesterol were 16.8, 20.0, 10.8, and 10.7 min, respectively. No oxidized sterol was detected in the lipid extracts, indicating that 1 mM EDTA is an effective agent in protection of cholesterol from oxidation, as shown previously.[17] Since underivatized oxysterols may decompose at the high temperature required for GC/MS analysis, we also examined the lipid extracts by preparative TLC on silica gel GF plates (1000 μm, Analtech). The total dpm of ^{14}C applied to the plates was ≥200 000. The plates were eluted twice with ethyl acetate–benzene (3:2, v/v), which resolved the sterols with the following R_f values: cholesterol, 0.92; 7-ketocholesterol, 0.71; 7α-hydroxycholesterol, 0.49; and 7β-hydroxycholesterol, 0.57. Zones corresponding to these standards were cut and eluted with chloroform–methanol (2:1, v/v). Analysis of the residues obtained after evaporation of the solvents was by liquid scintillation counting in 5 mL of Ecoscint. Only 1.2% of the total [4-^{14}C]cholesterol was converted to oxidized sterols, of which 92% was 7-ketocholesterol, 6% was 7β-hydroxycholesterol, and 2% was 7α-hydroxycholesterol. The polar lipid fraction at the origin of the plate was found to contain no ^{14}C dpm.

Analysis of Rates and Extents of Sterol Exchange. The fraction of labeled sterol undergoing exchange at time t is defined as α_t and is calculated as follows:

$$\alpha_t = [^{14}C/^3H]_t/[^{14}C/^3H]_{mix}$$

where $[^{14}C/^3H]_t$ and $[^{14}C/^3H]_{mix}$ represent the ratio of [^{14}C]sterol to [^{3}H]triolein in the eluate at time t and in the donor–acceptor vesicles or lyso-PC-containing dispersions without separation, respectively. The initial values of exchange of labeled sterol at times zero and infinity are α_0 and α_∞, respectively. These values were estimated by using a simplex iterative least-squares program.[18] In this analysis, all of the kinetic data (α_t), including experimental points obtained at times approaching the equilibrium value, were fit to eq 1:

$$F(t) = \alpha_\infty + [(\alpha_0 - \alpha_\infty)\exp(\text{slope} \times t)] \quad (1)$$

The rate constant and half-time of exchange were calculated with the following relationships:

$$k = -\text{slope}/1.1 \quad (2)$$

$$t_{1/2} = \ln 2/k \quad (3)$$

The size of the exchangeable pool of labeled cholesterol, X_{xch}, was calculated from the relationship

$$X_{xch} = [(\alpha_\infty - \alpha_0)/(1 - \alpha_0)] \times 1.1 \quad (4)$$

The factor of 1.1 is used in eq 2 to correct the rate constant of the unidirectional rate of [^{14}C]sterol movement from donor-to-acceptor species for back exchange of label; in eq 4, the factor of 1.1 is used to normalize the equilibrium value of 90.9% expected for random [^{14}C]sterol distribution between donor and acceptor particles (acceptor/donor, 10:1) to 100% exchange. The curves of fraction exchanged vs time (Figures 1–3) where obtained by an interpolative fit of $F(t)$, which was calculated by eq 1. In the semilog plots, the solid lines were obtained by an exponential fit of $1 - (X_t/X_\infty)$, which was calculated from the equation $1 - (X_t/X_\infty) = \exp(\text{slope} \times t)$, where $X_t = F(t) - \alpha_0$ and $X_\infty = (\alpha_\infty - \alpha_0)$.

Results

Kinetics of Exchange of Purified Sitosterol between Dipalmitoyl-PC and Egg PC Vesicles. To compare the rates of intermembrane movement of sitosterol and cholesterol we measured the time course of sterol exchange from donor to acceptor unilamellar vesicles of different PC acyl chain compositions and different PC/sterol molar ratios at 50 °C. Figure 1 gives the time course of exchange between vesicles containing 24 mol % sterol, and the insets show semilog plots of labeled sterol remaining in the donor vesicles as a function of time. Unlabeled sitosterol was purified from the commercially available, naturally occurring mixture of sitosterol/campesterol by HPLC (see Experimental Section). Both sterols undergo exchange between dipalmitoyl-PC vesicles at a much slower rate than between egg PC vesicles. In order to approach equilibrium in the dipalmitoyl-PC vesicle system it was necessary to carry out exchange measurements over a 5-day period. Nevertheless, the recovery of acceptor vesicles remained very high as judged by the retention of [^{3}H]triolein, indicating that fusion between donor and acceptor vesicles did not increase during the prolonged incubation; also, oxidation of cholesterol could not be detected (see Experimental Section). Exchange of both sterols between egg PC vesicles was relatively fast (Figure 1B), reaching equilibrium within a 7–48-h period for cholesterol and 50–100-h period for sitosterol. The extent of sterol exchange at equilibrium (X_{xch}) was similar for both sterols, and essentially all of the sterol was fully exchangeable. The inset to Figure 1 shows that the semilog plots are linear (correlation coefficients >0.99), indicating that both sterols undergo exchange in one kinetic pool. The rate of sterol exchange from PC bilayers containing unsaturated acyl chains is much faster than that from saturated PC bilayers; the half-times in egg PC bilayers are 296

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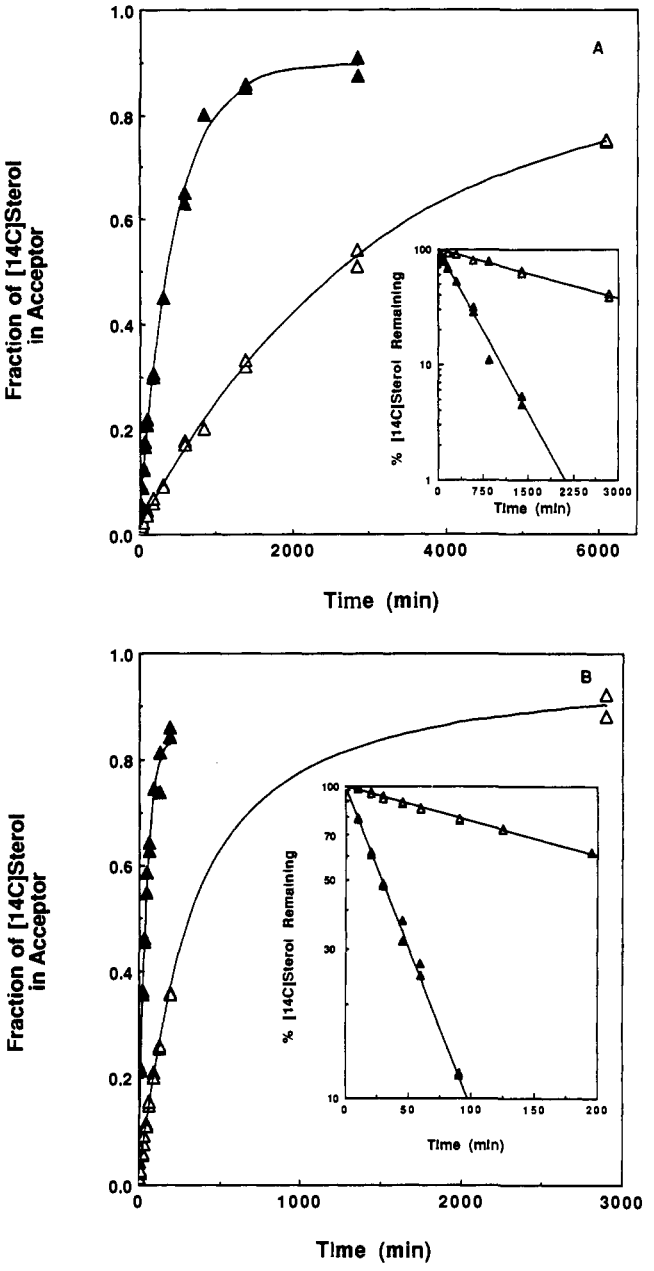

Figure 1. Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange between PC/sterol unilamellar vesicles at 50 °C. The vesicles contained 24 mol % sterol; sitosterol was purified by HPLC as described in the Experimental Section. Albumin was omitted in the incubation media. The host phospholipid in both donor and acceptor vesicles was (A) dipalmitoyl-PC and (B) egg PC. Inset, first-order plot of the exchange data: (a) (▲) $t_{1/2}$ 349.8 min (X_{xch} = 0.98), (△) $t_{1/2}$ 2337.5 min (X_{xch} = 0.95) and (B) (▲) $t_{1/2}$ 32.3 min (X_{xch} = 0.99), (△) $t_{1/2}$ 296 min (X_{xch} = 0.91).

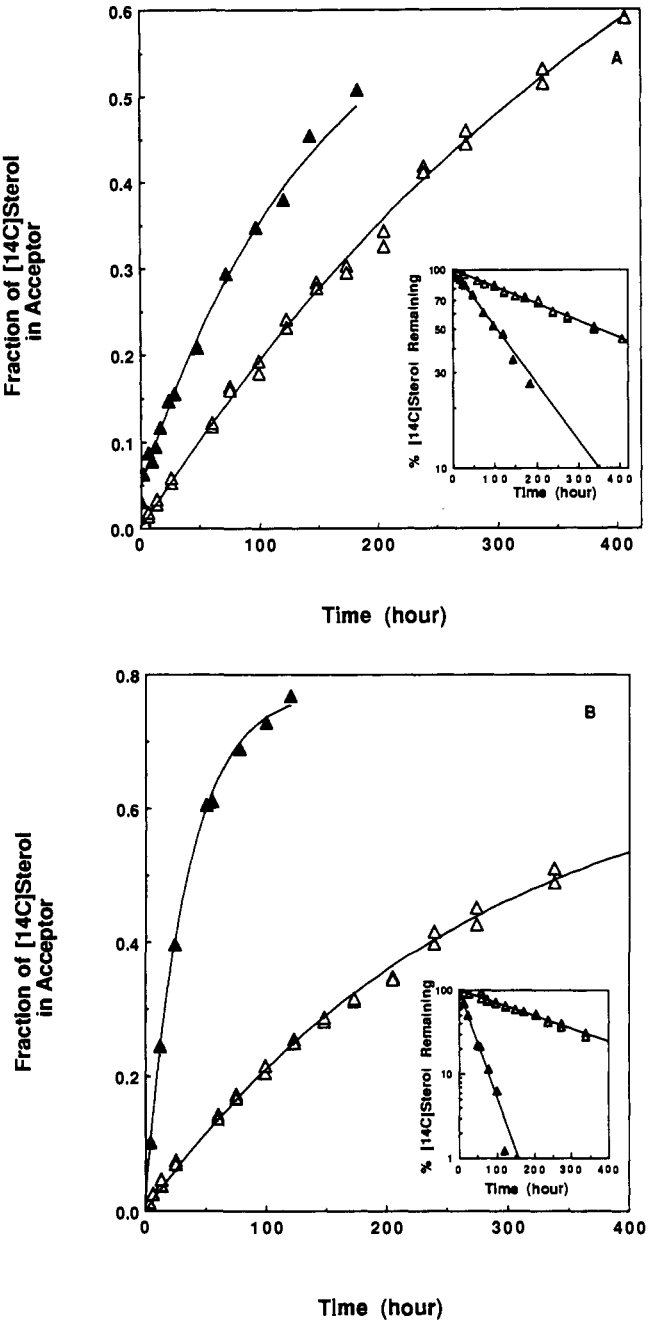

Figure 2. Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange at 50 °C between egg sphingomyelin unilamellar vesicles. The vesicles contained 10 mol % sterol. Sitosterol from Sigma contained 45 mol % campesterol and was used without further purification. Exchange experiments were run with albumin (2% w/v) (A) or without albumin (B). Inset, first-order plot of the exchange data: (A) (▲) $t_{1/2}$ 116 h (X_{xch} = 0.73), (△) $t_{1/2}$ 380 h (X_{xch} = 1.0) and (B) (▲) $t_{1/2}$ 26 h (X_{xch} = 0.86), (△) $t_{1/2}$ 216 h (X_{xch} = 0.78).

min for sitosterol and 32 min for cholesterol exchange, compared with 2338 min for sitosterol and 350 min for cholesterol exchange in dipalmitoyl-PC bilayers. These half-times for cholesterol exchange are consistent with data obtained in other studies of cholesterol exchange by several investigations (for reviews, see refs 19–21). The data are explained by reduced packing constraints in membranes prepared with phospholipids having unsaturated acyl chains.

Comparison of Cholesterol and Sitosterol Exchange Kinetics between PC/Sterol Vesicles at Various Mol %. Our previous study showed that even when present at only 1 mol % of the membrane lipid, sitosterol undergoes exchange between dipalmitoyl-PC vesicles and between egg PC vesicles more slowly than does cholesterol in the presence of albumin (2% w/v).[22] The vesicles in the previous study were prepared with the naturally occurring mixture of sitosterol and campesterol. Albumin was added because it is well-known that it binds to and modulates the biologic actions of many lipids,[23] including the stimulation of the rate of intermembrane cholesterol transfer without altering bilayer structure

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Table I. Influence of the 24-Alkyl Group on the Kinetics of Sterol Exchange between Dipalmitoyl-PC and Egg PC Vesicles at 50 °C[a]

mol % sterol	dipalmitoyl-PC vesicles: cholesterol $t_{1/2}$[c] (min)	dipalmitoyl-PC vesicles: sitosterol[b] $t_{1/2}$ (min)	dipalmitoyl-PC vesicles: sito/chol $t_{1/2}$ ratio	egg PC vesicles: cholesterol $t_{1/2}$ (min)	egg PC vesicles: sitosterol[b] $t_{1/2}$ (min)	egg PC vesicles: sito/chol $t_{1/2}$ ratio
1[c]	222.3 ± 43.2	990.8 ± 76.0	4.4	14.9 ± 1.3	109.1 ± 12.7	7.3
6				23.5 ± 10.6	136.6 ± 29.9	5.8
10	293.0 ± 48.1	1183.0 ± 60.2	4.0			
24	499.9 ± 78.4			26.1 ± 12.7	104.9 ± 19.8	4.0
50	60.7 ± 15.3	171.5 ± 20.7	2.8	31.2 ± 0.1	176.3 ± 12.6	5.7

B. Data Obtained with Purified Sitosterol in the Presence[d] or Absence[e] of Albumin

mol % sterol	dipalmitoyl-PC vesicles: cholesterol	dipalmitoyl-PC vesicles: sitosterol[f]	dipalmitoyl-PC vesicles: sito/chol $t_{1/2}$ ratio	egg PC vesicles: cholesterol	egg PC vesicles: sitosterol[f]	egg PC vesicles: sito/chol $t_{1/2}$ ratio
24	500 ± 78[d]	1707 ± 165[d]	3.4			
24	334 ± 19[e]	2338 ± 205[e]	7.0	32[e]	296[e]	9.2

[a] Vesicles were prepared with a constant total lipid concentration (donor vesicles, 1.0 mM; acceptor vesicles, 10.0 mM) and various concentrations of sterol and phospholipid. The $t_{1/2}$ values are the means ±SE of at least three different vesicle preparations. [b] A naturally occurring mixture obtained from Sigma was used; HPLC analysis indicated that it consisted of 55% sitosterol and 45% campesterol. [c] Data from ref 22. [d] Albumin (2%, w/v) was present in the incubation medium. [e] Albumin was omitted. [f] HPLC-purified sitosterol was used.

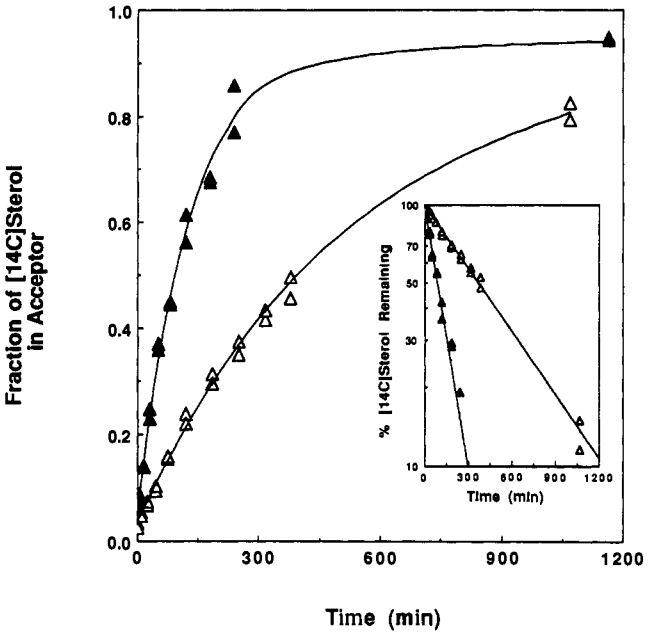

Figure 3. Time course of [^{14}C]cholesterol (▲) and [^{14}C]sitosterol (△) exchange at 37 °C between C18-lyso-PC-containing dispersions. Both donor and acceptor dispersions contained egg PC and 1.5 mol % sterol. Sitosterol was purified as described in the Experimental Section. Albumin was not used in the experiments. Inset, first-order plot of the exchange data: (▲) $t_{1/2}$ 100 min (X_{xch} = 1.0), (△) $t_{1/2}$ 409.6 min (X_{xch} = 1.0).

in the liquid-crystalline phase.[10b,24] Table I compares the half-times for sterol exchange at various molar ratios of sitosterol and cholesterol in dipalmitoyl-PC and egg PC bilayers in the presence and absence of albumin. The data in Table I show that the rate of [^{14}C]sitosterol exchange between dipalmitoyl-PC and between egg PC vesicles is markedly lower than that of [^{14}C]cholesterol, independent of whether purified sitosterol or the sitosterol/campesterol mixture is used in the vesicles and independent of whether albumin is present or absent. The exchange rates of both sitosterol and cholesterol increase markedly as an equimolar ratio of dipalmitoyl-PC to sterol is approached (Table IA). However, in egg PC/sterol vesicles the exchange rates of both sterols decrease slightly as the sterol content of the bilayer is increased from 1 to 50 mol %, suggesting a difference in phase structure in sterol/dipalmitoyl-PC vs sterol/egg PC vesicles.

Comparison of Cholesterol and Sitosterol Exchange Rates between Sphingomyelin/Sterol Vesicles. We also investigated the exchange of sterols between sphingomyelin bilayers in the presence or absence of albumin. The very slow rate of cholesterol exchange from egg sphingomyelin bilayers (Figure 2), indicative of tight packing between sphingomyelin and cholesterol, has been observed

Table II. Comparison of Cholesterol and Sitosterol/Campesterol Exchange Kinetics between Egg Sphingomyelin Vesicles and between Dipalmitoyl-PC Vesicles at 50 °C[a]

sterol (10 mol %)	egg sphingomyelin: half-time (h)	egg sphingomyelin: X_{xch}[b]	dipalmitoyl-PC: half-time (h)	dipalmitoyl-PC: X_{xch}
cholesterol	126 ± 15	0.73	4.9 ± 0.8	1.0
sitosterol	306 ± 104	0.96	19.7 ± 1.0	1.0

[a] Albumin (2% w/v) was present in the donor-acceptor incubation medium. [b] The fraction of exchangeable [^{14}C]sterol, X_{xch}, at equilibrium was calculated as described in the Experimental Section.

previously over a limited range of cholesterol concentrations in mixed glycerolipid-sphingomyelin bilayers.[13,25] We used incubation periods of about ~7 days in order to allow exchange to approach the equilibrium position. During this period we saw no visible flocculation or coalescence of vesicles, and we estimated that fusion of unilamellar vesicles and oxidation of cholesterol were minimal based on the recovery of ^{3}H dpm and TLC/GC analysis of the extracted lipids. Figure 2 shows that sitosterol at 10 mol % undergoes exchange more slowly than cholesterol at the same concentration, both in the presence and absence of albumin. The half-times for exchange of both sterols are much higher in sphingomyelin than in dipalmitoyl-PC bilayers (Table II), which reflects the high lateral packing density of sphingomyelin molecules relative to PC molecules.[25e] A comparison of the data in Figure 2 (parts A and B) indicates that the rates of exchange of sitosterol and cholesterol between sphingomyelin bilayers are decreased by factors of 2 and 4, respectively, when 2% w/v albumin is added; the X_{xch} values of the sterols are not markedly affected by addition of albumin. In contrast, albumin (at concentrations of 1 and 2% w/v) increased the rate of cholesterol movement between lipoproteins and fibroblasts[24c] and between mycoplasma membranes and PC vesicles.[10b,24a,b]

The extent of exchange of cholesterol between egg sphingomyelin bilayers is lower than that found between dipalmitoyl-PC bilayers at 10 mol % sterol, whereas sitosterol underwent essentially complete exchange. These results are in contrast to those obtained by Bar et al.,[26] who observed a much larger nonexchangeable pool in egg sphingomyelin bilayers at 50 °C (see Discussion section).

Kinetics of Sitosterol Exchange between C18-lyso-PC Aqueous Dispersions. In order to determine whether the rates of sitosterol

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and cholesterol exchange differ in surfactant-containing preparations, we used an experimental system containing 50 mol % C18-lyso-PC. Figure 3 shows that both sterols undergo first-order exchange from donor dispersions to an excess of acceptor dispersions at 37 °C. In agreement with the results obtained in PC and sphingomyelin vesicles, sitosterol underwent exchange between the C18-lyso-PC-containing dispersions much more slowly than cholesterol. The average $t_{1/2}$ values for sitosterol and cholesterol exchange are 438 ± 64 min and 105 ± 11 min, respectively, indicating that the 24α-ethyl group of sitosterol decreases the ease of sterol desorption from the lipid–water interfaces of these surfactant-enriched surfaces by a factor of 4 at 37 °C. As was observed in Figure 1, both sterols and fully exchangeable in a single kinetic pool.

Discussion

This study was carried out in order to determine the effects of alkylation at C_{24} of the sterol molecule on the kinetics of sterol exchange between phospholipid/sterol membranes and lysophospholipid/phospholipid/sterol dispersions. The influence of structural modifications in the sterol side chain on dynamic processes involving sterol movement between membranes or across the two monolayers of membrane bilayers has not been studied extensively, but several studies have demonstrated significant differences in intermembrane rates of sitosterol and cholesterol transfer. The rates of exchange of ^{14}C-labeled sterols from mycoplasma membranes to an excess of acceptor lipid vesicles were used previously to study the influence of sterol side-chain structure on the transbilayer distribution of sterols.[10] Sterols with alkyl groups at C_{24} were found to accumulate in the outer half of the bilayer, possibly because they experience steric interference during translocation from the outer to inner monolayer. Initial rates of transfer of cholesterol analogues between vesicles were also used to estimate the effects of changes in sterol side-chain structure on partitioning between dipalmitoyl-PC and egg PC bilayers.[27] These investigators found that cholesterol partitioned preferentially into dipalmitoyl-PC vesicles vs egg PC vesicles at 37 °C, whereas sitosterol and campesterol partitioned about equally into these phospholipids at this temperature. Cholesterol was incorporated into erythrocytes more readily than sitosterol, and the classes of phospholipids present in the erythrocyte membranes or in the micellar solution appeared to affect the degree of selectivity of sterol uptake.[28] A 4-fold greater uptake of 7-dehydrocholesterol over 7-dehydrositosterol into brush border membrane vesicles and rat erythrocyte membranes from bile salt-egg PC solution was observed.[29] The extent of cholesterol transfer between erythrocytes and plasma was greater than that of sitosterol, which was considered to be related to the higher rate of esterification of cholesterol by plasma lecithin:cholesterol acyltransferase.[30]

Ratio of Sitosterol/Cholesterol $t_{1/2}$ Values in PC, Sphingomyelin, and C18-lyso-PC Systems. The rate of sterol movement from saturated and unsaturated PC- or sphingomyelin–water interfaces is sensitive to the presence of the 24α-ethyl group of sitosterol (Figures 1 and 2). The ratios of the half-times for sitosterol/cholesterol exchange between dipalmitoyl-PC vesicles and between egg sphingomyelin vesicles containing 10 mol % sterol are about 4 and 2.4, respectively (Table II). This is consistent with our observation of a sitosterol/cholesterol $t_{1/2}$ ratio of ~6 from vesicles prepared from 6 mol % sterol, 44 mol % egg PC, 35 mol % egg sphingomyelin, and 15 mol % dicetyl phosphate.[22] At 24 mol % sterol, with HPLC-purified sitosterol, the ratio of the half-times for sitosterol/cholesterol exchange between egg PC vesicles is 9.2 in the absence of albumin (Figure 1); this ratio for exchange between dipalmitoyl-PC vesicles is 7.0 in the absence of albumin (Figure 1) and 3.4 in the presence of albumin (Table I). At 50 mol % sterol, with the naturally occurring mixture of 24-alkylated sterols, the ratios of the half-times for sitosterol/cholesterol exchange between dipalmitoyl-PC vesicles and egg PC vesicles are 3 and 6, respectively (Table I). The lower rate of sitosterol movement between vesicles prepared from dipalmitoyl-PC, egg PC, and egg sphingomyelin, both with and without albumin, indicates that membrane phospholipid composition and extent of saturation of fatty acyl chains did not dictate the sitosterol/cholesterol exchange rate ratio. The sitosterol/cholesterol $t_{1/2}$ ratio is 4 in aqueous dispersions containing 50 mol % C18-lyso-PC (Figure 3). These results differ from previous studies that showed a loss of selectivity of cholesterol vs sitosterol uptake into rat erythrocytes and upper villus cells when dipalmitoyl-PC and egg sphingomyelin were added to the micellar incubation medium in place of egg PC.[28b,31]

The model postulated for the transition state in cholesterol transfer between vesicles is that cholesterol is attached to the surface of the donor species by the tip of its hydrophobic side chain.[19] The rate-limiting step in sterol exchange or transfer from donor vesicles to an excess of acceptor vesicles is desorption from the outer monolayer of the donor membranes into the aqueous phase.[19] Our results indicate that the substitution of an ethyl group for a hydrogen at C-24 impedes the slow dissolution of the lipid from the surface of PC and sphingomyelin vesicles, probably by increasing the molecular packing in the bilayer and/or decreasing the aqueous phase solubility of the sterol. Additional van der Waals interactions are possible between phospholipid hydrocarbon chains and the sitosterol side chain compared with the cholesterol side chain; these favorable sterol–phospholipid interactions in the donor species may raise the energy of activation of the desorption process.

Influence of Sterol Mol % on Half-times. The rates of sitosterol and cholesterol exchange between dipalmitoyl-PC vesicles are much faster at 50 mol % sterol than at 1–10 mol % sterol (Table IA). In egg PC vesicles, however, the sterol exchange rates did not increase significantly on increasing the content of sterol from 6 to 50 mol %. The increase in exchange rate at high sterol content in dipalmitoyl-PC vesicles suggests that a region of sterol-rich clusters exists which facilitates sterol desorption. It is well-known that cholesterol is clustered in domains above ~33 mol % sterol.[32] McLean and Phillips[33] proposed that cholesterol desorbs from equimolar cholesterol-PC clusters in the bilayer, whereas Nemecz et al.[34] proposed that escape of cholesterol from cholesterol-rich clusters gives raise to a very slow exchange rate. We observe only one kinetic pool of cholesterol and sitosterol at both low and high sterol/phospholipid molar ratio. In order to account for the detection of only a single pool, sterol movement from interfacial regions between sterol-rich and sterol-PC phases must be rapid. The enhanced rates of sterol desorption at 50 mol % sterol in the donor dipalmitoyl-PC vesicles (sterol/PC molar ratio of 1.4) may arise from desorption from interfacial regions between phases. The absence of a similar rate enhancement at high sterol content in egg PC vesicles suggests that phase boundaries may not be present at 50 °C in this system. It should be noted that McLean and Phillips[33] also observed an increase in the rate of [^{14}C]-cholesterol efflux at 37 °C from dipalmitoyl-PC vesicles at very high sterol content (cholesterol/PC molar ratio of 3.4) compared with <50 mol % cholesterol. The rate enhancement at high cholesterol content was attributed to altered cholesterol–nearest neighbor interactions in the bilayer.

Extent of Sterol Exchange. Since all of the radiolabeled sitosterol and cholesterol underwent exchange between PC vesicles in a single kinetic pool, the rate of transbilayer sterol movement from the inner to outer leaflet of the donor vesicles must exceed that of exchange. Our observation that all of the cholesterol is exchangeable from vesicles prepared with saturated and unsaturated PCs is consistent with many earlier reports[19] but does not agree with recent studies in which a significant nonexchangeable

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pool of cholesterol was found in 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine vesicles at 37 °C[35] and at 24 °C[34] and in dimyristoyl-PC vesicles at 50 °C.[26] Although we did observe a sizeable (27%) nonexchangeable pool of cholesterol in egg sphingomyelin bilayers containing 10 mol % cholesterol (Table II), our results differ significantly from those of Bar et al.[26] who reported that 64% of the total [^{3}H]cholesterol in egg sphingomyelin vesicles containing 10 mol % cholesterol was not exchanged at 50 °C and from those of Bar et al.[36] who found that 47% of the cholesterol probe dehydroergosterol was nonexchangeable in bovine-brain sphingomyelin vesicles at 50 °C. Furthermore, we found near full exchangeability of cholesterol at other cholesterol/sphingomyelin molar ratios (Kan and Bittman, unpublished results).

Since our results are not strictly comparable to those reported by Bar et al.[26,35,36] and Nemecz et al.[34] because of differences in experimental design, we carried out the following experiments to examine possible sources of the discrepancy in the exchangeable cholesterol pool size. First, we attempted to measure radiolabeled cholesterol exchange from neutral donor vesicles containing sphingomyelin to acceptors containing egg PG or egg phosphatidic acid and sphingomyelin (as was done in refs 26 and 35) but were unsuccessful because of low recovery of the neutral vesicles. However, the discrepancy is unlikely to arise from the location of the negatively charged lipid since neutral and charged donor vesicles of PC gave similar rates of cholesterol exchange.[35,37] Another difference in experimental design that may affect exchangeability involves the presence of albumin, which was present in our exchange measurements (Figure 2A) but not in those of Bar et al.[26] and Nemecz et al.;[34] however, when we conducted exchange measurements without albumin (Figure 2B) we observed X_{xch} values of 0.86 and 0.78 for cholesterol and sitosterol, respectively. Third, the discrepancy in exchangeable pool size could arise from differences in methods of data analysis. However, some of our data sets were kindly analyzed by Dr. K. L. Bar with the iterative nonlinear least-squares program used by Bar et al.;[26,35] the values of $t_{1/2}$ and X_{xch} were quite similar to those obtained with our program. Finally, we considered the possibility that a difference in the time frame of the experiments affects the values of $t_{1/2}$ and X_{xch}. In the previous studies at 37,[35] 50,[26] and 24 °C,[34] exchange measurements were halted at about 8 h, whereas in the present studies aliquots were taken until equilibrium was approached and the endpoint was then estimated with the simplex program. We obtained lower values of X_{xch} and faster rates of exchange when data gathered before one half-time were used to compute these values. For example, by using the data obtained between 0 and 16.5 h for [^{14}C]cholesterol exchange from sphingomyelin bilayers we calculated $X_{xch} = 0.10$ and $t_{1/2} = 6.7$ h; for data points between 0 and 47.5 h, we calculated $X_{xch} = 0.15$ and $t_{1/2} = 11.1$ h; for data points between 0 and 97.6 h, we calculated $X_{xch} = 0.28$ and $t_{1/2} = 23.0$ h; for all of the experimental points (between 0 and 182 h) we calculated $X_{xch} = 0.73$ and $t_{1/2} = 116$ h. We therefore conclude that the apparent nonexchangeable pool size is increased when measurements are not extended to times that approach the equilibrium position. Other factors, presently unresolved, may also contribute to the differences in X_{xch} values determined in the present study compared with previous results.

Biological Relevance. Although exogenous sterol uptake in vivo is a complicated process, our results with sterol/phospholipid bilayers and sterol/lyso-PC/phospholipid dispersions have implications for the mechanism(s) by which dietary cholesterol is transferred preferentially into the outer leaflets of intestinal cell membranes compared with ingested phytosterols. The slower desorption of sitosterol we have observed from PC- and sphingomyelin-water interfaces over a wide range of sterol-to-phospholipid molar ratios suggests that slow spontaneous efflux of sitosterol relative to cholesterol contributes to the lower rate of sitosterol absorption by intestinal mucosal cells.

In assessing the biological relevance of this work, the possibility that cholesterol-binding protein(s) may be present in at least some intestinal cell membranes, as observed in brush border membranes from the small intestine of cholesterol-fed rabbits,[6b] should be considered. Membrane proteins that bind cholesterol preferentially may exist (see section IX of ref 21) and may have a physiological role in enhancing the extent of intestinal discrimination of sterol absorption. Indeed, protein-mediated transfer of cholesterol[15] and PC[38] into brush border vesicles derived from rabbit small intestine has recently been demonstrated. However, the finding that the membranes of red blood cells and brush borders showed similar preferential uptake of cholesterol over sitosterol[28b,29] casts some degree of doubt about the role of cholesterol-binding proteins in the absorptive *selectivity* of sterols. Furthermore, the exchange data presented here with both vesicles and lyso-PC-rich dispersions suggest that simple competition between mixed micelles and cell membranes can be an important factor in determining the relative rates of intestinal uptake of sterols; desorption of sitosterol from an "activated" micelle into an aqueous region may be significantly slower than desorption of cholesterol. It will be interesting to extend these studies to lipid mixtures containing glycolipids, which are also present in high concentration in intestinal epithelial membranes.

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Registry No. 4, 57-88-5; **1**, 83-46-5; C18-lyso-PC, 19420-57-6; dipalmitoyl-PC, 2644-64-6.

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