Interaction of Cholesterol with Sphingomyelin in Bilayer Membranes: Evidence That the Hydroxy Group of Sphingomyelin Does Not Modulate the Rate of Cholesterol Exchange between Vesicles†

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Received December 26, 1990; Revised Manuscript Received March 27, 1991

ABSTRACT: Cholesterol undergoes exchange between membranes containing sphingomyelin at a much slower rate than between membranes lacking sphingomyelin. To investigate the role of the hydroxy group at the 3-position of sphingomyelin in the interaction between sphingomyelin and cholesterol, we have measured the rates of [4-^{14}C]cholesterol exchange between unilamellar vesicles prepared with *N*-stearoylsphingomyelin or with synthetic analogues in which the hydroxy group is replaced with an *O*-alkyl group or with hydrogen. Vesicles prepared from 3-deoxy- and 3-*O*-methyl-*N*-stearoylsphingomyelin had the same rate of [^{14}C]-cholesterol desorption. The half-times for exchange from vesicles prepared with 3-*O*-methyl- and 3-deoxy-*N*-stearoylsphingomyelins and 10 mol % of cholesterol were only slightly faster (a factor of only 1.5) than that found from vesicles prepared from *N*-stearoylsphingomyelin and 10 mol % cholesterol. The rate of cholesterol desorption from vesicles could be accelerated by preparing vesicles from bulky 3-*O*-alkyl analogues of sphingomyelin. Vesicles containing 3-*O*-ethyl-*N*-stearoylsphingomyelin and 3-*O*-tetrahydropyranyl egg sphingomyelin gave rate enhancements of ~14 and 35, compared with the rates observed in vesicles made from *N*-stearoyl- and egg sphingomyelin, respectively. These data indicate that insertion of sterically bulky groups at the 3-position of sphingomyelin (such as ethoxy and tetrahydropyranyloxy) in place of hydroxy interferes markedly with the molecular packing of cholesterol and sphingomyelin in bilayer membranes; however, the hydroxy group of sphingomyelin is not critical for the strong interaction of cholesterol with sphingomyelin. These results suggest that van der Waals interactions are more important than hydrogen-bonding interactions involving the hydroxy group in contributing to tight lateral packing of cholesterol with sphingomyelin.

Cholesterol is a principal component of the plasma membrane of many mammalian cells. Among the major functions of cholesterol in plasma membranes is the modulation of the molecular motion of phospholipid acyl chains [e.g., see Hui (1988) and Yeagle (1988)]. Kinetic studies of radiolabeled cholesterol exchange between membranes have shown that the cholesterol molecules exchange more slowly in membranes containing SPM[1] than in those lacking SPM, indicating that cholesterol interfacial packing is tighter in SPM than in PC bilayers. In a binary mixture showing phase separation, differential scanning calorimetry studies indicated that SPM, either isolated from erythrocytes or with a chemically defined (*N*-palmitoyl) chain, interacted with cholesterol to a greater degree than did various synthetic phosphatidylcholines (Demel et al., 1977; Van Dijck, 1979). Fluorescence polarization measurements on phospholipid–cholesterol bilayers also indicated a higher degree of structural order in membranes from egg SPM than from various synthetic phosphatidylcholines (van Blitterswijk et al., 1987). Studies of the surface pressure–molecular area isotherms of mixed monolayers of cholesterol and SPM revealed that cholesterol has the capacity to condense bovine brain SPM to a greater extent than phosphatidylcholines (Lund-Katz, 1988; Grönberg & Slotte, 1990). The resistance of oxidation of cholesterol by cholesterol oxidase in monolayers is also related to strong sphingomyelin–cholesterol interactions (Grönberg & Slotte, 1990). Hence, there is evidence that the interaction of cholesterol with various naturally occurring (egg or bovine) or synthetic SPM is greater than that with egg or dipalmitoyl-PC in bilayer membranes.

Insights into the molecular basis for the higher apparent affinity of cholesterol for SPM have been lacking. Greater opportunities for van der Waals interactions have been considered for SPM compared with PC with similar acyl chain length and saturation (Lund-Katz et al., 1988), and hydrogen bonding has been postulated as a stabilizing force between the 3β-hydroxy group of cholesterol and sphingolipids (Boggs, 1987; Barenholz & Thompson, 1980). Although there is no *direct* evidence of such hydrogen bonding, the hydroxy and amide groups afford an important inter- and intramolecular hydrogen-bond capability (Pascher, 1976; Pascher & Sundell, 1977; Abrahamsson et al., 1977). In the current study, we explore the role of the 3-hydroxy group of SPM in the interaction with cholesterol by measuring the kinetics of [4-^{14}C]cholesterol exchange between vesicles. We synthesized SPM analogues in which the hydroxy group has been replaced by either a hydrogen atom or with a 3-*O*-methyl, 3-*O*-ethyl, or 3-tetrahydropyranyloxy group, with the amide linkage maintained (see Figure 1). Since the rate constant for cholesterol desorption is sensitive to differences in cholesterol–phospholipid interaction energy in bilayer membranes [e.g., see Phillips et al. (1987), Bittman (1988), and Lund-Katz et al. (1988)], our results reveal new insight into the extent to which the SPM hydroxy group contributes to SPM–cholesterol interactions.

† This research was supported by Grant HL-16660 from the National Institutes of Health.

[1] Abbreviations: PC, phosphatidylcholine; SPM, sphingomyelin; THP, tetrahydropyranyl.

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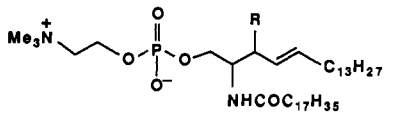


R = OH, *N*-stearoyl-SPM

R = OCH_3, 3-OMe-*N*-stearoyl-SPM

R = OC_2H_5, 3-OEt-*N*-stearoyl-SPM

R = H, 3-deoxy-*N*-stearoyl-SPM

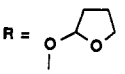
R =  (mixed-chain amide), 3-OTHP-egg SPM

FIGURE 1: Structures of the SPM derivatives used in this study.

Materials and Methods

Chemicals. Egg SPM, cholesterol, dicetyl phosphoric acid (DCP), and diethylaminoethyl-Sepharose CL-6B (DEAE-Sepharose CL-6B) were purchased from Sigma Chemical Co. (St. Louis, MO). [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 New England Nuclear (Boston, MA). Cholesterol was recrystallized twice before use from 95% ethanol–5% acetone. The purities of egg SPM and synthetic SPM analogues were assayed by thin-layer chromatography on silica G plates (Analtech, Newark, DE) in $CHCl_3$–CH_3OH–H_2O–NH_4OH (60:30:2:3 v/v). The SPM analogues migrated as single spots. The purity of cholesterol was assayed by TLC (Clejan et al., 1981) and by HPLC using a C18 column. [^{14}C]Cholesterol was analyzed by TLC as described by Clejan and Bittman (1984a); 98.2% of the total disintegrations per minute of [^{14}C]cholesterol was found to migrate as one hot zone.

Preparation and Characterization of Vesicles. Vesicles were made according to Fugler et al. (1985), with slight modification. The donor (1.0 mM, total lipid concentration) and acceptor (10.0 mM, total lipid concentration) vesicles contained 10 mol % of cholesterol. Acceptor vesicles were prepared with egg SPM rather than with the synthetic SPM analogues in order to keep the microenvironment in the acceptor vesicles similar to that in the donor vesicles. Donor vesicles contained 15 mol % DCP to confer negative charge and a trace of [^{14}C]cholesterol (0.08 μCi) as the exchangeable lipid. Acceptor vesicles contained a trace of [^{3}H]triolein (0.046 μCi) as a nonexchangeable marker to monitor the recovery of the neutral acceptors. To reduce oxidation of lipids during dispersion and incubation, EDTA (1 mM) was included in 20 mM sodium phosphate buffer, pH 6.0, as reported by Lijana et al. (1986). Aqueous dispersions were made from the lipid films by vortexing after incubation in a water bath at 50 °C for 10 min and were then sonicated in a Heat System Ultrasonics (Farmingdale, NY) Model W375 sonicator at room temperature. Donor vesicles were sonicated with a cup-horn for a continuous 1-h period; acceptor vesicles were sonicated with use of a microtip for 1 h at 50% duty cycle. The sonicated lipid dispersions were centrifuged for 10 min at 10000 rpm at 20 °C in a Du Pont Sorvall centrifuge Model RC5C (Sorvall Instruments, Wilmington, DE) with a SS-34 rotor. Pellets containing undispersed lipids, multilamellar liposomes, and titanium fragments were discarded.

The sizes of donor vesicles containing 10 mol % cholesterol were determined by dynamic light scattering by Chris Rutkowski using photon correlation spectroscopy as described by Hwang and Cummins (1982). Briefly, light from an argon ion laser was focused onto the vesicle sample in a glass cuvette maintained at 50 °C unless specified. The vesicles were made as described above except that the buffer was prefiltered five times through Millipore GS (0.2-μm) filters (Millipore Corp., Bedford, MA). The intensity of the scattered light was detected at a 90° angle to the incident beam. Deionized water was used for measuring refractive index and viscosity. Polystyrene spheres were used as size standards (diameter 129.2 ± 1.3 nm). The diameters of the vesicles were as follows (polydispersity index in parentheses): *N*-stearoyl-SPM, 177 nm (0.36); 3-deoxy-*N*-stearoyl-SPM, 206 nm (0.38); 3-*O*-methyl-*N*-stearoyl-SPM, 191 nm (0.24); 3-*O*-ethyl-*N*-stearoyl-SPM, 231 nm (0.4); egg SPM, 96.0 nm (0.23); 3-*O*-THP-egg SPM, 85.6 nm (0.34). Examination of the donor vesicles by negative-staining electron microscopy revealed that they were entirely unilamellar.

Analysis of Oxidation of Cholesterol. The extent of cholesterol oxidation in vesicles incubated at 50 °C for 4 days was examined as follows. In a typical experiment, donor vesicles containing 10 mol % cholesterol, 15 mol % DCP, 75 mol % *O*-ethyl-*N*-C_{18}-SPM, and traces of [^{14}C]cholesterol were incubated with acceptor vesicles containing 10 mol % cholesterol, 90 mol % egg SPM, and traces of [^{3}H]triolein at 50 °C for 4 days. The total lipids were extracted into $CHCl_3$–CH_3OH (2:1), and the concentrated extract was applied to silica GF plates (0.25-μm thickness; Analtech, Newark, DE) and eluted twice with ethyl acetate–benzene (3:2) (Theunissen et al., 1986). The R_f values for cholesterol, 7-ketocholesterol, and 7α- and 7β-hydroxycholesterol were 0.92, 0.71, 0.49, and 0.57, respectively. Zones corresponding to the R_f values of cholesterol, 7-ketocholesterol, and 7α- and 7β-hydroxycholesterol were scraped from the plates. Oxidation of cholesterol was examined by counting the scraped zones of silica gel directly in 5 mL of Ecoscint scintillation fluid (National Diagnostics, Highland Park, NJ). The total disintegrations per minute of ^{14}C applied to the plates was about 200000. Only 1.2% of the total [^{14}C]cholesterol was converted to oxidized products corresponding to the R_f values of the standards, of which 92% was 7-ketocholesterol, 2% was 7α-hydroxycholesterol, and 6% was 7β-hydroxycholesterol. The polar lipid fraction of the origin was also eluted and counted and was found to contain no ^{14}C disintegrations per minute.

Calorimetry. Differential scanning calorimetric measurements of the gel to liquid–crystalline phase transition of multilamellar dispersions of the phospholipids were performed with a Hart Scientific DSC calorimeter. Dry lipid films were dispersed above the phase transition temperature in 50 mM KCl solution. The lipid concentration was ~12 mg/mL. A scan rate of 15 °C/h was used. The T_m values given here were obtained from the cooling runs.

Measurement of [^{14}C]Cholesterol Exchange between Vesicles. We used the exchange methods of Fugler et al. (1985), who separated negatively charged donor from neutral acceptor in Pasteur pipets (0.6 × 3 cm) containing DEAE-Sepharose CL-6B, with a slight modification. Exchange of radiolabeled cholesterol was carried out at 50 °C. Acceptor vesicles and donor vesicles were preincubated separately at 50 °C in a Labline Model shaking water bath (Labline Instruments, Melrose Park, IL). Exchange was initiated by mixing and vortexing equal volumes of donor and acceptor vesicles. Aliquots (200 μL) of the incubation mixture were applied to DEAE-Sepharose CL-6B columns at room temperature after each column was prewashed with the buffer. The column was washed with 100 μL of buffer after the donor–acceptor mixture had entered into the column completely. An additional 1 mL of buffer was applied to the column to begin the collection of the eluate into the vial. To the eluate was added 8 mL of Scintiverse II (Fisher Scientific Co.) or 5 mL of Ecoscint

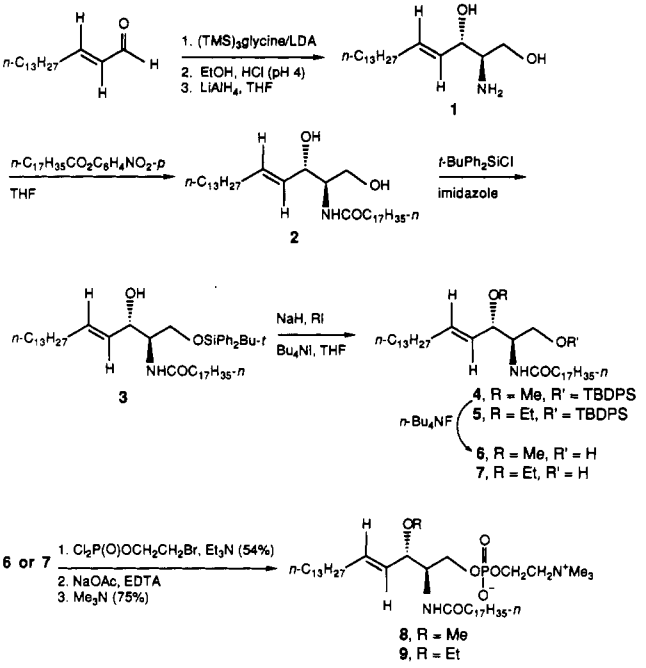
scintillation fluid. After the mixture was shaken by vortexing for about 30 s, the vials were counted in a Packard 2000 CA liquid scintillation counter (Packard Instrument Co., Downers Grove, IL) for 10 min. In order to correct data for spillover and background, spillover was calculated and background was counted. Spillover of ^{14}C disintegrations per minute into the ^{3}H channel was typically 0.0%, and the spillover of ^{3}H disintegrations per minute into the ^{14}C channel was typically 2.5%. The recovery of the neutral acceptor vesicles in the eluates was reported by Fugler et al. (1985) to be approximately 85–90%; also, <0.5% of the negatively charged donor vesicles were eluted. We found a similar recovery of acceptor vesicles, and about 0% of donor vesicles was eluted.

The kinetic analysis was modified from McLean and Phillips (1981). The fraction of [^{14}C]cholesterol in the acceptor at time t was displayed as $(^{14}C/^{3}H)_t/(^{14}C/^{3}H)_{mix}$, where $(^{14}C/^{3}H)_t$ is the ratio of [^{14}C]cholesterol/[^{3}H]glycerol trioleate in the eluate (acceptor vesicles) at time t and $(^{14}C/^{3}H)_{mix}$ is the ratio in an equal amount of reaction mixture not passing through the column. The $(^{14}C/^{3}H)_t/(^{14}C/^{3}H)_{mix}$ vs time plot demonstrates the extent of [^{14}C]cholesterol exchange. The percentage of [^{14}C]cholesterol remaining in the donor is defined as $1 - \{[(^{14}C/^{3}H)_t - (^{14}C/^{3}H)_0]/[(^{14}C/^{3}H)_\infty - (^{14}C/^{3}H)_0]\}$. Here, $(^{14}C/^{3}H)_0$ and $(^{14}C/^{3}H)_\infty$ represent the initial and infinity values obtained from fitting all of the $(^{14}C/^{3}H)_t$ data, including that at $t = 0$ and at time approaching equilibrium into a function of time, $F(t) = (^{14}C/^{3}H)_\infty + [(^{14}C/^{3}H)_0 - (^{14}C/^{3}H)_\infty] \exp(\text{slope} \times t)$, through nonlinear least-squares analysis using a simplex algorithm. The semilog plot of $1 - \{[(^{14}C/^{3}H)_t - (^{14}C/^{3}H)_0]/[(^{14}C/^{3}H)_\infty - (^{14}C/^{3}H)_0]\}$ vs time displays the rate of [^{14}C]cholesterol exchange. The half-time for cholesterol exchange is calculated from the quotient $(\ln 2)/k$, where k is the pseudo-first-order rate constant. Since the distribution of [^{14}C]cholesterol at equilibrium is 10:1 between acceptor and donor vesicles when a 10-fold excess of acceptor vesicles is used (McLean & Phillips, 1981), k is equal to −(slope)/1.1. The exchangeable pool of [^{14}C]cholesterol in the donor vesicles is calculated as $[(^{14}C/^{3}H)_\infty - (^{14}C/^{3}H)_0]/[(^{14}C/^{3}H)_{mix} - (^{14}C/^{3}H)_0] \times 1.1$.

Synthesis of Sphingomyelin Analogues. The synthetic routes we used to obtain the 3-substituted SPM analogues are outlined in Schemes I and II. Reactions were monitored by TLC on 0.25-mm-thick silica gel GF glass plates (Analtech, Newark, DE). Compounds were detected by spraying with 10% sulfuric acid in ethanol followed by charring on a hot plate. Phosphorus-containing compounds were detected with Dittmer–Lester reagent (Ryu & MacCoss, 1979). Flash chromatography was carried out with silica gel 60 (230-400 ASTM mesh) from E. Merck, purchased from Aldrich (Milwaukee, WI). ^{1}H NMR spectra were recorded with a 200-MHz IBM-Bruker or a 300-MHz GE spectrometer. Chemical shifts are given in parts per million downfield from tetramethylsilane as the internal standard. Infrared spectra were recorded on a Perkin-Elmer FT-IR 1600 spectrophotometer. Elemental analyses were done by Desert Analytics (Tucson, AZ). The solvents were dried as described elsewhere (Guivisdalsky & Bittman, 1989).

*N-Stearoyl-*DL*-erythro-sphingosine* (**2**). DL-*erythro*-Sphingosine (**1**) was obtained by aldol condensation of (*E*)-hexadec-2-enal with tris(trimethylsilyl)glycine, followed by reduction with lithium aluminum hydride, according to a modification of a procedure outlined previously (Schmidt & Kläger, 1982). To a solution of 0.70 g (2.32 mmol) of DL-*erythro*-sphingosine (**1**) in 15 mL of THF was added slowly a solution of 1.04 g (2.55 mmol) of *p*-nitrophenyl stearate in 4 mL of THF. After the mixture was stirred at room temperature for 22 h, the solvent was removed under vacuum. The residue was dissolved in 30 mL of chloroform and washed with 15% aqueous NaOH (2 × 20 mL) and water (3 × 20 mL) to remove *p*-nitrophenol. The organic layer was dried ($MgSO_4$), and the solvents were concentrated with a rotary evaporator. The crude product was purified by flash chromatography (elution with $CHCl_3$–MeOH, 95:5) to give 0.98 g (74%) of product as a white solid: mp 97–97.5 °C [lit. mp 97–98 °C for *N*-stearoyl-D-*erythro*-sphingosine (Hammarström, 1971; Julina et al., 1986)]; TLC ($CHCl_3$–EtOH, 9:1) R_f 0.62, ($CHCl_3$–EtOH, 100:7) R_f 0.45; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.85–0.91 (t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.25 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.60 (m, 2 H, CH_2CH_2CO), 2.01–2.05 (m, 2 H, CH_2CH=CH), 2.15–2.22 (t, J = 7.5 Hz, 2 H, CH_2CH_2CO), 3.61–3.67 (m, 1 H, CHNH), 3.85–3.90 (m, 2 H, CH_2OH), 4.10–4.18 (m, 1 H, CH=CHCHOH), 5.42–5.53 (dd, J = 15.4 Hz, 6.5 Hz, 1 H, vinyl H), 5.68–5.79 (dt, J = 15.4 Hz, 6.5 Hz, 1 H, vinyl H), 6.50 (d, J = 7.2 Hz, 1 H, NH). Anal. Calcd for $C_{36}H_{71}O_3N$: C, 76.40; H, 12.64; N, 2.47. Found: C, 76.46; H, 12.12; N, 2.48.

Scheme I: Reaction Sequence for the Syntheses of 3-*O*-Methyl-DL-*erythro*-*N*-stearoyl-SPM (**8**) and 3-*O*-ethyl-DL-*erythro*-*N*-stearoyl-SPM (**9**)



*1-O-(tert-Butyldiphenylsilyl)-2-N-stearoyl-*DL*-erythro-sphingosine* (**3**). Imidazole (0.22 g, 3.18 mmol) was dissolved in 25 mL of dry methylene chloride, and *tert*-butyldiphenylsilyl chloride (0.44 g, 1.59 mmol) was added. Immediately some white precipitate was formed (imidazolium chloride). After the mixture has stirred for 1 h at room temperature, a solution of protected ceramide **2** (0.9 g, 1.59 mmol) in 5 mL of methylene chloride was added slowly. The reaction mixture was stirred at room temperature overnight. Additional methylene chloride (15 mL) was added to dilute the mixture, and the mixture was washed with saturated sodium chloride solution (30 mL) and water (30 mL). The organic phase was dried ($MgSO_4$), and the solvent was removed by rotary evaporation. The residue was purified by flash chromatography (elution with hexane–ethyl acetate, 4:1) to give 1.04 g (81%) of the pure product: mp 33 °C; TLC (hexane–ethyl acetate, 4:1) R_f 0.26, ($CHCl_3$–EtOH, 98:2) R_f 0.43; IR

($CHCl_3$) 3600–3170, 3072, 3060, 2919, 2849, 1655, 1606, 1490, 1461, 1425, 1108, 903, 820, 732 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.85–0.91 (t, J = 6.3 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.07 (s, 9 H, $C(CH_3)_3$), 1.26 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.60 (m, 2 H, CH_2CH_2CO), 2.01–2.05 (m, 2 H, CH_2CH=CH), 2.11–2.19 (t, J = 7.6 Hz, 2 H, CH_2CH_2CO), 3.74–3.78 (m, 1 H, CHNH), 3.92–3.98 (m, 2 H, CH_2O), 4.19 (m, 1 H, CH=CHCHOH), 5.42–5.52 (dd, J = 15.4 Hz, 5.5 Hz, 1 H, vinyl H), 5.70–5.89 (dt, J = 15.6 Hz, 6.4 Hz, 1 H, vinyl H), 6.30–6.34 (d, J = 7.5 Hz, 1 H, NH), 7.39–7.46 (m, 10 H, Ar). Anal. Calcd for $C_{52}H_{89}O_3NSi$: C, 77.65; H, 11.15; N, 1.74. Found: C, 77.81; H, 11.02; N, 1.80.

*1-O-(tert-Butyldiphenylsilyl)-2-N-stearoyl-3-O-methyl-*DL*-erythro-sphingosine* (**4**). Sodium hydride (35 mg, 1.44 mmol) was added to a solution of 580 mg (0.72 mmol) of protected ceramide **3** in 15 mL of THF. After the reaction mixture was stirred at room temperature for 1 h, a trace of tetra-*n*-butylammonium iodide and 450 μL (7.2 mmol) of methyl iodide (10-fold molar excess) were added. The mixture was stirred at room temperature for 6 h. The solvent was removed under reduced pressure. The residue was dissolved in 25 mL of chloroform and washed with 10% ammonium chloride solution (20 mL). The organic phase was dried ($MgSO_4$). The residue was purified by flash chromatography (elution with hexane–ethyl acetate, 5:1) to give 380 mg (64%) of the pure product as a white solid: mp 49–49.5 °C; TLC (hexane–ethyl acetate, 5:1) R_f 0.48; IR ($CHCl_3$) 3425, 3072, 3060, 2919, 2849, 1661, 1590, 1490, 1461, 1378, 1261, 1108, 903, 820, 732, 703 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.85–0.91 (t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.07 (s, 9 H, $C(CH_3)_3$), 1.26 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.67 (m, 2 H, CH_2CH_2CO), 2.00–2.13 (m, 4 H, CH_2CH=CH, CH_2CH_2CO) 3.22 (s, 3 H, OCH_3), 3.65–3.72 (m, 2 H, $CHOCH_3$, CHNH), 3.95–4.09 (m, 2 H, CH_2O), 5.29–5.47 (m, 1 H, vinyl H), 5.61–5.91 (m, 1 H, vinyl H), 7.39–7.67 (m, 10 H, Ar). Anal. Calcd for $C_{53}H_{91}O_3NSi$: C, 77.78; H, 11.21; N, 1.71. Found: C, 77.90; H, 11.17; N, 1.84.

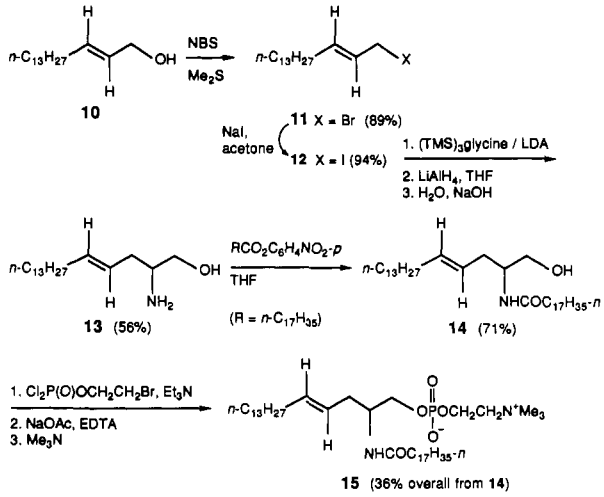
*1-O-(tert-Butyldiphenylsilyl)-2-N-*stearoyl-*3-O-ethyl-*DL*-erythro-sphingosine* (**5**). This compound was prepared from protected ceramide **3** in 54% yield by using the same procedure as that described for **4**: TLC (hexane–ethyl acetate, 6:1) R_f 0.55; IR ($CHCl_3$) 3440, 3071, 3050, 2926, 2854, 1655, 1590, 1514, 1465, 1428, 1361, 1263, 1216, 1113, 1008, 938, 872, 823, 759, 741, 701 cm^{-1}; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.86–0.90 (t, J = 6.5 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.07 (s, 9 H, $C(CH_3)_3$), 1.10–1.13 (t, J = 7.0 Hz, 3 H, OCH_2CH_3), 1.25 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.60 (m, 2 H, CH_2CH_2CO), 1.97–2.11 (m, 4 H, CH_2CH=CH, CH_2CH_2CO), 3.22–3.27 (q, J = 7.0 Hz, 2 H, OCH_2CH_3), 3.51–3.56 (m, 1 H, CHNH), 3.67–3.71 (dd, J = 9.9 Hz, 3.0 Hz, 1 H, $CHOCH_2CH_3$), 3.99–4.03 (m, 2 H, CH_2O), 5.26–5.34 (dd, J = 15.4 Hz, 7.0 Hz, 1 H, vinyl H), 5.57–5.69 (m, 2 H, vinyl H, NH), 7.37–7.64 (m, 10 H, Ar). Anal. Calcd for $C_{54}H_{93}O_3NSi$: C, 77.92; H, 11.26; N, 1.68. Found: C, 78.20; H, 11.52; N, 1.75.

*2-N-Stearoyl-3-O-methyl-*DL*-erythro-sphingosine* (**6**). This compound was prepared from **4** in 97% yield as follows. To a solution of 340 mg (0.42 mmol) of *tert*-butyldiphenylsilyl ether **4** in 10 mL of THF was added 0.83 mL (0.83 mmol) of a 1 M solution of tetra-*n*-butylammonium fluoride in THF. The mixture was stirred at room temperature for 30 min and then quenched with water and extracted with ether. The ether extract was dried ($MgSO_4$), the solvents were removed under reduced pressure, and the residue was purified by flash chromatography (elution with $CHCl_3$–EtOH, 98:2). There was obtained 233 mg (97%) of **6** as a white solid: mp 79.5 °C; TLC (hexane–ethyl acetate, 4:5) R_f 0.40; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.85–0.91 (t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.25 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.64 (m, 2 H, CH_2CH_2CO), 2.03–2.09 (m, 2 H, CH_2CH=CH), 2.18–2.26 (t, J = 7.5 Hz, 2 H, CH_2CH_2CO), 3.26 (s, 3 H, OCH_3), 3.57–3.62 (m, 1 H, CHNH), 3.83–3.99 (m, 3 H, CH_2O, $CHOCH_3$), 5.29–5.41 (dd, J = 15.4 Hz, 7.6 Hz, 1 H, vinyl H), 5.70–5.81 (dt, J = 15.4 Hz, 6.9 Hz, 1 H, vinyl H), 6.01–6.04 (d, J = 7.6 Hz, 1 H, NH). Anal. Calcd for $C_{37}H_{73}O_3N$: C, 76.62; H, 12.69; N, 2.41. Found: C, 75.17; H, 12.61; N, 2.23.

*2-N-Stearoyl-3-O-ethyl-*DL*-erythro-sphingosine* (**7**). This compund was prepared from **5** in (95%) yield by using the same procedure as that described for **6**: TLC (hexane–ethyl acetate, 4:5) R_f 0.52; ^1H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 (t, J = 6.9 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.14–1.19 (t, J = 7.1 Hz, 3 H, OCH_2CH_3), 1.25 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.57–1.66 (m, 2 H, CH_2CH_2CO), 2.03–2.10 (m, 2 H, CH_2CH=CH), 2.19–2.22 (t, J = 7.6 Hz, 2 H, CH_2CH_2CO), 3.17–3.29 (m, 2 H, OCH_2CH_3), 3.53–3.61 (m, 2 H, $CHOCH_2CH_3$, CHNH), 3.96–4.00 (m, 2 H, CH_2O), 5.33–5.41 (dd, J = 15.4 Hz, 7.4 Hz, 1 H, vinyl H), 5.72–5.77 (dt, J = 15.5 Hz, 6.7 Hz, 1 H, vinyl H), 6.28–6.30 (d, J = 7.6 Hz, 1 H, NH). Anal. Calcd for $C_{38}H_{75}O_3N$: C, 76.84; H, 12.73; N, 2.36. Found: C, 76.64; H, 13.00; N, 2.26.

*3-O-Methyl-*DL*-erythro-N-stearoyl-SPM* (**8**). This compound was prepared from **6** in 36% yield by using the following procedure for incorporation of the phosphocholine moiety. (2-Bromoethyl)phosphoric acid dichloride [0.5 M in THF, 900 μL, 0.45 mmol; prepared as described by Eibl and Nicksch (1978)] was cooled in an ice bath. After the addition of dry triethylamine (187 μL, 1.34 mmol), the phosphorylation mixture was kept at 25 °C and a solution of alcohol **6** (130 mg, 0.22 mmol) in 2 mL of dry THF was added dropwise with stirring. The reaction mixture was stirred for 1 day at room temperature. A precipitate (triethylammonium hydrochloride) formed, which was removed by filtration. The filtrate was evaporated to dryness. The residue was dissolved in 1.5 mL of THF. Sodium acetate (1.5 mL of a 0.5 M solution in water) and ethylenediaminetetraacetate disodium salt (0.1 mL of a 0.5 M solution in water) were added with stirring. After overnight hydrolysis, chloroform (10 mL) was added, and the mixture was extracted with water. The organic layer was dried ($MgSO_4$) and evaporated to dryness. The residue was purified by flash chromatography (eluted first with hexane–ethyl acetate, 4:5, then with $CHCl_3$–MeOH–7 N NH_4OH, 65:25:4) to give 70 mg of the bromoethyl ester intermediate as a yellow oil: TLC ($CHCl_3$–MeOH–7 N NH_4OH, 65:25:4) R_f 0.58. To a solution of 70 mg (0.090 mmol) of the bromoethyl ester in 0.9 mL of chloroform were added 1.5 mL of 2-propanol and 1.5 mL of acetonitrile, followed by 3 mL of a 25% aqueous solution of trimethylamine. The reaction was completed after stirring for 48 h at room temperature. The solvent was removed with use of a rotary evaporator (azeotropic distillation with excess 2-propanol). The crude product was purified by flash chromatography (elution first with $CHCl_3$–MeOH, 9:1, then with $CHCl_3$–MeOH–7 N NH_4OH, 65:25:4): yield (contains silica gel) 40 mg (61%); TLC ($CHCl_3$–MeOH–7 N NH_4OH, 65:25:4) R_f 0.36. Suspended silica gel was removed by passing a $CHCl_3$ solution of the product through a 0.45-μm Metricel filter (Gelman Sciences) three times to give the pure product **8**: TLC ($CHCl_3$–MeOH–7 N NH_4OH, 65:25:4) R_f 0.36; ^1H NMR (200 MHz, $CDCl_3$) δ (ppm)

Scheme II. Reaction Sequence for the Synthesis of 3-Deoxy-DL-*N*-stearoyl-SPM (**15**)



0.85–0.91 (t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.25 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.65 (m, 2 H, CH_2CH_2CO), 2.03–2.10 (m, 2 H, CH_2CH=CH), 2.22–2.30 (t, J = 7.4 Hz, 2 H, CH_2CH_2CO), 3.30 (s, 12 H, $N(CH_3)_3$, OCH_3), 3.78 (m, 3 H, CHNH, $CH_2N(CH_3)_3$), 3.95–4.10 (m, 4 H, $CH_2OP(O)(O^-)OCH_2$), 5.26–5.40 (dd, J = 15.4, 6.8 Hz, 1 H, vinyl H), 5.62–5.74 (dt, J = 15.4 Hz, 7.0 Hz, 1 H, vinyl H). Anal. Calcd for $C_{42}H_{85}O_6N_2P \cdot 3.5H_2O$: C, 62.42; H, 11.48; N, 3.46. Found: C, 62.34; H, 11.57; N, 3.09.

*3-O-Ethyl-*DL*-erythro-N-stearoyl-SPM* (**9**). This compound was prepared from **7** in 41% yield by using the same procedure as described above; TLC ($CHCl_3$–MeOH–7 N NH_4OH, 65:25:4) R_f 0.30; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.89 (t, J = 6.8 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.10–1.13 (t, J = 7.0 Hz, 3 H, OCH_2CH_3), 1.25 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.55–1.60 (m, 2 H, CH_2CH_2CO), 1.99–2.05 (m, 2 H, CH_2CH=CH), 2.12–2.17 (t, J = 7.6 Hz, 2 H, CH_2CH_2CO), 3.30–3.50 (m, 11 H, $N(CH_3)_3$, OCH_2CH_3), 3.70–3.85 (m, 4 H, CHNH, $CH_2N(CH_3)_3$, $CHOCH_2CH_3$), 4.23 (m, 4 H, $CH_2OP(O)(O^-)OCH_2$), 5.33–5.41 (dd, J = 15.4 Hz, 7.4 Hz, 1 H, vinyl H), 5.57–5.65 (dt, J = 15.4 Hz, 6.8 Hz, 1 H, vinyl H).

Synthesis of 3-Deoxy-N-stearoyl-SPM (Scheme II). 1-Bromo-(E)-hexadec-2-ene (**11**). To a flame-dried, 100-mL, two-necked round-bottom flask equipped with a magnetic stirrer, dropping funnel with rubber septum, and nitrogen inlet adapter was added 1.22 g (6.88 mmol) of *N*-bromosuccinimide (NBS). Methylene chloride (35 mL) was added, and the resulting solution was cooled to −30 °C with a dry ice–acetonitrile bath. Freshly distilled dimethyl sulfide (550 μL, 7.5 mmol) was added dropwise. The mixture was warmed to 0 °C with an ice bath, maintained at that temperature for 5 min, and cooled to −30 °C. To the resulting milky yellow suspension was added dropwise a solution of 1.5 g (6.25 mmol) of (*E*)-hexadec-2-en-1-ol (**10**) in 5 mL of methylene chloride. The suspension was warmed to 0 °C with an ice bath and stirred for 1.5 h. The ice bath was removed, the reaction mixture was allowed to warm to room temperature, and stirring was continued for an additional 15 min. The reaction mixture was poured into a 250-mL separatory funnel and washed with saturated sodium chloride solution (35 mL). The aqueous layer was washed with chloroform (2 × 20 mL). The combined organic phases were washed with saturated sodium chloride solution (25 mL), dried ($MgSO_4$), and concentrated on a rotary evaporator. The crude product was purified by flash chromatography. Elution with hexane afforded 1.68 g (89%) of pure **11**: TLC (hexane) R_f 0.59; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.80–0.92 (t, J = 7.0 Hz, 3 H, CH_3), 1.25 (m, 22 H, $(CH_2)_{11}$), 2.05 (m, 2 H, CH_2CH=CH), 3.98–4.08 (d, J = 3.6 Hz, 2 H, CH_2OH), 5.58–5.64 (dt, J = 15.5 Hz, 6.8 Hz, 1 H, vinyl H), 5.76–5.85 (dt, J = 15.4 Hz, 7.1 Hz, 1 H, vinyl H).

1-Iodo-(E)-hexadec-2-ene (**12**). To a solution of sodium iodide (619 mg, 4.13 mmol) in acetone (25 mL) in a 50-mL flask fitted with a reflux condenser protected by a calcium chloride guard tube was added 1.0 g (3.30 mmol) of 1-bromo-(*E*)-hexadec-2-ene (**11**). A precipitate of sodium bromide soon began to form. After the reaction mixture was left at room temperature overnight, sodium bromide was removed by filtration and the residue was washed with acetone. The filtrate was concentrated on a rotary evaporator, leaving a residue that was dissolved in 30 mL of hexane and then washed with 20 mL of water. The organic phase was dried ($MgSO_4$), and the solvents were removed under vacuum. The crude product was purified by flash chromatography (elution with hexane) to give 1.10 g (94%) of **12** as a light yellow oil: TLC (hexane) R_f 0.59. The ^{1}H NMR spectrum was identical with that of **11**.

*3-Deoxy-*DL*-sphingosine* (**13**). Diisopropylamine (192 μL, 1.37 mmol) was slowly dropped into 0.85 mL (1.37 mmol) of *n*-butyllithium (1.6 M in hexane) at −78 °C. Some white precipitate was formed immediately. After the mixture had stirred for 1 h at −78 °C, THF (15 mL) was added and the solution was stirred for 10 min. A solution of 365 mg (1.25 mmol) of tris(trimethylsilyl)glycine [$(TMS)_3$glycine] in 1.25 mL of THF was added slowly to the mixture. The reaction mixture was stirred for 1 h, and then a solution of 400 mg (1.14 mmol) of 1-iodo-(*E*)-hexadec-2-ene (**12**) in 1.5 mL of THF was added. The mixture was stirred for 2 h at −78 °C and for 30 min at 0 °C and then warmed to room temperature. Lithium aluminum hydride (216 mg, 5.70 mmol) was added slowly to the reaction mixture. The suspension was refluxed for 24 h and then was hydrolyzed with H_2O (216 μL), 15% NaOH (216 μL), and H_2O (3 × 216 μL). The reaction mixture was filtered and washed with chloroform (15 mL). Some isopropyl alcohol was added to the filtrate, and the solvents were removed under vacuum. The crude product was purified by flash chromatography (elution with $CHCl_3$–MeOH, 7:3) to afford 180 mg (56%) of pure 3-deoxy-DL-sphingosine (**13**): TLC ($CHCl_3$–MeOH, 7:3) R_f 0.51; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 (t, J = 6.8 Hz, 3 H, CH_3), 1.25 (m, 22 H, $(CH_2)_{11}$), 2.01–2.08 (m, 2 H, CH_2CH=CH), 2.15–2.11 (m, 2 H, CH=$CHCH_2$), 2.33 (s, 3 H, NH_2, OH), 3.09 (m, 1 H, $CHNH_2$), 3.58–3.66 (m, 2 H, CH_2OH), 5.35–5.40 (dt, J = 15.5 Hz, 7.1 Hz, 1 H, vinyl H), 5.54–5.67 (dt, J = 15.4 Hz, 6.8 Hz, 1 H, vinyl H).

*3-Deoxy-N-stearoyl-*DL*-sphingosine* (**14**). This compound was prepared from **13** in 71% yield by using the same procedure as that described for **2**: TLC (hexane–ethyl acetate, 4:5) R_f 0.32; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 (t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.25 (m, 50 H, $CH_2)_{11}$, $(CH_2)_{14}$), 1.58–1.64 (m, 2 H, CH_2CH_2CO), 1.99–2.10 (m, 2 H, CH_2CH=CH), 2.15–2.21 (m, 4 H, CH=$CHCH_2$, CH_2CH_2CO), 3.10 (m, 1 H, CHNH), 3.58–3.66 (m, 2 H, CH_2OH), 5.32–5.37 (dt, J = 15.5 Hz, 7.2 Hz, 1 H, vinyl H), 5.49–5.63 (dt, J = 15.4 Hz, 6.8 Hz, 1 H, vinyl H). Anal. Calcd for $C_{36}H_{71}NO_2$: C, 78.62; H, 13.01; N, 2.55. Found: C, 78.12; H, 13.26; N, 2.29.

*3-Deoxy-N-stearoyl-*DL*-SPM* (**15**). This compound was prepared from **14** in 36% overall yield by using the same procedure as that described for **8**: TLC ($CHCl_3$–MeOH–7

N NH_4OH, 65:25:4) R_f 0.32; ^1H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.91 (t, J = 7.0 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{14}$), 1.26 (m, 50 H, $(CH_2)_{11}$, $(CH_2)_{14}$), 1.58 (m, 2 H, CH_2CH_2CO), 1.96–1.99 (m, 2 H, CH_2CH=CH), 2.22–2.31 (m, 4 H, CH_2CH_2CO, CH_2CH=CH), 3.38 (s, 9 H, $N(CH_3)_3$), 3.86 (m, 3 H, CHNH, $CH_2N(CH_3)_3$), 4.25–4.36 (m, 4 H, $CH_2OP(O)(O^-)OCH_2$), 5.31–5.35 (dt, J = 15.5 Hz, 6.8 Hz, 1 H, vinyl H), 5.44–5.58 (dt, J = 15.4 Hz, 7.1 Hz, 1 H, vinyl H).

3-O-Tetrahydropyranyl-egg SPM. This compound was prepared from egg SPM in 62% yield by using the following procedure. To a solution of egg SPM (~1.2 mmol) in 12 mL of methylene chloride was added 182 μL (2.0 mmol) of dihydropyran. A few crystals of *p*-toluenesulfonic acid were added, which were dissolved by swirling. The reaction mixture was stirred at 45 °C for 6 h. The solution was diluted with ether and then washed with saturated sodium bicarbonate solution and water. The organic layer was dried ($MgSO_4$), and the solvents were removed with a rotary evaporator. The crude product was purified by flash chromatography: TLC ($CHCl_3$–MeOH–H_2O–concd NH_4OH, 65:25:2:2) R_f 0.36. The R_f value of egg SPM in the same solvent system was 0.24.

Results

Effect of Steric Bulk at the 3-Position of SPM on the Cholesterol Exchange Rate. Cholesterol undergoes very slow exchange from donor egg SPM vesicles to acceptor SPM vesicles in only one kinetic pool at 50 °C; however, the rate of cholesterol exchange from vesicles containing 3-OTHP-egg SPM is *much* faster than from vesicles containing egg SPM (the average $t_{1/2}$ values in 3-OTHP-egg SPM and egg SPM vesicles are 0.69 and 23.6 h, respectively). The size of the exchangeable pool is similar in these vesicles, i.e., 92% in 3-OTHP-SPM vesicles and 86% in egg SPM vesicles. Since the tetrahydropyranyloxy group is very bulky compared with the hydroxy group, steric hindrance may alter the molecular packing of 3-OTHP-SPM in the vesicle bilayer and impede tight contacts with cholesterol, thus accounting for the faster rate of cholesterol desorption from 3-OTHP-SPM bilayers. Calorimetric measurements indicate that the T_m value of the gel to liquid–crystalline phase transition is about 10 °C lower in 3-OTHP-egg SPM bilayers compared with that in egg SPM bilayers (the endotherms are broad because of the mixed chains present in SPM purified from natural sources; T_m: ~27 vs ~37 °C). In order to further explore the role of 3-hydroxy group of SPM in the retention of cholesterol molecules in the donor bilayer, SPM analogues bearing substituents with sizes more similar to that of the hydroxy group were tested.

Effects of Modifications at the 3-Position of N-Stearoyl-SPM on Cholesterol Exchange Rates between Vesicles. The time courses of [^{14}C]cholesterol exchange from vesicles containing *N*-stearoyl-SPM and 3-*O*-methyl-, 3-*O*-ethyl-, and 3-deoxy-*N*-stearoyl-SPM are shown in Figure 2. The rate of exchange from 3-*O*-ethyl-SPM vesicles (Figure 2A) is much faster than the rates measured in the other vesicles, whereas similar rates are found for vesicles prepared with *N*-stearoyl-SPM, 3-deoxy-*N*-stearoyl-SPM, and 3-*O*-methyl-*N*-stearoyl-SPM (Figure 2B). The size of the exchangeable pool is >84% for all of the vesicles with the exception of *N*-stearoyl-SPM vesicles, in which about 73% of the total labeled cholesterol was exchangeable. Again, only one kinetic pool of cholesterol is found (semilog plots not shown). The half-times for cholesterol exchange from vesicles containing *N*-stearoyl-SPM and the various analogues we made are listed in Table I. At 10 mol % sterol, cholesterol undergoes exchange at the same rate from vesicles prepared from 3-deoxy-

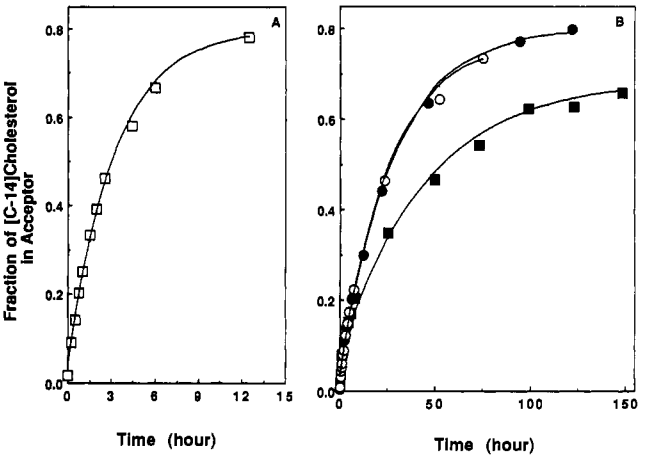


FIGURE 2: Influence of substitutions at the 3-position of *N*-stearoyl-SPM on the kinetics of [^{14}C]cholesterol exchange from vesicles containing 10 mol % cholesterol. Donor vesicles contained (A) 3-*O*-ethyl-SPM, (B) 3-*O*-methyl-SPM (●), 3-deoxy-SPM (○), and 3-hydroxy-SPM (unsubstituted *N*-stearoyl-SPM) (■). The acceptor vesicles contained egg SPM and 10 mol % cholesterol.

Table I: Effects of Modifications at the 3-Position of SPM on the Half-times for Cholesterol Exchange between SPM Vesicles at 50 °C

SPM analogue in the donor vesicles[a]	$t_{1/2}$ (h)
egg SPM	23.6 ± 3.6 (2)[b]
3-OTHP-egg SPM	0.69 ± 0.08 (2)
N-stearoyl-SPM	33.8 ± 0.8 (2)
3-deoxy-*N*-stearoyl-SPM	22.9 ± 4.3 (2)
3-*O*-methyl-*N*-stearoyl-SPM	23.0 ± 2.1 (2)
3-*O*-ethyl-*N*-stearoyl-SPM	2.4 ± 0.2 (2)

[a] The donor vesicles contained 75 mol % synthetic SPM, 10 mol % cholesterol, and 15 mol % dicetyl phosphate. The acceptor vesicles (10-fold excess relative to donor vesicles) contained 90 mol % egg SPM and 10 mol % cholesterol. [b] The number in parentheses indicates the number of times the experiments were run.

and 3-*O*-methyl-*N*-stearoyl-SPM ($t_{1/2}$ values of 22.9 ± 4.3 and 23.0 ± 2.1 h, respectively). These half-times are about 30% lower than that found with use of *N*-stearoyl-SPM vesicles ($t_{1/2}$ = 33.8 ± 0.8 h). In contrast, we found that the ratio of half-times for cholesterol exchange from *N*-palmitoyl-SPM vesicles relative to dipalmitoyl-PC vesicles is 8.8 at 50 °C and 6 mol % sterol. These results indicate that the 3-hydroxy group of SPM is not required for the slow rate of cholesterol desorption.

Discussion

The experiments described here were designed to explore the role of the hydroxy group of SPM in the very slow rates of desorption of cholesterol from SPM-containing surfaces. The rate-determining step for cholesterol exchange between vesicles is the desorption of cholesterol molecules from the lipid–water interface [reviewed by Phillips et al. (1987); Dawidowicz, 1987; Bittman, 1988]. The ease of desorption from the donor particle is dependent on the molecular packing in the membrane bilayer. Many investigators have compared the rates of exchange of cholesterol from PC and SPM bilayer membranes and have found that the half-times increase dramatically when SPM is present in the host phospholipid bilayers (Clejan & Bittman, 1984b; Fugler et al., 1985; Yeagle & Young, 1986; Bhuvaneswaran & Mitropoulos, 1986; Bar et al., 1987; Thomas & Poznansky, 1988; Lund-Katz et al., 1988; Kan & Bittman, 1990; Gold & Phillips, 1990). The molecular basis for the marked decrease of cholesterol exchange rate in the presence of SPM is not clear; the activation energy required to reach the transition-state complex from which cholesterol molecules desorb is considered to increase

when phospholipid–cholesterol interactions are strengthened (Phillips et al., 1987). Both inter- and intramolecular hydrogen bonding is possible in SPM bilayers, whereas less extensive intermolecular and no intramolecular hydrogen bonding is possible in PC bilayers; moreover, an intermolecular hydrogen bond has been postulated to contribute to the high affinity of SPM for cholesterol (Boggs, 1980, 1987; Barenholz & Thompson, 1980). The present data show that the rate of cholesterol exchange from vesicles containing 3-deoxy-*N*-stearoyl-SPM is the same as that from vesicles containing 3-*O*-methyl-*N*-stearoyl-SPM and is only 30% faster than the rate measured with *N*-stearoyl-SPM itself. 3-Deoxy-*N*-stearoyl-SPM has neither hydrogen-bond-donating nor hydrogen-bond-accepting properties at the 3-position but still displays a strong affinity for cholesterol, as reflected by a rate of desorption of cholesterol molecules that is nearly as slow as that from *N*-stearoyl-SPM–water interfaces. Therefore, we conclude that the 3-hydroxy group of SPM is not necessary for the strong interaction with cholesterol.

Cholesterol undergoes exchange faster from smaller vesicles than from large vesicles (McLean & Phillips, 1984; Fugler et al., 1985). The faster rate of exchange from 3-*O*-ethyl-SPM vesicles (Table I) does not arise from a significant difference in vesicle size since the vesicles prepared from the various *N*-stearoylsphingomyelins have similar sizes (see Materials and Methods); furthermore, the difference in rate does not reflect a difference in T_m value, since the 3-*O*-methyl- and 3-*O*-ethyl-SPMs have similar transition temperatures (~37 °C). Since the mean molecular area of 3-*O*-ethyl-*N*-stearoyl-SPM is significantly larger than that of 3-*O*-methyl-*N*-stearoyl-SPM (Grönberg, Ruan, Bittman, and Slotte, unpublished experiments), it is likely that steric hindrance introduced by the ethyl group induces loose packing, which enhances the rate of exchange. The marked enhancement of the exchange rate on introduction of the tetrahydropyranyloxy group into the 3 position of egg SPM (Table I) is another indication of the influence of steric bulk at the 3 position on the ease of cholesterol desorption. The mean molecular area of the 3-OTHP derivative is much larger than that of SPM itself (Grönberg, Ruan, Bittman, and Slotte, unpublished experiments).

Surface pressure–molecular area isotherms for SPM and PC monolayers indicate that the molecular area of SPM is smaller than that of PC (Lund-Katz et al., 1988). Lund-Katz et al. (1988) concluded that van der Waals attractive forces between SPM and cholesterol are apparently stronger than those between phosphatidylcholines (with similar acyl chain composition) and cholesterol because of the higher lateral cohesion in the SPM system. These interactions may be primarily responsible for the slow exchange rate of cholesterol in SPM-containing bilayers. It should be noted, however, that our exchange rate data obtained in vesicles with 10 mol % cholesterol are not closely correlated with the mean molecular areas of monolayers containing 30 mol % cholesterol. For example, the mean molecular areas of 3-deoxy-SPM and 3-methoxy-SPM monolayers containing 30 mol % cholesterol are 34.7 and 38.7 Å^2 at 15 mN/m and 25 °C (Grönberg, Ruan, Bittman, and Slotte, unpublished experiments), yet the rate of cholesterol exchange from 3-deoxy- and 3-methoxy-SPM vesicles is the same (Table I). Furthermore, the molecular area of 3-deoxy-SPM–cholesterol mixtures is considerably less than that of 3-hydroxy-SPM–cholesterol mixtures (34.7 and 40.0 Å^2, respectively, at 30 mol % cholesterol), but cholesterol undergoes exchange 30% faster from 3-deoxy-SPM bilayers. A correlation is anticipated between high lateral cohesion energies and slow rates of lipid exchange. When van der Waals interactions provide the dominant term in lateral cohesion energy, a decrease in molecular area is expected to result in a decrease in exchange rate. However, in vesicles prepared from some of the SPM analogues noted above the expected relationship between relative molecular area and exchange rate was not observed, implying that contributions from other sites in SPM may be significant. Although our data indicate that the free hydroxy group at the 3-position of SPM does not play a critical role in the rate of cholesterol exchange, it is possible that the amide group of SPM plays a role in stabilizing the interaction by hydrogen bonding to cholesterol (Sankaram & Thompson, 1990). However, we recently found that replacement of the glycerol oxygen atom at the *sn*-2 position of PC with a NH group did not have a marked effect on the rate of cholesterol exchange between PC vesicles (Kan et al., 1991). More direct information about the role of the amide group in SPM–cholesterol interactions requires investigations with amide-modified SPM analogues.

Physiological Significance. Cholesterol and SPM are important constituents of lipoproteins and plasma membranes of many mammalian cells. An understanding of the fluxes of free cholesterol between lipoproteins and cell membranes requires a detailed knowledge of the molecular interactions occurring between cholesterol and other components in lipoprotein and membrane surfaces. The present study indicates that intervesicle movement of cholesterol is not sensitive to the nature of the group at the 3-position of SPM unless the group is bulky. Replacement of the 3-hydroxy group of SPM by a small group (a hydrogen atom or an *O*-methyl group) results in only a 30% increase in the desorption rate from the vesicle–water interface; however, bulky groups probably cause spacing out of SPM molecules, resulting in enhanced rates of cholesterol movement. Additional synthetic SPM derivatives are required to gain a more complete understanding at the molecular level of the sites in SPM that participate in impeding cholesterol transfer between surfaces. The results reported here indicate that van der Waals interactions between SPM and cholesterol are more important than hydrogen-bonding interactions involving the 3-hydroxy group of SPM as a stabilizing force in both bilayers and monolayers.

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Conformation and Disulfide Bond Formation of the Constant Fragment of an Immunoglobulin Light Chain: Effect of Truncation at the C-Terminal Region

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Received February 5, 1991; Revised Manuscript Received April 16, 1991

ABSTRACT: Constant fragments with different carboxyl terminals, C_L(109–211), C_L(109–207), and C_L(109–200), were prepared by limited carboxypeptidase P or Y proteolysis of the constant fragment, C_L(109–214), of a type λ immunoglobulin light chain, and their conformations and stabilities, and formation of the disulfide bond from the reduced fragments, were studied. No change in conformation or stability was observed on removal of three residues from the C-terminal end. Removal of seven or more residues from the C-terminal end destabilized the C_L fragment. The rate of disulfide bond formation from reduced C_L(109–207) was about 7 times faster than that for C_L(109–214). These findings suggest that elongation of the polypeptide chain at least beyond the 207th residue is necessary for folding of the C_L fragment into a definite conformation.

Although many studies have been carried out on in vitro folding of proteins, the mechanism of folding in vivo is complicated and is not clearly understood. In addition to the problem of what factors are involved in the maturation and folding of nascent polypeptide chains in vivo, it is not clear whether the folding of proteins is initiated sequentially from the N-terminus (cotranslational folding) or after completion of the polypeptide chain (posttranslational folding) [see reviews: Jaenicke (1987), Tsou (1988), Wright et al. (1988), Baldwin (1989), and Fischer and Schmid (1990)]. Furthermore, it is unclear whether disulfide bonds are formed cotranslationally in vivo.

In our laboratory, the unfolding and refolding of the C_L[1] fragment of an immunoglobulin light chain have been studied in detail (Goto & Hamaguchi, 1982a,b, 1987; Tsunenaga et al., 1987; Goto et al., 1987; Kawata & Hamaguchi, 1991). In order to understand when folding of the C_L fragment is

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[1] Abbreviations: CD, circular dichroism; C_L, constant fragment of immunoglobulin light chain; C_L(109–x), fragment corresponding to sequence 109–x of type λ light chain (Nag); CPase, carboxypeptidase; DTT, dithiothreitol; Gdn-HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

0006-2960/91/0430-7766$02.50/0