Interaction of Cholesterol with Synthetic Sphingomyelin Derivatives in Mixed Monolayers†

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ABSTRACT: To study the structural requirements of the molecular interactions between cholesterol and sphingomyelins in model membranes, sphingomyelin derivatives were synthesized in which (a) the 3-hydroxy group was replaced with a hydrogen atom or with a methoxy, ethoxy, or tetrahydropyranyloxy group, (b) the *N*-acyl chain length was varied, and (c) the *N*-acyl chain length contained an α-hydroxy group. The chemical syntheses of these derivatives from DL-*erythro*-sphingosine are reported. The properties of these sphingomyelin derivatives were examined in monolayer membranes at the air/water interface. The mean molecular area of the pure *N*-stearoylsphingomyelin derivatives was determined, and the effects of cholesterol on the condensation of sphingomyelin packing in the monolayer were recorded. It was observed that replacement of the 3-hydroxy group of sphingomyelin with a hydrogen atom or its substitution with a methoxy or ethoxy group did not affect the ability of cholesterol to condense the molecular packing in monolayers. Even when a bulky tetrahydropyranyloxy group was introduced at the 3-hydroxy position of egg sphingomyelin, cholesterol was still able to condense the molecular packing of this derivative. The condensing effect of cholesterol on derivatives of *N*-stearoyl-SPMs was significantly larger than the comparable effect observed with 1,2-distearoyl-*sn*-glycero-3-phosphocholine or 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine. Our results with 3-hydroxysphingomyelins having differing *N*-acyl chain lengths (i.e., *N*-stearoyl, *N*-myristoyl, and *N*-lauroyl), and with 3-hydroxy-*N*-(α-hydroxypalmitoyl)sphingomyelin also indicated that cholesterol was able to induce condensation of the molecular packing. Another measure of the molecular packing in monolayers is the cholesterol oxidase susceptibility of cholesterol embedded in sphingomyelin-containing monolayers. The rate of enzyme-catalyzed cholesterol oxidation in monolayers containing 3-hydroxy-substituted *N*-stearoylsphingomyelins was about 30% lower than the comparable maximal rate measured in a monolayer of dipalmitoylphosphatidylcholine at the same surface pressure. Substitution of a hydroxy group at the α position of the amide chain of sphingomyelin did not perturb the projection of the sterol's 3β-hydroxy group toward the lipid/water interface. Cholesterol was, however, oxidized about 50% faster in monolayers containing 3-hydroxysphingomyelins with shorter acyl chains (i.e., *N*-lauroyl and *N*-myristoyl) than with a *N*-stearoyl chain, the rate being similar to that observed in a dipalmitoylphosphatidylcholine/cholesterol mixed monolayer. It is concluded that the 3-hydroxy group of sphingomyelin is not required for the efficient interaction between cholesterol and sphingomyelin in monolayer membranes. Furthermore, shortening the *N*-acyl group of sphingomyelin by four to six methylene groups had only a marginal effect on this interaction in monolayers.

Biological membranes are aggregates of proteins and various lipids. Membrane lipids include cholesterol and a heterogeneous group of complex lipids. It is well established that cholesterol interacts favorably with phospholipids and thereby stabilizes their membrane-building capacities (Phillips, 1972; Chapman, 1973; Nes, 1973; Huang, 1974). The specific interactions between cholesterol and different phospholipid classes appears to differ, however, and several lines of experimental approaches have suggested that cholesterol has a greater affinity for sphingomyelin (SPM)[1] compared with other phospholipid classes.

First, calorimetric studies on the interactions between cholesterol and different phospholipid classes have shown that cholesterol appears to mix more ideally with SPM than it does with phosphatidylcholine of equal chain lengths (Freire & Snyder, 1980). Earlier calorimetric studies indicating a preferential interaction between cholesterol and SPM in ternary mixtures of cholesterol, SPM, and phosphatidylcholine have been reported (Demel et al., 1977), but no preferential interaction between cholesterol and SPM or phosphatidylcholine was detected when the phospholipids were completely miscible with each other (Calhoun & Shipley, 1979).

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[1] Abbreviations: 3-deoxy-*N*-SPM, 3-deoxy-DL-*N*-stearoylsphingomyelin; 3-OMe-*N*-SPM, 3-*O*-methyl-DL-*erythro*-*N*-stearoylsphingomyelin; 3-OEt-*N*-SPM, 3-*O*-ethyl-DL-*erythro*-*N*-stearoylsphingomyelin; 3-OH-egg-SPM, 3-hydroxy-D-*erythro*-(egg sphingomyelin); 3-OH-*N*-stearoyl-SPM, 3-hydroxy-DL-*erythro*-*N*-stearoylsphingomyelin; 3-OH-*N*-myristoyl-SPM, 3-hydroxy-DL-*erythro*-*N*-myristoylsphingomyelin; 3-OH-*N*-lauroyl-SPM, 3-hydroxy-DL-*erythro*-*N*-lauroylsphingomyelin; 3-OH-*N*-(α-OH-palmitoyl)-SPM, 3-hydroxy-DL-*erythro*-*N*-[(2′-*R*,*S*)-hydroxyhexadecanoyl]sphingomyelin; egg 3-OTHP-SPM, egg 3-*O*-tetrahydropyranylsphingomyelin; DPPC, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine; DSPC, 1,2-distearoyl-*sn*-glycero-3-phosphocholine; POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; SPM, sphingomyelin; TBDPS, *tert*-butyldiphenylsilyl; TLC, thin-layer chromatography.

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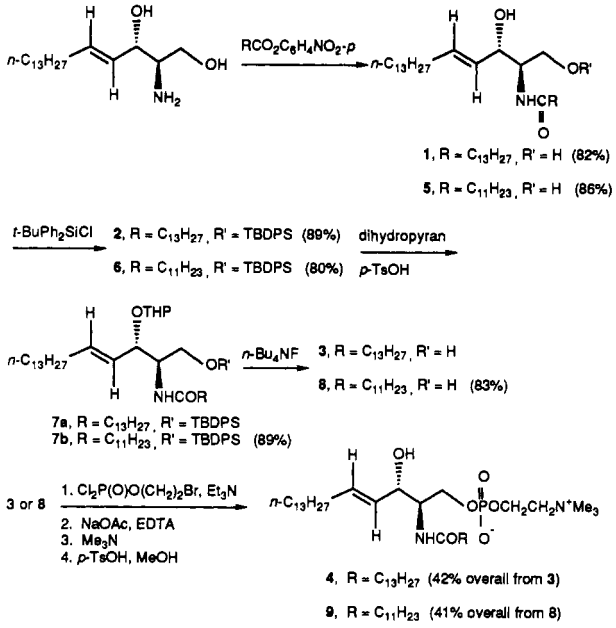
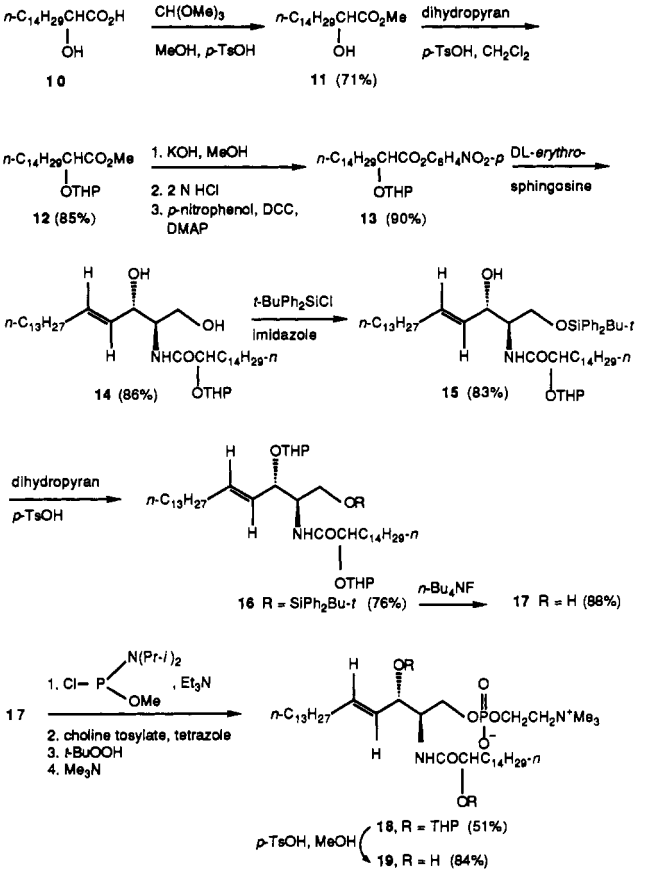
Scheme I: Reaction Sequence for the Syntheses of 3-OH-*N*-myristoyl-SPM (**4**) and 3-OH-*N*-lauroyl-SPM (**9**)



Scheme II: Reaction Sequence for the Synthesis of 3-OH-*N*-(α-OH-palmitoyl)-SPM (**19**)



Secondly, monolayer studies have indicated that cholesterol is able to condense the lateral packing density of SPM to a greater extent than is observed for phosphatidylcholine of the same acyl chain composition (Lund-Katz et al., 1988). SPM also has dramatic effects on the molecular dynamics of cholesterol in biological membranes, as it retards the rate of cholesterol desorption from lipid bilayers (Clejan & Bittman, 1984; Yeagle & Young, 1986; Lund-Katz et al., 1988; Gold & Phillips, 1990).

Finally, studies with cell culture systems indicated an apparent specific affinity of cholesterol toward SPM. Many years ago it was demonstrated that delipidated serum supplemented with SPM was far superior in extracting cholesterol from cultured cells compared with delipidated serum supplemented with other phospholipid classes (Burns & Rothblat, 1969). In addition, the selective removal of plasma membrane SPM by the action of sphingomyelinase has been shown to induce a dramatic redistribution of cholesterol from the cell surface into the cell (Slotte & Bierman 1988; Slotte et al., 1989, 1990; Pörn & Slotte 1990).

The higher affinity of cholesterol for SPM compared to other phospholipid classes could in part be the result of greater opportunities for van der Waals interactions between the two molecular species (Vandenheuvel, 1963; Lund-Katz et al., 1988), as well as to more favorable hydrogen-bonding opportunities between cholesterol and SPM (Boggs, 1987). Hydrogen bonding has been postulated as a stabilizing factor between the 3β-hydroxyl group of cholesterol and the amide oxygen in SPM (Boggs, 1987; Barenholz & Thompson, 1980), but experimental verification has been lacking. It is also possible that hydrogen bonds may form between cholesterol and the amide group of sphingomyelin, thus stabilizing the interaction.

The objective of this study was to examine the role of substitutions at the 3-hydroxy group of SPM on molecular interactions with cholesterol in mixed monolayer membranes at the water/air interface. We have determined the condensing effect of cholesterol (at variable mol %) on the molecular packing of these SPM derivatives. The effects of these SPM derivatives on the cholesterol oxidase susceptibility of cholesterol intercalated in SPM-containing monolayers (50 mol %) were also studied.

Material and Methods

Materials. The synthetic SPMs used in this study were prepared as 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 using 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).

Cholesterol (99% pure) and 5-cholesten-3-one (>98% pure) were purchased from Sigma Chemicals, St. Louis, MO, and their purities were determined by gas chromatography. 1-Palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine, 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, and 1,2-distearoyl-*sn*-glycero-3-phosphocholine were purchased from Sigma. The phosphatidylcholines were found to be chromatographically pure on thin-layer analysis by using Kiselgel 60 plates (Merck, Germany) with chloroform/methanol/acetic acid/water (50:30:8:4 v/v) as the solvent system (Skipski et al., 1967). Cholesterol oxidase (*Streptomyces sp.*) was obtained from Calbiochem, San Diego, CA. The organic solvents were at least 99% pure and were used without further purification. The water used was purified by using a Milli-Q Organex system to a resistivity greater than 15 MΩ/cm^2.

The syntheses of 3-OH-*N*-myristoyl-SPM (**4**) and 3-OH-*N*-lauroyl-SPM (**9**) were carried out as follows (see Scheme I).

*N-Myristoyl-*DL*-erythro-sphingosine* (**1**). This compound was prepared from DL-*erythro*-sphingosine and *p*-nitrophenyl myristate in 82% yield by using the same procedure as described by Kan et al. (1991) for *N*-stearoyl-DL-*erythro*-sphingosine; TLC ($CHCl_3$/EtOH, 10:1) R_f 0.47; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 [t, J = 6.5 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{10}$], 1.25 [m, 42 H, $(CH_2)_{11}$, $(CH_2)_{10}$], 1.56 (m, 2 H, CH_2CH_2CO), 2.00–2.07 (m, 2 H, CH_2CH=CH), 2.19–2.26 (t, J = 7.5 Hz, 2 H, CH_2CH_2CO), 3.68–3.72 (m, 1 H, CHNH), 3.89–3.98 (m, 2 H, CH_2OH), 4.07–4.14 (m, 1 H, CHOH), 5.47–5.58 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.75–5.87 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 6.20–6.25 (d, J = 7.2 Hz, 1 H, CHNH). Anal. calcd for $C_{32}H_{63}O_3N$: C, 75.38; H, 12.45; N, 2.75. Found: C, 75.29; H, 12.50; N, 2.69.

*1-(O-tert-Butyldiphenylsilyl)-2-(N-myristoyl)-*DL*-erythro-sphingosine* (**2**). This compound was prepared in 89% yield by using the same procedure as described by Kan et al. (1991) for the *N*-stearoyl derivative; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 [t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{10}$], 1.07 [s, 9 H, $C(CH_3)_3$], 1.25 [m, 42 H, $(CH_2)_{11}$, $(CH_2)_{10}$], 1.62–1.68 (m, 2 H, CH_2CH_2CO), 2.01–2.08 (m, 2 H, CH_2CH=CH), 2.15–2.22 (t, J = 7.5 Hz, 2 H, CH_2CH_2CO), 3.80–3.84 (m, 1 H, CHNH), 3.99–4.06 (m, 2 H, CH_2O), 4.20–4.25 (m, 1 H, CHOH), 5.49–5.58 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.77–5.86 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 6.13–6.17 (d, J = 7.5 Hz, 1 H, CHNH), 7.40–7.69 (m, 10 H, Ar). Anal. calcd for $C_{48}H_{81}O_3NSi$: C, 77.05; H, 10.91; N, 1.87. Found: C, 76.98; H, 11.06; N, 1.80.

*2-(N-Myristoyl)-3-(O-tetrahydropyranyl)-*DL*-erythro-sphingosine* (**3**). This compound was prepared from **2** in 72% yield; **2** was treated with dihydropyran and *p*-toluenesulfonic acid, giving **7a**, which was then treated with tetra-*n*-butylammonium fluoride, by using the same procedure as described by Kan et al. (1991) for the *N*-stearoyl derivative; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.91 [t, J = 6.4 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{10}$], 1.26 [m, 42 H, $(CH_2)_{11}$, $(CH_2)_{10}$], 1.51–1.78 [m, 8 H, CH_2CH_2CO, $(CH_2)_3$ of THP], 1.99–2.06 (m, 2 H, CH_2CH=CH), 2.15–2.22 (t, J = 8.0 Hz, 2 H, CH_2CH_2CO), 3.32 (m, 1 H, CHNH), 3.89–3.99 (m, 2 H, CH_2OH), 4.16–4.22 (m, 1 H CHOTHP), 4.45 (m, 1 H, O'CHO of THP), 5.31–5.43 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.64–5.78 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 6.36–6.40 (d, J = 7.6 Hz, 1 H, CHNH). Anal. calcd for $C_{37}H_{71}O_4N$: C, 74.82; H, 12.05; N, 2.36. Found: C, 75.29; H, 11.80; N, 2.21.

*N-Myristoyl-*DL*-erythro-SPM* (**4**). This compound was prepared in 41% overall yield by using the same procedure for incorporation of the phosphocholine moiety as described by Kan et al. (1991) for the *N*-stearoyl derivative. The THP protecting group was removed by heating overnight with *p*-toluenesulfonic acid in methanol; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 [t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{10}$], 1.25 [m, 42 H, $(CH_2)_{11}$, $(CH_2)_{10}$], 1.46 (m, 2 H, CH_2CH_2CO), 1.98–2.34 (m, 4 H, CH_2CH=CH, CH_2CH_2CO), 3.29 [s, 9 H, $N(CH_3)_3$], 3.77 [m, 3 H, $CH_2N(CH_3)_3$, CHNH], 4.04 [m, 4 H, $CH_2OP(O)(O^-)OCH_2$], 4.39 (m, 1 H, CHOH), 5.34–5.44 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.55–5.67 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H). Anal. calcd for $C_{37}H_{75}O_6N_2P\cdot 3H_2O$: C, 60.95; H, 11.20; N, 3.84; P, 4.25. Found: C, 60.80; H, 11.16; N, 3.98; P, 4.28.

*N-Lauroyl-*DL*-erythro-sphingosine* (**5**). This compound was prepared from DL-*erythro*-sphingosine and *p*-nitrophenyl laurate in 86% yield by using the same procedure as described for *N*-stearoyl-DL-*erythro*-sphingosine (Kan et al., 1991); ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.84–0.90 [t, J = 6.2 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_8$], 1.26 [m, 38 H, $(CH_2)_{11}$, $(CH_2)_8$], 1.49 (m, 2 H, CH_2CH_2CO), 1.93–1.97 (m, 2 H, CH_2CH=CH), 2.00–2.20 (t, J = 7.8 Hz, 2 H, CH_2CH_2CO), 3.42 (m, 1 H, CHNH), 3.81–3.91 (m, 2 H, CH_2OH), 4.03–4.11 (m, 1 H), CHOH), 5.40–5.51 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.68–5.80 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 6.13–6.18 (d, J = 7.5 Hz, 1 H, CHNH).

*1-(O-tert-Butyldiphenylsilyl)-2-(N-lauroyl)-*DL*-erythro-sphingosine* (**6**). This compound was prepared in 80% yield by using the same procedure as described for the *N*-myristoyl and *N*-stearoyl derivatives.

*1-(O-tert-Butyldiphenylsilyl)-2-(N-lauroyl)-3-(O-tetrahydropyranyl)-*DL*-erythro-sphingosine* (**7b**). This compound was prepared in 89% yield by using the same procedure as described for the *N*-stearoyl derivative (Kan et al., 1991); ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.84–0.91 [t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_8$], 1.07 [s, 9 H, $C(CH_3)_3$], 1.70–1.79 [m, 6 H, $(CH_2)_3$ of THP], 1.25 [m, 38 H, $(CH_2)_{11}$, $(CH_2)_8$], 1.51 (m, 2 H, CH_2CH_2CO), 1.97–2.09 (m, 2 H, CH_2CH=CH), 2.15–2.28 (t, J = 7.3 Hz, 2 H, CH_2CH_2CO), 3.33–3.44 (m, 1 H, CHNH), 3.70–3.89 (m, 2 H, CH_2O of THP), 3.98–4.03 (m, 2 H, CH_2O), 4.19–4.23 (m, 1 H, CHOTHP), 4.64–4.69 (m, 1 H, O'CHO of THP), 5.46–5.53 (dd, J = 15.4 Hz, 6.6 Hz, 1 H, vinyl H), 5.62–5.77 (dt, J = 15.4 Hz, 6.6 Hz, 1 H, vinyl H), 7.37–7.74 (m, 10 H, Ar).

*N-Lauroyl-3-(O-tetrahydropyranyl)-*DL*-erythro-sphingosine* (**8**). This compound was prepared in 83% yield by using the same procedure as described for the *N*-stearoyl derivative; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 [t, J = 6.4 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_8$], 1.26 [m, 38 H, $(CH_2)_{11}$, $(CH_2)_8$], 1.54–1.80 [m, 8 H, CH_2CH_2CO, $(CH_2)_3$ of THP], 2.02–2.04 (m, 2 H, CH_2CH=CH), 2.15–2.22 (t, J = 7.2 Hz, 2 H, CH_2CH_2CO), 3.34 (m, 1 H, CHNH), 3.47–3.63 (m, 2 H, CH_2O of THP), 3.89–4.00 (m, 2 H, CH_2O), 4.17–4.22 (m, 1 H, CHOTHP), 4.45 (m, 1 H, O'CHO of THP), 5.31–5.42 (dd, J = 15.5 Hz, 6.6 Hz, 1 H, vinyl H), 5.63–5.75 (dt, J = 15.5 Hz, 6.6 Hz, 1 H, vinyl H), 6.36–6.40 (d, J = 7.3 Hz, 1 H, CHNH).

*N-Lauroyl-*DL*-erythro-SPM* (**9**). This compound was prepared in 41% yield by using the same procedure as described for the corresponding *N*-stearoyl derivative (Kan et al., 1991); ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 [t, J = 6.3 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_8$], 1.25 [m, 38 H, $(CH_2)_{11}$, $(CH_2)_8$], 1.53 (m, 2 H, CH_2CH_2CO), 1.95 (m, 2 H, CH_2CH=CH), 2.12 (t, J = 7.2 Hz, 2 H, CH_2CH_2CO), 3.32 [s, 9 H, $N(CH_3)_3$], 3.66–3.79 [m, 3 H, CHNH, $CH_2N(CH_3)_3$], 3.97–4.19 [m, 4 H, $CH_2OP(O)(O^-)OCH_2$], 4.29 (m, 1 H CHOH), 5.39–5.46 (dd, J = 15.4 Hz, 6.6 Hz, 1 H, vinyl H), 5.61–5.72 (dt, J = 15.4 Hz, 6.6 Hz, 1 H, vinyl H), 7.04–7.08 (d, J = 7.5 Hz, 1 H, CHNH).

The synthesis of 3-OH-*N*-(α-OH-palmitoyl)-SPM (**19**) was carried out as follows (see Scheme II).

Methyl (2R,S)-Hydroxyhexadecanoate (**11**). (2*R*,*S*)-Hydroxyhexadecanoic acid (**10**; purchased from Lancaster Synthesis Ltd., Windham, NH) (2.0 g, 7.34 mmol) was dissolved in 35 mL of dry THF. Methanol (327 mL, 8.07 mmol), trimethyl orthoformate (3.21 mL, 29.4 mmol), and a trace of *p*-toluenesulfonic acid (monohydrate) were added. After the reaction mixture was refluxed overnight, the solvents were removed on a rotary evaporator. The crude product was pu-

rified by flash chromatography (elution with hexane/ethyl acetate, 3:1) to give 1.50 g of pure product **11**; yield, 71%; TLC (hexane/ethyl acetate, 3:1) R_f 0.44; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 (t, J = 6.3 Hz, 3 H, CH_3), 1.25 [m, 24 H, $(CH_2)_{12}$], 1.64 (m, 2 H, CH_2CHOH), 3.73 (t, J = 6.0 Hz, 1 H, CH_2CHOH), 3.79 (s, 3 H, CH_3).

*Methyl (2R,S)-O-(Tetrahydropyranyloxy)hexadecanoate (**12**).* Methyl (2*R*,*S*)-hydroxyhexadecanoate (**11**) 1.30 g, 4.54 mmol) was dissolved in 35 mL of methylene chloride. Dihydropyran (704 mL, 7.72 mmol) and a trace of *p*-toluenesulfonic acid were added. The reaction mixture was stirred at room temperature overnight. The solution was diluted with ether (15 mL) and washed with saturated sodium bicarbonate solution (25 mL) and water (25 mL). The organic phase was dried ($MgSO_4$), the solvents were removed on a rotary evaporator, and the residue was purified by flash chromatography (elution with hexane/ethyl acetate, 5:1) to give 1.33 g (79%) of 12; TLC (hexane/ethyl acetate, 5:1) R_f 0.52.

*p-Nitrophenyl [(2'R,S)-Tetrahydropyranyloxy]hexadecanoate (**13**).* A solution of potassium hydroxide (180 mg, 3.10 mmol) in 10 mL of methanol was added dropwise to 1.0 g (2.80 mmol) of methyl ester **12** in a 100-mL flask at room temperature. The reaction mixture was stirred at this temperature overnight, then heated at 60–70 °C for 3 h. The solvent was removed under reduced pressure. The residue was dissolved in 30 mL of water, cooled to 0 °C, and 2 N HCl was added slowly until the pH of the solution was 3. The product was isolated by extraction with methylene chloride (2 × 30 mL). The organic phases were combined, and the solvent was removed by rotary evaporation. To a solution of crude product in 20 mL of methylene chloride were added *p*-nitrophenol (442 mg, 3.08 mmol), *N*,*N*-dicyclohexylcarbodiimide (635 mg, 3.08 mmol), and 4-(dimethylamino)pyridine (34.2 mg, 0.28 mmol). The reaction mixture was allowed to stir at room temperature for 3 h. The *N*,*N*-dicyclohexyl urea precipitate was removed by filtration, and the filtrate was washed with 5% aqueous NaOH solution (3 × 20 mL). The organic phase was dried ($MgSO_4$), and the solvent was removed by rotary evaporation. The crude product was purified by flash chromatography (elution with hexane/ethyl acetate, 7:1) to give 1.2 g (90%) of **13**; TLC (hexane/ethyl acetate, 7:1) R_f 0.68; ^{1}H NMR (300 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 (t, J = 6.5 Hz, 3 H, CH_3), 1.20–1.33 [m, 24 H, $(CH_2)_{12}$], 1.55–2.00 (m, 8 H, $C_{13}H_{27}C$-H_2CHO, 3 CH_2 of THP), 3.65–3.80 (m, 2 H, CH_2O), 4.56–4.61 (t, J = 6.1 Hz, 1 H, $CHOCO_2$), 4.78–4.82 (m, 1 H, OCHO), 7.25–7.30 (m, 2 H, Ar), 8.25–8.30 (m, 2 H, Ar).

*N-[(2'-Tetrahydropyranyloxy)hexadecanoyl]-DL-erythro-sphingosine (**14**).* This compound was prepared from the reaction of DL-*erythro*-sphingosine (prepared as outlined by Kan et al. (1991)] with *p*-nitrophenyl ester **13** in 86% yield by using the same procedure as described for *N*-stearoylceramide; TLC ($CHCl_3$/EtOH, 10:1) R_f 0.45; ^{1}H NMR (200 MHz, $CDCl_3$, primes refer to the THP group in the amide chain) δ (ppm) 0.85–0.90 [t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{12}$], 1.29–1.37 [m, 46 H, $(CH_2)_{11}$, $(CH_2)_{12}$], 1.68–1.90 [m, 8 H, $(CH_2)_3$ of THP, CH_2CH_2CO], 2.05–2.12 (m, 2 H, CH_2CH=CH), 3.55–3.58 (m, 1 H, CHNH), 3.71–3.76 (m, 1 H, CHOH), 3.89–4.00 (m, 4 H, CH_2OH, CH_2O of THP), 4.22–4.26 (t, J = 7.2 Hz, 1 H, OCHCO), 4.63–4.66 (m, 1 H, O'CHO of THP), 5.51–5.59 (m, 1 H, vinyl H), 5.80–5.85 (m, 1 H, vinyl H), 7.19–7.22 (d, J = 7.5 Hz, 1 H, CHNH).

*1-(O-tert-Butyldiphenylsilyl)-N-[(2'-tetrahydropyranyloxy)hexadecanoyl]-DL-erythro-sphingosine (**15**).* This compound was prepared from **14** in 83% yield by using the same procedure as described for silylation of the *N*-stearoyl derivative (Kan et al., 1991); TLC (hexane/ethyl acetate, 4:1), R_f 0.44; ^{1}H NMR (200 MHz, $CDCl_3$, primes refer to the THP group in the amide chain) δ 0.85–0.90 [t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{12}$], 1.08 [s, 9 H, $C(CH_3)_3$], 1.25 [m, 46 H, $(CH_2)_{11}$, $(CH_2)_{12}$], 1.50–1.88 [m, 8 H, $(CH_2)_3$ of THP, $CH_2CHOTHP$], 1.97–2.03 (m, 2 H, CH_2CH=CH), 3.48–3.55 (m, 1 H, CHNH), 3.76–4.00 (m, 5 H, CHOH, CH_2O, CH_2O of THP), 4.20–4.24 (t, J = 7.2 Hz, 1 H, OCHCO), 4.60–4.63 (m, 1 H, O'CHO of THP), 5.40–5.52 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.70–5.82 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 7.10–7.13 (d, J = 7.5 Hz, 1 H, CHNH), 7.38–7.65 (m, 10 H, Ar). Anal. calcd for $C_{55}H_{93}O_5NSi$: C, 75.38; H, 10.70; N, 1.60. Found: C, 75.55; H, 10.83; N, 1.57.

*1-(O-tert-Butyldiphenylsilyl)-N-[(2'-tetrahydropyranyloxy)hexadecanoyl]-3-(O-tetrahydropyranyl)-DL-erythro-sphingosine (**16**).* This compound was prepared from **15** in 76% yield by using the same procedure for insertion of the THP group as described by Kan et al. (1991); TLC (hexane/ethyl acetate, 5:1) R_f 0.65.

*N-[(2'-Tetrahydropyranyloxy)hexadecanoyl]-3-(O-tetrahydropyranyl)-DL-erythro-sphingosine (**17**).* This compound was prepared from **16** in 88% yield by heating overnight with *p*-toluenesulfonic acid in methanol; TLC (hexane/ethyl acetate, 2:3) R_f 0.36; ^{1}H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 [t, J = 6.3 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{12}$], 1.26 [m, 46 H, $(CH_2)_{11}$, $(CH_2)_{12}$], 1.55–1.78 [m, 14 H, $(CH_2)_3$ of THP, $(CH_2)_3$ of THP in amide chain, and O'$CHCH_2$], 2.02–2.06 (m, 2 H, CH_2CH=CH), 3.21 (m, 1 H, CHNH), 3.51–3.60 (m, 1 H, CHOTHP), 3.71–4.02 (m, 6 H, CH_2OH, CH_2O of THP, and CH_2O of THP in amide chain), 4.13–4.18 (m, 1 H, OCHCO), 4.52 (m, 1 H, O'CHO of THP), 4.61 (m, 1 H, O'CHO of THP in amide chain), 5.30–5.42 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.65–5.76 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 7.15–7.19 (d, J = 7.7 Hz, 1 H, CHNH). Anal. calcd for $C_{44}H_{83}O_6N$: C, 73.18; H, 11.58; N, 1.94. Found: C, 72.57; H, 11.49; N, 1.84.

*N-[(2'-Tetrahydropyranyloxy)hexadecanoyl]-3-(O-tetrahydropyranyl)-DL-erythro-SPM (**18**).* To a solution of 160 mg (0.22 mmol) of ceramide **17** and 62 mL (0.44 mmol) of triethylamine in 5 mL of chloroform at 0 °C was added with syringe *N*,*N*-diisopropylmethylphosphoramidic chloride (54.3 mL, 0.26 mmol, 20% molar excess). After the mixture was allowed to react for 10 min at 0 °C, TLC (hexane/ethyl acetate, 1:1) indicated that no starting ceramide remained. After the mixture was concentrated to dryness under vacuum (without using a rotary evaporator in order to avoid water vapor), 1*H*-tetrazole (55.5 mg, 0.79 mmol, 3.6-fold excess) and dry choline tosylate [182 mg, 0.66 mmol, 3.0-fold excess; prepared as described by Rosenthal (1966) and dried over P_2O_5 at 78 °C] were added to the reaction flask. Acetonitrile/THF (1:1) (6 mL) was added, and the solution was stirred for 4 h at room temperature, after which time TLC analysis showed that the reaction was complete. The mixture was again evaporated to dryness under reduced pressure. The residue was dissolved in THF (5 mL) and added to *tert*-butyl hydroperoxide (81 mL of a 3 M solution in hexane, 0.25 mmol, 10% excess). The reaction mixture was stirred for 2 h at room temperature. Ethyl acetate (10 mL) was added, and the layers were separated. The organic layer was washed with 15 mL of 1 M triethylammonium hydrogen carbonate buffer, pH 7.5, to remove the excess of tetrazole and choline tosylate. The organic phase was concentrated to dryness, and the residue was dried thoroughly by repeated evaporation with dry toluene.

Finally, the toluene solution (5 mL) was treated with anhydrous trimethylamine (1 mL) in a 25-mL pressure bottle for 12 h at room temperature. After this period of time, the demethylation of the methyl ester of phosphocholine moiety was complete as judged by TLC ($CHCl_3$/MeOH/H_2O/conc. NH_4OH, 60:30:2:2). The solvent was removed using a rotary evaporator. The residue was purified by flash chromatography (elution with $CHCl_3$/MeOH/H_2O/conc. NH_4OH, 60:30:2:3). Suspended silica gel was removed by passing a chloroform solution of the product through a 0.45-mm Metricel filter (Gelman Sciences) three times to give 100 mg of **18** (51% overall yield from ceramide **17**).

N-[(2′*R*,*S*)-*Hydroxyhexadecanoyl*]-DL-*erythro-SPM* (**19**). To a solution of 100 mg (0.11 mmol) of **18** in 5 mL of dry methanol was added a trace of *p*-toluenesulfonic acid. After the reaction mixture was heated at 70 °C overnight, chloroform (15 mL) was added. The mixture was washed with saturated aqueous sodium bicarbonate solution (10 mL). The organic phase was dried by repeated evaporation with isopropyl alcohol. The crude product was purified by flash chromatography (elution first with $CHCl_3$/MeOH, 3:1, then with $CHCl_3$/MeOH/H_2O/conc. NH_4OH, 60:30:2:3). Suspended silica gel was removed by passing a chloroform solution of the product through a 0.45-mm Metricel filter three times to give 68 mg (84%) of the product; TLC ($CHCl_3$/MeOH/H_2O/conc. NH_4OH, 60:30:2:3) R_f 0.28; ^1H NMR (200 MHz, $CDCl_3$) δ (ppm) 0.85–0.90 [t, J = 6.6 Hz, 6 H, $CH_3(CH_2)_{11}$, $CH_3(CH_2)_{12}$], 1.25 [m, 46 H, $(CH_2)_{11}$, $(CH_2)_{12}$], 1.46 (m, 2 H, CH_2CHOH), 1.98 (m, 2 H, $CH_2CH{=}CH$), 3.29 [s, 9 H, $N(CH_3)_3$], 3.41 (m, 1 H, CHNH), 3.75–3.92 [m, 3 H, $CH_2N(CH_3)_3$, CHOH], 4.05–4.25 [m, 5 H, $CH_2OP(O)O^-O$-CH_2, CHOHCO], 5.34–5.49 (dd, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H), 5.69–5.80 (dt, J = 15.4 Hz, 6.7 Hz, 1 H, vinyl H). Anal. calcd for $C_{39}H_{79}O_7N_2P{\cdot}2H_2O$: C, 62.04; H, 11.08; N, 3.71. Found: C, 61.87; H, 10.84; N, 3.44.

Surface Pressure—Mean Molecular Area Isotherms. Surface pressure versus mean molecular area isotherms were measured for pure phospholipids and for various phospholipid/cholesterol and phospholipid/cholestenone mixtures with a computer-controlled KSV 5000 surface barostat (KSV Instruments, Helsinki). The lipids (50 μg) were spread from a hexane/ethanol (9:1, v/v) solution on water in a rectangular teflon trough. The trough had a total area of 487.5 cm^2 (6.5 cm × 75.0 cm), and it was thermostated to 25 °C. The solvent was allowed to evaporate, whereafter the monolayer was compressed with a barrier speed of 20 mm^2/s. Data were sampled every 2 s and were collected and analyzed with proprietary software (KSV Instruments).

Calculation of Percent Monolayer Condensation. The theoretical values for the mean molecular areas (A_{12}) of the mixed monolayers were calculated as follows (Chapman et al., 1969; Ibdah et al., 1989):

$$A_{12} = nA_1 + (1 - n)A_2 \quad (1)$$

A_1 and A_2 represent the mean molecular areas of the pure lipids and n and $(1 - n)$ represent their respective molar fractions in the mixed monolayer. The theoretical value (A_{12}) represents the area of a mixed monolayer with no condensation and is used as a zero value for calculation of the percent condensation of the mixed monolayer (observed mixed monolayer area = A_{mix}):

$$\% \text{ condensation} = [(A_{12} - A_{mix})/A_{12}] \times 100 \quad (2)$$

Cholesterol Oxidase Activity Measurements in Monolayers. The surface barostat and the method used for determination of cholesterol oxidase activity in monolayers have been described earlier (Grönberg & Slotte, 1990). The determinations were performed on water in a zero-order trough (Verger & de Haas, 1973), with a magnetically stirred (150 rpm) reaction compartment thermostated to 25 °C. The phospholipid/cholesterol mixture (50:50 mol %; 12.5 μg) in hexane/ethanol (9:1, v/v) was spread on the water, and the solvent was allowed to evaporate. The monolayer was compressed until a surface pressure of 15 mN/m was reached (barrier speed 6 mm^2/s), and this pressure was maintained by compensatory barrier movement (computer controlled) during the rest of the experiment. After the monolayer had stabilized at this surface pressure for about 5 min, 10 mUnits/mL cholesterol oxidase was added to the reaction compartment. The oxidation of cholesterol in the monolayer was registered as a backward movement of the barrier, as the reaction caused a monolayer area increase. Data were sampled every 10 s.

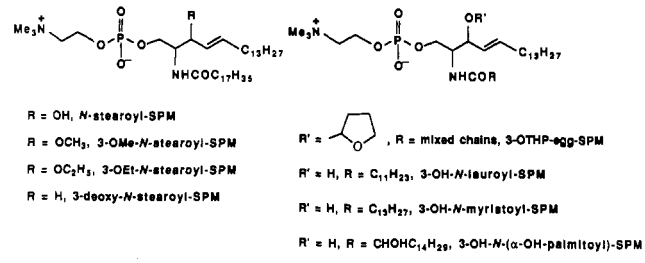


FIGURE 1: Structures of the SPM derivatives used in this study.

Calculation of Enzyme Activity. When cholesterol in a monolayer is oxidized to cholestenone the molecular interactions between the sterol and the phospholipid molecules are dramatically changed. It has been shown that cholestenone does not interact strongly with phospholipids in membrane systems (Bruckdorfer et al., 1969; Demel et al., 1972a, 1972b; Barenholz et al., 1981). The oxidation of cholesterol to cholestenone in monolayers can be observed as an increased monolayer area (Grönberg & Slotte, 1990). The enzyme activity was calculated as the percent of total cholesterol that was converted to cholestenone per second. The rate of conversion was calculated from the linear part of the area expansion curve representing the maximal oxidation rate. Since the condensation varied with surface pressure and with the use of different phospholipids, the measured activities were related to the corresponding isotherms. The area difference between the isotherms of corresponding cholesterol- and cholestenone-containing monolayers was used as a measure of total oxidation. The area increase caused by cholesterol oxidase was directly related to this measure of total oxidation.

Assays. The concentration of phospholipids in samples of the stock solutions was determined by using a commercial phospholipid/phosphorus assay kit (Boehringer Mannheim, Germany). The purity of the phospholipid samples was verified by TLC on Kiselgel 60 plates (Merck, Germany) with chloroform/methanol/acetic acid/water as eluent (50:30:8:4, v/v; Skipski et al., 1967). Each phospholipid class (and derivative) gave a single spot on the TLC plate.

RESULTS

Structures of the Synthetic SPMs Used. The structures of the different synthetic SPMs used in this study are shown in Figure 1. The first group consisted of *N*-stearoyl- and egg-SPMs with a substitution at the 3 position. The substitution of the smallest molecular size was the replacement of the 3-hydroxy group with a hydrogen atom (i.e., 3-deoxy-*N*-stearoyl-SPM), and the bulkiest substitution was the addition of a tetrahydropyranyloxy group at the 3 position of egg SPM. Other 3-hydroxy substitutions were the 3-methoxy and 3-

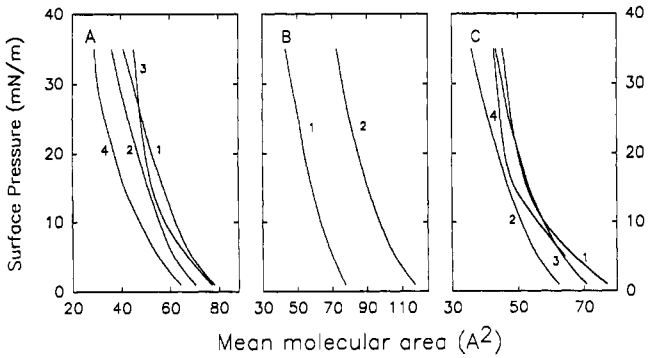


FIGURE 2: Isotherms of pure SPM derivatives on water at 25 °C. (A) Isotherms of 3-OH-substituted *N*-stearoyl-SPMs: 3-OH-*N*-stearoyl-SPM (line 3), 3-deoxy-*N*-SPM (line 4), 3-OMe-*N*-SPM (line 2), and 3-OEt-*N*-SPM (line 1). (B) Isotherms of 3-OH-substituted egg SPMs: 3-OH-egg-SPM (line 1) and 3-OHTP-egg-SPM (line 2). (C) Isotherms of 3-OH-SPMs with varying lengths of the amide-linked hydrocarbon chains: 3-OH-*N*-stearoyl-SPM (line 1), 3-OH-*N*-myristoyl-SPM (line 2), 3-OH-*N*-lauroyl-SPM (line 3), and 3-OH-*N*-(α-hydroxypalmitoyl)-SPM (line 4).

ethoxy derivatives of *N*-stearoyl-SPM. Another type of structural variation was to vary the length of the amide-linked hydrocarbon chains (*N*-lauroyl, *N*-myristoyl, and *N*-stearoyl), keeping the 3-hydroxy group without modification. In addition, 3-hydroxy-*N*-(α-hydroxypalmitoyl)-SPM was used to examine the effects of the α-hydroxy group in the fatty amide chain on cholesterol interactions. The latter compound was included since it has been demonstrated that naturally occurring SPMs contain α-hydroxy chains in some organisms (Karlsson, 1970).

Isotherms of Pure Synthetic SPMs. To examine how the substitutions of the 3-hydroxy group in SPM and how the length of the N-linked chain affected molecular packing in pure SPM monolayers, surface pressure versus mean molecular area isotherms were run for the different SPM derivatives. Figure 2 (panel A) shows the isotherms for 3-hydroxy-substituted *N*-stearoyl-SPMs. The 3-deoxy derivative had the smallest mean molecular area of the group, at all surface pressures examined (curve 4). Substitution of a 3-methoxy or 3-ethoxy group did not markedly change the mean molecular area compared with the parent compound (i.e., 3-hydroxy-*N*-stearoyl-SPM). With egg yolk derived SPMs (Figure 2, panel B), it was observed that the introduction of a tetrahydropyranyloxy group markedly increased the mean molecular area compared with the parent compound (Figure 2, panel B, curve 2).

The monolayer packing isotherms of SPM derivatives with variable N-linked hydrocarbon chain lengths (*N*-lauroyl, *N*-myristoyl, and *N*-stearoyl) are shown in panel C of Figure 2. 3-Hydroxy-*N*-myristoyl-SPM occupied the smallest area, whereas the *N*-lauroyl- and *N*-stearoyl-3-hydroxy-SPMs had mean molecular areas similar to each other; the latter compounds have mean molecular areas markedly larger than that of 3-OH-*N*-myristoyl-SPM. The mean molecular area of 3-OH-*N*-(α-OH-palmitoyl)-SPM was slightly smaller than that determined for 3-OH-*N*-stearoyl-SPM but larger than the value determined for 3-OH-*N*-myristoyl-SPM.

Properties of Cholesterol/SPM Monolayers. The molecular interactions between cholesterol and the various SPM derivatives were studied in mixed monolayers at the water/air interface. The sterol and the SPM components were mixed in different ratios (given as mole fraction of SPM), and the mean molecular area isotherms were recorded at various lateral surface pressures. Within the group of the 3-hydroxy-substituted *N*-stearoyl-SPMs, all compounds displayed decreased

Table I: Mean Molecular Areas of Various Synthetic Phospholipids in Pure Monolayers and in Mixed Monolayers Containing Equimolar Cholesterol at 25 °C and 15 mN/m

phospholipid monolayer	mean molecular area of phospholipid (Å²/mol) PL[a]	PL/Chol[b]	ΔÅ²	% condensation of PL packing
3-OH-*N*-stearoyl-SPM	52.7	41.3	11.4	13.0
3-OMe-*N*-stearoyl-SPM	51.0	39.3	11.7	13.5
3-OEt-*N*-stearoyl-SPM	57.3	35.9	21.4	22.3
3-deoxy-*N*-stearoyl-SPM	41.3	27.3	14.0	17.4
3-OH-egg-SPM	58.3	41.9	16.4	17.5
3-OTHP-egg-SPM	90.3	61.3	29.0	22.5
3-OH-*N*-stearoyl-SPM	52.7	41.3	11.4	13.0
3-OH-*N*-myristoyl-SPM	47.0	38.7	8.3	9.7
3-OH-*N*-lauroyl-SPM	53.3	40.7	12.6	13.7
3-OH-*N*-(α-OH-palmitoyl)-SPM	47.0	42.7	4.3	5.0
1,2-dipalmitoylphosphatidylcholine	46.6	41.2	5.4	6.5
1,2-distearoylphosphatidylcholine	48.2	42.2	6.0	7.1

[a] 100% phospholipid; [b] 50 mol % phospholipid.

mean molecular areas in the presence of cholesterol (Figure 3, panels A–D). This results from the well-known condensing effect of cholesterol on the packing of phospholipids in monolayers (Demel et al., 1967; Chapman et al., 1969; Phillips, 1972).

With egg yolk derived 3-hydroxy-SPM and 3-OTHP-SPM, cholesterol induced a comparable condensation of SPM monolayer packing (Figure 3, panels E and F). When the effects of variations in the length of the N-linked acyl chains on molecular packing were examined, it was again observed that cholesterol was able to condense the molecular packing in the mixed monolayer containing *N*-lauroyl, *N*-myristoyl, and *N*-stearoyl derivatives of 3-hydroxy-SPM (Figure 3, panels G, H, and J), as well as in a monolayer containing 3-OH-*N*-(α-OH-palmitoyl)-SPM (Figure 3, panel I). The percent condensation caused by incorporation of 50 mol % cholesterol (Table I) was smallest for 3-OH-*N*-(α-OH-palmitoyl)-SPM monolayers. The insertion of the polar hydroxy group into the N-linked chain of SPM near the sphingosine moiety apparently interferes with the strong interaction between cholesterol and SPM molecules, as reflected by the low value of ΔÅ².

Comparison of SPM/Cholesterol and PC/Cholesterol Monolayer Properties. The condensing effect of an equimolar amount of cholesterol in SPM and saturated PC (DPPC and DSPC) monolayers is shown in Table I. The mean molecular areas in equimolar films with cholesterol and phospholipids are lower in SPM monolayers than in PC monolayers at a surface pressure of 15 mN/m (Table I). These results indicate that the *N*-stearoyl-SPM molecules are more effectively packed than DPPC and DSPC molecules in the presence of equimolar cholesterol. The only exception to this general effect was observed with 3-OH-*N*-(α-OH-palmitoyl)-SPM, in which the hydroxy group of the N-linked chain may interefere with van der Waals attractive interactions with cholesterol.

Interestingly, the mean phospholipid molecular area of DPPC and DSPC also decreased to about 41–42 Å² in the presence of 50 mol % cholesterol (Table I), although the ΔÅ² was much smaller than observed with SPMs. Our values for the mean molecular areas of pure DPPC (at similar lateral surface pressures) are slightly lower than the values reported by Lund-Katz et al. (1988) but are very close to values reported by others (Birdi, 1987; Teissie et al., 1976). Our mean

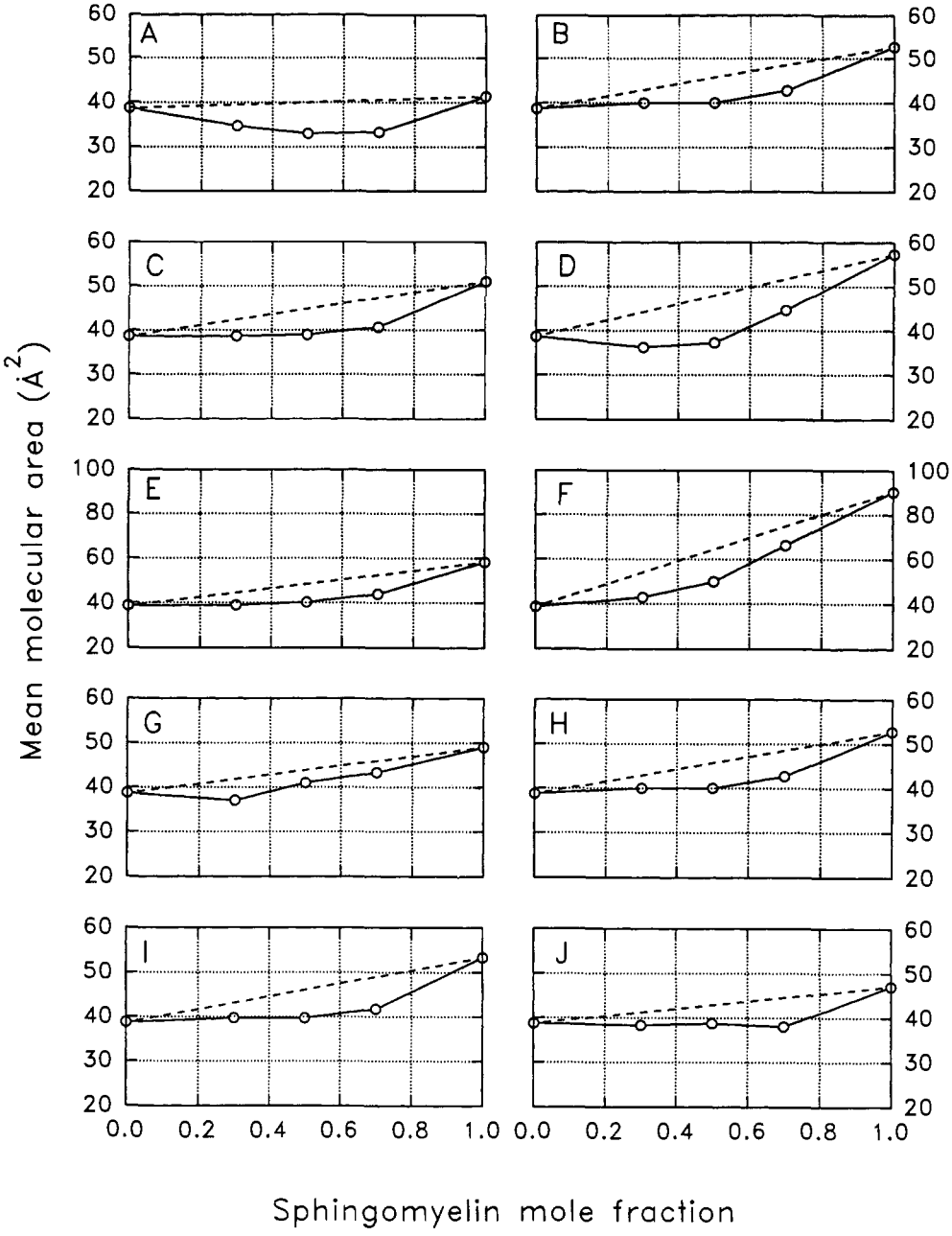


FIGURE 3: Relative condensations of mixed monolayers of cholesterol and sphingomyelin derivatives on water at 15 mN/m (25 °C). Mixtures of cholesterol with (A) 3-deoxy-*N*-stearoyl-SPM; (B) 3-OH-*N*-stearoyl-SPM; (C) 3-OMe-*N*-stearoyl-SPM; (D) 3-OEt-*N*-stearoyl-SPM; (E) 3-OH-egg-SPM; (F) 3-OTHP-egg-SPM; (G) 3-OH-*N*-lauroyl-SPM; (H) 3-OH-*N*-myristoyl-SPM; (I) 3-OH-*N*-(α-OH-palmitoyl)-SPM; (J) 3-OH-*N*-stearoyl-SPM. The dashed line indicates ideal mixing, where the mean molecular area was calculated by using eq 1.

molecular area of DSPC agrees well with value of 49–50 Å^2 reported by Paltauf et al. (1971) at the same surface pressure.

Cholesterol Oxidase Susceptibility of Cholesterol in SPM-Containing Monolayers. The oxidizability of cholesterol in a mixed monolayer can be considered as a measure of the packing of the monolayer, since looser packing allows higher rates of cholesterol oxidation (Grönberg & Slotte, 1990). The results of the enzyme-catalyzed oxidation of cholesterol in mixed monolayers are summarized Table II, and the rate is expressed as the percentage of the total cholesterol in the monolayer that was oxidized per unit time (s). The rates of cholesterol oxidation were remarkably similar for the various 3-hydroxy-substituted SPM derivatives. The rates were, however, only about 40% of the comparable oxidation rate observed in a more expanded mixed monolayer of 1-palmitoyl-2-oleoylphosphatidylcholine, and about 77% of the rate observed in a DPPC/cholesterol mixed monolayer. The 3-hydroxy-SPMs with shorter N-linked acyl chains (*N*-myristoyl and *N*-lauroyl) gave 40–50% higher cholesterol oxidation rates compared with 3-OH-*N*-stearoyl-SPM (Table II).

Discussion

This study has examined the effects of both the 3-hydroxy group of SPM as well as the length of the N-linked acyl chain on cholesterol/SPM interactions in mixed monolayers at the air/water interface. The main conclusion is that the replacement of the 3-hydroxy group with a hydrogen atom or with a methoxy, ethoxy, or a tetrahydropyranyloxy group did not markedly change the interactions between the SPM derivatives and cholesterol. This conclusion is drawn because cholesterol was able to condense the packing of the SPMs in the mixed monolayer, despite their 3-hydroxy substitutions. These substitutions also did not affect rates of enzyme-catalyzed oxidation of cholesterol in the monolayers, a process

Table II: Cholesterol Oxidase Catalyzed Oxidation of Cholesterol in Mixed Monolayers Containing Phosphatidylcholines or SPM Derivatives at 25 °C and 15 mN/m

phospholipid derivative	oxidation rate (% per s)[a]
3-OH-*N*-stearoyl-SPM	0.10 ± 0.01
3-OMe-*N*-stearoyl-SPM	0.10 ± 0.01
3-OEt-*N*-stearoyl-SPM	0.10 ± 0.02
3-deoxy-*N*-stearoyl-SPM	0.08 ± 0.00
3-OH-egg-SPM	0.09 ± 0.00
3-OTHP-egg-SPM	0.11 ± 0.02
3-OH-*N*-stearoyl-SPM	0.10 ± 0.01
3-OH-*N*-myristoyl-SPM	0.15 ± 0.01
3-OH-*N*-lauroyl-SPM	0.14 ± 0.01
3-OH-*N*-(α-OH-palmitoyl)-SPM	0.10 ± 0.00
1,2-dipalmitoylphosphatidylcholine	0.13 ± 0.01
1-palmitoyl-2-oleoylphosphatidylcholine	0.25 ± 0.01

[a] Each value is the average of at least three separate measurements ±SE.

known to be dependent on lipid packing parameters in the monolayer (Grönberg & Slotte, 1990).

The condensing effect of cholesterol on phospholipid packing in monolayers is a well-documented phenomenon (Demel et al., 1967, Chapman et al., 1969, Phillips, 1972). It appears that the 3β-hydroxy group of cholesterol is necessary for the effect, since cholestenone (which lacks the 3β-hydroxy group) also has a reduced capacity to condense the packing of phospholipids in monolayers (Demel et al., 1972a,b). In addition to the necessary 3β-hydroxy group, a condensing sterol must also have a fused tetracyclic ring system that is hydrophobic and stereochemically rigid (Butler et al., 1970; Demel et al., 1972a,b; de Kruijff et al., 1973a,b).

The molecular requirements for the condensing effect with respect to phospholipid structure are less clearly defined. It appears that the polar head group has some influence on the cholesterol-induced condensation of lipid packing, since cholesterol can condense the monolayer packing of dimyristoylphosphatidylcholine at a lateral surface pressure of 20 mN/m, whereas it cannot condense the packing of dimyristoylphosphatidylethanolamine under identical conditions (Chapman et al., 1969). The degree of unsaturation and length of the phospholipid acyl chains also appear to have marked effects on the cholesterol-induced condensation of molecular packing. Cholesterol is efficient in condensing the packing of unsaturated phosphatidylcholines (e.g., egg and dioleoyl), whereas it can only to a limited extent further condense the packing of saturated phosphatidylcholines (e.g., DPPC) at a lateral surface pressure above 20 mN/m (Lund-Katz et al., 1988). Other studies have indicated that phosphatidylcholines require hydrocarbon chains longer than 10 carbon atoms in order to be condensed by cholesterol (Joos & Demel, 1967; de Kruijff et al., 1973b). The present study shows that the extent of condensation induced by cholesterol is markedly larger for synthetic saturated SPMs (e.g., 3-OH-*N*-stearoyl-SPM) than it is for synthetic saturated phosphatidylcholines (.e.g., DSPC). This finding is consistent with monolayer data reported by Lund-Katz et al. (1988), which indicated that cholesterol increases the lateral cohesion energy of several SPMs more than with PCs of comparable acyl chain saturation.

The finding of this study that substitutions at the 3-hydroxy position of SPMs (*N*-stearoyl-SPM or egg SPM) did not abolish the cholesterol-induced condensation of molecular packing in mixed monolayers strongly suggests that the 3-hydroxy group of SPM is not centrally involved in those interactions with cholesterol that affect its condensing properties. The observations that the cholesterol oxidase susceptibility of cholesterol embedded in mixed monolayers was not markedly affected by the substitutions at the 3-hydroxy position in SPM further suggest that these functional groups did not interfere with the projection of the 3β-hydroxy group of cholesterol toward the lipid/water interface.

The rate of enzyme-catalyzed oxidation of cholesterol in mixed monolayers is known to depend both on the lateral surface pressure and on the phospholipid composition (Grönberg & Slotte, 1990). The optimal surface pressure for the cholesterol oxidase from *Streptomyces sp.* is around 20 mN/m. The rate of cholesterol oxidation was much faster in an unsaturated phosphatidylcholine monolayer compared to either a saturated phosphatidylcholine monolayer (e.g., DPPC) or a SPM monolayer. Within the group of 3-hydroxy-SPMs with differing lengths of N-linked acyl chain, cholesterol was oxidized significantly faster in 3-OH-*N*-lauroyl- and 3-OH-*N*-myristoyl-SPM monolayers. This suggests that the availability of cholesterol for oxidation by cholesterol oxidase at the monolayer/water interface was influenced not only by the phospholipid degree of saturation and polar head group composition but also (within the group of SPMs) by the length of the N-linked acyl chain in the hydrophobic region of the monolayer.

Another indicator of cholesterol/phospholipid interactions in membranes that can be examined is the kinetics of cholesterol exchange between donor and acceptor membranes with various phospholipid compositions. It has been observed that cholesterol exchange kinetics between lipid vesicles are markedly dependent on the phospholipid composition of the donor membranes [reviewed by Phillips et al. (1987) and Bittman (1988)]. The presence of SPM in the host membrane dramatically decreases the rate of cholesterol exchange from donor to acceptor vesicles (Clejan & Bittman, 1984; Fugler et al., 1985; Lund-Katz et al., 1988). In a recent paper (Kan et al., 1991), it was reported that the introduction of a bulky tetrahydropyranyloxy group at the 3 position of egg SPM led to an increase in cholesterol exchange rate by a factor of about 30. The replacement of the 3-hydroxy group of *N*-stearoyl-SPM with a hydrogen atom or a methoxy group had only a small effect (slightly increased exchange rate) on the rate of cholesterol exchange between vesicles at 50 °C, indicating that hydrogen-bonding interactions involving the 3-hydroxy group of SPM are not primarily responsible for the slow rate of cholesterol exchange from SPM vesicles (Kan et al., 1991).

In conclusion, monolayer studies on the effects of substitutions at the 3 position of SPM show that the free hydroxy group of SPM does not play a major role in contributing to the slow rate of cholesterol oxidation by cholesterol oxidase or to the increase in lateral packing density at the lipid/water interface on addition of cholesterol. The possible participation of the N-linked chain of SPM in interactions with cholesterol awaits further studies with synthetic amide-modified SPM analogues.

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