# Membrane Properties of D-*erythro*-*N*-acyl Sphingomyelins and Their Corresponding Dihydro Species

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ABSTRACT We have prepared acyl chain-defined D-*erythro*-sphingomyelins and D-*erythro*-dihydrosphingomyelins and compared their properties in monolayer and bilayer membranes. Surface pressure/molecular area isotherms of D-*erythro*-*N*-16:0-sphingomyelin (16:0-SM) and D-*erythro*-*N*-16:0-dihydrosphingomyelin (16:0-DHSM) show very similar packing properties, except that the expanded-to-condensed phase transition (crystallization) occurs at a lower surface pressure for 16:0-DHSM. The measured surface potential was generally about 100 mV less for 16:0-DHSM monolayers compared to 16:0-SM monolayers. The condensed domains (crystals) that formed in 16:0-SM monolayers as a function of compression displayed star-shaped morphology when viewed under an epifluorescence microscope. 16:0-DHSM monolayers did not form similar crystals upon compression. 16:0-DHSM was degraded much faster by sphingomyelinase from *Staphylococcus aureus* than 16:0-SM (10-fold difference in enzyme activity needed for comparable hydrolytic rate). Cholesterol desorption from 16:0-DHSM to cyclodextrin was slightly slower (~20%) than the rate measured from 16:0-SM monolayers (at 60 mol % cholesterol). The bilayer melting temperature of 16:0-DHSM was 47.7°C (ΔH 8.3 kcal/mol) whereas it was 41.2°C for 16:0-SM (ΔH 8.1 kcal/mol). Cholesterol/16:0-DHSM bilayers (15 mol % sterol) had more condensed domains than comparable 16:0-SM bilayers, as evidenced from the quenching resistance of DPH in DHSM membranes. We conclude that cholesterol interacts more favorably with 16:0-DHSM and that the membranes are more condensed than comparable 16:0-SM-containing membranes.

## INTRODUCTION

Sphingomyelin (SM) is the major sphingolipid class present in the external leaflet of cellular plasma membranes. It is highly enriched in the plasma membrane compartment, as is cholesterol (Lange and Ramos, 1983; Lange et al., 1989; Koval and Pagano, 1991). It also appears that sphingomyelin and cholesterol may colocalize in the cell membrane (Patton, 1970; Slotte and Bierman, 1988; Slotte, 1999), and that cholesterol prefers to interact with sphingomyelin, both in cell and model membranes (Pörn et al., 1993; Ohvo et al., 1997; Mattjus and Slotte, 1996; Ramstedt and Slotte, 1999a).

Naturally occurring sphingomyelins have the phosphocholine head group linked to the hydroxyl group on carbon one of a long-chain base (most often an 18-carbon amine diol), and have a long and highly saturated acyl chain linked to the amide group on carbon 2 of the long-chain base (for a review, see Barenholz, 1984). These sphingomyelins have the D-*erythro*-(2S, 3R) configuration of the long-chain base (Sarmientos et al., 1985). In cultured cells (i.e., human skin fibroblast and baby hamster kidney cells) about 90 to 95% of the sphingomyelins contain sphingosine (1,3-dihydroxy-2-amino-4-octadecene) as the long-chain base, whereas the remainder have sphinganine (1,3-dihydroxy-2-amino-4-octadecane) as the base (Ramstedt et al., 1999). The latter sphingomyelins are also called dihydrosphingomyelins (DHSM; see Scheme 1). Although it is currently not known why cells need both sphingomyelins and dihydrosphingomyelins, it is remarkable that DHSM accounts for 50% of all phospholipids in human lens membranes (Byrdwell and Borchman, 1997). The acyl chain composition of DHSM in human lens membranes is reported to be mainly 16:0 (57.8%) and 24:$1^{\Delta 15c}$ (23.3%; Byrdwell and Borchman, 1997). The lens membranes are also known to contain very high concentrations of cholesterol (about 3 cholesterol to 1 phospholipid; Li et al., 1985), and it is possible that DHSM may function in the human lens as an efficient solubilizer of cholesterol. Very little is known about the physico-chemical properties of DHSM as compared to those of sphingomyelins, except that the lack of the trans double bond between carbons 4 and 5 in DHSM is known to lead to a substantially higher melting temperature for DHSM as compared to an acyl-matched sphingomyelin (Barenholz et al., 1976). Consequently, DHSM is even more likely than sphingomyelin to undergo lateral segregation in glycerophospholipid-containing membranes, because of the huge difference in melting temperatures, and may thus contribute to the formation of laterally condensed domains in biomembranes (Brown, 1998).

In the present study, we have prepared enantiomerically pure (D-*erythro*) and acyl chain-defined sphingomyelins and dihydrosphingomyelins and compared their biophysical properties in both monolayer and bilayer membranes in the presence or absence of cholesterol.

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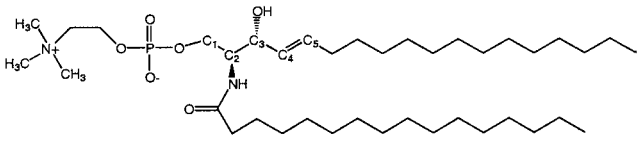
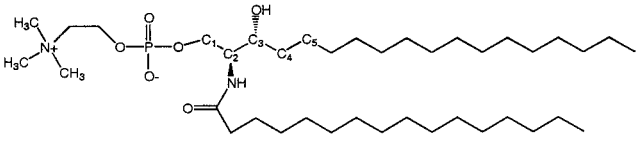


D-erythro-*N*-16:0-sphingomyelin



D-erythro-*N*-16:0-dihydrosphingomyelin

SCHEME 1

## EXPERIMENTAL PROCEDURES

### Materials

D-*erythro*-*N*-palmitoyl-sphingomyelin (16:0-SM) was purified from egg yolk sphingomyelin (Avanti Polar Lipids, Alabaster, AL) by reverse-phase HPLC (LiChrospher 100 RP-18 column, 5 $\mu$m particle size, 240 $\times$ 4 mm column dimensions; Merck, Darmstadt, Germany) using 5 vol-% water in methanol as eluent (at 1 ml/min, column temperature 40°C). D-*erythro*-*N*-palmitoyl-dihydrosphingomyelin (16:0-DHSM) was prepared from 16:0-SM by hydrogenation using palladium oxide (Aldrich Chemical Co., Milwaukee, WI), as catalyst (Schneider and Kennedy, 1967), and purified as described for 16:0-SM. D-*erythro*-*N*-oleoyl-sphingomyelin (18:1-SM) and D-*erythro*-*N*-oleoyl-dihydrosphingomyelin (18:1-DHSM) were prepared from the respective D-*erythro*-lysosphingomyelin/lyso-dihydrosphingomyelin, as described by Ramstedt and Slotte (1999a). D-*erythro*-lysodihydrosphingomyelin was prepared from D-*erythro*-lysosphingomyelin (Avanti Polar Lipids) by hydrogenation, as described above. The solvents used for synthesis of phospholipids were stored over Molecular Sieves 4A (Merck). 1,2-Dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1-palmitoyl-2-(12-doxyl)-stearoyl-sn-glycero-3-phosphocholine (12SLPC) were purchased from Avanti Polar Lipids. Cholesterol, sphingomyelinase (*Staphylococcus aureus*), and $\beta$-cyclodextrin (CyD) were obtained from Sigma Chemicals (St. Louis, MO). 1,6-Diphenyl-1,3,5-hexatriene (DPH) and 22-(N-(7-nitrobenz-2-oxa-1, 3-diazol-4-yl)amino)-23,24- bisnor-5-cholen-3$\beta$-ol (NBD-cholesterol) were obtained from Molecular Probes (Leiden, The Netherlands). Stock solutions of lipids were prepared in hexane/2-propanol (3/2, v/v), stored in the dark at $-20$°C, and warmed to ambient temperature before use. The CyD stock solutions were prepared in pure water to a concentration of 40 mM. The water used as subphase for the monolayers and for differential scanning calorimetry measurements was purified by reverse osmosis followed by passage through a Millipore UF Plus water purification system (Molsheim, France), to yield a product with a resistivity of 18.2 M$\Omega$cm.

### Force/area isotherms

Pure monolayers of each lipid were compressed (at 10 Å$^2$/molecule, min) on water at ambient temperature (20 $\pm$ 1°C) or at 37°C with a KSV surface barostat (KSV Instruments Ltd., Helsinki, Finland). Surface pressure or surface potential versus mean molecular area isotherms were acquired using proprietary KSV software. Surface potential was determined using a vibrating plate device (KSV Instruments Ltd.).

### Monolayer fluorescence microscopy

To follow the monolayer crystallization of pure sphingomyelins, or the formation of lateral cholesterol-rich domains (30 mol % cholesterol) in mixed cholesterol-sphingomyelin monolayers, we used monolayer fluorescence microscopy (Slotte, 1995a,b) with NBD-cholesterol as a fluorescent probe (at 1 mol %). Micrographs were obtained using a sensitive KP-M 1E/K video camera (Hitachi Denshi, Japan) attached to a DT3851 digitizing board (Data Translation, Marlboro, MA) in a personal computer. The lateral domains were documented at indicated mean molecular areas.

### Removal of monolayer cholesterol to the subphase by cyclodextrins

Mixed monolayers containing sphingomyelins and 60 mol % cholesterol were prepared at the air/water interface. The trough used was of a zero-order type, with a reaction chamber (volume, 23.9 ml; area, 28.3 cm$^2$) separated by a glass bridge from the lipid reservoir (Verger and De Haas, 1973; Ohvo and Slotte, 1996). CyD in a volume not exceeding 1 ml was injected into the reaction chamber, the content of which was continuously stirred (20°C). The final $\beta$-cyclodextrin concentration in the subphase was 1.7 mM. The removal of lipids from the monolayer to the subphase was determined from the area decrease of the monolayer at constant surface pressure (20 mN/m), as described by Ohvo and Slotte (1996).

### Differential scanning calorimetry

Sphingomyelins were dissolved in hexane and 2-propanol (3:2 v:v). The solvent was evaporated at 40°C under a stream of argon. Excess residual solvent was removed by vacuum drying at room temperature. The lipids were then suspended in water by warming the tube to 50°C and vortexing vigorously for about 30 s; final lipid concentration was 0.5 mg/ml. The water used as reference and the lipid suspensions were degassed under vacuum. The lipid suspension and water were loaded into the sample and reference cell, respectively, of a Nano II high-sensitivity scanning calorimeter (Calorimetric Science Corp., Provo, UT). Heating/cooling scans from 20 to 55°C at a scan rate of 0.3°C/min were performed. Three consecutive heating scans were made on both samples, giving identical thermograms. Data from two separate preparations of sphingomyelin liposomes were analyzed and averaged.

### Determination of the steady-state fluorescence anisotropy in unilamellar vesicles

Vesicles were prepared from pure sphingomyelins or from a mixture of 30 mol % cholesterol and 70 mol % sphingomyelins, using a Lipextruder (Lipex Biomembranes Inc., Vancouver, BC) and the extrusion technique described by Hope and coworkers (Hope et al., 1985). In brief, 100 nmol of the lipid, or lipid mixture, were dried under argon. The lipids were then sonicated in phosphate-buffered saline (2.5 ml) to yield multilamellar vesicles (MLV). MLVs were then extruded at 55°C through 400 nm polycarbonate filters (Costar Corp., Cambridge, MA) to yield the desired vesicles. DPH was used as a reporter molecule for steady-state anisotropy, and was included in the lipid mixture at 0.5 mol % before extrusion. Excitation was carried out at 360 nm and emission was recorded at 430 nm, at a temperature of 37°C, using a PTI Quantamaster 1 spectrofluorimeter (Photon Technology International, Surrey, England) operating in the T-format. The steady-state anisotropy, $r$, is defined as:

$$r = I_{\parallel} - GI_{\perp}/I_{\parallel} + 2GI_{\perp}$$

where $I_{\parallel}$ and $I_{\perp}$ are the fluorescence intensities with the analyzer parallel and perpendicular, respectively, to the vertical polarizer (Lakowicz, 1983). $G$ represents the ratio of the sensitivities of the detection system for vertically and horizontally polarized light (Lakowicz, 1983).

## Fluorescence quenching experiments

Essentially, we followed the procedure described by Xu and London (2000) for determining the quenching of DPH in sphingomyelin multilamellar vesicles by 12SLPC. Briefly, the multilamellar vesicles contained 50 $\mu$M total lipid and 1 mol % DPH and were prepared in 10 mM sodium phosphate and 150 mM NaCl (pH 7) at 55°C. The sample volume was 1 ml. Samples with quencher (F samples) contained an equimolar mixture of 16:0-SM (or 16:0-DHSM) and 12SLPC, or contained DOPC and 12SLPC, with or without 15 mol % cholesterol. Corresponding samples without quencher ($F_o$ samples) contained equimolar amounts of 16:0-SM (or 16:0-DHSM) and DOPC or only DOPC, respectively, with or without 15 mol % cholesterol. Background samples were prepared identically but without DPH. Fluorescence was measured at the indicated temperatures with a Hitachi F2000 spectrofluorimeter (Tokyo, Japan), with excitation and emission wavelengths at 358 nm and 427 nm, respectively.

## Sphingomyelin hydrolysis in monolayers

Sphingomyelins were hydrolyzed in monolayers by bacterial sphingomyelinase, essentially as described previously (Jungner et al., 1997). The hydrolysis experiments were carried out at 37°C and at a constant surface pressure of 20 mN/m. The buffer used contained 50 mM Tris/HCl (pH 7.4) with 140 mN NaCl and 5 mM $MgCl_2$. The final activity of enzyme in the subphase is indicated in each figure.

# RESULTS

## Force/area and surface potential/area isotherms of sphingomyelins

To characterize and compare the interfacial properties of the defined molecular species of 16:0-SM, 16:0-DHSM, 18:1-SM, and 18:1-DHSM, we prepared monolayers at the air/water interface at ambient temperature. Force/area compression isotherms of the saturated 16:0-containing species showed an expanded-to-condensed phase transition (Fig. 1 *A*), which occurred at a higher lateral surface pressure with 16:0-SM compared to the dihydro species 16:0-DHSM. The mean molecular area before onset of the transition was

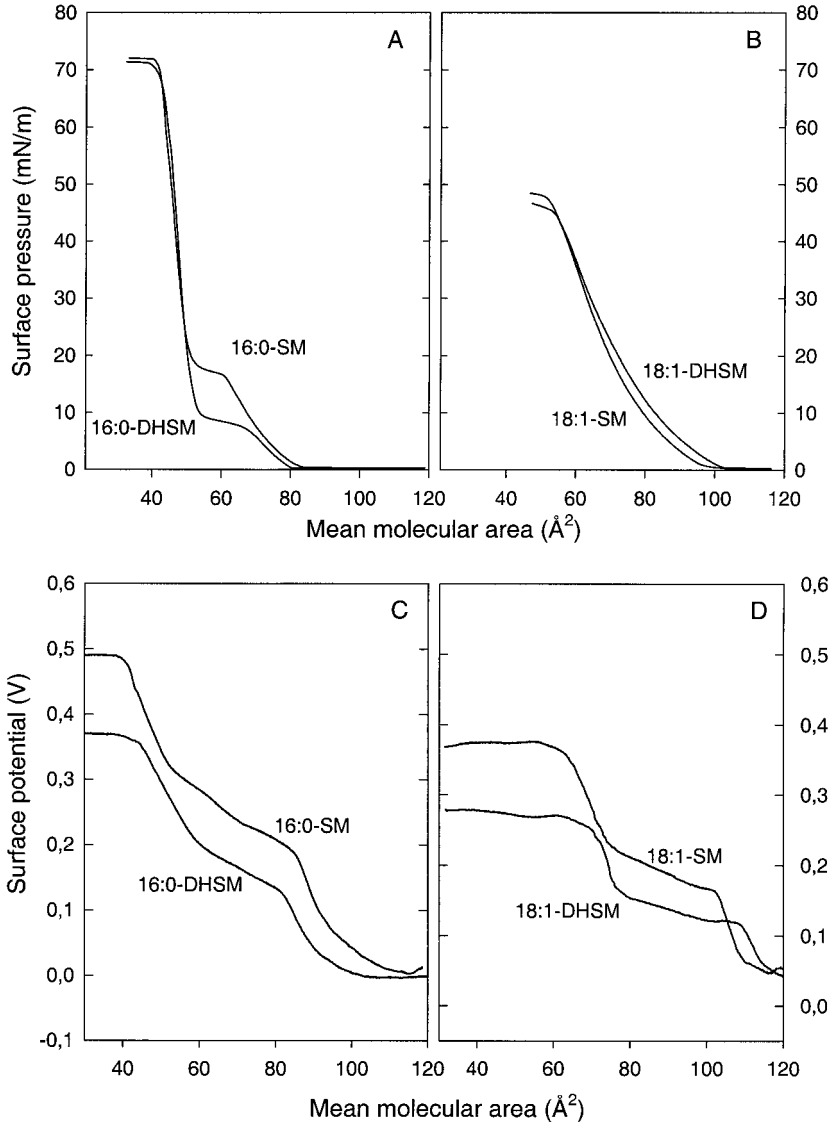



FIGURE 1 Force/area and surface potential/area isotherms for sphingomyelins. (*A*) Surface pressure versus mean molecular area isotherms for 16:0-SM and 16:0-DHSM. (*B*) Surface pressure versus mean molecular area isotherms for 18:1-SM and 18:1-DHSM. (*C*) Surface potential for the 16:0-sphingomyelins plotted against their mean molecular area. (*D*) Surface potential versus mean molecular area for 18:1$^{\Delta 9c}$-sphingomyelins. The pure monolayers were compressed on water at ambient temperature at a speed not exceeding 10 Å$^2$/molecule, min.

roughly similar for both lipid species, as it was also after the transition. Monolayers from both lipid species collapsed at about 72 mN/m. From a comparison of these two force/area isotherms, one can conclude that 16:0-DHSM formed crystals at lower pressure compared to 16:0-SM, and that for a bilayer system this would mean that the gel-liquid phase transition temperature of the dihydro species would be higher than for the acyl chain-matched sphingomyelin (see our differential scanning calorimetry results, which give the $T_m$ for these two lipids). Force/area isotherms of 18:1-SM and 18:1-DHSM showed that the introduction of an unsaturation into the amide-linked acyl chain removed the phase transition at ambient temperature (Fig. 1 *B*) from both lipid species. The isotherms in Fig. 1 *B* also show that 18:1-DHSM and 18:1-SM occupied roughly similar mean molecular areas at a given lateral surface pressure and that the monolayers were equally stable against compression-induced destabilization (collapse).

Surface potential versus mean molecular area isotherms of 16:0-SM and 16:0-DHSM monolayers indicate clearly the absence of the *trans* double bond in 16:0-DHSM reduced the measurable surface potential significantly (50–100 mV depending on packing density; Fig. 1 *C*). Fairly similar differences in surface potential were seen with 18:1-SM and 18:1-DHSM monolayers (Fig. 1 *D*).

## Monolayer fluorescence microscopy documentation of sphingomyelin monolayers

In order to visualize the formation of condensed phases in the monolayers of 16:0-containing sphingomyelins, monolayer fluorescence microscopy was used. In this method, the monolayer is doped with a small amount (0.5 mol %) of a fluorescent reporter molecule (NBD-cholesterol), which preferentially partitions into liquid-expanded phases (Slotte and Mattjus, 1995). Consequently, condensed phases appear as dark areas against a highly fluorescent matrix when the monolayer is viewed under the fluorescence microscope. When the pressure-induced crystallization of 16:0-SM was examined using NBD-cholesterol as reporter molecule, we could see clearly the formation of crystal nuclei (Fig. 2 *A*) at the onset of the expanded-to-condensed transition (see the isotherm in Fig. 1 *A*). As the surface pressure was increased (and the molecular area decreased), the crystals grew in size (Fig. 2, *B* and *C*). These crystals were not liquid-like, since they had a distinct star-like shape with 5 or 6 growth cones. Interestingly, although the force/area isotherm of 16:0-DHSM showed that this sphingomyelin species crystallized at even lower surface pressures than 16:0-SM, we could not see similar clearly defined crystals in the 16:0-DHSM monolayers (Fig. 2 *D*) as were seen with 16:0-SM (Fig. 2, *A–C*). The crystals that were formed in 16:0-DHSM monolayers were much smaller in size, and the resolution of the method did not allow us to observe details of their texture.

We have previously shown that the mixing of cholesterol into 16:0-SM monolayers results in the formation of macroscopic, cholesterol-rich condensed domains (Ramstedt and Slotte, 1999b). In this study very similar domain formation patters were observed in mixed monolayers containing 30 mol % cholesterol and either 16:0-DHSM (Fig. 3 *A*) or 16:0-SM (Fig. 3 *B*).

## Cholesterol desorption from mixed monolayers

Cholesterol desorption from monolayers to CyD in the subphase has been used by us as a measure of how well cholesterol interacts with other lipids in a mixed monolayer (Ohvo and Slotte, 1996; Ramstedt and Slotte, 1999a). We prepared mixed monolayers to contain 60 mol % cholesterol together with either 16:0-SM or 16:0-DHSM as the sphingomyelin species. We determined rates of cholesterol desorption to 1.7 mM $\beta$-CyD at a lateral surface pressure of 20 mN/m. The results show that desorption rates were slower from 16:0-DHSM mixed monolayers as compared to 16:0-SM mixed monolayers ($5.6 \pm 0.2$ and $6.9 \pm 0.2$ pmol/cm$^2$ · min, respectively). The observed desorption rate was practically zero with both SM types if the cholesterol concentration in the mixed monolayer was reduced to 50 mol% (data not shown). We previously measured desorption of cholesterol from 16:0-SM monolayers and observed a rate corresponding to 3.1 pmol/cm$^2$, min at 60 mol % cholesterol (Ramstedt and Slotte, 1999b). We consider the difference in rate for this study and the study of Ramstedt and Slotte (1999b) to be due to possible differences in cyclodextrin concentration (e.g., different moisture content).

## Sphingomyelinase-catalyzed hydrolysis of monolayer sphingomyelins

The degradation of sphingomyelin in monolayers by sphingomyelinase in the subphase can be followed as a time-dependent reduction in the monolayer area (at constant surface pressure), since the reaction product (i.e., ceramide) has a smaller molecular area requirement than sphingomyelin (Yedgar et al., 1982; Jungner et al., 1997). All sphingomyelinase experiments were performed at 37°C, because the reactions are very slow at ambient temperatures (data not shown). At 37°C all substrates used were in their expanded state at the lateral surface pressure used (the mean molecular area for 16:0-SM and 16:0-DHSM was 70.8 and 69.7 Å$^2$/molecule at 20 mN/m, respectively; isotherms not shown). We have previously shown that the degradation of 16:0-SM is a fairly slow process and that relatively high amounts of sphingomyelinase must be used in the subphase (Ramstedt and Slotte, 1999b). When the degradation of 16:0-SM and 16:0-DHSM by sphingomyelinase are compared (Fig. 4, *A* and *B*), it was clearly observed that 16:0-DHSM was degraded much faster than the 16:0-SM species

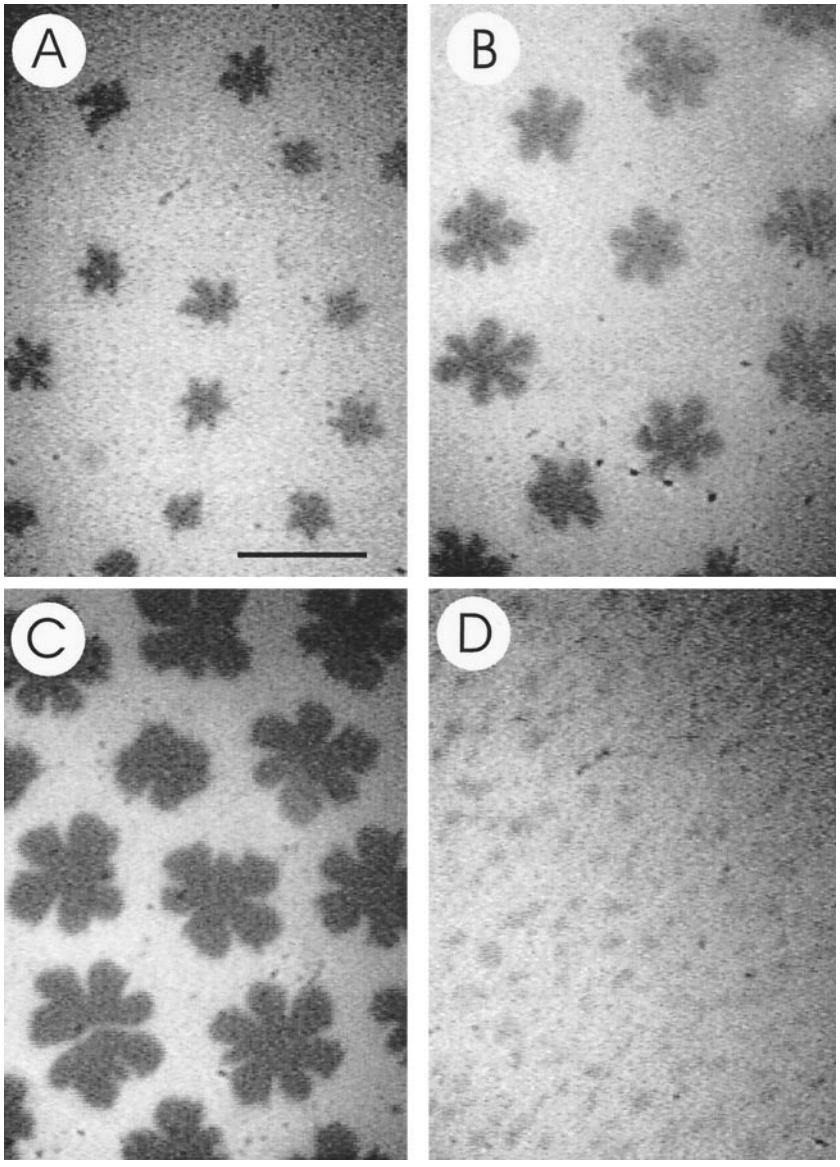



FIGURE 2 Visualization of laterally condensed domains in monolayers of 16:0-SM and 16:0-DHSM. Monolayers of the pure sphingomyelins were prepared to include 1.0 mol % NBD-cholesterol. The monolayers were compressed at a speed not exceeding 5 $Å^2$/molecule/min to the indicated mean molecular areas. The lateral domains were documented during the first compression. (*A*) Crystalline domains in a pure 16:0-SM monolayer right after the onset of the expanded-to-condensed phase transition (about 60 $Å^2$/molecule). The same system is shown after further compression to 58 $Å^2$/molecule (*B*) and 54 $Å^2$/molecule (*C*). (*D*) 16:0-DHSM after the onset of the transition (about 66 $Å^2$/molecule). Scale bar, 50 $\mu$m.

(10 times less enzyme needed for 16:0-DHSM compared to 16:0-SM). If degradations of 16:0-DHSM and 16:0-SM were performed at a similar enzyme concentration (i.e., 6.7 mU/ml), there was almost no degradation of 16:0-SM during the first hour (data not shown), whereas 16:0-DHSM was completely degraded in 20 min. Hydrolysis of 18:1-SM and 18:1-DHSM species by bacterial sphingomyelinase proceeded with roughly similar kinetics (using 6.7 mU/ml enzyme with both monounsaturated species, Fig. 4 *C*).

## Differential scanning calorimetry

To study the thermodynamics of the gel-to-liquid transition of 16:0-SM and 16:0-DHSM bilayers, differential scanning calorimetry was used. The lipids were hydrated thoroughly and dispersed by vigorous mixing at a temperature above 50°C. The thermograms were acquired with a heating or cooling scan rate of 0.3°C/min. The thermogram in Fig. 5 *A* shows the second heating scan obtained for dispersions of 16:0-DHSM. There is a small pre-transition centered at 44°C, whereas the main transition occurs at 47.7°C. The enthalpy of the pre-transition is about 0.7 kcal/mol (heating scan), whereas the enthalpy of the main transition is 8.3 kcal/mol (heating scan). On cooling, the 16:0-DHSM thermogram shows the main transition at 46.8°C ($\Delta$H 9.1 kcal/mol), whereas the pre-transition has moved to a significantly lower temperature (28.8°C, $\Delta$H 0.4 kcal/mol). The heating scan for 16:0-SM is shown in Fig. 5 *C* (main $T_m$ is at 41.2°C, $\Delta$H is 8.1 kcal/mol, the small pre-transition was centered at 28.2°C; not shown clearly in the figure because of the scale chosen). We recently published a similar heating scan for 16:0-SM (Ramstedt and Slotte, 1999b). A similar thermogram is shown here again, to allow easy comparison of the two lipid species. On cooling, the 16:0-SM

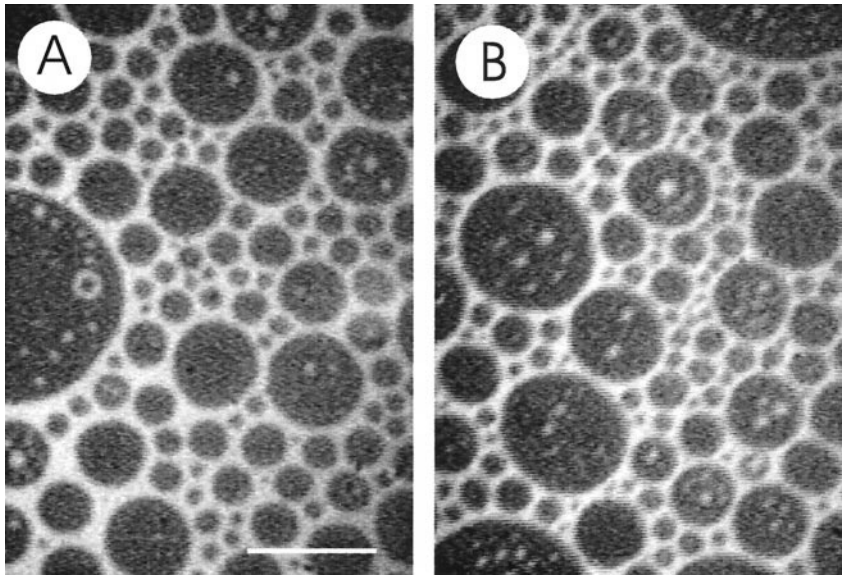



FIGURE 3 Visualization of laterally condensed domains in binary monolayers of 16:0-DHSM (*A*) and 16:0-SM (*B*) and cholesterol. The monolayers of the sphingomyelins ere prepared to include 30 mol % cholesterol together with 1.0 mol % NBD-cholesterol. The monolayers were compressed at a speed not exceeding 5 $Å^2$/molecule/min. The lateral domains were documented at a lateral surface pressure of 1 mN/m during the first compression. Scale bar, 50 $\mu$m.

displayed the liquid-to-gel transition at 40.3°C, whereas the mid-temperature for the pre-transition moved down to 25.2°C (thermogram not shown).

## Steady-state anisotropy of DPH in sphingomyelin vesicles

To obtain indirect information about sphingomyelin packing properties in bilayer membranes, we determined the anisotropy of DPH in both pure and cholesterol-containing sphingomyelin vesicles. DPH is a hydrophobic reporter molecule that orients itself along the acyl chains of the phospholipids in the bilayer (Shinitzky and Barenholz, 1974), and consequently is sensitive to packing properties in the hydrophobic core of the bilayer membrane (Shinitzky and Barenholz, 1978). The anisotropy of DPH, when plotted as a function of temperature in both 16:0-DHSM (Fig. 6 *A*) and 16:0-SM (Fig. 6 *B*) vesicles clearly reported the phase-transition that occurred in these vesicles. The mid-temperature of the transition, as reported by the anisotropy of DPH, was 48.2 and 41.1°C for 16:0-DHSM and 16:0-SM, respectively. The experimentally determined anisotropy of DPH at a given temperature below or above the phase transition was remarkably similar for 16:0-DHSM and 16:0-SM bilayers,

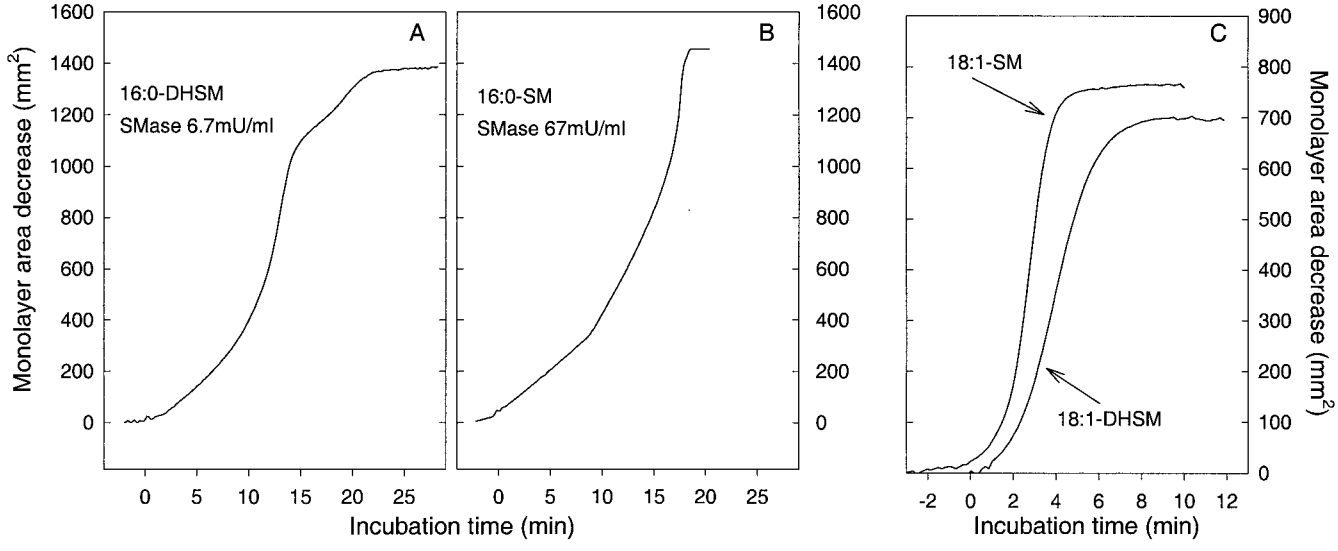



FIGURE 4 Hydrolysis of monolayer sphingomyelin by bacterial sphingomyelinase. Pure sphingomyelin monolayers were prepared at the air/buffer interface and were kept at a constant surface pressure of 20 mN/m. Sphingomyelinase was added at time zero to the stirred subphase (50 mM Tris/HCl, pH 7.4, 140 mM NaCl, 5 mM $MgCl_2$) to a final activity of 6.7 mU/ml for 16:0-DHSM, 18:1-DHSM and 18:1-SM, and 67 mU/ml for 16:0-SM. Degradation of sphingomyelin by the enzyme led to a monolayer area decrease that is plotted as a time function.

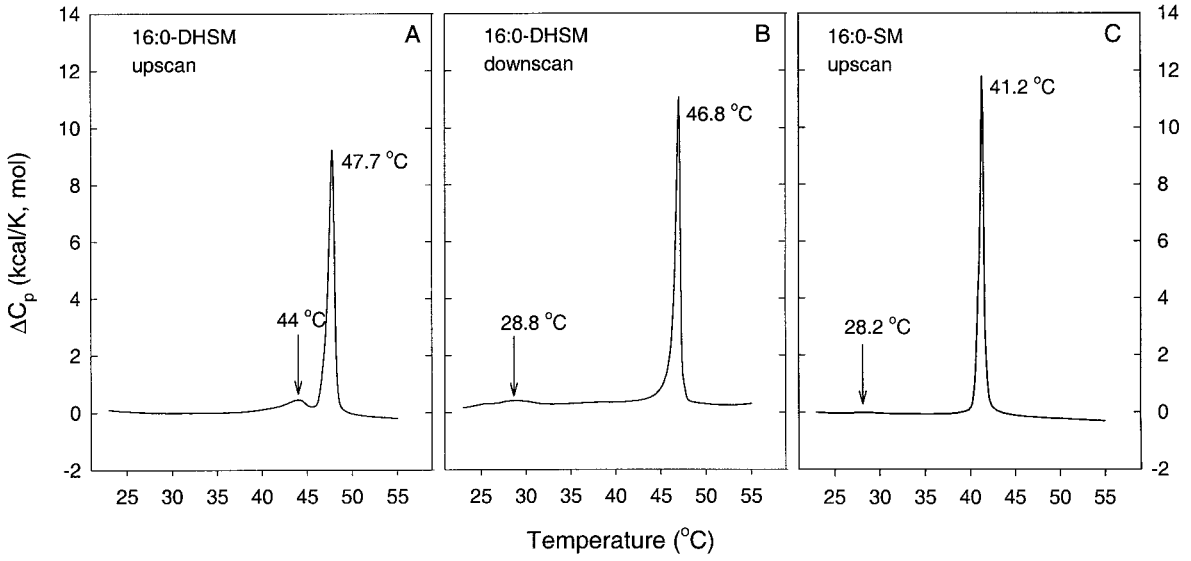



FIGURE 5 Heat capacity versus temperature curves for purified 16:0-SM and 16:0-DHSM. (*A*) Representative heating scan (0.3°C/min) of 16:0-DHSM, whereas (*B*) is the subsequent cooling scan (0.3°C/min). (*C*) The heating scan of 16:0-SM. The pre-transitions are indicated by arrows.

suggesting similar packing in the hydrophobic core of these membranes. Inclusion of 30 mol % cholesterol into the bilayer membranes removed the phase-transition (Fig. 6, *C* and *D*) for both sphingomyelins, whereas the overall DPH anisotropies remained similar for both sphingomyelin membranes.

In bilayers of either 18:1-SM or 18:1-DHSM (no cholesterol), the anisotropy of DPH was very similar in the temperature range of 10 to 40°C (Fig. 6, *E* and *F*). However, it appeared that 18:1-DHSM had its gel-to-liquid transition somewhere between 0 and 5°C (higher anisotropies, Fig. 6 *E*). 18:1-SM clearly had no observable transition above 0°C (Fig. 6 *F*).

### Formation of condensed domains in bilayers as determined from DPH quenching

Cholesterol has been shown to promote domain formation in model membrane systems (Ahmed et al., 1997; Xu and London, 2000). Ahmed and coworkers have recently developed a method to study sterol domain formation in vesicles (Ahmed et al., 1997). The method relies on the finding that DPH distributes almost equally between condensed and expanded domains, whereas 12SLPC (which is a DPH quencher) preferentially partitions into expanded domains (because of the bulky doxyl function). Consequently, if more condensed domains exist (compared to a control system) quenching of DPH by 12SLPC is less efficient. We studied the quenching resistance of DPH in multilamellar liposomes at different temperatures between 23 and 52°C. The liposomes contained either 16:0-SM or 16:0-DHSM with or without 15 mol % cholesterol. As seen in Fig. 7, in the absence of cholesterol the quenching of DPH was efficient and did not change markedly as the temperature was raised through the transition region. There was more quenching of DPH fluorescence in 16:0-SM liposomes as compared to 16:0-DHSM membranes, but the difference was not dramatic. Inclusion of 15 mol % cholesterol increased the quenching resistance of DPH markedly (e.g., at 23°C) for 16:0-SM, and even more dramatically in 16:0-DHSM liposomes (Fig. 7). With 16:0-SM liposomes, the cholesterol-induced domains melted as the temperature was increased; however, with 16:0-DHSM liposomes, the condensed domains persisted even at higher temperatures, although there was a gradual temperature-induced increase in DPH quenching.

## DISCUSSION

In this work we have compared the membrane properties of enantiomerically pure D-*erythro* sphingomyelins and dihydrosphingomyelins, containing either 16:0 or $18:1^{\Delta 9c}$ at the amide-linked position. The lack of the trans double bond at the carbon 4 position in 16:0-DHSM results in the molecule having a higher melting temperature in bilayers, and a lower crystallization pressure in monolayers, both indicative of stronger cohesive interactions between the molecules in the model membranes. It was shown already in 1976 that the gel-to-liquid phase transition in bilayer dispersions made of racemic palmitoyl sphingomyelin occurred at a 6.5°C lower temperature than what was observed for racemic palmitoyl dihydrosphingomyelin (Barenholz et al., 1976). In this study we are able to show for the first time that with enantiomerically pure 16:0-SM and 16:0-DHSM, the transition temperature difference is similar as that reported for racemic compounds (Barenholz et al., 1976). We also ob-

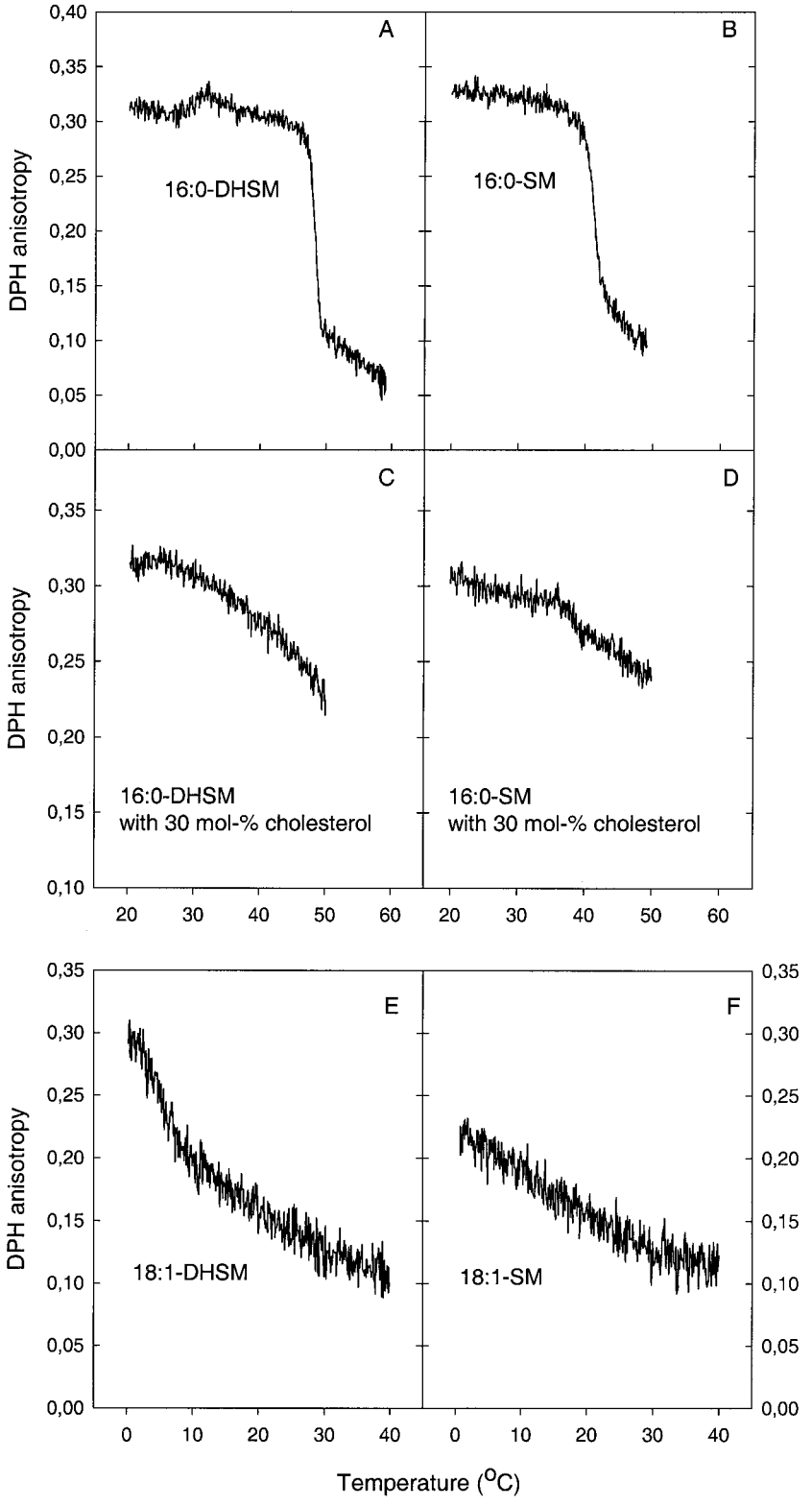



FIGURE 6 Anisotropy of DPH in large vesicles of sphingomyelins or dihydrosphingomyelins. Vesicles were prepared from either 16:0-SM, 16:0-DHSM, 18:1-SM, or 18:1-DHSM by extrusion through 400-nm diameter pores at a temperature above the respective transition temperature. The LUVs contained 0.5 mol % DPH as the reporter molecule. (*A*) and (*B*) show DPH anisotropy versus temperature for 16:0-SM and 16:0-DHSM large unilamellar vesicles. (*C*) and (*D*) show corresponding anisotropies for 16:0-SM and 16:0-DHSM, but with 30 mol % cholesterol. (*E*) and (*F*) show DPH anisotropies for 18:1-SM and 18:1-DHSM without cholesterol.

served that the heating curve of 16:0-DHSM showed a clear pre-transition at 44°C, a feature not seen in the heating scan of racemic palmitoyl dihydrosphingomyelin in the paper of Barenholz and coworkers (1976). A similar but smaller pre-transition is also seen for 16:0-SM, but at a much lower temperature (Fig. 3 and Ramstedt and Slotte, 1999b).

Borchman et al. (1996) have analogously studied the melting behavior of human lens sphingomyelins and dihy-

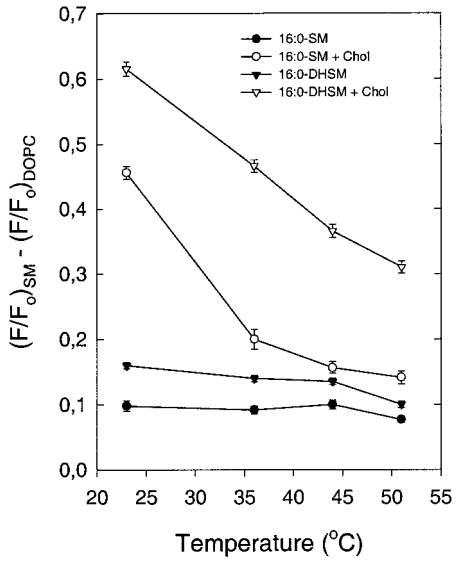



FIGURE 7 Formation of laterally condensed domains in 16:0-SM and 16:0-DHSM liposomes in the presence of cholesterol, as determined by quenching of DPH fluorescence by 12SLPC. The liposomes were prepared to contain the indicated sphingomyelin with or without 15 mol % cholesterol. The liposomes were equilibrated for 10 min at each indicated temperature before fluorescence intensity readings were taken similarly for all samples. The $(F/F_o)_{SM} - (F/F_o)_{DOPC}$ values were calculated and plotted as a function of temperature. Each value is the average from three similar experiments ± SEM.

drosphingomyelin using Fourier transform infrared measurements, and they were able to show a 9°C difference in the melting temperatures for the two sphingomyelin classes, although their derived transition temperatures (31 and 40°C, respectively) were surprisingly low and inconsistent with differential scanning calorimetry results for sphingomyelins and dihydrosphingomyelins with similar acyl chain compositions. Whereas egg yolk sphingomyelin (about 68% 16:0, 12% 18:0, 5% 20:0, 5% 22:0, and 10% 24:0 + 24:$1^{\Delta 15c}$, see Ramstedt et al., 1999) has a $T_m$ of 39.7°C, its $T_m$ as fully hydrogenated is shifted to 46°C (F. Ollila and J. P. Slotte, unpublished observations).

The analysis of the monolayer properties of sphingomyelins and dihydrosphingomyelins showed that their packing properties were very similar (with both 16:0 and 18:$1^{\Delta 9c}$ derivatives), except that the expanded-to-condensed phase transition (crystallization) occurred at a much lower pressure for 16:0-DHSM compared to 16:0-SM. The dihydrosphingomyelin monolayers (irrespective of acyl chain composition) had a 100 mV lower surface potential as compared to the corresponding trans-unsaturated sphingomyelin. Our results with enantiomerically pure and acyl-chain defined sphingomyelins and dihydrosphingomyelins agree surprisingly well with old results published by Shah and Shulman (1967) for natural sphingomyelins and their hydrogenated derivatives. The similar surface packing properties of 16:0-SM and 16:0-DHSM suggest that the head group orientation is similar in both monolayers (Fig. 1), and that the difference in surface potential therefore most likely originate from an inducible dipole due to the *trans* double bond present in sphingomyelin but absent in dihydrosphingomyelin (Shah and Shulman, 1967).

The formation of laterally condensed domains in monolayer membranes can be easily documented using monolayer fluorescence microscopy and a suitable fluorescence probe (Von Tscharner and McConnell, 1981; Slotte and Mattjus, 1995). The crystal domains present during compression of 16:0-SM could be visualized using NBD-cholesterol as a probe that was excluded from the condensed domains, and preferentially partitioned into the more expanded domains. These large star-shaped crystals of 16:0-SM have not previously been documented, and an earlier attempt by us to visualize them using NBD-phosphatidylcholine as a fluorescent reporter molecule failed, probably because the size of the domains in the presence of that probe most likely were too small compared to the resolution of the microscope used to visualize them (data not shown). Analogous asymmetric two-dimensional crystals have previously been observed when, e.g., dipalmitoylphosphatidylcholine is compressed on the air/water interface (for a review, see McConnell, 1991). 16:0-DHSM failed to produce large-scale crystals similar to those seen in 16:0-SM monolayers. It is known that dipole interactions in monolayers are important determinants of how the crystals can grow (McConnell, 1991), and clearly 16:0-SM and 16:0-DHSM differ in molecular dipole properties, as also clearly evidenced by the difference in surface potential. The macroscopic domain properties of cholesterol-containing sphingomyelin or dihydrosphingomyelin monolayers were very similar, and no differences between the two phospholipid species could be seen.

Because strong intermolecular interactions between cholesterol and a phospholipid affect the energy needed for cholesterol to desorb from the membrane, desorption rates can be used to estimate relative strengths of interactions in model and biological membranes (Phillips et al., 1987; Bittman, 1988; Ohvo and Slotte, 1996). Cholesterol desorption from monolayers to CyD acceptors is known to be much slower from sphingomyelin monolayers than from acyl-chain matched phosphatidylcholine monolayers (Ohvo and Slotte, 1996; Ramstedt and Slotte, 1999a). In this study we observed that cholesterol desorption to CyD in the subphase was significantly slower (about 20% at 60 mol % cholesterol and the conditions used) from mixed monolayers containing 16:0-DHSM than it was from monolayers with 16:0-SM. This finding clearly indicates a stronger interaction between cholesterol and 16:0-DHSM than between cholesterol and 16:0-SM. This effect of the *trans* double bond on cholesterol desorption is difficult to explain based on packing density differences, because none were observed. It is, on the other hand, clear that the trans double bond at carbon 4 in sphingomyelin is at a position where the

hydrophobic ring system of cholesterol is likely to be affected by its polar nature during close molecular interactions. The absence of this double bond in dihydrosphingomyelin may, therefore, result in a more favorable interaction with cholesterol, leading to observed decrease in desorption rates.

The hydrolysis of sphingomyelin in pure monolayers by bacterial sphingomyelinase was previously reported to be markedly affected by the stereochemistry of the sphingomyelin substrate molecules (Ramstedt and Slotte, 1999b). The differences in degradation rates were attributed to differences in packing properties for racemic and enantiomerically pure sphingomyelins (Ramstedt and Slotte, 1999b). Sphingomyelin hydrolysis has previously also been shown to be affected by the phase state of the molecules in the membrane, for both monolayers and bilayers (Yedgar et al., 1982; Jungner et al., 1997). We now observed, surprisingly, that 16:0-DHSM was degraded very much faster than 16:0-SM by the bacterial sphingomyelinase from *Staphylococcus aureus*, whereas rates of 18:1-DHSM and 18:1-SM were much more similar. Because the degradation assays were performed at 37°C and 20 mN/m (whereas isotherms were collected at ambient temperature), we separately verified that the 16:0-SM and 16:0-DHSM monolayers were both expanded at 20 mN/m at the higher temperature of 37°C. However, the 16:0-DHSM monolayer was still much closer to its crystallization pressure (at 20 mN/m and 37°C) than was true for 16:0-SM. Therefore, it is possible that the 16:0-DHSM monolayer was more ordered (even though the monolayer isotherm had expanded characteristics) at the given temperature than the 16:0-SM monolayer. It is known that at least phospholipase $A_2$ and cholesterol oxidase attack their monolayer substrates preferentially at boundaries between ordered and disordered states (Grainger et al., 1990; Slotte, 1995c). It is likely that the activity of sphingomyelinase at the water/monolayer interface is affected similarly by substrate packing heterogeneity in the membrane phase, although some fluidity is required for efficient adsorption of the enzyme to the interface (Jungner et al., 1997; Ramstedt and Slotte, 1999b).

Steady-state anisotropy measurements of DPH in multilamellar vesicles prepared from different sphingomyelins were also done in this study. DPH is known to partition equally between condensed and liquid domains, and is known to localize to the hydrocarbon core of the membrane bilayer (Shinitzky and Barenholz, 1974, 1978). The steady-state anisotropy of DPH was similar in 16:0-SM and 16:0-DHSM vesicles, both before and after the gel-liquid transition. Whereas cholesterol at 30 mol % abolished the melting transition, the anisotropy of DPH still remained similar in the two different sphingomyelins. With $18:1^{\Delta 9c}$-containing sphingomyelins, again no difference was seen for 18:1-SM and 18:1-DHSM. Clearly, the steady-state anisotropy of DPH failed to report differences in molecular environments that, e.g., cholesterol picked up (as evidenced from its desorption rate).

However, even though we could not see a difference in the properties of cholesterol/sphingomyelin domains in the two different monolayers (Fig. 3), differences could clearly be seen in bilayer membranes when the quenching susceptibility of DPH was determined according to the method of Ahmed et al. (1997). This method is based upon the observation that although DPH is distributed equally between expanded and condensed domains, the quencher (12SLPC) prefers to partition into expanded domains only (Ahmed et al., 1997). Xu and London (2000) showed very recently that the condensed sterol-rich domains that are formed in e.g., DPPC-bilayers are very dependent on the structure of the sterol, i.e., on how well a sterol can interact with a phospholipid. We observed that cholesterol was able to form condensed domains with 16:0-SM (at 15 mol % sterol and temperatures below 35°C). This finding is similar to the observed formation of condensed domains in cholesterol/DPPC bilayer systems (at 15 mol % sterol and below 35°C), as reported by Xu and London (2000). However, when 16:0-DHSM was used as the bilayer phospholipid, much more condensed domains were formed (as evidenced by the reduced quenching of DPH by 12SLPC, Fig. 7) than with 16:0-SM, and the domains formed were stable to much higher temperatures. These findings show that in bilayer membranes, cholesterol interacts favorably with 16:0-DHSM and that the condensed domains that form are fairly stable against changes in temperature. This implies that cholesterol and dihydrosphingomyelin may actually interact in biological membranes as well, since both components are present, and most likely contribute to the formation of condensed domains with specific functions in the plasma membranes. Such an interaction is also likely to be of great importance in the human lens membrane, where the concentrations of both lipids is particularly high.

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