Cholesterol Interacts with Lactosyl and Maltosyl Cerebrosides but Not with Glucosyl or Galactosyl Cerebrosides in Mixed Monolayers†

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ABSTRACT: Pure and mixed monolayers of mono- and dihexoside cerebrosides with cholesterol have been characterized at the air/water interface. Cholesterol oxidase was used as a reporter enzyme for the cholesterol–cerebroside interaction in the mixed monolayers. The cerebrosides either were derived from bovine brain extracts or were synthetic. The dihexoside cerebrosides were synthesized by coupling of the hepta-*O*-acetyl-α-lactosyl- or maltosylphosphoramidates with D-*erythro*-*N*-acylceramides in dichloromethane, in the presence of trimethylsilyl triflate and molecular sieves, followed by hydrolysis of the acetate-protecting groups. All of the bovine-brain-derived cerebrosides [galactosyl cerebroside (GalCer, types I and II), glucosyl cerebroside (GlcCer), and lactosyl cerebroside (LacCer)] had very condensed force–area isotherms (compressibility values of 3–5 × 10^{-3} m/mN at 20 mN/m), as did the synthetic *N*-stearoylmaltosylceramide (*N*-18:0 MaltCer). Shorter-chain synthetic cerebrosides (*N*-8:0 LacCer and *N*-8:0 MaltCer) had more expanded isotherms, with compressibility values of 15–17 × 10^{-3} m/mN. When cholesterol was included in mixed monolayers of monohexoside cerebroside, it did not induce significant condensation of packing (indicating that cholesterol did not increase the order of the acyl chains). However, with dihexoside cerebrosides, a cholesterol-induced condensing effect was observed, which amounted to a 11–19% reduction in the observed mean molecular area. When cholesterol oxidase was used to titrate the stoichiometry of cholesterol/cerebroside in mixed monolayers, at which pure cholesterol clusters appeared, it was observed that in monohexoside cerebroside monolayers cholesterol clusters were present even below a 1:1 molar stoichiometry. In contrast, with LacCer mixed monolayers, cholesterol did not appear to undergo phase separation until above a 1:1 molar ratio of cholesterol to LacCer. Interestingly, with MaltCer mixed monolayers, a stoichiometry of 2:1 (cholesterol to cerebroside) was observed, similar to that observed for cholesterol/sphingomyelin mixed monolayers [Slotte, J. P. (1992) *Biochemistry 31*, 5472–5477]. In conclusion, it appears that the polar head group of the cerebrosides was the most important determinant of how the molecules associated with cholesterol in mixed monolayers.

Glycosphingolipids (GSLs)[1] are present as components of the external leaflet of many membrane types (Thompson & Tillack, 1985; Hannun & Bell, 1989), as well as lipoproteins (Dawson et al., 1976; Clarke, 1981). GSLs are synthesized on the cytoplasmic side of the Golgi apparatus from activated sugars and ceramide (Coste et al., 1985, 1986; Futerman & Pagano, 1991; Jeckel et al., 1992) and are selectively transported to the plasma membrane compartment (Kok et al., 1991). The GSLs are thought to have important roles in cell–cell and cell–ligand interactions, as well as in cell differentiation and proliferation (Maggio et al., 1981; Bremer et al., 1986; Kojima & Hakomori, 1989; Nojiri et al., 1991; Springer & Lasky, 1991). GSLs are structurally related to sphingomyelin, since they share a common ceramide backbone. One important property of sphingomyelin is that it appears to regulate the cellular distribution of unesterified cholesterol, possibly because of favorable molecular interactions with cholesterol in membranes (Wattenberg & Silbert, 1983; Clejan & Bittman, 1984; Van Blitterswijk et al., 1987; Slotte & Bierman, 1988; Slotte et al., 1989). Since GSLs and sphingomyelin colocalize in the exoleaflet of the plasma membrane structure, it would be of interest to examine whether sphingolipids other than sphingomyelin have the ability to interact tightly with cholesterol.

In a recent monolayer study (Johnston & Chapman, 1988), it was shown that the molecular packing density of bovine brain GalCer (containing non-hydroxy *N*-acyl chains) was condensed by cholesterol in mixed monolayers, suggesting a molecular interaction similar in type to that seen with phospholipids (Chapman et al., 1969; Demel et al., 1972). No other monolayer studies have appeared, to our knowledge, of the interaction of cholesterol with neutral GSLs. Another measure of cholesterol–phospholipid (or colipid) interaction in monolayers is the susceptibility of cholesterol to oxidation by cholesterol oxidase (Slotte, 1992a,b). Cholesterol interacting strongly with a phospholipid is not readily susceptible to oxidation, whereas pure cholesterol clusters in a monolayer are readily oxidized to 4-cholesten-3-one (Slotte, 1992a). The enzyme-catalyzed conversion of monolayer cholesterol to 4-cholesten-3-one is easily determined using a surface barostat (Slotte, 1992a,b). We have recently used cholesterol oxidase to probe the stoichiometry at which free cholesterol clusters disappear in various cholesterol/phospholipid mixed monolayers with variable cholesterol/phospholipid molar ratios (Slotte, 1992b).

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[1] Abbreviations: GalCer, galactosylceramide; GlcCer, glucosylceramide; GSLs, glycosphingolipids, LacCer, lactosylceramide; MaltCer, maltosylceramide.

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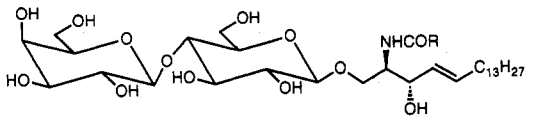
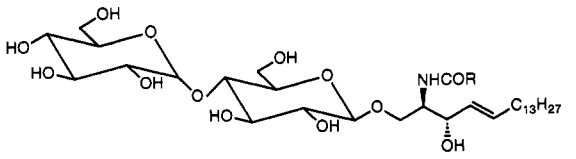
Chart I



N-8:0 LacCer, R = C_7H_{15}



N-8:0 MaltCer, R = C_7H_{15}

N-18:0 MaltCer, R = $C_{17}H_{35}$

Using this technique, we have in this study measured the extent of cholesterol interaction with both natural and synthetic mono- and dihexoside ceramides. On the basis of force–area isotherms of pure and mixed monolayer systems, we conclude that cholesterol interacts more strongly with dihexoside ceramides (LacCer and MaltCer) than it does with monohexoside ceramides (GalCer, with or without hydroxylated *N*-acyl chains, and GlcCer). The use of cholesterol oxidase to probe the interaction between cholesterol and cerebrosides also suggested that the interaction differed for mono- and dihexoside cerebrosides.

EXPERIMENTAL PROCEDURES

Materials. Cholesterol (99+%), bovine brain galactocerebrosides (type I contains about 98% α-hydroxy fatty acids; type II contains approximately 98% non-hydroxy fatty acids, primarily nervonic and lignoceric acids), glucosyl cerebrosides, lactosyl cerebrosides, lactose octaacetate, and maltose octaacetate were obtained from Sigma Chemical Co. (St. Louis, MO). Synthetic lactosyl and maltosyl cerebrosides (Chart I) were prepared as described below. Cholesterol oxidase (*Streptomyces* sp.) was purchased from Calbiochem (La Jolla, CA) and was used as delivered. Buffer salts were of *pro analysis* grade, and the water used was double-distilled and further purified with a Millex Q system (to better than 15 MΩ/cm). Aqueous hydrazine was purchased from J. T. Baker, trimethylsilyl triflate was from Petrarch Systems (Bristol, PA), and *N*,*N*,*N*′,*N*′-tetramethyldiamidophosphorochloriate was from Lancaster Synthesis (Windham, NH). Solvents for organic synthesis were dried as described elsewhere (Guivisdalsky & Bittman, 1989).

*Synthesis of N-Octanoyl-*D*-erythro-sphingosine.* To a solution of 200 mg (0.66 mmol) of D-*erythro*-sphingosine in 10 mL of dry THF was added 182 mg (0.68 mmol) of *p*-nitrophenyl caproate (Kan et al., 1991). The reaction mixture was stirred under nitrogen atmosphere overnight at room temperature. The reaction mixture was poured into water and extracted with ether and then washed with saturated aqueous sodium bicarbonate (4 × 30 mL) and with water (2 × 30 mL). The ether layer was dried over anhydrous sodium sulfate and concentrated under reduced pressure. The residue material was purified by column chromatography (elution with 0.5% methanol in chloroform), giving 168 mg (69%) of *N*-octanoyl-D-*erythro*-spingosine: R_f 0.60 (chloroform/methanol 9:1); ^{1}H NMR (200 MHz, $CDCl_3$) δ 0.87 (t, 6H, *J* = 6.73 Hz, $2CH_3$), 1.25 [br m, 30H, $(CH_2)_{11}CH_3$ and $(CH_2)_4CH_3$], 1.59 (br s, 2H, CH_2CH_2CO), 2.19 (m, 2H, $=CHCH_2$), 2.23 (t, 2H, *J* = 7.50 Hz, CH_2CO), 3.68 [d, 1H, *J* = 7.79 Hz, $HOCH_2CH(NH)$], 3.93 (m, 2H, CH_2OH), 4.33 [m, 1H, $=CHCH(OH)CH$], 5.55 (dt, 1H, *J* = 15.0, 6.39 Hz, vinyl proton), 5.75 (dd, 1H, *J* = 15.0, 6.5 Hz, vinyl proton), 6.26 (d, 1H, *J* = 7.11 Hz, CHN*H*CO).

*Synthesis of N-Octadecanoyl-*D*-erythro-sphingosine.* This compound was prepared from 350 mg (1.16 mmol) of D-*erythro*-sphingosine and 832 mg (2.05 mmol) of *p*-nitrophenyl stearate by using the same procedure as described above. There was obtained 450 mg (68%) of *N*-octadecanoyl-D-*erythro*-spingosine: R_f 0.60 (chloroform/methanol 9:1); ^{1}H NMR (200 MHz, $CDCl_3$) d 0.88 (t, 6H, *J* = 6.5 Hz, $2CH_3$), 1.25 [br m, 50H, $(CH_2)_{11}CH_3$ and $(CH_2)_{14}CH_3$], 1.63 (br s, 2H, CH_2CH_2CO), 2.19 (m, 2H, $=CHCH_2$), 2.23 (t, 2H, *J* = 7.50 Hz, CH_2CO), 3.68 [d, 1H, *J* = 8.14 Hz, $HOCH_2CH(NH)$], 3.93 (m, 2H, CH_2OH), 4.32 [m, 1H, $=CHCH(OH)CH$], 5.55 (dd, 1H, *J* = 15.4, 6.3 Hz, vinyl proton), 5.75 (dd, 1H, *J* = 14.58, 8.0 Hz, vinyl proton), 6.24 (d, 1H, *J* = 7.12 Hz, CHN*H*CO).

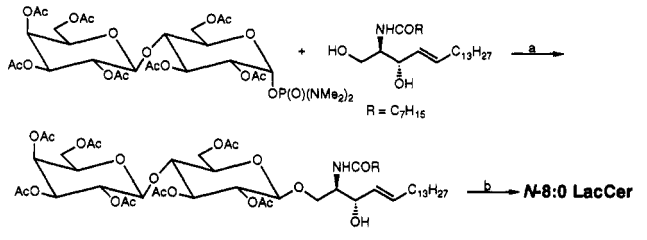
Synthesis of 2,3,6,2′,3′,4′,6′-Hepta-O-acetyllactose. To a solution of 1.0 g (1.47 mmol) of 1,2,3,6,2′,3′,4′,6′-octa-*O*-acetyl-β-lactose in 5 mL of DMF was added 140 mg of NH_2NH_2/AcOH (1:1 v/v) (Sadozai et al., 1986). The mixture was stirred for 3 h at room temperature. The reaction mixture was diluted with 100 mL of ethyl acetate, washed with water (3 × 100 mL), and dried over anhydrous sodium sulfate. The organic solvent was removed under reduced pressure to give crude 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyllactose in quantitative yield: R_f 0.22 (toluene/ethyl acetate 1:1); $[\alpha]^{25}_D$ +35.8° (*c* 2.45, $CHCl_3$).

Synthesis of 2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-α-lactosylphosphoroamidate. 2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyllactose (328 mg, 0.515 mmol) was dissolved in 10 mL of dry THF and cooled to −78 °C. *n*-Butyllithium (0.40 mL of a 1.57 M solution in *n*-hexane) was added, and the mixture was stirred for 15 min at the same temperature (Hashimoto et al., 1992). To this mixture was added tetramethyldiamidophosphorochloridate (87.5 mg, 0.515 mmol) in 0.5 mL of HMPA, and the reaction mixture was stirred for 2 h at −30 °C and at 0 °C for another 1 h. The reaction mixture was poured into water and extracted with ethyl acetate. The organic layer was washed with 2 N hydrochloric acid, water, sodium bicarbonate, and then with brine. The organic layer was dried over sodium sulfate, filtered, and distilled under reduced pressure. The crude material was purified by column chromatography (elution with 5% methanol in ethyl acetate), giving 157 mg (40%) of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-lactosylphosphoroamidate: R_f 0.65 (chloroform/methanol 8:2); $[\alpha]^{25}_D$ 38.3° (*c* 7.85, $CHCl_3$).

Synthesis of 2,3,6,2′,3′,4′,6′-Hepta-O-acetylmaltose. To a solution of 1.0 g (1.47 mmol) of 1,2,3,6,2′,3′,4′,6′-octa-*O*-acetyl-β-maltose in 5 mL of DMF was added 140 mg of NH_2NH_2/AcOH (1:1 v/v). After the reaction mixture was stirred for 3 h at room temperature, 100 mL of ethyl acetate was added, and the mixture was washed with water (3 × 100 mL). The organic layer was dried over anhydrous sodium sulfate. The solvent was removed under reduced pressure to give crude 2,3,6,2′,3′,4′,6′-hepta-*O*-acetylmaltose in quantitative yield: R_f 0.20 (toluene/ethyl acetate 1:1); $[\alpha]^{25}_D$ 87.6° (*c* 1.85, $CHCl_3$).

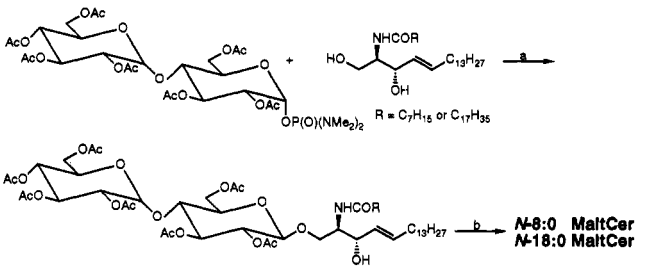
Synthesis of 2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-α-maltosylphosphoroamidate. This compound was prepared from 384 mg (0.60 mmol) of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetylmaltose and 102.5 mg (0.60 mmol) of tetramethyldiamidophosphoroch-

Scheme I



(a) TMSOTf, 4 A sieves, CH_2Cl_2; (b) 0.25 M KOH-MeOH

Scheme II



(a) TMSOTf, 4 A sieves, CH_2Cl_2; (b) 0.25 M KOH-MeOH

loridate in THF/HMPA as described above for 2,3,-6,2′,3′,4′,6′-hepta-*O*-acetyl-α-lactosylphosphoroamidate. There was obtained 232.5 mg (50%) of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-maltosylphosphoroamidate: R_f 0.65 (chloroform/methanol 8:2); $[\alpha]^{25}_D$ 90.5° (*c* 1.37, $CHCl_3$).

*Synthesis of 2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-β-lactosyl-N-octanoyl-*D*-erythro-sphingosine* (see Scheme I). A solution of 50 mg (0.117 mmol) of *N*-octanoyl-D-*erythro*-sphingosine in 10 mL of dry dichloromethane was stirred overnight under nitrogen atmosphere with activated powdered 4A molecular sieves at room temperature (Hashimoto et al., 1992). To the reaction mixture were added 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-lactosylphosphoramidate (82 mg, 0.106 mmol) and trimethylsilyl triflate (65.3 mg, 0.293 mmol), and the reaction flask was sealed and stirred under nitrogen atmosphere overnight at room temperature. The solvent was filtered and the mixture was concentrated under reduced pressure, giving a residue that was purified by column chromatography (elution with 75% ethyl acetate in hexanes). There was obtained 14.5 mg (12%) of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine: R_f 0.65 (ethyl acetate/hexanes 3:1).

*Synthesis of β-Lactosyl-N-octanoyl-*D*-erythro-sphingosine* (*N*-8:0 LacCer). 2,3,6,2′,3′,4′,6′-Hepta-*O*-acetyl-β-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine (14.5 mg, 13.89 μmol) was dissolved in 1.5 mL of 0.25 M methanolic KOH, and the mixture was stirred overnight at room temperature (Weber & Benning, 1986). The reaction mixture was neutralized with 2 N hydrochloric acid and extracted with chloroform. The chloroform layer was separated and concentrated under reduced pressure, and the residue was purified by column chromatography (elution with 10% methanol in chloroform), giving 10 mg (11%) of β-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine: R_f 0.51 (chloroform/methanol 8:2); $[\alpha]^{25}_D$ −4.3° (*c* 0.45, $CHCl_3$). The FAB mass spectrum gave MH^+ 750.

*Synthesis of 2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-β-maltosyl-N-octanoyl-*D*-erythro-sphingosine* (see Scheme II). This compound was prepared from 50 mg (0.117 mmol) of *N*-octanoyl-D-*erythro*-sphingosine, 90 mg (0.117 mmol) of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-maltosylphosphoroamidate, and 65.3 mg (0.293 mmol) of trimethylsilyl triflate in the presence of 4A activated molecular sieves as described above for 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine. There was obtained 68 mg of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-maltosyl-*N*-octanoyl-D-*erythro*-sphingosine, which was used in the next step without further purification: R_f 0.65 (ethyl acetate/hexanes 3:1).

*Synthesis of β-Maltosyl-N-octanoyl-*D*-erythro-sphingosine* (*N*-8:0 MaltCer). The *O*-acetyl groups were hydrolyzed in methanolic KOH as described above, giving 13 mg (14%) of β-maltosyl-*N*-octanoyl-D-*erythro*-sphingosine: R_f 0.51 (chloroform/methanol 8:2); $[\alpha]^{25}_D$ 23.2° (*c* 0.65, $CHCl_3$). The FAB mass spectrum gave MH^+ 750.

*Synthesis of 2,3,6,2′,3′,4′,6′-Hepta-O-acetyl-β-maltosyl-N-octadecanoyl-*D*-erythro-sphingosine.* This compound was prepared from 50 mg (0.117 mmol) of *N*-octadecanoyl-D-*erythro*-sphingosine, 68 mg (0.088 mmol) of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-α-maltosylphosphoroamidate, and 50 mg (0.22 mmol) of trimethylsilyl triflate in the presence of 4A activated molecular sieves as described above for 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-lactosyl-*N*-octanoyl-D-*erythro*-sphingosine. There was obtained 42.5 mg of 2,3,6,2′,3′,4′,6′-hepta-*O*-acetyl-β-maltosyl-*N*-octadecanoyl-D-*erythro*-sphingosine, which was used in the next step without further purification: R_f 0.65 (ethyl acetate/hexanes 3:1).

*Synthesis of β-Maltosyl-N-octadecanoyl-*D*-erythro-sphingosine* (*N*-18:0 MaltCer). The *O*-acetyl groups were hydrolyzed in methanolic KOH as described above, giving 12.6 mg (14%) of β-maltosyl-*N*-octadecanoyl-D-*erythro*-sphingosine: R_f 0.62 (chloroform/methanol 8:2); $[\alpha]^{25}_D$ 30.4° (*c* 0.57, $CHCl_3$). The FAB mass spectrum gave MH^+ 890.9.

Lateral Surface Pressure vs Mean Molecular Area Isotherms. Force–area isotherms were determined for pure cholesterol and cerebroside monolayers and for mixed monolayers containing varying molar ratios of cholesterol and cerebrosides with a KSV 3000 surface barostat (KSV Instruments, Helsinki). The isotherms were run in a rectangular Teflon trough (450 mm × 60 mm) on water at 22 °C. Stock solution of the lipids were made up in hexane/2-propanol (3:2 v/v) and were stored at −20 °C. The cerebroside stock solutions contained an additional drop (20 μL) of pure water to facilitate the formation of an optically clear solution. The lipid solution was spread on the aqueous surface in a volume of 10–100 μL, and the monolayer was then allowed to stabilize for 3–5 min before it was compressed at a barrier speed not exceeding 6 Å^2/(molecule min). Data were sampled every 2 s. At least two different runs were performed at each lipid composition, and the reproducibility was better than ±7%.

The compressibility of the monolayer films was calculated on the basis of data obtained from the force–area isotherms, according to

$$k = (-1/A)(dA/d\pi)$$

where A is the area per molecule (in Å^2) at a given surface pressure, π [mN/m; see also Ali et al. (1991)].

The extent of cholesterol-induced condensation of cerebroside packing in mixed monolayers is calculated from the deviation of the observed mean molecular area (in the mixed monolayer) from the theoretical molecular area in an ideal mixed monolayer, as detailed previously (Grönberg & Slotte, 1990).

Oxidation of Cholesterol in Mixed Monolayer Membranes. The oxidation of cholesterol in pure or mixed monolayers by cholesterol oxidase was determined in a thermostated zero-order Teflon trough (reaction chamber 30 mL, r = 29 mm; lipid reservoir 26 mm × 250 mm), with Tris buffer (50 mM

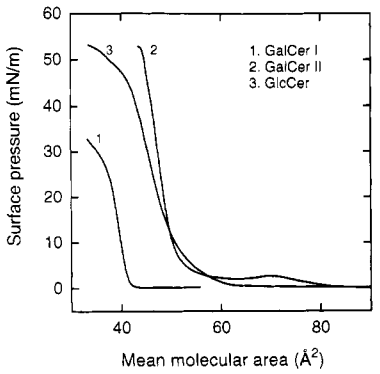


FIGURE 1: Force–area isotherms of neutral monohexoside ceramides. Pure GalCer (type I or II) or GlcCer was spread on the clean surface of pure water at 22 °C. After the film was allowed to stand for 5 min, it was compressed at a speed not exceeding 6 Å²/(molecule min). The isotherms shown are from a single run but are representative (within ±1 Å² at a given π above 5 mN/m) of several similar runs.

Tris-HCl, pH 7.4, 140 mM NaCl). The reaction compartment (2550 mm²) was magnetically stirred (100 rpm) and thermostated to 22 °C. The lipid solution was spread on the buffer surface, and the monolayer was compressed to 20 mN/m. Constant surface pressure was maintained by compensatory barrier movement (computer controlled) throughout the experiment. After the monolayer had stabilized for 5 min, cholesterol oxidase (50 millunits/mL) was added to the reaction compartment. The total time the monolayer was exposed to air at the air/water interface was about 15 min. The rate of the enzyme-catalyzed oxidation of cholesterol in the monolayer was registered (at constant surface pressure) as a backward movement of the barrier due to an oxidation-dependent increase in the monolayer area. Data were sampled every 10 s. Usually three experiments were performed with each mixed monolayer, and the measured oxidation times deviated less than ±15% of the calculated mean value. Since cholesterol oxidase is very unstable in solution, even at 0 °C, a 20 units/mL stock was prepared in Tris buffer, and aliquots of 300 μL were stored frozen at −20 °C. A new enzyme aliquot was used for every fourth monolayer, and the thawed enzyme was kept at 0 °C for a maximum of 60 min. The conversion of monolayer expansion to an average oxidation rate is described in previous studies (Slotte, 1992a,b).

RESULTS

Pure Monolayers of Monohexoside Ceramides. The force–area isotherms of pure monohexosylceramides were obtained on pure water at 22 °C by compressing the expanded monolayer at a compresson speed not exceeding 6 Å²/(molecule min) (Figure 1). Bovine brain GalCer (type I, containing α-hydroxy-substituted *N*-linked acyl chains) was observed to have a fairly condensed isotherm, with a liftoff area at 42 Å². The calculated compressibility (at 20 mN/m) for GalCer (type I) was 4×10^{-3} m/mN (Table I). No phase transitions were apparent during the compression, and the film started to collapse at 25 mN/m (smooth transition to a collapsed state). This early collapse was verified with different batches of bovine brain GalCer (type I; Sigma). GalCer (type II, containing nonhydroxylated *N*-linked acyl chains), on the other hand, displays a much larger mean molecular area compared to GalCer (type I) and also a previously described phase transition at a low surface pressure [at 70 Å² and 4 mN/m; see also Johnston and Chapman (1988)]. The monolayer collapsed rather sharply at 53 mN/m. The calculated compressibility value for GalCer (type II) at 20 mN/m was 3×10^{-3} m/mN (Table I). The GlcCer isotherm was more expanded at lower pressures compared to that of GalCer (type I) and indicated no phase transitions. The transition from an apparently stable GlcCer monolayer to a collapsed state occurred smoothly at a fairly high surface pressure (about 40 mN/m).

Table I: Properties of Pure and Mixed Monolayers of Cholesterol and Cerebrosides

	$X = 1$[a]		$X = 0.5$	
lipid species	$A^2_{20mN/m}$	$k_{20mN/m}$/ $\times 10^{-3}$ m/mM	$A^2_{20mN/m}$	% condensation[b]
cholesterol	39	1.3		
N-8:0 LacCer	75	17	51	11
LacCer (bb)[c]	52	3	37	19
N-8:0 MaltCer	71	15	49	11
N-18:0 MaltCer	52	4	38	17
GalCer type I (bb)	39	4	39	0
GalCer type II (bb)	49	3	43	2
GlcCer (bb)	49	5	42	5

[a] X gives the mole fraction of cerebroside in the monolayer. [b] Percent condensation at $X = 0.5$ is calculated as described under Experimental Procedures. [c] (bb), bovine brain.

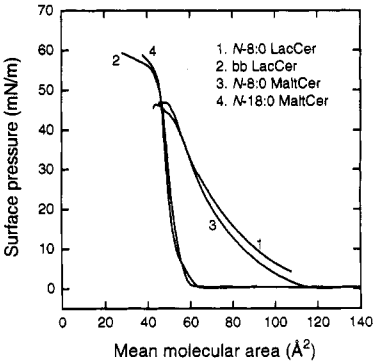


FIGURE 2: Force–area isotherms of synthetic and bovine-brain-derived lactosyl and maltosyl cerebrosides. Pure bovine-brain-derived LacCer, or synthetic *N*-8:0 LacCer, and *N*-8:0 MaltCer, or *N*-18:0 MaltCer, were spread on the clean surface of pure water at 22 °C. After the film was allowed to stand for 5 min, it was compressed at a speed not exceeding 6 Å²/(molecule min). The isotherms shown are from a single run but are representative (within ±1 Å² at a given π above 10 mN/m) of several similar runs.

Pure Monolayers of Dihexoside Ceramides. The force–area isotherms of all dihexoside ceramides tested had a significantly larger mean molecular area, as well as collapse area (Figure 2), compared with that of the monohexoside ceramides (Figure 1), possibly reflecting the space needed by the second sugar residue. Bovine brain LacCer displayed a fairly condensed isotherm with no apparent phase transitions. The compressibility value was low (3×10^{-3} m/mN at 20 mN/m) and comparable to that calculated for the cholesterol isotherm (1.3×10^{-3} m/mN; isotherm not shown). The synthetic *N*-18:0 MaltCer had a force–area isotherm very similar to that obtained for bovine brain LacCer with regard to the compressibility value (Table I), the collapse pressure, and the mean molecular area (Figure 2). The synthetic short-chain analogues of LacCer and MaltCer (i.e., *N*-8:0 derivatives) had very expanded (and similar) force–area isotherms (Figure 2). The transition from a stable monolayer to a collapsed state was fairly sharp and occurred at a much lower surface pressure as compared with either bovine brain LacCer or *N*-18:0 MaltCer.

Mixed Monolayers of Cholesterol and Mono- or Dihexoside

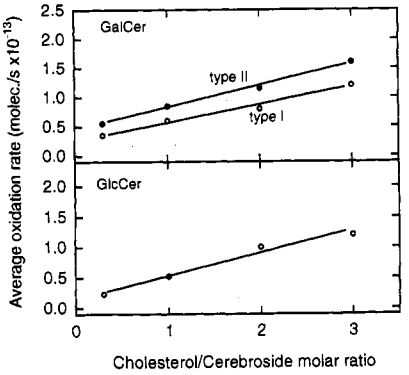


FIGURE 3: Stoichiometries of association between cholesterol and monohexoside cerebrosides, as probed by cholesterol oxidase. Mixed monolayers containing indicated cholesterol-to-cerebroside molar ratios (the cerebroside being either GalCer type I or II or GlcCer) were exposed to cholesterol oxidase in the subphase (50 mM Tris buffer, pH 7.4, with 140 mM NaCl) below the monolayer at 22 °C. The oxidation-dependent expansion of the monolayer was registered and converted to an average oxidation rate (molecules oxidized per second), as described under Experimental Procedures. Each value is the mean from two different monolayers at each molar ratio.

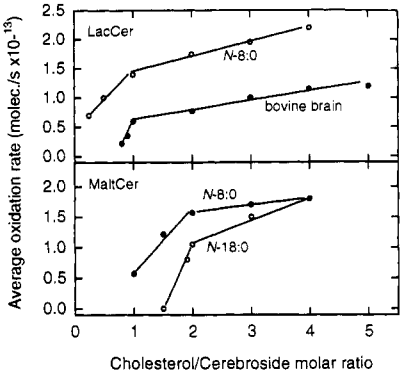


FIGURE 4: Stoichiometries of association between cholesterol and dihexoside cerebrosides, as probed by cholesterol oxidase. Mixed monolayers contained the indicated cholesterol-to-cerebroside molar ratios. The cerebrosides were bovine brain LacCer and synthetic *N*-8:0 LacCer (top) or *N*-8:0 MaltCer and *N*-18:0 MaltCer (bottom). Cholesterol oxidase was present in the subphase below the monolayer at 22 °C. The oxidation-dependent expansion of the monolayer was registered and converted to an average oxidation rate (molecules oxidized per second). Each value is the mean from two different monolayers at each molar ratio.

Ceramides. To gain information about the possible interactions between cholesterol and cerebrosides, force–area isotherms were obtained (on pure water at 22 °C) for the mixed monolayers. Table I lists the mean molecular areas for the various binary cholesterol/cerebroside complexes at 50 mol % each (at 20 mN/m). With the three monohexosylceramides tested, no or very limited condensation of molecular packing could be observed in the presence of cholesterol. However, all three dihexoside ceramides tested were significantly condensed in the presence of cholesterol, indicating that cholesterol was able to increase the order of the acyl chains of the dihexoside ceramides, similarly as previously shown for cholesterol/phospholipid monolayers (Chapman et al., 1969; Demel et al., 1972).

Oxidation of Cholesterol in Mixed Cerebroside Monolayers. The oxidation of cholesterol in pure cholesterol monolayers has been shown by this laboratory to be a fairly rapid process (Slotte, 1992a). Upon interaction of cholesterol with phospholipids in mixed monolayers, the oxidation rate is slowed down markedly, probably reflecting an increased difficulty of the sterol molecule to reach the active site of the enzyme (Slotte 1992a,b). When the ratio of cholesterol to a colipid in mixed binary monolayers is titrated (going from a high to a low cholesterol concentration), a stoichiometry is obtained at which the oxidation rate markedly decreases. This decrease in rate is interpreted to reflect the disappearance of pure cholesterol clusters in the mixed monolayers (Slotte, 1992b).

Using this technique with the different cerebrosides, we have determined the apparent average oxidation rate at different molar ratios of cholesterol to cerebroside. With all tested monohexosylceramides, the function of the average oxidation rate vs cholesterol/cerebroside molar ratio was linear and without a break (Figure 3). This linearity below a 1:1 molar ratio strongly suggests that cholesterol did not associate with the monohexosylceramides but rather separated into cholesterol clusters. This apparent lateral phase segregation is consistent with the lack of condensation reported in Table I for mixed monolayers of cholesterol and monohexosylceramides.

With dihexosylceramides, different stoichiometries were observed, depending on the sugar residues. With both bovine brain LacCer and *N*-8:0 LacCer, a break at a 1:1 stoichiometry was observed in the function of average oxidation rate vs cholesterol/cerebroside molar ratio (Figure 4). Cholesterol in the *N*-8:0 LacCer mixed monolayer was oxidized slightly faster as compared with the bovine brain LacCer mixed monolayer, possibly reflecting a looser association with *N*-8:0 LacCer compared with bovine brain LacCer. Surprisingly, both *N*-18:0 and *N*-8:0 MaltCer types displayed a 2:1 stoichiometry (Figure 4). This 2:1 stoichiometry is similar to that observed for sphingomyelin-containing mixed monolayers (Slotte, 1992b), whereas a 1:1 stoichiometry is believed to represent a thermodynamically stable association in phosphatidylcholine-containing membranes (Phillips & Finer, 1974; Huang, 1977; Collins & Phillips, 1982; Slotte, 1992b). As with the lactosyl cerebrosides, cholesterol appeared to be oxidized slightly faster in an *N*-8:0 MaltCer mixed monolayer than in an *N*-18:0 MaltCer monolayer.

DISCUSSION

The objective of this study was to examine the mode of cholesterol interaction with neutral GSLs in mixed monolayers at the air/water interface at 22 °C, to compare the results obtained with our previously published results on the interaction of cholesterol with sphingomyelins in mixed monolayers (Grönberg & Slotte, 1990; Slotte, 1992b). The extent of molecular interactions in the mixed monolayers was deduced from the mixing behavior of the components (i.e., from the effects of cholesterol on packing density in mixed monolayers) and from the use of cholesterol oxidase, which probed the oxidation susceptibility of cholesterol in the mixed monolayers of the various neutral GSLs (Slotte, 1992a,b).

Force–area isotherms of pure monohexosyl cerebroside on water at 22 °C displayed solid-condensed behavior at lateral surface pressures above 10 mN/m (Figure 1), with compressibility values (at 20 mN/m) ranging over 3–5 × 10^{-3} m/mN (Table I). These observations are generally in good agreement with previously published force–area isotherms of bovine-brain-derived GalCer at similar temperatures (Oldani et al., 1975; Ries, 1982; Ali et al., 1991). The low-pressure part of the GalCer (type II) displayed a transition from a liquid-expanded to a liquid-condensed state at a mean molecular area of 70 Å^2 and a lateral surface pressure of 4 mN/m. This phase transition observed for GalCer (type II) at 22 °C occurred at a slightly lower surface pressure (4 mN/

m) than the corresponding transition reported at 37 °C (~8 mN/m; Johnston & Chapman, 1988). Whereas GalCer (type II) displayed a fairly sharp transition from a stable monolayer to a collapsed state (at 53 mN/m), both GalCer (type I) and GlcCer monolayers had poorly defined transitions to a collapsed state (starting at lateral surface pressures of 25 and 40 mN/m, respectively). Johnston and Chapman (1988) have suggested that the poorly defined collapse in GalCer (type I) monolayers is due to the formation of bilayer structures (at 37 °C). A similar collapse mechanism is likely to occur even at slightly lower (22 °C) experimental temperature. It should be kept in mind, however, that differences in transitions of monolayers to collapsed states are likely to be very dependent on compression speed, trough design, and other factors related to the equipment used.

The force–area isotherms of long-chain dihexosyl cerebrosides also exhibited solid-condensed behavior (Figure 2), with compressibility values of 3–4 × 10^{-3} m/mN at 20 mN/m (Table I). These monolayers of bovine-brain-derived LacCer and *N*-18:0 MaltCer also displayed a fairly smooth transition to a collapsed state (at about 55 mN/m). In contrast to the long-chain dihexosyl cerebrosides, short-chain dihexosyl cerebrosides (*N*-8:0 LacCer and MaltCer) had a liquid-expanded type of force–area isotherm, with compressibility values at 15–17 × 10^{-3} m/mN. The finding that short-chain cerebrosides (e.g., *N*-8:0 MaltCer) had a more expanded force–area isotherm compared with their longer-chain analogue (e.g., *N*-18:0 MaltCer) is consistent with the expected loss in van der Waals attractive forces between the acyl chains as their length decreases (Salem, 1962). An analogous situation has been reported for the force–area isotherms of *N*-20:$1^{\Delta 11}$ and *N*-18:$1^{\Delta 9}$ galactosylsphingosines (Ali et al., 1991).

The degree of interaction between cholesterol and cerebrosides in binary mixed monolayers was estimated on the basis of the condensing effect of cholesterol on the packing of cerebrosides in binary mixed monolayers. The observed mean molecular area in the equimolar mixed monolayer (at 20 mN/m) was compared with the calculated mean molecular area based on simple additivity, with deviations from this value suggesting lipid mixing and direct molecular interactions (Goodrich, 1957). In our hands, none of the monohexoyl cerebrosides appeared to interact very strongly with cholesterol in equimolar mixed monolayers at 20 mN/m (and 22 °C). The observed mean molecular area in mixed monolayers of cholesterol and GalCer (type I) or GalCer (type II) was very close to that expected from simple additivity, whereas GlcCer mixed monolayers were slightly more condensed (about 5%) by cholesterol. Johnston and Chapman (1988) have reported that cholesterol does not condense the packing or GalCer (type I) above 10 mN/m (37 °C), whereas they have reported that GalCer (type II) was significantly condensed, even at 35 mN/m (also at 37 °C). This discrepancy in results could arise from the difference in the temperature (22 vs 37 °C), since the galactosyl cerebrosides are more expanded at higher temperatures (Johnston & Chapman, 1988). In contrast to the findings with monohexosyl cerebrosides, cholesterol appeared to readily interact with dihexosyl cerebrosides, even at 22 °C (Table I). Both lactosyl and maltosyl cerebrosides were effectively condensed by cholesterol in equimolar mixed monolayers (at 20 mN/m). These results would suggest that the *polar head group* of the cerebrosides is the most significant determinant of how cholesterol mixes or interacts with cerebrosides in monolayers.

The results obtained with cholesterol oxidase clearly indicate that cholesterol interacts differently with monohexosyl cerebrosides compared with dihexosyl cerebrosides. In monohexosyl cerebroside monolayers, cholesterol oxidase was able to oxidize cholesterol at all cholesterol-to-cerebroside ratios tested (even at 0.33 mol of cholesterol to 1 mol of cerebroside) (Figure 3), suggesting that no strong associations exist between cholesterol and the monohexosyl cerebrosides. We found that the average oxidation rate vs molar ratio function was linear well below a 1:1 stoichiometry in all monohexosyl cerebroside mixed monolayers, suggesting that cholesterol clusters were present at all ratios tested. The existence of apparent lateral phase separation, as detected by cholesterol oxidase, is consistent with the lack of condensation for equimolar mixed monolayers of cholesterol and monohexosyl cerebrosides (Table I).

In mixed monolayers of cholesterol and dihexosyl cerebrosides, cholesterol oxidase revealed some interesting findings (Figure 4). With lactosyl cerebrosides, a 1:1 molar stoichiometry was observed, at which the linear average oxidation rate vs molar ratio function displayed a break (Figure 4, top). This 1:1 stoichiometry suggests that no cholesterol clusters were present below this molar ratio. Therefore, the mixed cholesterol/LacCer monolayer behaved similarly to the previously reported cholesterol/phosphatidylcholine mixed monolayer, with regard to how cholesterol oxidase recognizes its substrate (Slotte, 1992b). In contrast to what was seen with LacCer monolayers, mixed monolayers of MaltCer were characterized by a 2:1 cholesterol/cerebroside molar stoichiometry (Figure 4, bottom), similar to what has been reported by us for cholesterol/sphingomyelin mixed monolayers (Slotte, 1992b). The stoichiometries are not affected by changing the *N*-acyl chain structure. The only difference in the structure between *N*-8:0 LacCer and *N*-8:0 MaltCer resides in the sugar residues, i.e., in the orientation of the hydroxyl group at carbon 4 of the distal sugar unit, and the β1,4-glycosidic linkage in LacCer vs the α1,4 linkage in MaltCer; thus, the polar region of these cerebrosides appears to determine the stoichiometry of association with cholesterol. It is presently unclear how polar head-group interactions influence the two-dimensional structure of the mixed monolayer in such a way that the cholesterol oxidase susceptibility of cholesterol is so markedly affected; differences in hydration, hydrogen bonding, and spatial orientation of the disaccharide moiety may affect lateral packing of cerebrosides with neighboring cholesterol molecules.

In conclusion, the present results have indicated that cholesterol is miscible in dihexosyl cerebroside monolayers. Of the dihexosyl cerebrosides studied, LacCer is found both in plasma membranes and in lipoproteins. We used MaltCer to determine if the orientation of the carbohydrate residues in the dihexoside cerebroside affects the interaction with cholesterol. Since both plasma membranes and the lipoprotein polar shell contain significant amounts of unesterified cholesterol, it is at least in theory possible that cholesterol may interact with LacCer in these structures. Although it has been fairly well established that sphingomyelin is the quantitatively most dominant effector of cholesterol distribution in cells (Slotte et al., 1989; Pörn et al., 1993), at this stage we cannot rule out the possibility of physiological cholesterol–LacCer interactions. To test for the possibility of such interactions in biological membranes, one would need to examine the effects of enzymatic LacCer degradation on cholesterol distribution, in analogy with cell experiments that have been done with sphingomyelinase (Slotte et al., 1989; Gupta & Rudney, 1991).

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