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# Influence of Molecular Packing and Phospholipid Type on Rates of Cholesterol Exchange†

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ABSTRACT: The rates of [$^{14}$C]cholesterol transfer from small unilamellar vesicles containing cholesterol dissolved in bilayers of different phospholipids have been determined to examine the influence of phospholipid–cholesterol interactions on the rate of cholesterol desorption from the lipid–water interface. The phospholipids included unsaturated phosphatidylcholines (PC's) (egg PC, dioleoyl-PC, and soybean PC), saturated PC (dimyristoyl-PC and dipalmitoyl-PC), and sphingomyelins (SM's) (egg SM, bovine brain SM, and *N*-palmitoyl-SM). At 37 °C, for vesicles containing 10 mol % cholesterol, the half-times for exchange are about 1, 13, and 80 h, respectively, for unsaturated PC, saturated PC, and SM. In order to probe how differences in molecular packing in the bilayers cause the rate constants for cholesterol desorption to be in the order unsaturated PC > saturated PC > SM, nuclear magnetic resonance (NMR) and monolayer methods were used to evaluate the cholesterol physical state and interactions with phospholipid. The NMR relaxation parameters for [4-$^{13}$C]cholesterol reveal no differences in molecular dynamics in the above bilayers. Surface pressure ($\pi$)–molecular area isotherms for mixed monolayers of cholesterol and the above phospholipids reveal that SM lateral packing density is greater than that of the PC with the same acyl chain saturation and length (e.g., at $\pi$ = 5 mN/m, where both monolayers are in the same physical state, dipalmitoyl-PC and palmitoyl-SM occupy 87 and 81 Å$^2$/molecule, respectively). The greater van der Waals interaction in the SM monolayer (or bilayer) compared to PC gives rise to a larger condensation by cholesterol (e.g., in equimolar monolayers at $\pi$ = 5 mN/m, the condensations of dipalmitoyl-PC and palmitoyl-SM are 16 and 31 Å$^2$/molecule, respectively). This is a direct demonstration of the greater interaction of cholesterol with SM compared to PC. An estimate of the van der Waals interactions between cholesterol and these phospholipids has been used to derive a relationship between the ratio of the rate constants for cholesterol desorption and the relative molecular areas (lateral packing density) in two bilayers. This analysis suggests that differences in cholesterol–phospholipid van der Waals interaction energy are an important cause of varying rates of cholesterol exchange from different host phospholipid bilayers.

In order for cholesterol molecules to exchange or transfer between membranes or lipoproteins, they must desorb from the lipid–water interface of the donor particle into the aqueous phase before diffusing to and adsorbing into the acceptor particle [for a review, see Phillips et al. (1987)]. Since the desorption step is rate limiting (McLean & Phillips, 1981), it follows that a complete understanding of the transfer process requires elucidation of the influence of cholesterol–phospholipid molecular packing on the rate of desorption of cholesterol molecules from lipid–water interfaces.

Here we compare the rates of transfer of [$^{14}$C]cholesterol from the bilayer membrane of small unilamellar vesicles containing 10 mol % cholesterol in egg phosphatidylcholine (egg PC),[1] dipalmitoyl-PC, and egg or brain sphingomyelin. The cholesterol interfacial packing is monitored in terms of the rotational freedom of cholesterol molecules in each system, as measured by nuclear magnetic resonance (NMR) spin-

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[1] Abbreviations: DCP, dicetyl phosphate; DMPC, 1,2-dimyristoyl-3-*sn*-phosphatidylcholine; DOPC, 1,2-dioleoyl-3-*sn*-phosphatidylcholine; DPPC, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; SM, sphingomyelin.

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lattice relaxation times ($T_1$) and line widths ($\nu_{1/2}$) of [4-$^{13}$C]cholesterol. In addition, surface pressure-molecular area isotherms of mixed cholesterol–phospholipid monolayers are used to quantitate the lateral packing density in these cholesterol–phospholipid model membranes. This information is integrated in a treatment of the probable influence of phospholipid–cholesterol van der Waals interactions on the rate constant ($k$) for cholesterol exchange from phospholipid–water interfaces.

## Experimental Procedures

*Materials.* Crystalline [4-$^{13}$C]cholesterol with 90 atom % $^{13}$C enrichment was obtained from Merck Co. (Montreal, Canada), cholesterol and oleic anhydride were used as supplied by Sigma Chemical Co. (St. Louis, MO), and [7-$^{3}$H(N)]-cholesterol (specific activity 23 Ci/mmol) and [4-$^{14}$C]cholesterol (specific activity 55 mCi/mmol) were purchased from New England Nuclear (Boston, MA). [7-$^{3}$H(N)]Cholesteryl oleate was prepared to a specific activity of 55 mCi/mmol by reaction of oleic anhydride and [7-$^{3}$H(N)]cholesterol (the specific activity was reduced by mixing with unlabeled cholesterol in chloroform) according to the procedure of Lentz et al. (1975). Egg yolk phosphatidylcholine (egg PC), dimyristoyl-PC (DMPC), dipalmitoyl-PC (DPPC), dioleoyl-PC (DOPC), and soybean PC were obtained from Calbiochem-Behring Corp. (La Jolla, CA). The egg and bovine brain sphingomyelin (SM) samples were also supplied by the same company; representative fatty acid compositions for these naturally occurring SM's have been reported (Barenholz, 1984). Dicetyl phosphate (DCP), *N*-palmitoyl-D-, and *N*-oleoyl-D-sphingomyelin were purchased from Sigma Chemical Co. (St. Louis, MO).

The lipids were stored under nitrogen at −20 °C as stock solutions in chloroform–methanol (1:1). The purities of the lipids were assayed by thin-layer chromatography on silica gel G plates (Analtech, Newark, DE) in two solvent systems: (1) petroleum ether–diethyl ether–acetic acid (75:24:1 v/v) and (2) chloroform–methanol–water (65:25:4 v/v). The purity of dicetyl phosphate was assessed by chromatography in chloroform–methanol–ammonium hydroxide (56:18:3 v/v). Lipids were visualized by spraying developed thin-layer plates with a 50% sulfuric acid solution and charring the sprayed plates at 200 °C for 15 min. Application of 100-$\mu$g quantities of the unlabeled lipids gave single spots by charring. The purities of the radiolabeled sterols were determined by thin-layer chromatography using ITLC SA plates (Gelman Sciences, Ann Arbor, MI). The plates were cut, at positions conforming to standards, placed in liquid scintillation vials, and analyzed for radioactivity by liquid scintillation counting in 10 mL of scintillation cocktail (ScintiVerse; Fisher Scientific Co., Philadelphia, PA). The radiolabeled sterols were judged to be >98% pure. $D_2O$, purchased from Stohler Isotopes (Waltham, MA) or Merck (Rahway, NJ), was routinely deoxygenated and stored under nitrogen. Manganous chloride ($MnCl_2$; ACS grade), obtained from Fisher Scientific Co. (Fairlawn, NJ), was dried for 18 h at 110 °C before use. Other reagents were analytical grade. All organic solvents were redistilled prior to use.

*Assay of Cholesterol Exchange between Small Unilamellar Vesicles.* Neutral and negatively charged unilamellar vesicles were prepared in 20 mM sodium phosphate, pH 6.0, by a modification of the procedure of Barenholz et al. (1977), as described previously (McLean & Phillips, 1981). Phospholipids whose gel to liquid-crystal transition temperatures are below room temperature [see Silvius (1982) and Barenholz (1984)] were sonicated on ice (McLean & Phillips, 1981; Lund-Katz & Phillips, 1984) while those with higher transition temperatures were sonicated at 50 °C (Bittman et al., 1984). Donor phospholipid vesicles (negatively charged) were prepared with [4-$^{14}$C]cholesterol as the exchangeable lipid; neutral acceptor vesicles contained [7-$^{3}$H]cholesteryl oleate as a nonexchangeable marker to monitor their recovery. Neutral vesicles were separated from negatively charged vesicles containing 15 mol % dicetyl phosphate on short columns of DEAE-Sepharose (Pharmacia) by a modification of the procedure of Hellings et al. (1974) and van den Besselaar et al. (1975) as described previously (McLean & Phillips, 1981). The ratio of acceptor to donor vesicles was 10:1, and, typically, <3% of the negatively charged vesicles and about 80% of the neutral vesicles were recovered in the column eluate. The time courses of [4-$^{14}$C]cholesterol transfer between the two vesicle populations were analyzed according to isotope exchange kinetics (McKay, 1938) [see McLean and Phillips (1981) and Phillips et al. (1987) for further details]. Because the exchange from some donor vesicles was very slow, experimental estimates of the equilibrium distribution ($X_\infty$) of [$^{14}$C]cholesterol could not be obtained, so the theoretical $X_\infty$ was used in all cases to compute $k$. Neutral and charged donor small unilamellar vesicles exhibited similar rates of cholesterol exchange (McLean & Phillips, 1984).

*Nuclear Magnetic Resonance Measurements.* The preparation of vesicles containing 10 mol % [4-$^{13}$C]cholesterol and 90 mol % phospholipid used in the NMR measurements has been described previously (Bittman et al., 1984; Lund-katz & Phillips, 1984). To examine the effect of dicetyl phosphate, vesicles containing 10 mol % [4-$^{13}$C]cholesterol, 15 mol % dicetyl phosphate, and 75 mol % of the phospholipid under investigation were prepared in a similar fashion. Twenty microliters of 1,4-dioxane and $D_2O$ as the NMR lock compound was added to the solution of vesicles to increase the volume by 20% to 1.5 mL. The vesicles were transferred to 10-mm NMR tubes under $N_2$, and NMR analysis was completed within 24 h. The procedures used to obtain $^{13}$C NMR spectra and to measure $T_1$ values have been described elsewhere (Lund-Katz & Phillips, 1984). All proton-decoupled $^{13}$C NMR spectra were recorded at 90.55 MHz by employing a Bruker WH 360 spectrometer and broad-band, phase-modulated proton decoupling. $Mn^{2+}$ in the form of its chloride was added to the aqueous phase of some NMR samples as a relaxation enhancement agent to study the exposure of the phospholipid polar group and the cholesterol C-4 atom to the aqueous phase. The mole ratio of cation to phospholipid was 0.06; when $Mn^{2+}$ was added to the vesicles, the solution was sonicated briefly to allow the cation to enter the aqueous phase encapsulated in the vesicles.

*Surface Pressure–Molecular Area Isotherms.* The surface pressure ($\pi$) as a function of average molecular area ($A$) of either pure lipid or mixed lipid monolayers spread at the air–water interface was measured at 25 °C in a surface balance using a modification of the procedure of Phillips and Chapman (1968). The apparatus and methods used have been described elsewhere (Laboda et al., 1986). Briefly, the lipids were spread quantitatively from a hexane–ethanol (9:1 v/v) solution onto the surface of the subphase buffer (10 mM phosphate–150 mM NaCl, pH 7.6). After 5 min, the films were compressed by using a movable Teflon barrier at a rate <10 Å$^2$/(min·molecule). Surface pressures for a given molecular area (angstroms squared per molecule) were reproducible to within 1 mN/m.

*Analytical Procedures.* The concentration of cholesterol in all of the vesicles was determined by gas–liquid chroma-

Table I: Half-Times for Exchange of Cholesterol from Small Unilamellar Phospholipid Vesicles

| host phospholipid[a] | half-time, $t_{1/2}$ (h)[b], at 37 °C | at 45 °C |
|---|---|---|
| phosphatidylcholine | | |
| dipalmitoyl | 16 ± 3 ($n$ = 10) | 5.5 ± 0.8 ($n$ = 20) |
| dimyristoyl | 11 ± 1 ($n$ = 9) | |
| egg | 1.2 ± 0.05 ($n$ = 19) | 0.5 ± 0.04 ($n$ = 6) |
| dioleoyl | 0.8 ± 0.09 ($n$ = 11) | 0.15 ± 0.03 ($n$ = 4) |
| soybean | 0.3 ± 0.06 ($n$ = 13) | |
| sphingomyelin | | |
| egg | | 19 ± 1 ($n$ = 10) |
| bovine brain | | 14 ± 3 ($n$ = 10) |
| palmitoyl | 77 ± 4 ($n$ = 7) | 11 ± 2 ($n$ = 12) |

[a] The donor vesicles comprised 10 mol % [$^{14}$C]cholesterol, 15 mol % dicetyl phosphate, and 75 mol % phospholipid. The donor vesicles were used in cholesterol exchange experiments with 10-fold excess acceptor vesicles (10 mol % cholesterol, 90 mol % egg PC, and a trace of [$^{3}$H]-cholesteryl oleate) present so that the $t_{1/2}$ values are characteristic of the rate-limiting desorption of cholesterol from the phospholipid–water interface [cf. McLean and Phillips (1981)]. [b] The half-times are derived from the first-order rate constants ($k_1$) describing the kinetics of cholesterol exchange using the relationship $t_{1/2} = \ln(2/k_1)$. The tabulated $t_{1/2}$ values are mean values (±SEM). $n$ is the number of kinetic experiments performed; at least three different preparations of vesicles were used except in the experiments with unsaturated PC at 45 °C.

tography using coprostanol as an internal standard (Bates & Rothblat, 1974). Protein determination was carried out following the Lowry method as adapted by Markwell et al. (1978), and phospholipid content was monitored by phosphorus analysis (Sokoloff & Rothblat, 1974).

Negative-stain electron microscopy (Collins & Phillips, 1982) was used to assess the particle sizes of the various vesicles.

## Results

*Rate of Cholesterol Exchange as a Function of Phospholipid Acyl Chain Saturation and Phospholipid Class.* Table I gives the rates of cholesterol transfer from negatively charged 10 mol % cholesterol–PC unilamellar vesicles of different acyl chain compositions to acceptor vesicles at two different temperatures, 37 and 45 °C. The exchange kinetics were identical with neutral donor vesicles in which the dicetyl phosphate was omitted; this is consistent with the observation that dicetyl phosphate has a negligible effect on the acyl chains in PC and SM bilayers (Shinitzky & Barenholz, 1974). Charged and neutral small unilamellar vesicles composed of either saturated PC, unsaturated PC, or SM had similar size distributions with diameters of 23 ± 2 nm. The exchange data indicate that the rate of cholesterol transfer from lipid–water interfaces is a function of the acyl chain composition of the donor bilayer. The rate of cholesterol exchange from bilayers of unsaturated phospholipids where essentially all of the unsaturated acyl chains contain a single cis double bond (egg PC or DOPC) is approximately an order of magnitude faster than the rate of exchange from saturated PC bilayers such as DMPC and DPPC at 37 °C (approximately 1.0 vs 14.0 h). The transfer half-time ($t_{1/2}$) for cholesterol decreases to 0.3 h when polyunsaturated soybean PC is used as the host phospholipid.

These data are consistent with reports from several laboratories showing that cholesterol exchanges relatively rapidly from unsaturated bilayers [for a review, see Phillips et al. (1987)]. However, the 37 °C data in Table I are complicated by the fact that dipalmitoyl-PC is not above its gel to liquid-crystal phase transition temperature. At 45 °C, all the phospholipid bilayers reported in Table I are in the liquid-crystal phase [see transition temperature data reported by Silvius (1982) and Barenholz (1984)]. Consequently, the $t_{1/2}$ values show how acyl chain composition and phospholipid type can influence cholesterol desorption from bilayers in a given physical state. Comparison of the $t_{1/2}$ values for cholesterol exchange from PC bilayers at 45 °C (Table I) confirms the ranking of the $t_{1/2}$ values made at 37 °C, although the rate of desorption of cholesterol molecules from the lipid–water interfaces is approximately 3 times faster at the elevated temperature due to the high activation energy associated with this process [cf. McLean and Phillips (1982)]. The rate of cholesterol exchange from dioleoyl-PC bilayers at 45 °C is so rapid that it is at the limit of the assay.

It is apparent from Table I that the $t_{1/2}$ for cholesterol exchange from SM bilayers is much longer than the value for PC bilayers. Although naturally occurring SM's tend to have a higher content of long acyl chains than PC (Barenholz, 1984), the $t_{1/2}$ for cholesterol exchange from *N*-palmitoyl-SM is about twice that from dipalmitoyl-PC at 45 °C where bilayers of both lipids are in the liquid-crystal state. The slowing of the cholesterol exchange rate by SM molecules has been reported for mixed SM–PC and SM bilayers (Fugler et al., 1985; Bhuvaneswaran & Mitropoulos, 1986; Yeagle & Young, 1986). It is interesting to note that bovine brain SM, whose fatty acid composition is heterogeneous, forms sealed, intact vesicles while the other two sphingomyelins whose fatty acid composition is more homogeneous apparently do not form sealed vesicles (Cohen et al., 1984).

*NMR of [4-$^{13}$C]Cholesterol in Small Unilamellar Vesicles.* In principle, cholesterol interfacial packing in saturated PC, unsaturated PC, and SM small unilamellar vesicles can be monitored in terms of the rotational freedom of cholesterol molecules in each system, as reflected by the NMR spin–lattice relaxation time ($T_1$) and line width ($\nu_{1/2}$) of [4-$^{13}$C]cholesterol [e.g., see Brainard et al. (1981)]. The line widths for the phospholipid polar group $-N(CH_3)_3$ carbons and the 4-$^{13}$C atom of the cholesterol molecules (present at 10 mol %) in small unilamellar vesicles of DMPC, DPPC, egg PC, and bovine brain SM at 45 °C are similar in all four systems. The line widths are 13 Hz for the polar head group carbons and in the range 35–43 Hz for the 4-$^{13}$C atom of cholesterol. It follows that the segmental motions of these groups in all four types of bilayer are similar. The spin–lattice relaxation times for the 4-$^{13}$C atom of the cholesterol molecules solubilized in the bilayer of DMPC, DPPC, egg PC, and bovine brain SM small unilamellar vesicles are in the range 140–160 ms. Thus, it is apparent that the nucleus in question relaxes similarly in all four types of vesicles. It was not possible to evaluate the rotational freedom of cholesterol molecules in egg and palmitoyl-SM small unilamellar vesicles because at the concentrations needed for NMR measurements (~50 mg of phospholipid/mL) these lipids aggregated strongly, preventing reliable assessments of $\nu_{1/2}$ and $T_1$. The addition of 15 mol % dicetyl phosphate to the four types of small unilamellar vesicles does not affect cholesterol fast and slow molecular motions as reflected by $\nu_{1/2}$ and $T_1$ (data not shown); this is consistent with the fluorescence polarization studies of Shinitzky and Barenholz (1974).

The spin–lattice relaxation enhancement induced by paramagnetic ions such as $Mn^{2+}$ is a function of the $Mn^{2+}$–$^{13}$C distance and has been used to obtain distance information in PC bilayers (Weinstein et al., 1980; Bittman et al., 1984) and to probe the proximity to the aqueous interface of cholesterol molecules in high- and low-density lipoproteins (Lund-Katz & Phillips, 1984, 1986). The relaxation enhancements observed when $Mn^{2+}$ was added to vesicles at a $Mn^{2+}$:phospholipid molar ratio of 0.06 were similar to those reported pre-

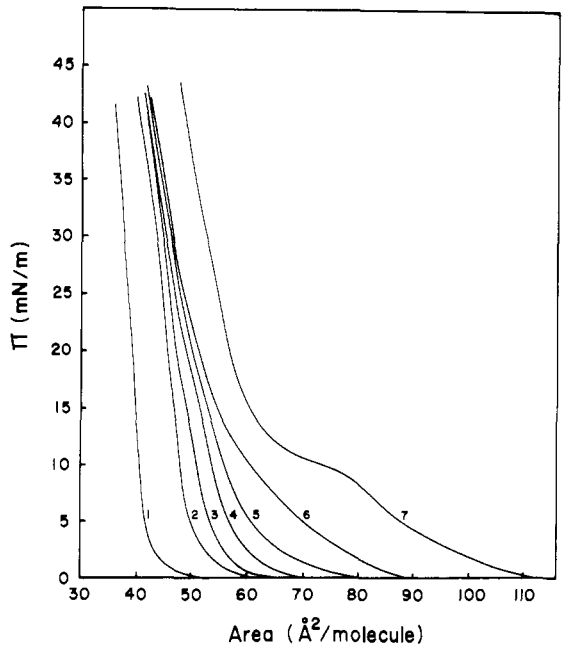


FIGURE 1: Surface pressure–mean molecular area isotherms for mixed monolayers of 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine and cholesterol on a subphase of 10 mM phosphate in 0.15 M NaCl (pH 7.6) at 25 °C. Mole fraction of phosphatidylcholine: curve 1, 0.0 (i.e., pure cholesterol monolayer); curve 2, 0.2; curve 3, 0.4; curve 4, 0.5; curve 5, 0.6; curve 6, 0.8; curve 7, 1.0.

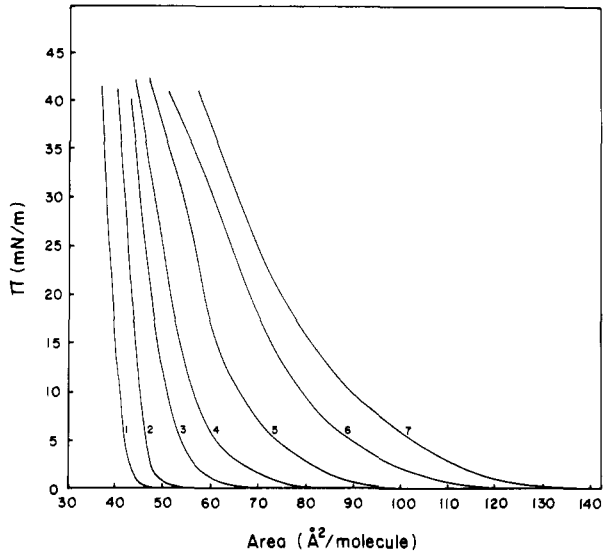


FIGURE 2: Surface pressure–mean molecular area isotherms for mixed monolayers of egg phosphatidylcholine and cholesterol. See legend to Figure 1 for further details.

viously from this laboratory for [4-$^{13}$C]cholesterol and N($CH_3$)$_3$ resonances from lipoproteins and PC vesicles (Bittman et al., 1984; Lund-Katz & Phillips, 1984, 1986). The [4-$^{13}$C]cholesterol and N($CH_3$)$_3$ $T_1$ values were reduced from 140 to 80 ms and from 590 to 160 ms, respectively. Application of the analysis presented before (Lund-Katz & Phillips, 1984) to these $T_1$ data enables the distance ratio $r_{Mn\text{-}chol}/r_{Mn\text{-}N(CH_3)_3}$ to be computed. The distance ratio in vesicles of DMPC, DPPC, egg PC, and bovine brain SM has a value of unity, which is the same as that observed for lipoproteins.

*Monolayer Studies.* The various PC and SM molecules utilized in this study all form stable monomolecular films at the air–water interface as indicated by the surface pressure ($\pi$)–molecular area ($A$) isotherms depicted in Figures 1–4. The $\pi$–$A$ curves for pure dipalmitoyl-PC (Figure 1) and egg PC (Figure 2) are consistent with previous reports in that the

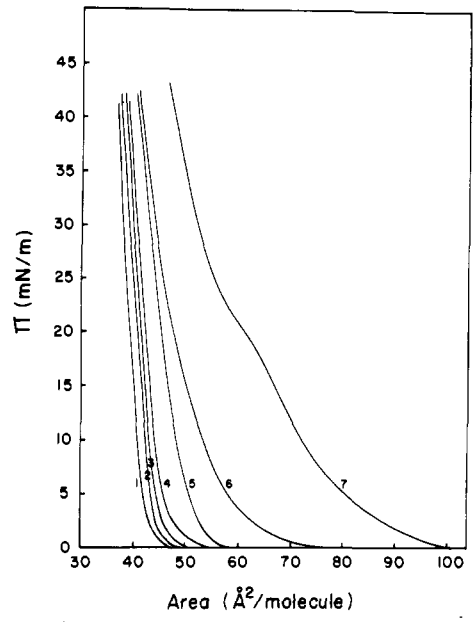


FIGURE 3: Surface pressure–mean molecular area isotherms for mixed monolayers of *N*-palmitoylsphingomyelin and cholesterol. See legend to Figure 1 for further details.

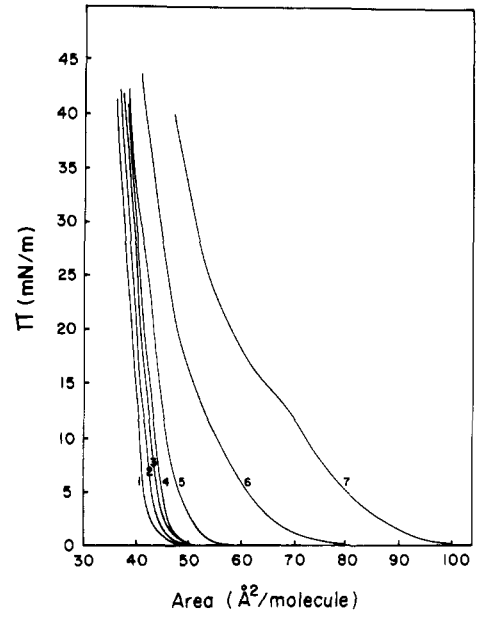


FIGURE 4: Surface pressure–mean molecular area isotherms for mixed monolayers of bovine brain sphingomyelin and cholesterol. See legend to Figure 1 for further details.

dipalmitoyl-PC monolayer exhibits a phase transition from an expanded to a condensed state at 9 mN/m whereas the egg PC monolayer is expanded at all $\pi$ values [cf. De Bernard (1958), Phillips and Chapman (1968), Phillips (1972), and Demel and DeKruijff (1976)]. SM monolayers have not been examined as extensively as PC monolayers. However, the $\pi$–$A$ curve for palmitoyl-SM in Figure 3 is similar to that of Yedgar et al. (1982) in that a phase transition is apparent and the limiting area is about 50 Å$^2$/molecule. At 25 °C, the phase transition in a palmitoyl-SM monolayer occurs at $\pi$ = 19 ± 1 mN/m; the temperature dependence of the phase transition $\pi$ (data not shown) is similar to that reported earlier for saturated PC homologues (Phillips & Chapman, 1968). An egg SM monolayer has a similar $\pi$–$A$ curve to that shown in Figure 3 for palmitoyl-SM. Comparison with Figure 4 indicates that bovine brain SM also exhibits similar monolayer properties with a phase transition occurring at $\pi$ = 14 ± 1

Table II: Comparison of Condensation of Phosphatidylcholines and Sphingomyelins in Mixed Monolayers with Cholesterol

| phospholipid (PL) monolayer[a] | surface pressure (mN/m) | PL molecular area (Å²/mol) pure PL monolayer | PL molecular area (Å²/mol) equimolar PL–chol monolayer[b] | condensation of PL packing (Å²/mol) |
|---|---|---|---|---|
| phosphatidylcholine | | | | |
| dipalmitoyl | 5 | 87 | 71 | 16 |
| | 20 | 57 | 57 | 0 |
| | 30 | 52 | 52 | 0 |
| egg | 5 | 102 | 81 | 21 |
| | 20 | 75 | 63 | 12 |
| | 30 | 65 | 58 | 7 |
| dioleoyl | 5 | 105 | 83 | 22 |
| | 20 | 79 | 66 | 13 |
| | 30 | 70 | 59 | 11 |
| sphingomyelin | | | | |
| palmitoyl | 5 | 81 | 50 | 31 |
| | 20 | 61 | 45 | 16 |
| | 30 | 52 | 43 | 9 |
| egg | 5 | 83 | 51 | 32 |
| | 20 | 62 | 45 | 17 |
| | 30 | 54 | 45 | 9 |
| oleoyl | 5 | 87 | 63 | 24 |
| | 20 | 68 | 54 | 14 |
| | 30 | 61 | 51 | 10 |
| brain | 5 | 80 | 48 | 32 |
| | 20 | 58 | 43 | 15 |
| | 30 | 51 | 42 | 9 |

[a] Monolayers spread at 25 °C on phosphate-buffered subphase (10 mM phosphate 0.15 M NaCl, pH 7.6). [b] Areas occupied by cholesterol molecules are 42, 39, and 38 Å² at surface pressures of 5, 20, and 30 mN/m, respectively.

mN/m at 25 °C. An oleoyl-SM monolayer is expanded at all $\pi$ values at 25 °C (data not shown).

SM monolayers occupy lower molecular areas at the same surface pressures (i.e., they are more condensed) than monolayers of the corresponding PC. For example, at $\pi$ = 5 mN/m, palmitoyl-SM occupies 81 Å²/molecule while dipalmitoyl-PC occupies 87 Å²/molecule, and an oleoyl-SM monolayer is more condensed than a dioleoyl-PC monolayer at all $\pi$ values (Table II). It is apparent that the presence of the sphingosine moiety in the SM molecule gives rise to a closer molecular packing than occurs in the equivalent PC molecule which contains the diacylglycerol group. In addition, naturally occurring SM molecules contain longer acyl chains which also tend to be more saturated than the acyl chains of naturally occurring PC molecules (e.g., egg SM contains primarily N-linked palmitate whereas egg PC is primarily 1-palmitoyl-2-oleoyl-PC). The lateral packing density of SM in bilayers also appears to be greater than that of PC. Thus, in the liquid-crystal state, the surface area per molecule in fully hydrated bovine brain SM bilayers is about 60 Å² (Shipley et al., 1974) whereas the equivalent figure for dipalmitoyl-PC bilayers is about 70 Å² (Chapman et al., 1967; Phillips & Chapman, 1968).

The isotherms for mixed cholesterol monolayers depicted in Figures 1–4 show that cholesterol can condense PC and SM monolayers. The relative condensations at $\pi$ = 20 mN/m are compared in Figure 5. It is apparent that the dipalmitoyl-PC monolayer which is above its phase transition at this surface pressure is not condensed further by cholesterol [cf. Chapman et al. (1969)]. However, the expanded SM and PC monolayers are significantly condensed (Figure 5) [cf. De Bernard (1958) and Phillips (1972)]. The interaction of SM and cholesterol in monolayers has not been examined previously. The mean molecular areas in equimolar films of cholesterol and various

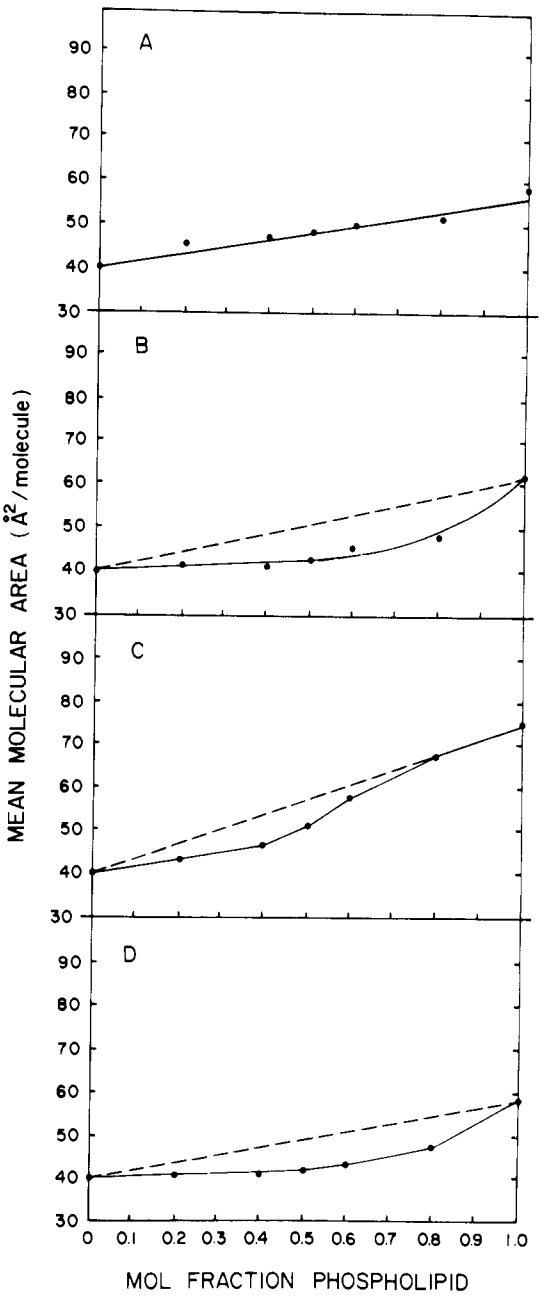


FIGURE 5: Mean molecular area–mole fraction plot for the mixed monolayers of cholesterol and phosphatidylcholine or sphingomyelin described in Figures 1–4. The surface pressure is 20 mN/m throughout, and the dashed lines represent the mean molecular areas for ideal mixing of cholesterol and phospholipid. Panel A, 1,2-dipalmitoyl-3-*sn*-phosphatidylcholine; panel B, *N*-palmitoylsphingomyelin; panel C, egg phosphatidylcholine; panel D, bovine brain sphingomyelin.

Table III: Molecular Packing Densities in Equimolar Mixed Monolayers of Cholesterol with Phosphatidylcholines and Sphingomyelins

| host phospholipid | mean molecular area (Å²/mol) $\pi$ = 5 mN/m | $\pi$ = 20 mN/m | $\pi$ = 30 mN/m |
|---|---|---|---|
| phosphatidylcholine | | | |
| dipalmitoyl | 56 | 49 | 45 |
| egg | 61 | 51 | 48 |
| dioleoyl | 63 | 53 | 48 |
| sphingomyelin | | | |
| palmitoyl | 46 | 42 | 40 |
| egg | 46 | 43 | 41 |
| oleoyl | 53 | 46 | 44 |
| brain | 45 | 41 | 40 |

PC and SM molecules (Table III) indicate that at all $\pi$ values the monolayers containing SM are more closely packed. There are two contributions to this effect. (1) SM molecules occupy smaller surface areas than PC molecules in pure phospholipid monolayers, when monolayers in the same physical state are compared (Table II). (2) The SM molecules are condensed to a greater degree when cholesterol is present in the monolayer (e.g., in equimolar monolayers at $\pi$ = 5 mN/m, dipalmitoyl-PC is condensed by 16 Å$^2$/molecule while palmitoyl-SM is condensed by 31 Å$^2$/molecule; see Table II, last column). This result is consistent with fluorescence polarization measurements on phospholipid–cholesterol bilayers which show that in the fluid state cholesterol has a relatively high affinity for egg SM and dipalmitoyl-PC compared to unsaturated phospholipids (Van Blitterswijk et al., 1987). Studies of the relative affinities of SM and PC for cholesterol in phospholipid bilayers have been complicated by variations in the physical states of the bilayers so that conflicting conclusions have been drawn [for a review, see Barenholz (1984)]. The monolayer molecular area data summarized in Figures 1–5 and Tables II and III show that in the expanded fluid state, cholesterol condenses and interacts with SM more strongly than with the corresponding PC. The condensation of SM is greater when SM and PC are compared at the same molecular area (i.e., at different $\pi$ values); for instance, oleoyl-SM at $\pi$ = 20 mN/m and dioleoyl-PC at $\pi$ = 30 mN/m occupy 69 ± 1 Å$^2$/molecule and are condensed by 14 and 11 Å$^2$/molecule, respectively. The relatively large condensation of SM is even more dramatic when the decreases in molecular area of palmitoyl-SM and dipalmitoyl-PC at $\pi$ = 30 mN/m are compared (Table II).

## Discussion

It is now established that unesterified cholesterol molecules can transfer passively between membranes by diffusion through the intervening aqueous phase. Desorption of cholesterol molecules from the donor lipid–water interface is rate limiting for the overall transfer process. The rate of exchange is strongly temperature dependent, and transfer is presumed to proceed through a transition-state complex where the cholesterol molecule is attached to the donor membrane by the tip of its hydrophobic alkyl moiety [for a review, see Phillips et al. (1987)]. The thermodynamic functions associated with formation of the transition-state complex can be derived by using a model for the kinetics of micellization developed by Aniansson and colleagues (Aniansson et al., 1976). This approach has permitted a semiquantitative prediction of the relative rates of transfer of lipids based upon their aqueous solubilities. In addition, the free energy of activation and the rate of transfer are affected by the interactions of the desorbing cholesterol molecule with its phospholipid neighbors in the lipid–water interface. This is the basis of the varying $t_{1/2}$ values reported in Table I for exchange of cholesterol from different host phospholipid bilayers. When cholesterol is present in a bilayer where strong phospholipid–cholesterol intermolecular interactions occur (i.e., the cholesterol is dissolved in a good solvent), then its free energy is reduced. The activation energy required to form the transition-state complex is relatively high in this case. This qualitative reasoning rationalizes the rate constants for cholesterol desorption in Table I being in the order unsaturated PC > saturated PC > SM.

The differences in cholesterol–phospholipid interaction energy in the donor bilayer, which give rise to the above ranking of rate constants for cholesterol exchange, may be a consequence of variations in the following factors. (1) One factor is the relative juxtaposition of cholesterol and phospholipid molecules along their long axes (i.e., normal to the bilayer plane). However, besides demonstrating that the segmental motions of the cholesterol C-4 atom are essentially the same in all bilayers studied, the NMR data also indicate that the exposure of cholesterol molecules to the aqueous phase is similar in all the bilayers. The results of the relaxation enhancement experiments using [4-$^{13}$C]cholesterol and $Mn^{2+}$ indicate that the cholesterol hydroxyl group is located in the region of the PC carbonyl groups [cf. Bittman et al. (1984) and Lund-Katz and Phillips (1984)] and in the corresponding region of the SM bilayer. In the case of PC bilayers, the cholesterol molecule extends to about the C-12 atom of the acyl chains (Darke et al., 1972). This location means that the unsaturated chains in, for example, brain and egg SM are effectively saturated as far as their interaction with cholesterol is concerned because the only unsaturated fatty acid is nervonic acid (Barenholz, 1984) and the double bond is at the C-15 atom. (2) The lateral packing density of the cholesterol and phospholipid molecules in the bilayer is another factor. In relatively expanded bilayers, the van der Waals attractive interaction energy between cholesterol and the phospholipid hydrocarbon chains will be lower. The molecular area data summarized in Table III indicate that the relative expansions of the phospholipid monolayers (in an equimolar mixture with cholesterol) are in the rank unsaturated PC > saturated PC > SM. This indicates that the van der Waals attraction between cholesterol and the phospholipid increases in the same order (see below). It should be noted that the rather weak dependence of the rate constant for cholesterol exchange on the cholesterol:phospholipid molar ratio in the donor bilayer suggests that cholesterol desorbs from a region of the bilayer which is laterally phase separated from the free-melting phospholipid (McLean & Phillips, 1982). This region is thought to comprise equimolar PC–cholesterol clusters whose fluidity is invariant to temperature over a wide range (Darke et al., 1972). As a consequence of this lateral phase separation, the rate constant for cholesterol desorption is not sensitive to the physical state of the free phospholipid regions.

The fact that the lateral packing density of SM molecules in monolayers (Table II) and bilayers (Schmidt et al., 1977) is high relative to PC molecules means that the van der Waals attractive interactions are stronger so that the condensation by cholesterol is enhanced. In a monolayer in a given physical state and at a given $\pi$, SM molecules are more closely packed than the PC with the same acyl chain saturation and length, and this enhances the cholesterol–SM interaction when cholesterol is added at the same $\pi$. It is interesting that cholesterol condenses SM somewhat more than the corresponding PC even when the SM and PC are compared at the same molecular area (i.e., at different $\pi$ values). Differences in hydrogen bonding may contribute to these effects [cf. Schmidt et al. (1977)].

If the simplifying assumption is made that the differences in the free energy of activation, which underlie the ranking of rate constants for cholesterol exchange from bilayers in the order unsaturated PC > saturated PC > SM, arise solely from variations in cholesterol–phospholipid van der Waals interaction energy, then some more quantitative insights can be gained from the following treatment. Salem (1962) has derived an approximate expression (estimated to be accurate to within 20%) for the calculation of the dispersion energy between two close-packed hydrocarbon chains. The model ignores entropic effects, so the enthalpic contribution ($W$) to the van der Waals attractive interaction energy is derived. If the preexponential factor is the same for cholesterol desorbing from

bilayers of two different phospholipids, then the ratio of the rate constants for this process is $k_1/k_2 = e^{-W_1/RT}/e^{-W_2/RT}$. Rearrangement gives

$$\ln (k_1/k_2) = \frac{1}{RT}(W_2 - W_1) \quad (1)$$

Salem (1962) has shown that for two methylene groups $W = 1340/d^6$ kcal/mol where $d$ is the intermolecular separation in angstroms. In order to estimate $W$ for cholesterol–phospholipid interaction at various interfacial areas, we take the cholesterol to be equivalent to two hydrocarbon chains [the limiting area of cholesterol in a monolayer is 40 Å$^2$/molecule which is similar to that of close-packed phospholipid molecules [cf. Chapman et al. (1969)]] and assume that the interaction extends over 12 methylene groups of the hydrocarbon chain [cf. Darke et al. (1972)]. For a hexagonal array of vertically orientated, cylindrical hydrocarbon chains, the interfacial area per chain $A = \sqrt{3}d^2/2$. Substitution of these conditions into the expression for $W$ between two hydrocarbon chains [eq 8 in Salem (1962)] gives the van der Waals attractive interaction energy between cholesterol and phospholipid as

$$W_{C\text{-}P} \simeq 10^4/A^{5/2} \text{ kcal/mol} \quad (2)$$

The length of a $CH_2$ group is taken as 1.25 Å, and $A$ (in angstroms squared) is taken as half the molecular area occupied by a phospholipid or cholesterol molecule. Substitution of eq 2 into eq 1 gives

$$\ln (k_1/k_2) = \frac{10^4}{RT}\left(\frac{1}{A_2^{5/2}} - \frac{1}{A_1^{5/2}}\right) \quad (3)$$

where $R$ is the gas constant and $T$ is the absolute temperature (the product is expressed in kilocalories per mole). Equation 3 gives the ratio of the rate constants for cholesterol desorption as a function of the relative molecular areas in two bilayers.

The $t_{1/2}$ values for cholesterol exchange from vesicles of unsaturated PC, saturated PC, and SM are about 1, 13, and 80 h, respectively (Table I). For the present order of magnitude calculations, we assume that the rate constants ($k$) for desorption of cholesterol from bilayers of unsaturated PC, saturated PC, and SM are in the ratio 100:10:1. The values of $A$ to be employed in eq 3 are derived from the surface pressure–molecular area isotherms of the equimolar cholesterol–phospholipid monolayer at $\pi = 30$ mN/m [this is similar to the lateral pressure in a bilayer (Phillips & Chapman 1968)]. This is in recognition of the fact that exchange data are consistent with cholesterol desorption from such equimolar clusters (McLean & Phillips, 1982). The cholesterol and phospholipid are assumed to have the same value of $A$ in these equimolar monolayers. Knowing the ratio of the rate constants for cholesterol exchange from two different phospholipid bilayers, and the value of $A$ in one such bilayer, it is possible to compute $A$ for the other phospholipid bilayer from eq 3. For instance, comparing unsaturated PC (component 1) to saturated PC (component 2) bilayers, $k_1/k_2 = 10$ and $A_1 = 24$ Å$^2$ [the molecular area of an equimolar egg PC–cholesterol monolayer at $\pi = 30$ mN/m is 48 Å$^2$ (Figure 2)]. Substitution into eq 3 gives $A_2 = 21$ Å$^2$ which is equivalent to a molecular area in an equimolar dipalmitoyl-PC–cholesterol monolayer of 42 Å$^2$; the experimental value is 45 Å$^2$/molecule (Figure 1). Similar calculations using egg SM rather than dipalmitoyl-PC as component 2 give a computed value of $A_2 = 19$ Å$^2$ (38 Å$^2$/molecule) compared to an experimental molecular area for an equimolar cholesterol–egg (or palmitoyl) SM monolayer of 41 Å$^2$ at $\pi = 30$ mN/m (Figure 3). It is apparent that for a given ratio of rate constants for cholesterol desorption, eq 3 somewhat overpredicts the difference in lateral packing density between the two types of phospholipid bilayer. Nonetheless, the analysis suggests that differences in cholesterol–phospholipid van der Waals interaction energy are the cause of varying rates of cholesterol exchange from different host phospholipid bilayers. A more refined treatment will necessitate a more rigorous treatment of the van der Waals interactions in bilayers.

In conclusion, increases in the lateral packing density in the lipid–water interface decrease the rate of cholesterol desorption because the higher cholesterol–phospholipid van der Waals attraction raises the activation energy barrier opposing desorption. The slower desorption from saturated PC bilayers relative to unsaturated PC bilayers is presumably solely due to the greater van der Waals attraction in the former system. Increased van der Waals interaction between SM and cholesterol relative to PC and cholesterol is also significant in explaining the slower exchange from the SM system. However, it is likely that other interactions such as hydrogen bonding also contribute to the greater affinity between SM and cholesterol. Variations in the degree of phospholipid saturation and the SM:PC ratio in the plasma membrane may be among the ways that cells regulate cholesterol efflux to extracellular lipoproteins [cf. Phillips et al. (1987)].

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**Registry No.** Dioleoyl-PC, 4235-95-4; dimyristoyl-PC, 18194-24-6; dipalmitoyl-PC, 63-89-8; cholesterol, 57-88-5.

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# Structure of a Bent DNA: Two-Dimensional NMR Studies on $d(GAAAATTTTC)_2$†

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ABSTRACT: Intrinsic DNA bending is caused by specific DNA sequences. The decamer $d(GA_4T_4C)_2$, when it repeats in a synthetic polymer or in kinetoplast DNA, results in a macroscopic bending of the molecule as a whole. We employed high-resolution two-dimensional NMR methods to examine the intrinsic structural properties of the $d(GA_4T_4C)_2$ duplex in solution. Examination of the NOESY data at 50- and 100-ms mixing times indicated that the kinds of observed NOEs can originate if each of the ten nucleotidyl residues belongs to the B-DNA family, i.e., C2′-endo,anti. However, the *degree* of observed NOE intensities from the A-T junction as well as the observed AH2–AH2 cross-peaks from adjacent AT pairs could not be rationalized on the basis of a straight B-DNA model but could be explained by only a B-DNA model with some structural discontinuity at the A-T junction—the site of 2-fold symmetry in the molecule. In view of the fact that the degree of observed NOE intensities can be complicated by spin diffusion and by fine structural distortion, we have resorted to the use of quantitative theoretical NOESY simulation (which takes into account primary, secondary, and higher orders of NOE) to delineate the structural discontinuity at the A-T junction and to arrive at a structure for the duplex $d(GA_4T_4C)_2$. We propose a "junction B-DNA model" which can quantitatively explain the 2D NOESY data at 100- and 50-ms mixing times. In this model the two structural blocks in the molecule, i.e., $d(GA_4)$·$d(T_4C)$ and $d(T_4C)$·$d(GA_4)$, are conformationally equivalent and are connected at the A-T junction where the base pairs are stably stacked, but the two local structural frames do not coincide in space. This model can create an overall bending of 10° with a center of curvature 50 Å away from the center of the duplex. It is the thesis of this paper that the observed bending in polymers with a repeat of $d(GA_4T_4C)_2$ and the bending in natural DNAs where $A_nT_n$·$A_nT_n$ repeats are present originate at the oligonucleotide repeat level.

DNA bending refers to the phenomenon in which the long axis of a DNA duplex is curved. Bending of DNA can be classified in two broad categories: (i) intrinsic bending due to specific base sequences, e.g., bending of kinetoplast DNA (Hagerman, 1984; Kifchin et al., 1986; Marini et al., 1982), and (ii) induced bending, e.g., DNA bending in a protein–DNA complex (Frederick et al., 1984). However, fine structural details of a bent DNA in solution have not been determined so far experimentally. We believed that the decamer $d(GA_4T_4C)_2$, a prototype of a bending center in the kinetoplast DNA, would be an ideal model system to work

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