Sphingomyelin–Lecithin Bilayers and Their Interaction with Cholesterol†

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ABSTRACT: Utilizing X-ray diffraction and differential scanning calorimetry (DSC), we have studied (1) the structure and thermotropic properties of hydrated *N*-palmitoylsphingomyelin, (2) the interaction of *N*-palmitoylsphingomyelin with dimyristoyllecithin, and (3) the interaction of cholesterol with *N*-palmitoylsphingomyelin and dimyristoyllecithin, both individually and in a 50:50 (mol/mol) mixture. *N*-Palmitoylsphingomyelin forms bilayers which undergo a thermotropic order–disorder (gel–liquid crystalline) transition at 40.5 °C (ΔH = 5.8 kcal/mol). The bilayer repeat distance is 66.8 Å at 10 °C and 61.6 Å at 50 °C. *N*-Palmitoylsphingomyelin exhibits miscibility with dimyristoyllecithin in both the gel and liquid-crystalline phases, and no lateral phase separation occurs. Scanning calorimetry indicates that interaction with cholesterol is similar for both *N*-palmitoylsphingomyelin and dimyristoyllecithin and that in a 50:50 (mol/mol) mixture cholesterol shows no preferential affinity for either phospholipid.

Lecithin (PC),[1] sphingomyelin (SM), and cholesterol are major lipid constituents of many plasma membranes (Rouser et al., 1968; Nelson, 1972), and there is growing evidence particularly for the red cell membrane (Bretscher, 1972a,b; Verkleij et al., 1973) for a preferential location of PC and SM on the external monolayer. Furthermore, red cells from different mammalian species are characterized by differences in the ratio of PC to SM, while the total contribution of the two phospholipids to the membrane surface is relatively constant (deGier & van Deenen, 1961; Zwaal et al., 1973). In addition, PC, SM, and cholesterol are important constituents of lipid-transporting plasma lipoproteins (Skipski, 1972) and there is convincing evidence that these lipids are present as a monolayer on the surface of the spherical particles. These polar surface lipid components, together with proteins, surround the nonpolar lipids (cholesterol esters and triglycerides) and are responsible for the overall stability of the lipoprotein particles in aqueous media (Shipley et al., 1972; Deckelbaum et al., 1977; Shen et al., 1977; Bradley & Gotto, 1978).

Although much information is available on the structure and properties of hydrated lecithin systems, as well as lecithin–cholesterol interactions, until recently much less was known about the corresponding properties of sphingomyelin. Our initial X-ray diffraction and differential scanning calorimetry (DSC) study of bovine brain sphingomyelin (Shipley et al., 1974) showed that, although its hydration properties and bilayer structure were similar to those of lecithins, its transition from an ordered gel structure to the liquid-crystalline bilayer phase occurred at a surprisingly high temperature for a naturally occurring lipid (approximately 30–40 °C). This behavior was confirmed by the detailed calorimetry studies of Barenholz et al. (1976) and, in addition, these authors characterized the thermotropic properties of some synthetic sphingomyelins.

The high chain melting behavior causes bovine brain SM to undergo lateral phase separation as hydrated mixtures of SM and egg PC are cooled to temperatures below about 40 °C (Untracht & Shipley, 1977). A similar study utilizing the fluorescence probe diphenylhexatriene to monitor lipid bilayer properties resulted in similar conclusions concerning SM phase separation (Hertz & Barenholz, 1977). Van Deenen and co-workers (van Dijck et al., 1976; Demel et al., 1977) have used laterally phase separated systems to examine the affinity of cholesterol for different membrane phospholipids. Utilizing DSC, these authors showed a preferential affinity of cholesterol for sphingomyelin in mixed SM–PC bilayers, irrespective of whether the SM was the higher or lower chain-melting lipid (Demel et al., 1977).

Recently, we have been studying the interactions of sphingomyelins containing specific N-acylated fatty acids with synthetic lecithins in both the presence and absence of cholesterol. This paper characterizes the structure and thermotropic properties of hydrated *N*-palmitoylsphingomyelin (PSM) and then describes the interaction of cholesterol with PSM, dimyristoyllecithin (DML), and a 50:50 (mol/mol) mixture of PSM and DML.

Materials and Methods

Materials. Dimyristoyllecithin (DML) was purchased from Sigma Chemical (St. Louis, MO) and was further purified by silicic acid chromatography eluting with a chloroform–methanol gradient. It was then determined to be greater than 99% pure by thin-layer chromatography (TLC). Palmitoylsphingosinephosphorylcholine (PSM) was prepared from bovine brain sphingomyelin and palmitic acid. Bovine brain sphingomyelin was purchased from Lipid Products (Surrey, England) and palmitic acid and cholesterol were obtained from Nu Chek Prep (Austin, MN).

Sphingosinephosphorylcholine (SPC) was prepared by deacylation of bovine brain sphingomyelin by a modified version of the method described by Kaller (1961). The heterogeneous acyl chains were hydrolyzed by dissolving sphingomyelin (1 g) in a 1:1 6 N HCl/1-butanol solution (5 mL) and heating in a water bath (100 °C) for 1 h. The reaction was neutralized with ammonium hydroxide. The separation of SPC was accomplished by solvent extraction using 25 mL of a biphasic mixture of 0.1 N HCl, methanol, 1-butanol, and benzene (5:5:1:9) per g of sphingomyelin. The lower, aqueous phase was then examined by TLC, developing in 1-butanol–ethanol–acetic acid–water (8:2:1:3) to show three

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[1] Abbreviations used: PC, lecithin; SM, sphingomyelin; DSC, differential scanning calorimetry; PSM, *N*-palmitoylsphingomyelin; DML, dimyristoyllecithin; TLC, thin-layer chromatography; SPC, sphingosinephosphorylcholine.

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or four spots corresponding to fatty acids, sphingosine or sphingosine phosphate, unhydrolyzed sphingomyelin, and sphingosinephosphorylcholine. These reaction products were separated on a silicic acid column (Bio-Sil A 200–400 mesh) and monitored by TLC using 5 × 10 mm precoated silica gel F-254 plates. Visualization of the spots was achieved by developing in an iodine tank or the plates were sprayed with either the Dragendoff reagent, molybdenum blue, or ninhydrin to identify the choline-containing products, the phosphate-containing products, and those with a free amino group, respectively.

The SPC was acylated with palmitic acid using 1,1'-carbonyldiimidazole (Aldrich Chemical, Metuchen, NJ) as a condensing agent activator (Boss et al., 1975). A fivefold excess of the fatty acid and activator was allowed to react in benzene at 40 °C for about 20 min. The SPC was taken to dryness and then the fatty acid–imidazole complex in benzene was added. This reaction mixture was heated to 72 °C in vacuo for an hour and a half. After about 5 or 10 min, the benzene was evaporated to allow the fatty acid–activator complex to react more efficiently with the SPC.

After acylation, 0.1 N KOH in methanol was added to the reaction mixture for 1 h in order to cleave the *O*-acyl bond formed during the nonspecific acylation of SPC. The amide linkage is stable to mild base hydrolysis. The PSM was eluted from a silicic acid column with a chloroform–methanol gradient. The fraction containing PSM was spotted on a TLC plate and developed in chloroform–methanol–water (65:25:4). Visualization in an iodine tank showed it to be greater than 99% pure. In addition, it was ninhydrin negative.

Sample Preparation. PSM and DML in Excess Water. Aliquots from a stock solution of PSM in chloroform–methanol (2:1, v/v) were transferred to preweighed tubes with a central constriction. The solvent was evaporated under nitrogen and placed in a vacuum overnight. The tubes were reweighed to determine the amount of lipid added. Specific aliquots from a stock solution of DML in chloroform–methanol (2:1, v/v) were then added to these PSM-containing tubes to give various molar ratios of PSM:DML. The tubes were dried down under nitrogen, placed in a vacuum overnight, and reweighed to determine the total amount of phospholipid and the exact PSM:DML molar ratio. An excess amount of doubly distilled water (40–50% of the total weight; phospholipid plus water) was added to each dry mixture using a microsyringe. The tubes were purged with nitrogen and immediately flame sealed. Equilibration was accomplished by repeatedly centrifuging the mixture through the central constriction for 3 or 4 h at 50 °C. Each mixture was examined by differential scanning calorimetry (DSC) and X-ray diffraction. Immediately after opening the tube, a well-mixed sample of the mixture was weighed, heated in vacuo, and reweighed to determine, gravimetrically, the percentage of water.

PSM–DML plus Cholesterol in Excess Water. Binary mixtures of PSM–DML (1:1, mol/mol) were prepared as before, but, before the system was hydrated, specific aliquots from a stock solution of cholesterol in chloroform were added to yield various mole ratios of phospholipid to cholesterol (i.e., 1:1:1, 1:1:0.5, etc.). The solvent was then evaporated and the mixtures were hydrated, equilibrated, and sealed as before. Again, each mixture was examined by DSC, by X-ray diffraction, and by gravimetric analysis to determine the water content.

Differential Scanning Calorimetry. Samples (2–8 mg) were hermetically sealed in stainless steel pans and heating and cooling runs over the temperature range −5 to +60 °C were performed on a Perkin-Elmer (Norwalk, CT) DSC-2 calorimeter, calibrated with cyclohexane and indium. Heating rates were either 2.5 or 5.0 °C/min. In addition to the peak temperature, the onset and completion temperatures were determined from the intersection of the base line with the extrapolated line of the ascending and descending slopes, respectively. Enthalpy measurements were obtained from the area under the transition compared with those for known standards. The area was determined by planimetry utilizing a base line connecting the pre- and post-transition base lines.

X-ray Diffraction. Nickel-filtered Cu Kα radiation (λ = 1.5418 Å) from an Elliot GX-6 rotating anode X-ray generator was focused by X-ray cameras using either toroidal mirror (Elliot, 1965) or double mirror optics (Franks, 1958). Samples were sealed in thin-walled capillaries (Charles Supper Co., Natick, MA) in a sample holder kept at constant temperature by a circulating solvent/water bath. Diffraction patterns were recorded on Ilford Industrial G X-ray film and the intensity distribution was derived using a Joyce-Loebl Model III-CS scanning microdensitometer.

Results

N-Palmitoylsphingomyelin–Water. A mixture of PSM–water (50:50, w/w) was examined by DSC and X-ray diffraction. Its thermal behavior is quite similar to that of the structurally related dipalmitoyllecithin (DPL). PSM exhibits a sharp endotherm with its maximum at 41.5 °C, a value similar to that of DPL, although its transition enthalpy of 5.8 kcal/mol is significantly lower (cf. 8.7 kcal/mol for DPL). In addition, PSM exhibits a small transition endotherm (ΔH = 0.40–0.85 kcal/mol) centered at 31 °C.

X-ray diffraction of PSM–water at 10 °C showed sharp low angle reflections in the ratio 1:0.5:0.33..., corresponding to a lamellar repeat distance, d, of 66.8 Å. In the wide angle region, a strong diffraction line was observed at 4.14 Å. With the assumption that maximum hydration was identical with that of bovine brain SM (42 wt % water), the lipid thickness, d_l, and surface area per hydrophilic group at the water interface, S, were calculated to be 38.4 Å and 54.8 Å^2, respectively. These values suggest an angle of tilt of the PSM molecules to the bilayer normal larger than that recorded for synthetic lecithins, 47° as compared with about 35° (Janiak et al., 1976). Above the transition at 50 °C, the interlamellar repeat distance decreased to 61.6 Å, the thickness (d_l) decreased to 35.4 Å, and the surface area (S) increased to 59.4 Å^2. So far, X-ray diffraction patterns recorded at temperatures intermediate between the two transitions of PSM show only lamellar diffraction lines.

N-Palmitoylsphingomyelin–Dimyristoyllecithin–Water. The interaction of dimyristoyllecithin (DML) and PSM in excess water was investigated by DSC and X-ray diffraction. As shown in Figure 1, DML exhibits a sharp endothermic transition with a peak maximum at 26 °C (onset = 23.8 °C) and a pretransition at about 15 °C. As PSM is added, the main transition broadens and initially the peak temperature decreases slightly to approximately 24.5 °C. As more PSM is added, the peak temperature progressively increases as does the transition width, the latter being maximal at ~50 mol % PSM. Further addition of PSM produces a decrease in the transition width. With the possible exception of the small enthalpy pretransitions, only a single fairly sharp symmetrical transition endotherm is observed in the temperature range 0–60 °C for all mixtures. A plot of the onset, maximum, and completion temperatures vs. mole fraction of PSM is shown in Figure 2. Except perhaps at low sphingomyelin contents, the DSC data suggest that the two lipids exhibit complete

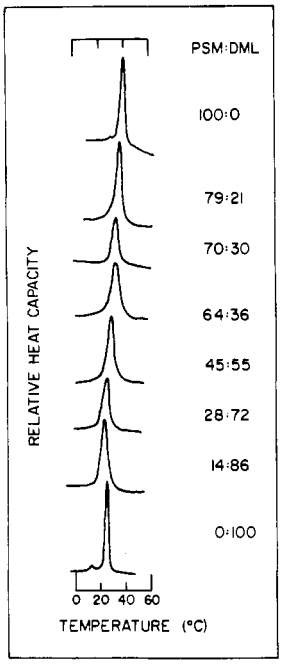

FIGURE 1: Representative DSC heating curves of *N*-palmitoylsphingomyelin (PSM)–dimyristoyllecithin (DML) mixtures in excess water. Heating rate: 5 °C/min.

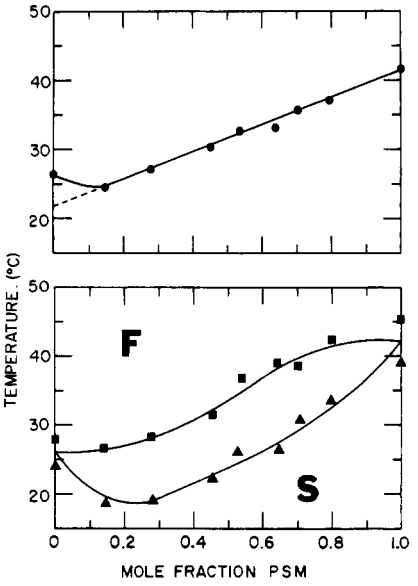

FIGURE 2: Plot of DSC peak transition temperature (●, top) and onset (▲, bottom) and completion (■, bottom) temperatures vs. mole fraction of *N*-palmitoylsphingomyelin for PSM–DML mixtures in excess water. S represents solid (gel) phase and F fluid (liquid-crystalline) phase. In the transition zone, S and F coexist.

miscibility in both the high temperature and low temperature phases. The plot of transition enthalpy vs. mole fraction of PSM shown in Figure 3 also indicates complete miscibility since the transition enthalpy increases linearly with PSM content.

X-ray diffraction data were recorded for all DML–PSM mixtures at temperatures below (10 °C) and above (50 °C) the transition. At 50 °C, all mixtures exhibited a diffuse wide angle reflection at 4.6 Å characteristic of the melted chain Lα structure. In the low angle region only one set of lamellar diffraction lines was exhibited by each mixture and the bilayer periodicity, *d*, increased linearly with PSM content from 57 to 62 Å (Figure 4B). At 10 °C, all mixtures gave a relatively sharp diffraction maximum between 4.1 and 4.2 Å. In the

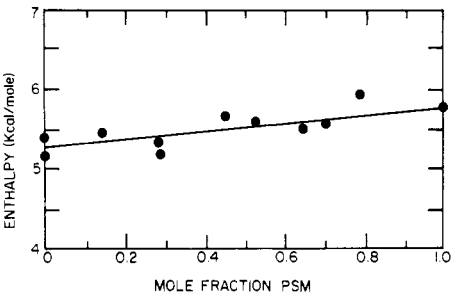

FIGURE 3: Plot of transition enthalpy (kcal/mol) vs. mole fraction of *N*-palmitoylsphingomyelin for PSM–DML mixtures in excess (50 wt %) water.

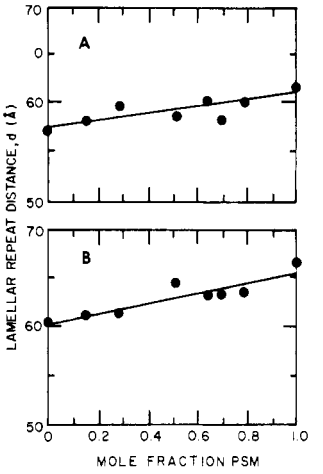

FIGURE 4: Plot of lamellar repeat distance *d* (Å) vs. mole fraction of *N*-palmitoylsphingomyelin for PSM–DML mixtures in excess (50 wt %) water at 50 °C (A) and 10 °C (B).

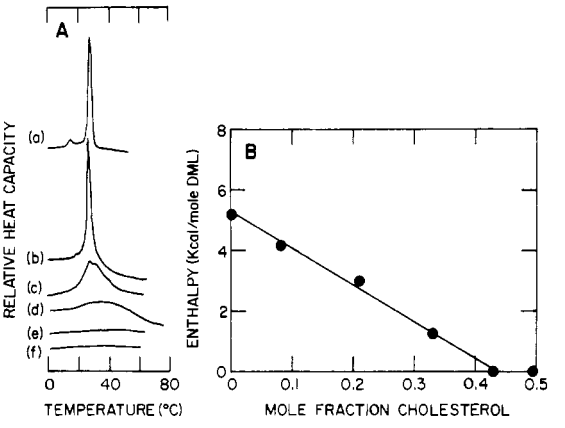

FIGURE 5: (A) DSC heating curves of hydrated (50 wt % water) dimyristoyllecithin–cholesterol mixtures with (a) 0.00, (b) 0.083, (c) 0.21, (d) 0.34, (e) 0.43, and (f) 0.49 mole fraction of cholesterol. Heating rate: 2.5 °C/min. (B) Plot of total transition enth (kcal/mol of DML) vs. cholesterol mole fraction.

low angle region, again only a single set of lamellar diffraction lines was exhibited by each mixture and the bilayer periodicity increased linearly from 61 to 67 Å with increasing PSM content (Figure 4A). These data argue against lateral phase separation and suggest that complete miscibility of DML and PSM occurs above and below the transition for all mixtures.

Interaction of Cholesterol with Hydrated N-Palmitoylsphingomyelin and Dimyristoyllecithin. Prior to examining the interaction of cholesterol with mixtures of the two phospholipids, we had examined its interaction with hydrated PSM and hydrated DML. As shown previously (Hinz & Sturtevant, 1972), addition of cholesterol to fully hydrated

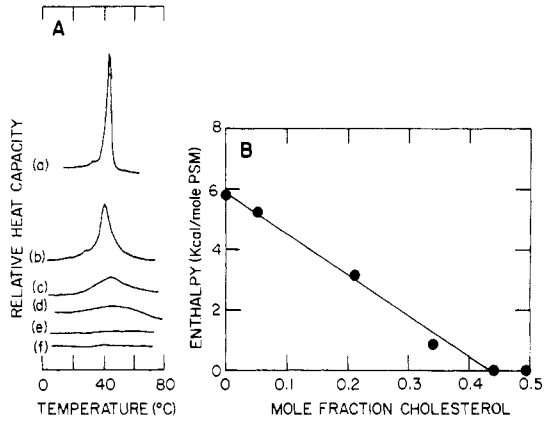

FIGURE 6: (A) DSC heating curves of hydrated (50 wt % water) *N*-palmitoylsphingomyelin–cholesterol mixtures with (a) 0.00, (b) 0.052, (c) 0.21, (d) 0.33, (e) 0.44, and (f) 0.49 mole fraction of cholesterol. Heating rate: 2.5 °C/min. (B) Plot of total transition enthalpy (kcal/mol of PSM) vs. cholesterol mole fraction.

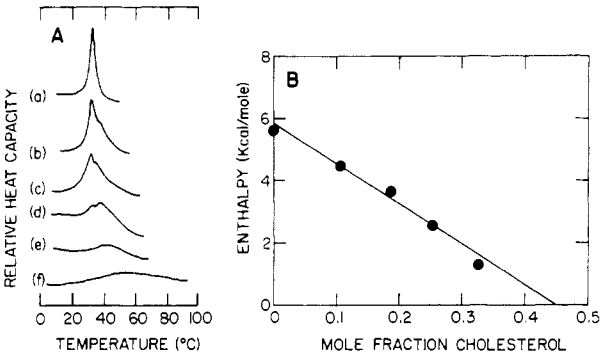

FIGURE 7: (A) DSC heating curves of hydrated (50 wt % water) *N*-palmitoylsphingomyelin–dimyristoyllecithin mixtures (50:50, mol/mol) with increasing cholesterol content with: (a) 0.00, (b) 0.103, (c) 0.15, (d) 0.183, (e) 0.255, and (f) 0.324 mole fraction of cholesterol. Heating rate: 2.5 °C/min. (B) Plot of total transition enthalpy (kcal/mol of phospholipid) vs. cholesterol mole fraction.

DML progressively decreases the enthalpy of the chain melting transition at ~23 °C (see Figure 5A). It is clear that this sharp transition is no longer present for mixtures containing >21 mol % cholesterol. As cholesterol is added, a new transition appears at slightly higher temperatures. This transition gets progressively broader; its peak maximum shifts progressively to higher temperatures and is no longer visible with mixtures containing >43 mol % cholesterol. The total enthalpy associated with both transitions decreases linearly as the cholesterol content is increased (see Figure 5B) reaching zero at approximately 42 mol % cholesterol (see Discussion).

Addition of cholesterol to fully hydrated PSM shows qualitatively similar behavior to DML. The chain order–disorder transition decreases in enthalpy and this sharp transition is not present at 21 mol % cholesterol (Figure 6A). Again, a transition that becomes progressively broader appears on the higher temperature side of the initial transition and the total enthalpy decreases linearly reaching zero at 42 mol % cholesterol (see Figure 6B and Discussion).

Interaction of Cholesterol with Hydrated N-Palmitoylsphingomyelin–Dimyristoyllecithin (50:50, mol/mol). Having demonstrated that PSM and DML are completely miscible in hydrated bilayers below and above their order–disorder transition, it was of interest to investigate the affinity of cholesterol for the two phospholipids in a miscible rather than immiscible system. Utilizing scanning calorimetry we have monitored the overall effect of cholesterol on a fully hydrated equimolar mixture of PSM and DML, paying particular attention to the influence of cholesterol on the residual sharp order–disorder transition at 32 °C. Representative DSC heating curves together with a plot of the total enthalpy (expressed in kcal/mol phospholipid) vs. mole fraction of cholesterol (expressed with respect to total phospholipid) are shown in Figure 7. The overall pattern is identical with the effect of cholesterol on the individual phospholipids PSM and DML. The enthalpy associated with the sharp melting component decreases with increasing cholesterol, reaching zero at a mole fraction of cholesterol between 0.2 and 0.25. It should be noted that the *peak temperature* of this transition, i.e., 32 °C, is unaltered at all cholesterol contents in the range 0–0.25 mole fraction. In addition as cholesterol is added a broad transition is clearly visible on the high temperature side of the initial transition. This broad transition is the only transition detectable at cholesterol mole fractions > 0.25 and, with increasing cholesterol, becomes progressively broader extending greater than 50 °C at a cholesterol mole fraction of 0.33. This broad transition is difficult to detect and to quantitate at higher cholesterol contents.

Discussion

Although hydrated multilamellar liposomes made from sphingomyelin isolated from brain tissue, red cells, etc. exhibit rather complex thermotropic behavior (Shipley et al., 1974; Barenholz et al., 1976), if the amide-linked fatty acyl composition is controlled, a much simpler thermal behavior results (Barenholz et al., 1976). In this paper we have described the partial synthesis of *N*-palmitoylsphingomyelin and examined (i) its thermotropic properties, (ii) its interaction with dimyristoyllecithin, (iii) its interaction with cholesterol (compared with the DML–cholesterol system), and (iv) the ability of cholesterol to distinguish between (or preferentially interact with) sphingomyelin and lecithin in a bilayer system in which the two phospholipids are miscible.

Hydrated PSM exhibits a simple chain-melting behavior with its transition temperature (40.5 °C) quite similar to that of its lecithin analogue dipalmitoyllecithin. By X-ray diffraction we have demonstrated that PSM below and above its transition temperature exists in a hydrated lamellar bilayer phase. At 10 °C, the sharp 4.14 Å line is indicative of both an ordered chain configuration and an ordered chain packing arrangement. At 47 °C, above the transition, the PSM remains in a lamellar system; typically, the lamellar repeat distance decreases by approximately 5 Å; and the broad 4.6 Å line characterizes the change to a less ordered chain configuration and chain packing arrangement. More details of the molecular arrangement in both the low temperature ("gel") and high temperature ("liquid-crystalline") forms of PSM will come from a more detailed X-ray diffraction study of this system (W. Calhoun and G. G. Shipley, work in progress).

We have shown previously that mixtures of natural sphingomyelins and lecithins which differ considerably in their hydrocarbon chain transition temperature form completely miscible phospholipid bilayers at high temperatures and undergo lateral phase separation of the higher melting sphingomyelin component as the temperature is lowered (Untracht & Shipley, 1977). As shown in Figures 1–3, mixtures of PSM and DML, their transition temperatures differing by approximately 17 °C, exhibit only a single order–disorder transition at all molar ratios. Except at low PSM contents, where the presence of the $P_{\beta'}$ phase of DML may complicate the phase diagram (Janiak et al., 1976; Luna & McConnell, 1977), complete miscibility of the two phos-

pholipids PSM and DML is indicated. The transition enthalpy increases linearly with PSM content (Figure 3), as does the lamellar repeat distance for both the low temperature (gel) and high temperature (liquid-crystalline) forms (Figure 4). Furthermore, the X-ray diffraction data demonstrate that lamellar bilayer phases are present at all DML:PSM molar ratios. Apart from possible immiscibility in the low temperature, low PSM region due to the presence of the $P_{\beta'}$ phase of DML, the phase behavior is quite similar to that of the much studied DML–dipalmitoyllecithin system where complete solid (gel) and fluid (liquid-crystalline) phase miscibility has been demonstrated (Phillips et al., 1970; Mabrey & Sturtevant, 1976). Thus, not all lecithin–sphingomyelin systems exhibit temperature dependent phase immiscibility, leading to lateral phase separation. As with other phospholipid mixtures, the contributions of differences in chain-melting temperature, chain-length differences, and polar head-group differences may all play a role. It remains to be established what effects the unusually long fatty acids ($C_{24:0}$ and $C_{24:1}$) present in natural sphingomyelins have on lipid mixing and demixing.

Addition of cholesterol to either DML or PSM produces quantitatively similar effects when examined by DSC. For both phospholipids, cholesterol contents up to approximately 0.25 mole fraction result in a reduction of the enthalpy associated with the sharp order–disorder transition (peak temperature at 41.0 °C for PSM and 26.0 °C for DML). At the same time, a broad transition appears on the high temperature side of the initial sharp transition. At mole fractions of cholesterol greater than 0.25, the initial sharp transition is not observed, only the broad transition being present. With increasing cholesterol, this transition becomes progressively broader, its peak temperature shifts to higher values, and no detectable enthalpy can be measured at mole fractions cholesterol greater than 0.42.

Similar behavior has been observed using high precision calorimeters for dipalmitoyllecithin–cholesterol (Estep et al., 1978; Mabrey et al., 1978) and DML–cholesterol (Mabrey et al., 1978). These authors have attempted to quantitate the enthalpic contribution of the sharp and broad transitions to the overall enthalpy change utilizing different methods of curve decomposition. Although neither method appears entirely satisfactory, both groups show a linear decrease in the enthalpy associated with the initial transition reaching zero at approximately 0.20–0.25 mole fraction of cholesterol.

On the basis of the calorimetric data, our results would suggest that in all respects the interaction of cholesterol with PSM and DML is similar. However, the precise mechanism for the interaction, the stoichiometry of putative phospholipid–cholesterol complexes, the structures present at intermediate cholesterol contents, etc. remain to be determined.

Finally, we wished to examine further the interesting concept that cholesterol has a different affinity for different phospholipids (van Dijck et al., 1976) and that of the phospholipids examined cholesterol has the greatest affinity for sphingomyelin (Demel et al., 1977). These conclusions were based upon DSC experiments on systems in which the two lipid components (lecithin and sphingomyelin) undergo lateral phase separation. At low cholesterol contents, progressive and exclusive removal of the DSC peak associated with the chain melting of the sphingomyelin-rich phase occurs whether or not this phase is the higher or lower melting phase. Our own experiments were designed to evaluate whether or not cholesterol could recognize and interact preferentially with either DML or PSM in a mixed bilayer in which the two phospholipids are miscible above and below the order–disorder transition. In the 50:50 (mol/mol) DML–PSM system showing a peak transition temperature at 32 °C, we would predict that cholesterol could produce the following effects as monitored by DSC. (1) If cholesterol interacts with DML, the lower melting component, a population of DML molecules will be "removed" from the mixture giving the fairly sharp transition at 32 °C. By analogy with the DML–cholesterol system, a broad transition should result and the residual phospholipid mixture, uninfluenced by cholesterol, should become progressively enriched with the higher melting PSM. This would result in a residual lipid population that will melt at a progressively higher temperature as cholesterol is added. Thus, a progressive increase in the peak temperature of the initial sharp transition would be predicted. (2) Conversely, if cholesterol preferentially interacts with PSM, the higher melting component, the residual lipid population should become enriched in DML and a progressive decrease in the peak temperature of the initial sharp transition is expected. (3) However, if cholesterol cannot distinguish between PSM and DML, on the average an equal amount of the two phospholipids will interact with cholesterol, their contribution to the enthalpy of the initial sharp transition will be removed, but the residual phospholipid composition will be identical with that of the initial mixture, equimolar, and the peak transition temperature should remain unaltered at 32 °C.

As shown in Figure 7A, our DSC data show that addition of cholesterol *produces no shift, up or down in temperature, of the initial transition peak*. This result argues strongly for the explanation given in 3 above and demonstrates that in a miscible bilayer system cholesterol has no preferential affinity for PSM over DML, or vice versa. In this system cholesterol appears to have an equal affinity for either phospholipid. Thus, although cholesterol can recognize and interact preferentially with sphingomyelin in a laterally phase separated system (Demel et al., 1977), it cannot do so when the two lipids are intimately mixed. Clearly it is possible that whatever molecular feature is responsible for inducing lateral phase separation may also endow sphingomyelin with a specific affinity for cholesterol. A likely candidate for inducing lateral phase separation is a long fatty acid ($C_{24:0}$ or $C_{24:1}$) amide-linked to sphingomyelin. It remains to be seen whether or not cholesterol has differing affinities for sphingomyelin dependent upon the unusually extreme differences in the chain length of its N-acylated fatty acids.

Acknowledgments

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Kinetics and Mechanism of Phosphatidylcholine and Cholesterol Exchange between Single Bilayer Vesicles and Bovine Serum High-Density Lipoprotein†

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ABSTRACT: We investigated the exchange of lipids between sonicated, single bilayer vesicles containing egg phosphatidylcholine and cholesterol in a molar ratio of 2:1 and bovine serum high density lipoprotein, free of lecithin:cholesterol acyltransferase activity. Lipid exchange was followed by incubating radiolabeled vesicles ([^{14}C]cholesterol and [^{3}H]-phosphatidylcholine) with the lipoprotein (preequilibrated with excess unlabeled vesicles) at a constant temperature of 37 °C and by fractionating the reaction mixtures on Sepharose CL-4B columns at 5 °C. The proportion of radiolabeled lipid appearing in the lipoprotein elution peak, with time, was used in the kinetic analysis based on the radioisotope exchange treatment. Results indicate that there is no net transfer of lipids under our experimental conditions. All of the vesicle cholesterol is exchangeable, with a radiolabel exchange half-life of 1 h 8 min. In contrast, only 69% of the vesicle phosphatidylcholine exchanges with the high density lipoprotein, suggesting that only the outer half of the vesicle bilayer participates in the exchange; the half-life for [^{3}H]phosphatidylcholine exchange is 6 h. Reverse exchange from radiolabeled, preequilibrated high density lipoprotein to unlabeled vesicles was also observed. In the reverse exchange experiments, all of the lipoprotein cholesterol participates in the exchange, but only 49% of the lipoprotein phosphatidylcholine is involved. The exchange of both lipids is temperature dependent and has activation energies of 17.0 ± 2.8 kcal/mol for cholesterol and 12.5 ± 2.0 kcal/mol for phosphatidylcholine. A linear concentration dependence of the exchange rates indicates that both lipids exchange between vesicles and high density lipoprotein by a "bimolecular" process with second-order rate constants of 1.44 ± 0.56 mM^{-1} h^{-1} for cholesterol and 0.164 ± 0.045 mM^{-1} h^{-1} for phosphatidylcholine.

Serum lipoproteins, the lipid carriers in blood, are capable not only of solubilizing lipids but also of exchanging lipids with various membranes, cells, and tissues of the organism. A number of studies over the years have demonstrated that all classes of lipoproteins can exchange "structural" lipids, i.e., phospholipids and cholesterol with cell membranes (reviewed by Jackson et al., 1976; Bruckdorfer & Graham, 1976; Bell, 1976). Reed (1968) showed that phosphatidylcholine (PC)[1] and sphingomyelin of erythrocytes exchange only partially with the corresponding phospholipids in serum and that phos-

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[1] Abbreviations used: PC, phosphatidylcholine; HDL, high-density serum lipoprotein; TLC, thin-layer chromatography; Ans, 8-anilino-1-naphthalenesulfonate.

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