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Phospholipid Structure and the Packing of Cholesteryl Oleate at the Lipid/Water Interface†

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ABSTRACT: The miscibility and packing characteristics of cholesteryl oleate in films of phospholipids at the air/water interface were measured as a function of phospholipid structure. For phospholipids which differ only in the polar substituent on the phosphate, miscibility is positively correlated with the molecular areas of the pure phospholipids under comparable conditions. For choline phospholipids which differ in apolar substituents, miscibility is negatively correlated with the number of aliphatic groups and with the proportion of those groups containing cis unsaturation. Phospholipids containing saturated aliphatic moieties and lysophosphatidylcholines exhibited nonideal mixing in the monolayer phase. The miscibility data can be described by a geometric model in which cholesteryl esters are accommodated in the potential free area available in the apolar region of the surface phase. When applied at a surface pressure of 20 mN/m, it yields 18, 35, and 116 Å^2/molecule for the partial molecular areas of saturated chains, cis-unsaturated chains, and cholesteryl oleate, respectively. Thus, cholesteryl ester miscibility is regulated by phospholipid head group and aliphatic composition insofar as they determine the space available in the aliphatic region of the interface.

Long-chain esters of cholesterol are often perceived only as constituents of bulk lipid phases but do exhibit small solubilities in phospholipid bilayer membranes [e.g., see Janiak et al. (1974) and Gorrissen et al. (1980)] and in lipid films at the air/water interface. In the latter system, they are solubilized by a variety of non-cholesterol lipids (colipids) provided the acyl moiety of the cholesteryl ester contains cis unsaturation (Smaby et al., 1979). The position and extent of this unsaturation, the molar ratio of lipid components, and the packing density of the lipid components determine the distribution of cholesteryl ester between a bulk lipid phase and two immiscible

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surface phases (Smaby & Brockman, 1981a,b).

If these results are extrapolated to natural emulsions such as intestinal fat droplets, lipoproteins, and arterial lipid deposits, the mass of cholesteryl ester present in interfacial phases is a small fraction of the total. However, this small quantity of interfacial ester may have a much larger biological importance. This arises because some, and possibly all, enzymatic reactions involving cholesteryl esters and water-soluble proteins are interfacial [e.g., see Bhat & Brockman (1982)].

To better understand the regulation of substrate availability for interfacial reactions, we have investigated in greater detail the physical behavior of cholesteryl oleate and phospholipids in lipid films. The results reveal a linear relation between cholesteryl ester miscibility and the potential "free space" in the apolar region of the surface phase and the existence of nonideal mixing in the monolayer phase.

Materials and Methods

Lipids. Cholesteryl oleate was purchased from Nu-Chek Prep, Inc., Elysian, MN. Its purity was checked by thin-layer chromatography. From measured detection limits, after spraying with chromic/sulfuric acid followed by charring, the purity was shown to be greater than 99.5%. Bovine liver phosphatidylinositol (PI), egg sphingomyelin (Sph), egg phosphatidylethanolamine (PE), bovine brain phosphatidylserine (PS), 1-palmitoyl-*sn*-glycero-3-phosphocholine, 1-oleoyl-*sn*-glycero-3-phosphocholine, and 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine were purchased from Avanti Biochemicals, Birmingham, AL. 1,2-Ditetradecanoyl-*sn*-glycero-3-phosphocholine was purchased from Supelco, Inc., Bellefonte, PA. Phosphatidylethanolamine was repurified by thin-layer chromatography in a solvent system of chloroform/methanol/water (65:25:4). The purity of each phospholipid was greater than 99% when analyzed by thin-layer chromatography. Phospholipid concentration in stock solutions for surface studies was determined by assaying aliquots for organic phosphate (Bartlett, 1959).

Solvents. Petroleum ether was purified as previously described (Smaby & Brockman, 1981a). Ethanol and methanol were distilled from KOH and zinc, and chloroform was redistilled.

Surface Pressure–Molecular Area Isotherms. Surface pressure was measured as a function of area by using a computerized Langmuir film balance (Brockman et al., 1980). In all cases, the lipids were spread onto a 10 mM potassium phosphate/0.1 M sodium chloride subphase, pH 6.6, at 24 °C. The lipids were spread in 50 μL of petroleum ether (dimyristoylglycerophosphocholine), petroleum ether/ethanol (9:1) (sphingomyelin, phosphatidylserine, phosphatidylethanolamine, dipalmitoylglycerophosphocholine, and phosphatidylinositol), or petroleum ether/chloroform/methanol (50:33:17) (1-palmitoylglycerophosphocholine and 1-oleoylglycerophosphocholine). After the film stood at a large molecular area for 4 min, it was compressed at ≤5 $Å^2$ min^{-1} $molecule^{-1}$. Phase transitions were identified by using second and third derivatives as previously described (Brockman et al., 1980).

Phospholipid Analysis. Fatty acid composition was determined by gas–liquid chromatography as previously described (Smaby et al., 1983).

Results

The importance of the polar head-group substituent in regulating cholesteryl ester miscibility was determined by using a series of four glycerol-based phospholipids differing primarily in the polar substituent attached to the phosphoryl group. As shown in Table I, the phosphatidylethanolamine, -choline, -serine, and -inositol used had roughly equal percentages of saturated and cis-unsaturated acyl groups. The miscibility limit for cholesteryl oleate in the monolayer surface phase with each phospholipid is shown as a function of surface pressure in Figure 1. The data for egg phosphatidylcholine (PC) containing mixtures are taken from Bhat & Brockman (1981). The phase boundaries are similar but show quantitative differences in cholesteryl oleate miscibility at any particular surface pressure. The relative solubilities of cholesteryl oleate at 20 mN/m are PE < PS ≤ PC < PI. This order approximates that for the molecular areas of the pure colipids at 20 dyn/cm, PE < PS < PC < PI. Thus, for these diacyl phospholipids, the larger the area occupied by the pure phospholipid at a particular surface pressure, the greater is its ability to solubilize cholesteryl oleate in the monolayer phase.

In other respects, the surface behavior of these mixtures is quite similar to that of other cholesteryl ester containing systems studied (Smaby & Brockman, 1981b). At mole fractions of cholesteryl oleate between 0.5 and 1.0, a second phase boundary is observed which is approximately the same for all four mixtures and occurs to surface pressures greater than 30 mN/m. Also, as before, average molecular area–composition isobars are linear throughout the monolayer region.

If the glycerol-based egg phosphatidylcholine is replaced by egg sphingomyelin, the phase behavior with cholesteryl oleate is markedly altered (Figure 2a). At all pressures, the

Table I: Acyl Compositions of Phospholipids

chain length:no. of double bonds	mol %				
	PE	PC	PS	Sph	PI
16:0	21.8	35.9	1.8	86.9	5.3
16:1 + 17:0	0.4	1.9	0.5	6.8	0.9
18:0	30.8	12.2	49.4	1.3	56.3
18:1	21.6	29.9	33.5	2.7	13.6
18:2	13.1	16.5	0.4	1.3	4.5
20:1		0.1	3.5	1.1	
20:2	0.2	0.1	0.6		
20:3	0.4	0.3	0.9		6.5
20:4	9.4	2.5	0.7		9.4
20:5					2.0
22:4	0.2		1.6		1.5
22:5 (ω6)	1.1	0.4	0.9		
22:5 (ω3)	0.1		0.1		
22:6	0.9	0.3	6.3		
	47.4[a]	51.9[a]	48.8[a]	5.9[a,b]	38.4[a]

[a] Percent unsaturated chains. [b] Includes the alkyl chain of sphingosine, i.e., 100 − [(% saturated + 100)/2].

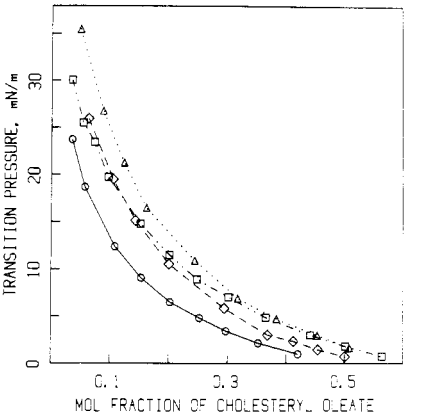

FIGURE 1: Miscibility limits for cholesteryl oleate in the monolayer phase in mixtures with phosphatidylethanolamine (○), phosphatidylserine (□), phosphatidylinositol (△), and phosphatidylcholine (◇).

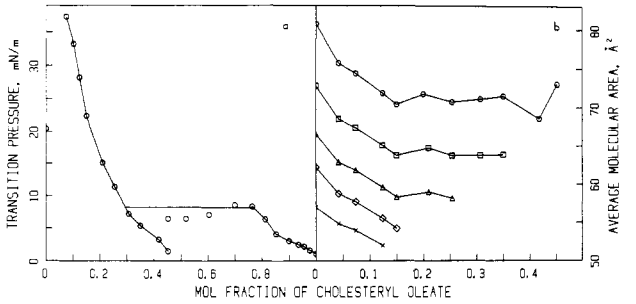

FIGURE 2: Cholesteryl oleate in mixtures with sphingomyelin. (a) Phase diagram; (b) average molecular area–composition isobars at representative surface pressures: 1 (○), 5 (□), 10 (△), 15 (◇), and 20 mN/m (×).

solubility of cholesteryl oleate in the monolayer phase is enhanced relative to the mixtures shown in Figure 1. This is more apparent at higher pressures which correspond to higher lipid packing densities. For example, at 20 mN/m, solubility is 2.0 times that with egg phosphatidylcholine and 1.5 times that with phosphatidylinositol. Also, the double layer is destabilized, existing only at surface pressures below 8 mN/m. Examination of average molecular area–composition isobars (Figure 2b) in the monolayer region of Figure 2a reveals a second difference. In contrast to data obtained with the glycerol-based phospholipids, the plots are not linear in the monolayer region but show marked deviations from ideal mixing behavior. Moreover, there is an apparent discontinuity at 0.15 mol fraction above which the average molecular area remains constant.

From the data shown, the structural determinants of the enhanced miscibility behavior and nonideal mixing cannot be assessed. They could conceivably arise from the replacement of the glycerol of egg phosphatidylcholine by the dihydroxyaminopropyl end of the sphingosine molecule or by differences in aliphatic chain structure (Table I). To help determine if the novel physical behavior of sphingomyelin/cholesteryl oleate mixtures was related to the apolar part of the molecule, disaturated phosphatidylcholines were substituted for egg phosphatidylcholine. The phase diagram and average molecular area–composition plots for dimyristoylphosphatidylcholine/cholesteryl oleate mixtures showed the same features observed with sphingomyelin, namely, enhanced solubility in the monolayer phase relative to egg phosphatidylcholine, decreased stability for the double-layer phase, and nonlinear isobars in the monolayer region. Similar results were obtained with dipalmitoylphosphatidylcholine. For this system, we also observed the liquid-expanded–liquid-condensed phase transition for dipalmitoylphosphatidylcholine which increased from about 8 to 15 mN/m with increasing cholesteryl oleate in the monolayer region. This indicates preferential stabilization of the liquid-expanded state by cholesteryl oleate. The nonideal behavior of the average molecular area–composition isobars was phase dependent, showing an apparent discontinuity at about 0.27 mol fraction in the liquid-expanded state and at 0.1 mol fraction in the liquid-condensed state. Overall, the data suggest that the degree of aliphatic group unsaturation, not the phospholipid backbone, is a major determinant of cholesteryl ester solubility in phospholipid films.

The role of the number of acyl chains of choline phospholipids in cholesteryl ester solubilization was examined with lysophosphatidylcholines containing either palmitate or oleate in the *sn*-1 position. As shown by the superimposed monolayer phase boundaries in Figure 3, 1-oleoylglycerophosphocholine solubilized more cholesteryl oleate at higher surface pressures than did 1-palmitoylglycerophosphocholine, and both solubilized as much, or more, at 20 mN/m as the disaturated phospholipids. This shows again that aliphatic chain saturation is a determinant of cholesteryl ester solubilization but that the number of acyl chains is also important. The average molecular area–composition isobars for these lysophospholipid/cholesteryl oleate mixtures show nonlinear behavior similar to that observed with the saturated phospholipids.

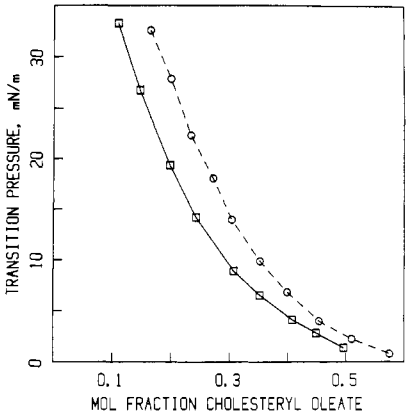

FIGURE 3: Miscibility limits for cholesterol oleate in the monolayer phase in mixtures with 1-palmitoylglycerophosphocholine (○) and 1-oleoylglycerophosphocholine (□).

Discussion

For the most part, previous quantitative studies of cholesteryl ester miscibility were performed with colipids containing cis-unsaturated acyl moieties (Smaby et al., 1979; Smaby & Brockman, 1981a,b). In the present study, the role of colipid structure in determining the extent of miscibility was investigated more systematically. The results obtained with a series of phospholipids with different head groups but similar acyl compositions show that cholesteryl oleate miscibility in the monolayer phase correlates positively with the cross-sectional area of the parent colipid under similar conditions. In contrast, with a series of phosphatidylcholines and sphingomyelin, miscibility shows a negative, approximately linear correlation with the molecular area of the colipid alone (graphs not shown). The areas of the colipids in this latter group differ primarily as a result of the proportion of saturated and cis-unsaturated aliphatic moieties. Thus, solubility varies inversely with the degree of cis unsaturation. Because cis-unsaturated acyl groups have greater cross-sectional areas than saturated chains, our results suggest that miscibility is regulated by the amount of unoccupied, or potentially unoccupied, space in the apolar region of a colipid surface phase. Consistent with this concept are the relatively high miscibilities of cholesteryl oleate in films of lysophosphatidylcholines regardless of acyl composition. The molecular areas of the parent lysophospholipids (55–60 Å²/molecule at 20 mN/m) are apparently determined largely by the head group, enabling far greater solubilization of cholesteryl oleate than for the corresponding diacyl species with similar molecular areas.

Quantitatively, the relation between solubility and free space can be expressed at any surface pressure by

$$S = \frac{A_{CL} - n[fA_u + (1-f)A_s]}{A_{CE}} \quad (1)$$

where S is the solubility of cholesteryl oleate per mole of colipid, A_{CE}, A_{CL}, A_u, and A_s are the areas occupied by one molecule of cholesteryl oleate, colipid, a cis-unsaturated aliphatic moiety, and a saturated aliphatic moiety, respectively, and f is the fraction of the n aliphatic moieties of the colipid which are cis unsaturated. For all nine pairs of compounds

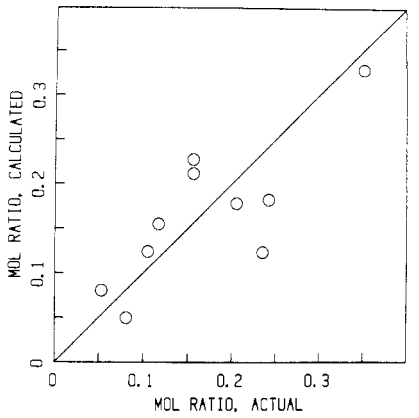

FIGURE 4: Calculated vs. measured solubilities of cholesteryl oleate per mole of phospholipid at 20 mN/m for cholesteryl oleate/phospholipid mixtures. Calculated solubilities were obtained by using eq 1 with A_{CE} = 115.9, A_u = 35.2, and A_s = 18.4 Å^2/molecule as described in the text.

presented in this paper, stable surface films were obtained up to 20 mN/m. At this surface pressure, the solubility data for these and dioleoylphosphatidylcholine (Smaby et al., 1979) were fitted to compositions (Table I) and molecular areas of the pure colipids by using a modified Marquardt strategy. A best fit was obtained with A_{CE} = 116, A_u = 35, and A_s = 18 Å^2/molecule, and the correlation coefficient of the line (Figure 4) was 0.85. In particular, the reasonable values of A_u and A_s support the hypothesis that cholesteryl ester miscibility is determined principally by the amount of free space which can be created in the apolar region of the colipid surface phase.

In the region of monolayer phase formation, the average molecular area–composition isobars obtained with the more saturated colipids and lysophosphatidylcholine were not linear and showed one or more discontinuities at lower surface pressures. In general, deviations from linearity of such plots within a single surface phase indicate nonideal mixing (Gaines, 1966), and discontinuities suggest the formation of preferred packing arrays (Dervichian, 1958). Although our results demonstrate nonideal behavior, the data are insufficient at present to attempt any detailed analysis of packing trends.

A retrospective examination of isobars for other binary cholesteryl ester/colipid mixtures studied previously reveals discontinuities at very low surface pressures. Furthermore, the cross-sectional areas of cholesteryl oleate previously calculated by extrapolation of data from linear isobars (Smaby et al., 1979) are considerably lower than the value of 116 Å^2 suggested by analysis of phase boundary data at 20 mN/m. This would suggest that with other colipids which we studied previously the absence of discontinuous average molecular area–composition isobars at most surface pressures in the monolayer region does not indicate ideal mixing, as we had assumed (Smaby et al., 1979), merely the inability to detect deviations from it. This occurred because of relatively low solubility limits for cholesteryl ester in the monolayer phase, particularly at higher surface pressures.

In living systems, the surfaces of both intracellular and extracellular lipid deposits and lipoproteins surround a bulk lipid phase, commonly referred to as the core of the particle. With large particles, for which the radius of curvature is a minor determinant of the physical state, the surface of the particle should be modeled along the phase boundary lines we observe in the monolayer to double layer or bulk transitions shown in the phase diagrams. Thus, our results would imply that the surface availability of cholesteryl esters for enzyme-catalyzed reactions such as hydrolysis is strongly influenced by the number and composition of the aliphatic moieties of the other surface lipids. Indirectly, head-group area and packing are important as they can regulate the spacing of the aliphatic groups. It should be noted that for anionic lipids this spacing is dependent on pH and Ca^{2+} concentration [e.g., see Shimojo & Ohneshi (1967) and Patil et al. (1979)], indicating a possible connection between miscibility and metabolic states.

Registry No. Cholesteryl oleate, 303-43-5; 1-palmitoyl-*sn*-glycero-3-phosphocholine, 17364-16-8; 1-oleoyl-*sn*-glycero-3-phosphocholine, 19420-56-5; 1,2-dipalmitoyl-*sn*-glycero-3-phosphocholine, 63-89-8; 1,2-ditetradecanoyl-*sn*-glycero-3-phosphocholine, 18194-24-6.

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