Novel Surface Phase Containing Cholesteryl Esters. 2. Nonequivalence of Cholesteryl Arachidonate and Those with 18-Carbon, Cis-Unsaturated Acyl Groups†

Janice M. Smaby and Howard L. Brockman*

ABSTRACT: Surface pressure–area isotherms for binary mixtures of cholesteryl octanoate, elaidate, stearate, oleate, linoleate, linolenate, and arachiodonate in mixtures with dioleoyllecithin, triolein, oleic acid, and oleoyl alcohol were measured at 24 °C. Analysis of the pressure and area characteristics as a function of composition showed that double-layer surface phase formation is primarily dependent on the structure of the acyl moiety of the cholesteryl ester. Cholesteryl esters with saturated or trans-unsaturated acyl chains apparently do not form double-layer surface phases. The esters of oleate, linoleate, and linolenate formed double-layer as well as monolayer phases and their properties in these phases were similar. In contrast to other cis-unsaturated esters, cholesteryl arachidonate formed a mixed monolayer phase with miscibility in all proportions and did not form a double-layer phase. Our results show that the polar lipid monolayer separating bulk cholesteryl ester from the aqueous milieu not only solubilizes finite amounts of cholesteryl esters but also can contribute to the organization of lipid adjacent to the monolayer. That such organization is observed with the predominant cholesteryl ester species of blood and aorta suggests a role for double-layer structure in regulating the transport and metabolism of cholesteryl esters in lipoproteins, arterial lipid deposits, and adrenal cortex. The absence of double-layer formation and high monolayer solubility of cholesteryl arachidonate suggest that it should be more abundant than other cholesteryl esters in bilayers and in monolayers surrounding bulk lipid phases.

Cholesteryl esters are normal constituents of lipoproteins and are the principal lipid to accumulate in the arterial wall during the progression of atherosclerosis. In the arterial wall they are subject to metabolic transformation, being hydrolyzed at both acid and neutral pHs by lysosomal and cytoplasmic hydrolases (Brockman, 1979). In serum they are synthesized by lecithin:cholesterol acyltransferase (Glomset, 1979) and can be exchanged between lipoproteins via complexation with a water-soluble exchange protein (Barter & Jones, 1980). Each of these reactions presumably occurs at a lipid–water interface and, therefore, requires the availability of cholesteryl esters at or near the interface. This interface is normally depicted as comprised of (apolipo)proteins and polar lipids such as lecithin, sphingomyelin, and cholesterol. However, it has been shown that some cholesteryl esters may reside in this monolayer of lipids which separates the core of the lipid droplet or lipoprotein from the aqueous milieu (Smaby et al., 1979). More recently, it has been shown that cholesteryl myristoleate (9–14:1) could form a second, double-layer phase with dioleoyl lecithin at the air–water interface (Smaby & Brockman, 1980). This phase is miscible with the monolayer phase and can accommodate greater amounts of cholesteryl myristoleate than the monolayer phase. The importance of cholesteryl ester availability for metabolic reactions occurring at the lipid–water interface has prompted a more thorough study of the formation and properties of the novel, double-layer phase. Using the same model system with which the phase behavior of dioleoyl lecithin–cholesteryl myristoleate was described, we have examined the properties of the more physiologically relevant stearoyl (18:0), oleoyl (9–18:1), linoleoyl (9,12–18:2), linolenoyl (9,12,15–18:3), and arachidonoyl (5,8,11,14–20:4) esters of cholesterol in mixtures with other lipids (colipids). The results of this study show that cholesteryl esters possessing cis-unsaturated acyl moieties with methylene-interrupted unsaturation beginning at position 9 are similar to the myristoleoyl derivative previously described. However, the elaidoyl, the stearoyl, and, in particular, the arachidonyl esters of cholesterol are markedly different in their phase behavior, showing the absence of double-layer formation at all pressures and compositions.

Experimental Procedures

Materials

Reagents. Lipids. All cholesteryl esters, oleyl alcohol, oleic acid, and triolein (trioleoyl glycerol) were purchased from NuChek Prep. Inc., Elysian, MN. The purity of each compound was checked by thin-layer chromatography. Each showed only one spot after detection with sulfuric–chromic acid, and, from measured detection limits, the lipids were shown to be >99.5% pure. Dioleoyllecithin (1,2-dioleoyl-*sn*-glycero-3-phosphocholine) was purified as previously described (Smaby & Brockman, 1980).

Spreading Solvent. Petroleum ether, free of surface active impurities, was purified as previously described (Smaby & Brockman, 1980).

Water. Water was purified by reverse osmosis, deionization, and distillation from alkaline permanganate.

Other Chemicals. All other compounds were reagent grade and used without further purification.

Methods

Force–Area Measurements. Surface pressure–area determinations were made as previously described (Smaby & Brockman, 1980). Unless otherwise specified, lipids were spread in petroleum ether onto a 10 mM potassium phos-

† From the Hormel Institute, University of Minnesota, Austin, Minnesota 55912. *Received June 4, 1980.* This investigation was supported specifically by U.S. Public Health Service Research Grant HL 23003 from the National Heart, Lung and Blood Institute. General support was provided by Program Project Grant HL 08214 and by the Hormel Foundation. This work was done during the tenure of an Established Investigatorship (H.L.B.) of the American Heart Association with funds contributed in part by the Minnesota Affiliate. Portions of this work were presented at the Golden Jubilee International Congress on Essential Fatty Acids and Prostaglandins, May 4–7, 1980, Minneapolis, MN.

0006-2960/81/0420-0724$01.00/0

Table I: Comparison of Parameters Describing Properties of Cholesteryl Esters in Pure and Mixed Monolayer States Comprising the Double-Layer Phase

		double-layer phase				mixed double-layer state				pure ester double-layer state	
colipid[a]	cholesteryl ester[b]	collapse pressure (mN/m)	r_m	m	transition area (Å²/molecule)	r_n	A_u	n	transition area (Å²/molecule)	collapse area (Å²/molecule)	collapse pressure (mN/m)
PC	14:1[c]	38.6	1.000	1.95	29.6	1.000	38.2	9.50	29.9	24.5	3.0
PC	18:1	26.6	1.000	1.94	25.8	0.998	36.6	8.79	33.7	25.0	0.8
PC	18:2	20.6	0.999	1.84	31.7	0.999	39.2	10.87	38.7	25.5	1.3
PC	18:3	14.2	0.997	2.04	34.6	0.999	40.1	12.68	35.4	25.6	1.3
TO	14:1	8.4	0.989	2.59	39.2	1.000	33.1	11.01	54.1		
TO	18:1	7.2	0.996	2.26	34.1	1.000	32.8	11.35	62.4		
TO	18:2	6.3	0.987	2.96	45.7	1.000	36.6	16.01	41.9		
TO	18:3	4.6	0.976	2.21	51.2			20.79			
OA	18:1		1.000	0.58	28.6			6.63			
OL	18:1		0.998	0.50	31.8			6.53			

[a] PC = dioleoylphosphatidylcholine; TO = trioleoyl glycerol; OA = oleic acid; OL = oleyl alcohol. [b] 14:1 = cholesteryl myristoleate; 18:1 = cholesteryl oleate; 18:2 = cholesteryl linoleate; 18:3 = cholesteryl linolenate. [c] Data for 14:1 with PC are from Smaby & Brockman (1980).

phate–0.1 M sodium chloride subphase, pH 6.6, 24 °C. Compression rates varied from 1 to 8 Å² min⁻¹ molecule⁻¹, and compression was generally from very large molecular areas to an area less than the collapse area of the colipid alone. Phase transitions were identified by using second and third derivatives of the surface pressure–area isotherms (Brockman et al., 1980).

Results

To examine the effects of colipid structure on phase distribution, we used triolein, dioleoyllecithin, oleic acid, and oleyl alcohol as representative cis-9-unsaturated tri-, di-, and monochain lipids of varying head-group structure. These were studied in binary mixtures with cholesteryl myristoleate, oleate, linoleate, linolenate, arachidonate, elaidate, octanoate, and stearate to determine how structural changes in the acyl moiety of the cholesteryl ester affected the phase behavior of the system. It is known that cholesteryl stearate does not form mixed monomolecular films at low mole fractions of cholesteryl ester with a wide variety of colipids (Smaby et al., 1979). Likewise, in the present study using higher mole fractions, it showed no surface activity in mixtures with either triolein or dioleoyl lecithin. Cholesteryl elaidate and octanoate, which form metastable monomolecular films (Smaby et al., 1979), did not cause any additional expansion of the surface at high mole fractions when mixed with dioleoyl lecithin, and, in fact, at high mole fractions the monomolecular phase was destabilized. In contrast, all of the cholesteryl esters with cis-unsaturated acyl moieties did form mixed surface films over the entire range of mole fractions, and, except for cholesteryl arachidonate, the families of surface pressure–area curves obtained were qualitatively similar to those reported for cholesteryl myristoleate and dioleoyllecithin mixtures (Smaby & Brockman, 1980). The surface pressure–area isotherms for the cis-unsaturated cholesteryl esters are not shown here but are available as supplementary material (see paragraph at end of paper regarding supplementary material). The collapse areas of the pure cholesteryl esters which formed double-layer surface films are given in Table I.

From the surface pressure–area data, phase diagrams were constructed and are shown in Figures 1-3. Figure 1 shows the superimposed phase diagrams for cholesteryl oleate, linoleate, and linolenate in mixtures with dioleoyllecithin. Figure 2 shows a similar set of data with the inclusion of cholesteryl myristoleate for mixtures with triolein. Individual phase diagrams for cholesteryl oleate mixed with oleic acid and oleyl alcohol are shown in Figure 3a,b. A generalized schematic of the type of phase behavior exhibited in Figures 1, 2, and 3a,b is shown in Figure 4 as solid lines. By analogy with the cholesteryl myristoleate–dioleoyllecithin system (Smaby & Brockman, 1980), region I is a mixed monolayer phase, region

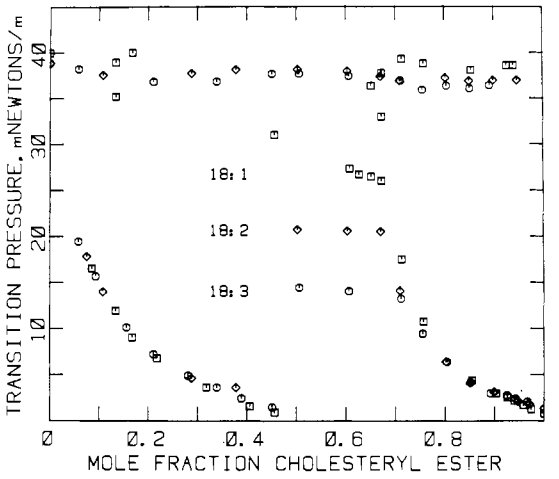

FIGURE 1: Composition dependence of phase transition pressures for cholesteryl oleate (□), linoleate (◇), and linolenate (○) in mixtures with dioleoyllecithin. Transition pressures were obtained from surface pressure–area isotherms available as supplementary material.

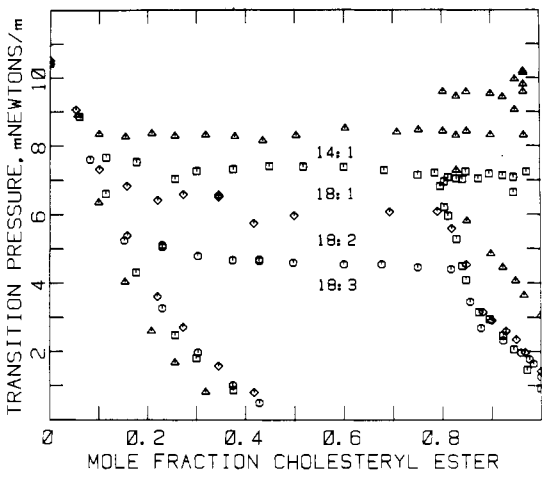

FIGURE 2: Composition dependence of phase transition pressures for cholesteryl myristoleate (△), oleate (□), linoleate (◇), and linolenate (○) in mixtures with triolein. Transition pressures were obtained from surface pressure–area isotherms available as supplementary material.

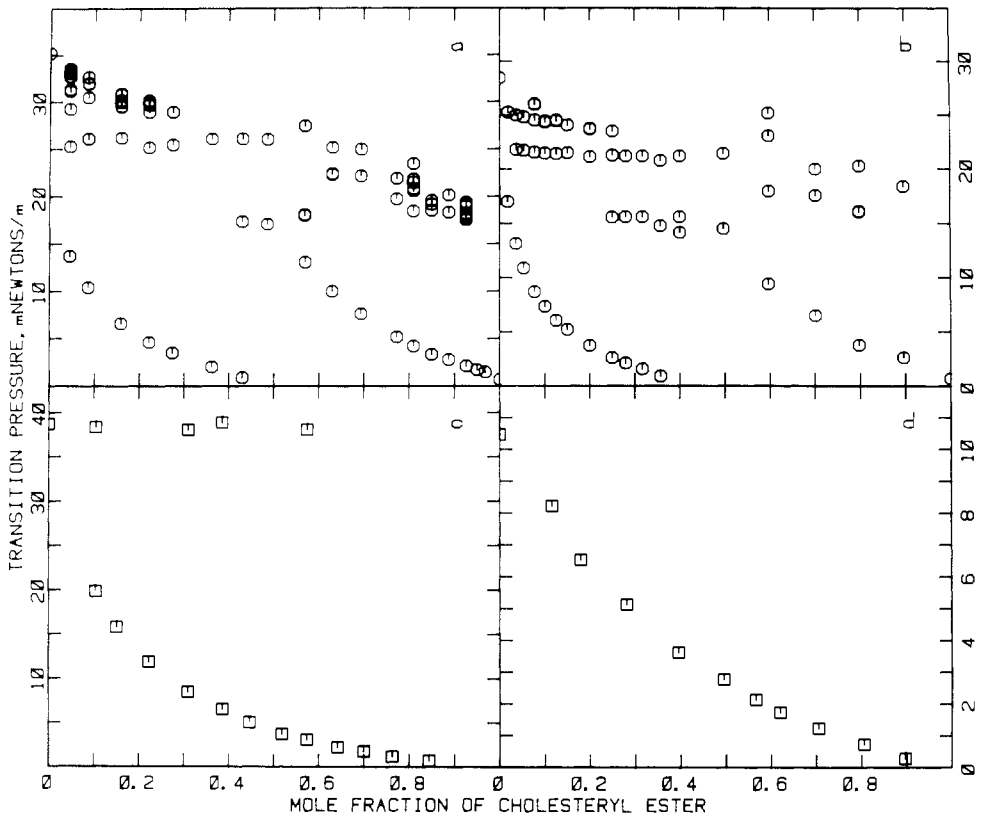

FIGURE 3: Composition dependence of phase transition pressures for cholesteryl oleate in mixtures with (a) oleic acid and (b) oleoyl alcohol and for cholesteryl arachidonate in mixtures with (c) dioleoyllecithin and (d) triolein. Transition pressures were obtained from surface pressure–area isotherms available as supplementary material.

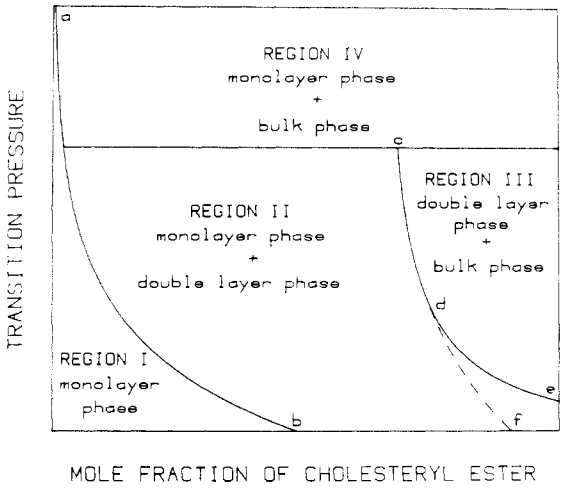

FIGURE 4: Generalized phase diagrams representing data of the type shown in Figures 1, 2, and 3a,b.

II is a mixture of monolayer phase and a double-layer surface phase containing both components, and region III is a mixture of double-layer phase and bulk cholesteryl ester. In contrast to myristoleate, Figure 1 shows that the 18-carbon, cis-unsaturated esters of choleserol do not form the double-layer phase up to the collapse pressure of the colipid. Instead, they exhibit a maximum pressure above which the phase is not stable. This gives rise to a region IV in the phase diagram in which the mixed monolayer phase coexists with bulk cholesteryl ester. The superposition of the phase diagrams in Figure 1 shows that the collapse pressure of the double-layer phase is the only major difference among members of the set. Note also that each of the pure cholesteryl esters forms a surface phase with a small, but finite, collapse pressure. The values of the maximum collapse pressures for the double-layer phase and pure cholesteryl ester are summarized in Table I. Limited surface pressure–area data have been obtained for the lecithin with cholesteryl oleate and with cholesteryl linolenate at 37 °C. These indicate that temperature has little effect on the shape of the phase diagram. it does decrease by ~50% the maximum pressure at which the double-layer phase is stable, and the collapse pressures of the pure cholesteryl esters are 0 within the accuracy of our measurements (±0.2 mN/m).

For the same cholesteryl esters mixed with triolein (Figure 2), the phase transitions differentiating regions II and III from region IV were more readily detectable, and the collapse of the pure colipid generally did not exhibit a sharp identifiable transition. This behavior indicates miscibility of the components in the bulk phase (Crisp, 1949). Otherwise, the phase behavior with triolein is essentially that observed with the lecithin as colipid. Cholesteryl oleate with the monochain colipids, oleyl alcohol and oleic acid, shows regions I–III clearly, but the phase transitions between II or III and region IV were not well-defined as shown in Figure 3a,b. This lack of sharpness at higher pressures, possibly the result of metastable states or miscibility of colipid with bulk cholesteryl ester, is responsible for the multiple phase transitions detected by the automated system.

In the portion of region II to the left of line cdf in Figure 4, the cholesteryl ester and colipid in the double-layer phase exist in a preferred packing arrangement or state of variable stoichiometry. The minimum number of cholesteryl ester molecules per colipid in this state, m, can be obtained from the phase-transition pressures and compositions for the system along cde (Figure 4) (Smaby & Brockman, 1980). Mathematically, the transition pressure is plotted against the negative log of the mole fraction of uncomplexed cholesteryl ester, $[X(1 + m) - m]/[mX - m + 1]$, where X is the mole fraction of

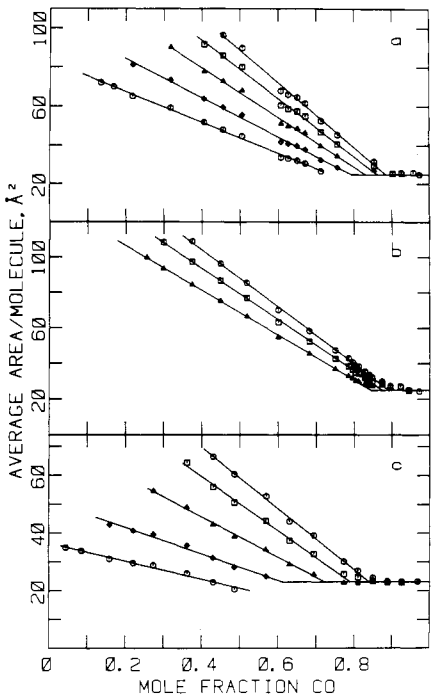

FIGURE 5: Average molecular area in Å² as a function of composition for (a) cholesteryl oleate–dioleoyllecithin mixtures at 1, 2, 4, 8, and 16 mN/m, (b) cholesteryl oleate–triolein mixtures at 1, 2, and 4 mN/m, and (c) cholesteryl oleate–oleic acid mixtures at 1, 2, 4, 8, and 16 mN/m. Pressures of 1 and 16 mN/m are represented by (○), 2 by (□), 4 by (△), and 8 by (◇). The data are from force–area curves (supplementary material), and the least-squares lines were calculated by using data between lines ab and cde in Figure 4, with the exception of areas < 30 Å²/molecule and mole fractions > 0.8 for panels a and b and areas < 30 Å²/molecule and mole fractions > 0.7 for panel c.

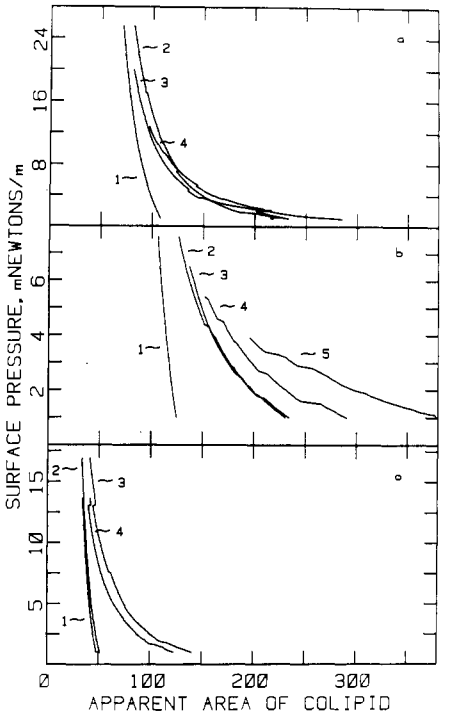

FIGURE 6: Apparent molecular area in Å² of colipid as a function of surface pressure in pure monolayer phase and in mixed, double-layer state. In the double-layer state, molecular areas were calculated every 0.1 mN/m between 1 and 0.5 mN/m below the maximum pressures at which the double-layer states were stable. (a) (1) dioleoyllecithin, with (2) cholesteryl oleate, (3) cholesteryl linoleate, and (4) cholesteryl linolenate; (b) (1) triolein, with (2) cholesteryl myristoleate, (3) cholesteryl oleate, (4) cholesteryl linoleate, and (5) cholesteryl linolenate; (c) (1) oleyl alcohol, (2) oleic acid, (3) oleic acid with cholesteryl oleate, and (4) oleyl alcohol with cholesteryl oleate.

cholesteryl ester in the system. The moles of uncomplexed ester are the total in the system less m moles per mole of colipid. The data corresponding to line cde (Figure 4) in Figures 1–3 were analyzed in this manner to determine the best value of m. As indicated by the coefficients of correlation, r_m, in Table I, all the data from Figures 1–3 are well described by the model. The values of m which gave the best fit and the collapse areas of the cholesteryl esters calculated from the slopes are also given in the table.

The similarities in form between the phase diagrams also extend to plots of average molecular areas of the surface, as shown by the representative data in Figure 5. Such plots are comparable to those described for cholesteryl myristoleate and dioleoyllecithin, particularly in region II; they are uniformly linear at higher pressures, but at lower pressures they are biphasic, with the average molecular area not falling below the collapse area of the pure ester. This behavior has been previously related to the preferential formation of a mixed double-layer state or packing arrangement which coexists with the monolayer phase in region II (Smaby & Brockman, 1980). The ends of the linear segments correspond on the left to the phase boundary ab in Figure 4 and on the right to line cdf. To the right of df and below de (Figure 4), a double-layer, pure cholesteryl ester state also coexists and is miscible with the mixed state. The data defining df can be fitted to the same form of equation as the phase transition pressures along cde (Smaby & Brockman, 1980). At any pressure, the mole fraction of cholesteryl ester relative to colipid in the interfacial layer of the double-layer state is given by $[A_u R_2 - \bar{A}(1 + R_2)]/[(A_u - \bar{A})(1 + R_2)]$, where R_2 is the ratio of cholesteryl ester to colipid at that pressure along df, $\bar{A}$ is the constant average molecular area along df which equals the collapse area of the pure cholesteryl ester (Table I), and A_u is the molecular area of cholesteryl ester comprising the upper layer of each of the double-layer states (Smaby & Brockman, 1980). For each of the mixtures described in Figures 1 and 2, the data corresponding to df in Figure 4 were obtained from average area plots of the type shown in Figure 5 and were fitted to obtain the best values of A_u. The coefficients of correlation, r_n, for these plots and the values of A_u which gave the best fit are shown in Table I. The x intercept of each line is the solubility limit of cholesteryl ester in the preferred state and yields the maximum ratio of cholesteryl ester to colipid, n. The slope of each line yields the molecular area of the cholesteryl ester at the state transition. These are also given in Table I. Although most mixtures with lecithin and triolein as colipid were consistent with the mathematical model describing line df, the data for cholesteryl linolenate with triolein as well as for cholesteryl oleate with oleic acid and oleoyl alcohol did not give reasonable convergence, and the parameters describing the state could not be directly obtained. The values of n shown for these mixtures in Table I were, therefore, esimated by extrapolation (Smaby & Brockman, 1980).

The slopes and intercepts of the linear segments of the average area plots of the type shown in Figure 5 were obtained each 0.1 mN/m and used to compute apparent surface pressure–area isotherms for the colipid along line cdf (Figure 4), i.e., when only a single, mixed, double-layer surface phase is present (Smaby & Brockman, 1980). These are shown in Figure 6 and are plotted as surface pressure vs. the apparent molecular area of the colipid. For comparison, the isotherm for the pure colipid is also shown. For each colipid, the apparent surface pressure area isotherms are similar, and, as with cholesteryl myristoleate, the apparent force–area curve par-

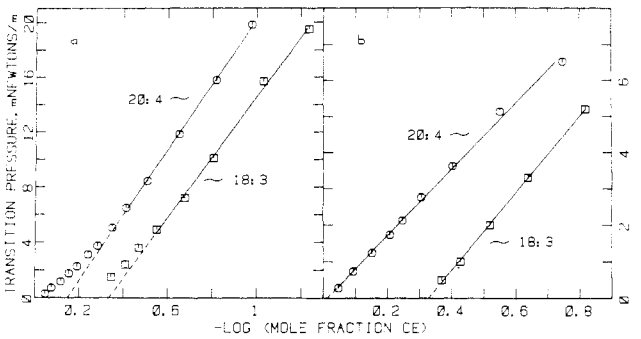

FIGURE 7: Phase-transition pressure vs. negative logarithm of mole fraction of cholesteryl esters with (a) dioleoyllecithin and (b) triolein. The cholesteryl esters are cholesteryl linolenate (□) and cholesteryl arachidonate (○). lines were fitted by the method of least squares using data from Figure 3c,d.

allels that of the colipid at high surface pressure but is expanded at low pressures.

As seen in Figures 1 and 2 data obtained with cholesteryl linolenate fall in the same pattern as the other cis-unsaturated esters. Cholesteryl arachidonate, however, exhibits markedly different phase behavior; parts c and d of Figure 3 show only a single set of transitions delineating the boundary between two phases. The components are miscible in all proportions, and, as shown earlier, the pure ester does not exhibit a measurable collapse pressure at 24 °C (Kwong et al., 1971). To characterize the surface phase formed with cholesteryl arachidonate, we plotted the phase-transition pressure–composition data as previously described (Smaby et al., 1979), and the results are shown in Figure 7. With both colipids, the solubility limit is essentially 1, and from the linear regions indicated by the least-squares lines in Figure 7 the molecular areas at monolayer collapse were calculated to be 39.4 and 99.8 Å^2 with lecithin and triolein as colipids. For comparison the corresponding data for cholesteryl linolenate are shown in each case. For cholesteryl linolenate, the solubility limits with the two colipids are 0.447 and 0.471 mole fraction, and the calculated molecular areas are 41.0 and 88.8 Å^2. The nonlinearity exhibited by both cholesteryl esters at high mole fractions (low transition pressures) with dioleoyl lecithin is characteristic of the monolayer phase for other cholesteryl esters mixed with this colipid (Smaby et al., 1979).

Average molecular area–composition isobars for mixtures with cholesteryl arachidonate were constructed for each data set in that part of the phase diagram (Figure 3c,d) which appears to correlate with region I of Figure 4. These were linear ($r \geq 0.981$), and extrapolation of each line to pure cholesteryl ester gives the apparent molecular area of cholesteryl arachidonate in the phase at that pressure (Smaby et al., 1979). The apparent force–area isotherms so generated are shown in Figure 8. Also shown in Figure 8 are the apparent force–area curves for cholesteryl linolenate obtained in the same manner from raw data representing region I of the phase diagrams for mixtures with dioleoyllecithin and triolein. With lecithin the two cholesteryl esters exhibit essentially the same apparent force–area curve, whereas with triolein the cholesteryl linolenate curve is somewhat more expanded.

Discussion

The formation of mixed monolayers containing cholesteryl esters is independent of head-group structure but markedly dependent on the structure of the cholesteryl ester acyl moiety (Smaby et al., 1979). In the present study, this work was extended by examining the phase behavior of cholesteryl li-

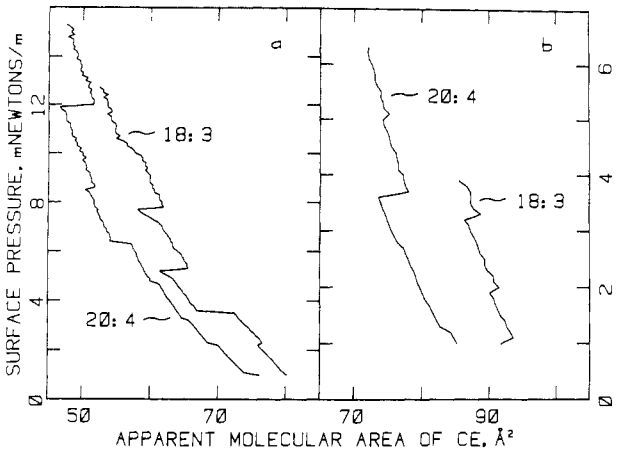

FIGURE 8: Apparent force–area curves for the cholesteryl esters (CE), cholesteryl arachidonate (left) and cholesteryl linolenate (right) with (a) dioleoyllecithin and (b) triolein.

nolenate and arachidonate. In the monolayer region cholesteryl linolenate showed properties essentially identical with the less saturated homologues, whereas cholesteryl arachidonate did not. It was miscible with the colipids in all proportions, and, therefore, at any given mole fraction it exhibits approximately twice as high a concentration in the monolayer phase as the 18-carbon acid esters. Otherwise, its properties are essentially identical with those of the 18-carbon cholesteryl esters in the monolayer phase. The high solubility of cholesteryl arachidonate cannot simply be related to its being isotropic at 24 °C because, above its melting point, cholesteryl linolenate did not exhibit significantly increased solubility in dioleoyllecithin. Under physiological conditions, the increased availability of cholesteryl arachidonate may increase its relative rate of utilization in reactions involving cholesteryl esters in the monolayer phase and for hydrolysis may, therefore, lead to a perferential release of arachidonic acid over oleate, linoleate, or linolenate. This may be of special importance in adrenal cortex in which cholesteryl ester hydrolysis supplies significant amounts of arachidonic acid for prostaglandin synthesis (Chanderbhan et al., 1979) as well as free cholesterol for steroidogenesis (Vahouny et al., 1978).

A second feature of cholesteryl arachidonate phase behavior is its inability to form the double-layer surface phase exhibited by the other cis-unsaturated esters. For cholesteryl linolenate, double-layer phase formation is not eliminated above its melting point, suggesting a relationship between the double-layer phase and bulk cholesteryl ester mesophases. Cholesteryl arachidonate forms neither cholesteric nor smectic mesophases, whereas the oleoyl, linoleoyl, and linolenoyl esters do (Small, 1970). Because our data are consistent overall with the formation of a defined layer of cholesteryl ester above the interfacial layer in the double-layer phase, the data suggest that the structure of the upper layer is related to smectic mesophase structure. Indeed, in lipoproteins this ordered layer of cholesteryl ester adjacent to the surface lipid layer is associated with the presence of a smectic-like structure in the core (Atkinson et al., 1978).

The increased solubility of cholesteryl arachidonate in the monolayer phase and its inability to form the double-layer phase seem not to be related simply to the number of double bonds in the acyl chain but more likely to their location. In building space-filling models of this compound, we noted that if the acyl chain is placed adjacent to the cholesterol ring system, as we envision it in the monolayer phase (Smaby & Brockman, 1980), the double bonds are adjacent to the rings. For the esters with cis unsaturation beginning at position 9,

the double bonds are all in the region of the junction of the cholesterol side chain and its ring system (C_{17}). It will be of interest to study positional isomers of cholesteryl oleate and linoleate to test this hypothesis.

The inability of saturated and trans-unsaturated cholesteryl esters to reversibly form monolayer phases (Smaby et al., 1979) or to form double-layer phases suggests that their incorporation into arterial lipid deposits may be less reversible than for cis-unsaturated esters. It should be noted, however, that the present experiments were confined to binary mixtures of pure compounds. Previous studies of the monolayer phase have suggested that in more complex mixtures these esters might be mobilized by the presence of cis-unsaturated cholesteryl esters (Smaby et al., 1979).

The solubility of cholesteryl esters in lecithin bilayers, as opposed to surface layers on bulk lipid droplets, indicates that only the monolayer phase is present. This conclusion is based on the phase diagrams presented herein and in previous work (Smaby et al., 1979) which indicate that at surface pressures above 30 mN/m only a few percent of the cholesteryl linoleate is solubilized, similar to its measured solubility in bilayers (Janiak et al., 1974). The extremely small bilayer solubility of cholesteryl palmitate and its spin-labeled analogue in other than microcrystalline form (Grover & Cushley, 1979; Valic et al., 1979) is also consistent with our observation that saturated cholesteryl esters do not form mixed surface phases. On the basis of these similarities, we would expect a solubility for cholesteryl arachidonate in synthetic bilayers and natural membranes of between 5 and 10 mol %.

The double-layer phase is formed by cholesteryl myristoleate, oleate, linoleate, and linolenate with all colipids with which they were examined. For 18:1, 18:2, and 18:3, the maximum pressures at which this phase was stable have values of 0.69:0.54:0.37 relative to the collapse pressure of dioleoyllecithin and 0.69:0.60:0.44 relative to triolein. This indicates that double-layer phase formation, like mixed monolayer formation (Smaby et al., 1979), is primarily the result of interactions between the apolar portions of the cholesteryl esters and colipids.

If the proposed model for the structure of the double-layer phase is correct (Smaby & Brockman, 1980), then the values of m for the cholesteryl esters mixed with a given colipid should be similar and the molecular areas of the individual cholesteryl esters in the upper layer, A_u, should be approximately the same. As shown in Table I, these relationships are well obeyed for mixtures with lecithin and reasonably well obeyed with triolein as a colipid. The lack of any trend in A_u values with increasing chain length or unsaturation indicates that the end of the acyl chain is not a prime determinant of packing in the upper layer of the double-layer phase and the values reflect the cross-sectional area of cholesterol within the error of our measurements and fitting procedure. Likewise, the constancy of the collapse areas of the pure cholesteryl esters, ranging only from 24.5 to 25.6 Å^2/molecule, shows that the degree of unsaturation is also not important in the packing of the interfacial layer. As opposed to m values, n definitely increases with increasing unsaturation. Given the constancy of A_u, this indicates a greater solubility of cholesteryl ester in the interfacial layer with an increase in unsaturation.

The transition areas of the cholesteryl esters obtained from slopes of the linear plots of phase and state transition pressures are summarized in Table I and are approximately the same for all esters with lecithin but show more scatter with triolein. This area represents the apparent molecular area of the ester just prior to the transition (Crisp, 1949). If, as suggested (Smaby & Brockman, 1980), the double-layer, pure cholesteryl ester state is metastable, the similarity of the values obtained from the two linear plots is not surprising. At high pressures, double-layer cholesteryl ester is being forced from its association with colipid to a bulk phase, and at lower pressures, the transition is to a surface state. For each case, the cholesteryl ester is in a similar, double-layer state just prior to the transition. The area in this state is the measured parameter (Crisp, 1949). The transition areas are also similar to the collapse area of pure cholesteryl ester. Thus, the transition from the mixed double-layer state to the pure ester double-layer state does not involve a significant rearrangment of cholesteryl ester packing. Rather, the major change in the system is absence of association with colipid. This may explain why we do not observe a discontinuity in our force–area curves concomitant with the state transition.

Our studies have shown that at a lipid–water interface polar lipids can solubilize cis-unsaturated cholesteryl esters. Moreover, if the unsaturation begins at position 9 on the acyl chain, a double-layer phase can also be formed. In this phase, the cholesteryl esters are structured in a layer or layers above an interfacial mixed monolayer of polar lipid and cholesteryl ester. It should be noted that the oleoyl, linoleoyl, and linolenoyl esters of cholesterol which form both monolayer and double-layer phases comprise two-thirds of the cholesteryl esters of lipoproteins and aorta as well as other tissues (Cornwell et al., 1975). In the aorta, fatty streaks are largely cholesteryl oleate in the smectic state, but more advanced plaques contain more unsaturated cholesteryl esters and are isotropic (Katz et al., 1976). The packing arrangements which we have shown are formed by the biologically predominant cholesteryl esters may reflect stages in the conversion of nascent to spherical HDL's or in the formation of arterial inclusions. In the latter case, for example, cholesterol oleate esterified in arterial cell membranes (Brockman, 1979) would at first be incorporated in the leaflets of the bilayer membrane in the equivalent of a monolayer phase. At physiological packing densities, which are equivalent to 30–35 mN/m (Van Deenen et al., 1977), the solubility of cholesteryl oleate would be only a few percent (Figure 1). Continued cholesteryl oleate synthesis could result in the bilayer becoming a "double–double layer". In this regard it should be noted that for cholesteryl oleate–egg lecithin films the mixed double-layer phase is stable up to 38 mN/m (S. G. Bhat and H. L. Brockman, unpublished results) compared to 27 mN/m for the same ester with dioleoyllecithin (Table I). Beyond 60–70 mol % cholesteryl oleate a smectic bulk phase would be formed, cholesteryl ester in the monolayer phase would be absent, and the geometry of the system would become spherical with the inclusion becoming distinct from the parent membrane.

Supplementary Material Available

Surface pressure–area isotherms on which the analysis presented herein is based (13 pages). Ordering information is given on any current masthead page.

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Reversed Cubic Phase with Membrane Glucolipids from *Acholeplasma laidlawii*. ^1H, ^2H, and Diffusion Nuclear Magnetic Resonance Measurements†

Åke Wieslander, Leif Rilfors, Lennart B.-Å. Johansson, and Göran Lindblom*

ABSTRACT: Monoglucosyl diglyceride and diglucosyl diglyceride are the dominant lipids of the *Acholeplasma laidlawii* membrane. Diglucosyl diglyceride forms a lamellar liquid crystalline phase with water while monoglucosyl diglyceride forms a reversed hexagonal phase. Depending on the amounts of unsaturated acyl chains of the lipids, a *mixture* of monoglucosyl diglyceride and diglucosyl diglyceride forms lamellar or reversed cubic phases at physiological temperatures. A high degree of cis unsaturation favors formation of the cubic phase with increasing monoglucosyl diglyceride content. The structure of the cubic phase is composed of aggregates, where the lipids can diffuse over macroscopical distances. A structure containing close-packed spherical *micelles* is therefore ruled out, and the NMR diffusion data are compatible with other previously proposed cubic bicontinuous structures [Luzzati, V., & Spegt, P. A. (1967) *Nature (London) 215*, 701; Scriven, L. E. (1976) *Nature (London) 263*, 123; Lindblom, G., Larsson, K., Johansson, L. B.-Å., Fontell, K., & Forsén, S. (1979) *J. Am. Chem. Soc. 101*, 5465]. Monoglucosyl diglyceride/diglucosyl diglyceride ratios forming cubic phases have not been observed in vivo. It is concluded that formation of the cubic phase is strongly dependent on the molecular shape of the lipids. The results are significant for the physiological regulation of the lipid composition in *A. laidlawii* membranes as well as for the function and organization of biological membranes in general.

All biological membranes contain a number of different lipids (Ansell et al., 1973), most of which form lamellar liquid crystalline phases together with water (Luzzati & Tardieu, 1974). However, exceptions to formation of the bilayer structure have been found for some lipids. Hence it has been shown that phosphatidylethanolamine (PE)[1] from various organisms (Shipley, 1973) and monoglucosyl diglyceride (MGDG) from *Acholeplasma laidlawii* (Wieslander et al., 1978) form reversed hexagonal (H_{II}) mesophases. The only lipid structure compatible with a nonleaky and functioning membrane is the lamellar phase, and lipid bilayer stability thus is of crucial importance for the living cell. It cannot be excluded, however, that other mesophase structures may form within the membrane during short time periods or in the proximity of integral membrane proteins. Local regions forming such structures have been proposed to be advantageous to cell functions like membrane fusion (Lucy, 1975), exo- and endocytosis, and transbilayer movement of lipids (Cullis & De Kruijff, 1979). The very rapid translocation of PE from the inner to the outer leaflet in *Bacillus megaterium* membranes has been shown to be independent of the synthesis of lipid and protein and of sources of metabolic energy, probably excluding a "flip-flop" mechanism (Langley & Kennedy, 1979). Instead, lateral diffusion along transient lipid "hairpin" structures in the vicinity of hydrophilic transmembrane channels is suggested. Since the hairpin bend has a small radius of curvature, it must be stabilized by lipids forming highly curved aggregates (Langley & Kennedy, 1979). However, large amounts of such lipids destabilize the membrane, eventually leading to a membrane disrupture. Therefore, the balance between lipids forming lamellar and nonlamellar phases can only vary within certain limits in order to

† From the Department of Microbiology, University of Lund, S-223 62 Lund, Sweden (Å.W. and L.R.), and Division of Physical Chemistry 2, Chemical Centre, University of Lund, P.O. Box 740, S-220 07 Lund, Sweden (L.B.-Å.J. and G.L.). *Received March 12, 1980; revised manuscript received August 1, 1980.* This work was supported by the Swedish Natural Science Research Council.

[1] Abbreviations used: MGDG, monoglucosyl diglyceride; DGDG, diglucosyl diglyceride; NMR, nuclear magnetic resonance; H_{II}, reversed hexagonal; PE, phosphatidylethanolamine.

0006-2960/81/0420-0730$01.00/0