Lipid structure and the behavior of cholesteryl esters in monolayer and bulk phases

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Abstract The behavior of cholesteryl esters at the air–buffer interface was studied as a function of molecular area and the presence of noncholesterol-containing lipids (colipids). The data obtained indicate that cholesteryl esters with other than long, saturated acyl groups can be present in surface phases up to packing densities approximately those in natural membranes. Their apparent molecular areas in such phases, which are largely determined by colipid structure, suggest their orientation with the ester function toward the interface. The extent of miscibility in the surface phase is also a strong function of colipid structure. Reversibility of the monolayer to bulk phase transition is determined exclusively by the acyl structure of the cholesteryl ester. Of the esters examined, only those with *cis* unsaturation collapsed reversibly. Our data predict that cholesteryl esters should be present in small, but finite amounts on the surface of arterial lipid deposits and that a prerequisite for the removal of such deposits is that the bulk lipid phase be in a liquid or liquid crystalline state.—**Smaby, J. M., W. J. Baumann, and H. L. Brockman.** Lipid structures and the behavior of cholesteryl esters in monolayer and bulk phases. *J. Lipid Res.* 1979. **20:** 789–795.

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The importance of cholesteryl esters in the etiology of atherosclerosis has prompted many studies of their properties in bulk phases. Such studies have demonstrated a clear relationship between the bulk properties of the esters in model systems and the formation of lipid deposits in the arterial wall (1).

We recently reported the results of studies using cholesteryl oleate and triolein in monolayers at the air–buffer interface which showed these compounds to be miscible up to approximately a 1:1 molar ratio (2). The cholesteryl oleate was shown to be oriented with its ester function at the air–buffer interface and the cholesterol and fatty acyl moieties occupied approximately the same cross-sectional areas, and presumably orientation, that they do alone and in triglycerides, respectively.

In this work we extend our initial observations to include a number of cholesteryl esters with different acyl groups in mixtures with other lipids (colipids) of different polar and apolar structures. The results obtained help to define the types of interactions necessary for mixed monolayer formation, the structural features governing the degree of miscibility in the surface phase, and the structural requirements for the transport of cholesteryl esters from bulk to surface phases.

MATERIALS AND METHODS

Reagents

Lipids. All cholesteryl esters, cholesterol, and triolein (trioleoyl glycerol) were purchased from Nu-Chek Prep, Elysian, MN. Egg lecithin was from Grand Island Biological Co., and dioleoyl lecithin (1,2-dioleoyl-*sn*-glycerol-3-phosphocholine) dissolved in chloroform was from Supelco. The concentration of dioleoyl lecithin in the chloroform solution was determined by assaying aliquots for phosphorus. Trioctanoin (trioctanoyl glycerol, Eastman) was purified by distillation, bp 197–199°C at 0.5 mm Hg. 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 neutral lipids were shown to be greater than 99.5% pure.

Dioleyl ethanediol (1,2-di-*O*-cis-9′-octadecenyl ethanediol) was prepared by alkylation (3) of 1-*O*-tetrahydropyranyl (THP) ethanediol (ref. 4; bp 44°C, 0.04 mm) with *cis*-9-octadecenyl methanesulfonate (3), removal of the protective THP group by treatment with methanol–diethyl ether–conc. hydrochloric acid 25:25:1 (by vol) at room temperature, followed by alkylation (3) of the glycol monoether (5) with *cis*-9-octadecenyl methanesulfonate. The dioleyl ether of ethanediol was purified by chromatography on thin layers of Silica Gel H; developing solvent, hexane–diethyl ether 85:15 (v/v), R_f 0.7; mp 14°C (5). ^{13}C NMR at 20 MHz (CDCl$_3$/TMS), 129.8 ppm (CH=CH), 71.5

(OCH$_2$ of chain), 70.2 (OCH$_2$ of diol), 32.0 (ω-2 CH$_2$), 29.8 – 29.4 (CH$_2$ envelope), 27.3 (=CH—CH$_2$), 26.2 (O—CH$_2$—CH$_2$ of chain), 22.7 (ω-1 CH$_2$), 14.1 (ω-CH$_3$).

Spreading solvent. Petroleum ether (bp 60–70°C) was stirred with 98% sulfuric acid for 20 hr and washed once with water, once with 0.1 M sodium bicarbonate, and twice with water. The organic layer was dried overnight over calcium chloride and distilled (65–68°C) from calcium hydride. The product was tested for surface active impurities by spreading 100 μl on to a clean air–buffer interface and recording its force–area curve (see below). No surface pressure could be measured, even at the minimum usable trough area, indicating the absence of surfactant.

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

Experimental procedures

Force–area measurements. Surface pressure–area determinations were made using a Lauda recording film balance (Brinkmann Instruments, Westbury, NY). An air atmosphere was used for all determinations. Within the time of our experiments we saw no evidence for significant oxidation of the lipids employed nor did we detect any aging of the stock solutions over the several days each was used. Furthermore, the surface behavior of cholesteryl linoleate was comparable to that of cholesteryl oleate, presumably a more stable lipid. When not being used, the solutions were stored at −20°C.

The instrument used was a Langmuir type balance in which surface pressure is measured using a floating barrier attached to an inductive linear transducer. Calibration of surface pressure readings was made with reference to the collapse pressures of trioctanoin, triolein, and dioleyl ethanediol as determined from surface tension measurements made using the deNöuy ring method (6). Unless otherwise indicated, lipids were spread in 50 μl of petroleum ether on to a 10 mM potassium phosphate, 0.1 M sodium chloride subphase, pH 6.6, 24°C. After standing at a molecular area of 64–92 Å^2/acyl moiety of the colipid for 3 min, the monolayer was compressed at approximately 15 Å^2/min per molecule of colipid to an area/molecule of 30 Å^2 per acyl group, and then expanded to the original area at the same rate. Force–area curves were obtained by recording surface pressure vs. area/molecule of colipid during the compression–expansion cycle.

Phosphorus determination. Aliquots of dioleoyl lecithin in chloroform were reduced to dryness with N$_2$. Total phosphate in each sample was assayed after perchlorate digestion according to Bartlett (7).

RESULTS

Our previous study (2) described in detail the application of the two-dimensional phase rule (8, 9) to monolayers comprised of triolein and cholesteryl oleate and is a model for the present study. Our results showed that cholesteryl oleate would expand monolayers of triolein in a manner that indicated miscibility of the components in two dimensions (2). Confirming this at temperatures of 24, 30, and 37°C was the linearity of plots of the negative log of mole fraction of cholesteryl oleate vs. surface pressure (critical pressure) for the monolayer phase to collapsed phase transition. That the system was at equilibrium was suggested by the independence of the results from the previous history of the monolayer and the reversibility of compression curves after 30 min in the collapsed state (~90 Å^2/molecule of triolein).

In the present work we have measured compression and expansion force–area curves at the air–buffer interface as a function of composition for triolein, dioleyl ethanediol, trioctanoin, and dioleoyl lecithin in binary mixtures with the oleoyl, linoleoyl, myristoleoyl, octanoyl, stearoyl, and elaidoyl esters of cholesterol. Also studied were the binary mixtures of egg lecithin and cholesteryl oleate and the ternary mixtures triolein–cholesteryl oleate–cholesteryl elaidate, trioctanoin–cholesteryl oleate–cholesteryl elaidate, and triolein–cholesteryl myristoleate–cholesterol. Data were acquired from 0 dynes/cm through the collapse pressure of the colipid except for dioleoyl lecithin curves which were limited to 31 dynes/cm. For all colipids alone the force–area curves were completely reversible; i.e., no irreversible collapse or significant hysteresis was observed.

Monolayer properties. The compression curve for the colipid in each binary mixture was expanded as a direct function of the mole fraction of cholesteryl ester except for mixtures containing cholesteryl stearate, which showed no expansion. Compression curves for cholesteryl linoleate with four colipids are shown in **Fig. 1.** The curves for cholesteryl oleate–triolein mixtures have been presented previously (2) and the other isotherms are available on microfiche.[1] Note that increasing the mole fraction of cholesteryl ester pro-

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[1] The isotherms not shown in Fig. 1 are available through NAPS document #03481 for 44 pages of supplementary material. Order from ASIS/NAPS, Microfiche Publications, P.O. Box 3513, Grand Central Station, New York, New York 10017. Remit in advance, in U.S. funds only, $11.00 for photocopies or $3.00 for microfiche. Outside the U.S. and Canada add postage of $3.00 for photocopy and $1.00 for microfiche.

duces a family of curves similar to those observed with triolein–cholesteryl oleate mixtures (2).

Miscibility of all cholesteryl esters used (except stearate) with all colipids is shown by the linearity of the plots in **Fig. 2** (8, 9). As before (2), the fit of the points was excellent, the lowest coefficient of correlation being 0.998. Note that there is a deviation of the points from the lines with the phospholipid below a critical pressure of 2 dynes per cm, suggesting a more open molecular packing in the monolayer at very low surface pressures. At critical pressures higher than those shown linearity was not observed, presumably because the colipid is partially soluble in the collapsed phase (9). From the data shown in Fig. 2 the area/molecule of each cholesteryl ester at the critical transition and its solubility in each colipid were calculated as previously described (2) and the results are summarized in **Table 1**. Assuming a zero collapse pressure for pure cholesteryl ester (2, 10), the solubility limit is the mole fraction of ester represented by the x-intercept and the slope of the line is the area of the cholesteryl ester at collapse (8, 9).

An alternate way to obtain information on the state of cholesteryl ester molecules in the mixed monolayers is to examine the average molecular area of the monolayer as a function of composition at pressures below the critical pressure. In all cases where cholesteryl ester induced monolayer expansion, such plots were linear, indicating uniform molecular packing over the entire miscibility range. **Fig. 3** shows typical average molecular area–composition plots for cholesteryl linoleate in mixtures with each of the four colipids at a pressure approximately equal to the critical pressure at one-half the solubility limit for each binary mixture. By extrapolating such plots to a mole fraction of one for the cholesteryl ester, its apparent area can be obtained at that pressure. If for each binary mixture a series of such plots is made over a range of surface pressures, the resulting areas can be used to construct an apparent force–area curve for the cholesteryl ester (2). The apparent force–area curves obtained for each of the cholesteryl esters (except stearate) with each colipid are shown in **Fig. 4.** Note the anomalous behavior of the curves obtained with trioctanoin as colipid at low pressures. They suggest that at a surface pressure of approximately 1 dyne/cm there is a marked rearrangement of packing in which the cholesteryl ester greatly increases its area/molecule.

The groupings of curves in Fig. 4 at markedly different areas/molecule suggest that the packing of molecules in the mixed monolayers is governed largely by colipid structure. The effect of introducing free cholesterol into the system was examined with mix-
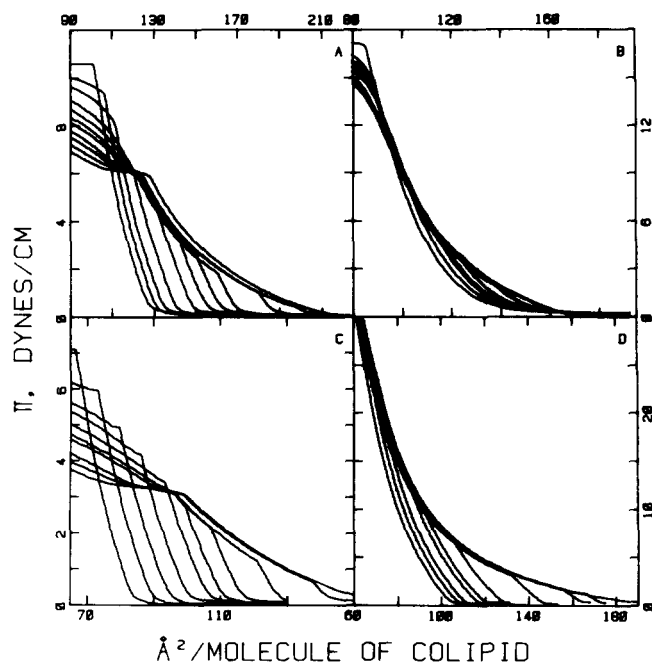

Fig. 1. Force–area curves for cholesteryl linoleate with four colipids. The subphase was 0.01 M potassium phosphate, 0.1 M NaCl, pH 6.6, 24°C. For each colipid the mole fractions of cholesteryl linoleate were, from left to right (a) triolein, 0.0, 0.038, 0.073, 0.136, 0.191, 0.239, 0.272, 0.321, 0.386, 0.440, 0.486; (b) trioctanoin, 0.0, 0.018, 0.036, 0.052, 0.069, 0.095, 0.129, 0.156, 0.181; (c) dioleyl ethanediol, 0.0, 0.056, 0.106, 0.150, 0.191, 0.228, 0.262, 0.321, 0.371, 0.415; (d) dioleoyl lecithin, 0.0, 0.049, 0.093, 0.134, 0.171, 0.236, 0.340, 0.419, 0.452, 0.507.

tures of triolein and cholesteryl myristoleate. The mole fraction of cholesterol plus cholesteryl myristoleate was held constant at 0.13 relative to triolein and force–area curves were obtained with mixtures having free to esterified cholesterol ratios from zero to one. The critical pressures obtained, *plotted as a function of the mole fraction of cholesteryl myristoleate relative to triolein plus cholesteryl myristoleate*, are shown in **Fig. 5** together with comparable data obtained in the absence of free cholesterol. The agreement of the data from ternary mixtures with that obtained with binary mixtures shows that the presence of free cholesterol in the monolayer has no measurable effect on the two-dimensional solubility or packing of cholesteryl myristoleate in triolein monolayers.

Bulk surface transfer. For each compression curve the corresponding expansion curve was also measured. With the compounds employed three types of behavior were observed. The compression–expansion cycle is said to exhibit type A behavior when compression and expansion curves are essentially identical. Reversibility of this type was previously shown for cholesteryl oleate–triolein mixtures (2).

A type B cycle occurs when the cholesteryl ester
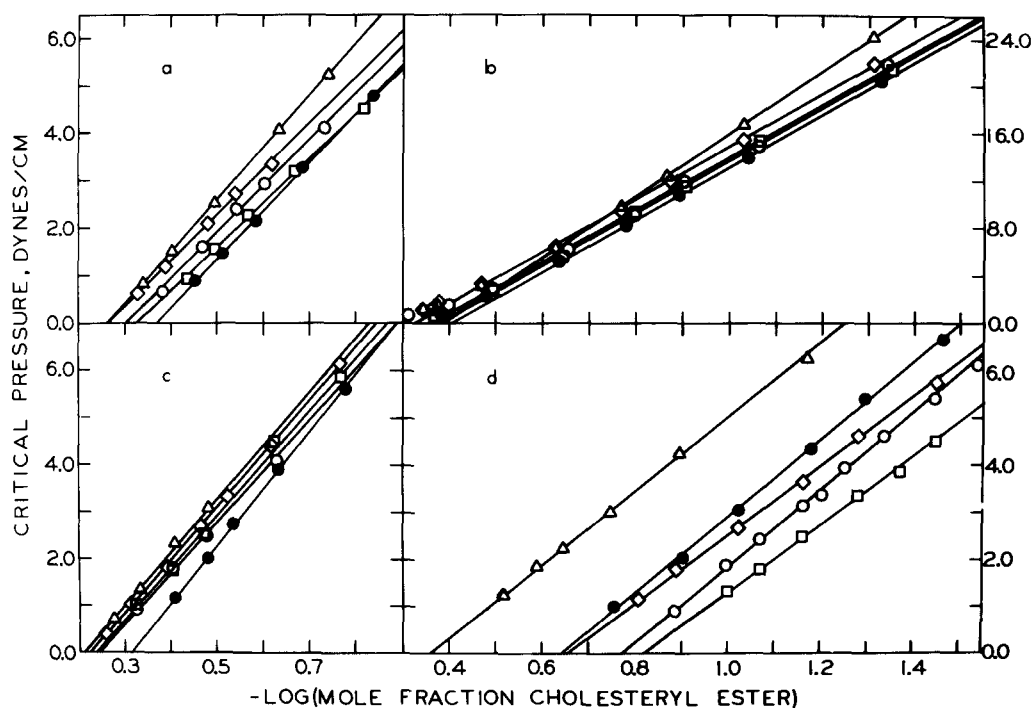
Fig. 2. Critical pressures vs. negative logarithm of the mol fraction of the cholesteryl esters with colipids dioleyl ethanediol (*a*), dioleoyl lecithin (*b*), triolein (*c*), and trioctanoin (*d*). The cholesteryl esters are cholesteryl oleate (O), cholesteryl elaidate (□), cholesteryl linoleate (◇), cholesteryl octanoate (△), and cholesteryl myristoleate (●). Lines were fitted by the method of least squares. Data are taken from sets of force–area curves similar to Fig. 1.

expands the colipid monolayer when spread at large molecular area but causes little or no expansion after the monolayer has been compressed beyond the collapse area for the colipid alone. This behavior indicates that the collapse phenomenon observed at higher pressures is essentially irreversible within the time scale of our experiment, which is approximately 10 min. Decreasing the decompression rate or stopping the decompression at a surface pressure within the monolayer miscibility range did not result in any significant transfer of molecules from the collapsed to the monolayer phase. Thus, bulk phase to surface phase transfer is very slow or the mixed monolayer formed at large area/molecule and low surface pressure is in a metastable state.

Type C behavior is that exhibited by cholesteryl stearate, i.e., there was no miscibility in surface phases, nor did it form any separate, immiscible surface phase. Table 1 shows the type of behavior observed for each colipid–cholesteryl ester pair and for ternary

TABLE 1. Molecular areas, solubility limits and reversibility of collapse for cholesteryl esters in monolayers with noncholesterol-containing lipids
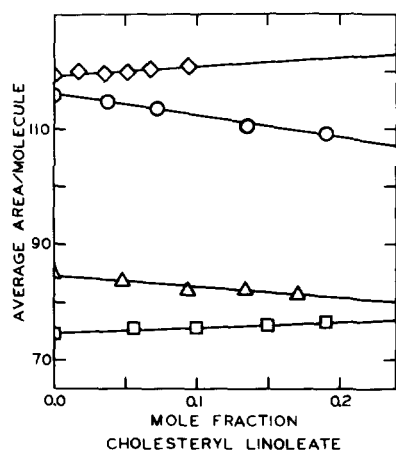
	Colipid											
	Triolein			Trioctanoin			Dioleoyl Lecithin			Dioleyl Ethanediol		
Cholesteryl Ester	Area[a]	Sol.[b]	Rev.[c]	Area	Sol.	Rev.	Area	Sol.	Rev.	Area	Sol.	Rev.
Oleate	87.0	0.452	A	118.5	0.171	A	42.8	0.429	A	96.6	0.393	A
Linoleate	83.8	0.472	A	128.5	0.221	A	42.9	0.474	A	97.6	0.431	A
Elaidate	84.6	0.457	B	130.2	0.151	B	41.4	0.416	B	101.1	0.371	B
Octanoate	83.3	0.489	B	119.7	0.436	B	35.9	0.405	B	85.0	0.430	B
Myristoleate	78.0	0.385	A	116.4	0.229	A	42.5	0.392	A	91.8	0.337	A
Stearate			C			C			C			C
Oleate + elaidate	83.0	0.422	A	132.1	0.149	A						

[a] A²/molecule at the critical pressure as calculated from Fig. 2 (see text).
[b] Solubility limit, mole fraction, as calculated at zero surface pressure from Fig. 2 (see text).
[c] Reversibility type as defined in text.

Fig. 3. Typical plots of average area/molecule for cholesteryl linoleate mixtures with each of the four colipids. Triolein at a surface pressure (π) of 3.5 dynes/cm (O), trioctanoin at 2.5 dynes/cm (◇), dioleyl ethanediol at 3 dynes/cm (□), and dioleoyl lecithin at 7 dynes/cm (△).

mixtures composed of triolein or trioctanoin with a 1:1 mixture of cholesteryl elaidate and oleate.

DISCUSSION

The ability of cholesteryl esters to form mixed monolayers with colipids of different structures shows clearly that no specific head group interactions are required. Both neutral and phosphodiacyl lipids were effective as was a dialkyl neutral lipid. Nor was it necessary to have *cis* unsaturation in the acyl moiety. It should be noted, however, that each of the colipids used in this study exhibits "liquid" type monolayer behavior (11) when spread alone.

For the colipids with Δ9 unsaturation in the acyl moieites, the solubilities of all the miscible cholesteryl esters are essentially identical with an average of 0.422 ± 0.042 (SD). On the other hand, solubilities in trioctanoin are less than 0.23 except for the cholesteryl octanoate–trioctanoin pair which shows a solubility of 0.436. Thus, the solubilities depend largely on acyl structure of the colipid and only slightly on colipid head group structures.

The effect of the structure of the acyl group of the cholesteryl ester on solubility is minimal. For any given colipid, changing the Δ9 unsaturation of the cholesteryl ester from *cis* to *trans* has no significant effect nor, with one exception, does shortening the chain from 18 to 8 carbons and eliminating the double bond. In contrast, reversibility of monolayer collapse shows essentially a complete dependence on the acyl group of the cholesteryl ester; those containing *cis*-unsaturation exhibited identical compression and expansion
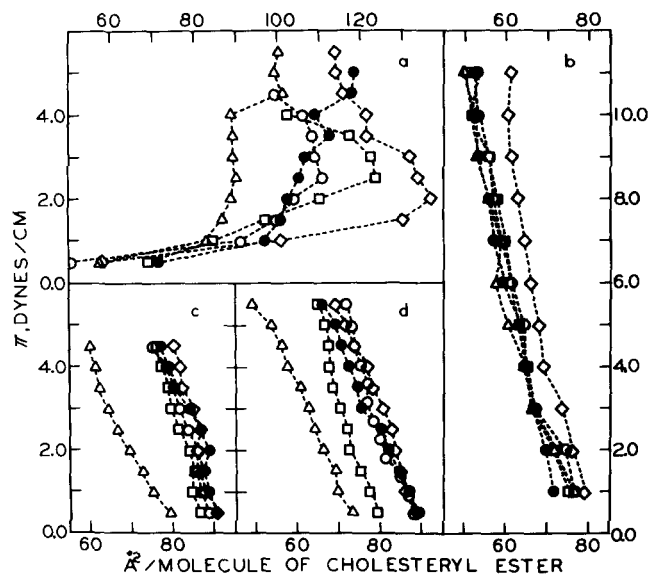
Fig. 4. Extrapolated force–area curves for cholesteryl esters with trioctanoin (*a*), dioleoyl lecithin (*b*), dioleyl ethanediol (*c*), and triolein (*d*). The cholesteryl esters were cholesteryl oleate (O), cholesteryl elaidate (□), cholesteryl linoleate (◇), cholesteryl octanoate (△), and cholesteryl myristoleate (●). The curves were obtained from plots of the type shown in Fig. 3.

force–area curves whereas the octanoate and elaidate esters of cholesterol collapsed irreversibly. The irreversibility exhibited with elaidate was eliminated when cholesteryl oleate was present in a 1:1 molar ratio. These observations indicate that the state of the cholesteryl esters in the bulk phase determines reversibility and are consistent with the studies of Small (12) and Loomis et al. (13) which show that
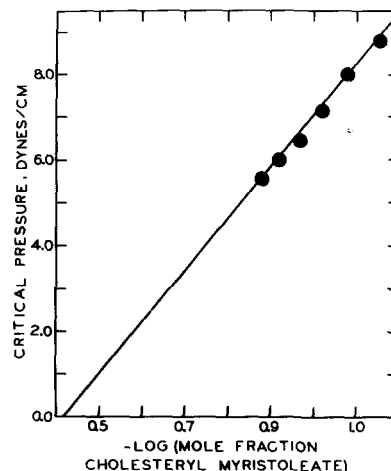
Fig. 5. Critical pressure of cholesteryl myristoleate–triolein mixtures vs. negative logarithm of mol fraction of cholesteryl myristoleate from Fig. 2, *c* is shown by the straight line. The closed circles are the critical pressure of cholesteryl myristoleate–triolein obtained from the force–area curves of cholesteryl, cholesteryl myristoleate, and triolein.

cholesteryl esters containing *cis* unsaturation exhibit low solid to smectic and smectic to isotropic transition temperatures relative to those with more saturated acyl moieties. The results are also consistent with our earlier suggestion (2) that the critical transition measured with cholesteryl oleate–triolein mixtures was to an isotropic or liquid crystalline phase.

The reversibility of our data with cholesteryl esters containing a *cis* unsaturated acyl group does not prove unequivocally that the system is in true equilibrium (14). However, for those curves defined as reversible, the observed reversibility suggests that even if the surface phases are metastable, they are sufficiently long lived to be of possible physiological significance.

The molecular areas of cholesteryl esters (Fig. 4) obtained as a function of surface pressure from average area plots (Fig. 3) are necessarily "apparent" areas, because the monolayer phase does not exist at mole fractions of cholesteryl ester greater than 0.5. For each colipid the apparent areas of the cholesteryl ester at any surface pressure tend to cluster in a narrow range of areas, particularly those with $\Delta 9$ unsaturation. For example, the data for triolein shown in Fig. 4 fall in the range of 70–80 Å^2/molecule whereas with trioctanoin as the colipid, the range is approximately 100–120 at equivalent surface pressures. Note that within each colipid set, cholesteryl octanoate occupies the smallest area, cholesteryl elaidate is larger, and the three esters with *cis*-unsaturation are largest and approximately equal, showing how the acyl group of the cholesteryl ester affects its packing in the monolayer.

Molecular areas were also calculated from mole fractions and measured critical surface pressures as summarized in Table 1. For the four colipids these critical molecular areas were even more tightly clustered with averages ($\pm$SD) of 83 $\pm$ 3, 123 $\pm$ 7, 41 $\pm$ 3, and 94 $\pm$ 4 for triolein, trioctanoin, dioleoyl lecithin, and dioleyl ethanediol, respectively. For the egg lecithin–cholesteryl oleate mixtures the area/molecule from a plot of the type shown in Fig. 2 was 36.1 Å^2/molecule. If these averages are compared to the clusters shown in Fig. 4 at very low surface pressures, the agreement is very good for each set, except that obtained with dioleoyl lecithin.

The unusually small average molecular areas calculated from the log plots obtained with egg and dioleoyl lecithin at higher pressures suggest that near monolayer collapse cholesteryl esters can, like free cholesterol (15), occupy a unique packing arrangement with the phospholipids. Supporting this are the small apparent areas shown in Fig. 4 at the higher pressures. Note that sufficient average area data for calculating areas were available only up to 11 dynes/cm. At

pressures between 20 and 30 dynes/cm the apparent areas would be near the 41 Å^2/molecule calculated from Fig. 2. Thus, our data indicate that, contrary to a recently proposed model (15), the classical cholesterol–phospholipid "condensation" phenomenon does not require hydrogen bond formation between the hydroxyl group of cholesterol and an acceptor on the phospholipid molecule. Supporting this is an earlier investigation of dialkyl lecithin analogs which showed that hydrogen bond formation between cholesterol and acyl oxygen is not essential for condensation to occur (16). It should be emphasized that these observations do not preclude hydrogen bond formation between cholesterol and lecithin, they merely show that it is not required for condensation. That measurements of condensation in monolayers may reflect nonequilibrium conditions due to slow demixing has been recently shown by Tajima and Gershfeld (14). If demixing were sufficiently slow the phenomenon would even appear reversible and could possibly explain why we observed a discrepancy between two methods of calculating the areas for cholesteryl esters in mixtures with dioleoyl lecithin.

The absence of any unique interaction between cholesterol and a nonlecithin colipid was shown with cholesterol–triolein–cholesteryl myristoleate mixtures. As shown in Fig. 5, cholesterol in no way affects solubility or molecular area of the other two lipids.

The magnitudes of the apparent areas and those measured from critical transitions are consistent with the cholesteryl esters being oriented with their ester functions toward the interface and their apolar moieties roughly normal to the plane. For the cholesteryl oleate–triolein and cholesteryl oleate–dioleyl ethanediol pairs, this has been more directly demonstrated by the susceptibility of the cholesteryl ester to attack by a secondary ester hydrolase (2).

Although the mixtures described herein cover only a few of those possible, the results have implications for the study of cholesteryl ester deposition and removal during the formation and regression of the atherosclerotic plaque. That miscibility of cholesteryl esters in monolayers is a general phenomenon, governed largely by colipid structure, increases the value of the system as model for studying the effects of lipid structure on the ability of cholesteryl esters to serve as substrates for the hydrolases necessary for their removal from the arterial wall. Particularly interesting is the ability of lecithin to solubilize cholesteryl ester at packing densities approximating (17) or higher than (18) those believed to exist in biological membranes. This indicates that cholesteryl esters should be accessible to water-soluble enzymes on the surface of atherosclerotic lipid deposits. The

extent of solubility at a more physiological packing density would be only a few mole percent and is consistent with the observed low solubility of cholesteryl esters in phospholipid bilayers (19, 20). Another possibility suggested by our data is that the surface of lipoproteins could also contain a few mole percent of cholesteryl ester, in contrast to most models for lipoprotein structure (21).

Our studies of the reversibility of monolayer collapse show that *cis*-unsaturation in the acyl chain is necessary for cholesteryl esters to be rapidly transported from a bulk phase to the lipid–water interface and that cholesteryl oleate can reverse the collapse of cholesteryl elaidate. This dependence on lipid fluidity in the bulk phase suggests that a liquid or liquid crystalline bulk phase may be a necessity for the transport of cholesteryl esters from the inside to the surface of arterial deposits. Once on the surface, they would then be susceptible to hydrolysis and removal of the more polar reaction products.▮

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