Properties of cholesteryl oleate and triolein in mixed monolayers at the air–water interface

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Abstract The properties of cholesteryl oleate and triolein in mixed monolayers at the air–water interface have been measured between 24 and 37°C. Analysis of force–area curves obtained as a function of the mol fraction of cholesteryl oleate indicates that at relatively low surface pressures these compounds are miscible in two dimensions up to a limit of about 0.5 mol fraction. At higher pressures either cholesteryl oleate or both lipids are expelled from the monolayer to form a bulk phase which is in rapid equilibrium with the surface phase. In the monolayer phase, orientation of the ester function of cholesteryl oleate is toward the aqueous phase, interaction with triolein is minimal, and packing is uniform over the solubility range. This, together with the susceptibility of the cholesteryl oleate to enzymatic hydrolysis, suggests the applicability of monolayer systems to the study of cholesterol esterase activity. Comparison of our results with the bulk properties of these lipids suggests that the expelled cholesteryl oleate exists as a smectic mesophase and thus the system may provide a model for studying the transfer of molecules between the interior and surface of lipid deposits of the type found in atherosclerotic lesions.

Supplementary key words miscibility · phase transition · liquid crystal · cholesterol esterase

Long chain cholesteryl esters are relatively nonpolar molecules (1) which exhibit limited solubility in binary mixtures with more polar lipids such as triglycerides (2, 3) and phospholipids (4). In ternary systems containing a low concentration of water, the solubility of the cholesteryl esters in the lamellar lipid phase is even lower, but it increases when the water content of the system is above 15% by weight. Concomitant studies with polarizing light microscopy, calorimetry, X-ray diffraction, and monolayer expansion suggest that, at the higher water concentration, the cholesteryl ester is located at the lipid–water interface (5). This conclusion is reinforced by enzymatic studies which show that small amounts of cholesteryl oleate incorporated into unilamellar lecithin liposomes are hydrolyzed by extracts from liver and aorta (6, 7).

At an air–water interface the behavior of cholesteryl esters depends largely on the structure of the acyl function. The short chain esters, cholesteryl formate (8) and acetate (9, 10), readily form monolayers which, at low surface pressures, exhibit force–area isotherms quite similar to that of cholesterol alone. This similarity has been taken as an indication that the acyl function protrudes into the aqueous phase and thus does not contribute to the area occupied by the molecule in the monolayer. Cholesteryl butyrate, which has a longer acyl chain, exhibits an unstable isotherm on an aqueous subphase and collapses at 7 dynes per cm (approximately 42 Å² per molecule). Making the subphase 2 M in sodium chloride increases the stability of the film while changing the limiting area only slightly (11). The similarity of these areas to that of cholesterol alone has led to the conclusion that at collapse the butyl group is forced down into the aqueous phase. Increasing the acyl chain to six carbons results in even more unstable films, even on 2 M sodium chloride.

The surface properties of long chain cholesteryl esters, which are more relevant to biological systems, have also been investigated. Both saturated and unsaturated esters yield pressure–area curves when compressed at an air–water interface, but the monolayers are unstable and give limiting areas for these molecules at collapse in the range of 25–30 Å² per molecule, values far below the collapse area of cholesterol alone (9, 10, 12). For the esters containing oleate, linoleate, linolenate, and arachidonate, it has been suggested that the isotherms are produced by oxidation products rather than by the esters themselves (9). In mixed monolayers with more surface active lipids such as lecithin, triglycerides, or cholesterol, long chain cholesteryl esters produce an expansion effect, indicating their presence in the monolayer phase (9, 10, 12). As the mixed monolayers are compressed, the cholesteryl ester is extruded into a bulk phase at a pressure that is dependent upon its solubility in the other lipids.

The relevance of the surface behavior of cholesteryl esters to an understanding of their deposition and

removal from the arterial wall has prompted us to study the expansion effects in more detail. Noting, as previous authors have (10), the behavioral similarities between these lipids and apolar hydrocarbons, we have applied to our data the mathematical treatment employed by Davis, Krahl, and Clowes (13) and Clowes (14), and later by Snart (15), for mixed monolayers containing hydrocarbons. Our results show a two-dimensional miscibility (16, 17) between cholesteryl oleate and triolein in the range from 24 to 37°C and provide information about the arrangement of molecules in both the surface and collapsed phases.

MATERIALS AND METHODS

Reagents

Lipids. Cholesteryl oleate and triolein were obtained from Nu-Chek Prep, Elysian, MN. They had a stated purity of 99+% and gave a single spot when analyzed by thin-layer chromatography with a solvent system of petroleum ether–ether–acetic acid 85:15:1 (v/v/v).

[^{3}H]Cholesteryl oleate was synthesized on a micro scale from oleoyl chloride (Nu-Chek Prep), cholesterol (Nu-Chek Prep), and [1,2-^{3}H]cholesterol (New England Nuclear, lot 853-154, 60 Ci/mmol) as previously described (18).

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 from calcium hydride. The product had a boiling range of 65–68°C and gave no measurable force–area curve when spread at twice the level normally used for force–area measurements (see below).

Cholesterol esterase. The preparation of this enzyme from porcine pancreas has been previously described. The sample used was 94% pure as determined by photodensitometry of stained gels from polyacrylamide gel electrophoresis (18).

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

Experimental procedures

Force–area curves. Surface pressure–area determinations were made using a Lauda recording film balance (Brinkmann Instruments, Westbury, NY). This instrument is a Langmuir type balance in which surface pressure is measured using a floating barrier attached to an inductive linear transducer. 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. After standing at 195–240 Å^2/molecule of triolein for 3 min, the monolayer was compressed at approximately 15 Å^2/min per molecule of triolein to an area/molecule of 90 Å^2, and then expanded to the original area at the same rate. Force–area curves were obtained by recording surface pressure vs. area/molecule of triolein during the compression–expansion cycle.

Surface tension measurements. During enzymatic digestion of mixed monolayers, surface tension was monitored at 24°C using a duNoüy ring attached to a Cahn RG recording electrobalance (19) equipped with a T-Y strip chart recorder. Maximum pull on the ring was maintained by manually moving the balance, mounted on a rack and pinion transport, up and down as required.

Enzymatic digestion of monolayers. Hydrolysis was measured in a circular Teflon trough with a diameter of 4 cm and a volume of 10 ml. For each assay 10 mM phosphate buffer, 0.1 M NaCl, pH 7.5, was added, the surface was cleaned by aspiration, and a solution of triolein and cholesteryl oleate in petroleum ether containing approximately 1.5×10^4 dpm of [^{3}H]cholesteryl oleate was added to give a surface pressure of approximately 4 dynes/cm. After allowing 3 min for complete evaporation of solvent, the solution was magnetically stirred, cholesterol esterase (85 μg in 100 μl of buffer) was added and after 1 min stirring was stopped. After 30 min the monolayer was collected and percent hydrolysis of the cholesteryl ester was determined by thin-layer chromatography–scintillation counting as previously described for the hydrolysis of glycerides by pancreatic lipase (19).

Measurement of radioactivity. Radioactivity was measured using a Packard Tri-Carb liquid scintillation spectrometer and a toluene–Triton X-100-based scintillation fluid (20).

RESULTS

The ability of cholesteryl oleate to increase the apparent area/molecule of triolein in monolayers is shown in **Fig. 1a–c**. Such expansion is in qualitative agreement with previous studies (9, 10, 12) and shows that the cholesteryl ester is occupying space in the monolayer. As the surface pressure is increased in the region from approximately 0.15 to 0.5 mol fraction of cholesteryl oleate, each curve shows a phase transition above which the curves form a common

envelope. This is consistent with the miscibility of cholesteryl oleate and triolein in the surface phase where the phase transition pressure, or critical pressure, is the point at which cholesteryl oleate is ejected to form a bulk phase (16, 17). Although not shown in Fig. 1a–c, the envelope curve approached the abscissa at large molecular areas, showing that the equilibrium spreading pressure of cholesteryl oleate is near zero dynes/cm. The possible existence of a mixed, gaseous phase at large areas is not excluded, but our instrument is not sufficiently sensitive to study this region of the force–area curves.

If the triolein–cholesteryl oleate system behaves ideally, the envelope curve should extend up to and collapse at the same point as triolein. As the figures show, increasing the mol fraction of cholesteryl oleate decreases the collapse pressure toward a lower limit. Similar behavior has been observed for hydrocarbons in monolayers of cholesterol (14) and should indicate the formation at higher pressure of a collapsed phase

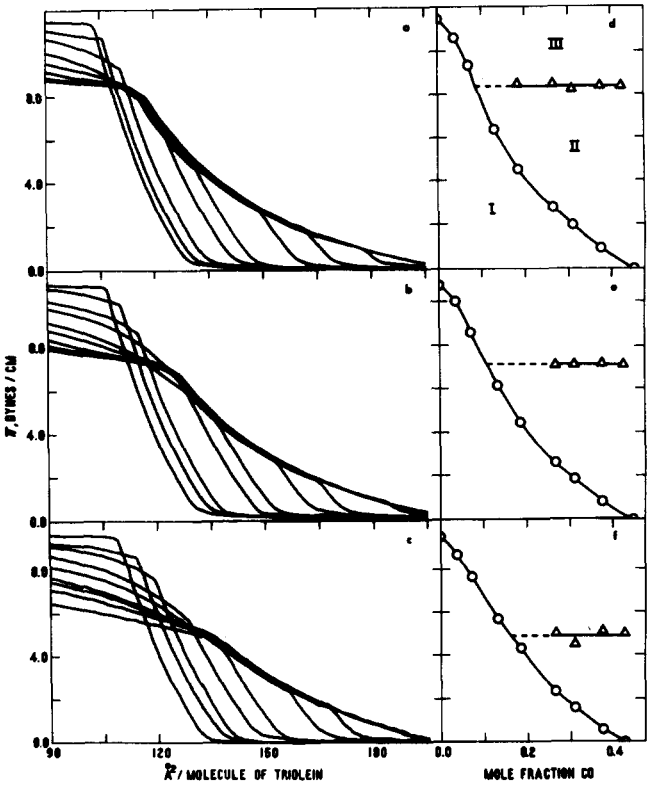

Fig. 1. Force–area curves (a–c) and monolayer phase diagrams (d–f) for cholesteryl oleate (CO) triolein mixtures. Subphase was 0.01 M potassium phosphate–0.10 M NaCl, pH 6.6. Temperatures were 24°C (a and d), 30°C (b and e) and 37°C (c and f). For each set of curves the mol fractions of cholesteryl oleate were, from left to right, 0, 0.037, 0.072, 0.133, 0.186, 0.265, 0.311, 0.374, 0.425, and the number of triolein molecules was held constant at 2.41×10^{16}. Circles (d–f) show critical pressures and triangles are collapse pressures for curves exhibiting two phase transitions. Data are from Fig. 1, a–c.

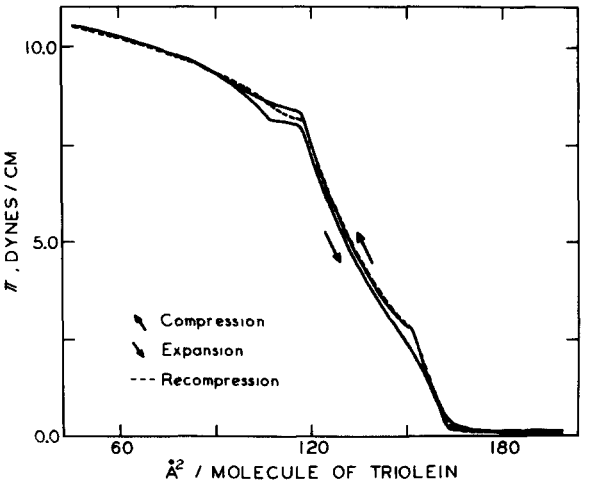

Fig. 2. Reversibility of collapse for a typical cholesteryl oleate–triolein monolayer. Subphase was 0.01 M potassium phosphate–0.10 M NaCl, pH 6.6, 24°C. The mol fraction of cholesteryl oleate was 0.267. Initial compression is upper solid line; expansion curve is lower solid line; and recompression is dashed line.

containing both triolein and cholesteryl oleate (17). A consequence of the bulk phase solubility of these lipids is that the force–area curves obtained at the lower mol fractions of cholesteryl oleate do not show a well defined critical pressure apart from the collapse pressure.

If the interpretation of the data shown in Fig. 1 is correct, the surface and bulk phases should be in equilibrium (16, 17) and the force–area curves should, therefore, be reversible. The curves in **Fig. 2** show that this criterion is fulfilled. A mixed monolayer was formed at an area of 210 Å²/molecule of triolein and then compressed to 45 Å²/molecule. The surface was then expanded to its original area and recompressed. There is a small hysteresis observable in comparing expansion with compression but recompression of the monolayer gives a force–area curve essentially identical to the initial compression curve. The near identity of two compression curves also shows that molecules were not lost during the experiment through barrier leakage or dissolution into the aqueous phase. Expansion curves are not shown for each compression curve shown in Fig. 1, but they were recorded. At 24 and 30°C, results similar to those given above were obtained, whereas, at 37°C, the expansion curves frequently gave values up to 0.5 dyne/cm below zero at large areas. This reflects an observed instability of the zero point at this temperature.

In addition to measurement of reversibility, other control experiments were performed to test the stability and reproducibility of the system. The following changes in procedure had no significant

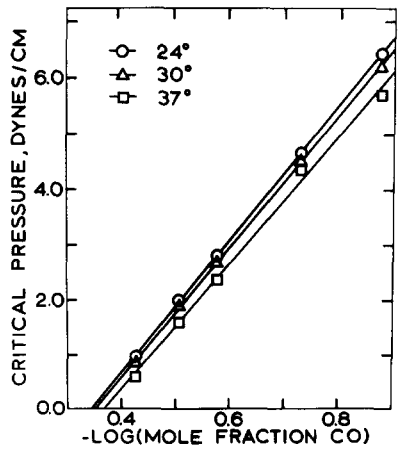

Fig. 3. Critical pressures vs. negative logarithm of the mol fraction of cholesteryl oleate at 24°C (circles), 30°C (triangles) and 37°C (squares). Lines were fitted by method of least squares. Data are taken from Fig. 1, a–c.

effects on either compression or expansion curves obtained at 24°C: *a*) varying the compression speed from 8.0 to 33.6 Å²/min per molecule of triolein; *b*) initially spreading the monolayer at 360 Å²/molecule instead of 195–240 Å²/molecule; *c*) initiating compression after 14 min instead of the usual 3 min; *d*) allowing the monolayer to stand at 90 Å²/molecule of triolein before recording the expansion curves; and *e*) spreading the monolayer at 45 Å²/molecule of triolein instead of 195–240 Å², expanding it to 195 Å²/molecule then compressing it, and reexpanding it. Compression–expansion curves determined at 24°C on several days with different solutions, each containing 0.186 mol fraction of cholesteryl oleate, were reproducible with percent standard deviations for area/molecule of triolein at a given surface pressure of 0.7% for compression and 0.9% for expansion, both below and above the critical pressure. The collapse pressures measured at 33 Å²/molecule of triolein had a percent standard deviation of 0.5%. The pH dependency of the system was obtained by measuring force–area curves for a mixture containing 0.261 mol fraction of cholesteryl oleate on subphases of 10 mM potassium phosphate, 0.1 M NaCl at pH values from 3.5 to 9.5 in 1.0 pH increments. The set of curves obtained (not shown) was essentially identical with percent standard deviations at 3.0 and 7.0 dynes/cm of 1.0 and 1.1 percent. All curves were reversible. Thus, the data from Figs. 1 and 2, together with the control experiments, show that at temperatures from 24–37°C and over a wide range of pH values, cholesteryl oleate and triolein form a miscible surface phase which, within the time course of our experiments, exists in equilibrium with a bulk phase of cholesteryl oleate, or cholesteryl oleate and triolein, depending on the surface pressure.

From the interpretation given above of the data from Figs. 1a–c, a phase diagram can be constructed for each temperature. These are shown in Fig. 1d–f and each shows three distinct regions. In region I, only a single, mixed surface phase is present; in region II, both the surface phase and a cholesteryl oleate bulk phase coexist; and in region III, the surface phase can coexist with a mixed triolein–cholesteryl oleate bulk phase.

For such a miscible system at equilibrium, it has been shown that a plot of the negative $\log_{10}$ of the mol fraction of the expressed component, cholesteryl oleate, vs. the critical pressure should be linear for values of the mol fraction over which the activity coefficient of the expressed component is constant (16, 17). Usually, this occurs near saturation. **Fig. 3** shows that, for mixed monolayers of triolein and cholesteryl oleate, this relationship is obeyed not only near saturation but at all values of mole fraction of cholesteryl oleate above 0.13. The data are from Fig. 1a–c and the coefficient of correlation for each line was ≥0.99. The slope of each line should be $2.303RT/A_c$ where R is the gas constant, T is the absolute temperature and A_c is the molecular area of cholesteryl oleate at its point of expression from the monolayer. In addition, the intercept of the line with the equilibrium spreading pressure of the expressed component gives the solubility limit for that component in the mixed monolayers (16, 17). The data show that pure cholesteryl ester in the monolayer phase does not exhibit a measurable spreading pressure; hence, the negative log of the solubility limit is given by the x-intercept of the line. **Table 1** shows the values of A_c and the solubility limits calculated from the slopes and intercepts of the lines shown in Fig. 3. The solubility decreases only slightly with temperature and, as would be expected, increasing temperature causes an expansion of the area occupied by cholesteryl oleate at collapse.

The free energy required to expel cholesteryl oleate from the mixed monolayer can be calculated for each

TABLE 1. Solubility limit and area/molecule of cholesteryl oleate at the critical pressure for triolein–cholesteryl oleate monolayers

Parameter, °C	Temperature, °C		
	24	30	37
Solubility limit, mol fraction	0.452	0.445	0.427
Å²/molecule of cholesteryl oleate	78.2	81.2	85.6

Data are calculated from the slopes and intercepts of the lines in Fig. 3 as described in the text.

TABLE 2. Work required to eject cholesteryl oleate from triolein–cholesteryl oleate monolayers

Mol Fraction Cholesteryl Oleate	Temperature					
	24°C		30°C		37°C	
	π_c Dynes/cm	ΔF Cal/mol	π_c Dynes/cm	ΔF Cal/mol	π_c Dynes/cm	ΔF Cal/mol
0.133	6.40	720	6.20	725	5.70	702
0.186	4.70	529	4.50	526	4.35	536
0.265	2.80	315	2.65	310	2.35	290
0.311	2.00	225	1.90	222	1.65	203
0.374	0.95	107	0.85	99	0.60	74

Data are taken from Fig. 1, a–c. ΔF was calculated as $NA_c'\pi_c$ where N is Avagadro's number, A_c' is the molecular area of cholesteryl oleate at the critical pressure, π_c. A_c' was calculated as the difference between the molecular areas of the mixed monolayer and triolein alone at π_c, divided by the mol fraction of cholesteryl oleate in the monolayer.

curve from the relationship, $\Delta F = NA_c'\pi_c$, where N is Avagadro's number and A_c' is the molecular area of cholesteryl ester at the critical pressure, π_c (14). **Table 2** shows that, as the mol fraction of cholesteryl oleate in the monolayer is increased, the free energy change decreases from approximately 0.7 to 0.1 Kcal/mol, indicating a relatively weak interaction between cholesteryl oleate and triolein in the monolayer.

Although the system behaves ideally from about 0.1 to 0.5 mol fraction of cholesteryl oleate the points from 0.0 to 0.1 mol fraction do not fit the lines shown in Fig. 3. The deviation is probably due to the solubility of the triolein in the bulk cholesteryl oleate phase, but could be due to the existence of a different molecular arrangement of triolein and cholesteryl oleate in the monolayer at low mol fraction of cholesteryl oleate. To test this hypothesis, we determined the average area/molecule of lipid in the monolayer for 0.5 dyne/cm intervals from 0.5 to 6.0 dynes/cm as a function of the mol fraction of cholesteryl oleate. It can be readily shown that if the packing of the molecules is uniform over the entire solubility range then at any surface pressure below the critical pressure a plot of average area/molecule of lipid vs. mol fraction will be linear, up to a 1:1 ratio of cholesteryl oleate to triolein. Each data set was reasonably linear (coefficient of correlation ≥ 0.97) and **Fig. 4** shows three such plots at 24°C for surface pressures of 1.5, 2.5, and 5 dynes/cm. This linearity indicates that at any surface pressure the packing of molecules in the monolayer is the same over the entire solubility range.

The apparent area per molecule of cholesteryl oleate in the monolayer can be obtained at any pressure by mathematical extrapolation of the line to 1.0 mol fraction. Using the apparent molecular areas determined every 0.5 dynes/cm, a force–area curve for cholesteryl oleate was calculated at each temperature as shown in **Fig. 5** (circles). Shown for comparison (solid line) for each is the predicted force–area curve for cholesteryl oleate which was calculated as the sum of measured area/molecule of cholesterol and one-third of the measured area/molecule of triolein at each pressure and temperature. In each case, the agreement between curves is good, considering the limited number of points, the extrapolation necessary to determine the actual force–area curve, and the implicit assumptions necessary to calculate the predicted curve. This agreement, together with the low free energies of solution, suggests that "condensation" between cholesteryl oleate and triolein is minimal and that the cholesterol and acyl moieties of cholesteryl oleate in the monolayer are oriented as they would be in more polar molecules, i.e., with the ester group facing the aqueous phase and the apolar groups away from it.

To test this orientation hypothesis, we measured the susceptibility of [^{3}H]cholesteryl oleate in the mixed monolayer to hydrolysis of cholesterol esterase from

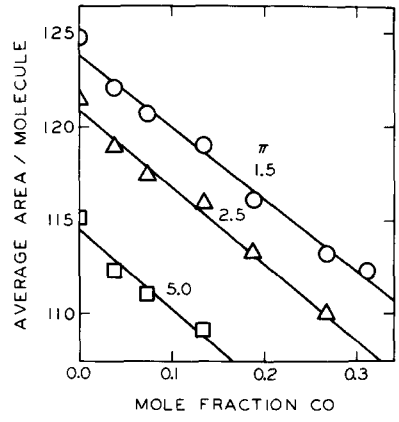

Fig. 4. Typical plots of average area/molecule for triolein–cholesteryl oleate (CO) mixtures at 24°C. Surface pressures (π) were 1.5, 2.5, and 5.0 dynes/cm. See Fig. 1 for experimental conditions and raw data.

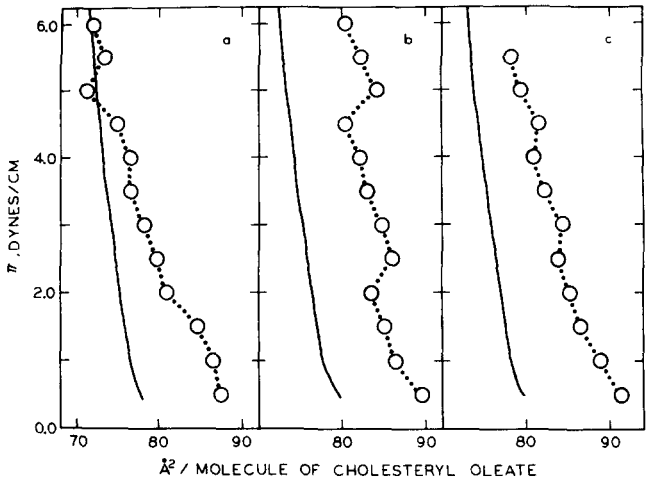

Fig. 5. Extrapolated and predicted force–area curves for cholesteryl oleate. Predicted curves (solid lines) were generated by summing areas/molecule of cholesterol and ⅓ triolein obtained under conditions identical to those of Fig. 1. Extrapolated curves were obtained from plots of the type shown in Fig. 4 as described in the text. Left to right, 24°C, 30°C, and 37°C.

porcine pancreas in the aqueous phase. This enzyme readily hydrolyzes trioctanoin and triolein in monolayers at an air–water interface[1] and should hydrolyze cholesteryl oleate in a mixed monolayer if the ester assumes an orientation at the interface similar to that of the triglyceride. The mol fraction of cholesteryl oleate was 0.186, a value that insured that the initial pressure of 4 dynes/cm was below the critical pressure for that mixture. After 30 min incubation with enzyme at 24°C, 86% of the cholesteryl ester was hydrolyzed, whereas less than 1% was consumed in the absence of enzyme. Essentially identical results (75% hydrolysis) were obtained with dioleyl ethane diol, which forms mixed monolayers with cholesteryl oleate under the same conditions but cannot be attacked by the enzyme. These data support the results of the physical studies and indicate that the ester function is oriented toward the air–water interface as it is for triglycerides.

DISCUSSION

Our results show clearly that cholesteryl oleate and triolein are miscible at the air–water interface. Not only do the combined curves form an envelope, but also the system is completely reversible and independent of the previous history of the monolayer.

The solubility of cholesteryl oleate in triolein approaches 1:1 at all temperatures studied. This value is considerably higher than was observed for the ternary system of lecithin–cholesteryl linoleate–water where one part in 32 of the cholesteryl ester could be dissolved in lecithin bilayers in the presence of excess water (5). This difference in solubility limits probably reflects the difference in packing densities between lecithin in a bilayer phase and triolein in the monolayer system. In the monolayers the highest solubilities are at surface pressures approaching zero dynes/cm whereas in the erythrocyte membrane, a natural bilayer, packing densities are comparable to those in a phospholipid monolayer at 31–34 dynes/cm (21).

As in the bilayer system, our physical and enzymatic data indicate that cholesteryl oleate in the monolayer phase has its ester function oriented toward the aqueous phase. Furthermore, the agreement between the predicted force–area curve for cholesteryl oleate and that from extrapolation indicates that the overall orientation of the molecules differs little from that of cholesterol and the acyl moieties of glycerides under equivalent conditions. Our values of 70–90 Å² per molecule for cholesteryl oleate do, however, differ markedly from the 25–30 Å² per molecule obtained with cholesteryl esters alone or in mixed monolayers containing cholesterol (12), indicating that the nature of the other lipid has a large influence on the state and/or orientation of the cholesteryl ester at the interface.

The linearity of the average area plots shows that the packing of the molecules is uniform over the entire miscibility range and the similar solubility limits and A_c values at 24, 30, and 37°C show that there is no major change in that arrangement with temperature. Likewise, alteration of subphase pH has, as would be expected for a neutral monolayer, a negligible effect. This uniformity of molecular arrangement over a range of experimental conditions together with the ability to vary monolayer composition and packing density independently suggest that monolayer systems of the type described will be valuable tools for studying the hydrolysis of cholesteryl esters by water soluble enzymes.

The reversibility of the force–area curves shows that the molecules in the collapsed phase are in rapid equilibrium with those in the surface phase. This suggests that the cholesteryl oleate collapsed phase exists as an isotropic liquid or mesophase below its normal transition temperatures of 51°C (crystalline to isotropic), 47.5°C (isotropic to cholesteric), and 41°C (cholesteric to smectic) (2). It has been shown that cholesteryl oleate can be dispersed into an excess of water as small droplets of approximately 1 μ diameter. These droplets do not show the cholesteric to smectic transition and the smectic

[1] H. L. Brockman, unpublished experiments.

mesophase is stable at temperatures "far below that of the smectic to crystalline transition" (22). This suggests that, in our system at pressures below the triple point (see Fig. 1d–f), the collapsed cholesteryl oleate exists as a smectic mesophase. The author further notes that the suspension particles are virtually identical to spherulites, a lipid mesophase of predominantly cholesteryl esters, which can be isolated from early atherosclerotic lesions (23), and other authors have shown that liquid or liquid crystalline phases are present in more advanced lesions (24, 25). Thus, mixed monolayer systems may be valuable as models for studying the exchange of molecules between the surface and interior of atherosclerotic lesions as well as for studying the interactions of cholesteryl esters with hydrolytic enzymes.

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