Properties of cholesteryl esters in pure and mixed monolayers

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ABSTRACT The surface properties of cholesteryl palmitate, stearate, linoleate, linolenate, arachidonate, and acetate were investigated. Long-chain esters were not surface-active and force-area (π-A) isotherms were not obtained. Unsaturated cholesteryl esters were oxidized at the air–water interface and these oxidized lipids gave expanded π-A isotherms. Cholesteryl acetate had an equilibrium spreading pressure of 14.0 dynes/cm and formed a stable monolayer indistinguishable from cholesterol below that surface pressure. Cholesteryl linoleate formed mixed monolayers with surface-active lipids, and the amount of cholesteryl linoleate in the monolayer depended both on its solubility in the other lipid and on the surface pressure. Even at moderate surface pressures cholesteryl linoleate was extruded from the monolayer into a bulk phase. Cholesteryl acetate exhibited the well-known condensing effect of cholesterol in mixed monolayers with egg lecithin.

SUPPLEMENTARY KEY WORDS palmitate · stearate · linoleate · linolenate · arachidonate · acetate · surface area · equilibrium spreading pressure · mixed monolayer · condensing effect · stability · oxidation

FREE CHOLESTEROL is an important component of mammalian cell membranes (1) and it is found at other interfaces such as the surface coat of the chylomicron (2, 3). Cholesteryl esters have not been reported as membrane components. Zilversmit (2) and Huang and Kuksis (3) found that cholesteryl esters were localized in the interior lipid core of chylomicrons. These investigators showed that the lipids of the surface fraction of chylomicrons included phospholipids, cholesterol, and triglyceride, but virtually no cholesteryl ester. Cholesteryl esters are generally more soluble than cholesterol in lipid and other nonaqueous phases (4), and these differences in lipid solubility have been suggested by Zilversmit (2) as an explanation for the cholesteryl ester distribution in the interior core of the chylomicron.

The localization of lipids in mixed films at an interface depends not only on solubility but also on surface properties. Two immiscible compounds form a mixed monolayer when both compounds are surface-active. Thus, cholesterol and triolein, which are virtually immiscible (5), form mixed monolayers at the air–water interface (6) because both triglycerides and cholesterol are surface-active molecules (7). On the other hand, a compound which is not itself surface-active may form a mixed monolayer if it is miscible with a surface-active compound. Thus, polycyclic hydrocarbons which do not form stable monolayers are miscible with cholesterol and form mixed monolayers with this compound (6). The surface properties of cholesteryl esters have not been investigated extensively. An early report indicated that the short-chain esters cholesteryl formate and cholesteryl acetate formed stable monolayers (8). Surface properties of long-chain cholesteryl esters have not been described, probably because the somewhat analogous esters of long-chain alcohols and fatty acids do not form stable monolayers (9). We have examined the surface properties of cholesteryl esters as well as the surface properties of films which contained cholesteryl linoleate mixed with the membrane surfactants lecithin, cholesterol, and triglyceride.

MATERIALS AND METHODS

Cholesterol was purchased from Applied Science Laboratories Inc. (State College, Pa.) or the Hormel Institute (Austin, Minn.), cholesteryl esters and triglycerides from the Hormel Institute, and egg lecithin from General Biochemicals (Chagrin Falls, Ohio). The egg lecithin gave a single spot for choline phosphoglycerides on Silica Gel H developed in chloroform–

methanol–water 65:25:4 (v/v/v). *n*-Hexane was purified as previously described (10). All lipids were dissolved in hexane, and their concentrations were determined by cholesterol (11), fatty acid ester (12), or phosphorus (13) analyses. Water was double distilled from glass into polyethylene containers and had a specific conductance of 1.6×10^{-6} mho.

Surface pressure (π) was measured by the Wilhelmy plate technique (14), utilizing a Cahn RG recording balance and a Varian model 20 recorder. The Langmuir trough, inner dimensions $1 \times 9.8 \times 50$ cm, was milled from a solid Teflon block. A platinum foil 1 cm wide was used as the dipping plate. The movable bar was milled from Teflon and was propelled by a high-torque, variable-speed motor. The trough and weighing assembly were enclosed in a Plexiglas case. The π-A isotherms were generated at a compression rate of from 2–5 A^2 per molecule per min. All measurements were made at the ambient temperature.

RESULTS

The surface properties of both saturated and unsaturated long-chain cholesteryl esters were studied. π-A isotherms typical of lipids such as cholesterol or unsaturated fatty acids and their methyl esters were not generated with the unsaturated esters cholesteryl linoleate, linolenate, and arachidonate (Fig. 1). Very low and variable surface pressures were obtained when these compounds were spread as hexane solutions on the Langmuir trough and the trough surface was compressed to a small area. When cholesteryl linoleate and linolenate were applied to the trough as solids, surface pressures of from 1–2.5 dynes/cm were obtained after equilibration of from 19–24 hr. An appreciable π, 23 dynes/cm, was obtained with solid cholesteryl linoleate after equilibration was continued for 66 hr, and a high π was maintained when the dipping plate was removed and cleaned. Expanded π-A isotherms were generated when the unsaturated cholesteryl esters were spread as hexane solutions and equilibrated for 16 hr at a calculated surface area of 100 A^2/molecule prior to compression (Fig. 1). High surface pressures were maintained in these experiments when the dipping plate was removed and cleaned after compression.

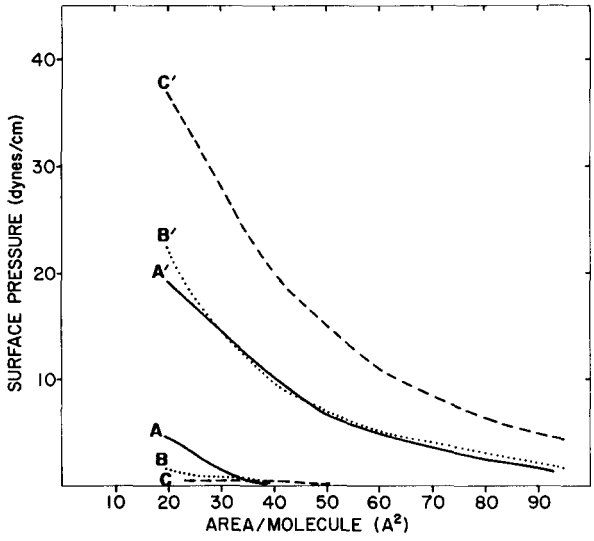

FIG. 1. π-A isotherms for cholesteryl esters spread on 0.01 M sodium chloride at the ambient temperature, 25–27°C. Cholesteryl linoleate (*A*), linolenate (*B*), and arachidonate (*C*) were spread and compressed immediately, or the same esters (*A′*, *B′*, *C′*) were first equilibrated for 16 hr at 100 A^2/molecule and then compressed.

The saturated esters cholesteryl palmitate and stearate behaved in a strikingly different manner from the unsaturated cholesteryl esters. No surface pressures were generated when these compounds were spread as solids and equilibrated for a number of hours. When cholesteryl palmitate and stearate were spread as hexane solutions and either compressed immediately or first equilibrated at 100 A^2/molecule for 16 hr, rigid and sometimes cloudy films which tilted the dipping plate appeared as the films were compressed. Large surface pressures were generated between 20 and 10 A^2/molecule but π decreased on standing and, in contrast to the unsaturated cholesteryl esters, π decreased to zero when the dipping plate was removed and cleaned after compression.

The interactions between cholesteryl linoleate and surface-active lipids were investigated with mixed films of cholesteryl linoleate and cholesterol, triolein, or egg lecithin (Fig. 2). At low surface pressures the surface-active molecules in the mixed films apparently occupied larger areas than they occupied in pure films. The expanding effect of cholesteryl linoleate decreased as π increased and cholesteryl linoleate had little effect on the films as they approached collapse. The expanding effect of cholesteryl linoleate on egg lecithin and triolein films was very much greater than the expanding effect of cholesteryl linoleate on a cholesterol film.

The surface properties of short-chain cholesteryl esters were quite different from those of long-chain cholesteryl esters. Cholesteryl acetate formed a typical monolayer. π-A isotherms for cholesteryl acetate (Fig. 3) and cholesterol (Fig. 2) were similar until π approached the 13–16 dynes/cm region. At this point cholesteryl acetate underwent a phase transformation and on further compression gave an unexpected high π and low surface area phase. When excess cholesteryl acetate was spread as a hexane solution, the film generated an equilibrium spreading pressure, π_E, of 14.0 dynes/cm. Subsequent experiments showed that cholesteryl acetate films were metastable at pressures above π_E, and these

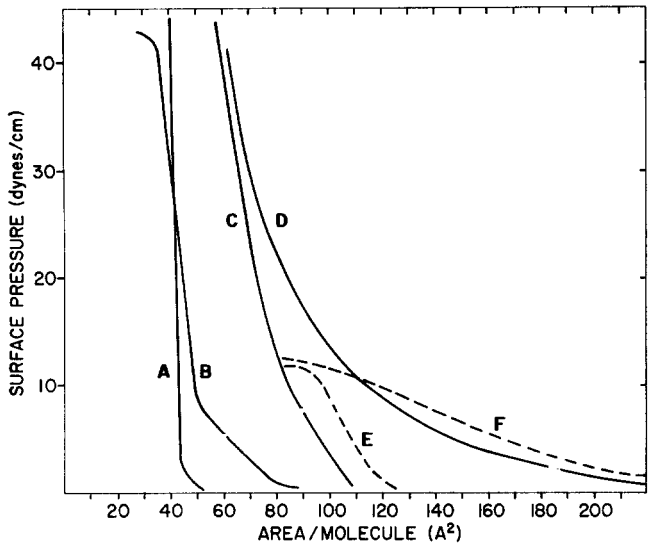

FIG. 2. π-A isotherms for cholesterol (A) and an equal molar mixture of cholesterol and cholesteryl linoleate (B), egg lecithin (C) and an equal molar mixture of egg lecithin and cholesteryl linoleate (D), triolein (E), and an equal molar mixture of triolein and cholesteryl linoleate (F). Surface areas were calculated from the number of cholesterol, egg lecithin, or triolein molecules applied to the trough. Lipids were spread on 0.01 M sodium chloride at the ambient temperature.

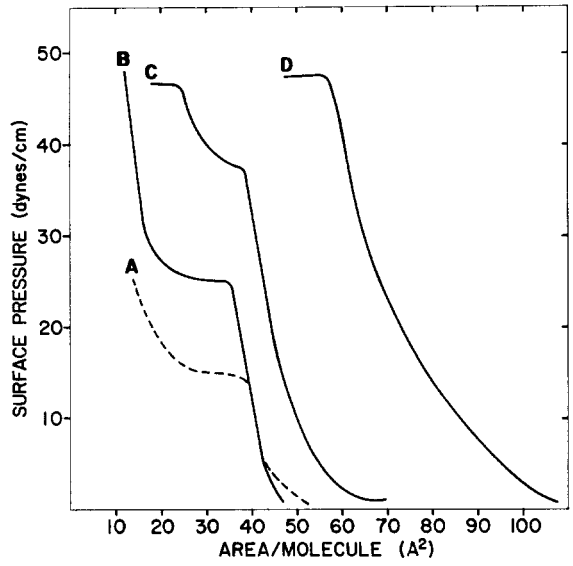

FIG. 3. π-A isotherms for cholesteryl acetate (A), mixtures of egg lecithin and cholesteryl acetate where the mole fraction of egg lecithin was 0.25 (B) and 0.50 (C), and egg lecithin (D). Surface areas were calculated from the total number of egg lecithin and cholesteryl acetate molecules applied to the trough, and represent mean molecular areas. Lipids were spread on 0.01 M sodium chloride at the ambient temperature.

films collapsed toward π_E whenever compression was stopped above π_E.

When cholesteryl acetate was mixed with egg lecithin the shape of the π-A isotherm was altered (Fig. 3). The mixed monolayers exhibited phase transformations, and the π at which these phase transformations occurred decreased and approached the π_E for cholesteryl acetate as the mole fraction of cholesteryl acetate in the mixture was increased.

The mean molecular areas for the molecules in the cholesteryl acetate–egg lecithin films were measured at two π values, 5 and 10 dynes/cm, below the π_E for cholesteryl acetate. Mean molecular areas were also obtained from the equation (7):

$$A_{12} = N_1A_1 + N_2A_2 \qquad \text{Eq. 1}$$

where A_{12} is the calculated mean molecular area in the mixed film, N_1 and N_2 are the mole fractions of the two components, and A_1 and A_2 are the molecular areas of the two components in pure films. Mean molecular areas calculated from this equation were greater than the experimental mean molecular areas found with mixed films (Fig. 3), and it appeared that cholesteryl acetate condensed the egg lecithin monolayer (Fig. 4).

DISCUSSION

The esters of long-chain fatty acids and alcohols do not have sufficient polarity to form monolayers (9). Cholesteryl esters behave in the same manner. Thus, π_E values for cholesteryl palmitate and stearate were zero, and when hexane solutions of these compounds were spread and compressed the films developed the rigid and cloudy characteristics of scum-like multilayers. π-A isotherms were not generated with unsaturated cholesteryl esters; however, these compounds did generate surface pressures and form expanded curves after they had been equilibrated at the air–water interface for a number of hours. We attribute these expanded curves to autoxidation of the unsaturated fatty acid moieties of the cholesteryl esters because stable surface pressures and expanded curves were not generated with saturated cholesteryl esters. An alternative explanation, the hydrolysis of cholesteryl esters, is unlikely for the following reasons. Unsaturated fatty acids such as linoleic acid are relatively soluble in an aqueous subphase and rapidly dissolve out of a monolayer into the subphase (15). Thus, cholesteryl ester hydrolysis would yield cholesterol–cholesteryl ester mixtures, and the π-A isotherms of these mixtures (Fig. 2) are quite different from the expanded π-A isotherms of unsaturated cholesteryl esters (Fig. 1). Small amounts of oxidized cholesteryl ester may explain the very low and variable surface pressures which were generated when these compounds were spread from hexane solutions and compressed immediately to small surface areas (Fig. 1).

Early studies with polycyclic hydrocarbons by Harkins and Morgan (16) and Davis, Krahl, and Clowes (17),

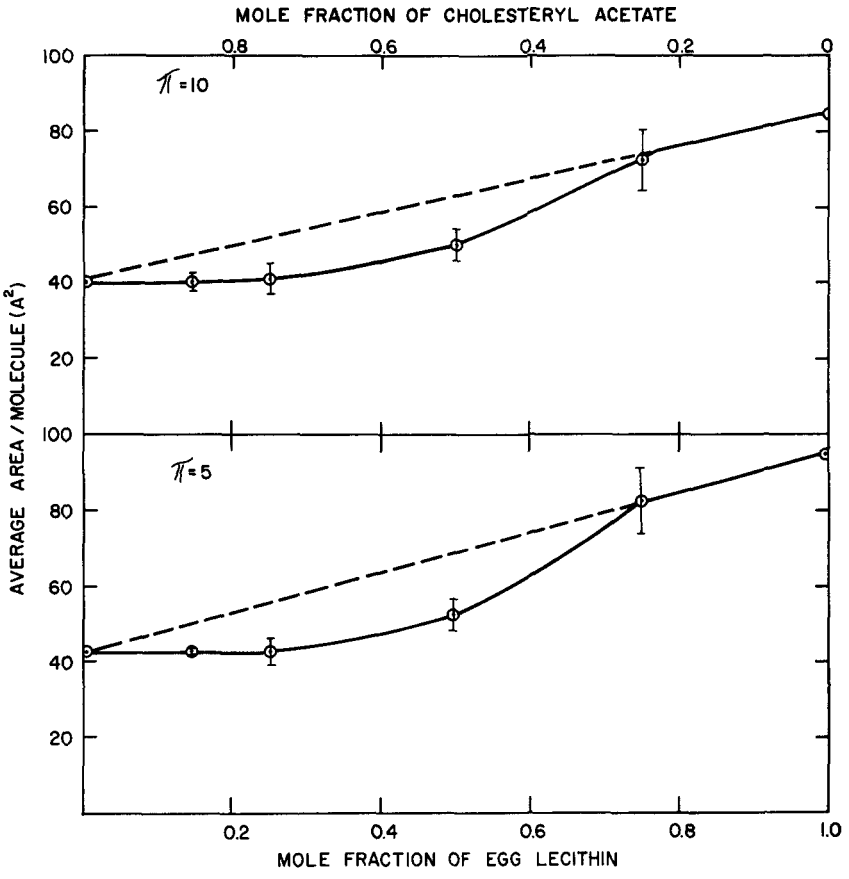

FIG. 4. Variation of mean molecular area with composition and π for mixed monolayers of cholesteryl acetate and egg lecithin. Mean molecular areas were calculated from pure films by Equation 1 (- - -) or were measured with mixed films (——) in the film balance (see Fig. 3 for typical measurements).

and a recent study by Snart (18), showed that hydrocarbons which did not themselves form monolayers expanded cholesterol, cholestanol, stearic acid, and lecithin monolayers. Cholesteryl linoleate behaves in the same manner as the polycyclic hydrocarbons. Mixed films of cholesteryl linoleate and egg lecithin or triolein are greatly expanded at low surface pressures (Fig. 2). Cholesteryl linoleate is apparently squeezed out of the mixed monolayer during compression, generating the envelope curve predicted from the application of a two-dimensional phase rule by Crisp (6) to a mixed monolayer in the presence of an excess bulk phase containing one component. This tendency for cholesteryl esters to form a bulk phase may explain in part the location of cholesteryl esters in the lipid core of the chylomicron and the formation of amorphous cholesteryl ester globules in atheroma. Small (5) has recently suggested that these globules represent cholesteryl esters in a cholesteric liquid–crystalline phase.

Cholesteryl linoleate expanded the cholesterol monolayer much less than it expanded either triolein or phospholipid monolayers (Fig. 2), and indeed the limited expansion of the cholesterol monolayer may represent the contribution of oxidized cholesteryl linoleate (Fig. 1) rather than the formation of a miscible cholesterol–cholesteryl linoleate film. These data suggest that cholesteryl linoleate is much less soluble in cholesterol than in lipids such as triolein or egg lecithin. Solubility data for a different cholesteryl ester, cholesteryl oleate, have been obtained by Small (5) from condensed-phase diagrams, and his studies show that cholesteryl oleate, like cholesteryl linoleate, is much less soluble in cholesterol than in triolein at the ambient temperature. These data show that mixed monolayers are formed with immiscible components only if both components of the mixture, for example cholesterol and triolein (6), are surface-active. Since melting curves for cholesteryl oleate mixtures in condensed phases are now available, further studies on the properties of mixed cholesteryl oleate films may provide experimental verification for the two-dimensional phase rule proposed by Crisp (6) and later utilized by Snart (18).

In early studies, Adam and Jessop (8) found that the short-chain esters cholesteryl formate and acetate formed monolayers indistinguishable from cholesterol. We found, in contrast to Adam and Jessop, that cho-

lesteryl acetate films were stable and indistinguishable from cholesterol only up to a surface pressure of 14.0 dynes/cm, the π_E for cholesteryl acetate, while cholesterol films were stable at much higher pressures in the 36–39 dynes/cm region. The anomaly which exists between our results and those of Adam and Jessop suggests that these workers used a faster compression rate and compressed the cholesteryl acetate film beyond its collapse point (10).

The similarity in cross-sectional area between short-chain cholesteryl esters and cholesterol in the stable parts of the π-A isotherms may be explained by the vertical orientation of the ester group into the subphase so that the molecular dimensions of the ester are determined by the cholesterol portion of the molecule. This orientation was suggested by Alexander and Schulman (19) for the acetate esters of long-chain alcohols. The unexpected high π and low surface area phase observed with films of pure cholesteryl acetate (Fig. 3) may be explained by the formation of multilayers analogous to the multilayers observed by Larsson, Lundquist, Ställberg-Stenhagen, and Stenhagen (20) when ethyl stearate was compressed beyond its collapse point. These multilayers may also explain the unstable high π and low surface area phase for cholesteryl palmitate and stearate.

Mixed films of cholesteryl acetate and egg lecithin showed both a decrease in the monolayer collapse point (Fig. 3) and a condensing effect (Fig. 4). The decrease in the collapse point and the extrusion of cholesteryl acetate into a bulk phase at higher surface pressures is characteristic of partially miscible films as predicted by Crisp (6). The condensing effect in the stable or miscible part of the π-A curve is characteristic of cholesterol in mixed films (21) and further supports the hypothesis that the acetate group is vertically oriented into the aqueous subphase.

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