Bile Acid/Phosphatidylcholine Interactions in Mixed Monomolecular Layers: Differences in Condensation Effects but Not Interfacial Orientation between Hydrophobic and Hydrophilic Bile Acid Species†

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ABSTRACT: Monomolecular layers of undissociated bile acids and membrane lipids at the air/water interface serve as useful models for the interactions between fully ionized bile salts and physiological membranes. Employing an automated Langmuir−Pockels surface balance, surface pressures and dipole moments were measured as functions of molecular area for six dihydroxy bile acids: ursodeoxycholic acid (UDCA), deoxycholic acid (DCA), chenodeoxycholic acid (CDCA), hyodeoxycholic acid (HDCA), allodeoxycholic acid (alloDCA), and 7α,12α-dihydroxycholanoic acid (7,12-OH-DCA), individually and in mixed monomolecular layers with 1-palmitoyl-2-oleoyl-*sn*-3-glycerophosphatidylcholine (POPC) with and without cholesterol on a 5 M NaCl subphase at pH 2. In general, monolayers of hydrophilic bile acids (UDCA, HDCA) had lower collapse pressures and surface dipole moments than hydrophobic bile acids (CDCA ≈ DCA ≈ alloDCA < 7,12-OH-DCA). In binary mixtures with POPC, all bile acids including the synthetic 7,12-OH-DCA and the uncommon alloDCA condensed mixed monomolecular layers, with the degree of condensation correlating positively with bile acid hydrophobicity. In contrast, none of the bile acids caused further condensation of condensed POPC/cholesterol monomolecular layers. Surface dipole moments of binary bile acid/POPC ± cholesterol mixtures demonstrated strict additivity, implying that no change in molecular orientation of the interface occurred over film compositions that varied from 0 to 100% bile acid. We conclude that the long axes of the steroid nuclei of dihydroxy bile acids remain parallel to the interface in mixed bile acid/POPC ± cholesterol monomolecular layers under all conditions. We infer that fully dissociated hydrophobic and, to a lesser extent, hydrophilic bile salts condense phospholipid monomolecular layers and membranes in a similar fashion.

Monomolecular layers (monolayers) of biological lipids can be studied as models for biological membranes. With knowledge of the precise number of molecules at a defined air−water interface, measurements of the lateral pressure exerted by molecules can allow determination of amphiphile orientation, as well as the nature of the surface phases present (Gaines, 1966). Surface chemistry techniques have established that undissociated bile acids orient with their sterol nuclei parallel to the aqueous interface, in a manner that allows their hydrophilic groups to interact with water (Ekwall et al., 1957; Miyoshi et al., 1991; Shibata et al., 1991; Small, 1971). Studies of bile acid/phosphatidylcholine mixed monolayers demonstrate that mixtures of these two dissimilar molecules do not behave ideally, in that average molecular areas are not a weighted average of molecular areas of the two components (Minoñes Trillo et al., 1968; Small, 1971). Because of the apparent small cross-sectional areas of bile acids when mixed with phosphatidylcholine monolayers, it was proposed that ionized bile salts are oriented with their long axes perpendicular to the membrane surface (Small, 1971). Bile salts could then form reverse dimers or tetramers in biological membranes, with their hydrophilic surfaces in apposition, and their hydrophobic surface interacting with the acyl moieties of long-chain phospholipids (Small, 1971). Cholesterol is well-known to interact with phosphatidylcholine monolayers and bilayers (Phillips, 1972), with its steroid nucleus oriented perpendicularly to the interface. Cholesterol condenses the phosphatidylcholine monolayer and decreases the apparent cross-sectional areas of phospholipid molecules (Chapman et al., 1969).

We hypothesized that monolayers of undissociated bile acids mixed with POPC;[1] with and without cholesterol would provide insights into the physical chemical interactions of fully ionized bile salts with biological membranes and their differing cytotoxic and cytoprotective effects *in vivo* (Coleman, 1988; Heuman et al., 1991a,b; Kanai et al., 1990). Specifically, we examined how bile acids interacted with POPC ± cholesterol monolayers, and by inference physiological membranes, and determined whether hydrophilic and hydrophobic bile acids differed in their interactions with a typical membrane lipid. To compare the mechanisms of interactions of hydrophobic and hydrophilic bile acids with

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-3-*sn*-glycerophosphatidylcholine; UDCA, ursodeoxycholic acid; DCA, deoxycholic acid; HDCA, hyodeoxycholic acid; CDCA, chenodeoxycholic acid; alloDCA, allodeoxycholic acid; 7,12-OH-DCA, 7α,12α-dihydroxycholanoic acid; π−*A*, surface pressure−area.

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physiological membranes, we have examined these interactions systematically employing an automated Langmuir–Pockels surface balance. We demonstrate that the degree of monolayer condensation decreased in the rank order deoxycholate > chenodeoxycholate > hyodeoxycholate > ursodeoxycholate, in proportion to bile acid hydrophobicity (Heuman, 1989). We have also studied allodeoxycholic acid, which differs from deoxycholic acid in having a trans rather than a cis A/B ring junction in the sterol nucleus, and the nonphysiological 7α,12α-dihydroxycholanoic acid, which, unlike all naturally occurring bile acids (Hagey, 1992), lacks an hydroxyl group in the 3-carbon position. These systematic studies challenge the concept that bile acids self-associate within the monolayer as "reverse micelles" (Small, 1971). In contrast, we demonstrate that, at all relative proportions of POPC molecules, dihydroxy bile acids remain parallel to the air–water interface.

EXPERIMENTAL PROCEDURES

Materials. Deoxycholic acid (DCA; 3α,12α -dihydroxy-5-β-cholanoic acid) was obtained from Aldrich Pharmaceuticals (Milwaukee, WI). Chenodeoxycholic acid (CDCA; 3α,7α-dihydroxy-5β-cholanoic acid) was obtained from Pharmazal Diamalt (courtesy of Dr. Herbert Falk, Freiburg, Germany). Ursodeoxycholic acid (UDCA; 3α,7β-dihydroxy-5β-cholanoic acid) was obtained from Sigma Chemicals (St. Louis, MO). Hyodeoxycholic acid (HDCA; 3α,6α-dihydroxy-5β-cholanoic acid) was obtained from Calbiochem (La Jolla, CA). 7,12-OH-DCA (7α,12α-dihydroxy-5β-cholanoic acid) was synthesized at Calbiochem circa 1970. 1-Palmitoyl, 2-oleoyl-*sn*-glycero-3-phosphatidylcholine (POPC) was purchased from Avanti Polar Lipids (Alabaster, AL). All bile acids and POPC gave a single spot on thin layer chromatography (butanol/acetic acid/water, 10:1:1, 200 μg application) with the exception of 7,12-OH-DCA, which was 98% pure. Allodeoxycholic acid (alloDCA; 3α,12α-dihydroxy-5α-cholanoic acid) was a generous gift of Dr. Alan F. Hofmann (San Diego, CA). Cholesterol (Nu-Chek, Austin, MN), its reduced derivative cholestanol, and cholanoic acid (both from Sigma Chemicals) were greater than 99.9% pure by GLC. All HPLC grade solvents were shown to be free of surface-active impurities by control surface film compressions. Talc (Merck) and sodium chloride were roasted at 600 °C for 6 h to remove oxidizable and surface-active impurities. Water was double-distilled and filtered through activated charcoal. Glassware was alkali/acid washed overnight in EtOH/2 N KOH (50:50 by volume) and in 1 N HNO_3, followed by thorough rinsing in distilled water.

Spreading Solutions. Approximately 400 mg of a bile acid or POPC was dissolved in 10 mL of methanol or chloroform and filtered through a 0.22 μm filter (Millipore, Bedford MA). Dry weights were obtained in triplicate at 1 min intervals over 4 min, with extrapolation to zero time. The calculated dry weight error (99% confidence interval) was within 1%. To prepare spreading solutions, a 200 μL aliquot of a stock solution was diluted to 50 mL with hexane/ethanol (3:1, v/v). From an ~0.4 mM solution, a spreading volume of ~170 μL produced an initial area of 200 Å^2/molecule, as calculated from the total surface area, spreading solution molarity, spread volume, and Avogadro's number. Between applications of a spreading solution, syringes were flushed thrice with chloroform/methanol (1:1). Spreading solutions were stored at −5 °C for up to 2 months and were allowed to come to room temperature before use. For bile acid/POPC mixtures, volumes of stock spreading solutions necessary to produce 1 mL of total solution were dispensed with a Hamilton syringe into a 2 mL volumetric flask and mixed immediately prior to spreading.

Surface Pressure and Surface Potential Measurements. To prevent bulk solubilization of dihydroxy bile acids, studies were carried out on a aqueous subphase containing 5 M NaCl, pH 2.0. After water was titrated to pH 2.0, roasted NaCl was added to achieve the desired final concentration, which was checked by dry weight. Preliminary experiments demonstrated that the pH of water was not appreciably altered by addition of NaCl. All surface balance studies were conducted at 22 °C, with the exception of measurements of CDCA and UDCA multilayers, which were carried out at 37 °C by means of a circulating thermostated water bath. For studies of the behavior of POPC as a function of pH, subphase pH was varied from 1.3 to 10 and utilized immediately after preparation.

Isothermal compression of monomolecular films was carried out with an automated Langmuir–Pockels surface film balance (KSV, Oy, Finland), consisting of a 15 × 54 cm Teflon trough and an electrobalance interfaced with a digital computer. Surface tension was measured with a platinum Wilhelmy blade of 19.6 ± 0.02 mm in width, and 1 cm in depth (generous gift of Dr. Jean-Claude Montet, Marseille, France). After rinsing with distilled water and heating to incandescence, the Wilhelmy blade was fully wetted and positioned so that one-quarter of the blade was submerged to ensure a 0° contact angle. Following spreading of the lipid solution, 5 min was allowed for solvent evaporation, and then the spread film was compressed at 12 Å^2 molecule^{-1} min^{-1}. In preliminary studies, pressure–area (π–A) isotherms of pure bile acids as well as binary and ternary mixtures with POPC and cholesterol obtained at other compression rates (6 and 18 Å^2 molecule^{-1} min^{-1}) did not show variation with compression rate or hysteresis, indicating near-equilibrium experimental conditions. Collapse pressures were reproducible to within 0.2 mN/m. Isotherms were reproducible consistently to within 1% (area at 2.5 mN/m), and from one to three isotherms were obtained for each bile acid/POPC ± cholesterol mixture, with representative isotherms shown in Results. To monitor viscosity qualitatively, roasted extraclean talcum powder was sprinkled on the surface during monolayer compression and a fine jet of air directed at the talc was used to assess particle movement.

Surface potential was measured with a home-built polonium-210 air electrode (NRD, Inc. Grand Island NY), a KCl reference electrode, and a Model PHMM64 pH/mV meter (Radiometer America, Inc., Westlake OH), with an analog to digital interface (Data Translation, Marlboro, MA) to an IBM personal computer for data acquisition. After equilibration for 10 min, a baseline value of surface potential (V) for a clean water subphase was measured. Surface dipole moment (ΔV), which normalizes surface potential per molecule, was calculated from measured surface potential and molecular area (Gaines, 1966):

$$\Delta V(\text{mD}) = \frac{V\,(\text{mV}) \times \text{molecular area (Å}^2)}{12\pi}$$

Experimental Design. Monolayers of binary bile acid and POPC mixtures were studied at relative compositions that

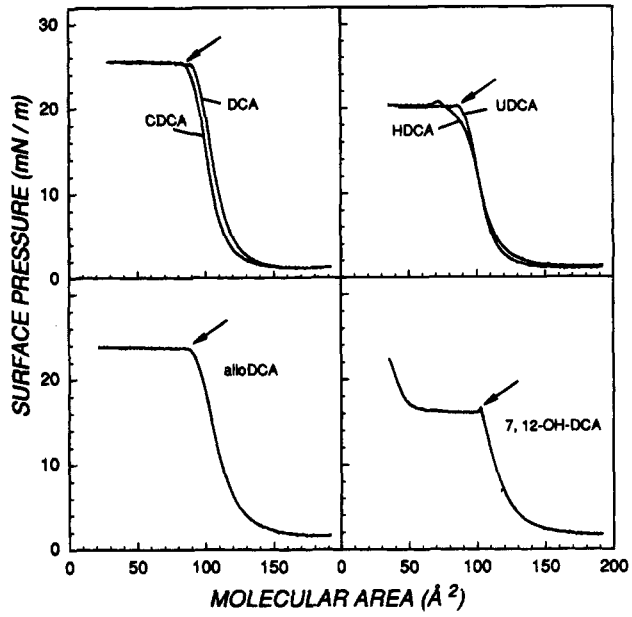

FIGURE 1: Representative π–A isotherms for monolayers of CDCA, DCA, HDCA, UDCA, alloDCA, and 7,12-OH-DCA on a 5 M NaCl, pH 2, subphase at 22 °C. As the bile acids films are compressed, liftoff occurs at an area of between 145 and 130 Å^2/molecule. With further compression, the surface pressure rises steeply to pressures between 16 and 25 mN/m. At the arrows, the monolayers collapse, with formation of multilayers of bile acids at the surface. Because two phases coexist at the interface, further compression does not lead to an increase in surface pressure while the more expanded phase is still present. Surface monolayers and multilayers remained fluid throughout compression, except for HDCA and the 7,12-OH-DCA, which solidified at low molecular areas at 22 °C. As discussed in footnote 3, when compressed beyond the collapse point, all the bile acids crystallize into surface multilayers when sufficient time is allowed to elapse, especially at 37 °C.

varied from 0 to 100 mol % bile acid. Various proportions (0–100 mol %) of bile acid were also studied in mixtures with POPC/cholesterol premixed in 1:1 or 2:1 molar ratios. To compare the effects of cholesterol with those of bile acids, binary mixtures of cholesterol and POPC in compositions that varied from 0 to 100 mol % POPC were also examined.

Bile Acid Hydrophobicity Index. Retention times of the six unconjugated bile acids were determined by reversed phase HPLC, with an evaporative light scattering detector (Varex ELSD II, MD). The method of Roda and colleagues (1992) was modified slightly, with the use of 15 mM ammonium acetate, pH 5.4 in MeOH/H_2O (75:25, v/v), and a solvent flow rate of 0.5 mL/min at 22 °C. Approximately 5 nmol of each bile acid was injected, and the hydrophobic index was calculated (Heuman, 1989), utilizing values of 0.0 for taurocholate and 1.0 for taurolithocholate. Standards included the taurine and glycine conjugates and unconjugated forms of cholate, DCA, CDCA, and UDCA, all as sodium salts (Sigma or Calbiochem).

Data Analysis. For a bile acid/POPC ± cholesterol monolayer, surface pressure was recorded as a function of the average molecular area of the species present, as calculated from the number of molecules spread on the surface and the known surface area of the trough. Data were analyzed using a spreadsheet program to determine the average molecular area when the surface pressure initially reached a fixed value, e.g., 2.5 mN/m. Extrapolations of the average molecular area of bile acids or cholesterol in POPC monolayers were calculated from binary additivity curves by linear least-squares analysis of the portions of the curves with ≥85 mol % POPC.

Table 1: Monolayer Collapse Pressures and Areas at Collapse for Steroids Spread on 5 M NaCl, pH 2.0, 22 °C

bile acid or sterol	collapse pressure (mN/m)	area at collapse (Å^2/molecule)
DCA	25	91
CDCA	25	86
UDCA	20	87
HDCA	20	86[a]
alloDCA	23	91
7,12-OH-DCA	16	102
cholesterol	>40	37[b]
cholestanol	>40	37[b]
cholanoic acid	15	49[c]

[a] Because there was no sharp collapse point in this π–A isotherm, the area at collapse was taken to be the intercept of the extrapolated vertical and horizontal portions. [b] Values agree with Demel and colleagues (1972a) (water, pH 5.4, 20 °C) and Ekwall and colleagues (1957) (3 M NaCl, pH 2.0, 22 °C). [c] This value differs from that of Ekwall and colleagues (1957), who obtained a collapse pressure of 20 mN/m and a collapse area of 41 Å^2 (3 M NaCl, pH 2.0, 22 °C).

Molecular Models. Three-dimensional models of each bile acid, POPC, and cholesterol were produced using the software ChemDraw and Chem3DPlus (Cambridge Scientific, Cambridge MA). Atomic coordinates were used to calculate the solid angles between the carbon–oxygen bonds for the two hydroxyl groups of each bile acid.

RESULTS

Monolayer Stability. CDCA and UDCA transfer into the bulk subphase was assessed by compressing monolayers to a pressure of 13 mN/m and observing the surface pressure over 60 min.[2] The surface pressure of CDCA decreased from 13 to 12 mN/m, whereas in the case of UDCA, surface pressure decreased from 13 to 8 mN/m, indicating a very slow solubilization rate into the 5 M NaCl, pH 2.0, subphase.

Dihydroxy Bile Acid Monolayers. Figure 1 displays π–A isotherms, reflecting the dependence of surface pressure on molecular area, for the six dihydroxy bile acids studied. All bile acids show similar characteristics: liftoff occurs at ~130–145 Å^2/molecule, and all collapse to surface multilayers (shown by arrows) at pressures between 16 and 25 mN/m. Molecular areas at collapse lie between 86 and 102 Å^2/molecule (see Table 1) and are consistent with the sterol nucleus lying flat at the interface (Demel et al., 1972a; Small, 1971). Areas at collapse of hydrophilic bile acid (UDCA, HDCA) monolayers were lower than those of the hydro-

[2] Attempts to study trihydroxy bile acid monolayers were unsuccessful. As was found in earlier studies (Minoñes Trillo et al., 1968; Miyoshi et al., 1991; Shibata et al., 1991; Small, 1971), π–A isotherms for α–muricholic, cholic, ursocholic, and hyocholic acids all lacked a pressure-invariant two-phase region below 80 Å^2/molecule with a subphase of 5 M NaCl at pH 2, indicating that significant bulk bile acid solubilization occurred. Since precise knowledge of the number of surface molecules is required, this negated interpretation of additivity curves (Llopis et al., 1973; Small, 1971). We hypothesized that a 7.8 M LiCl subphase, because of the larger hydrated radius of the lithium ion, might more effectively salt out hydroxyl and carboxyl groups and render trihydroxy bile acids insoluble. However, a pressure-invariant two-phase region in the π–A isotherm was not observed for the trihydroxy bile acids under these conditions. Slopes of the π–A isotherms did not correlate with bulk solubilities of the trihydroxy bile acids.

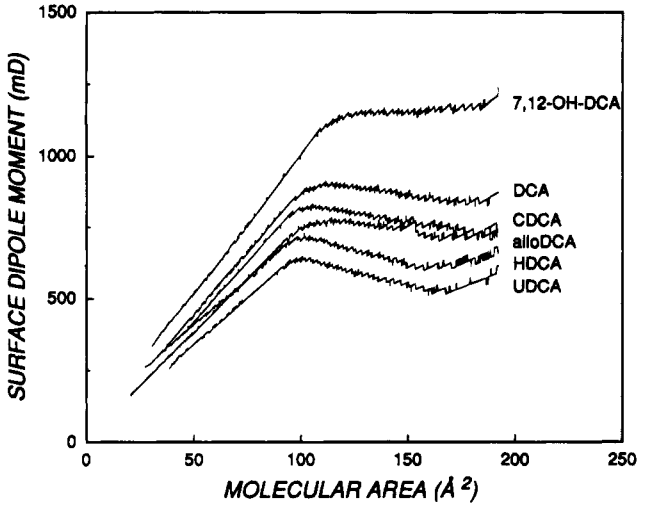

FIGURE 2: $\Delta V-A$ isotherms for monolayers of CDCA, DCA, HDCA, UDCA, alloDCA, and 7,12-OH-DCA on a 5 M NaCl, pH 2, subphase at 22 °C. As the monolayers are compressed, the dipole moments do not change appreciably until the collapse point is reached at a molecular area of ~100 Å². Following collapse, the dipole moments decrease linearly, presumably because pairing in the multilayers cancels the dipoles. At all molecular areas, the synthetic 7,12-OH-DCA has the largest dipole moment, whereas the hydrophilic bile acids (UDCA, HDCA) have the smallest.

phobic bile acids, CDCA and DCA as noted previously (Carey et al., 1981; Miyoshi et al., 1991), although molecular areas at collapse did not correlate with the hydrophobic indexes (see below). In contrast, monolayers of cholesterol, cholestanol, and cholanoic acid had much smaller areas (37, 37, and 49 Å², respectively) at collapse (Table 1), consistent with previous observations (Chapman et al., 1969; Small, 1971) indicating that the steroid nuclei are oriented perpendicularly to the interface. Whereas monolayers remained fluid throughout compression, all bile acid multilayers crystallized when held at a constant area above the collapse pressure for a sufficient length of time.[3]

Figure 2 depicts surface dipole moments as functions of molecular area for all six bile acids. Surface dipole moments did not vary substantially during monolayer compression at larger molecular areas (200−100 Å), but following monolayer collapse, as determined by surface pressure measurements (Figure 1), the surface dipole moment decreased linearly with decreasing molecular area, apparently reflecting cancellation of molecular dipole moments in multilayers composed of alternating layers of bile acids (Ekwall et al., 1957; Gaines, 1966; Small, 1971). Bile acids with an equatorial hydroxyl function (HCDA, UDCA) exhibited smaller dipole moments than their α-axial congeners. Further, the 5αH (A/B trans) junction of alloDCA generated a smaller dipole moment than DCA, which has a 5βH (A/B cis) ring junction.

Mixed Monomolecular Films with POPC. Figure 3 displays selected $\pi-A$ isotherms of binary mixtures of UDCA with POPC, varying from 0 to 100 mol % POPC. At all pressures, mixed monolayers were in a two-

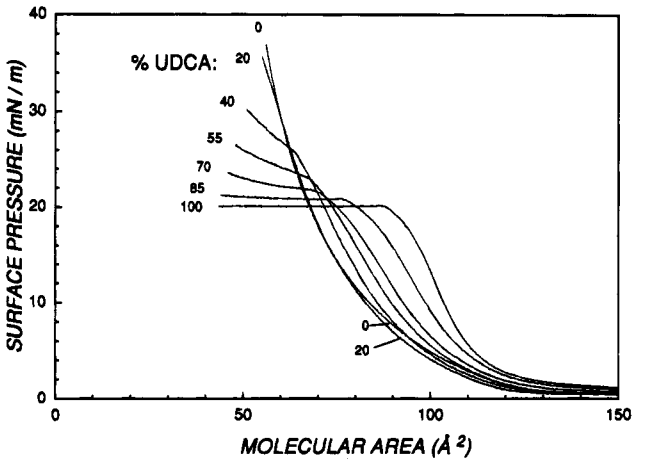

FIGURE 3: $\pi-A$ isotherms of monolayers of binary mixtures of UDCA and POPC ranging from 0 to 100 mol % UDCA, spread on a 5 M NaCl, pH 2, subphase at 22 °C. The 20 mol % UDCA curve is shifted to the left of the 0 mol % UDCA $\pi-A$ isotherm because of monolayer condensation by UDCA. As the relative mole percent of POPC rises, the collapse pressure of UDCA increases, implying intimate miscibility of both molecular species.

dimensional liquid state uniformly. As the relative UDCA content decreases (labeled curves), the collapse pressures of UDCA increase, e.g., 21 mN/m at 85 mol % UDCA. In contrast, the $\pi-A$ isotherm of POPC alone does not show a collapse point below 40 mN/m. Isotherms of other dihydroxy bile acid/POPC mixtures showed similar or larger increases in collapse pressure as relative POPC content was increased (data not shown). When the mole fraction of UDCA increases to 20% at low pressures (<10 mN/m), the $\pi-A$ isotherm shifts to the left, that is, to smaller molecular areas for all surface pressures. Therefore, despite addition of UDCA, which has a larger surface area than the phospholipid, the POPC monolayer becomes condensed.

Figure 4 depicts the average molecular area for binary UDCA/POPC and DCA/POPC monolayers as functions of mole percent POPC at fixed surface pressures (2.5−40 mN/m). In the absence of bile acid/POPC interactions, the average molecular area would be a weighted average of the values for the pure components, as shown by the dashed lines for ideal mixtures of POPC and DCA or UDCA at 2.5 mN/m. For all surface pressures displayed, there is a negative deviation from ideality, indicating that the mixed monolayer is condensed as compared with the individual pure components. The deviation from ideality is larger for the hydrophobic bile acid DCA than for the hydrophilic bile acid UDCA. With increasing surface pressure, average molecular areas decrease, and the negative deviation from ideality is less pronounced for both bile acids (Figure 4). Above 20 mol % bile acid at surface pressures of 30 and 40 mN/m, the binary additivity curve becomes concave downward. Since the precise number of molecules in the surface film is unknown above the collapse point, molecular areas are artifactually small at high surface pressures for mixtures with higher bile acid contents.

Figure 5 shows the average molecular areas for mixtures of CDCA, HDCA, 7,12-OH-DCA, and alloDCA with POPC at a surface pressure of 2.5 mN/m. As illustrated by the negative deviation of the average molecular areas from additivity (dashed lines), all dihydroxy bile acids condense POPC monolayers. In each panel, the four points nearest the POPC axis (85−100 mol % POPC) were extrapolated

[3] Surface crystallization, as reflected by an abrupt decrease in surface pressure to approach the equilibrium spreading pressure, occurred after 20 and 45 min at 37 °C with UDCA and CDCA, respectively. Surface viscosity (see Methods) revealed a fluid surface both prior to and after the decrease in surface pressure, suggesting that crystalline islands of bile acids coexisted within a fluid monolayer film.

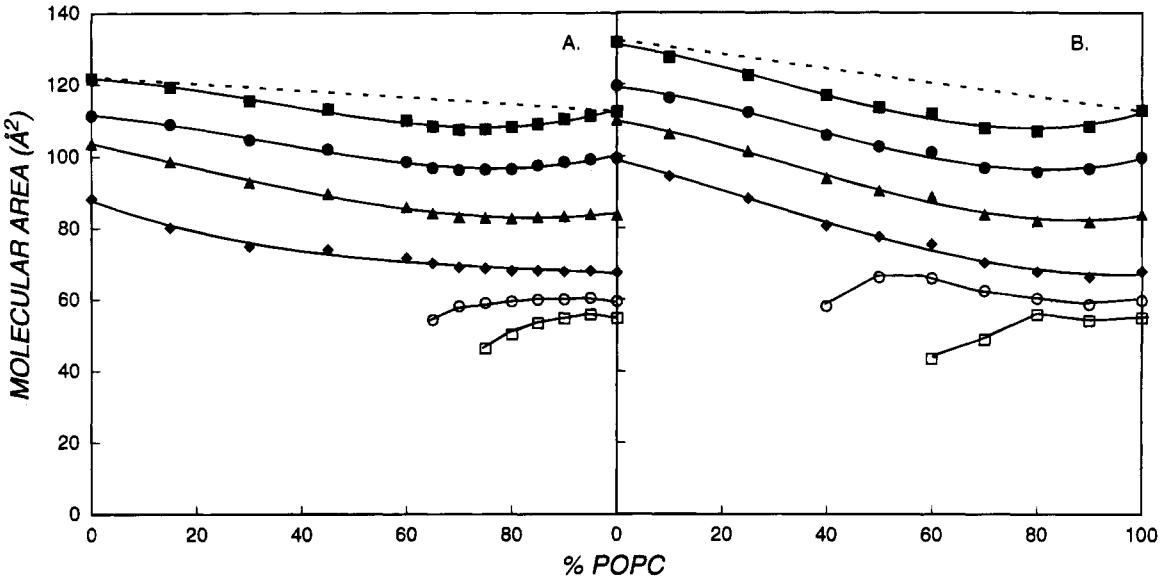

FIGURE 4: Dependence of average molecular area on relative bile acid composition, as determined from $\pi-A$ isotherms of binary mixtures of (A) UDCA/POPC and (B) DCA/POPC for various surface pressures: ■, 2.5; ●, 5.0; ▲, 10; ◆, 20; ○, 30; and □, 40 mN/m. For ideal mixtures, the average molecular area would be the weighted average of the areas of the pure components, as shown by the dashed lines for a surface pressure of 2.5 mN/m. At all pressures there is a negative deviation from ideality, implying that DCA more than UDCA condenses the POPC monolayer. Because bile acid-rich monolayers collapse at high pressures (30−40 mN/m), average molecular areas could not be determined for all compositions. Curves shown are third-degree polynomial fits.

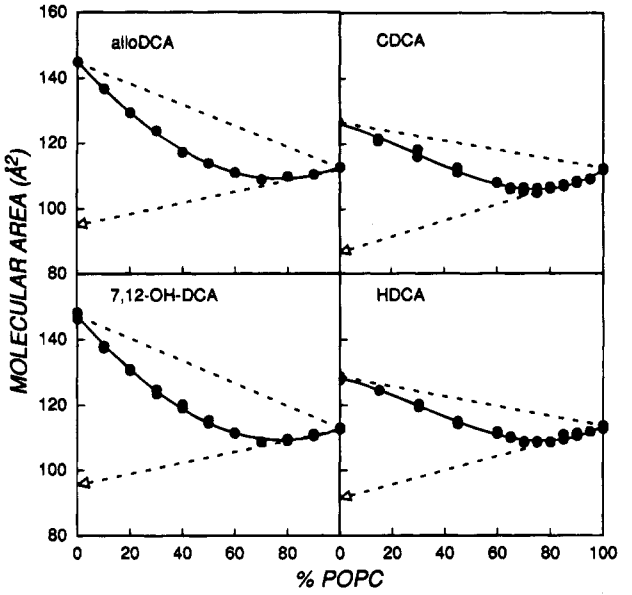

FIGURE 5: Area-additivity curves for POPC with CDCA, HDCA, 7,12-OH-DCA, and alloDCA. Molecular areas are plotted against mole fraction of POPC at a fixed pressure of 2.5 mN/m. At this relatively low pressure, the monolayer is in a liquid state. Upper dashed lines indicate perfect additivity of the molecular area. All bile acids show a negative deviation from ideal additivity lines. In each panel, the initial four points nearest the POPC axis (85−100 mol % POPC) are extrapolated to the opposite axis, indicated by the dashed arrow. This intercept on the bile acid axis is the "effective area" per bile acid molecule in the POPC-rich region (implications discussed in text).

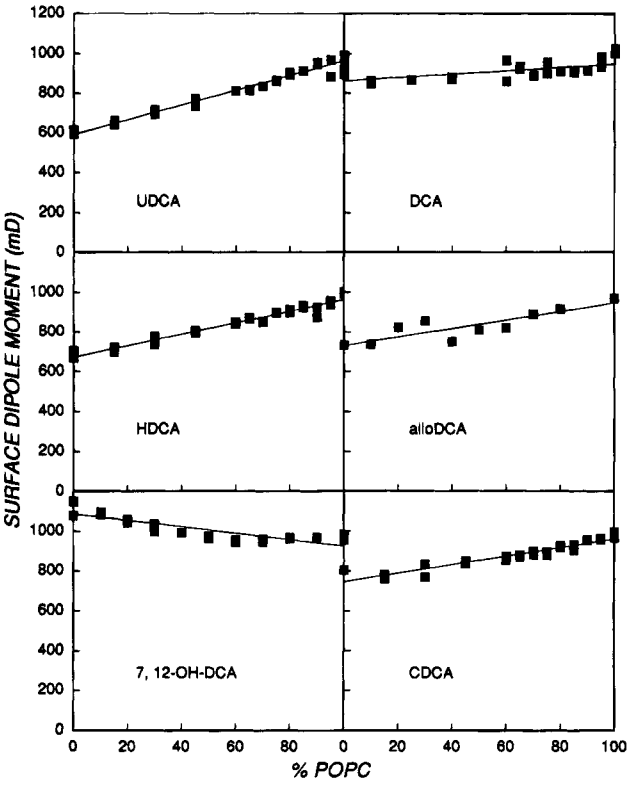

FIGURE 6: Dependence of the surface dipole moment on mole percent POPC for binary mixtures with UDCA, DCA, HDCA, alloDCA, 7,12-OH-DCA, and CDCA at a surface pressure of 2.5 mN/m. Solid lines indicate the linear least-squares fits. The surface dipole moment displayed near perfect additivity for all bile acids.

to the opposite axis, indicated by the lower dashed arrow. The intercept on the bile acid axis has been suggested to yield the "effective" area of a bile acid molecule added to a phospholipid monolayer (Small, 1971) (see Discussion).

Figure 6 displays the dependence of the surface dipole moment on mole percent POPC for binary mixtures of bile acids with POPC at a surface pressure of 2.5 mN/m. In contrast to the negative deviation from ideal behavior of the molecular areas (Figure 5), surface dipole moments show ideal behavior. Hence, neither the surface dipole moment of the bile acid nor the POPC is affected by interactions with each other, suggesting strongly that the molecular orientation does not depend on POPC content. We infer below that bile acid molecules remain with the long axes of the sterol nuclei parallel to the air−water interface (see Discussion).

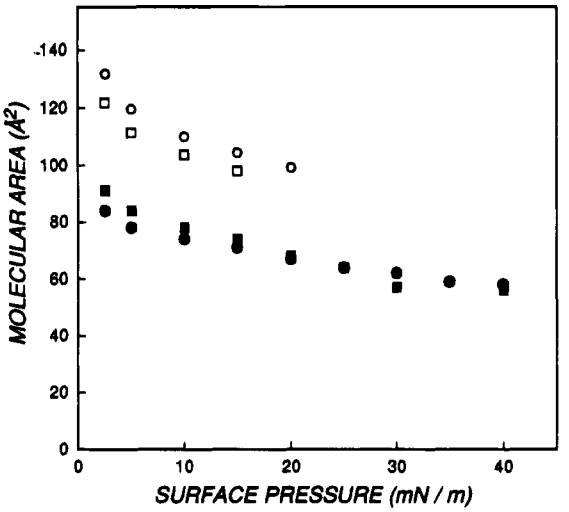

FIGURE 7: Effects of surface pressure on molecular areas of bile acids in pure monolayers and in binary mixtures with POPC. Molecular areas are shown for pure bile acid monolayers of (○), DCA and (□), UDCA. Bile acid areas were extrapolated from the binary curves of (●), DCA/POPC and (■), UDCA/POPC, as shown in Figure 4, over the range 85−100% POPC (see text for details). Molecular area decreases monotonically with increasing surface pressure for pure bile acid monolayers and binary mixtures with POPC.

Figure 7 displays the surface pressure dependence of pure UDCA and DCA molecular areas and the extrapolated areas or "effective" areas of bile acids in binary bile acid/POPC monolayers. Both extrapolated areas of bile acids in binary mixtures and actual areas of pure bile acid monolayers decrease gradually with increasing surface pressure. Differences between the actual area of the pure bile acid and the extrapolated area are greater for DCA than for UDCA, implying that DCA has a more potent condensing effect. However, the effective areas of DCA and UDCA are similar and decrease with increasing surface pressure, implying that the orientations of both bile acids in POPC monolayers are similar. For all pressures, the effective areas (~60−90 Å^2) are substantially larger than the cross-sectional area of the sterol nucleus (~37−49 Å^2) [Small (1971); this work]. Indeed, the minimum areas are very similar to those calculated by Ekwall and colleagues (1957) for a close packed bile acid monolayer with all hydroxyl groups interacting with water.

To better understand the meaning of the effective area (Figure 7), a comparison with the condensing effects of cholesterol on POPC molecules is useful. Figure 8A displays the average molecular area of binary mixtures of POPC and cholesterol on a 5 M NaCl, pH 2, subphase, for surface pressures varying from 2.5 to 40 mN/m. As is well-known for other phosphatidylcholine species (Chapman et al., 1969; Ghosh et al., 1971, 1973), cholesterol condenses the POPC monolayer; i.e., there is a negative deviation from ideality as shown by the dashed line at 2.5 mN/m (Figure 8A). Extrapolation of the average molecular area obtained at high POPC content to 0 mol % POPC yields values for the effective areas of cholesterol molecules as shown in Figure 8B. The extrapolated molecular area of cholesterol ranges from −4 to +25 Å^2 and therefore does not correspond to physical reality. As discussed below, the effective area cannot be correlated with a particular dimension of the cholesterol or bile acid molecule, but reflects the proficiency with which these molecules alter the molecular packing of the hydrocarbon chains of the phospholipid.

Figure 8C displays the average surface dipole moment at several surface pressures for binary mixtures of POPC and cholesterol. Unlike measurements of surface area, the surface dipole moment of binary mixtures exhibits only minimal deviations from ideal behavior. This suggests that there is no change in orientation of the molecules in the monolayer as the relative amount of POPC increases from 0 to 100%.

Dihydroxy Bile Acid/POPC/Cholesterol Monolayers. Figure 9 displays $\pi-A$ isotherms for various relative compositions of UDCA and a 1:1 molar ratio of POPC/cholesterol. Compared with UDCA/POPC (Figure 3), several differences are apparent in these $\pi-A$ isotherms. Small amounts of UDCA do not condense the POPC/cholesterol monolayer, whereas in the case of pure POPC monolayers (Figure 3), 20 mol % UDCA condenses the monolayer. The collapse pressure of UDCA increases as the mole percent POPC increases, indicating that equimolar ratios of POPC/cholesterol form a fully miscible monolayer with UDCA (Crisp, 1949) (see Discussion). However, the increment in collapse pressure of POPC/cholesterol monolayers with UDCA is less than for UDCA in POPC monolayers (Figure 3). This suggests that POPC/cholesterol does not interact as effectively as pure POPC with UDCA to prevent multilayer collapse of the bile acid monolayer.

Figure 10 shows area-additivity curves for a ternary mixture of POPC/cholesterol (1:1 and 2:1 ratios) with UDCA and DCA molecules. Molecular areas are plotted against mole fraction of the POPC/cholesterol mixture, with 100% bile acid on the left, and 100% POPC/cholesterol mixture on the right. The areas of the POPC/cholesterol monolayers are considerably smaller than those for pure POPC (Figure 5). The 1:1 POPC/cholesterol system (Figure 10C,D) shows perfect additivity with the bile acids, whereas the 2:1 POPC/cholesterol mixture (Figure 10A,B) is only condensed minimally by addition of bile acids. As was shown for bile acid/POPC mixtures in Figure 5, mixtures of bile acids with POPC and cholesterol (2:1 or 1:1) display strict additivity of the surface dipole moments (data not shown). When the bile acid molecular area at high POPC/cholesterol content is extrapolated to the bile acid axis, the effective area is identical to that for the pure bile acid. Therefore, in contrast to the case with POPC monolayers, when bile acids interact with an already condensed POPC/cholesterol monolayer, no further condensation occurs.

Hydrophobicity Values of Bile Acids. Table 2 displays hydrophobic indexes for all six bile acids studied, as determined by reversed phase HPLC with evaporative light scattering detection (pH 5.4). For comparison, values are shown for common unconjugated species, as previously determined by reversed phase HPLC by Heuman (1989) at higher ionic strengths. The hydrophobic indexes of alloDCA and DCA are similar, indicating that the orientation of the A/B junction does not greatly affect hydrophobicity. In contrast to DCA and CDCA, both of which have two axially oriented hydroxyl groups including a 3-OH function, 7,12-OH-DCA is extremely hydrophobic. Thus, the 3-OH group of the common bile acids is an important contributor to bile acid hydrophilicity and to interfacial anchoring (Figure 1).

Figure 11 shows the relationships between hydrophobicity and the molecular areas of the pure bile acids as well as the differences between the areas of pure bile acids and their

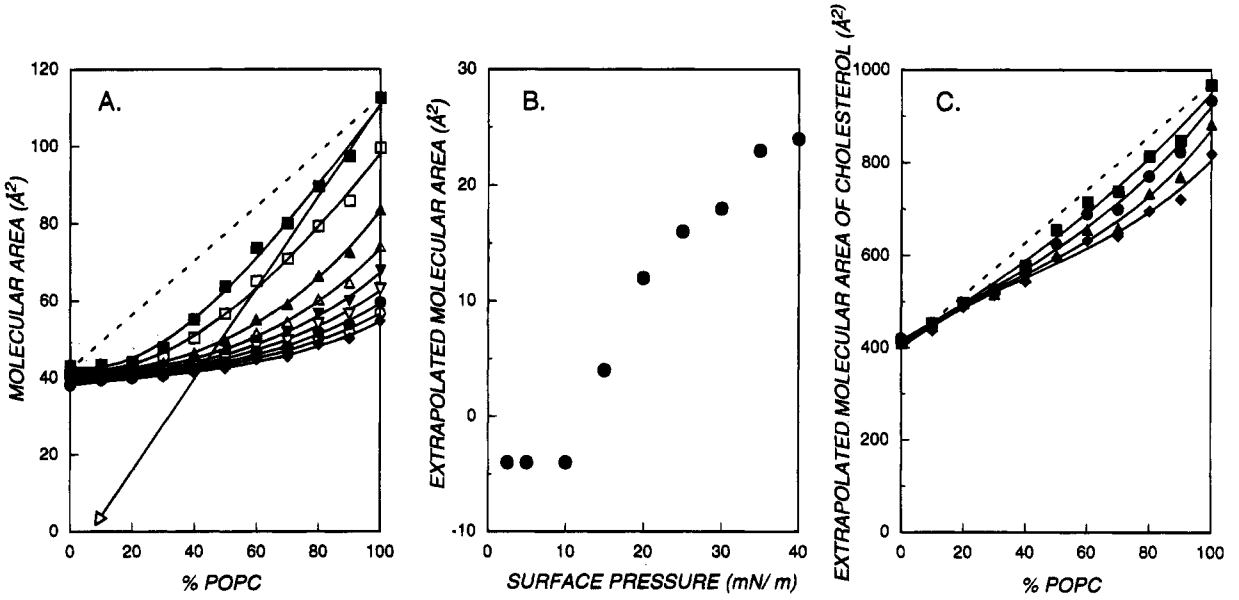

FIGURE 8: (A) Dependence of average molecular area of POPC/cholesterol monolayers on relative lipid composition, as determined from π–A isotherms of binary mixtures at incremental surface pressures: ■, 2.5; □, 5.0; ▲, 10; △, 15; ▼, 20; ▽, 25; ●, 30; ○, 35; and ♦, 40 mN/m. Curves are third-degree polynomial fits. As observed for bile acid/POPC mixtures, cholesterol condenses POPC monolayers at all relative compositions. (B) Molecular areas of cholesterol in POPC monolayers, as extrapolated from 85 to 100 mol % POPC monolayers, as a function of surface pressure. At all surface pressures, the extrapolated area of a cholesterol molecule is considerably less than its cross-sectional area of 40 Å² (Demel et al., 1972a). Indeed, at surface pressures of ≤25 mN/m, values for the extrapolated area of a cholesterol molecule are less than zero, a value without physical meaning. (C) Values of the surface dipole moment for POPC/cholesterol binary monolayers with relative compositions from 0 to 100 mol % POPC. Different curves represent values at surface pressures of (■), 2.5; (●), 5.0; (▲), 10; and (◆), 20 mN/m. There is only minimal deviation from ideality, suggesting that the orientation of the molecules does not change during compression or with changes in relative composition.

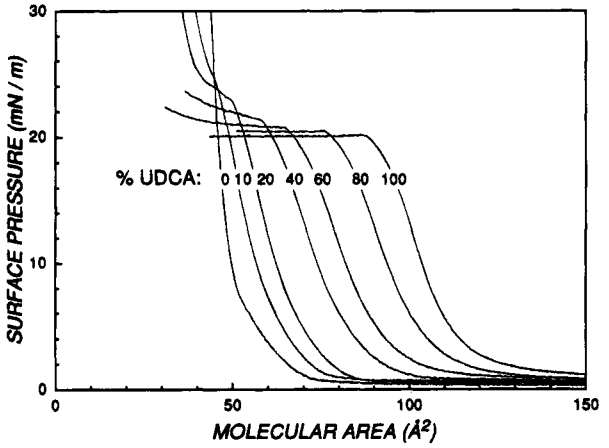

FIGURE 9: π–A isotherms of monolayers of mixtures of 0–100 mol % UDCA with a 1:1 (mol) mixture of POPC/cholesterol, spread on a 5 M NaCl, pH 2, subphase at 22 °C. As POPC/cholesterol is added, the collapse pressure of UDCA increases, but to a lesser extent than for UDCA with pure POPC (see Figure 3).

effective areas in POPC monolayers (all at 2.5 mN/m). Assuming no change in bile acid orientation, the difference between these areas corresponds to a decrease in the molecular area of the surrounding POPC molecules. Molecular areas of the six dihydroxy bile acids in expanded monolayers (Figure 11, closed circles) correlate positively with hydrophobic index ($r^2 = 0.61$) with more hydrophobic bile acids having more expanded π–A isotherms. A stronger correlation ($r^2 = 0.91$) exists between the condensing effect of bile acids and their hydrophobicity (Figure 11, closed squares). The more hydrophobic bile acids have a greater molecular area, and we infer, they interact more effectively with the acyl chains of POPC (see Discussion).

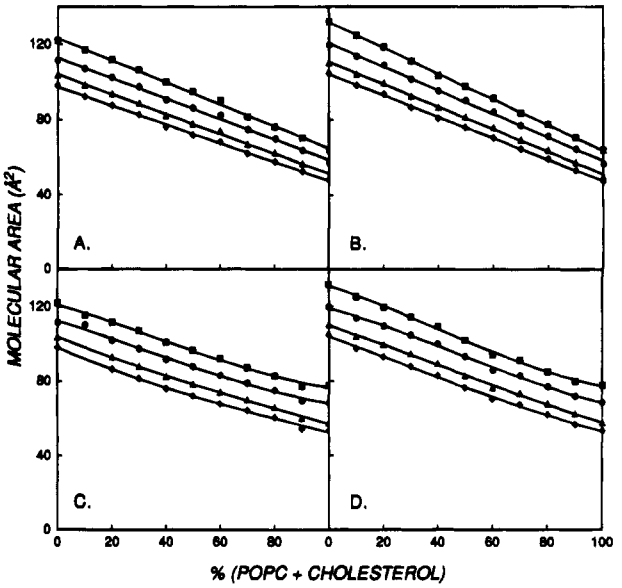

FIGURE 10: Dependence of average molecular area on relative lipid composition, as determined from π–A isotherms of binary mixtures of (A) UDCA with a 2:1 (mol) mixture of POPC and cholesterol, (B) DCA with a 2:1 (mol) mixture of POPC and cholesterol, (C) UDCA with a 1:1 (mol) mixture of POPC and cholesterol, and (D) DCA with a 1:1 (mol) mixture of POPC and cholesterol. Data at incremented surface pressures of (■), 2.5; (●), 5.0; (▲), 10; and (◆), 20 mN/m are shown. Neither UDCA nor DCA produces substantial deviations from ideality when mixed with POPC/cholesterol monolayers. Curves shown in (A) and (B) are third-degree polynomial fits, and in (C) and (D) are least-squares fits.

DISCUSSION

Surface chemical techniques provide information on molecular orientation as well as the strength of interactions between amphiphilic molecules at an interface (Carey, 1982,

Table 2: Hydrophobic Indexes of Bile Acids As Determined by HPLC[a]

bile acid	retention time (min)	hydrophobic index	hydrophobic index[b]
DCA	61.6	1.67	1.46
CDCA	59.4	1.58	1.37
UDCA	20.5	0.67	0.49
HDCA	26.4	0.90	c
alloDCA	67.0	1.68	c
7,12-OH-DCA	152	2.31	c

[a] Conditions were 0.025 M ammonium acetate, MeOH/H_2O (75:25 v/v), pH 5.4, 22 °C, 0.5 mL/min, with detection by an evaporative light scattering detector. [b] Data from Heuman (1989). [c] Not available.

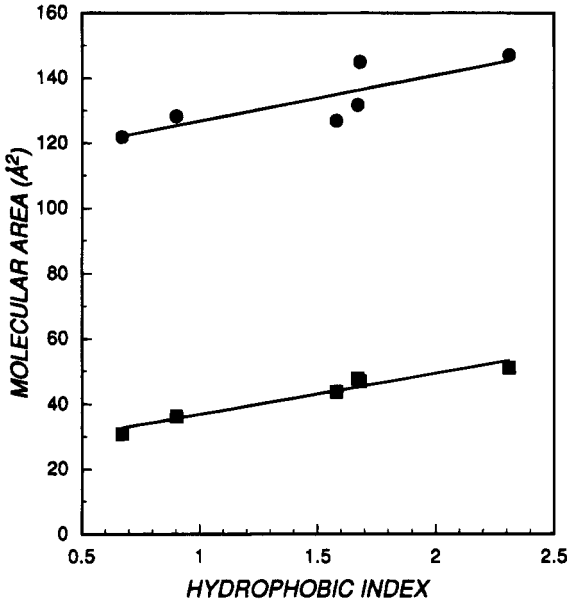

FIGURE 11: Bile acid hydrophobicity dependence of molecular areas of pure bile acids (●) and differences between the molecular areas of a pure bile acid monolayer and the effective areas of the bile acid in the POPC monolayer (■). Linear least-squares fits to the data are shown.

Small, 1971). The automated technique employed in this work facilitates systematic and highly precise study of bile acids with a range of hydrophobicities and provides information on bile acid orientation, affinity for the aqueous subphase, and condensing effect on POPC, the second most-abundant phospholipid in hepatocyte plasma membranes and mammalian bile (Hay et al., 1993). The most abundant species in bile, 1-palmitoyl-2-linoleoyl-*sn*-glycero-3-phosphatidylcholine, was not utilized because of concerns of oxidation during study. However, it is known that 1-palmitoyl-2-linoleoylphosphatidylcholine and POPC have comparable surface properties (Demel et al., 1972b) and hydrophobicities (Hay et al., 1993).

Pure Bile Acid Monolayers. To accurately interpret molecular areas derived from surface balance studies, the number of molecules at the air/water interface must be known precisely. In contrast to trihydroxy bile acids (see footnote 2), the number of dihydroxy bile acid molecules dissolved into the subphase over the 12 min compression time was less than 2% of the total in the monolayer and, therefore, did not significantly affect interpretation of the $\pi-A$ isotherms. Although the aqueous solubility of UDCA in 0.15 M NaCl at pH 3.1 is 9 μM, a value substantially lower than the solubility of CDCA, 27 μM (Roda & Fini, 1984), the rate of dissolution of UDCA from the surface monolayer was 3−4 times greater than that of CDCA.

Measurements of surface pressure did not depend upon rate of compression at surface pressures below the collapse pressure (see Methods), indicating that $\pi-A$ isotherms were measured at equilibrium. However, when compressed multilayers of UDCA and CDCA were allowed to equilibrate at 37 °C for times longer than the compression times, a dramatic drop in the surface pressure took place at 20 and 45 min, respectively, consistent with surface crystallization. CDCA required a longer time to crystallize compared with UDCA, most likely due to its more complicated crystal structure. Crystallographic studies indicate that UDCA molecules crystallize with their long axes parallel whereas CDCA molecules crystallize with their long axes perpendicular to each other (Carey, 1985; Lindley & Carey, 1987). The similarity of the crystal structure of UDCA to previously proposed structures of surface multilayers (Ekwall et al., 1957; Small, 1971) probably facilitates crystallization for UDCA as compared with CDCA. The longer time for crystallization at the lower temperature is consistent with decreased thermal molecular motion that facilitates molecular rearrangement and crystallization.

Several of the common bile acids were found previously to have similar properties in their surface behavior (Carey, 1985; Minoñes Trillo et al., 1968; Miyoshi et al., 1991; Shibata et al., 1991; Small, 1971). In general, dihydroxy bile acids exhibit liftoff areas of 120−140 Å^2 and collapse at molecular areas of 85−100 Å^2 (Carey, 1985; Small, 1971). As can be inferred from molecular models in Figure 11, the molecular areas of the hydrophilic surface of all dihydroxy bile acids are comparable, and indeed, the molecular areas at collapse are similar (Table 1). The presence of an equatorial hydroxyl group (UDCA, HDCA) forces a slightly different orientation of the steroid nucleus with respect to the interface to allow both hydroxyl groups to interact with the aqueous subphase.

Collapse pressures (see Table 1) are indicative of the strength of the hydrophilic attachment to the subphase, as well as the interactions between hydrophilic head groups within the monolayer. Zwitterionic POPC[4] and neutral cholesterol, both with a single hydrophilic moiety, exhibit

[4] The molecular area of POPC in the monolayer was sensitive to changes in the pH of the 5 M NaCl subphase. At a surface pressure of 2.5 mN/m, the molecular area of POPC decreased from 119 Å^2/molecule at pH 5 and 10 to 112 Å^2/molecule at pH 2, and to 107 Å^2/molecule at pH 1.3. We infer from these data that POPC may be partially protonated at pH 2.0 and thus carries a partial net positive charge. Shah and Schulman (1967) also observed that the $\pi-A$ isotherms of dipalmitoyl, dioleyl, and egg phosphatidylcholines were invariant between pH 2 and 8, but that a small increase in ionic strength (0.01−0.1 M NaCl) increased phosphatidylcholine molecular area by 5 Å^2/molecule. In contrast, on a NaCl-free subphase at pH 2.0, the molecular area of POPC was invariant at 93 Å^2/molecule at 2.5 mN/m over the pH range from 1.3 to 11 (unpublished observations). Electrophoretic curves of bulk dispersions of phosphatidylcholine molecules (Bangham, 1968) have indicated that the head group, which is zwitterionic at pH 3.0, gains a net positive charge at pH 2.0. However, the predicted expansion (Gaines, 1966) for a charged monolayer was not observed with a 5 M NaCl subphase in our studies. In fact, we found that, at pH values consistent with partial ionization, the POPC monolayer was condensed. It is likely that at neutral pH, association of sodium ions with the negatively charged phosphate head group of POPC leads to an expansion of the POPC monolayer as compared with pH values at which the phosphate moiety becomes protonated. Since all studies in this work were done at pH 2.0 and 5 M NaCl, the minor effects of pH and ionic strength on POPC molecular area can be neglected.

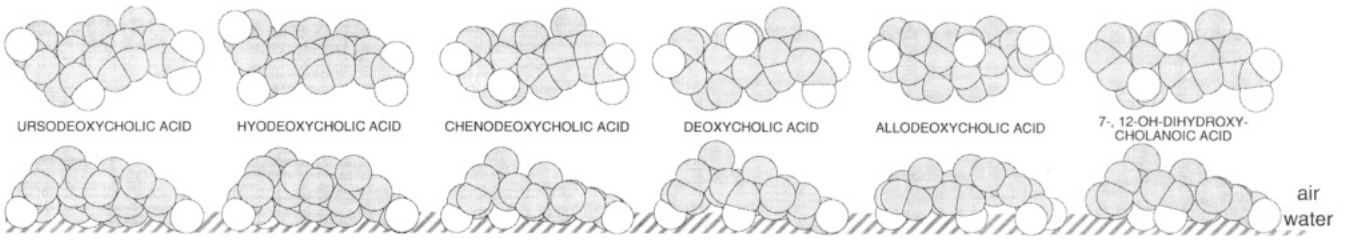

FIGURE 12: Three-dimensional representations of the six dihydroxy bile acids studied. Oxygen atoms are white and carbon atoms are gray; hydrogen atoms have been omitted. The top view depicts the hydrophilic (α) surface of the steroid nucleus, and the bottom view depicts the lateral aspect of the bile acid molecules as they interact with an aqueous interface (shaded area). The hydrophilic bile acids, which are less potent in condensing POPC monolayers, are rotated around their long axes, and have hydroxyl groups that exhibit more lateral orientation than the hydroxyl groups in more hydrophobic bile acids.

collapse pressures above 40 mN/m, whereas cholanoic acid has a collapse pressure of 15 mN/m. As more hydrophilic groups are added to the steroid nucleus of cholanoic acid, the strength of the overall hydrophilic anchor increases. UDCA and HDCA display lower collapse pressures than DCA and CDCA, suggesting that the 7β- and 6α-OH groups (both equatorial) interact less effectively with the aqueous subphase than the 7α- and 12α-OH groups. In contrast, the 3α- and 7β-OH of UDCA and the 3α- and 6α-OH of HDCA are oriented at an angle to each other, with hydroxyl groups projecting axially and equatorially, respectively, from the steroid nucleus. Thus, at the interface, these molecules have relatively large areas of hydrocarbon/water contact. This results in lower collapse pressures but greater bulk hydrophilicities since both arise from decreased contiguous hydrocarbon area on the hydrophobic (β) surface of the molecules.

Surface dipole moment is a measure of the change in electrical potential at the water/air interface induced by the presence of the monolayer as compared with the aqueous surface alone (Brockman, 1994). The dipole moment cannot be directly correlated with molecular dipole moments of the individual molecules in the monolayer since the structuring effects of their polar functions on water molecules contribute. However, because of similarities in the molecular structure of the bile acids examined, differences in surface dipole moments between bile acids are likely to reflect differences in the orientation of the hydroxyl groups and their interactions with the aqueous subphase. Because their side chains are identical and flexible, the contribution of the carboxylic acid group is unlikely to differ among bile acids. Indeed, the similarity in adsorbed molecular areas of glycine and unconjugated bile acids suggests that the side chain may not be at the surface, but within the aqueous subphase (Carey et al., 1981).

To compare the relative contributions of the hydroxyl groups in various orientations, three-dimensional molecular models were oriented with both hydroxyl groups flat (see Figure 12), and the coordinates of the oxygen−carbon bond were used to calculate the relative magnitude of the net dipole moments of the two hydroxyl groups. These three-dimensional molecular models (showing carbon and oxygen atoms only) demonstrate that the C−OH bonds for CDCA and DCA are oriented similarly, with angles of 6 and 11° between the C−OH bonds, respectively, whereas the two C−OH bonds of UDCA and HDCA are oriented closer to a perpendicular arrangement, subtending angles of 111 and 64°, respectively (see methods and Figure 12). The widely spaced hydroxyl groups of UDCA and HDCA molecules compared with CDCA and DCA result in less distinct hydrophobic and hydrophilic surfaces (Figure 12). Consistent with the concept that measurements of the surface dipole moment reflect the relative orientation of the hydroxyl groups, the dipole moments of the hydroxyl groups of CDCA and DCA are additive, whereas those of UDCA and HDCA partially cancel each other. With the exception of 7,12-OH-DCA, the rank ordering of the vector components of the two hydroxyl groups perpendicular to the surface agrees with the rank ordering of increases in surface dipole moments as shown in Figure 2.

The importance of the 3-OH group in anchoring cholanoic acid to water is highlighted by the low collapse pressure and large molecular area of 7,12-OH-DCA (Figure 1). Moreover, the 3-OH critically affects intermolecular interactions, as inferred from its ubiquity in natural bile acids (Hagey, 1992), and underscored by recent infrared spectroscopic studies showing dramatic shifts in the C−OH vibrations and C−H skeletal structure vibrations caused by the absence of the 3-OH group (Okido et al., 1993). Synthetic monohydroxy bile acids such as 7α- and 12α-monohydroxycholanoic acids that lack the 3-OH group have much smaller molecular areas at collapse, suggesting that the 3-OH group anchors bile acids parallel to the interface (Bogle et al., 1995).

Although alloDCA and DCA differ in conformation of the junction of the A and B rings in the steroid nucleus, their surface properties in pure monolayers are similar and consistent with both molecules lying flat at the water/air interface, as for other bile acids studied. AlloDCA generates a smaller dipole moment than DCA, presumably due to differences in the A/B junction of the sterol nucleus and consequently, the orientation of the hydroxyl groups, with the interface.

Monolayers of Bile Acids with POPC. All bile acids examined shared similarities in their interactions with POPC. POPC monotonically increased the collapse pressure at which bile acids are forced out of the monolayer (see Figure 3). This behavior implies intimate miscibility; i.e., the bile acids do not undergo lateral phase separation from POPC in the monolayer (Small, 1971). If separate "islands" of bile acids were present in the mixed monolayer, they would be forced out of the monolayer at the same pressure as for the pure bile acid, contrary to our observations. For all bile acid/POPC mixtures, molecular areas decreased monotonically during compression, without discontinuities that would suggest an abrupt change in bile acid orientation at the surface or any second-order phase transition.

POPC monolayers were condensed by addition of the bile acids examined as well as by cholesterol (Figure 8) as shown schematically in Figure 13. In the absence of cholesterol, POPC monolayers are expanded, and consequently, the acyl

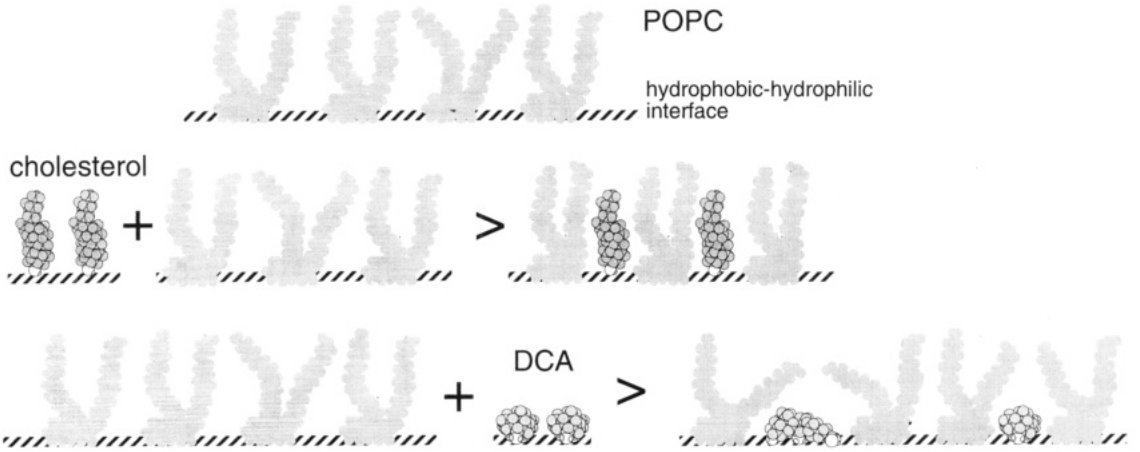

FIGURE 13: Schematic depiction of proposed mechanisms by which cholesterol and bile acid molecules condense POPC monolayers. Lateral views of three-dimensional representations of POPC, cholesterol, and DCA (with all atoms depicted) at a hydrophobic/hydrophilic interface are shown. The relative molecular area is depicted by the length of the shaded interface. On the left are schemata of the relative areas occupied by the lipids in pure monolayers. On the right, mixtures are depicted, with the interacting lipids occupying smaller surface areas. DCA is shown end on in addition to the side view as shown in Figure 12. See text for details.

chains are relatively fluid. However, when cholesterol is added, the steroid moiety intercalates between phospholipid acyl chains with its long axis parallel to the acyl chains (Chapman et al., 1969). This configuration has two major effects: the head group areas of both molecular species are not additive, but pack together more efficiently than either one alone (see Figure 8A); moreover, the acyl chains of the phospholipid become more ordered, since they are constrained by the presence of the relatively rigid sterol nucleus of cholesterol (Phillips, 1972). It should be noted that condensation of POPC by cholesterol occurs without a major change in the orientation of either molecular species, as evidenced by binary additivity curves of the surface dipole moments that show only minimal deviations from ideality (see Figure 8C).

How do bile acids, with their cholesterol-derived sterol nucleus, interact in terms of molecular packing with POPC monolayers? There is general agreement that bile acid molecules in pure monolayers lie flat at the air/water interface and do not lie perpendicular to the interface as do the monofunctional cholesterol, cholestanol, and cholanoic acid molecules (Figure 1, Table 1) (Carey, 1985; Small, 1971). However, the generally accepted view has held that bile acid molecules and the fully ionized bile salts associate to form dimers or larger aggregates, i.e., "reverse micelles", within the hydrophobic portion of the membranes, and are thus oriented with the steroid nuclei perpendicular to the interface (Carey, 1985; Small, 1971). Because extrapolated areas of bile acid molecules in phospholipid monolayers are smaller than the most closely packed monolayer, i.e., the area at collapse pressure (see Figure 7 and Table 1), it has been inferred that bile acid molecules within phospholipid membranes could not be oriented with their steroid nucleus parallel to the surface (Small, 1971). However, the small or negative values of the extrapolated areas for cholesterol molecules in POPC monolayers (Figure 8B) demonstrate that these magnitudes do not necessarily have a physical reality. Therefore, the actual values of the extrapolated areas of bile acid molecules in POPC monolayers do not appear to give direct information on bile acid molecular orientation.

We believe that several lines of evidence presented herein support the concept that bile acid molecules in POPC monolayers are oriented with the sterol nucleus parallel to the interface at all surface pressures. First, as noted above, the effective areas of all bile acids studied in POPC monolayers exceed the cross-sectional area of the steroid nucleus, which is ~40 Å^2 as inferred from cholesterol, cholanoic acid, and cholestanol (Table 1). Second, additivity curves of the surface dipole moments for binary bile acid/POPC mixtures (Figure 6) demonstrate essentially ideal behavior, from which we infer than there are no orientational changes in bile acid conformation throughout the range of relative bile acid/POPC compositions. Moreover, none of the bile acid/POPC $\pi-A$ isotherms (e.g., Figure 3) provide evidence for a first- or second-order phase transition (Dervichian, 1939). This should be evidenced by an invariant pressure with monolayer compression, as is seen for the pure bile acids at areas below the collapse pressure, or a discontinuity in the $\pi-A$ isotherm, as has been observed during changes in molecular orientation with other compressed monolayers (Fahey & Small, 1986). Thus, we observe no evidence for a change in bile acid orientation during monolayer compression in any of the $\pi-A$ isotherms. Last, bile acids do not condense POPC/cholesterol monolayers (Figure 10). Thus, extrapolated bile acid molecular areas are identical to those in pure bile acid monolayers. It is unlikely, therefore, that bile acid orientation differs in POPC and POPC/cholesterol monolayers.

What is the molecular packing of bile acid and POPC molecules in monolayers, and by extrapolation, in bilayers? Examination of three-dimensional bile acid structures (Figure 11) demonstrates that bile acid molecules with different orientations and positions of the hydroxy groups could not pack in a similar arrangement as reverse dimers with their hydrophilic groups shielded from hydrocarbon chains. Bile acid molecules with their long axes lying flat at the interface present a packing problem, since the acyl chains of phospholipids are much longer (~20 Å) than the thickness of the bile acid molecule (~8 Å). Because POPC molecules occupy a larger area than the head group occupies at maximal compression (~42 Å^2), the acyl chains are not fully extended, but are in a fluid state. We postulate that this fluid nature of the acyl chains allows the chains to interact with the hydrophobic surface of individual bile acid molecules, as depicted schematically in Figure 13. In this orientation, the acyl chains interact most likely with the sides as well as the

hydrophobic surfaces of the bile acids (see Figure 13). Although both DCA and UDCA molecules condense POPC monolayers, the more lateral equatorial position of the hydroxyl groups of UDCA molecules prevents interaction with the acyl chains of POPC to the same degree as with DCA.

In support of this concept, Ulmius and colleagues (1982) found that the trihydroxy bile salt sodium cholate dramatically decreased the order parameter of the acyl chains of egg yolk phosphatidylcholine, particularly for carbon atoms near the methyl terminus. This is consistent with the terminal parts of the acyl chains interacting with the hydrophobic face of the bile acid. The idea that the effective area of bile acid molecules depends upon their interaction with phosphatidylcholine acyl chains rather than their orientation in the monolayer is also supported by the findings of Minoñes Trillo and colleagues (1968). They observed that dihydroxy bile acids condensed a relatively condensed phosphatidylcholine monolayer (unspecified structure with molecular area of 78 $Å^2$ at 2 mN/m) less than shown here for POPC. If phosphatidylcholine molecules are already tightly packed in a monolayer, because of saturated acyl chains, the cholesterol content (Figure 9), or of high surface pressure (Figure 4), then bile acids are generally ineffective at condensing the monolayer further. Recent studies of Smaby and colleagues (1994) demonstrate that the structure of the *sn*-1 acyl (or its analog) chain of phospholipids is crucial for determining the degree to which cholesterol molecules condense phosphatidylcholine and sphingomyelin monolayers. Small and colleagues (Small & Bourgès, 1966; Small et al., 1966) concluded from X-ray diffraction measurements of the interbilayer spacing of lamellar liquid crystals that ionized bile salts increased the thickness of an egg phosphatidylcholine bilayer and, hence, must be oriented with their long axis perpendicular to the bilayer. However, if bile acid molecules decreased the average areas of phosphatidylcholine head groups, but the lipid density remained constant, then bile acids must increase the thickness of the bilayer, regardless of bile acid orientation.

There is ample precedent in the literature for the concept that amphiphilic molecules can penetrate in a bilayer structure to different depths relative to the length of the acyl chains of membrane molecules (Gabriel & Roberts, 1984; Hamilton et al., 1991; Rowe & Campion, 1994). One mechanism facilitating incorporation and packing of molecules with less hydrophobic penetration or mismatch compared to the length of the acyl chains of phospholipids is interdigitation of the methyl termini (Slater & Huang, 1988). It remains to be evaluated whether extension of the methyl termini beyond the midpoint of the bilayer occurs in the presence of bile salts in a membrane.

Physiological Correlations. The surface monolayer studies presented herein provide a well-defined system, where all bile acid molecules are in the lipid monolayer at the water/air interface, and partitioning into the bulk aqueous phase does not have to be considered. Nonetheless, we studied a surface monolayer in this work, rather than a bilayer, and the bile acids were protonated, rather than being fully ionized as occurs physiologically. The ionization state of the carboxylic side chain most likely does not influence the surface properties since surface adsorption studies have shown that glycine conjugated and unconjugated bile salts have similar molecular areas (Carey et al., 1981). Moreover, the fully ionized 3-monohydroxy bile salt (sodium lithocholate) displayed similar condensation effects on POPC monolayers, without exhibiting discontinuities in the dependence of surface pressure or potential on molecular area that would suggest a change in bile salt orientation (Bogle et al., 1995). Extrapolation of such monolayer studies to the biological world must be considered with caution since packing constraints are likely to be different for monolayers compared to bilayers. Nonetheless, as evidenced by the ability of phospholipids with widely varying acyl chain lengths to form interdigitated bilayers (Boggs & Koshy, 1994) as well as membrane proteins to be of a thickness different from that of the phospholipid bilayer, it is reasonable to conclude that bile acids can lie parallel to the membrane interface and interact with the much longer acyl chains of POPC.

Bile salt hydrophobicity correlates with cytotoxicity *in vivo* and *in vitro* as well as with the ability of bile salts to dissolve membranes (Coleman, 1988; Heuman et al., 1991a,b; Kanai et al., 1990). The ability of a bile acid to condense a lipid monolayer appears to correlate with the ability of the fully ionized bile salt to dissolve lipid from membranes. More hydrophobic bile salt species added to membranes with a lower cholesterol content are associated with more rapid membrane dissolution, and we have shown here hydrophobic bile acids induce a greater degree of condensation with phospholipid monolayers. The key factor in common may be the ability of the convex hydrophobic surface of the bile acid molecule to interact with the acyl chains of the phospholipid. With increased cholesterol content, the acyl chains are more ordered and extended, so bile acids and salts are less able to interact with and hence condense and/or solubilize the phospholipid molecules.

This study provides strong evidence against the formation of bile acid dimers, or "reverse micelles", within either phosphatidylcholine or cholesterol/phosphatidylcholine monolayers, in contrast to the formation of "reverse micelles" by bile acids in organic solvents (Vadnere & Lindenbaum, 1982). The hydrophilic interior of these proposed entities has been hypothesized to facilitate transport of hydrophilic substances across physiological membranes (Abramson & Shamoo, 1979; Carey, 1985). However, we have recently shown that bile salts do not increase osmotic water permeability at concentrations up to those which dissolve membranes (Donovan et al., 1993; Zucker et al., 1993). This would be expected if molecules of bile acids or salts lie flat at the aqueous interface and if the hydrophilic portions of the molecule do not penetrate the hydrocarbon portion of the bilayer.

In conclusion, the more potent condensing effects of hydrophobic bile acids, and consequent more intimate interactions with the hydrocarbon chain portions of lipid monolayers, correlate with the enhanced detergent and cytotoxic capabilities of hydrophobic bile acids and salts (Coleman, 1988; Heuman et al., 1991a,b; Kanai et al., 1990). When bile acids are fully dissociated as bile salts, we infer that the steroid nuclei of hydrophobic and hydrophilic bile acid species are identical in their orientations but differ in their hydrophobic interactions with the hydrophobic portion of membranes, a property that correlates with their respective abilities to damage or protect biological membranes. It is highly likely that pathophysiological differences in membrane effects between hydrophilic and hydrophobic bile salts may be fundamentally related to their differing abilities to interact

with the acyl chains of phospholipids and condense membranes. Nonetheless, it is clear that when mixed with membrane lipids, all di- and trihydroxy bile acids remain oriented so that they lie flat at the lipid−water interface.

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