# Spread Monomolecular Films of Monohydroxy Bile Acids and Their Salts: Influence of Hydroxyl Position, Bulk pH, and Association with Phosphatidylcholine†

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ABSTRACT: Undissociated dihydroxy bile acids, alone or with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC), lie with their long axes parallel to aqueous−lipid interfaces [Fahey, D. A., Carey, M. C., and Donovan, J. M. (1995) *Biochemistry 34*, 10886−10897]. To test the generality of this orientation, we used an automated Langmuir−Pockels surface balance to examine pressure−molecular area isotherms and dipole moments of insoluble monohydroxy bile acids and their salts, which are sparingly soluble because of their presumed high Krafft points. We studied lithocholic acid (LCA) (the natural 3α-OH isomer), glycolithocholic acid (GLCA) (its glycine conjugate), and the semisynthetic isomers, 7α-OH- and 12α-OH-cholanoic acids with and without POPC, at pH values ranging from 2 to 12. Monolayer collapse pressures increased sigmoidally with ionization, giving apparent p*K* values of 7.0−8.5 and implying a stronger affinity of the bile salt anions for the interface. At monolayer collapse, the molecular area of LCA was ∼85 Å$^2$ independent of pH, consistent with the steroid nucleus lying flat. In contrast, the interfacial area of 7-OH-cholanoic acid decreased from ∼80 Å$^2$ at pH 2 to ∼40 Å$^2$ above pH 9, consistent with a more vertical orientation and approximating 12-OH-cholanoic acid, which exhibited a molecular area of ∼45 Å$^2$ at all pH values. All monohydroxy bile acids condensed POPC monolayers more effectively at low than at high (ionized) pH. We conclude that the 3-OH group is crucial for anchoring bile acids and their salts to the aqueous interface, with all monohydroxy species condensing phospholipid membranes regardless of ionization state.

Spread monomolecular films (monolayers) of biologically important lipids are useful models to study physical−chemical properties of biological membranes. In particular, measurement of the lateral pressure exerted at defined molecular areas allows insights into the structure of the surface phases present at an air−water interface (*1*). Monolayers of common undissociated bile acids orient with their steroid nuclei parallel to the aqueous−air interface, allowing interaction of their α- and β-oriented hydroxyl groups with the bulk aqueous phase (*2*−*4*). However, it was suggested earlier (*5*, *6*) that, in mixed monolayers with long-chain phosphatidylcholines, bile acids are oriented with their long axes perpendicular to the interface and their hydrophilic surfaces interacting as dimers. This molecular arrangement was based largely on the nonideal mixing behavior of bile acid/phosphatidylcholine monolayers, where the average molecular areas were significantly smaller than the weighted average of the molecular areas of the two components (*3*, *7*).

On the basis of several lines of evidence, we concluded from previous surface balance studies (*4*) that the long axis of the steroid nucleus of dihydroxy bile acids in mixed monomolecular layers with 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine (POPC)[1] with and without cholesterol remains parallel to the interface irrespective of the surface pressure. As we and others have observed (*3*, *4*), the effective molecular area[2] of dihydroxy bile acids in POPC monolayers is greater than the cross-sectional area of the steroid nucleus, which is ∼40 Å$^2$ (*4*). Moreover, the effective area of bile acids is identical whether in condensed POPC/cholesterol monolayers or in a pure monolayer (*4*) at an air−water interface, wherein bile acids lie flat (*3*, *8*). Third, from the ideal mixing behavior of surface potential measurements of dihydroxy bile acid/POPC monolayers, we inferred (*4*) that

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[1] Abbreviations: POPC, 1-palmitoyl-2-oleoyl-*sn*-glycero-3-phosphocholine; π−A, surface pressure−molecular area; LCA, lithocholic acid (3α-monohydroxy-5β-cholanoic acid); GLCA, glycolithocholic acid (glycyl amidate of LCA); TC, sodium taurocholate (tauryl amidate of cholate (3α,7α,12α-trihydroxy-5β-cholanoic acid, sodium salt)); 7-OH-CA, 7α-monohydroxy-5β-cholanoic acid; 12-OH-CA, 12α-monohydroxy-5β-cholanoic acid; TLC, thin-layer chromatography; $pH_{app}$, apparent pH; DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid).

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no change in orientation takes place and that the long axis of the steroid nucleus must therefore remain parallel to the interface at all POPC/bile acid ratios. Last, neither first- nor second-order phase transitions (*9*) were observed in any bile acid/POPC surface pressure−molecular area ($\pi$−A) isotherm (*3*, *4*). Condensation of a mixed monolayer, i.e., negative deviation from ideality, was ascribed to more efficient molecular packing of the fluid acyl chains of phosphatidylcholine with the hydrophobic surfaces of bile acid molecules (*4*). In an analogous fashion, cholesterol molecules condense phosphatidylcholine monolayers without a change in interfacial orientation (*10*, *11*). Although reverse bile acid dimers were not observed with POPC/dihydroxy bile acid interactions, methyl esters of lithocholic acid (LCA) self-associate in $CCl_4$ (*12*). We hypothesized that the greater hydrophobicity (*13*) of the monohydroxy bile acids might make these molecules more likely to penetrate into the hydrophobic domains of lipid monolayers and bilayers and form reverse micelles.

Because the p$K_a$ values of unconjugated bile acids in phosphatidylcholine vesicles are ~7, both ionized and fully protonated bile acids are present at physiological pH values (*14*). Previous systematic studies of surface properties have focused on fully protonated dihydroxy bile acids because the more soluble ionized dihydroxy bile salts and trihydroxy bile acids and salts form unstable monolayers (*4*), precluding accurate measurement of molecular areas. However, the extremely limited aqueous solubility of ionized LCA at ambient temperature (*15*) facilitates investigation of insoluble ionized monolayers of this bile salt (*2*, *3*).

Herein we compare surface properties of the physiologic LCA and glycolithocholic acid (GLCA), both containing a single 3α-OH group, with the nonphysiologic monohydroxy isomers containing single hydroxyl groups at the 7α or 12α positions. To compare the surface behavior of unconjugated monohydroxy bile salts and GLCA in their undissociated and fully ionized states, we examine the monohydroxy bile acids alone and in mixed monolayers with POPC as functions of bulk pH (2−12). We provide evidence that the steroid nucleus of 7α- or 12α-monohydroxy bile acid/salt can assume an orientation perpendicular to the aqueous interface but only at high surface pressures. This behavior contrasts with the invariably parallel orientation of molecules of the 3α-monohydroxy bile acids LCA or GLCA at the aqueous−air interface. In addition, we show that all monohydroxy bile acids, regardless of interfacial orientation, condense POPC monolayers with undissociated being more effective than ionized species.

## EXPERIMENTAL PROCEDURES

*Materials*. LCA (3α-hydroxy-5$\beta$-cholanoic acid) and GLCA (3α-hydroxy-5$\beta$-cholanoic acid *N*-[carboxymethyl]-amide), sodium taurocholate (3α,7α,12α-trihydroxy-5$\beta$-cholanoic acid *N*-[2-sulfoethyl]amide, sodium salt) (TC), and sodium taurolithocholate (3α-hydroxy-5$\beta$-cholanoic acid *N*-[2-sulfoethyl]amide, sodium salt) were obtained from Sigma Chemical Co. (St. Louis, MO). Synthetic 7α-hydroxy-5$\beta$-cholanoic acid (7-OH-CA) and 12α-hydroxy-5$\beta$-cholanoic acid (12-OH-CA) were generous gifts of Drs. Claudio Schteingart, Huong-Thu Ton-Nu, and Alan F. Hofmann (San Diego, CA). Cholanoic acid (CA) (5$\beta$-cholanoic acid) was graciously donated by Dr. Donald M. Small (Boston, MA). All unconjugated bile acids gave a single spot on thin-layer chromatography (TLC) ($BuOH/CH_3COOH/H_2O$, 10:1:1, v/v/v) of 200 $\mu$g applications, and GLCA was 99% pure by both TLC and HPLC. POPC, purchased from Avanti Polar Lipids (Alabaster, AL), was found also to be >99% pure by TLC ($CHCl_3$/MeOH/$NH_4OH$, 65:25:4, v/v/v). Sodium chloride, phosphoric acid, and mono-, di-, and tribasic sodium phosphate were of the highest purities. Talc (hydrous magnesium silicate) was obtained from J. T. Baker (Phillipsburg, NJ). Sodium chloride and talc were roasted at 600 °C for 6 h to remove organic impurities. All solvents were of HPLC grade and were free of surface-active impurities as demonstrated by a constant surface pressure upon compression of the pure subphase. Water was purified by reverse osmosis, filtered through activated charcoal, and distilled. Glassware was cleaned by successive overnight soaking in EtOH/2 M KOH (50:50, v/v) and 1 M $HNO_3$, followed by thorough rinsing with distilled water.

*Spreading Solutions.* Stock solutions of unconjugated bile acids in MeOH were heated gently to facilitate dissolution and then filtered through a 0.22-$\mu$m Millex-GV filter (Millipore Corp., Bedford, MA). GLCA and POPC were solubilized in $CHCl_3$/MeOH (1:1, v/v) and $CHCl_3$, respectively, and filtered through 0.5-$\mu$m Millex-LCR filters (Millipore Corp., Bedford, MA). Dry weights were determined as previously described (*4*). To prepare spreading solutions (0.15−0.30 mM), stock solutions were diluted with hexane/EtOH (3:1, v/v) for bile acids and with $CHCl_3$/MeOH (1:1, v/v) for POPC. Solutions were stored in sealed flasks under argon at −5 °C for up to 3 months; however, limited supplies of 7-OH-CA and 12-OH-CA permitted preparation of only one batch of spreading solutions, which were stored for the duration of the study.

Spreading volumes for an intended initial molecular area of ~150 Å$^2$/molecule were determined from solution molarity, surface area of the Langmuir−Pockels trough, and Avogadro's number. After prolonged storage, variations in concentration (<10%) of 7-OH-CA or 12-OH-CA caused by microprecipitation or evaporation of solvent were corrected by comparing the molecular area to that of the original $\pi$−A isotherms at a pressure of 2.5 mN/m. With adjustment in this manner, isotherms were superimposable with those of freshly prepared solutions.

*Surface Pressure and Surface Potential Measurements*. Bulk solubilization of bile acids was minimized by using a 5 M NaCl subphase (*4*). To stabilize bulk phase pH, we added solutions of sodium phosphate buffer to roasted NaCl, verifying the pH of the subphase indirectly by measuring the pH of the buffer solution (without NaCl) at the end of each day's experiments. Although 10 mM phosphate buffer was adequate to maintain low pH values, it became necessary to employ 100 mM phosphate buffer to maintain pH values at 9 and 10.[3] We therefore utilized subphases buffered with

[2] The effective molecular area of bile acids in POPC monolayers is obtained from bile acid/POPC condensation curves plotted for a given surface pressure: A linear regression line is developed for the points plotting in a straight line on the POPC-rich right side of the graph and extrapolating that line to the *y*-axis (0% POPC). Because of the intimate miscibility of bile acids with POPC in monolayers, average molecular areas in a binary bile acid/POPC mixture are smaller than would be expected for an ideal mixture in which molecules do not interact (*3*). Also see Experimental Procedures.

100 mM phosphate in all subsequent studies. Surface balance measurements were carried out only when both subphase and spreading solution had equilibrated to 21 ± 1 °C.

Surface pressures and surface potentials were measured on a computer-controlled Langmuir−Pockels film surface balance (KSV Instruments, Helsinki, Finland). The apparatus consisted of a Teflon minitrough with symmetrically moving barriers (maximum dimensions between barriers: 30 cm × 75 mm) and a dipping unit modified to hold a home-built $^{210}$Po air electrode for surface potential measurements, as previously described (*4*). Spread monolayers were allowed to equilibrate for 5 min before initiating compression at a rate of 12 Å$^2$ molecule$^{-1}$ min$^{-1}$ (*4*). Collapse pressures were reproducible to within 0.3 mN/m, and molecular areas were reproducible to within ±2% at 2.5 mN/m. Reported results are representative of a group of individual isotherms ($n$ = 2−5). Qualitative assessments of surface viscosity were obtained by sprinkling roasted talc on the surface during monolayer compression and subsequently observing particle movement under the influence of a fine air jet directed at a glancing angle to the surface.

*Experimental Design.* We examined the effects of hydroxyl group position and ionization state for each bile acid/salt, both alone and in one mixture with POPC (intended molar composition: 70% bile acid/30% POPC). Individual bile acids were studied at bulk pH values that varied from pH 2 to pH 12, but because of the potential for POPC hydrolysis, bile acid/POPC mixtures were not examined at pH values above 10. Molecular areas at film collapse were determined from the intersection of tangents drawn on either side of the collapse pressure. Because of the dependence of $\pi$−A isotherms on pH, we determined each interfacial p$K_a$ directly from the midpoint of the sigmoidal curves plotting collapse pressure against pH. We also examined the effects of ionization on the additivity behavior of binary mixtures for each unconjugated monohydroxy bile acid with POPC (0−100 mol %) at pH 2 and pH 10.

GLCA showed no appreciable dissolution in the subphase at pH values of 2 and 6 but at pH 8 displayed increasing solubility near the collapse pressure, dissolving completely at pH 10 within 10 min. Studies of the surface behavior of GLCA only encompassed pH values from 2 to 8. Binary mixtures of GLCA with POPC were studied at pH 4 and pH 8 under conditions where the bile acid is predominantly undissociated or predominantly ionized, respectively.

*Potentiometric Titration.* The bulk p$K_a$ values of the three monohydroxy bile acids were determined by potentiometric titration in 50 or 60 mM TC solution (molar ratio TC: monohydroxy bile acid, 9−18:1) with continuous stirring at 22 °C, as described previously (*16*). An excess of the fully ionized TC maintained the otherwise insoluble monohydroxy bile acids in mixed micellar solution, as demonstrated by their optical clarity during several hours of titration (*17*).

*Bile Acid Hydrophobicity Index.* Retention times of the unconjugated monohydroxy bile acids were determined by reverse-phase HPLC utilizing an evaporative light scattering detector (Varex ELSD II, MD) with the following modifications of the method of Roda and colleagues (*18*). The mobile phase was isocratic with 15 mM ammonium acetate in MeOH/$H_2O$ (90:10; v/v), titrated to apparent pH (p$H_{app}$) values between 4.6 and 9.0 at 22 °C. Hydrophobic indices were calculated (*13*) utilizing arbitrary values of 0.0 for TC and 1.0 for sodium taurolithocholate.

*Data Analysis.* Average molecular areas of bile acids and POPC in monolayers were calculated from the number of molecules spread on a known surface area (*4*). Extrapolations of the effective molecular area of bile acids in 80−100 mol % POPC monolayers were calculated from binary additivity curves by linear least-squares analysis.

*Molecular Models.* To assist with molecular interpretation of the surface balance data, three-dimensional models of each monohydroxy bile acid and POPC were examined using the software ChemDraw and Chem3D Plus (Cambridge Scientific, Cambridge, MA).

## RESULTS

*Monolayer Stability.* To determine bile acid transfer into the bulk subphase, we compressed monohydroxy bile acid monolayers at pH 2 and pH 10 (or pH 2 and pH 8 for GLCA) to a pressure of ∼12 mN/m and monitored surface pressure for 15 min, the approximate time necessary to obtain an isotherm. For unconjugated bile acids, the loss from the surface into the subphase, calculated from the decrease in surface pressure, ranged from <0.5% to 5.0% but was substantially less at lower pressures. This is consistent with the extremely low bulk aqueous solubility reported for LCA ["insoluble" (*19*) and 0.05 $\mu$M (*20*)] and for 7-OH-CA [0.2 $\mu$M (*20*)] at room temperature and pH values of ∼3.0. Under similar conditions, the loss of GLCA into the subphase was essentially identical (2−4.5%). However, at pH 8, when pressures were raised to 20−30 mN/m and held for 15 min, GLCA transfer into the bulk increased to ∼12%, consistent with greater solubility upon increased ionization.

*Surface Behavior of Unconjugated Monohydroxy Bile Acids.* Figure 1 displays $\pi$−A isotherms for LCA, 7-OH-CA, and 12-OH-CA at pH 2, 21 °C. Shown for comparison are the isotherms for cholanoic acid (no hydroxyl function) and the dihydroxy bile acid deoxycholic acid (DCA) from ref *4*. At molecular areas of ∼100−125 Å$^2$, all three monohydroxy bile acids reach "lift-offs", where further compression of the closely packed monolayer initiates an increase in surface pressure. As with DCA (lift-off area ∼145 Å$^2$), these values are consistent with the monohydroxy bile acid molecules lying flat at the air−water interface at low surface pressures (*3*). With further compression, surface pressures of LCA and 7-OH-CA increase markedly to collapse pressures of ∼18.5 and 23.5 mN/m, respectively. In contrast, 12-OH-CA undergoes a second-order phase transition between 45 and 90 Å$^2$/molecule, with monolayer collapse occurring at ∼23.5 mN/m, identical to the collapse pressure of 7-OH-CA. Collapse pressures of all three monohydroxy bile acids are appreciably higher than that of cholanoic acid (∼15mN/m) but in a similar range as those (20−25 mN/m) (*4*) of dihydroxy bile acids (Figure 1). When monolayer compression is continued beyond the collapse area, all pressures remain invariant (Figure 1). From this behavior, we infer that two surface phases are in equilibrium,

[3] Representative $\pi$−A isotherms at pH 2 and pH 5, utilizing either 10 or 100 mM phosphate buffer, were superimposable. At pH values above 10, isotherms obtained on 100 mM phosphate buffer were expanded minimally as compared with those on a 10 mM phosphate subphase.

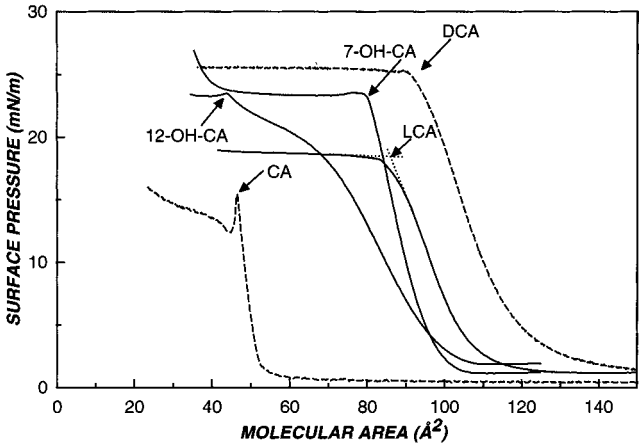


FIGURE 1: Representative $\pi-A$ isotherms for monolayers of LCA, 7-OH-CA, and 12-OH-CA on a subphase of 5 M NaCl, 10 mM phosphate buffer, pH 2, at 21 °C. For comparison, $\pi-A$ isotherms of cholanoic acid and the dihydroxy bile acid DCA (both represented with dashed lines) from ref *4* are shown. The collapse point in each isotherm was determined by drawing tangents to the curve on either side of collapse (illustrated with dotted lines for LCA). Lift-off for all three monohydroxy bile acids occurs between ~100 and 125 Å²/molecule, followed by a steep rise to collapse pressures of 18.5 and 23.5 mN/m for LCA and 7-OH-CA, respectively. At the collapse point (indicated by arrows), monolayers become bile acid multilayers. In contrast to LCA and 7-OH-CA, 12-OH-CA undergoes a second-order phase transition between areas of 45−90 Å²/molecule before reaching its collapse point. Collapse areas of LCA and 7-OH-CA are approximately 85 and 80 Å², respectively, whereas monolayer collapse of 12-OH-CA and cholanoic acid occurs at ~45 Å².

a monolayer coexisting with an accumulating multilayer (*1*, *9*). The presence of this invariant region demonstrates the negligible solubility of the monohydroxy bile acids under these conditions. With further compression to areas smaller than approximately 40 Å², the multilayer collapses and there is an additional increase in pressure (*3*), as seen in Figure 1 for 7-OH-CA. Throughout compression and for 30 min after monolayer collapse, surface films of all three unconjugated monohydroxy bile acids remain liquid as assessed qualitatively (see Experimental Procedures).

Molecular areas of LCA and 7-OH-CA at monolayer collapse are ~85 and 80 Å², respectively. These values are consistent with those observed (86−91 Å²) for dihydroxy bile acids (*4*). In contrast, the molecular collapse area for 12-OH-CA is ~45 Å², similar to that of cholanoic acid (*4*) and consistent with the cross-sectional area of the steroid nucleus (*3*). We infer that, at low surface pressures, the steroid nucleus of 12-OH-CA lies parallel to the aqueous interface but assumes a vertical position upon compression. Because the lift-off area for cholanoic acid (52 Å²) is only slightly greater than the collapse area (Figure 1), the steroid nucleus of this bile acid appears to remain approximately perpendicular to the aqueous interface at all pressures.

Figure 2 displays the dependences of $\pi-A$ isotherms of each monohydroxy bile acid on pH values between 2 and 12. For LCA (Figure 2A), baseline pressure, area at lift-off, and collapse pressure increase with pH and therefore progressive ionization of the carboxylic acid. The collapse areas remain relatively constant to pH 7, decrease slightly between pH 7 and pH 8, and again remain constant at higher pH values. Collapse pressures of 7-OH-CA (Figure 2B) also increase concomitantly with increases in bulk pH. However, the shapes of 7-OH-CA isotherms change markedly with dissociation of the bile acid: At pH values of 9−12, a second-order phase transition occurs in the isotherms between molecular areas of ~45−90 Å². Collapse areas also shift with changes in the shape of the isotherm: Between pH values of 2 and 7, collapse areas remain constant at ~80 Å² but decrease sharply between pH 7 and pH 9, leveling off at ~40 Å² at pH values ranging from 9 to 12. Collapse pressures of 12-OH-CA (Figure 2C) also increase progressively with ionization. However, areas of monolayer collapse remain relatively constant (40−46 Å²) at all pH values. The second-order phase transition in the 12-OH-CA isotherm, observed at pH 2, 5, and 7, diminishes with further increases in surface ionization.

As observed for all these monohydroxy bile acids, increasing collapse pressures upon side chain ionization reflect the molecules' heightened affinity for the aqueous interface. At pH values of 2−7, i.e., in mostly undissociated states, the

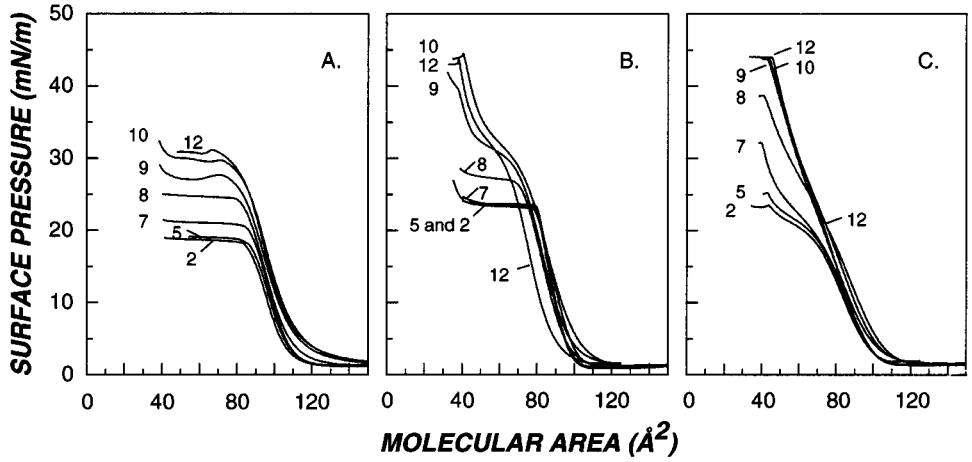


FIGURE 2: pH dependences (inscribed values) of $\pi-A$ isotherms for monolayers of (A) LCA, (B) 7-OH-CA, and (C) 12-OH-CA on a 5 M NaCl subphase at 21 °C. The subphase contained 10 mM phosphate buffer at pH 2, 5, and 12 but 100 mM phosphate buffer at pH 7−10. For all monohydroxy bile acids, areas at lift-off as well as collapse pressures increase with increasing pH. At pH values of 2−7, i.e., in the mostly undissociated state, $\pi-A$ isotherms of 7-OH-CA (B) resemble those of LCA (A), but with further ionization, i.e., progressively higher pH values, the shape of the curve shifts to resemble those of 12-OH-CA (C). At pH values of 9−12, 7-OH-CA (B) undergoes a second-order phase transition between molecular areas of ~45−90 Å². The second-order phase transition of 12-OH-CA (C) observed at pH 2 lessens with increasing pH and degree of ionization.

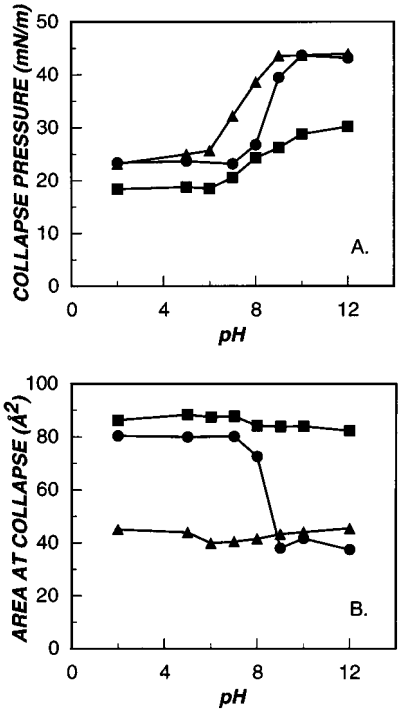


FIGURE 3: pH dependences of (A) collapse pressure and (B) area at collapse for monolayers of (■) LCA, (●) 7-OH-CA, and (▲) 12-OH-CA. (A) With increasing ionization, collapse pressures become elevated in sigmoid fashion for all monohydroxy bile acids. Midpoints of the sigmoidal curves, i.e., approximately pH 8.2 (LCA), 8.5 (7-OH-CA), and 7.5 (12-OH-CA), correspond to the p$K_a$ of the bile acids in a pure monolayer. (B) Molecular areas of LCA and 12-OH-CA at collapse are only minimally affected by increasing pH values. In contrast, the area at collapse of 7-OH-CA shows an abrupt decrease between pH values of 7 and 9.

shape of $\pi-$A isotherms of 7-OH-CA (Figure 2B) resembles that of LCA (Figure 2A) and the dihydroxy bile acids studied previously (Figure 1) (*4*). In contrast, with increasing ionization, the shape of the 7-OH-CA isotherm resembles that of 12-OH-CA (Figure 2C). Areas at collapse for LCA and undissociated species of 7-OH-CA are much larger than for 12-OH-CA and the ionized species of 7-OH-CA.

Figure 3 summarizes the alterations in monolayer characteristics of pure monohydroxy bile acids as the bulk pH is varied from 2 to 12. Figure 3A shows that the collapse pressure of each bile acid increases in sigmoidal fashion with increasing pH corresponding to ionization of the carboxylic acid side chain. Because these are one-phase surface systems, the midpoints of the sigmoid curves, i.e., approximately 8.2, 8.5, and 7.5, correspond to the p$K_a$ in a pure monolayer for LCA, 7-OH-CA, and 12-OH-CA, respectively. Figure 3B shows that the collapse area for LCA and 12-OH-CA changes only minimally with increasing pH. However, the collapse area of 7-OH-CA decreases sharply between pH 7 and pH 9, reflecting the pronounced change in 7-OH-CA isotherms over the same pH range (Figure 2B).

Figure 4 displays surface dipole moments as functions of molecular area for the monohydroxy bile acids, with DCA (*4*) shown for comparison (inset), all at pH 2, 21 °C. Surface dipole moment is a measure of the change in surface dipole normalized for the number of molecules in the monolayer (*1*, *4*). Figure 4 shows that the largest surface dipole moments of all three monohydroxy bile acids are slightly less than half the value for DCA. Most likely this reflects the presence

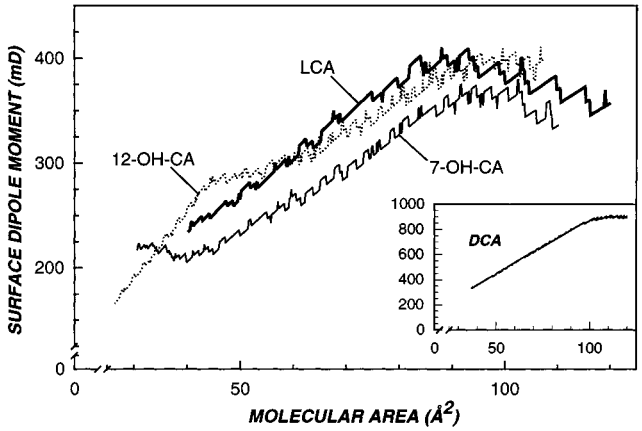


FIGURE 4: Surface dipole moments (mD) of LCA (bold line), 7-OH-CA (light line), and 12-OH-CA (dotted line) on a 5 M NaCl/10 mM phosphate buffer subphase at pH 2. Peak surface dipole moments of all three monohydroxy bile acids are slightly less than half the value for DCA (*4*), shown in the inset. In the case of LCA and 7-OH-CA as well as DCA, surface dipole moments begin to decline near the collapse point of the monolayer at approximately 90, 90, and 100 Å$^2$, respectively. Surface dipole moments of 12-OH-CA undergo a gradual inflection between molecular areas (45−90 Å$^2$) corresponding to the second-order phase transition (see Figure 1), followed by a pronounced discontinuity and a steeper decline following monolayer collapse.

of one hydroxyl group, irrespective of position, in comparison to two hydroxyl groups (as in DCA) interacting with the aqueous interphase. Because surface dipole moments of the monohydroxy bile acids are so much smaller than those of dihydroxy bile acids, the magnitude of changes observed was also more subtle. For DCA (Figure 4 inset), surface dipole moments are constant between molecular areas of 125 and 100 Å$^2$ and then decrease sharply and linearly following monolayer collapse at 91 Å$^2$ (see Figure 1). The displayed surface dipole moments of LCA and 7-OH-CA correspond to a similar $\pi-$A profile (see Figure 1). However, in the case of 12-OH-CA, there is a broad inflection in the dipole moment between molecular areas of 45−90 Å$^2$ corresponding to the second-order phase transition observed in the $\pi-$A isotherm (see Figure 1). Following monolayer collapse at 45 Å$^2$ (see Figure 1), there is a marked decrease in dipole moment, consistent with fewer molecules interacting with the aqueous subphase as further compression results in progressive buildup of a multimolecular layer (*3*).

*Surface Behavior of Monohydroxy Bile Acid/POPC Monolayers*. Figure 5 displays representative $\pi-$A isotherms for binary mixtures of the monohydroxy bile acids with varying proportions of bile acid to POPC at pH 2 (upper panels: A, LCA; B, 7-OH-CA; and C, 12-OH-CA) and 10 (lower panels: D, LCA; E, 7-OH-CA; and F, 12-OH-CA), respectively. With decreasing molar percentage of bile acid (inscribed values), monolayer collapse pressures increase until, at the highest relative POPC contents, the isotherms resemble those of POPC alone with no collapse below 40 mN/m. The progressive increases in collapse pressure imply that bile acids and POPC are completely miscible in the monolayer. If this were not the case, bile acids would collapse out of the mixed monolayer at the same pressures as in pure systems (see Figure 1).

Figure 6 shows mean molecular areas of LCA/POPC monolayers as functions of mol % POPC at two pH values [panels A (pH 2) and D (pH 10)], 7-OH-CA/POPC [panels

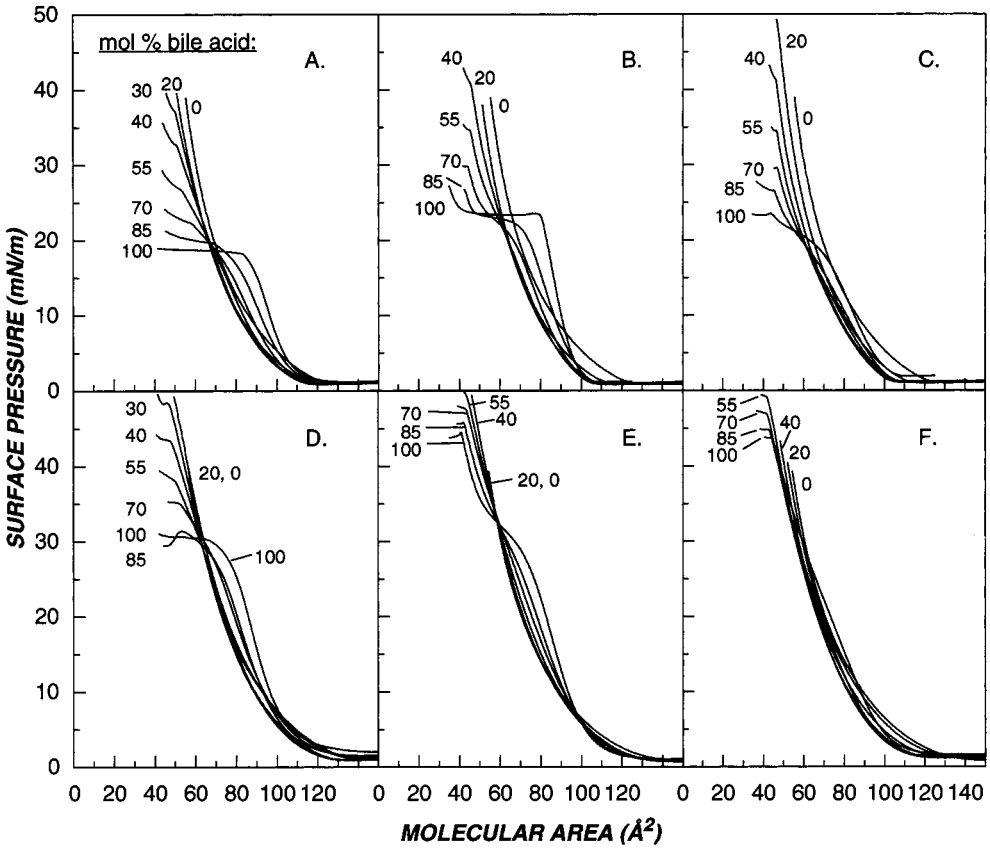


FIGURE 5: $\pi$−A isotherms for binary mixtures of the monohydroxy bile acids LCA (A and D), 7-OH-CA (B and E), and 12-OH-CA (C and F) with varying proportions of POPC at pH 2 (A−C) and pH 10 (D−F). The inscribed percentages show bile acid compositions, which range from 0 to 100 mol %. Monolayers were spread on a 5 M NaCl subphase prepared with 10 mM phosphate buffer (pH 2) or 100 mM phosphate buffer (pH 10) at 21 °C. With increases in the relative mol % POPC, collapse pressures rise progressively, implying intimate miscibility of the bile acids and POPC. For example, for LCA/POPC mixtures at pH 2 (A), the collapse pressure increases from 18 mN/m for 100% LCA to 37 mN/m for 30% LCA/70% POPC. At 80 mol % POPC (A), monolayer collapse does not occur below 40 mN/m, and the isotherm of the mixed monolayer is similar to that of POPC alone.

B (pH 2) and E (pH 10)], and 12-OH-CA/POPC [panels C (pH 2) and F (pH 10)], all at surface pressures ranging from 2.5 to 40 mN/m. These comparisons for various relative compositions of bile acid and POPC allow evaluation of the degree to which each bile acid condenses the monolayer. Dashed lines at 2.5 mN/m connect the mean molecular areas of pure bile acid and pure POPC monolayers and depict the weighted average, i.e., ideal mixing of the pure components. Negative deviations from the dashed line indicate condensation of the POPC monolayer by bile acids. LCA, 7-OH-CA, and 12-OH-CA at pH 2 demonstrate similar negative deviations, indicating that all three undissociated bile acids condense POPC monolayers to the same degree. In general, the deviation from ideality becomes less pronounced at higher surface pressures, as was observed for dihydroxy bile acids (*4*). At the highest surface pressures nearing the collapse pressure of the bile acid-rich monolayer, some curves (e.g., 20−40 mN/m for LCA/POPC at pH 2) become concave downward, preventing precise determination of apparent molecular areas. At pH 10 (lower panels), similar but lesser effects were observed for fully ionized LCA and 12-OH-CA, whereas fully ionized 7-OH-CA condensed the POPC monolayer only marginally. Therefore, although monohydroxy bile acids condense POPC monolayers, the un-ionized species do so to a greater degree than the ionized species.

We proposed earlier that condensation of POPC monolayers by dihydroxy bile acids is facilitated by more efficient packing of POPC acyl chains around the hydrophobic surface of the steroid nuclei of the bile acids lying flat at the interface (*4*). The effectiveness of 12-OH-CA in condensing POPC monolayers suggests that condensation is independent of the interfacial orientation of the bile acid. Since the orientation of the steroid nucleus of cholanoic acid is perpendicular, with the COOH-group anchored in the interface (*3*, *4*), we tested the hypothesis that steroid orientation is immaterial to the condensation process by examining $\pi$−A isotherms of monolayers of binary mixtures of cholanoic acid and POPC (primary data not displayed). Figure 7 plots mean molecular areas for various relative compositions of cholanoic acid and POPC at pH 2 at a surface pressure of 2.5 mN/m. The degree of negative deviation from ideality approximates that of 12-OH-CA at pH 2 (see Figure 6C), indicating that the ability of bile acids to condense POPC monolayers is independent of their orientation within the monolayer. It is notable that 12-OH-CA at pH 2, despite the position of its OH group, condenses POPC monolayers as effectively as cholanoic acid (Figures 6C and 7).

To determine the p$K_a$ of binary mixtures of monohydroxy bile acids in a POPC mixed monolayer, we obtained $\pi$−A isotherms for mixtures containing ~30 mol % POPC at pH values ranging from 2 to 10 (primary data not shown). All isotherms lacked an invariant intermediate region prior to collapse, from which we inferred full miscibility of the components. Figure 8 displays the dependence of collapse

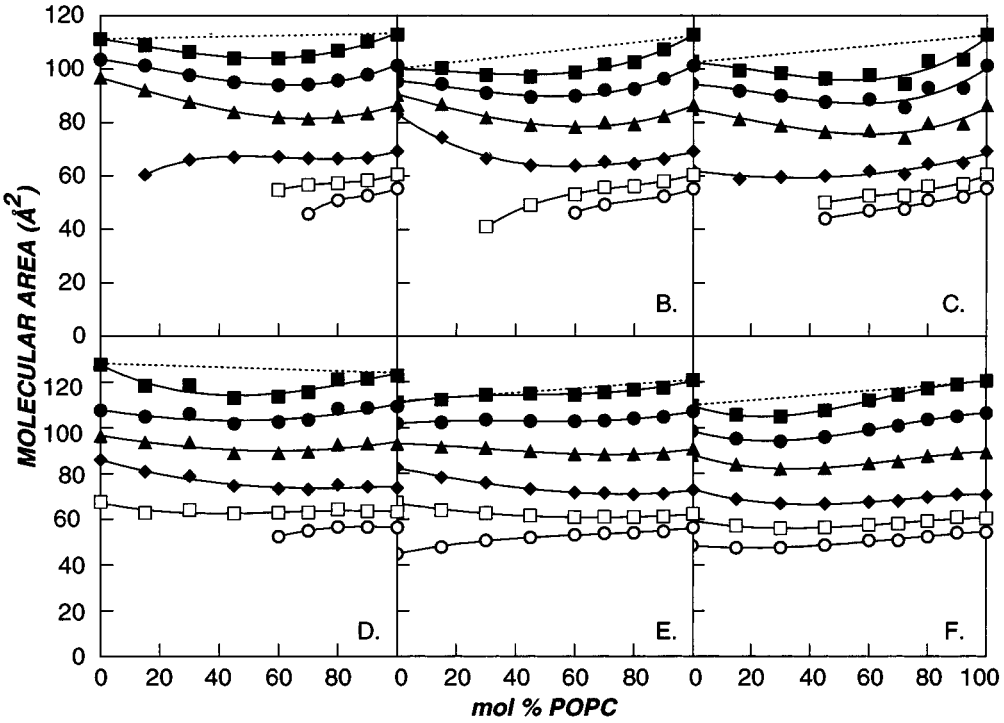


FIGURE 6: Dependences of average molecular areas on relative bile acid compositions, as determined from $\pi-A$ isotherms of binary mixtures of LCA/POPC (A and D), 7-OH-CA/POPC (B and E), and 12-OH-CA/POPC (C and F) at pH 2 (A−C) and pH 10 (D−F). Determinations were made for several surface pressures: (■) 2.5, (●) 5.0, (▲) 10, (◆) 20, (□) 30, and (○) 40 mN/m. (Curves are third-degree polynomial fits.) The dashed lines at 2.5 mN/m connecting 0 and 100% POPC indicate the anticipated behavior if ideal mixing of the two components occurred. At pH 2, all bile acids produce negative deviations from ideality, indicating condensation of the POPC monolayer. As pressures increase, condensation decreases, particularly at pH 10. When nearing the collapse pressure of the bile acid-rich monolayer, the curves become concave downward at the highest surface pressures, precluding accurate determination of apparent molecular areas. All three bile acids condense the monolayers to lesser degrees when fully ionized at pH 10 (D−F) than when un-ionized at pH 2 (A−C).

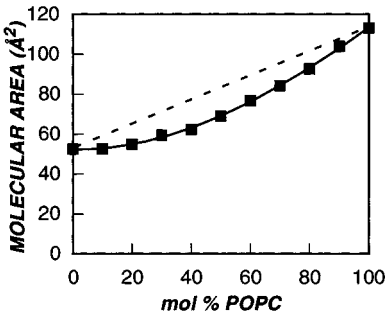


FIGURE 7: Dependence of average molecular area on relative % POPC composition for binary mixtures of cholanoic acid and POPC at a surface pressure of 2.5 mN/m, as determined from $\pi-A$ isotherms (5 M NaCl, pH 2, at 21 °C). Although cholanoic acid (no hydroxyl function) is oriented perpendicularly to the aqueous interface at all relative POPC compositions, there is substantial negative deviation from ideality in the binary mixtures, approximating that of 12-OH-CA at pH 2 (Figure 6C), indicating condensation of the POPC monolayer.

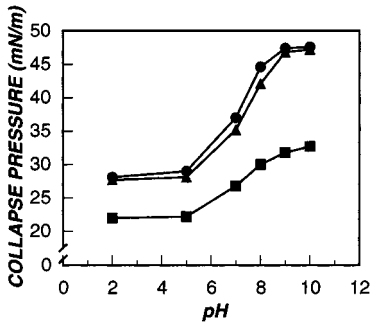


FIGURE 8: Dependences of collapse pressures on bulk pH for binary mixtures of monohydroxy bile acids [(■) LCA, (●) 7-OH-CA, and (▲) 12-OH-CA] and ~30 mol % POPC (5 M NaCl/100 mM phosphate buffer, at 21 °C). All bile acids display a sigmoidal increase in collapse pressure with increasing pH values. The p$K_a$ values of LCA, 7-OH-CA, and 12-OH-CA in a mixed monolayer (i.e., approximately 7.5, 7.0, and 7.5, respectively) were determined from the midpoints of the curves.

pressures on pH for these bile acid/POPC mixtures. As with pure bile acids (see Figure 3A), collapse pressures increase with progressive ionization of the carboxylic acid side chain. From the midpoints of the sigmoidal curves (Figure 8), we inferred that the p$K_a$ of each bile acid in a mixed monolayer lies between 7.0 and 7.5.

*Ionization of Monohydroxy Bile Acids in Mixed Micellar Solution.* The p$K_a$ values of monohydroxy bile acids were estimated by potentiometric titration of each bile acid in metastable TC mixed micellar solutions (data not displayed). Estimated p$K_a$ values derived from the midpoint of each titration curve were 6.1−6.3, somewhat lower than those determined for monohydroxy bile acids in pure or mixed POPC monolayers.

Hydrophobic indices, as determined by HPLC retention times, decreased in a sigmoidal fashion with increasing $pH_{app}$ of the methanolic eluent solution from 2.2 to 1.5 for LCA, from 2.8 to 2.2 for 7-OH-CA, and from 2.8 to 2.3 for 12-OH-CA (data not displayed). Therefore, loss of the 3α-OH group increases molecular hydrophobicity markedly, as was observed previously for the synthetic 7α,12α-dihydroxycholanoic acid (*4*). By comparing HPLC retention times of

Table 1: p$K_a$ Values of Monohydroxy Bile Acids under Various Physical−Chemical Conditions

| physical−chemical state | LCA | 7-OH-CA | 12-OH-CA |
|---|---|---|---|
| monomer, bulk phase[a] | ∼4.5 | ∼4.5 | ∼4.5 |
| in TC micelles, bulk phase[b] | 6.3 | 6.1 | 6.1 |
| in pure monolayers[c] | ∼8.2 | ∼8.5 | ∼7.5 |
| in mixed POPC monolayers | ∼7.5 | ∼7.0 | ∼7.5 |

[a] Determined by retention times on HPLC as a function of $pH_{app}$ of MeOH/$H_2O$ (75:25, v/v) and by comparison with dihydroxy bile acids under the identical conditions. [b] Determined by potentiometric titration of monohydroxy bile acids solubilized in TC micellar solutions (see Experimental Procedures). [c] Determined by variation of collapse pressure with pH of subphase (Figure 3).

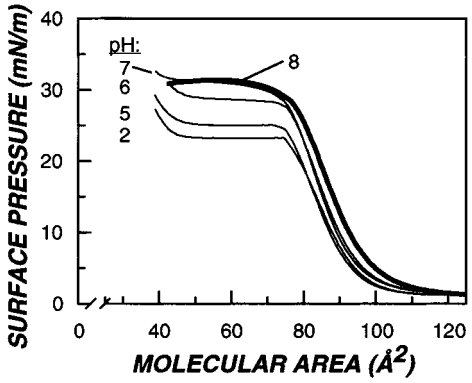


FIGURE 9: Representative $\pi$−A isotherms of GLCA at pH values (inscribed numbers) from 2 to 8 on a subphase of 5 M NaCl/100 mM phosphate buffer at 21 °C. At all pH values, baseline pressures are essentially identical, but molecular areas at lift-off gradually expand with increasing pH. At pH 2, a sharp monolayer collapse occurs at a molecular area of 75 Å$^2$ and a surface pressure of 23 mN/m. As pH is increased progressively, collapse pressures also increase, and a more gradual collapse occurs with molecular areas remaining essentially constant at ∼75 Å$^2$. At pH 8, the downward slope of the $\pi$−A isotherm for GLCA (bold curve) provides graphic evidence of increasing solubility at high surface pressures.

cholic acid [p$K_a$ of ∼5 (*3*)] as functions of $pH_{app}$ (*13*, *21*), we found that all monohydroxy bile acids gave slightly lower p$K_a$ values of ∼4.5 (data not shown). Table 1 summarizes p$K_a$ values of the monohydroxy bile acids as determined under various physical chemical conditions.

*Monolayers Containing Glycolithocholic Acid*. Figure 9 presents representative $\pi$−A isotherms of GLCA at pH values ranging from 2 to 8. At pH 2, monolayer collapse occurs at a molecular area of 75 Å$^2$ and surface pressure of 23 mN/m. With increasing pH, molecular areas at lift-off increase and collapse pressures become elevated, but molecular areas at collapse remain essentially identical at ∼75 Å$^2$. The isotherm at pH 8 (bold line) becomes concave downward at molecular areas beyond the collapse point, i.e., surface pressure falls with continuing compression, indicating solubilization of GLCA from the surface into the bulk phase and rendering the calculation of molecular areas no longer accurate. Similar to the behavior of the unconjugated species (Figure 2), the collapse pressures of GLCA increase with increasing pH. At pH 2, GLCA multilayers after collapse remain liquid for at least 30 min but solidify prior to collapse at pH values of 4 and 6.

Figure 10A shows $\pi$−A isotherms for mixed GLCA/POPC monolayers at pH 4. There is an increase in the pressure at which monolayer collapse of GLCA occurs, indicating complete miscibility of the bile acid and bile salt with POPC. (See also LCA in Figure 5A,D.) Figure 10B summarizes mean molecular areas for mixed GLCA/POPC monolayers at pH 4 as functions of mol % POPC at surface pressures of 2.5−40 mN/m (consecutive curves from top to bottom). Negative deviations from ideality indicate condensation of the POPC monolayer by the fully protonated GLCA, although the magnitude is less than that observed with unconjugated LCA (see Figure 6A).[4]

## DISCUSSION

Studies of spread monomolecular films provide well-defined models for lipid bilayers and can contribute information, otherwise difficult to obtain, on molecular orientation and intermolecular interactions at the air (hydrophobic)−aqueous interface. Previously, inferences regarding the physical−chemical state of fully ionized bile salts have necessarily been extrapolated from studies of protonated dihydroxy bile acids at an air−water interface because of the comparatively greater aqueous solubility of trihydroxy bile acids (*3*) and dihydroxy bile salts (*4*). Undissociated dihydroxy bile acids form stable monolayers on high ionic strength subphase but not when they are ionized (*4*). In contrast, neither trihydroxy bile salts nor conjugated dihydroxy bile acids form stable monolayers, even for the fully protonated glycine-conjugated form, on a 5 M NaCl subphase (*4*, *22*, *23*).

Undissociated monohydroxy bile acids are extremely insoluble in bulk aqueous solution because of the single hydroxyl function and remain insoluble when fully ionized due to their high Krafft points[5] (*3*, *8*). Therefore, unlike other bile acids, unconjugated monohydroxy bile acids, whether protonated or fully ionized, should form stable monolayers. As we have shown here, the strengthening of hydrophilicity with the glycine side chain renders GLCA monolayers unstable in the ionized form (Figure 9). Furthermore, as the most hydrophobic of the naturally occurring bile acids, LCA is the most likely candidate to form dimers within monolayers or bilayers as previously postulated (*3*). Based on the enhanced hydrophobicity of the synthetic 7α,12α-dihydroxycholanoic acid as compared with common dihydroxy bile acids (*4*), the 7α and 12α isomers of LCA were predicted to be even more hydrophobic than LCA, as confirmed by HPLC determinations of hydrophobic indices (see Results).

*Orientation of Monohydroxy Bile Acids and Their Salts in Monolayers.* LCA shares key surface characteristics with dihydroxy bile acids (*4*). The lift-off area at ∼125 Å$^2$ (pH 2) is similar, indicating that at low surface pressures the long axis of the steroid nucleus lies parallel to the interface (*3*). The collapse area is ∼85 Å$^2$, similar to the areas (86−91 Å$^2$) at collapse of dihydroxy bile acids (*4*). Upon full

[4] Studies with GLCA and POPC monolayers at pH 8 showed a decrease in surface pressure with compression in the range of 45−100 mol % GLCA, implying our inability to determine the number of molecules at the interface accurately (data not shown). Although bulk GLCA solubility at pH 8 precludes precise knowledge of the molecular area at the interface, minimal condensation of the binary mixture did occur at 2.5 and 5 mN/m.

[5] Krafft points are known to be ∼50 °C for the sodium salts of LCA and GLCA (*3*). We inferred that the Krafft points of the sodium salts of 7-OH-CA and 12-OH-CA were greater than ambient temperature (∼21 °C) from the inability of their surface films to solubilize in the bulk phase upon monolayer compression at pH 10.

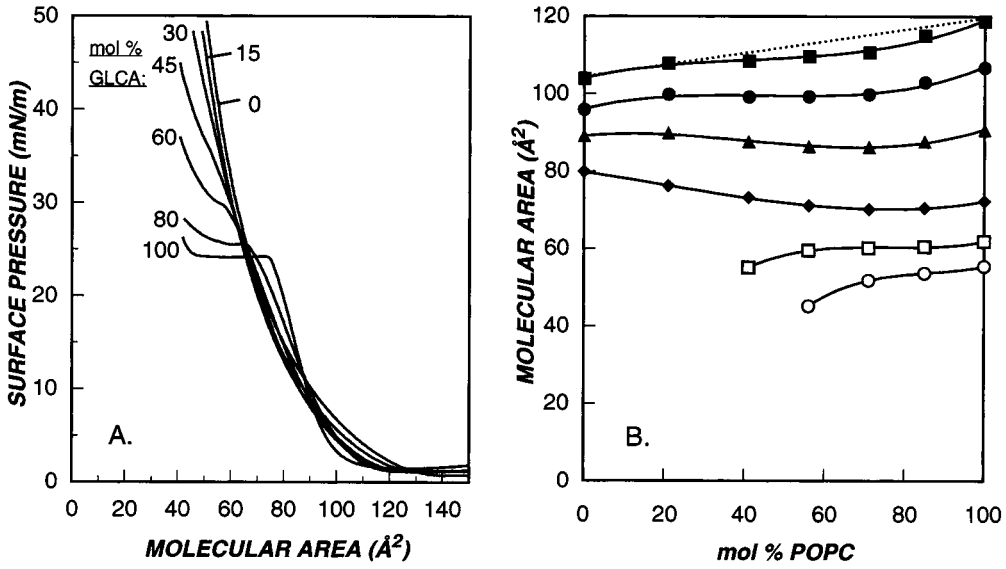


FIGURE 10: Behavior of GLCA/POPC mixed monolayers at pH 4. (A) $\pi-A$ isotherms of GLCA compositions ranging from 0 to 100 mol % (inscribed percentages). The collapse pressures of GLCA in the monolayer increase with increasing % POPC, indicating total miscibility of the conjugated bile acid with POPC. (See also Figure 5A,D for unconjugated LCA.) (B) Dependences of average molecular areas on relative bile acid composition at several surface pressures: (■) 2.5, (●) 5.0, (▲) 10, (◆) 20, (□) 30, and (○) 40 mN/m. The dashed line at 2.5 mN/m represents the ideal mixing behavior of the two components. Negative deviations from ideality indicate that the protonated GLCA condenses POPC monolayers, although to a lesser degree than unconjugated LCA (Figure 6A); GLCA decreases the effective molecular area by ~15 Å$^2$ as compared to ~35 Å$^2$ for LCA. The curves become concave downward at 30 and 40 mN/m, indicating that the effective molecular areas could no longer be determined.

ionization, the area at collapse changes only minimally (Figure 3B), contrary to earlier observations on LCA (*3*). However, the collapse pressure does increase with ionization (Figure 3A), a trend also noted previously (*3*). The behavior of cholanoic acid at pH 2 differs strikingly in that no substantial increase in surface pressure occurs until molecular areas are reached approaching that of the cross-sectional area of the steroid nucleus (Figure 1) (*3*).

The behavior of 12-OH-CA contrasts with that of both LCA and 7-OH-CA at pH 2. The lift-off pressure above 100 Å$^2$ (Figure 1) implies that the initial orientation of the steroid nucleus is parallel to the interface, but with compression, the steroid nucleus assumes a more perpendicular orientation. The gradual increase in surface pressure over the broad range of molecular areas between 100 and 45 Å$^2$ suggests that a change in the structure of the monolayer occurs, analogous to the increase in surface pressure for POPC as acyl chains reorient during compression. Because there is no invariant pressure region until collapse at 45 Å$^2$, we infer that only one surface phase is present at greater molecular areas (*9*). Whether protonated or ionized, 12-OH-CA undergoes a second-order phase transition during compression of its monolayer, most likely caused by a change in molecular orientation.

The behavior of 7-OH-CA is intermediate between that of LCA and 12-OH-CA. Apparently the affinity of the 7α-OH group for the aqueous interface lies between the aqueous affinities of the 3α- and 12α-OH groups, corresponding to an intermediate configuration in three-dimensional representations (see Figure 11). We speculate that ionization of the carboxylic acid side chain may alter the balance of hydrophilicity between opposite ends of the molecule to allow lift-off of the hydroxyl group and, at high pressure, assumption of a more vertical orientation, as occurs with 12-OH-CA (Figure 2).

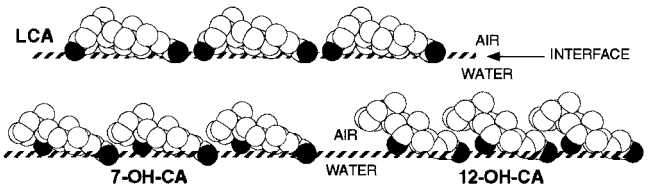


FIGURE 11: Three-dimensional representations of the monohydroxy bile acids (LCA, 7-OH-CA, and 12-OH-CA) studied. Carbon atoms are depicted in white, oxygen atoms are depicted in black, and hydrogen atoms have been omitted for clarity. At pH values of 2−12 and at low surface pressures, the steroid nucleus of all three monohydroxy bile acids lies flat at the aqueous−air interface. The 3α-OH group of LCA strongly anchors the end opposite the carboxyl side chain to the aqueous interface. In the case of the 7α-OH and especially the 12α-OH group, these permit slight tilts of the molecule at the interface without withdrawing the corresponding hydroxyl group from the interface. However, with increasing compression (12-OH-CA) and higher pH values (7-OH-CA and 12-OH-CA), molecular reorientations force the molecules to become perpendicular, and the 7α-OH and 12α-OH groups are withdrawn from the interface.

Molecular models of the monohydroxy bile salts highlight the crucial role of the 3α-OH group in orienting the steroid nucleus parallel to the interface (Figure 11). The 3α-OH group interfacially anchors the end of the molecule opposite the carboxyl side chain. Consequently, the lifting out of a 3α-OH function on a bile acid molecule from the interface is energetically highly unfavorable as compared to the corresponding 7α- or 12α-OH groups. Additionally, the position of the 3α-OH group is less sterically hindered than the 7α- or 12α-OH group (*3*), presumably allowing more interaction with the aqueous interface. As we observed previously for the synthetic bile acid 7α,12α-dihydroxycholanoic acid (*4*), decreasing the hydrophilic area by eliminating the 3α-OH group produces a more hydrophobic molecule. Furthermore, 7α,12α-dihydroxycholanoic acid collapses out of the interface at 16 mN/m in contrast to 25 mN/m as seen with DCA

and CDCA (*4*). Moreover, the lack of the 3α-OH group allows both 7-OH-CA and 12-OH-CA to assume more vertical orientations without withdrawing the corresponding hydroxyl group from the interface (Figure 11).

Surface dipole moments reflect the changes in electrical potential at the aqueous−air interface for the monolayer alone as compared with the aqueous interface (*24*). The net magnitude is the sum of molecular dipole moments of individual molecules as well as their ordering effects on the subphase. Comparison of surface dipole moments of dihydroxy bile acids suggested that the contribution of the hydroxyl groups predominates, since the rank order can be predicted from the resultant vector of the sum of the hydroxyl groups (*4*). Furthermore, the surface dipole moment of an unstable monolayer (*4*) of the trihydroxy bile acid cholic acid is 1300 mD (*22*), which is ∼50% greater than values for dihydroxy bile acids and approximately three times those of monohydroxy bile acids (Figure 4). Therefore, differences in dipole moments reflect changing orientation and number of hydroxyl groups rather than contributions of the carboxylic side chain (*4*). Both LCA and 7-OH-CA show a gradual decline in surface dipole moment during the invariant region of the surface pressure isotherm (Figure 4), consistent with cancellation of individual molecular dipoles during conversion from a monolayer to a multilayer. For 12-OH-CA, the decrease in surface dipole moment between ∼90 and 45 Å$^2$, observed in the absence of an invariant region of the $\pi-A$ isotherm, may be attributed to reorientation of the molecules at the interface.

The wide range of molecular areas between lift-off and collapse for 12-OH-CA is characteristic of a molecular rearrangement within the monolayer (*1*). In contrast, compression of expanded monolayers of phosphatidylcholine occurs over a broad range of areas, i.e., POPC has a lift-off at ∼120 Å$^2$, rising gradually to values of 40 mN/m at ∼55 Å$^2$. This is attributed to the fluid nature of the acyl chains, which undergo a change in orientation from a random chain with predominantly gauche $CH_2$ conformations at the lift-off point to a minimum approximating the cross-sectional area of two acyl chains with mainly trans conformations. In contrast, more rigid molecules containing a steroid nucleus, such as cholanoic acid (Figure 1) and cholesterol (*3, 10*), exhibit very little increase in surface pressure with compression of the condensed monolayer until values close to their cross-sectional areas (*2, 3*).

*Interactions of Monohydroxy Bile Acids and Their Salts with Phosphatidylcholine.* Both the undissociated form of LCA and its fully ionized sodium salt induce condensation in POPC monolayers, i.e., negative deviations from average molecular areas predicted by ideal mixing (Figure 6A,D). Our results agree with those of Small (*3*) measured at pH 2 on a $H_2O$ subphase but disagree with those obtained by Gálvez Ruiz and Cabrerizo Vilchez (*25*), who obtained isotherms for LCA/L−α-phosphatidylcholine mixtures (molecular species not specified) on a $H_2O$ subphase, pH ∼6, at 25 °C, showing molecular areas that differ markedly, i.e., LCA bigger and phosphatidylcholine smaller, from those in Figure 5A,D. As a result, their additivity plots showed positive deviations from ideality at surface pressures displayed in Figure 6A,D.

In our previous work (*4*), the degree of condensation for a series of dihydroxy bile acids correlated with bile acid hydrophobicity. Indeed, at pH 2, the more hydrophobic 7-OH-CA and 12-OH-CA induce a greater degree of condensation in mixed monolayers than does LCA, and condensation was less for the fully ionized species than for the undissociated form. The effective molecular area (see Experimental Procedures) of LCA in the POPC monolayer was ∼35 Å$^2$ less than that of the pure bile acid, whereas the areas of 7-OH-CA and 12-OH-CA were ∼45 Å$^2$ smaller. However, LCA induces the same degree of condensation of the POPC monolayer as found previously for the hydrophilic ursodeoxycholic acid or hyodeoxycholic acid molecules (*4*). This may be due to differences in regional hydrophobicity not revealed by measurements of overall hydrophobicity by the HPLC method (*13*). Possibly, the somewhat smaller molecular area of LCA molecules as compared with dihydroxy bile acids (see Figure 1) precludes the even tighter molecular packing that condensation implies.

*p$K_a$ Values of Bile Acids in Monolayers.* It is well-established that the ionization constants of carboxylic acids depend on the local environment. We observed progressive increases in the apparent p$K_a$ of monohydroxy bile acids from ∼4.5 for the monomer, to 6.1−6.3 for bile acids metastably solubilized in TC micelles, to ∼7.0−8.5 for bile acids in pure or mixed monolayers with POPC (Table 1). The p$K_a$ of cholic acid in small unilamellar phosphatidylcholine vesicles has been determined by NMR to lie above 6.8 (*14*). Other amphiphiles such as fatty acids also exhibit an increase in p$K_a$ in bilayers as compared to monomers in aqueous solution (*14*). For example, the p$K_a$ values of lauric and oleic acids are 8.0 and 8−8.5, respectively, as inferred by titration in bulk solution when a lamellar fatty "acid-soap" phase is present (*26*). In phosphatidylcholine bilayers, the p$K_a$ of oleic acid is ∼7.5, but when bound to albumin or in monomeric solution, the p$K_a$ is ∼4.2 (*27*). Monolayers exhibit p$K_a$ values similar to those of bilayers: For example, p$K_a$ values of fatty acids in monolayers were estimated to be 7.9−9.7, based on rates of dissolution into the bulk solution (*28*). It has been suggested that the high negative surface charge density sequesters protons in an electrical double layer, leading to an increase in pH for a fixed degree of ionization (*14, 19*). Additionally, changes in the local dielectric constant due to high ionic strength may increase the apparent p$K_a$. As expected for an ionized as compared with an uncharged monolayer (*1*) and as previously observed for fatty acids (*29*), LCA and 12-OH-CA monolayers expand upon ionization (Figure 2).

*Pathophysiological Correlations*. In a systematic analysis of vertebrate and fish biles across phylae, Hagey has shown that all natural bile salts contain a 3α-OH group (*30*). The physical−chemical importance of this functional group was suggested by our earlier observation that the synthetic 7α,-12α-dihydroxycholanoic acid has an anomalously low collapse pressure in monolayers (*4*), yet extensive pharmacophoric work by Kramer et al. (*31*) has revealed that the 3α-OH group is not essential for active intestinal or hepatic uptake of bile acids. The present work predicts that, under physiological conditions, the 3α-OH group of all naturally occurring bile acids and their salts will allow the molecules to interact with the bulk aqueous solution surrounding membranes, while the hydrophobic portion of the bile acid/salt molecules will interact with and condense the liquid acyl chains of membrane phospholipids.

At physiological concentrations, bile salt molecules partition preferentially into the phospholipids of membranes (*3*−*6*). Therefore, the mechanisms of interactions of bile salts with lipid membranes are fundamental to understanding their physiological properties. Indeed, the functional consequences of bile salt adsorption to membranes are predicted to be a direct consequence of the molecular structure of bile salts in lipid bilayers. We previously utilized mixed monomolecular layers of dihydroxy bile acids and POPC to deduce that the steroid nucleus remains oriented with the long axis parallel to the lipid bilayer over all relative bile acid/POPC compositions (*4*). This contrasts with previous conclusions that bile acids form dimers with the hydrophilic surfaces apposed, oriented with the steroid nucleus perpendicular to the lipid bilayer (*3*). The functional consequence of the hydrophilic surface of bile acids remaining at the aqueous interface is that no hydrophilic domain is created within the hydrophobic interior of the membrane. Indeed, bile salts do not produce aqueous channels that increase membrane permeability to water or small nonionic solutes (*32*).

In conclusion, the exceptional behavior of monohydroxy bile acids lacking the 3-OH group provides definitive support for our previous conclusions that naturally occurring bile acids (all with 3α-OH functions) are oriented uniquely with the steroid nucleus parallel to a hydrophobic−aqueous interface. Moreover, we have extended our observations on the surface behavior of bile acids and POPC and have obtained similar behavior with the fully ionized monohydroxy bile salts. We conclude that under physiological conditions, naturally occurring ionized bile salts, similar to the undissociated bile acids, are also oriented with their hydrophilic hydroxyl groups interacting with bulk aqueous solution. Furthermore, their hydrophobic surfaces will interact with the hydrocarbon chain portions of lipid monolayers, effectively condensing bilayers. We believe that differences in membrane condensing effects between hydrophilic and hydrophobic bile acids, irrespective of their ionization state, as exemplified here by 3α-, 7α-, and 12α-monohydroxy cholanoic acids and their salts, are related to subtle differences in their common abilities to interact with the acyl chains of phospholipids.

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