The ionization behavior of bile acids in different aqueous environments

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Abstract The ionization behavior of cholic acid, deoxycholic acid, and chenodeoxycholic acid in a variety of physiologically important molecular environments was studied using ^{13}C NMR spectroscopy. The apparent pKa of the carboxyl group was determined from titration curves obtained from the dependence of the carboxyl carbon chemical shift on pH. Using 90% ^{13}C isotopic substitution of the carboxyl carbon, a complete titration curve was obtained for cholate at a concentration below its critical micelle concentration and solubility limit in water. Incorporation of 12 mole % bile acid into mixed micelles with its taurine conjugate prevented precipitation of the unconjugated bile acid, and titration curves for cholic, deoxycholic, and chenodeoxycholic acids in the mixed micelles were obtained. The apparent pKa was also determined for ^{13}C-enriched bile acids complexed with bovine serum albumin and in egg phosphatidylcholine vesicles. For monomers, micelles, and BSA complexes of all three bile acids and for deoxycholic and chenodeoxycholic acid in vesicles, one magnetic environment was observed. In contrast, two environments, both titratable, were detected for cholic acid in phosphatidylcholine vesicles. The apparent pKa's of the bile acids in the different environments ranged from 4.2 to 7.3. At pH 7.4, as monomers or bound to albumin, the bile acids were fully ionized, but when associated with phosphatidylcholine vesicles they were only partially ionized. In addition, aspects of the molecular motion and relative hydrophobicity of the bile acid carboxyl group in the environments studied were discerned from chemical shift, linewidth, and lineshape data. — **Cabral, D. J., J. A. Hamilton, and D. M. Small.** The ionization behavior of bile acids in different aqueous environments. *J. Lipid Res.* 1986. **27:** 334–343.

Supplementary key words bile salts • ^{13}C NMR spectroscopy • apparent pKa • monomers • micelles • phospholipid vesicles • protein complexes

Bile salts are important physiologic solubilizers of other lipids (1–3). Bile salts readily dissolve in aqueous media while the corresponding bile acids have limited solubility and must be solubilized by other molecules. Bile acids and salts may exist in monomeric form, in simple and mixed bile salt micelles, in mixed micelles with other lipids, in phospholipid vesicle bilayers (membranes), or bound to proteins (4–6). In most species the bile salts in hepatic and gallbladder bile exist mostly as taurine and glycine conjugates. The bile salts are secreted into the duodenum where they encounter large changes in pH (pH 3–8) and aid in the digestion and absorption of fat (7). In man and some animals, some of the bile salts are hydrolyzed by intestinal bacteria to the corresponding unconjugated bile salt. These are reabsorbed by the intestine and transported in the portal vein bound to albumin to the liver where they are effectively extracted and taken up by hepatic cells (4). The free bile salts must then traverse cellular membranes and interact with enzymes which reconjugate them with glycine or taurine. Finally, the conjugated bile salts are resecreted into hepatic bile where they may be present as monomers or in mixed micelles or even in vesicles. Thus, both free and conjugated bile salts may encounter different pHs during their enterohepatic recycling and also interact with proteins. Because of possible variation in the carboxyl group microenvironment, it may not be accurate to extrapolate the ionization behavior of monomeric bile acids to the more complex systems. It is important, therefore, to determine the ionization state of the unconjugated bile acids in these different molecular environments.

The ionization behavior of many bile acids has been studied potentiometrically (8–11). However, it has been difficult to assess the ionization state of bile acids when present in small amounts in other environments, particularly when these environments have other titratable groups. ^{13}C NMR spectroscopy is well suited for studying the ionization states of carboxylic acids by measurement of the carboxyl ^{13}C chemical shift versus pH (12). The use of ^{13}C enrichment of the carboxyl carbon provides a

Abbreviations: NMR, nuclear magnetic resonance; CA, cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid); DCA, deoxycholic acid (3α,12α-dihydroxy-5β-cholanoic acid); CDCA, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); PC, phosphatidylcholine; BSA, bovine serum albumin; NaTC, sodium taurocholate; NaTDC, sodium taurodeoxycholate; NaTCDC, sodium taurochenodeoxycholate; [24-^{13}C], a bile acid 90% isotopically enriched in ^{13}C at carbon 24 (standard numbering); NaC, cholic acid, sodium salt; NaDC, deoxycholic acid, sodium salt; NaCDC, chenodeoxycholic acid, sodium salt; CMC, critical micelle concentration; S/N, signal to noise ratio; TLC, thin-layer chromatography.

means of monitoring small amounts of the acids in solution alone (12) or with other molecules (13–15). In this study we have used ^{13}C NMR spectroscopy to study aqueous carboxyl (C24) ^{13}C-enriched cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CDCA) below the critical micelle concentration (CA only), in mixed micelles with their taurine conjugates, in egg phosphatidylcholine (PC) unilamellar vesicles, and in complexes with bovine serum albumin (BSA). The apparent pKa's of the bile acids were determined in each environment. In addition, features of the bile acid molecular environments were assessed from the carboxyl ^{13}C chemical shift, linewidth, and lineshape.

MATERIALS AND METHODS

Materials

Sodium taurocholate (NaTC), sodium taurodeoxycholate (NaTDC), and sodium taurochenodeoxycholate (NaTCDC) were purchased from Calbiochem-Behring (San Diego, CA). Egg PC was obtained from Lipid Products (South Nutley, Surrey, UK), and crystalline fatty acid-free BSA (fraction V) was from Sigma Chemical Company (St. Louis, MO). CDCA and methyl cholate with 90% ^{13}C isotopic substitution at carbon 24 were gifts from Dr. Peter Klein. Ninety percent [24-^{13}C]DCA was purchased from Merck & Company (St. Louis, MO). All non-enriched bile salts were >95% pure by TLC. NMR spectra of ^{13}C-enriched bile salts with signal to noise ratios >20:1 (in H_2O at pH 10.0) showed only a single carboxyl peak. D_2O, $CDCl_3$, and $(CH_3)_4Si$ were obtained from Stohler Isotope Chemicals (Waltham, MA).

NMR spectroscopy

Proton-decoupled Fourier transform ^{13}C NMR spectra were obtained at 50.3 MHz with a Bruker WP-200 spectrometer equipped with a Bruker B-VT-1000 variable temperature unit and an Aspect 2000 data system. Aqueous samples were placed in 10-mm NMR tubes with D_2O included as an internal lock for the PC and BSA samples. For monomeric and micellar samples, $CDCl_3$ with $(CH_3)_4Si$ in a coaxial insert served as an external lock and reference for chemical shift assignments. The terminal methyl of the PC acyl chains at 14.10 ppm (16) or the relatively narrow amino acid resonance at 39.84 ppm (17) in the BSA spectra was used as an internal chemical shift reference. Chemical shifts were accurate to ± 0.05 ppm except for BSA spectra (± 0.1 ppm). Broad-band proton decoupling (1.0 W) centered 3.4 ppm downfield from $(CH_3)_4Si$ was used. Spectra were obtained at 35°C using 16K data points and a pulse interval of 2s or 3s.

Sample preparation

To prepare [24-^{13}C]sodium cholate (NaC), [24-^{13}C]methyl cholate was hydrolyzed in excess NaOH with heating. After 2 hr the reaction mixture was cooled to 20°C and spun at 2000 rpm for 15 min, and the clear supernatant was separated from any unreacted crystalline methyl cholate. The cholate preparation showed a single spot by thin-layer chromatography (cyclohexane–ethyl acetate–acetic acid 7:23:3). A ^{13}C NMR spectrum at pH 10 gave a single resonance in the carboxyl region at the expected chemical shift (18, 19). Stock solutions of [24-^{13}C]NaC, [24-^{13}C]sodium deoxycholate (NaDC), and [24-^{13}C]sodium chenodeoxycholate (NaCDC) at 15 mg/ml, pH 10, were used for sample preparation.

An aliquot of the [24-^{13}C]NaC stock was diluted with H_2O to yield aqueous cholate (0.2 mM) below its critical micelle concentration (CMC) and below the solubility limit of CA. For mixed bile salt micelles, 10 mg of the appropriate bile salt (natural abundance) and 90 mg of its taurine conjugate were dissolved in 2.0 ml of H_2O and the pH was adjusted to 11. This 7:1 NaTC-NaC mol ratio adequately maintains the cholic acid in solution at low pH (8, 9). All micellar and monomeric solutions were clear and colorless at pH 11.

Bile acid-BSA complexes were prepared by the procedure of Parks et al. for the preparation of fatty acid-BSA complexes (13). An aliquot (1.3 ml; 1.6×10^{-3} mmol) of aqueous 8% (w/v) BSA (pH 7.4) was added with mixing to 0.1 ml (3.2×10^{-3} mmol) [24-^{13}C]NaC, NaDC, or NaCDC stock solution in an NMR tube. Samples with higher mol ratios of bile acid/BSA were prepared for DCA. The pH was adjusted to 10; the sample was clear and slightly viscous.

Unilamellar vesicles were prepared by two methods. In the first method, described previously (20), 97 mg of egg PC and 3 mg of [24-^{13}C]bile salt were mixed in chloroform-methanol 2:1 and the solvent was evaporated under N_2. The sample was hydrated, adjusted to pH 10, and ultrasonically irradiated using a Branson W-350 Sonifier in a pulsed mode, 35% duty cycle, and 3.5 output level. The sonication vial was suspended in an ice bath to keep the sample temperature at 25–30°C. Sonication was carried out for 45 min, titanium was removed by low speed centrifugation, and the translucent supernatant (pH 10) was transferred to an NMR tube. For the second method, 97 mg of PC was hydrated and sonicated as before; then 3 mg of [24-^{13}C]NaC (pH 10) was added to the vesicle preparation and mixed by vortexing for 1 min. The concentration of egg PC in the NMR tube was 6.06 g/dl (78 mM). The mol ratio of PC to bile salt was ~20:1. The size of the vesicles was monitored by negative stain electron microscopy using 1% phosphotungstic acid on a 1/50 dilution of 78 mM (3% NaC–97% PC) preparation on carbon-coated Formvar grids at 97,875 × magnification.

Titration

Samples were titrated from high to low pH with 1.0 N HCl, except for 0.2 mM NaC which was titrated with 0.1 N HCl. The pH was monitored using a Beckman model 3560 digital pH meter equipped with a Markson microtip probe to allow measurement in the NMR tube. At selected pH values, the amount of HCl added was recorded, the sample appearance was noted, and ^{13}C NMR spectra were obtained. The pH measured before and after each NMR run was the same (± 0.1 pH units). The C24 chemical shift was plotted versus pH, and the

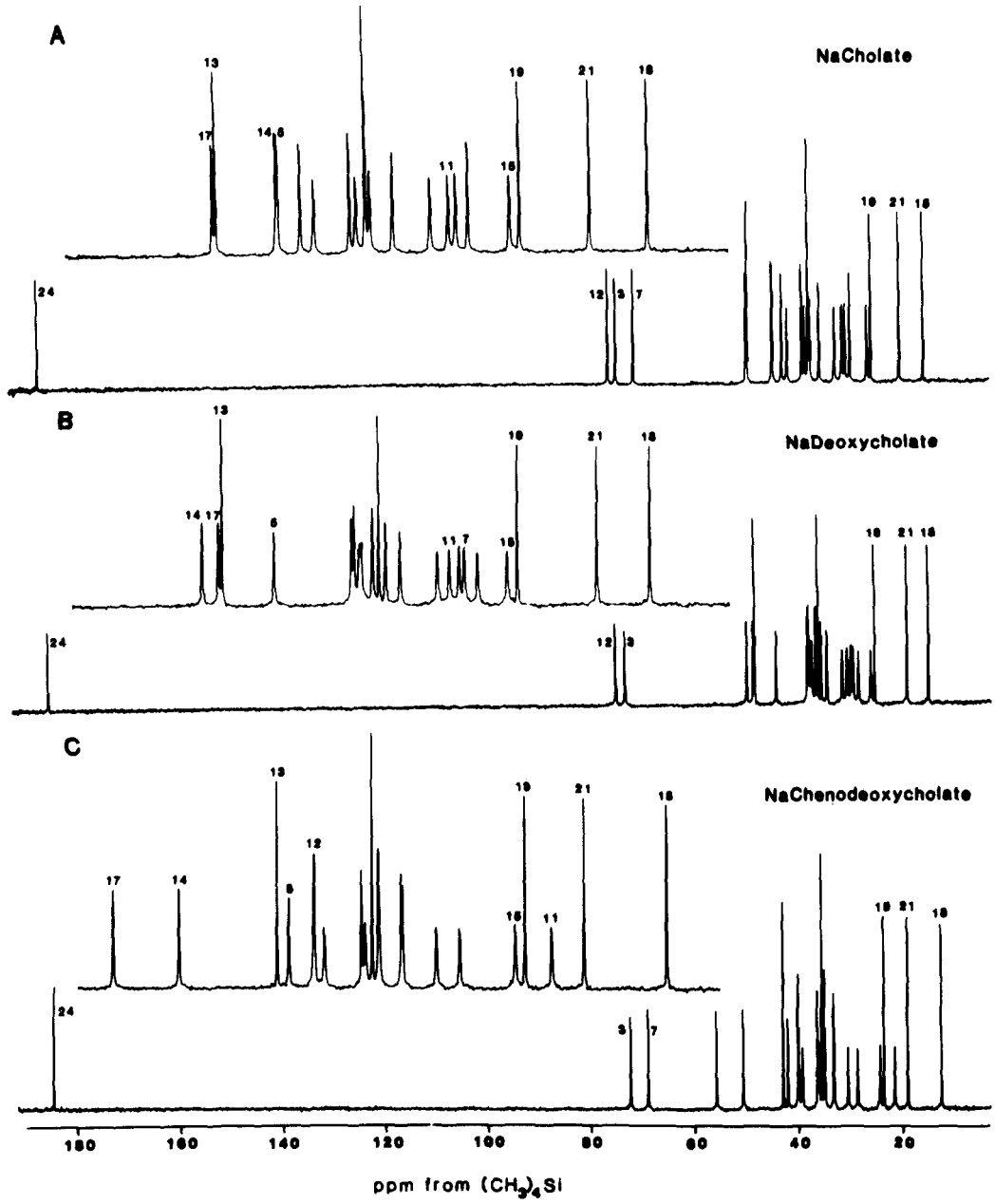

Fig. 1. ^{13}C NMR spectra at 50.3 MHz of aqueous bile salts; 5% w/v, pH 9.3. Chemical shifts are relative to $(CH_3)_4Si$ and selected assignments of individual resonances indicated are based on Barnes and Geckle (16) and Leibfritz and Roberts (17). Note that assignments of the hydroxyl carbons (3, 7, 12) are according to Leibfritz and Roberts. A, NaC; B, NaDC; C, NaCDC. Inserts are expanded hydrocarbon region of the bile salts from 11 ppm to 60 ppm.

apparent pKa was defined as the pH corresponding to the chemical shift at one-half the maximum chemical shift difference (12).

RESULTS

Peak assignment

Natural abundance ^{13}C NMR spectra of 5% aqueous solutions of NaC (116 mM), NaDC (120 mM), and NaCDC (120 mM) at pH 9 are shown in **Fig. 1.** All 24 carbons yielded resolved resonances; selected assignments indicated are based on those of Barnes and Geckle (18) for aqueous micellar cholate and on those of Leibfritz and Roberts (19) for the acids in methanol. Concentrations of NaC and NaDC were adjusted from 5 mM to 116 or 120 mM, and the pH was maintained at 9.0 throughout. The chemical shift of numerous peaks showed a concentration dependence, the magnitudes and directions of which were in agreement with those of Conte et al. (21) and Murata et al. (22) for NaDC. The concentration-dependent chemical shift change for the carboxyl resonance of NaC and NaDC, ~0.5 ppm, was small compared with those produced by titration (4.5–5.3 ppm).

Chemical shift of monomeric cholic acid as a function of pH

A dilute solution of [24-^{13}C]NaC (0.2 mM) below its CMC (9 mM in H_2O at 20°C) and below the solubility limit of CA (9) was titrated from pH 10 to pH 2 and gave no visible precipitate during titration. The NMR titration curve (chemical shift vs pH) given in **Fig. 2** shows that the chemical shift decreased from 184.7 ppm at high pH to 180.0 at pH 2. The apparent pKa was 4.6. At the low concentration used, an adequate signal to noise ratio (S/N) was obtained for the ^{13}C-enriched carboxyl carbon only after 14–20 hr (25,000–35,000 accumulations). The S/N of this peak was not sufficient for measuring an accurate linewidth, although the resonance was narrow (<10 Hz) at all pH values. Resonances from the carbons at natural abundance were not detected. A micellar solution (116 mM) of NaC at pH 10 was also titrated. Natural abundance ^{13}C spectra taken at pH 10.0–7.0 gave an invariant C24 chemical shift of 184.30 ppm (Fig. 2). However, at pH 6.5 the acid precipitated and no NMR signal was detected from the solution containing the precipitated CA.

Chemical shift of micellar bile acids as a function of pH

Natural abundance ^{13}C spectra of mixed micelle systems containing a mol ratio of one unconjugated to seven taurine-conjugated bile salts (NaC/NaTC, NaDC/NaTDC, and NaCDC/NaTCDC) were obtained as a function of pH. The height of the unconjugated carboxyl resonance, ~0.1 that of its taurine conjugate carbonyl resonance, was proportional to its concentraton in the mixed micelle. A single, narrow (<10 Hz) carboxyl carbon peak was observed at each pH, but the S/N was not sufficient to measure an accurate linewidth. The pKa values (5.3, 6.1, and 6.3) for CA, DCA, and CDCA, respectively, were in good agreement with potentiometrically determined values for simple micelles of each acid (8–11). The titration curve for CA in mixed micelles with NaTC is shown in Fig. 2. The mixed micelles were back-titrated from low pH to two intermediate pH values and to pH 9. The measured carboxyl chemical shifts fell on the titration curves.

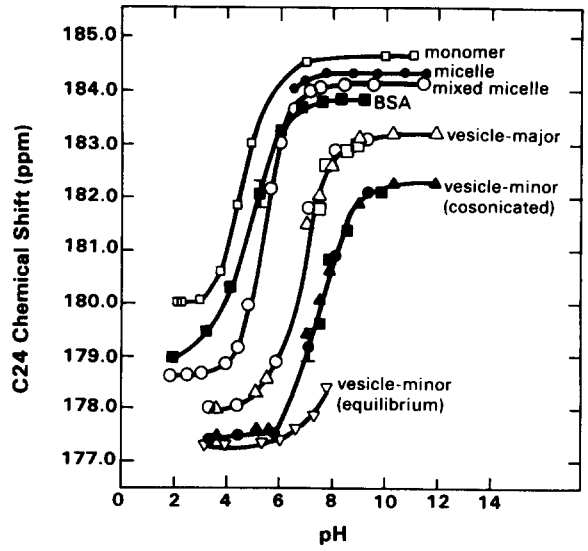

Fig. 2. Titration curves for CA in the molecular environments studied. Aqueous monomeric CA (0.2 mM below its CMC and solubility limit). Aqueous micellar CA (116 mM). CA/NaTC mixed micelles (1:7 mol/mol). CA/BSA complexes (2:1 mol/mol). CA/egg PC vesicles (1:20 mol/mol), major peak. CA/egg PC cosonicated vesicles (1:20 mol/mol), minor peak. NaC added to PC vesicles at pH 9 (1:20 mol/mol), minor peak.

Chemical shift of bile acids bound to BSA as a function of pH

^{13}C NMR spectra of the bile salt/BSA complexes (2:1 mol/mol, respectively) gave a single peak from the ^{13}C-enriched carboxyl carbon for each bile salt at pH 9. **Fig. 3** shows spectra for [24-^{13}C]CDCA bound to BSA at three pH values. The CDCA carboxyl peak was well resolved from the protein resonances except for the small glutamate carboxyl resonance (see Fig. 3A). In addition to chemical shift changes with pH, there werre marked changes in the linewidth of the carboxyl peak. As illustrated in the CDCA/BSA spectrum, the C24 peak was slightly asymmetric at pH 7.4 (Fig. 3A). It showed a greater assymmetry and broadened to 53 Hz at pH 3.9 (Fig. 3B). After complete protonation of the bile acid (Fig. 3C), the carboxyl peak became very narrow (4 Hz) and

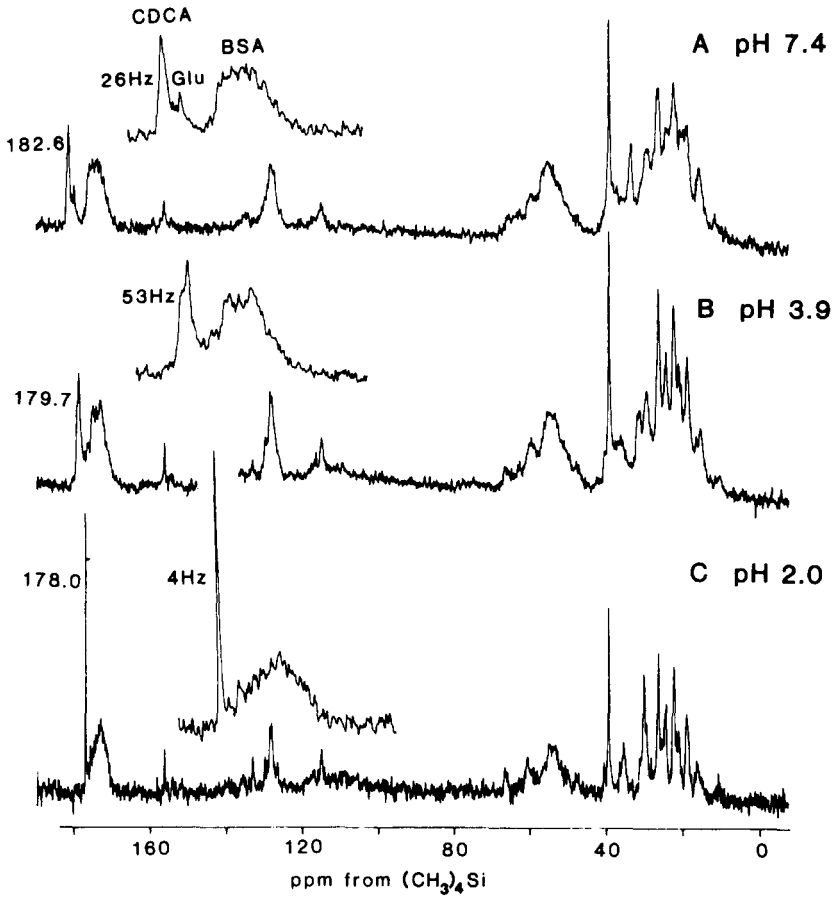

Fig. 3. ^{13}C NMR spectra at 50.3 MHz of [24-^{13}C]CDCA in complexes with BSA (2:1 mol/mol; 3.2×10^{-3} mM CDCA and 1.6×10^{-3} mM BSA in 1.4 ml): pulse interval 2.0 s, 3.0 Hz line-broadening used in processing. Chemical shifts relative to the narrow BSA peak at 39.84 ppm (17). A, pH 7.4; B, pH 3.9; C, pH 2.0. Inserts show expanded carboxyl/carbonyl region (170 ppm–184 ppm) for the indicated pH values. BSA and Glu indicate the carbonyl and glutamate carboxyl peaks from BSA, respectively. Note: these spectra and those in Fig. 4 were not obtained under spectrometer conditions that allow peak intensities to be directly related to the number of carbons contributing to that peak.

symmetrical. In the absence of BSA, CDCA would have precipitated and no peak would be observed below pH ~6.5. Therefore, CDCA was bound to the BSA even at low pH. Linewidth changes similar to those for CDCA occurred for CA/BSA and DCA/BSA. The apparent pKa's of CA, DCA, and CDCA when bound to BSA (4.5, 4.9, and 4.2, respectively) did not differ significantly.[1] The titration curve for CA bound to BSA is shown in Fig. 2. After titration of each bile salt/BSA sample to low pH, the sample was back titrated to two intermediate pH points, and the measured chemical shifts fell on the titration curve. ^{13}C NMR spectra of DCA/BSA complexes were also obtained as a function of DCA/BSA mol ratio at fixed pH (pH 9). A single carboxyl peak at 182.7 ppm was observed for DCA/BSA mol ratios of 2:1, 3:1, 4:1, and 6:1.

Chemical shift of bile acid in phospholipid membranes as a function of pH

Vesicles were prepared by cosonication of CA, DCA, or CDCA with egg PC (~1:20 mol/mol) and were stable at all pH values investigated (pH 11–3), showing only a slight increase in turbidity by visual inspection at low pH values. Spectra obtained for the same sample up to 48 hr apart were similar. Spectra of PC vesicles with DCA or CDCA gave a single carboxyl peak throughout the titration (**Fig. 4** for CDCA). The carboxyl peaks of both DCA and CDCA broadened slightly at and below the pKa. The apparent pKa's for DCA (6.5) and CDCA (6.6) were not significantly different. Spectra of the CA/PC cosonicated vesicle samples gave two carboxyl peaks, a major peak and a minor peak (varying from <3% to 10% of the major peak at high pH, depending on the particular sample preparation). **Fig. 5** shows spectra of the CA/PC cosonicated vesicles at selected pH values (pH 10.2, 7.0, and 3.4). In addition to chemical shift changes, the CA peaks exhibited linewidth

[1]Because of the greater linewidth and asymmetric lineshape, the uncertainty in measuring chemical shift in the spectra of bile acid-BSA complexes was much greater than that for the other systems reported herein.

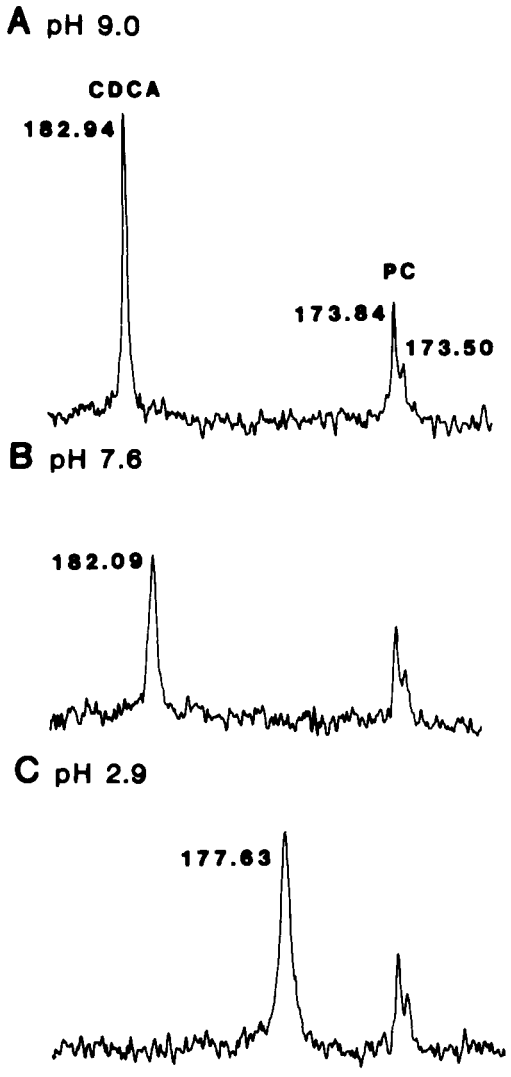

Fig. 4. ^{13}C NMR spectra of the carboxyl/carbonyl region (170 ppm–184 ppm) of [24-^{13}C]CDCA-egg PC vesicles, showing inner and outer carbonyl peaks for egg PC and a single carboxyl peak for CDCA at selected pH values. The pulse interval was 2.0 s, 2.0 Hz line-broadening used in processing. The CDCA and PC (1:22 mol/mol) were hydrated in 1.6 ml and sonicated until clear. Chemical shifts are relative to the terminal methyl of the PC acyl chains at 14.10 ppm (15). A, pH 9.0; B, pH 7.6; C, pH 2.9.

changes during titration. At high and low pH the CA carboxyl resonances were narrow; as the pKa was approached the peaks broadened. The two CA carboxyl peaks gave titration curves with differing apparent pKa's (Fig. 2). The major peak had an apparent pKa (6.8) similar to CDCA and DCA. The minor CA peak had a significantly higher pKa of 7.3. After complete protonation of CA, the pH was immediately raised to pH 10, where only the major peak was seen at 183.2 ppm (spectrum not shown). Since [24-^{13}C]CA below its CMC would not be detected in the number of accumulations (~500) used to obtain the vesicle spectra and would have a very different chemical shift if present (~184.7 ppm) and since the relative concentrations of CA and PC were such that CA micelles would not be present (9), we reasoned that the two CA peaks in the vesicle spectrum resulted from CA in two different magnetic environments within the PC bilayer. Negative stain electron micrographs of BA/PC (3% NaC–97% PC) samples showed a homogeneous population of unilamellar vesicles with a diameter of 229 ± 54 Å (mean ± SD, 199 particles counted). Vesicle diameter was the same at low and high pH.

A second method for NaC/PC vesicle preparation was used to help identify the two CA environments. In this method PC vesicles were prepared and [24-^{13}C]NaC was added at pH 9.3. At this pH, CA in the absence of vesicles would be completely ionized and would have a chemical shift of ~184.7 ppm (see Fig. 2). However, the ^{13}C spectrum showed a single carboxyl resonance at 183.3 ppm (**Fig. 6A**), the same chemical shift as the major peak in the spectrum of PC cosonicated with CA at high pH (Fig. 5). Thus at this pH, CA was incorporated into the vesicle and was completely ionized. After 24 hr of incubation, a single peak at the same chemical shift and intensity was seen. After decreasing the pH to 3.1, two peaks were seen immediately at 177.36 ppm and 177.97 ppm (Fig. 6B), identical to the chemical shifts of the minor and major peaks of the cosonicated system at the same pH. Titration of this sample from pH 3.1 to pH 7.2 (Fig. 6C–E) with 1.0 N NaOH yielded two titration curves. The titration points for the outer CA peak fell on the titration curve of the major peak in the cosonicated system at all pH values (Fig. 2). The minor peak, identified as CA in the inner monolayer (see Discussion), gave a titration curve from pH 3.1 to 7.2 before broadening beyond detectability at pH 7.8 (Fig. 6F). The chemical shifts above pH 5 were lower than those of the minor peak for the cosonicated vesicles (Fig. 2). In addition, the minor peak broadened and decreased in intensity as the pH was raised; the area ratio of the major peak to minor peak increased from ~2:1 at pH 3.1 to >10:1 at pH 7.8. When 3 mg of [24-^{13}C]NaC was added to vesicles at pH 10 and then titrated to pH 3, two titration curves identical to those found when titrating from pH 3 to 9 were obtained. This reversibility suggests that the partial titration curve for the CA minor peak represented the equilibrium ionization behavior of CA in the inner monolayer of small unilamellar phospholipid vesicles.

DISCUSSION

The apparent pKa of a carboxyl group in a simple or complex environment can be determined from pH-dependent measurements of the carboxyl ^{13}C chemical shift (12, 23). **Table 1** summarizes the apparent pKa values of CA, DCA, and CDCA in several molecular

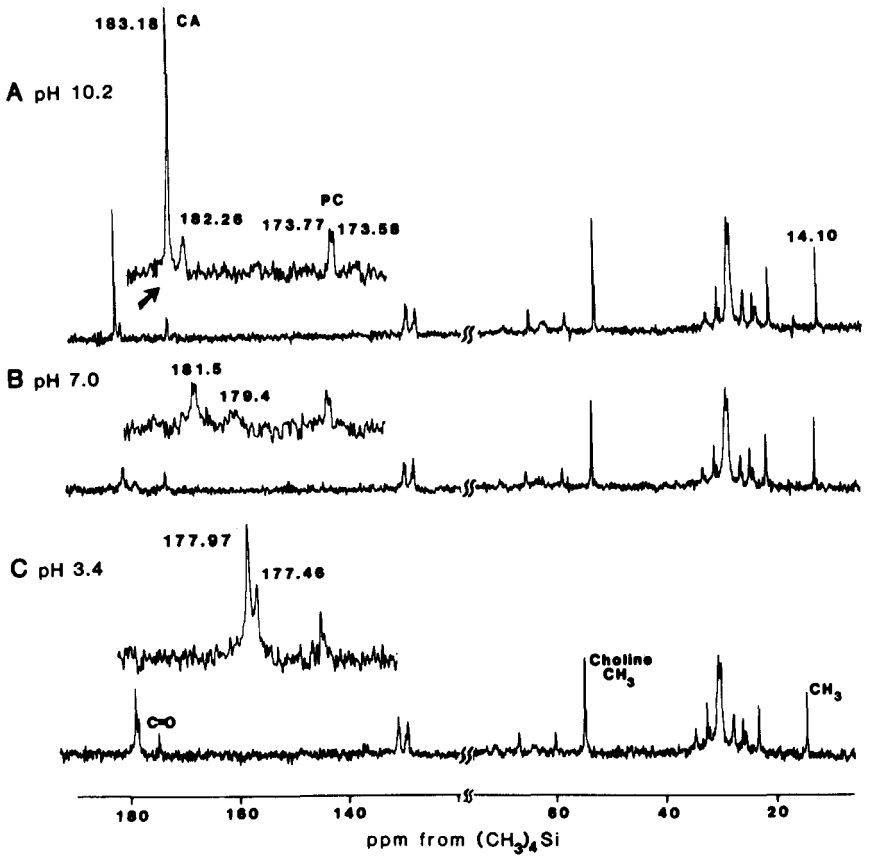

Fig. 5. ^{13}C NMR spectra of [24-^{13}C]CA-egg PC cosonicated vesicles (3:97 w/w; ~1:21 mol/mol in 1.6 ml). Chemical shifts relative to the terminal methyl of the PC acyl chains at 14.10 ppm (15). Spectra were accumulated with a pulse interval of 2.0 s, and processed using 2.0 Hz line-broadening. Major and minor peaks refer to CA in two different magnetic environments. Selected resonances of the PC spectrum labeled: carbonyl (C=O), choline methyl (choline CH_3), and fatty acyl methyl (CH_3). Inserts show expanded carbonyl region 170 ppm–184 ppm for each pH. A, pH 10.2; B, pH 7.0; C, pH 3.4.

environments as measured by ^{13}C NMR spectroscopy. Aqueous CA below its CMC and solubility limit had an apparent pKa of 4.6, a significantly lower value than previously reported from potentiometric titration of 3 mM CA (pKa 4.98; ref. 11) and from extrapolation of apparent pKa values for CA in methanol–water solutions (pKa 5.06; ref. 24). Our value is only slightly lower than the values for dilute aqueous carboxylic acids (pKa 4.7–4.9; ref. 12). In that case, the pKa value measured by NMR corresponded precisely to the value determined potentiometrically on the same sample (12). Our lower pKa may reflect the lower concentration used to prevent precipitation of the acid form. The very low water solubilities of DCA and CDCA prevented determination of the monomeric pKa values of these acids by NMR.

Micellar solutions of unconjugated bile acids precipitate at intermediate pH (pH 6–7) on formation of the acid in amounts above what the micelle can solubilize (8, 9). In contrast, the taurine conjugates have low pKa's (pKa <2) and remain soluble at all pH values (9). Incorporation of 10 wt% of the unconjugated bile acid into micelles of the taurine conjugate will prevent precipitation of the free acid (8, 9), allowing determination of the pKa of the unconjugated acid in the taurine-conjugate micelle. The pKa's determined from the complete NMR titration curves for CA, DCA, and CDCA in the mixed micelles were quite similar to those determined potentiometrically for micelles of the unconjugated bile acids alone (9–11). The apparent pKa of CA in the mixed micelle was significantly higher than that for monomeric CA. Thus, comparison of pKa values in these systems suggests that the local environment of the carboxyl group was altered to a greater extent on going from monomer to micelle than from simple micelle to mixed (taurine conjugate) micelle.

The apparent pKa values for the three bile acids bound to BSA are similar to those determined for long-chain fatty acids bound to BSA in titratable sites (14, 15). ^{13}C NMR has detected one titrating site and several nontitrating sites for fatty acids bound to BSA. In contrast,

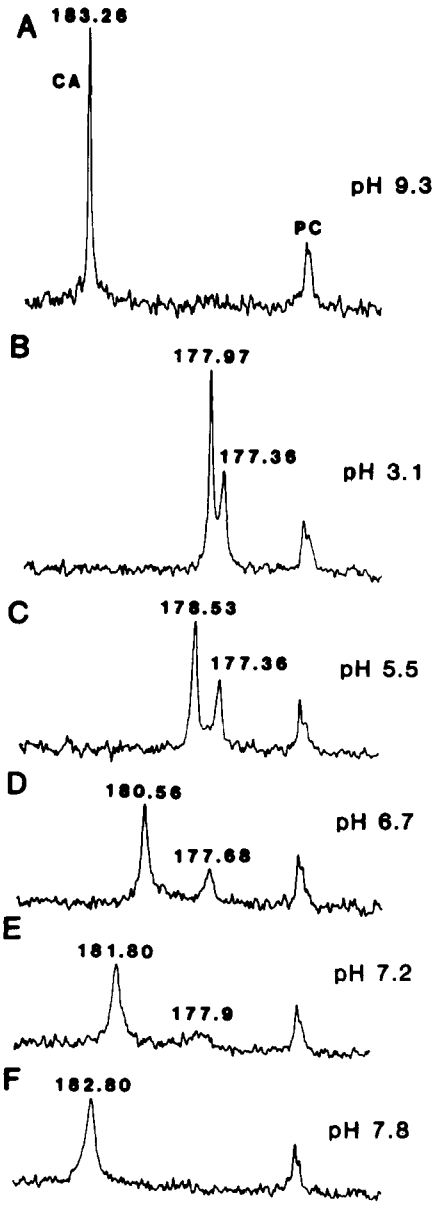

Fig. 6. ^{13}C NMR spectra of the carboxyl/carbonyl region (170 ppm–184 ppm) of [24-^{13}C]NaC added to preformed PC vesicles (~1/20 mol/mol, NaC/PC). The pulse interval was 2.0 s, and 2.0 Hz line-broadening used in processing A, [24-^{13}C]NaC added at pH 9.3; B, pH reduced from 9.3 to 3.1 with the addition of 1.0 N HCl; C–F, titration of sample B with 1.0 N NaOH to pH 5.5, pH 6.7, pH 7.2, pH 7.8, respectively.

only a titrating site was present for the bile acids. In egg PC vesicles, the apparent pKa values of CA, DCA, and CDCA were increased relative to all other systems. The pKa's of DCA, CDCA, and the CA major peak were not greatly different. However, they were significantly lower than the pKa for small concentrations of oleic acid in PC vesicles (pKa 7.4; ref. 15, 23).

Details of the molecular environments and motions of the bile acid carboxyl carbon can also be obtained from the ^{13}C NMR results. In monomeric and micellar systems, the bile acid carboxyl resonance was narrow, indicative of a relatively mobile carboxyl group and a relatively homogenous environment. The carboxyl peak for bile acids complexed to BSA was quite broad and somewhat asymmetric except at low pH. These results suggest that the bile acids bound to BSA might be present in slightly different magnetic environments in slow exchange. In addition, the rotational motion of the carboxyl may be inhibited by interaction with amino acid groups. In contrast, long-chain fatty acids showed multiple narrow resonances when complexed with BSA (13, 14). Our results demonstrated binding of DCA to BSA at molar ratios up to 6:1 DCA/BSA, but even at the higher DCA/BSA molar ratios, multiple environments such as occurred with oleic acid (13) or myristic acid (14) were not clearly seen. Equilibrium binding studies have shown a single high affinity site and several low affinity sites for DCA binding (7). The intense, narrow bile acid C24 resonance at low pH, where the bile acid is fully protonated, shows that the bile acid was bound to albumin and that its local magnetic environment was more homogenous and/or its rotational motion was more rapid. Thus, both hydrophobic and hydrophilic interactions appear to be important in the binding of bile acids to BSA.

In the CA/PC vesicle system, two environments in slow exchange on the NMR time scale were detected. The relative population of the two environments changed with pH, showing the greatest disparity at high pH. From the chemical shift (see below) and pH-dependent intensity changes, these environments were identified as the outer and inner monolayers of the bilayer. When the vesicles were prepared by cosonication of CA and PC at pH 11 (to allow CA to distribute to both inside and outside monolayers of the vesicles) two peaks were seen—a peak at 183.2 ppm and a small peak at 182.1 ppm. However, when NaC was added to preformed vesicles as NaC monomers, only the large peak at 183.3 ppm was seen. In this case NaC was present only in one magnetic environment, presumably the outer monolayer of the vesicle. These spectra were stable for >12 hr indicating very slow exchange of cholate between the outer and inner monolayers. Reducing the pH from 9.3 to 3.1 caused immediate protonation of the external CA, which could then flip to the inner monolayer of the vesicle and establish an equilibrium ratio (~2/1), similar to the ratio of PC in the outer and inner monolayers of a small vesicle (25). The exchange between the two monolayers was much less than 30 exchanges/sec but faster than the time needed to accumulate the spectra (~40 min).[2] At pH 5.5, when CA in the outer monolayer began to titrate, the resonances

[2]The upper limit for the exchange rate may be determined from the chemical shift difference or the linewidth, whichever is more stringent.

TABLE 1. Apparent pKa's of bile acids in different molecular environments

	Cholic	Deoxycholic	Chenodeoxycholic
Below CMC and solubility limit[a] (0.2 mM)	4.6	ND[b]	ND[b]
Bile acid/BSA complex (2:1 mol/mol)[c]	4.5	4.9	4.2
Mixed micelles (1:9 w/w) bile acid/taurine conjugate[a]	5.3	6.1	6.3
Bile acid/egg PC vesicles (3:97 w/w, ~1:21 mol/mol)[a]	6.8 major peak 7.3 minor peak, cosonicated	6.5	6.6

[a]Apparent pKa ± 0.2.
[b]ND, not determined.
[c]Apparent pKa ± 0.3.

became clearly separated. Between pH 6 and 8, CA in *both* environments titrated, showing that the proton flux through the vesicle was relatively rapid. However, the relative peak area of the CA in the inner monolayer decreased markedly in this pH range, indicating a net flux of CA to the outer monolayer. As the outer monolayer began to become ionized, the amount of CA in the inner monolayer diminished. This would occur if the protonated form of the bile acid exhibited rapid flip-flop relative to the ionized form. Thus, the titration to the ionized form on the outside depleted the protonated CA on the inner monolayer, since once the protonated form flipped out it became ionized and remained in the outer monolayer. The reversibility of this titration series and the inability to observe the CA minor peak above pH 7.8, except when CA and PC are cosonicated at high pH (Figs. 5 and 6), suggest that adding NaC to preformed vesicles represented the equilibrium location and ionization state of CA in the outer and inner monolayers of small sonicated vesicles. We were not able to identify the reason for the differences in chemical shift of the CA minor peak from pH 5.5–7.2 between the two methods of vesicle preparation. It is possible, however, that cosonication of CA with PC forced a small amount of NaC into the inner monolayer of the vesicle (which resulted in the minor peak in the cosonicated spectra at high pH) and that this "trapped" NaC altered the titration behavior of cholic acid in the inner monolayer. Our results may be relevant for the transport of CA across physiologic membranes because small changes in pH near 7.4 could significantly increase or decrease the net rate of bile acid movement across the bilayer.

Table 2 summarizes the maximum, minimum, range of chemical shifts, and the chemical shift at the pKa for the CA carboxyl resonance in the systems studied. At a fixed ionization state the chemical shift of the carboxyl carbon will be directly proportional to the net H-bonding of the carboxyl group, and as the environment of the carboxyl carbon becomes more hydrophobic, the carboxyl resonance will shift upfield (12, 26, 27). The observed chemical shifts for CA suggest that the monomeric environment is the most hydrophilic and the PC inner monolayer is the most hydrophobic. This general result would be expected since maximum hydration will occur for monomers, and since the phospholipid bilayer will provide a strongly hydrophobic environment for the bile acid causing partial dehydration of the carboxyl group. The carbonyl groups of PC molecules on the outer monolayer have more extensive H-bonding interactions with H_2O (greater degree of hydration) and these resonances are therefore shifted downfield relative to resonances for carbonyl groups on the inner monolayer (28). A close

TABLE 2. Chemical shift ranges (ppm) for CA

	Maximum Chemical Shift	Minimum Chemical Shift	Chemical Shift Range	Chemical Shift at pKa
0.2 mM	184.68	180.00	4.68	182.34
5% w/v (116 mM)	184.30			
CA/NaTC micelles (1:9 w/w)	184.12	178.59	5.58	181.38
CA/BSA complexes (2:1 mol/mol)	183.52	178.65	4.87	181.08
CA/egg PC vesicles (3:97 w/w)				
Major peak	183.18	177.98	5.21	180.58
Minor peak; cosonicated	182.26	177.46	4.80	179.86
Minor peak; equilibrium		177.31		

examination of the NMR chemical shift data suggests that for ionized CA, the order of increasing hydrophobicity is monomer < micelle < mixed micelles < BSA-bound < PC outer monolayer < PC inner monolayer cosonicated system. The hydrophobicity of protonated CA increases in the order; monomer < BSA-bound < mixed micelle < PC outer monolayer < PC inner monolayer.

It is also known that, in the absence of complicating factors, the apparent pKa of a carboxyl group increases with increasing hydrophobicity (29). Monomeric CA was found to have the lowest pKa and CA in the inner monolayer of PC vesicles had the highest. With the exception of CA/BSA complexes, an upfield shift for the CA carboxyl peak correlated with an increase in the apparent pKa. Thus the relative hydrophobicity estimated from the apparent pKa corresponded to that estimated from the chemical shift values.

We gratefully acknowledge Donald Gantz for performing the electron microscopy and thank Anne Gibbons and Irene Miller for preparation of the manuscript. This work was supported by grants HL-26335, HL-07291, and HL-07429 from the United States Public Helth Service.

Manuscript received 29 August 1985.

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