Effects of side chain length on ionization behavior and transbilayer transport of unconjugated dihydroxy bile acids: a comparison of nor-chenodeoxycholic acid and chenodeoxycholic acid

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Abstract ^{13}C-NMR spectroscopy was used to examine the effect of side chain length on the ionization properties and transmembrane transport rate of 3α,7α-dihydroxy bile acids. When solubilized in taurocholate micelles, [23-^{13}C]nor-chenodeoxycholic acid (nor-CDCA) had a pK_a of 6.1, similar to that of CDCA (pK_a 6.2), its C_{24} homologue. In unilamellar phosphatidylcholine vesicles, the pK_a of nor-CDCA was 7.0, whereas that of CDCA was 6.6. Lineshape analysis indicated that the rate of ionization of nor-CDCA as a micellar solute or as a vesicle component was very slow (0.4×10^5 sec^{-1}) compared to that of acetic acid in water (8.7×10^5 sec^{-1}). Lineshape analysis of spectra of the protonated form of nor-CDCA at acidic bulk pH indicated that the transbilayer transport rate of nor-CDCA (580 sec^{-1}) was six times faster than that of CDCA (100 sec^{-1}). It is proposed that the shorter side chain of the nor-CDCA molecule causes it to reside more deeply inside the vesicle bilayer than CDCA, explaining its weaker ionization and more rapid flip-flop rate. These in vitro experiments imply that, in vivo, a given C_{23} nor-dihydroxy bile acid will ionize less readily when present in membranes, and it will also flip-flop faster than its C_{24} homologue.—**Ko, J., J. A. Hamilton, H-T. Ton-Nu, C. D. Schteingart, A. F. Hofmann, and D. M. Small.** Effects of side chain length on ionization behavior and transbilayer transport of unconjugated dihydroxy bile acids: a comparison of nor-chenodeoxycholic acid and chenodeoxycholic acid. *J. Lipid Res.* 1994. **35:** 883-892.

Supplementary key words apparent pK_a • bile acids • bile salts • ^{13}C-NMR spectroscopy • mixed micelles • phospholipid vesicles • transbilayer diffusion • bile acid transport

In vertebrates, the major primary and secondary bile acids are conjugated efficiently in N-acyl linkage with glycine or taurine during hepatic transport. As a result, unconjugated bile acids are usually present in bile in only trace proportions (1). Conjugated bile acids are secreted into the small intestine during digestion and then absorbed efficiently from the distal intestine mostly by an active transport process. A small fraction of conjugated bile acids escapes ileal absorption and undergoes deconjugation by bacterial enzymes either in the distal small intestine or in the large intestine. Some of these unconjugated bile acids are absorbed, return to the liver, are reconjugated during their transport through the hepatocyte, and are resecreted into bile. As a result of this continuous conjugation, deconjugation, and reconjugation in the normal enterohepatic cycling of bile acids, both the enterocyte and hepatocyte are continuously exposed to a flux of unconjugated bile acids in the healthy organism (2).

A considerable body of experimental data has provided strong evidence for rapid, passive absorption of unconjugated C_{24} dihydroxy bile acids into the hepatocyte (3-5), enterocyte (6-12), and biliary ductular epithelial cell (13-16). Cellular entry of unconjugated bile acids has been presumed to be passive by flip-flop across the lipid domains of cell membranes (17). The rate of passive uptake of unconjugated bile acids has been shown to be inversely proportional to the number of hydroxy groups, that is monohydroxy > dihydroxy > trihydroxy (6-8, 10). This is in agreement with the theory that predicts that the absorption rate should be proportional to the partitioning

Abbreviations: CDCA, chenodeoxycholic acid; norCDCA, nor-chenodeoxycholic acid; ^{13}C-NMR, ^{13}C-nuclear magnetic resonance; ^{1}H-NMR, ^{1}H-nuclear magnetic resonance; TC, taurocholate; C, cholic acid; PC, phosphatidylcholine; Cl_3CD, deuterated chloroform; TMS, tetramethylsilane; MS, mass spectrometry; ACOH, acetic acid; NMR and MS abbreviations: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; b, broad; J, coupling constant; M, molecular ion; ei, electron impact.

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into the membrane and the rate of diffusion through the membrane, that is, the rate of transport across the lipid bilayer of the membrane (18, 19).

The ^{13}C-NMR spectroscopy technique is well suited for studying the behavior of weak acids such as bile acids (17, 20, 21) or fatty acids (22) when solubilized in micelles or incorporated into lipid bilayers. If the carboxyl carbon is enriched with ^{13}C, the chemical shift of the ^{13}C carboxyl can be monitored at relatively low concentrations. The chemical shift of the carboxylic carbon is sensitive to the state of ionization of the carboxyl group as well as its chemical microenvironment. Under appropriate conditions, analysis of the ^{13}C-NMR spectra can provide information on the pK_a as well as the distribution of labeled molecules between the outer vesicle surface and the inner vesicle surface. In addition, it is possible to determine approximately the transbilayer transport rate, or "flip-flop" rate, of the labeled molecules between the outer and inner monolayers of the vesicle (17, 21).

In this paper we report studies aimed at determining whether the length of the bile acid side chain influences the ionization properties and the transbilayer transport rate of unconjugated dihydroxy bile acids. To do this, we performed experiments with nor-chenodeoxycholic acid (nor-CDCA), a C_{23} bile acid containing a side chain with only four carbon atoms instead of the five carbon atoms that are present in most natural bile acids. The ionization properties and transmembrane transport rates of this bile acid, labeled with ^{13}C on its carboxyl group, were compared with data obtained with chenodeoxycholic acid (CDCA), its C_{24} homologue in this study and in a previous study (17, 20, 21). One stimulus for this work was a body of studies in the intact animal or perfused liver that reported a marked difference in the physiological properties and metabolism of these two homologues (14).

MATERIALS AND METHODS

Experimental design

The extent of ionization in relation to bulk pH was measured for the two bile acids when present as a micellar solubilizate in taurocholate (TC) micelles. This approach was taken rather than simple aqueous titration (23, 24) because the acid form of CDCA (and presumably of nor-CDCA) is quite insoluble in water (25). In addition, the ionization in relation to bulk pH and the flip-flop rate of the ^{13}C-enriched bile acids, when present in egg phosphatidylcholine (PC) unilamellar vesicles, were determined.

Materials

Sodium TC (NaTC) was purchased from Calbiochem (San Diego, CA). Egg PC of 99% purity was purchased from Avanti Polar-Lipids (Alabaster, AL). Both were quite pure by chromatography and used without further purification. D_2O and $CDCl_3$ were purchased from Wilmad Glass Co. (Buena, NJ). Tetramethylsilane (TMS) was purchased from Stohler Isotope Chemicals (Rutherford, NJ). ^{13}C-enriched CDCA and ^{13}C-enriched cholic acid (CA) were purchased from MSD Isotopes (Montreal, Canada). [^{13}C]KCN, 99 atom % ^{13}C, was purchased from Aldrich Chemical Company (Milwaukee, WI).

Synthesis of [^{13}C]carboxyl nor-CDCA

3α,7α-Dihydroxy-24-nor-5β-cholan-23-oic acid (nor-CDCA) was prepared from CDCA using a method previously reported (26).

3α,7α-Diacetyloxy-24-nor-5β-cholan-23-oic acid: 3α,7α-dihydroxy-24-nor-5β-cholan-23-oic acid (1.83 mmol) was stirred in a mixture of acetic acid (7 ml), acetic anhydride (2 ml), and 5 N $HClO_4$ (0.070 ml) for 30 min at room temperature. The reaction mixture was poured on ice and the product was extracted with ether; the organic layer was washed with water to neutrality, dried, and evaporated (yield 92%). A small sample was recrystallized from ethyl acetate for analytical purposes: mp 185–186°C (Lit. 213–214°C) (EtOH) (27).

^1H-NMR (360 MHz, Cl_3CD): 0.692 (s, 3H, Me-18), 0.933 (s, 3H, Me-19), 1.035 (d, J=6.1 Hz, 3H, Me-21), 2.034 (s, 3H, CH_3COO^-), 2.056 (s, 3H, CH_3COO^-), 2.472 (dd, J=14.8 and 3.2 Hz, 1H, H-22), 4.589 (m, 1H, H-3), 4.879 (bs, 1H, H-7).

3α,7α-Diacetyloxy-22-iodo-23,24-dinor-5β-cholane was prepared from 3α,7α-diacetyloxy-24-nor-5β-cholan-23-oic acid according to Concepción et al. (28) and purified by silica gel column chromatography (yield 65%).

^1H-NMR (360 MHz, Cl_3CD): 0.690 (s, 3H, Me-18), 0.932 (s, 3H, Me-19), 1.024 (d, J=5.4 Hz, 3H, Me-21), 2.034 (s, 3H, CH_3COO^-), 2.074 (s, 3H, CH_3COO^-), 3.170 (dd, J=9.6 and 4.5 Hz, 1H, H-22), 3.327 (bd, J=9.6 Hz, 1H, H-22'), 4.583 (m, 1H, H-3), 4.876 (bs, 1H, H-7).

MS (EI, 70 eV, Hewlett-Packard 5890 Series II gas chromatograph/5970 Mass Selective Detector), m/z (%): 484 (M-60 (AcOH), 1), 424 (M-2×60, 100), 409 (M-2×60-15, 28), 370 (M-2×60-[C_1–C_4]), 357 (M-127 (I)-60, 4), 297 (M-127-2×60, 57), 255 (M-side chain-2×60, 13), 228 (255-[C_{16}–C_{17}], 10), 213 (228-15, 13).

[23-^{13}C]3α,7α-dihydroxy-24-nor-5β-cholan-23-oic acid: 3α,7α-diacetyloxy-22-iodo-23,24-dinor-5β-cholane (0.45 mmol) was heated with [^{13}C]KCN (0.58 mmol) in DMF at 100°C for 3 h. The intermediate [^{13}C]22-nitrile was isolated by partition between ethyl ether and water, purified by column chromatography, and hydrolyzed with 3 N KOH in ethanol–water 1:1, for 96 h at 80°C. The product was

isolated by conventional means and purified by adsorption chromatography using silica gel column chromatography (yield 68%).

^{1}H-NMR (Cl_3CD-d_4-MeOH, 360 MHz): 0.703 (s, 3H, Me-18), 0.907 (s, 3H, Me-19), 1.021 (d, J=5.8 Hz, 3H, Me-21), 2.144 (bq, J=12.6 Hz, 1H, H-4α), 2.436 (bdd, $J_{H\text{-}22\text{-}H\text{-}22'}$=11.5 Hz, $J_{H\text{-}22\text{-}C\text{-}23}$=7.2 Hz, 1H, H-22), 3.83 (bs, 1H, H-7); H-3 obscured by the CD_2HOH signal.

^{13}C-NMR (Cl_3CD-d_4-MeOH, 50 MHz): 176.35 (C-23). The [23-^{13}C]nor-chenodeoxycholic acid was greater than 97% pure.

NMR methods

^{13}C-NMR spectra were obtained on a Bruker WP-200 spectrometer with an Aspect 2000A computer at 50.3 MHz. Aqueous samples were placed in 10 mm NMR tubes with D_2O included as an internal lock. For the PC samples, the terminal methyl group of the PC acyl chains at 14.10 ppm served as an internal chemical shift reference (20). For the mixed micellar samples, external TMS inserts were used as the chemical shift reference. Unless stated otherwise, spectra were obtained at 28°C with a pulse interval of 2 seconds.

Sample preparations

[^{13}C]nor-CDCA in TC micelles: 4.5 μl of a 264 mM solution of the potassium salt of [^{13}C]nor-CDCA in H_2O was added to 1.6 ml H_2O and 0.2 ml D_2O in a 10 mm NMR tube. Solid NaTC (40 mg, 74 μmol) was added, the pH was adjusted to 10.0 with 1.0 N KOH in H_2O, and an external probe containing TMS was inserted. The final concentration of [^{13}C]nor-CDCA was 0.64 mM and the final concentration of TC was 40.2 mM. The critical micelle concentration of TC in this system is about 10 mM (29), so that the concentration of TC in micellar form was 30 mM.

[^{13}C]CDCA in TC micelles: 0.45 mg (1.14 μmol) of [^{13}C]CDCA and 40 mg (74 μmol) of NaTC were added to 1.6 ml H_2O and 0.2 ml D_2O in NMR tubes. The pH was adjusted to 10.0 as above, and an external probe containing TMS was inserted. The final concentration of [^{13}C]CDCA was 0.62 mM; and the final concentration of TC, sodium salt, was 40.2 mM.

[^{13}C]nor-CDCA in PC vesicles: unilamellar vesicles were prepared by taking an aliquot of egg PC in chloroform (dry weight of 97 mg) and evaporating the solvent under N_2. The sample was hydrated with 1.6 ml of a 0.56% w/w KCl solution in H_2O and 0.2 ml D_2O, adjusted to pH 7.4, and ultrasonically irradiated using a Branson W-350 sonifier in a pulsed mode at 25–30°C. Sonification was carried out for 60 min and the titanium debris from the sonifier was removed by spinning the samples for 15 min using a low-speed table-top centrifuge. The translucent supernatant was transferred to an NMR tube. Then, 3 mg of [^{13}C]nor-CDCA was added to the vesicle preparation, mixed by vortexing for 1 min, after which the pH was adjusted to 7.0 with 0.1 N KOH in H_2O. The final concentration of PC in the sample was 54 g/l, and the mol ratio of nor-CDCA to PC was 1:16.

Titrations

Samples of the bile acids in TC micelles were titrated from high to low pH with 1.0 N HCl in H_2O. The sample of [^{13}C]nor-CDCA in unilamellar vesicles was titrated from neutral pH to low pH with 1.0 N HCl in H_2O and back to high pH with 1.0 N KOH in H_2O. The pH was monitored using a Beckmann model 3560 digital pH meter equipped with a Microelectrodes microtip probe (model MI-412, Londonderry, NH) to allow measurement in the NMR tubes.

Transbilayer movement rate experiments

A sample of [^{13}C]nor-CDCA in unilamellar vesicles was adjusted to pH 4.0 with 1.0 N HCl in H_2O and ^{13}C-NMR spectra were taken at 9.2°C, 14.5°C, 19.8°C, 28.4°C, 35.9°C, and 41.2°C with a pulse interval of 2 sec. These results were compared to experiments on [^{13}C]NaCDC using the same experimental protocol and the same spectrometer carried out previously in this laboratory (17, 21).

Time required for equilibration

The equilibration times in the experiments reported here are influenced by at least two effects: the first is the time needed for protonated molecules to flip-flop until the inside and outside concentrations are in equilibrium; the second is the time required for the intravesicular pH to equilibrate with the external medium.

The transbilayer diffusion (flip-flop) of bile acids is a relatively rapid process with a half time of less than a second and thus this process is clearly at equilibrium in our experiments.

To establish that the ^{13}C-NMR spectra were acquired after the internal pH had equilibrated with the external pH we carried out the following experiment using [^{13}C]cholic acid. [^{13}C]cholic acid was chosen because its transbilayer diffusion rate is much slower than that of nor-CDCA or CDCA (17) and consequently, at 35°C its exchange process is slow enough on the NMR time scale to permit the detection of distinct resonances for the bile acid molecules located on either side of the vesicle membrane (21). [24-^{13}C]cholic acid in the form of its sodium salt was added to preformed egg PC vesicles, and the pH was adjusted to 7.4, 5.0, or 3.0. The bile salt initially binds to the external surface of the vesicle. Then the protonated bile acid rapidly diffuses across the bilayer to the internal leaflet. There some of it ionizes giving its proton

to the internal compartment causing the pH to fall inside the vesicle. This creates a pH gradient (30, 31) and generates a transmembrane potential that dissipates by a counterflow of cation such as K^+ with a half life of a few minutes (30). After the addition of [^{13}C]cholate, ^{13}C-NMR spectra were recorded at 10-min intervals until the chemical shifts for the inside and outside signals showed no further changes. The external peak remained constant. A constant internal chemical shift is evidence for pH equilibration as both internal and external [^{13}C]carboxyl chemical shifts are very sensitive to changes in pH (20). Equilibrium was obtained by 10 min for the samples at pH 3.0 and 5.0. At pH 7.4 a steady state was not obtained until between 20 and 30 min, presumably because of the low concentration of protonated molecules present at this pH. As nor-CDCA and CDCA are known to flip-flop much more rapidly than cholic acid, and as the preparation of the NMR samples required more than 30 min (due to time required for transport and loading of the sample as well as shimming of the magnet), all the samples would have achieved equilibrium before any data were acquired.

Temperature calibration

Because of the difference between actual sample temperature and the temperature measured by the heating coil thermocouple, a calibration of the thermocouple reading had to be performed in the range of 2°C (275°K) to 37°C (310°K). Samples were allowed to equilibrate in the spectrometer probe at a given temperature. They were then rapidly removed from the spectrometer and their temperature, as the samples equilibrated with room temperature, was monitored with an Omega Trendicator thermocouple (model 410A-T-C, Stamford, CT). The temperature of the sample in the spectrometer probe was obtained by extrapolation to time zero.

Data analysis

Titration of [^{13}C]nor-CDCA and [^{13}C]CDCA in mixed micellar solution. pKa values were calculated by fitting the chemical shift for the carboxyl carbon obtained at different pH values to the theoretical Henderson-Hasselbalch curve (20). Lineshape analysis to determine the kinetics of ionization was carried out by simulation of spectra using the solution to the Bloch equations for exchange between two unequally populated sites (32). For a bile acid in a micellar phase in aqueous solution, the ionization process can be represented by

$$BAH_{mic} + H_2O \underset{k_2}{\overset{k_1}{\rightleftarrows}} BA^-_{mic} + H_3O^+$$

where BAH_{mic} and BA^-_{mic} represent the protonated bile acid and the conjugated base in the micelle.[2] The kinetic expressions for the rate of change of magnetically labeled species are:

$$\frac{d[BAH_{mic}]}{dt} = -k_1[BAH_{mic}][H_2O] = -k'_1[BAH_{mic}]$$

and

$$\frac{d[BA^-_{mic}]}{dt} = -k_2[H_3O^+][BA^-_{mic}] = -k'_2[BA^-_{mic}]$$

As $[H_2O]$ is constant, the population of BAH_{mic} decays exponentially with a first order rate k'_1, and the average life of a ^{13}C nucleus in BAH_{mic} is $\tau_1 = 1/k'_1$. The population of BA^-_{mic} will decay with a pseudo first order constant $k'_2 = k_2[H_3O^+]$ which depends on the pH of the experiment. The average life of a ^{13}C nucleus in BA^-_{mic} is then $\tau_2 = 1/k'_2$. For a given k'_1 and pH, k'_2 can be calculated from the steady state relationship, $p_1 \cdot k'_1 = p_2 \cdot k'_2$, and the expression for the acidity constant, $p_2 \cdot [H_3O^+] = K_a \cdot p_1$:

$$k'_2 = k'_1 [H_3O^+]/K_a$$

where p_1 and p_2 are the populations at site 1 ($[BAH_{mic}]$) and site 2 ($[BA^-_{mic}]$).

The frequencies and line widths for the two sites were obtained from the spectra at low and high pH values, and a k'_1 was sought that best approximated the spectra at all pH values.

Titration of [^{13}C]nor-CDCA in PC vesicles. The pK_a was determined as in the case of nor-CDCA as a micellar solute (see above).

Flip-flop rates of [^{13}C]nor-CDCA in PC vesicles. The kinetics of flip-flop were measured at equilibrium and

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[2]In theory, the equilibrium could include a term for free ionized monomers in solution, i.e.,

$$BAH_{mic} + H_2O \rightleftarrows BA^-_{mic} + BA^-_{monomer} + H_3O^+.$$

We have attempted to estimate how much is in the micelle by using a monomeric concentration of nor-chenodeoxycholate at high pH (9.5), where it is ionized (A^-), and then adding at pH 9.5 increasing amounts of taurocholate and following the chemical shift. The chemical shift falls from 184.08 to become nearly constant at about 183.2 above 100 mM TC, indicating that virtually all of the ionized nor-chenodeoxycholate must be in the micelle. At 116 mM TC, the highest concentration studied, the chemical shift is 183.18 ppm. When no taurocholate is present, the chemical shift is 184.08. Assuming that the chemical shift reflects the distribution, at 42 mM (the concentration used in Fig. 1) we estimate that >70% of the ionized form is in the micelle and <30% is present as monomer. We have therefore ignored the small contribution from monomeric ionized bile salt.

considered to be first order or pseudo first order reactions. The assignment of the signals corresponding to the bile acid present on either side of the vesicle membrane was based on previous studies that had shown that the signal resonating at lower field and with a larger area corresponded to the bile acid inserted in the outer leaflet of the membrane, whereas the smaller peak at higher field corresponds to the bile acid inserted in the inner leaflet (17, 21). Accordingly, the signal at 176.44 ppm was assigned to nor-CDCA molecules in the outer layer and the signal at 175.28 ppm (at 9.2°C) was assigned to the bile acid molecules inserted in the inner layer of the vesicle membrane. Flip-flop rates at different temperatures were determined by lineshape analysis using the solution of the Bloch equations for two unequally populated sites (32). The ratio of populations of bile acid outside/inside of the vesicle (R = 1.7) was obtained from the analysis of line shapes of the low temperature spectra (9.2 and 19.8°C). At each temperature, exchange rates $k_{out \rightarrow in}$ were calculated for a range of line widths which resulted in a simulated spectrum identical to the experimental one (see Results). The activation energy (E_a) was calculated from the Arrhenius plot $\ln(k_{out \rightarrow in})$ versus 1/T for temperatures between 9.2 and 28.4°C.

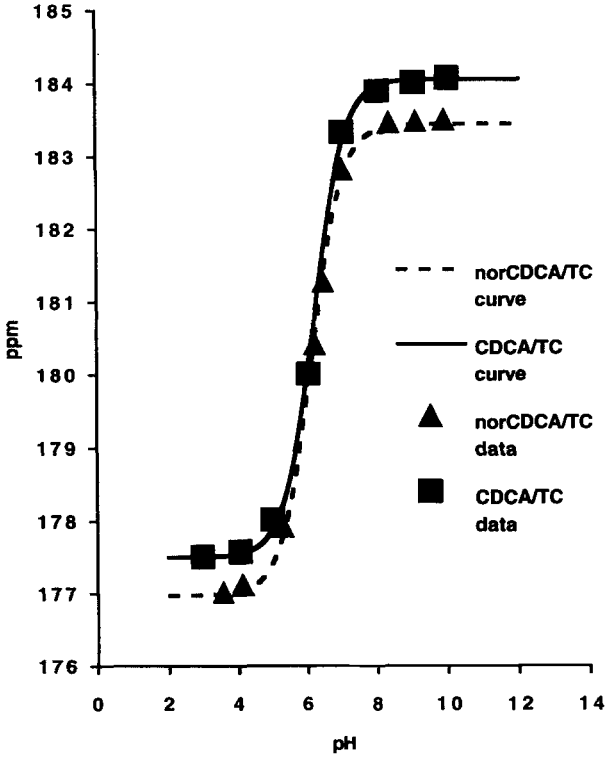

Fig. 1. Titration curve for [23-^{13}C]nor-CDCA and [24-^{13}C]CDCA in mixed micelles with NaTC. Chemical shift of the carboxyl carbons of [^{13}C]nor-CDCA and [^{13}C]CDCA as a function of pH. The points represent actual data points. The lines represent the best fit for each bile acid using the theoretical Henderson-Hasselbalch equation; nor-CDCA: pK_a 6.1, CDCA: pK_a 6.2.

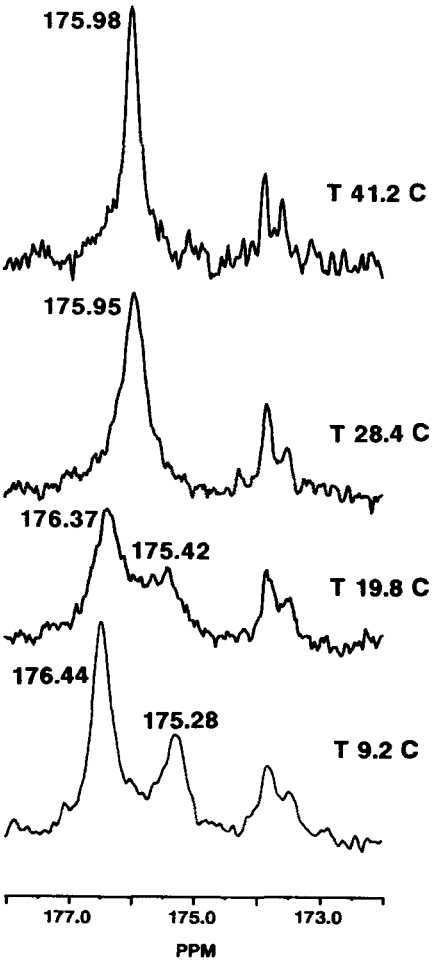

Fig. 2. Effect of temperature on the spectrum of [^{13}C]nor-CDCA in egg PC unilamellar vesicles: determination of flip-flop rate. ^{13}C-NMR spectra of [^{13}C]nor-CDCA in PC unilamellar vesicles at pH 4 at four selected temperatures. The two small peaks at about 173.5 and 173.8 ppm are from the inner and outer PC carbonyls carbons.

RESULTS

Properties of nor-CDCA and CDCA as micellar solutes

The ^{13}C-NMR chemical shifts of the carboxyl carbons of nor-CDCA and CDCA in mixed TC micelles as a function of pH are shown in **Fig. 1.** Fitting of theoretical Henderson-Hasselbalch curves to the experimental points gave a pK_a value of 6.1 for nor-CDCA and a pK_a value of 6.2 for CDCA. The resonances were narrow ($\nu_{1/2} <$ 6 Hz) at high and low pH, but at pH values close to the pK_a the peaks broadened ($\nu_{1/2}$ 12 Hz for nor-CDCA, $\nu_{1/2}$ 6 Hz for CDCA). This broadening suggested a decreased protonation/deprotonation (on the NMR time scale) near the pK_a. Line shape analysis for the nor-CDCA case, assuming a natural line width of 6 Hz, gave a value of approximately 40,000 sec^{-1} for the pseudo first order ionization constant k'_1.

Properties of nor-CDCA when present in PC vesicles

Rate of transbilayer transport. The effect of selected temperatures on the ^{13}C-NMR spectra of [^{13}C]nor-CDCA in egg PC unilamellar vesicles at pH 4.0 is shown in **Fig. 2.** Six temperatures ranging from 9.2 to 41.2°C were studied. The spectra are characteristic of an unequally populated two-site exchange process with coalescence temperature slightly below 28°C. The maximum separation between the peaks corresponding to the two sites was obtained at 14.5°C. The observed exchange process corresponds to the unfacilitated movement of protonated bile acid molecules across the bilayer (flip-flop). Calculation of flip-flop rates was complicated by the fact that the T^*_2 values for each site at the different temperatures were not known. Analysis of the line shapes of the spectra at low temperature permitted the calculation of values for $k_{out \rightarrow in}$ which were relatively insensitive to T^*_2. However, at 28.4, 35.9 and 41.2°C the exchange rates obtained depended strongly on the assumed T^*_2s. For these temperatures, $k_{out \rightarrow in}$ was calculated for a range of reasonable line widths (6 to 13 Hz) (Fig. 2 and **Table 1**). The energy of activation calculated from the effect of temperature on flip-flop rate was 30 ± 3 kcal/mol.

Ionization. The chemical shift values of the carboxyl carbon of [^{13}C]nor-CDCA in PC vesicles at different pH values could be well fitted by the Henderson-Hasselbalch equation and gave a value for the apparent pK_a of 7.0 (**Fig. 3**). The signal, however, broadened considerably (almost 10-fold) and became asymmetric at pH values close to the pK_a (**Fig. 4**), suggesting slow exchange. This behavior has also been observed for fatty acids bound to phospholipid vesicles (33). Lineshape analysis was not possible in this case because the chemical shift of the ionized carboxyl group in the internal side of the bilayer was not observed, and the pK_a values for the bile acid inserted on either side of the membrane were not known.

The broadening of the signal at pH values close to the apparent pK_a indicates that not only the transmembrane flip-flop of the protonated bile acid but also the ionization/protonation steps were slow on the NMR time scale.

TABLE 1. Transbilayer movement rates for nor-CDCA and CDCA

Temperature	$k_{out \rightarrow in}$ (sec^{-1})[a]	$t_{1/2}$ (ms)[d]
Norchenodeoxycholic acid	E_a = 30 ± 3 kcal/mol	
9.2°C	10 ± 5	69.3
14.5°C	25 ± 5	27.7
19.8°C	55 ± 5	12.6
28.4°C	320 ± 90	2.2
35.9°C	580 ± 160	1.2
41.2°C	750 ± 250	0.9
Chenodeoxycholic acid[b]	E_a = 20 kcal/mol[c]	
35.0°C	100	6.9

[a]Due to the uncertainty in the natural line widths of the resonances, $k_{out \rightarrow in}$ was obtained by line shape analysis of the experimental spectra assuming a range of reasonable $\nu_{1/2}$ values. Results for $k_{out \rightarrow in}$ are expressed as the mean of the obtained range of values ± one-half of the spread between the maximum and minimum value at each temperature. Line widths used: 9.2°C: 10–14 Hz (only range compatible with spectrum); 14.5°C: 8–12 Hz; 19.8°C: 4–14 Hz (line shape relatively insensitive to line width); 28.4°C: 6–12 Hz; 35.9°C: 6–10 Hz; 41.2°C: 6–10 Hz.

[b]The kinetic analysis for the transbilayer movement of CDCA was obtained from ref. 17.

[c](Note: The E_a given in ref. 17 is in error. A calculation error was detected concerning the equation: slope = $-E_a/R$. Instead of multiplying the slope by R to obtain E_a, the slope was divided by R. We have recalculated and our value for E_a is correct.)

[d]The half-life ($t_{1/2}$) was calculated from the flip-flop rate (k) with the assumption that each rate was first order or pseudo first order ($t_{1/2}$ = 0.693/k).

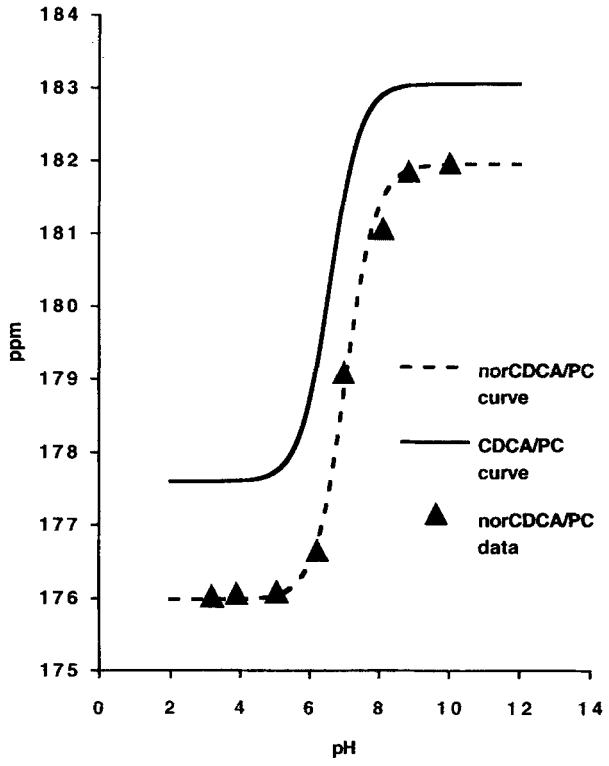

Fig. 3. Titration curves of [^{13}C]nor-CDCA and [^{13}C]CDCA in unilamellar vesicles: chemical shift of the carboxyl carbons of [^{13}C]nor-CDCA and [^{13}C]CDCA in PC vesicles as a function of pH. The points represent actual data points. The lines are the best fits of the theoretical Henderson-Hasselbalch equations; nor-CDCA: pK_a 7.0; CDCA: pK_a 6.6. Data for CDCA were obtained on the same spectrometer using the same protocol and are reported in ref. 21 (copies of ref. 21 are available upon request from University Microfilms International, Dissertation Information Services, Ann Arbor, MI 48106.)

DISCUSSION

^{13}C-NMR spectroscopy was used to define the ionization properties and flip-flop rates of two dihydroxy bile acids that are identical in structure except for the length of the branched side chain that ends in a carboxyl group. When present as a solute in TC micelles, the pK_a value

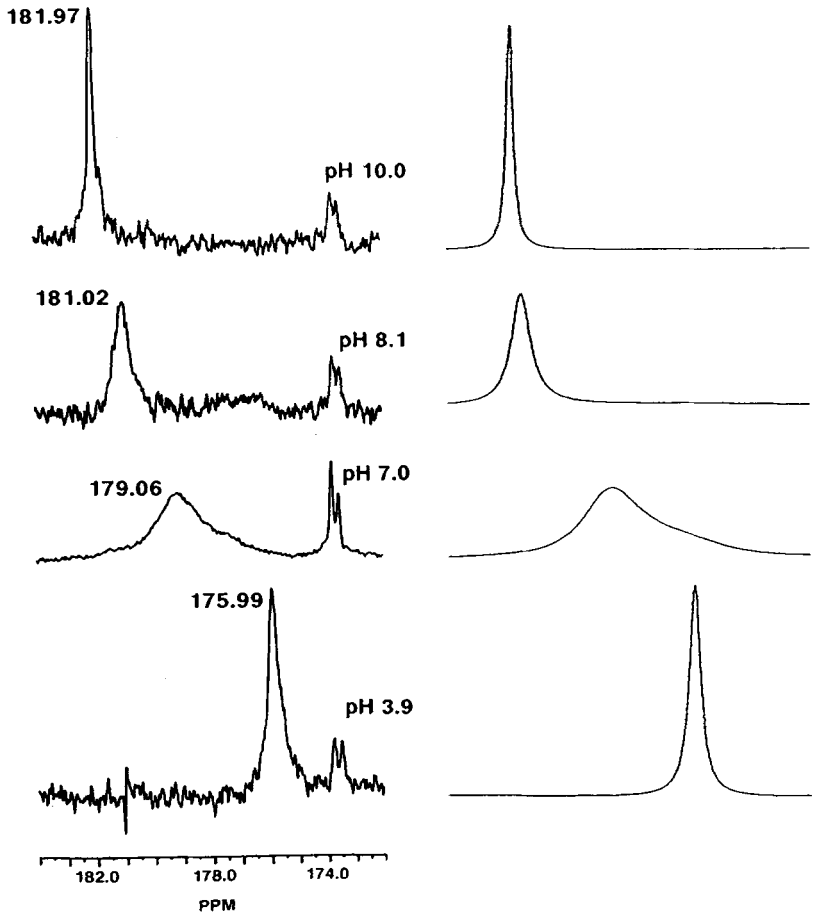

Fig. 4. Effect of pH on ^{13}C-NMR spectra of [^{13}C]nor-CDCA in PC unilamellar vesicles: ^{13}C-NMR spectra of [^{13}C]nor-CDCA in PC unilamellar vesicles at 28°C at different pH values. Curve simulations are on the right.

of nor-CDCA (6.1) did not differ significantly from the pK_a of CDCA (6.2). In dilute aqueous solution, the (intrinsic) pK_a value of CDCA, as that of all unconjugated bile acids with an aliphatic side chain, is about 5 (23, 34). The intrinsic pK_a of nor-CDCA has not been determined, but is likely to be quite similar. For example, the intrinsic pK_a values of the simple carboxylic acids with the chemical structure corresponding to the side chain of the two bile acids are virtually identical, pK_a 4.9 for isobutyric acid and pK_a 4.8 for isovaleric acid (35). The similarity of pK_a values and of the chemical shifts of the carbonyl carbon atom of the two bile acid homologues suggests that there is no great difference in the environments of the C_{24} and the C_{23} bile acids in the TC mixed micelle. The 0.5 ppm difference in chemical shifts of the protonated or the ionized forms of CDCA and nor-CDCA in mixed micelles should be attributed to the difference in the side chain length per se. This reasoning is based on the observation that the same chemical shift difference is observed between C_{24} and C_{23} bile acids when dissolved in d_4-methanol (36), as well as for their methyl esters dissolved in Cl_3CD (37).

Nor-CDCA and CDCA had similar pK_a values when present as micellar solutes, but the observed pK_a values were more than one unit higher than the pK_a of bile acids when present in monomeric solution in water. The increase in pK_a values of bile acids (20, 23, 38), long chain fatty acids (39), or acid–base indicators (40), when solubilized in anionic micelles, is well known (41). The magnitude of this effect depends on the surface potential of the micelle (41).

Nor-CDCA exhibited marked spectral differences from CDCA when inserted in PC vesicles. The chemical shifts of the anion and protonated forms of nor-CDCA were substantially lower (upfield) than for CDCA (Fig. 3); and its pK_a (7.0) was higher than for CDCA (pK_a 6.6). We have previously observed that the insertion of C_{24} bile acids in PC vesicles results in an upfield shift of the carboxyl group resonance (both the protonated form and the anion). Because the chemical shift of a carboxyl group is known to be proportional to its net hydrogen bonding, we suggested that this upfield shift is due to partial dehydration of the bile acid carboxyl group upon insertion in the hydrophobic environment of the phospholipid bilayer

(20). The fact that the resonance of the carboxyl group of nor-CDCA in PC vesicles is shifted upfield relative to CDCA suggests that a given nor-bile acid carboxyl undergoes greater dehydration when present in a bilayer than its corresponding C_{24} homologue. We speculate that this occurs because the nor-CDCA carboxyl group is located more deeply in the bilayer than that of the CDCA molecule. This model also offers an explanation for the lower acidity of nor-CDCA in PC vesicles: because of its deeper position in the bilayer, the carboxylate ion resulting from dissociation of nor-CDCA is likely to be less stabilized by hydration than the carboxylate ion of CDCA.

Lineshape analysis of the spectra obtained during the titration of nor-CDCA in micelles and in PC vesicles indicated that the dissociation of the bile acid and the reverse reaction were relatively slow processes. The kinetic dissociation constant of nor-CDCA in mixed micelles was found to be 0.4×10^5 sec^{-1}, which is about 20 times slower than the dissociation rate of acetic acid in water, 8.7×10^5 sec^{-1} (42). As the speed of proton transfer toward the micelle is unlikely to be different from that in bulk water, the measured rate is likely to indicate a slow rearrangement of the bile acid in the micelle to give the final NMR observable entities. A similar rearrangement appears to be occurring during the ionization of the bile acid inserted in the PC vesicle bilayer. With ionization, the carboxyl group would be expected to move closer to the hydrophilic micelle/water interface or bilayer/water interface to permit greater hydration and minimize charge separation from counterions.

The flip-flop rate for nor-CDCA at 35°C was six times higher than the rate for CDCA. The transmembrane movement of a protonated bile acid molecule inserted in the outside leaflet of a vesicle requires four steps: *1)* the breaking of hydrogen bonds (with external water) as well as the disruption of electrostatic forces (with dipoles or charges on the outside surface of the layer), permitting the compound to move to the hydrophobic core of the bilayer; *2)* diffusion of the solute across the bilayer to the inside of the vesicle; *3)* rotation of the molecule so that its polar group faces the internal side of the vesicle; and *4)* restoration of hydration and electrostatic interactions with dipoles and charges on the inside surface of the vesicle. Nor-dihydroxy bile acids appear to possess properties that would favor speedier movement through steps 1 and 2. If the carboxyl group of the nor-bile acid is located deeper in the membrane, then the nor-bile acid would have fewer hydrogen bonds and electrostatic interactions to break to achieve dissolution in the hydrocarbon portion of the membrane. Diffusion within the hydrocarbon region of biological and artificial membranes exhibits a very strong dependency on molecular volume. Assuming that the correlation found for relatively small molecules can be extended to molecules of medium size, shortening a bile acid by one methylene group (~18 cm^3/mole) (43) could increase the diffusion coefficient in a membrane by as much as a factor of 3 (18). In addition, the overall more spherical shape of the nor-bile acid might speed up the rotation movement to face the other side of the bilayer.

In the biliary fistula animal, infusion of nor-CDCA induces a much greater bile flow than CDCA (14). The greater choleretic effect of nor-CDCA is explained by nor-CDCA being a poor substrate for cholanoyl-CoA ligase in the hepatocyte (44). As a result, nor-CDCA is secreted into bile in unconjugated form and therefore undergoes cholehepatic circuiting within the liver. With each cycle, bile flow is induced by the osmotic effects of the secreted bile acid anion. In contrast, CDCA is conjugated with glycine or taurine before secretion into bile. The secreted N-acyl conjugates are not absorbed by the biliary ductular epithelium, and each molecule stimulates bile flow only once (45). In the hepatocyte, a fraction of nor-CDCA is not only conjugated with taurine, but also conjugated with glucuronate in the endoplasmic reticulum (46). The data presented here indicate that nor-CDCA should flip-flop rapidly across the lipid bilayer of the smooth endoplasmic reticulum to enter the inner compartment where glucuronidation occurs.

In summary, these in vitro studies indicate that the length of the side chain influences both ionization and flip-flop rates for dihydroxy bile acids that have been previously incorporated into model membranes. Although the two homologues had considerably different ionization and transport properties in vitro, both homologues are absorbed at about the same rate from the perfused small intestine in the anesthetized rodent (8). Thus, in vivo, transmembrane permeation does not appear to be a rate-determining step in epithelial transport. Rather, two other factors are likely to be key determinants of epithelial permeation. The first is diffusion through the unstirred water layer which for lipophilic molecules may be rate-limiting when intestinal absorption is studied in the anesthetized animal (17, 47). The second factor is adsorption to the membrane which should be less for nor-dihydroxy bile acids because of their lower lipophilicity (48).

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