Transbilayer Movement of Bile Acids in Model Membranes†

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ABSTRACT: The ability of bile acids to traverse membranes has important implications for their reabsorption from the gut, recirculation to and uptake into the liver, and resecretion into bile. The rate constant for transbilayer movement, or "flip-flop", of three common, unconjugated bile acids was determined by ^{13}C nuclear magnetic resonance spectroscopy. At high pH, the sodium salts of the bile acids did not appreciably traverse the bilayer; however, upon protonation a rapid equilibration between the inner and outer monolayers occurred. The rate of flip-flop of each bile acid at 37 °C was found to be dependent on both number and location of hydroxyl groups but not on concentration in the bilayer over the range studied (2–4 wt %) nor on the presence of a different bile acid in the same bilayer.

The primary bile salts cholate (C)[1] and chenodeoxycholate (CDC) are secreted into bile, mainly as glycine and taurine conjugates, where they aid in the solubilization of cholesterol and phospholipids. In the intestine, a fraction of the bile salts are deconjugated to form the free salts, and the secondary bile salts deoxycholate (DC) and lithocholate (LC) are formed by the action of 7α-dehydroxylases on the primary bile salts (Paumgartner & Paumgartner, 1982). The relative hydrophobicity of these common bile salts, often referred to as the hydrophobic/hydrophilic balance, is related to the amount of cholesterol solubilized by a given amount of bile salt (Armstrong & Carey, 1982). The more hydrophobic bile salts DC and CDC can solubilize more cholesterol than C. Cholic acid (CA), chenodeoxycholic acid (CDCA), and deoxycholic acid (DCA) are reabsorbed in the gut by both active and passive transport mechanisms, recirculated to the liver bound to protein, and taken up into the hepatocytes to be reconjugated in the microsomal compartments and resecreted into bile (Lack & Weiner, 1966). These bile acids must traverse the membranes of the intestine and liver during recirculation, associate with microsomes during conjugation, and move across the cannilicular membrane during secretion. The details of the transmembrane movements involved have not been determined.

There are few examples where rates of transbilayer movement of biological molecules have been accurately determined by nonperturbing methods (DeKruijff et al., 1977). More often, a bulky probe is incorporated into the molecule of interest, and the flip rate of the probe is measured on the assumption that the presence of the added group does not greatly alter the movement of the biomolecule. The present study examines the kinetics of transbilayer movement, "flip-flop", of three unconjugated bile acids (CA, CDCA, and DCA) in model bilayers by use of ^{13}C nuclear magnetic resonance (NMR) spectroscopy. The rate constants for flip-flop are discussed in terms of the structure and ionization state of the bile acids.

Experimental Procedures

Materials. Egg PC was purchased from Lipid Products (South Nutley, Surrey, U.K.) $Dy(NO_3)_3$ was obtained from Alfa Division, Morton Thiokol (Danvers, MA). Cholic, deoxycholic, and chenodeoxycholic acids, 90–94% ^{13}C enriched at carbon 24, were purchased from MSD Isotopes (St. Louis, MO). All lipids were >95% pure by thin-layer chromatography and were used without further purification.

Sample Preparation. Bile acid/egg PC unilamellar vesicles were prepared as previously described (Cabral et al., 1986). PC (98, 97, or 96 mg) was hydrated in H_2O and then sonicated at 25–30 °C with a Branson W350 sonifier in a pulsed mode. Titanium was removed by low-speed centrifugation. Then 2, 3, or 4 mg of 24-^{13}C-labeled bile salt was added to the preformed vesicles from a stock solution of the sodium salt of the acid (pH 10.0). D_2O (0.1 mL) was added as an internal lock signal. The total volume of the sample was 1.7 mL. To obtain vesicles containing fully protonated bile acid, the vesicle preparations were titrated with 1.0 N HCl to pH 3.5 ± 0.5 (Cabral et al., 1986).

Temperature-Dependent Spectral Changes. ^{13}C NMR spectra were obtained at selected temperatures at 50.3 MHz with a Bruker WP200 spectrometer equipped with an Aspect 2000 or 2000A data system and a Bruker B-VT-1000 variable temperature unit (temperatures accurate to ±0.5 °C). Broad-band ^1H decoupling (1.0 W) centered 3.4 ppm downfield from tetramethylsilane was used. The terminal methyl peak of the egg PC fatty acyl chains at 14.10 ppm was used as a chemical shift reference (Hamilton et al., 1974), and spectra were processed with 2.0-Hz line broadening. The rate constants for flip-flop of the bile acids were estimated from the temperature-dependent spectral changes according to the modified Bloch equations for exchanging systems for a simple, first-order, two-site exchange (James, 1985). A rate constant was calculated for each temperature point, and the activation energy for flip-flop was determined from plots of ln k vs. $1/T$. Spectra were obtained as a function of temperature for vesicles with CA, CDCA, DCA, or a mixture of CA + DCA (1:1 w/w), starting at ambient probe temperature, ~35 °C. The results for the different concentrations of bile acid used were not significantly different.

Inversion Transfer. The resonance from the carboxyl carbon of CA in the inner or outer monolayers of the vesicles was

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[1] Abbreviations: DC, 3α,12α-dihydroxy-5β-cholanoate (deoxycholate); DCA, deoxycholic acid; C, 3α,7α,12α-trihydroxy-5β-cholanoate (cholate); CA, cholic acid; CDC, 3α,7α-dihydroxy-5β-cholanoate (chenodeoxycholate); CDCA, chenodeoxycholic acid; k, rate constant for transbilayer movement; LC, lithocholate; NMR, nuclear magnetic resonance; PC, phosphatidylcholine; T_1, spin–lattice relaxation time.

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selectively inverted in the ^1H decoupled spectrum by using a Dante pulse sequence (Morris & Freeman, 1978) consisting of 16 short, low-power pulses of 5.9-μs duration, separated by 0.001 s. The low-power pulse was generated by connecting an attenuator (6-dB attenuation) in series with the pulse transmitter. This pulse was sufficient to invert selectively the desired peak at the transmitter frequency; side bands occurred at intervals of $\pm$1000 Hz (1/0.001 s), well outside the region of interest. A standard 90° observe pulse was executed after a variable delay time (from 0.003 to 6.0 or 7.5 s) that allowed CA to exchange between monolayers. This sequence was repeated to achieve 1000 scans for each delay time; the total time for the experiment (using 15 delay times) was 44 h. The magnetization of the resonances at each delay time was measured as the integrated area under the peaks (areas are accurate to $\pm$20%). The nuclear Overhauser effect for the two peaks due to broad-band decoupling was the same, and the total time between inversion pulses, 8 times the spin–lattice relaxation time (T_1), allowed complete return to equilibrium. Spectra were processed with 5.0-Hz line broadening. Rate constants were determined by nonlinear least-squares regression according to the solutions to the modified Bloch equations of McConnell (1958), correcting for incomplete inversion (Alger & Prestegard, 1977; Malloy et al., 1985). Analysis was done for the two-site inversion transfer according to the NLIN procedure of the SAS statistical package (SAS Institute, Carey, NC) at the Boston University Academic Computing Center (Boston, MA) for either a two- ($k_{\text{inner}\rightarrow\text{outer}}$, $k_{\text{outer}\rightarrow\text{inner}}$) or four- ($k_{\text{inner}\rightarrow\text{outer}}$, $k_{\text{outer}\rightarrow\text{inner}}$, $T_{1,\text{outer}}$, $T_{1,\text{inner}}$) parameter fit.

Results and Discussion

We have previously shown that at 35 °C protonation of NaC (3 wt %) in small unilamellar egg phosphatidylcholine (egg PC) vesicles results in the appearance of a second, more shielded magnetic environment for the ^{13}C-enriched carboxyl group of CA (see Figure 1a) (Cabral et al., 1986; Small et al., 1984). The two carboxyl resonances were proposed to represent CA in the outer and inner monolayers of the vesicle bilayer because the peaks had an $\sim$2/1 intensity ratio and their chemical shifts were different from that of aqueous CA (Cabral et al., 1986). These assignments have subsequently been verified by using the lanthanide shift reagent dysprosium (Dy^{3+}). Addition of Dy^{3+} [pH 4.0, 0.2 mM $Dy(NO_3)_3$ added to preformed CA/egg PC vesicles] selectively broadened the more intense downfield CA carboxyl peak at 178.0 ppm (spectra not shown). Sonication in the presence of 5.0 mM Dy^{3+} gave a ^{13}C NMR spectrum in which both CA carboxyl peaks were broadened to base-line level. After dialysis of the vesicles for 24 h against distilled H_2O at pH 4.0, a single resonance at 178.0 ppm, corresponding to the carboxyl peak of CA in the outer monolayer, was seen (not shown).

In contrast to CA, ^{13}C NMR spectra at 35 °C of CDCA and DCA added to egg PC vesicles gave a single carboxyl peak at all pH values studied (Cabral et al., 1986), suggesting either that the exchange rate between monolayers for the dihydroxy bile acids was fast on the NMR time scale or that these bile acids did not cross the bilayer. Upon reducing the temperature of the DCA and CDCA vesicle samples at pH 4, two carboxyl peaks were seen, indicating flip-flop does occur but at a much faster rate than for CA at 35 °C. The rate constants, k, for the flip-flop of the three bile acids at various temperatures were estimated from solutions to the modified Bloch equations as described under Experimental Procedures. As the sample temperature was increased, the flip-flop rate increased. The two resolved carboxyl peaks broadened and then collapsed into a single peak that narrowed to a limiting line width (see Figure 1 for CA). The k's for CA ranged from $\sim$3 s^{-1} at 30 °C to $\sim$180 s^{-1} at 70 °C. Rate constants of a similar range were found for CDCA and DCA, but at a lower temperature for a given k. Doubling the concentration from 2% to 4% did not affect k. The energy of activation for transbilayer movement of each bile acid was estimated from plots of ln k vs. $1/T$ (see Table I for a comparison of kinetic parameters).

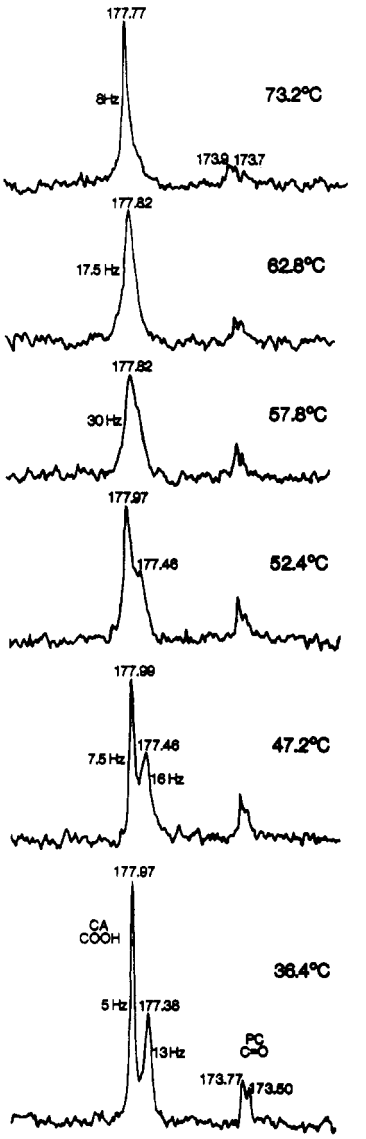

FIGURE 1: ^{13}C NMR spectra of the carboxyl/carbonyl region of egg PC vesicles containing cholic acid as a function of increasing temperature. Egg PC vesicles were prepared as described. The terminal methyl peak of the egg PC fatty acyl chains at 14.10 ppm was used as a chemical shift reference (Hamilton et al., 1974), and spectra were processed with 2.0-Hz line broadening. The rate constants for transbilayer movement were estimated from the solution to the modified Bloch equations for exchanging systems for a simple, first-order, two-site exchange (James, 1985). A rate constant was calculated for each temperature point. Similar spectra were obtained for CDCA and DCA in egg PC vesicles.

When the two populations in exchange are of unequal size, as in this case, the error in k estimated by temperature variation can be as high as 200%. Therefore, k's for CA flip-flop from outer → inner and inner → outer monolayers at 35 °C were also measured by a two-site ^{13}C inversion transfer as described. It was not feasible to maintain the low temperatures necessary to resolve the CDCA and DCA in inner and outer peaks for the period (44 h) required to obtained inversion transfer data. Figure 2 shows spectra at 35 °C of the CA

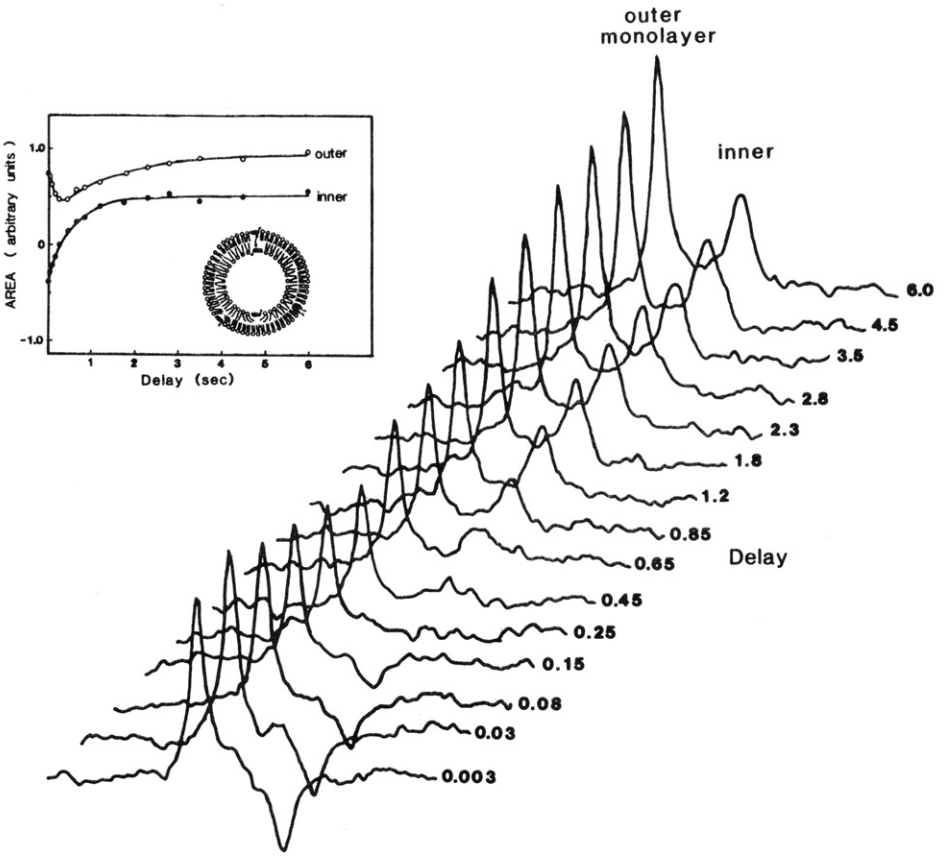

FIGURE 2: Inversion transfer spectra of the carboxyl peaks of cholic acid in the outer and inner monolayers of egg PC vesicles. Egg PC/CA vesicles were prepared as described. Spectra were processed with 5.0-Hz line broadening. A similar set of spectra were obtained by selectively inverting the outer monolayer CA carboxyl peak. Rate constants (k) were determined by nonlinear least-squares regression according to the solutions to the modified Bloch equations of McConnell (1958), correcting for incomplete inversion (Alger & Prestegard, 1977; Malloy et al., 1985). The inset shows a plot of the integrated areas of the carboxyl resonances at each delay time. The decrease in area for the outer monolayer CA peak is due to exchange with inverted CA molecules in the inner monolayer. A representation of an egg PC/bile acid containing vesicle is also shown.

Table I: Kinetic Analysis of Transmembrane Movement of Bile Acids at 35 °C[a]

bile acid	k (s^{-1})[b]	k (s^{-1})[c]	E_{act}[d] (kcal/mol)	$t_{1/2}$[e] (ms)	$t_{1/2}$[e] (h), pH 10.0 ± 0.2
cholic (3α,7α,12α-trihydroxy)	outer → inner, 3.4 ± 2.1 inner → outer, 5.9 ● 4.3	5	7.2	139.0	>24
chenodeoxycholic (3α,7α-dihydroxy)		100	5.1	6.9	>24
deoxycholic (3α,12α-dihydroxy)		150	4.2	4.6	>24

[a] All studies were done at pH 3.5 ± 0.5 unless indicated otherwise. [b] Rates determined from two-site inversion transfer method as described in Figure 2. Rate given is the average of two-parameter and four-parameter nonlinear least-squares regression (mean ± SE). [c] Rates are for movement from the outer monolayer to the inner monolayer estimated by temperature variation as described in Figure 1; mean of three trials. k's for 2%, 3%, and 4% bile acid were not significantly different. Rates for CDCA were determined at 3% CDCA only. [d] Activation energy for flipping was estimated by plotting ln k vs. $1/T$ by use of data from three trials (not shown: r = 0.94, 0.91, and 0.81 for CA, CDCA, and DCA, respectively). [e] Rates are assumed to be first order or pseudo first order ($t_{1/2} = 0.693/k$).

carboxyl peaks at selected delay times after the inner monolayer CA carboxyl peak was selectively inverted. The integrated area of the noninverted peak was the same (±20%) at zero delay (extrapolated from the shortest delay, 0.003 s) and at equilibrium intensity (from the spectrum with the longest delay, 6.0 or 7.5 s). The percent inversion achieved was 55% for the outer monolayer CA carboxyl peak and 80% for the inner monolayer peak. The equations used to relate magnetization (i.e., integrated area) to k for flip-flop correct for incomplete inversion (Alger & Prestegard, 1977). The data were analyzed by using a nonlinear least-squares regression program for either a two- or four-parameter fit. The k's calculated (Table I) are in good agreement with the temperature variation method for $k_{outer \rightarrow inner}$. Further, if we assume that (i) the rate fo flip-flop is first order or pseudo first order and (ii) the equilibrium amount of bile acid in each monolayer at pH 3.5 is known,[2] we can predict that $k_{inner \rightarrow outer}$ should be twice $k_{outer \rightarrow inner}$. The k's obtained from the two-site inversion transfer agree with this prediction within experimental error.

The k's for transbilayer movement for CDCA and DCA were greater than for CA at any given temperature over the range studied. With only two hydroxyl groups, these bile acids are more hydrophobic than CA and had a lower activation energy for traversing the bilayer. There was not, however, a simple relationship between the number of hydroxyl groups and k. The position of the OH group was also important. The

[2] This was estimated as 1.9 ● 0.1 from the equilibrium intensities of the two resolved peaks with the GLINFIT program by Alex D. Bain, Bruker Spectrospin Canada, Ltd., Ontario, Canada. The nuclear Overhauser enhancement and the T_1 value (measured by inversion recovery) were the same for the inner and outer peaks.

more hydrophobic 3α,12α-dihydroxy-DCA flip-flops faster than 3α,7α-dihydroxy-CDCA at a given temperature. Therefore, the overall hydrophobicity of the bile acid is important in determining its rate of transbilayer movement. Furthermore, extrapolation of these data to the more hydrophobic steroid cholesterol (whose rate of flip-flop has not been directly measured as of yet) would predict a flip rate in egg PC vesicles much faster than 1 ms.

Since more than one bile acid is present in the same membrane in vivo, it was important to test whether the presence of one bile acid affected the k of a second bile acid in the same vesicles. CA and DCA in the same vesicle sample in equal amounts (3 mg each) had rate constants for flip-flop, estimated from temperature-dependent spectral changes, that were not significantly different from the rates found when only one bile acid was present (data not shown). The rate of flip-flop of DCA did not affect the rate for CA (k for DCA was greater than 10 times k for CA at 35 °C in all cases), indicating that heterodimers of the two bile acids did not form and flip as a unit.

Several conclusions can be drawn from these results. First, the time scale for transbilayer movement of unconjugated bile acids in small unilamellar vesicles is such that the kinetics can be determined by NMR spectroscopy. The k's for flip-flop can be determined over a range of temperatures and bile acid concentrations. The kinetics for this process are dependent on both the number and position of hydroxyl groups but not on the concentration of bile acid over the range studied. Also, different bile acids present in the same bilayer appear to flip independently. The kinetics of transbilayer movement and the noncooperativity between bile acid species may be important in the selective reabsorption of CA and CDCA in the intestine, the quick and efficient uptake into the liver, and the reconjugation of these acids in the endoplasmic reticulum of the liver.

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