

# The hydrophobic-hydrophilic balance of bile salts. Inverse correlation between reverse-phase high performance liquid chromatographic mobilities and micellar cholesterol-solubilizing capacities

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**Abstract** To examine quantitatively the hydrophobic-hydrophilic properties of bile salts, we determined the reverse-phase high performance liquid chromatographic (HPLC) mobilities of monomeric bile salt solutions and the equilibrium cholesterol-solubilizing capacities of 100 mM micellar solutions. Studies with the common bile salts (ursodeoxycholate, UDC, cholate, C, chenodeoxycholate, CDC, and deoxycholate, DC) demonstrated that HPLC mobility, which correlates with hydrophilicity, was markedly influenced by both position and orientation, in addition to number, of hydroxyl functions, in that mobility decreased in the order UDC > C > CDC > DC. Conjugation of the bile salt was also important, in that the HPLC mobility of the taurine (T)-conjugates was greater than the glycine (G)-conjugates which in turn was greater than that of the free bile salts. Equilibrium micellar cholesterol solubilities were also influenced by bile salt structure and correlated inversely with hydrophilicity, in that solubility decreased in the order DC > CDC > C > UDC with free bile salts > G-conjugates > T conjugates. For each bile salt series, double logarithmic plots of the cholesterol-solubilizing capacities expressed in mole fraction units versus the HPLC retention factors (*k'*) gave linear relationships. Linear regression equations were employed to predict the equilibrium cholesterol-solubilizing capacities of a number of less common bile salts from their HPLC retention factors. Each theoretical value agreed closely with that derived entirely by experiment. A comparison of the HPLC mobilities of the less common bile salts with the more common species revealed that not only were sulfate and oxo substituents more hydrophilic than  $\alpha$ -oriented hydroxyl functions, but, in the dihydroxy species, a single equatorial hydroxyl function such as in UDC ( $3\alpha,7\beta$ ) and in hyodeoxycholate ( $3\alpha,6\alpha$ ) was more hydrophilic than two or three hydroxyl functions at 3, 7, and/or 12 $\alpha$  (axial) positions. These studies taken together suggest that reverse-phase HPLC mobility and equilibrium cholesterol-solubilizing capacities are inverse functions of each other and correlate closely with the hydrophilicity of bile salt molecules. In addition, the evidence here deduced further strengthens our recent deductions based on an evaluation of a number of other physical-chemical properties (Carey, M. C., J.-C. Montet, M. C. Phillips, M. J. Armstrong, and N. A. Mazer, 1981. *Biochemistry*. 20: 3637–3648.) that cholesterol may be solubilized in micellar bile salt solutions by both hydrophobic and hydrophilic association with the external (“hydrophilic”) sur-

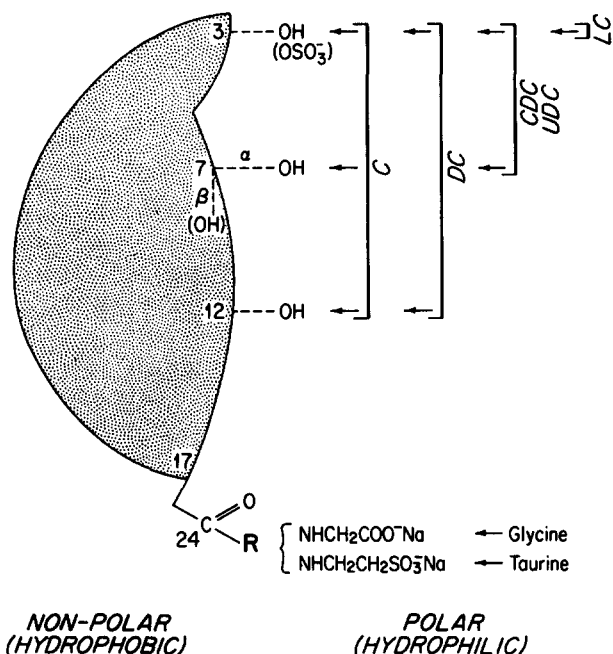
face of bile salt micelles rather than with the hydrophobic surface of the micelle's interior.—**Armstrong, M. J., and M. C. Carey.** The hydrophobic-hydrophilic balance of bile salts. Inverse correlation between reverse-phase high performance liquid chromatographic mobilities and micellar cholesterol-solubilizing capacities. *J. Lipid Res.* 1982. 23: 70–80.

**Supplementary key words** bile salt conjugation • critical micellar temperature • retention factor • uncommon bile salts • hydroxyl, oxo, and sulfate functions • UV-absorption spectra

The common bile salts are a family of soluble amphiphilic molecules that possess two distinct hydrocarbon surfaces (**Fig. 1**). The more hydrophobic hydrocarbon surface lies on the convex ( $\beta$ ) side of the steroid nucleus and is devoid of hydrophilic substituents. In contrast, the hydrocarbon surface on the concave ( $\alpha$ ) side is less hydrophobic due to its smaller total surface area and by the presence of one, two, or three hydroxyl (less commonly sulfate or glucuronidate) functions. Furthermore, the aliphatic side chain and conjugating amino function (taurine or glycine) terminates in a strong ionic polar group which, when extended and oriented at an interface, contributes a strong hydrophilic moiety to the less hydrophobic side (1). Hence, bile salts are biplanar molecules with two functionally different sides. In recent studies (1) we have shown that the two “sides” of a bile salt molecule each play important but distinct roles in its physical-chemical properties. The desire of the more hydrophobic (in earlier nomenclature “hydrophobic”)

Abbreviations: HPLC, high performance liquid chromatography; CMC, critical micellar concentration; CMT, critical micellar temperature; ChM, cholesterol monohydrate; C, cholate; CDC, chenodeoxycholate; DC, deoxycholate; UDC, ursodeoxycholate; LSCSO<sub>4</sub>, lithocholate sulfate; T- and G-prefixes, taurine and glycine conjugates, respectively; TLC, thin-layer chromatography; GLC, gas-liquid chromatography.

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**Fig. 1.** Schematic representation of the amphiphilic structure of bile salts (See list for abbreviations). The hydrophobic convex ( $\beta$ ) side is devoid of hydrophilic substituents. The hydrophilic concave ( $\alpha$ ) side is spiked with hydroxyl groups at the 3, 7, and/or 12 positions and may be conjugated with taurine or glycine at C 24 (indicated by arrows). In UDC the  $7\beta$ -OH function is equatorial and in LC the 3-OH group may be conjugated with sulfate ( $\text{SO}_4$ ).

side of the bile salt monomer to eliminate its contact with the aqueous solvent is reflected in primary micelle formation (2). This takes place at a fairly well-defined concentration known as the critical micellar concentration (CMC) (1, 3). The hydrophobic properties that are responsible for primary micelle formation can be measured experimentally by the magnitude of the CMC, the mean micellar weight, and the aggregation number (number of monomers per primary micelle) just above the CMC and mean hydrodynamic radius of the primary micelles measured at, or extrapolated to, the CMC (1–3). While these primary micellar properties differ somewhat between bile salts, their similarities, especially the CMC values ( $\approx 1\text{--}5$  mM in  $0.15$  M  $\text{Na}^+$  at  $37^\circ\text{C}$ ) (1)) suggest that similar areas of hydrocarbon are removed from contact with the aqueous solvent when primary micelles form. Conversely, since the less hydrophobic (in earlier nomenclature “hydrophilic”) side of the bile salt monomer forms the external surface of the primary micelle, this side governs micellar growth and secondary micelle formation (2). This side also determines, in large measure, interfacial or surface properties (surface tension lowering, collapse pressure, and equilibrium spreading pressure) (1) and more or less classical thin-layer and gas-liquid chromatographic properties of bile salts (4,

5). All of these properties differ markedly from bile salt to bile salt as does a key physiological property of bile salt micelles, that of solubilizing cholesterol (1, 6–9).

To test quantitatively the interrelationships between the hydrophobic-hydrophilic balance of bile salt molecules and their micellar capacities to solubilize cholesterol, we compared equilibrium micellar solubilities of cholesterol with the chromatographic mobilities of bile salt monomers measured by reverse-phase high performance liquid chromatography (HPLC). A marked inverse correlation was found between HPLC mobility ( $\text{retention}^{-1}$ ) and the cholesterol-solubilizing capacity. These observations taken together with other physicochemical evidence (1) suggest that the less hydrophobic (“hydrophilic”) surface of the bile salt molecules was importantly involved in both the micellar association with cholesterol and the molecular association with the hydrocarbon stationary phase of the HPLC column. This relationship allows one to predict with considerable accuracy the micellar cholesterol-solubilizing capacity of any bile salt species from its ‘retention factor’ (defined below) by reverse-phase HPLC.

## THEORY

### HPLC retention ( $\text{mobility}^{-1}$ ) of bile salt monomers

The reverse-phase HPLC retention of a bile salt species is determined by the partitioning of the bile salt molecules between the hydrocarbon surface of the stationary phase ( $\text{BS}_{\text{hc}}$ ) of the column and the more polar mobile phase ( $\text{BS}_{\text{mp}}$ ):

$$\text{BS}_{\text{mp}} \stackrel{K_p}{\rightleftharpoons} \text{BS}_{\text{hc}} \quad \text{Eq. 1}$$

The partition coefficient ( $K_p$ ) in Equation 1 is defined by the law of mass action (10) as:

$$K_p = \frac{[\text{BS}]_{\text{hc}}}{[\text{BS}]_{\text{mp}}} \quad \text{Eq. 2}$$

where  $[\text{BS}]_{\text{hc}}$  and  $[\text{BS}]_{\text{mp}}$  are the bile salt concentrations in the hydrocarbon and more polar phases, respectively. The HPLC retention factor,  $k'$ , of the bile salt can then be derived (11) from:

$$k' = K_p \frac{V_{\text{hc}}}{V_{\text{mp}}} \quad \text{Eq. 3}$$

where  $V_{\text{hc}}$  and  $V_{\text{mp}}$  represent the volumes of the hydrocarbon and polar phases, respectively. An expansion of this relationship expresses the mass ratio of bile salt in both phases.

The Gibbs free energy change ( $\Delta G_p$ ) for partitioning of the bile salt between the phases can be related to the equilibrium partition coefficient ( $K_p$ ) by:

$$\Delta G_p = -RT \ln K_p \quad \text{Eq. 4}$$

where R and T are the gas constant and absolute temperature, respectively.

### Cholesterol solubility in bile salt micelles

The equilibrium solubility of cholesterol monohydrate (ChM) in a micellar solution of bile salts can be described by the relationship:



where  $\text{ChM}_{\text{aq}}$  and  $\text{ChM}_{\text{mic}}$  are aqueous and micellar forms of cholesterol monohydrate and  $K_b$  is the equilibrium constant.  $K_b$  can be related to the concentrations of both species by the law of mass action:

$$K_b = \frac{[\text{ChM}]_{\text{mic}}}{[\text{ChM}]_{\text{aq}}} \quad \text{Eq. 6}$$

The mole fraction  $\chi_{\text{ChM}}$  ( $\chi_{\text{ChM}}$ ) solubilized in a particular bile salt solution is given by:

$$\chi_{\text{ChM}} = \frac{[\text{ChM}]_{\text{mic}}}{[\text{ChM}]_{\text{mic}} + [\text{BS}]} \quad \text{Eq. 7}$$

and by substituting Equation 6 in Equation 7 and rearranging, we obtain:

$$\chi_{\text{ChM}} = \frac{K_b[\text{ChM}]_{\text{aq}}}{K_b[\text{ChM}]_{\text{aq}} + [\text{BS}]} \quad \text{Eq. 8}$$

Under experimental conditions of constant temperature, ionic strength, and bile salt concentration, the Gibbs free energy change ( $\Delta G_b$ ) for micellar solubilization of ChM is given by:

$$\Delta G_b = -RT \ln K_b \quad \text{Eq. 9}$$

Since the free energy of bile salt hydrophobic interactions is involved in both cases, then  $K_p$  should be linearly related to  $K_b$  or:

$$-RT \ln K_p = m(-RT \ln K_b) + b \quad \text{Eq. 10}$$

Since  $K_p \propto k'$  (Equation 3) and  $K_b \propto \chi_{\text{ChM}}$  (Equation 7) then it follows that:

$$\log k' = m \log \chi_{\text{ChM}} - \text{constant} \quad \text{Eq. 11}$$

Hence, if the assumptions implied above are correct, the logarithm of the HPLC retention factors of a particular bile salt series should be related linearly to the logarithm of the mole fraction of cholesterol solubilized by the bile salt micellar solutions at equilibrium.

## EXPERIMENTAL PROCEDURE

### Materials

Unconjugated chenodeoxycholate (CDC) and ursodeoxycholate (UDC) were kindly provided by Dr. Herbert Falk (Freiburg, West Germany). The glycine (G) conjugates of CDC and UDC were generously custom-synthesized by the Tokyo Tanabe Co. (Tokyo, Japan). The taurine (T) and glycine conjugates of hyodeoxycholate ( $3\alpha,6\alpha$ -dihydroxy-5 $\beta$ -cholanoate) were obtained from Steraloids (Wilton, NH).  $7\alpha,12\alpha$ -Dihydroxy-5 $\beta$ -cholanoate was obtained from Pharmazell (Raubling, West Germany) and  $3\alpha$ -hydroxy-7-oxo-5 $\beta$ -cholanoate was the generous gift of Roussel Uclaf (Romainville, France). Unconjugated cholate (C), deoxycholate (DC), hyodeoxycholate, hyocholate ( $3\alpha,6\alpha,7\alpha$ -trihydroxy-5 $\beta$ -cholanoate),  $3\alpha,7\alpha$ -dihydroxy-12-oxo-5 $\beta$ -cholanoate, and all other taurine and glycine conjugates including the taurine conjugate of lithocholate sulfate ( $\text{TLC SO}_4$ ) were obtained from Calbiochem-Behring, Inc. (San Diego, CA). The taurine conjugate of hyocholate was synthesized by the method of Hofmann (12). Tauroolithocholate sulfate was converted from the diammonium to the disodium salt by the method of Tserng and Klein (13). The sodium salts of CDC, UDC, hyocholate, hyodeoxycholate,  $7\alpha,12\alpha$ -dihydroxy-5 $\beta$ -cholanoate, and  $3\alpha$ -hydroxy-7-oxo-5 $\beta$  cholanoate were prepared from the crystalline acids as previously described (14). Purity of the bile salts was assessed by thin-layer chromatography (TLC) and HPLC. Purity of DC, CDC, UDC, GCDC, GUDC, GC,  $7\alpha,12\alpha$ -dihydroxy-5 $\beta$ -cholanoate, and  $3\alpha$ -hydroxy-7-oxo-5 $\beta$ -cholanoate were found to be greater than 98% and therefore were not subjected to further purification. All other bile salts (TC, TDC, GDC, C, hyocholate, hyodeoxycholate,  $\text{TLC SO}_4$ ) were less than 98% pure and were recrystallized from aqueous ethanol (12, 15, 16) until satisfactory purity by TLC and HPLC was achieved.

Cholesterol (Nu-Chek Prep, Austin, MN) was recrystallized three times from hot 95% ethanol, followed by preparation of the crystalline monohydrate as described by Igimi and Carey (6). Radiolabeled cholesterol used in a number of the dissolution experiments was prepared by mixing  $[1,2\text{-}^3\text{H}]$ cholesterol (New England Nuclear Co., Boston, MA) with an ethanolic solution of pure unlabeled cholesterol prior to recrystallization to give a specific activity of 0.39 mCi/mmol. After recrystallization, the cholesterol was chemically and radiochemically pure by TLC with zonal scanning (6). HPLC grade methanol (MeOH) was purchased from Waters, Inc. (Milford, MA). The NaCl (Certified Grade, Fisher Scientific, Pittsburg, PA) was roasted at 600°C for 3 hr in a muffle furnace to oxidize and remove

organic impurities.  $\text{KH}_2\text{PO}_4$ ,  $\text{H}_3\text{PO}_4$ ,  $\text{NaHCO}_3$ , and  $\text{Na}_2\text{CO}_3$  were Fisher Certified Grade reagents. Ion-exchanged and glass-distilled water and acid-alkali washed glassware were used throughout.

## Methods

**Solutions.** For HPLC studies, bile salts were dissolved in 75% MeOH:25%  $\text{H}_2\text{O}$  to give a final concentration of 1 mg/ml. For equilibrium ChM solubility studies, 100-mM solutions of each bile salt were prepared on a wt/vol basis either in 0.15 M NaCl at pH 7.0 (taurine conjugates) or in carbonate-bicarbonate buffer (0.05 M with added NaCl to give a final  $\text{Na}^+$  concentration of 0.15 M) at pH 10.0 (unconjugated bile salts and glycine conjugates).

**UV Absorption.** UV absorption spectra were determined for representative bile salts in 75% MeOH using a Cary Model 118-C variable wavelength scanning spectrophotometer (Varian Associates, Palo Alto, CA). The monochromator, light source, photomultiplier tube and sample chambers were purged with nitrogen to permit accurate measurements of absorbances at low wavelengths.

**HPLC.** HPLC was performed on an isocratic liquid chromatograph (Beckman Instruments, Inc., Fullerton, CA, Model 330) with a Model 110A pump and a Model 210 injection valve. The reverse-phase column was an Altex Ultrasphere-ODS column,  $250 \times 4.6$  mm (Altex, Inc., Palo Alto, CA) with octadecylsilane (C18) coated  $5 \mu\text{m}$  silica particles as the stationary phase. Flow rates were set at 1.0 ml/min to generate a pressure of 3000 psi. The bile salt elution profiles were monitored with a variable wavelength UV-visible spectrophotometer (Hitachi, Ltd., Tokyo, Japan, Model 100-40) set at 210 nm, a wavelength at which there was significant absorption by the weakly chromophoric groups ( $C=0$ ,  $S=0$ ) of each bile salt. Peak retention times and areas were recorded with a computing integrator (Shimadzu Corp., Kyoto, Japan, Model CR1-A). The mobile phase consisted of 75% MeOH:25%  $\text{H}_2\text{O}$  (by volume) buffered with 0.005 M  $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$ . The apparent pH of the final solution measured with a glass electrode (Radiometer, Copenhagen) was  $5.0 \pm 0.1$ .

The HPLC retention factor ( $k'$ ) was calculated from the normalized peak retention times according to Bloch and Watkins (17):

$$k' = \frac{t_r - t_0}{t_0} \quad \text{Eq. 12}$$

where  $t_0$  = retention time of the solvent front and  $t_r$  = retention time of the bile salt elution profile measured at 210 nm.

*Equilibrium cholesterol monohydrate solubility.* Equi-

librium cholesterol solubility studies were performed in triplicate according to the powder dissolution method of Igimi and Carey (6). Cholesterol crystals were ground to a fine powder in an agate mortar and 100 mg was added to 10 ml of a 100 mM bile salt solution. The tubes were sealed under nitrogen and incubated in a  $37^\circ\text{C}$  water bath. Tubes were vortex-mixed twice daily and samples were withdrawn at 24-hr intervals for 7 days. The samples were passed through a pre-warmed ( $37^\circ\text{C}$ )  $0.22 \mu\text{m}$  filter (Millipore Corp., Bedford, MA) to remove excess crystalline cholesterol, and were analyzed in duplicate for cholesterol content with a cholesterol oxidase kit (Kyowa Hakko Kogyo Co., Ltd., Tokyo, Japan). In certain dissolution studies (with UDC, TUDC, GUDC, and all the uncommon bile salts) where radiolabeled cholesterol was employed, the solutions were filtered as described above and the filtrates were analyzed in duplicate for radioactivity employing a LS-230 Liquid Scintillation Spectrometer (Beckman Instruments, Fullerton, CA). The cholesterol concentration solubilized by solutions of CDC, TCDC, and GCDC was assayed both by the cholesterol oxidase method and radiochemically.

*Measurement of critical micellar temperatures.* Since certain bile salts (taurohyodeoxycholate, glycohyodeoxycholate, hyodeoxycholate, and  $7\alpha,12\alpha$ -dihydroxy- $5\beta$ -cholanoate) were found to be sparingly soluble within the ambient temperature ranges, the critical micellar temperatures (CMT's) of 100 mM solutions were determined in triplicate at pH 10.0 by the clearing point method (18).

*Prediction of equilibrium cholesterol monohydrate solubility.* The logarithm of the ChM solubility (in mole fraction units) by 100 mM micellar solutions was plotted versus the logarithm of the HPLC retention factor ( $k'$ ) according to each mode of conjugation and for each homologous series of common bile salts. The linear regression equations generated for these relationships were employed to predict the equilibrium cholesterol monohydrate solubilities of several less common bile salts from their HPLC retention factors.

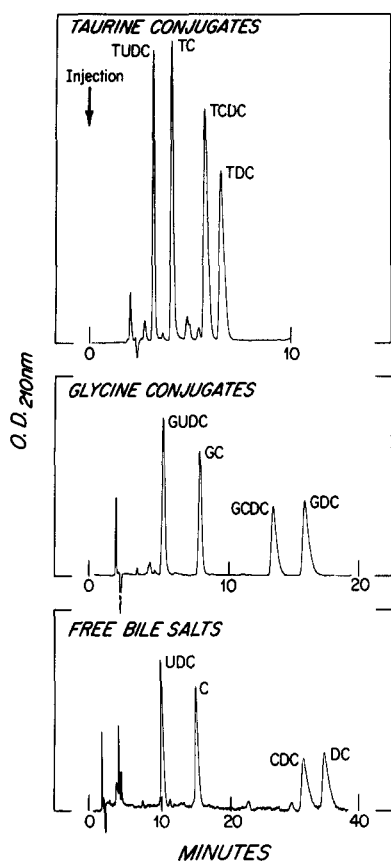
*Statistics.* Mean  $\pm$  standard deviations were computed for retention factors, micellar bile salt:ChM molar ratios, and for the ChM mole fraction solubilized at equilibrium. Double logarithmic linear regression analyses were performed by the method of least squares (19).

## RESULTS

### UV absorption spectra

Ultraviolet adsorption spectra were obtained in 75% MeOH for cholate and its taurine and glycine conjugates (data not shown). The absorbance in each case increased





**Fig. 2.** Reverse-phase high performance liquid chromatography (HPLC) of bile salts performed in 75% MeOH–25% 0.005 M  $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$  at pH 5.0 with an Ultrasphere ODS  $250 \times 4.6$  mm column. Within each series the ordering of the bile salt mobilities is  $\text{UDC} > \text{C} > \text{CDC} > \text{DC}$ .

to a peak of  $\approx 195$  nm. Detection of UV absorption at lower wavelengths was precluded by the optical properties of MeOH and the spectrophotometer. Absorption coefficients ( $\epsilon$ ) were determined at both 210 nm and 195 nm and were (in units of  $\text{L} \cdot \text{M}^{-1} \cdot \text{cm}^{-1}$ ) as follows:  $\epsilon_{210 \text{ nm}}$ : TC,  $1.74 \times 10^3$ ; GC,  $1.19 \times 10^3$ ; C,  $8.28 \times 10^2$ ;  $\epsilon_{195 \text{ nm}}$  (estimate): TC,  $1.21 \times 10^4$ ; GC,  $9.08 \times 10^3$ ; C,  $4.14 \times 10^3$ . The molar absorptivities indicate that the sulfate or the carboxyl groups of the peptide conjugated bile salts are more strongly chromophoric than the single carboxyl group of the unconjugated bile salt. The HPLC elution profiles were therefore monitored at 210 nm. This wavelength afforded reasonable extinction coefficients and was greater than the UV cutoff for methanol ( $\approx 200$  nm).

### HPLC

HPLC chromatograms of free and conjugated UDC, C, CDC, and DC are shown in **Fig. 2**. The retention (mobility $^{-1}$ ) of the bile salt species on the reverse-phase HPLC column follows the rank order  $\text{UDC} < \text{C} < \text{CDC} < \text{DC}$  regardless of whether the bile salt is conjugated or not. When conjugated, the ordering of HPLC retention factors is T-conjugates  $<$  G-conjugates  $<$  free bile salts. The specific values for the retention factors ( $k'$ ) are listed in **Table 1** and vary from  $0.55 \pm 0.02$  for TUDC (most mobile) to  $17.09 \pm 0.14$  for DC (least mobile).

### Equilibrium cholesterol monohydrate solubilities

Representative plots of the cholesterol concentration solubilized in 100 mM solutions of DC and its conjugates

**TABLE 1.** HPLC retention factors ( $k'$ ) and equilibrium cholesterol monohydrate solubilities of the common bile salts<sup>a</sup>

Bile Salt	$k'^b$	$\frac{\text{Mol BS}^c}{\text{Mol ChM}}$	$X_{\text{ChM}}^d$	$X_{\text{ChM}}^e$
<i>Means <math>\pm</math> SD (n = 3)</i>				
TUDC <sup>f</sup>	$0.55 \pm 0.02$	$658 \pm 136^g$	$0.0016 \pm 0.0003$	
TC	$1.04 \pm 0.09$	$54 \pm 5$	$0.018 \pm 0.002$	0.016 0.027
TCDC	$1.80 \pm 0.18$	$58 \pm 9^g$	$0.017 \pm 0.003$	0.021 0.023
TDC	$2.21 \pm 0.13$	$24 \pm 1$	$0.040 \pm 0.002$	0.032 0.037
GUDC	$1.71 \pm 0.01$	$495 \pm 156^g$	$0.0022 \pm 0.0007$	
GC	$3.43 \pm 0.18$	$41 \pm 4$	$0.024 \pm 0.002$	0.019 0.036
GCDC	$6.27 \pm 0.51$	$41 \pm 4^g$	$0.024 \pm 0.002$	0.029 0.029
GDC	$7.89 \pm 0.27$	$19 \pm 1$	$0.051 \pm 0.003$	0.050 0.046
UDC	$4.75 \pm 0.17$	$324 \pm 49^g$	$0.0031 \pm 0.0004$	
C	$7.12 \pm 0.07$	$30 \pm 2$	$0.032 \pm 0.002$	
CDC	$15.28 \pm 0.19$	$17 \pm 1^g$	$0.056 \pm 0.003$	
DC	$17.09 \pm 0.14$	$14 \pm 2$	$0.069 \pm 0.010$	

<sup>a</sup> HPLC performed in 75% MeOH/25% 0.005 M  $\text{KH}_2\text{PO}_4/\text{H}_3\text{PO}_4$  at pH 5.0; ChM solubility in 100 mM bile salts at 37°C.

<sup>b</sup> HPLC retention factor (see Methods for derivation).

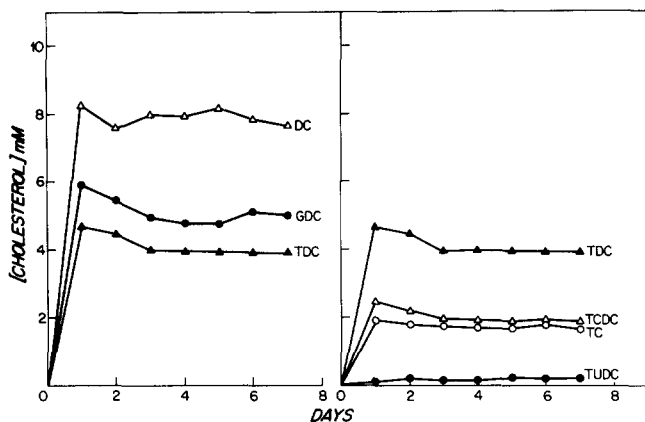
<sup>c</sup> Moles of bile salt necessary to solubilize 1 mole ChM.

<sup>d</sup> ChM mole fraction solubilized ( $[\text{ChM}]/([\text{ChM}] + [\text{BS}])$ ).

<sup>e</sup> "Best" literature values: left column from Ref. (7) and right column from Ref. (8).

<sup>f</sup> Abbreviations as in text.

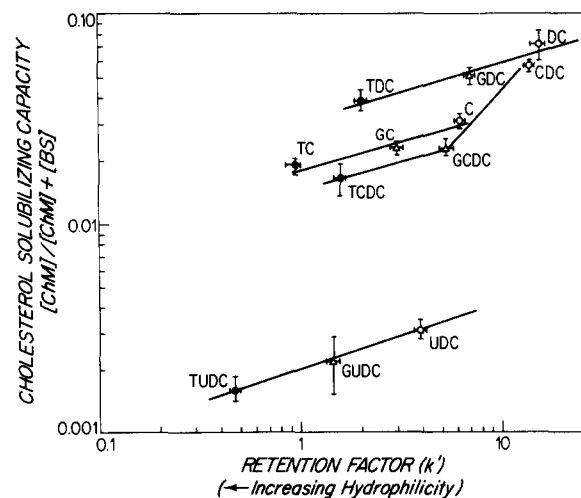
<sup>g</sup> Includes data from Ref. (6).



**Fig. 3.** Plots of cholesterol monohydrate (ChM) solubility as functions of time in 100 mM bile salt solutions at pH 10.0 (glycine conjugates and unconjugated bile salts) and at pH 7.0 (taurine conjugates). In the left panel, the ChM-solubilizing capacities of deoxycholate (DC) and its conjugates are compared. In the right panel, the ChM-solubilizing capacities of taurine conjugated ursodeoxycholate (UDC), cholate (C), chenodeoxycholate (CDC), and deoxycholate (DC) are compared. The values corresponding to the flat portions of the curves were taken as equilibrium ChM solubilities.

and all the common taurine conjugates at different time points are shown in **Fig. 3**. The initial peak in solubility apparently reflects the initial higher solubility of an anhydrous cholesterol contaminant (6). The equilibrium solubility of ChM obtained from the flat portion of the curves is reached by 3–4 days in all cases. The rank ordering for equilibrium ChM solubilities in each study is: T-conjugates < G-conjugates < free bile salts, e.g. **Fig. 3** (left panel) with  $UDC < C < CDC < DC$ , **Fig. 3** (right panel). All solubility values are listed in **Table 1** together with reliable literature values (7, 8) with which the present values are comparable.

In **Figs. 4** and **5**, double logarithmic plots of ChM solubilities in mole fraction units are displayed versus the HPLC retention factors according to **Equation 11**. In **Fig. 4**, the relationship between the two properties is examined to show the influence of variations in the pattern of conjugation. These relationships are linear and are essentially parallel from species to species with the exception of CDC which unaccountably does not fit this linear relationship. In **Fig. 5**, the relationship between ChM solubilizing capacity and HPLC mobility is examined to show the influence of variations in bile salt hydroxyl substitution for each form of conjugation. It is apparent that the ChM-solubilizing capacity increases as the HPLC mobility decreases, i.e. the more ChM a given bile salt solubilizes at equilibrium, the longer it is retained on the HPLC column. Conversely, the less ChM solubilized, the more mobile the bile salt is on the HPLC column. These data fall on three straight lines, whose regression coefficients are tabulated in **Table 2**. Cholate and its conjugates do not fall on the best fit regressions for the dihydroxy bile salts. Hence the “A”

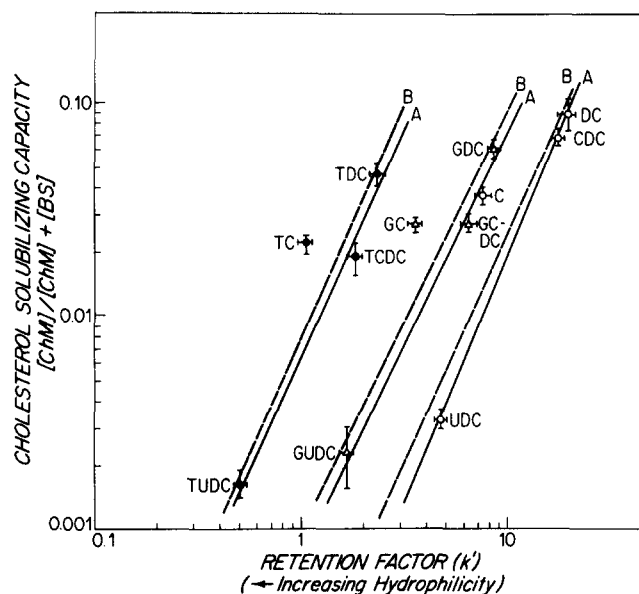


**Fig. 4.** Double logarithmic plots of the cholesterol monohydrate (ChM)-solubilizing capacities (expressed as ChM mole fraction solubilized) versus the HPLC retention factor ( $k'$ ) of the bile salts. The linear relationships show the influence of varying the conjugation of each bile salt species.

regressions are for the dihydroxy bile salts only and the “B” regressions include the trihydroxy species.

#### Critical micellar temperature

The CMT for a 100 mM solution of taurohyodeoxycholate was 25°C; glycohyodeoxycholate, 65°C; hyodeoxycholate, 32°C; and 7 $\alpha$ ,12 $\alpha$ -dihydroxy-5 $\beta$ -cholan-



**Fig. 5.** Double logarithmic plots of the cholesterol monohydrate (ChM)-solubilizing capacities expressed as ChM mole fraction solubilized, versus the HPLC retention factor ( $k'$ ) of the bile salts. The linear relationships show the influence of variations in bile salt hydroxyl substituents for each form of conjugation. The solid lines (A) are regressions derived from the dihydroxy bile salts ursodeoxycholate (UDC), chenodeoxycholate (CDC), and deoxycholate (DC) and their conjugates. The interrupted lines (B) are regressions which include the trihydroxy bile salt cholate (C) and its conjugates.

TABLE 2. Regression coefficients for cholesterol solubilizing capacity  $([ChM]/[ChM] + [BS])$  versus HPLC retention factors ( $k'$ )

	A	B
Taurine conjugates	$y = 160x + 0.006$ $r = 0.993$	$y = 112x + 0.008$ $r = 0.921$
Glycine conjugates	$y = 95x + 0.0008$ $r = 0.996$	$y = 71x + 0.001$ $r = 0.924$
Free bile salts	$y = 279x + 0.00007$ $r = 0.999$	$y = 120x + 0.0002$ $r = 0.896$

A, Regressions utilizing only dihydroxy bile salts ( $n = 3$ ); B, regressions utilizing dihydroxy and trihydroxy bile salts ( $n = 4$ );  $y$ , cholesterol solubilizing capacity;  $x$ , HPLC retention factors ( $k'$ );  $r$ , correlation coefficient.

oate, 64°C. The CMT of 90 mM TLC $SO_4$  has been reported to be  $\approx 37^\circ C$  (20); in this study, 100 mM solutions just achieved optical clarity at 37°C. The CMT values of 100 mM solutions of all the other bile salts studied were less than 23°C and for the common bile salts are known to be less than 0°C (21).

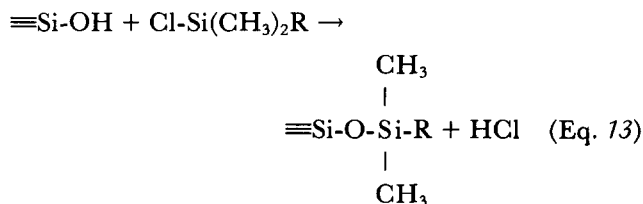
### Comparison of theory with experiment for less common bile salts

The retention factors for several less common bile salts, including those with oxo functions in the 7 and 12 positions, and those hydroxylated at the 6 $\alpha$ -position, are listed in Table 3. The HPLC retention factors were all measured (Table 3) and the regression coefficients in Table 2 were used to predict equilibrium ChM-solubilizing capacities for these bile salts. Both predicted and observed ChM solubilities give satisfactory agreement.

### DISCUSSION

The definition of precise metabolic and physical-chemical roles for the different molecular species of bile

salts has been hampered by the difficulty involved in separating and quantifying the configurationally similar bile salt species. The development of sophisticated HPLC technology has provided novel possibilities for rapid and precise bile salt separation and measurement (17, 22, 23). Reverse-phase HPLC separates molecules on the basis of affinity for a polar mobile phase versus a non-polar stationary phase (10). The stationary phase in this type of chromatography consists of silica particles to which dimethyl-chloroalkylsilanes, generally eighteen carbons long, have been covalently bonded (10, 24):



where



Due to steric hindrance, not all silanol (Si-OH) groups on the particle surface will react with long chain chlorosilanes (24). Therefore, the silica is further reacted with trimethylchlorosilane in an end-capping reaction (24). By reacting the silica with both chlorosilanes the number of accessible free silanol groups is minimized and the column surface is rendered essentially completely hydrophobic in nature. The ability of a column to separate isomeric forms of a molecule (e.g., chenodeoxycholate and deoxycholate) depends upon the selectivity and efficiency of the column packing (10, 23) which in turn are functions of the extent of the coverage of the silica particle with the bonding matrix (see above), the size regularity of the packing material, and the manner in which the column is packed (24).

TABLE 3. HPLC retention factors and ChM equilibrium solubilities of less common bile salts

Bile Salt	$k'^a$	$X_{ChM}^b$		Observed
		Predicted <sup>c</sup>		
		A	B	
Taurolithosulfate (3 $\alpha$ -sulfate)	0.33	.0005	.0008	.0012
Taurohyodeoxycholate (3 $\alpha$ ,6 $\alpha$ -dihydroxy)	0.71	.0027	.0038	.0039
Taurohyocholate (3 $\alpha$ ,6 $\alpha$ ,7 $\alpha$ -trihydroxy)	0.61	.0019	.0028	.0017
Glycohyodeoxycholate (3 $\alpha$ ,6 $\alpha$ -dihydroxy)	1.87	.0026	.0037	.0016
Hyodeoxycholate (3 $\alpha$ ,6 $\alpha$ -dihydroxy)	6.20	.0060	.0098	.0029
Hyocholate (3 $\alpha$ ,6 $\alpha$ ,7 $\alpha$ -trihydroxy)	5.04	.0036	.0064	.0050
3 $\alpha$ ,7 $\alpha$ -dihydroxy-12-oxo-5 $\beta$ -cholanoate	2.94	.0010	.0021	.0005
3 $\alpha$ -hydroxy-7-oxo-5 $\beta$ -cholanoate	6.05	.0056	.0093	nd <sup>d</sup>
7 $\alpha$ ,12 $\alpha$ -dihydroxy-5 $\beta$ -cholanoate	0.13	.0000	.0000	nd <sup>d</sup>

<sup>a</sup> HPLC retention factor (see Methods for derivation).

<sup>b</sup> ChM mole fraction solubilized  $([ChM]/[ChM] + [BS])$ .

<sup>c</sup> Predicted from regressions based on dihydroxy bile salts only (A) or regressions based on dihydroxy plus trihydroxy bile salts (B).

<sup>d</sup> nd: Not determined.

The polar mobile phases used in bile salt HPLC separations have been generally short-chain aliphatic alcohols in acidic (17, 22, 23) or basic (25) buffers. The most successful mobile phases have been methanol–acetate buffer 65:35 at pH 4.7 (17) or isopropanol–phosphate buffer at moderate ionic strength (10 mM) and pH 7.0 (25). We have developed here an HPLC method that is highly selective between isomers as well as between conjugates (Fig. 2). The separation between  $3\alpha$ ,  $7\alpha$  and  $3\alpha$ ,  $12\alpha$  isomers, which has frequently been unobtainable (23) or cumbersome (22) is well resolved in each series (Fig. 2). The decision to use phosphate rather than an acetate buffer (17) was dictated by the use of a UV detector (210 nm). This type of detection system is more sensitive than a refractive index detector (11, 23) such as that used by Bloch and Watkins (17), with the caveat that the mobile phase buffers have a UV absorbance cut-off at  $\approx 200$  nm. This method works extremely well when examining standard-grade bile salts as was done here. These solutions contain no interfering chromophores such as bile pigments which might interfere with the detection of the most highly mobile bile salts (e.g. TUDC) since they elute close to the solvent front in native bile. Both pH and ionic strength have been found before to be critical factors in obtaining good separations (17, 22). In the present study, a change in pH of 0.3 units above or below that employed (pH = 5.0) caused a significant decrease in the selectivity between glycine and taurine conjugates, as well as between the common dihydroxy species, i.e., CDC and DC.

### Equilibrium cholesterol monohydrate solubility

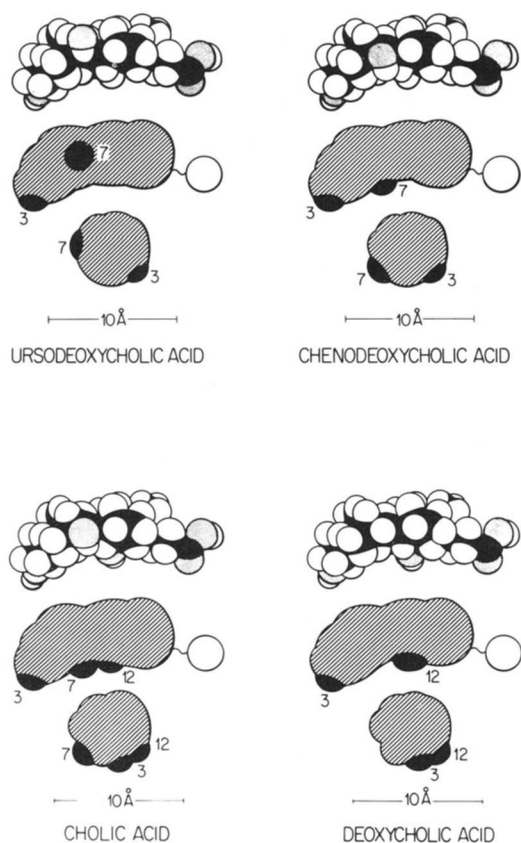
The equilibrium cholesterol solubilities presented here agree in large part with those obtained by other authors (7, 8). The present values tend to be slightly lower, particularly when compared with Neiderhiser and Roth's (8) values (e.g.,  $\chi_{\text{ChM}}$  for TC: 0.018 vs. 0.027). Recent work from this laboratory (6) has shown that the ChM-solubilizing capacities of equimolar concentrations of UDC and CDC epimers as well as their T- and G-conjugates are distinctly different; a result confirmed here. All bile salts were studied at 100 mM concentrations to ensure comparability of the data from study to study. However, two bile salts investigated, glycohyodeoxycholate and  $7\alpha,12\alpha$ -dihydroxy- $5\beta$ -cholanoate gave CMT values (often called Krafft points, the temperature at which a bile salt solution clarifies (20)) that were well above  $37^\circ\text{C}$ . For this reason, ChM solubilities in 100 mM solutions of these bile salts were performed at  $67^\circ\text{C}$ . Since Igimi and Carey (6) have shown that the cholesterol solubility increases in pure bile salt systems only slightly between 20 and  $50^\circ\text{C}$ , our results were employed in this analysis without adjustment.

### Correlation between HPLC mobility and ChM solubility

The equilibrium ChM solubilities and the retention factor  $k'$  demonstrate a significant correlation for the dihydroxy bile salts (UDC, CDC, and DC and their conjugates) (Fig. 5). However, the incorporation of the results for the trihydroxy bile salt, cholate, disimproved the correlation (Table 3). This result suggests that cholate and its conjugates are rendered more hydrophilic than expected due to the addition of a third hydroxyl group. When equilibrium ChM solubility and  $k'$  are examined on the basis of bile salt conjugation (Fig. 4), the relationships are linear with the exception of CDC. This bile salt solubilizes more ChM than expected based on the extrapolation of a linear relationship between TCDC and GCDC. The explanation for this anomaly is unknown, but could conceivably have pathophysiological relevance.

Generally, the retention of a solute on an HPLC column is a function of the hydrophobic-hydrophilic balance of the solute (26). Our findings suggest that this relationship holds for the bile salts examined here, and further, that the cholesterol-solubilizing capacity of a bile salt is a precise function of its hydrophobic-hydrophilic balance as well. The striking correlation between the HPLC retention and cholesterol-solubilizing capacity suggests that if the affinity of the hydrophilic bile salt surface for the C18 column is low then its affinity for cholesterol will also be low. Further, since the areas of hydrophobic ( $\beta$ ) surface of the bile salts which are removed from aqueous contact upon micellization appear to be similar (1), then cholesterol is probably bound in some fashion to the outer "hydrophilic" surface of the micelle (1). Since equilibrium ChM solubility differs from one bile salt species to another (Table 1), this is the most obvious explanation for such dissimilarities. Obviously, more refined physical-chemical methods will be required to further verify this model of ChM solubility by bile salt micelles. Shaw and Elliott (25) have recently published work on a number of bile salts relating CMC and HPLC retention factors. They suggest that their data provide an index of bile salt hydrophobicity that is predictive of the CMC. However, the CMC values reported by these authors were determined by methylcholanthrene solubilization and are not in agreement with CMC values obtained by non-invasive methods (1). We suggest, on the basis of the present data, that such apparent CMC values from bile salt solubilization of a hydrophobic molecule are more related to the mole fraction of methylcholanthrene solubilized than to the true CMC, and hence are consistent with the deductions in the present experiments relating the binding of highly hydrophobic molecules to the surface rather than the interior of the micelles.





**Fig. 6.** Stuart-Briegleb molecular models of the common unconjugated bile acids with schematic longitudinal and cross-sectional representations of the models. The stippled atoms and closed circles and ovals show the positions and orientations of the hydroxyl functions (from Small (37) and Carey et al. (1) with permission of the copyright owners and the authors).

Many of the separations performed on HPLC to date have been empirically derived. Melander, Chen, and Horváth (26) were the first to propose that the relationship between solute structure and the energetics of retention should provide the basis for developing an indexing system based on HPLC retention data in order to understand the hydrophobic nature of organic compounds. The findings presented here confirm this prediction and demonstrate that HPLC retentions can be applied in order to comprehend the hydrophobic-hydrophilic balance of a particular class of biological compounds, which in turn is related to an important physiological physical-chemical property of bile salts, that of solubilizing cholesterol.

#### Influence of molecular structure: studies with uncommon bile salts

The HPLC mobilities of the uncommon bile salts were generally good predictors of their equilibrium cholesterol solubilizing capacities (Table 3). The regressions that were derived for the dihydroxy bile salts ("A" regres-

sions, Table 3 and Fig. 5) provided somewhat better predictions than did those that included cholate and its conjugates ("B" regressions, Table 3). The less common bile salts were very hydrophilic in nature, suggesting that the molecular modifications ( $6\alpha$ -OH,  $3\alpha$ -SO<sub>4</sub>, 7-oxo and 12-oxo functions) changed the hydrophobic-hydrophilic balance markedly toward the more hydrophilic side. The double logarithmic transformation of the data renders the predictions sensitive to small changes in the HPLC retention factor  $k'$ . These differences are larger than the experimental error of both HPLC and ChM solubility measurements.

The hydrophilicity of the common bile salts is strongly influenced by subtle variations in molecular structure. Thus UDC and its G- and T-conjugates are not only more hydrophilic than their  $7\alpha$  epimers, CDC, GCDC, and TCDC, but are even *more* hydrophilic than cholate and its conjugates. The difference in the hydrophobic-hydrophilic balance of UDC and CDC, which differ only in the orientation of the 7-hydroxyl group, warrants particular emphasis. The  $7\beta$ -OH in UDC, when compared to the  $7\alpha$ -OH in CDC, results in a significant increase in the hydrophilicity as measured by the retention factor  $k'$ . This high degree of hydrophilicity is also reflected in a marked decrease in micellar ChM solubility. An examination of Stuart-Briegleb space-filling models (Fig. 6) shows that the  $7\beta$ -hydroxy group of UDC is equatorial. Recent mass spectroscopic findings indicate that the equatorial  $7\beta$ -hydroxyl group is more resistant to the loss of associated water than is a  $7\alpha$ -hydroxyl group,<sup>2</sup> suggesting that the  $7\beta$ -hydroxyl group is intrinsically more hydrophilic. We have emphasized earlier (1) that, since the CMC values and micellar sizes at the CMC are not different between CDC and UDC or between their conjugates, the hydrocarbon area that loses contact with water when micelles form must be the same.

While other explanations may be possible, it appears that the only reasonable explanation for the profound differences in micellar ChM solubility from these data is that ChM binding possibly occurs on the "hydrophilic" surface of the micelles (1). In our earlier work (1) we have suggested a molecular model for this binding that is consistent with the properties of the hydrophilic surface of primary bile salt micelles in general and with thermodynamic predictions. The addition of a  $6\alpha$ -OH to form the trihydroxy ( $3\alpha,6\alpha,7\alpha$ ) hyocholate or the dihydroxy hyodeoxycholate ( $3\alpha,6\alpha$ ) makes the compounds more hydrophilic than cholate and slightly less so than UDC (Tables 1 and 3). It is obvious, by the same token, that hyodeoxycholate is much more hydrophilic than either CDC or DC ( $k' = 6.20$  vs. 15.28 and 17.09, re-

<sup>2</sup> Macdonald, I., Dalhousie University, Halifax, Nova Scotia, Canada. Personal communication.

spectively). Similar relationships apply to the taurine conjugates. This suggests that the  $6\alpha$ -OH group is intrinsically more hydrophilic than other OH substituents with the exception of a  $7\beta$ -OH function (UDC). Like UDC, this may also be due to decreased steric hindrance for interaction with water. Both model building and x-ray diffraction studies (27) clearly demonstrate that a  $6\alpha$ -hydroxyl group is also *equatorial* to the plane of the steroid ring system.

### Pathophysiological implications

The relationship described here between HPLC retention and cholesterol solubility provides novel insights into the hydrophobic-hydrophilic balance of bile salt molecules and the relationship of this property to pathophysiological function. A knowledge of the phase-partitioning properties of both synthetic and uncommon naturally occurring bile salts, such as that obtained with HPLC retention data, allows the accurate prediction of the physical-chemical function of these molecules and perhaps ultimately of their physiological function. For example, the cholestatic potential of bile salts decreases in the order lithocholate > DC > CDC, while C reverses cholestasis (28) and even near its  $T_m$  is still choleric (28–30). Tauro- and glycolithocholate are also cholestatic, but the  $3\alpha$ -sulfation of these compounds reduces their cholestatic potential (28). Thus, the more hydrophobic the bile salt, the more likely is the bile salt to induce cholestasis. In prolonged cholestasis, bile salts are modified in several ways that render them more polar (31–35). Specifically, in intrahepatic cholestasis increased synthesis of tri- and tetra-hydroxylated bile salts (with OH functions appearing at C1 and C6) occurs (31–33), sulfation of all bile salts is frequent (33, 34), and even appreciable glucuronidation (35) of the common bile salts occurs. High levels of these unusual derivatives may be found in bile, blood, and urine. All of these molecular modifications serve to markedly increase the hydrophilicity of bile salts. One consequence of such modifications would be to decrease the membranolytic potential of the more hydrophobic common bile salts, in particular decreasing their ability to solubilize and transport membrane cholesterol. In agreement with this, Hardison, Weiner, and Hatoff (36) found in the rat that TUDC infusions were less hepatotoxic than TCDC infusions and that the  $T_m$  of TUDC was greater than that of TCDC, possibly reflecting a reduced membranolytic potential for the more hydrophilic TUDC. However, other systematic studies along these lines are required to relate the hydrophobic-hydrophilic balance of bile salts to their physiological membranolytic functions, particularly the solubilization of hepatic lecithins for secretion into bile. ■

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