pH-Solubility relations of chenodeoxycholic and ursodeoxycholic acids: physical–chemical basis for dissimilar solution and membrane phenomena

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Abstract We examined by titration the electrochemical properties, apparent pK (pK′a), precipitation pH, and undissociated bile acid solubilities of chenodeoxycholic acid and its 7β epimer, ursodeoxycholic acid and their glycine conjugates as functions of a number of physical–chemical variables. Despite comparable pK′a values, ursodeoxycholic acid and its glycine conjugate precipitated from H_2O (37°C) at pH values of 8.0–8.1 and 6.5–7.4 whereas chenodeoxycholic acid and its glycine conjugate precipitated at pH values of 7.0–7.1 and 4.8–5.0, respectively. These differences were related to the low solubility of undissociated ursodeoxycholic acid in water (53 μM) and in ursodeoxycholic micelles (saturation ratio of anion:acid, 90–400:1) compared with the higher solubility of chenodeoxycholic acid in water and in chenodeoxycholate micelles (250 μM and 5–25:1, respectively). In model bile systems including those composed of conjugated ursodeoxycholate–chenodeoxycholate mixtures, ursodeoxycholic acid was less soluble than chenodeoxycholic acid and induced the mixtures to gel between pH 7.0 and 4.5–6.5. These results suggest that in vivo *1*) the solubility and absorption of oral ursodeoxycholic acid from the duodenum–jejunum may be limited, *2*) ursodeoxycholic acid will precipitate in the colon at pH values <8.0 but chenodeoxycholic acid is soluble at pH values >6.9 and hence is capable of eliciting a secretory diarrhea, *3*) the precipitation pH of glycoursodeoxycholic acid, the predominant bile acid in bile during therapy with ursodeoxycholic acid, falls within the physiological range, thus it is possible that this bile acid may short-circuit the entero-hepatic circulation and even precipitate from bile or gut luminal contents as crystals.—**Igimi, H., and M. C. Carey.** pH Solubility relations of chenodeoxycholic and ursodeoxycholic acids: physical–chemical basis for dissimilar solution and membrane phenomena. *J. Lipid Res.* 1980. **21:** 72–90.

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Since the original report (1) that chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid, CDCA) desaturated bile with cholesterol and induced cholesterol gallstone dissolution (2), it has become generally recognized that certain bile acids, when given orally, can be delivered to the enterohepatic circulation, concentrate therein and influence the secretory rates of other biliary lipids (3). In 1975 Makino et al. (4) reported that the administration of the 7β epimer of CDCA, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid, UDCA), a major constituent of the Chinese drug Yûtan (dried bear's bile), also desaturated bile with cholesterol and dissolved cholesterol gallstones (4, 5), a result confirmed in a number of studies (6–14). This bile acid, in contrast to CDCA, rarely induces diarrhea, serum and biliary lithocholate levels remain normal and hypertransaminasemia is not observed (5–18). Further, UDCA is not hepatotoxic in laboratory animals (19–22) except in the rabbit (23). Hence, UDCA promises to be the bile acid of choice for medical gallstone dissolution.

In spite of the burgeoning wealth of data on the biochemical, metabolic and clinical aspects of UDCA, information on the physical–chemical properties of this bile acid is limited. We demonstrated in independent studies (18, 24, 25) that the cholesterol-solubilizing capacity of micellar solutions of unconjugated UDCA and conjugated UDCA-lecithin mixtures is far inferior to that of CDCA conjugates and of the other common bile salts (25, 26). Moreover, we documented this observation in systematic phase equilibria studies (25, 27), and demonstrated that in the presence of physiological amounts of lecithin, the glycine conjugate of UDCA (GUDCA) solubilized significantly less cholesterol than equimolar concen-

Abbreviations: CDCA, chenodeoxycholic acid (prefixes T and G indicate taurine and glycine conjugates respectively); UDCA, ursodeoxycholic acid (prefixes T and G indicate taurine and glycine conjugates respectively); A^-, fully ionized bile salt anion; HA, undissociated bile acid; CMC, critical micellar concentration; TLC, thin-layer chromatography.

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trations of the taurine conjugate (TUDCA) (27, 28). This result led us to advocate (28) dietary taurine supplementation for patients on urso-therapy as a possible stratagem for improving biliary cholesterol solubility, because glycine conjugation increases with oral bile acid therapy (29) and dietary taurine can reverse this trend (30). In light of these considerations, we hypothesized that, since micellar solutions of pure sodium UDCA and its conjugates solubilize minimal amounts of cholesterol, the micellar solubility of the sparingly soluble protonated species of UDCA might be similarly reduced. If, then, the pKa's of UDCA and its glycine conjugate fell within the ranges reported for the common dihydroxy bile acids (31), it was possible that the pH values at which UDCA and GUDCA precipitated from aqueous solution might exceed physiological values.

This report, therefore, describes a systematic potentiometric titration study of UDCA and GUDCA under a variety of physical chemical conditions including those of physiological relevance. We also compare our results with systematic studies on CDCA and its glycine conjugate (GCDCA) and have measured the equilibrium aqueous solubility of the protonated species of each bile acid by an independent method. Our findings not only confirm our hypothesis, but demonstrate striking differences in the general electrochemical properties and solubilities of the two bile acids. Our results provide a physicochemical basis for the infrequency of diarrhea with UDCA, suggest that jejunal adsorption of UDCA may be limited and provide additional evidence why patients on urso-therapy should supplement their diets with free taurine since, under certain conditions, GUDCA might precipitate from bile and from gut luminal contents.

EXPERIMENTAL PROCEDURE

Materials

Non-radioactive bile acids. Several preparations of UDCA and its conjugates were supplied by the Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan). Samples of CDCA were generous gifts from Dr. Falk, GmbH and Co. (Freiburg, Germany) and the conjugates of CDCA were obtained as sodium salts from Calbiochem (San Diego, CA). All preparations were initially checked by thin-layer chromatography using a 250 μg application, and only those preparations which gave single spots were recrystallized for electrochemical study. Recrystallization was carried out thrice from 95% ethanol (v/v) (UDCA, CDCA), 10% ethanol (v/v) (GUDCA, TUDCA) or anhydrous diethyl ether–95% ethanol mixtures (v/v) (GCDCA, TCDCA). The preparations were then rechecked by thin-layer chromatography, gas–liquid chromatography (UDCA, GUDCA and TUDCA) (32) and high performance liquid chromatography (TCDCA, TUDCA) (courtesy of Dr. John B. Watkins, Children's Hospital Medical Center, Boston, MA) and were found to be >99% pure. The experiments described also provided an internal check of bile salt purity as deduced from the shape and percent equivalence of the potentiometric titration curves.

Radioactive bile acids. [11,12-^{3}H]UDCA (sp act 2.37 mCi per μmol) was synthesized by Dainabot Radioisotope Labs Ltd. (Tokyo, Japan) and graciously supplied to us by Dr. Isao Makino (Hokkaido University School of Medicine, Sapporo, Japan). [11,12-^{3}H]-CDCA as the sodium salt (sp act 43 mCi per μmole) was prepared by the New England Nuclear Co. (Boston, MA) and was generously supplied from Inventory Stock. Both materials were better than 99% chemically and radiochemically pure by TLC and zonal scanning. All other radioactive bile acids, cholic (3α,7α,12α-trihydroxy,5β-cholanoic), deoxycholic (3α,12α-dihydroxy,5β-cholanoic) and lithocholic (3α-monohydroxy,5β-cholanoic) acids were labeled with ^{14}C in the 24 (carbonyl) position and were supplied as sodium salts (California Bionuclear Corporation, Sun Valley, CA; The Radiochemical Center, Amersham, U.K.; and New England Nuclear Co., Boston, MA). Specific activities ranged from 10 mCi/mmol to 58 mCi/mmol and all the compounds were at least 98% chemically and radiochemically pure in appropriate TLC systems with zonal scanning.

Miscellaneous chemicals. Monoolein (rac-glyceryl-1(2)-oleate) was obtained from Nu-Chek-Prep (Austin, MN) and was 99% pure by TLC. Egg yolk phosphatidylcholine (1,2-diacyl-*sn*-glycero-3-phosphocholine, lecithin) was obtained from Lipid Products, Redhill, Surrey, U.K. and was >99% pure as described (26). HCl and NaOH (Natl. Bur. Std.) were A.C.S. reagent grade (Fisher Scientific, Boston, MA). NaCl (A.C.S.) was roasted in a muffle furnace at 600°C for 6 hr to oxidize and remove organic impurities. Water was filtered, passed through an ion exchanger and distilled from an all-Pyrex automatic distillation apparatus (Corning Glass Works, Corning, NY). To eliminate the possibility of bile salt hydrolysis by atmospheric CO_2, distilled water was boiled for 30 min and then cooled at 4°C in air-tight flasks prior to each experiment. Glassware was washed in sulfochromic acid (24 hr) and thoroughly rinsed in running distilled water to ensure a final pH of 6.8–7.2. The glassware was then wrapped in aluminum foil and dried in an oven.

Methods

Bile salt solutions. Solutions were prepared on a w/v basis in volumetric flasks with anhydrous bile acids (or salts) to which was added distilled water containing, in certain experiments, NaCl to achieve the desired ionic strength. In the case of unconjugated bile acids and glycine conjugates, dissolution was aided with a few drops of 2M NaOH. Mixed micellar solutions of individual bile salts or bile salt mixtures together with monoolein or lecithin were prepared by drying a homogeneous lipid mixture in 95% ethanol (v/v) to constant weight followed by constitution with aqueous solvent to achieve the desired concentration (w/v) (26).

Potentiometric titrations. Proton titrations with 0.5, 1.0 or 2.0 M HCl were carried out to equilibrium as described earlier (31, 33). The H^+ ion activity (pH) was measured with a No. 65 research pH meter (Radiometer, Copenhagen, Denmark) and a H^+-sensitive glass electrode. In brief, 5 ml of a bile salt solution was placed in a glass cup of a manual titration assembly (TTTI, Radiometer, Copenhagen, Denmark) through which water-saturated N_2 was circulated continuously. The pH of the solution was first adjusted to about 11–12 with 2M NaOH and the volume of added base was recorded. After each addition of HCl, the pH values were monitored as a function of time until constant (equilibrium) values were obtained. Depending upon the bile salt species, bile salt concentration and degree of supersaturation, the time required for equilibration varied from a few minutes to several hours. For this reason, the time required for the consumption of an entire equivalent of HCl, i.e. to bring the pH of each system to ≈pH 2.0 averaged 6–14 hr. The temperature of the samples was kept constant (10–60°C) by circulating water (Haake Model FE water bath, Haake, Inc., Saddlebrook, NJ) through a thermostatted jacket which enclosed the glass titration cup. To achieve a constant temperature below room temperature, the heat exchanger was coupled to a Neslab bath cooler (Neslab Instruments, Inc., Portsmouth, NH). Continuous rapid stirring was achieved with a corrugated Teflon-coated magnetic stirrer which also provided a qualitative estimate of the viscosity of the solutions. The pH of precipitation and the pH at the first appearance of a Tyndall phenomenon (bluish opalescence) was recorded by visual observation with the aid of the narrow beam from a high intensity light source (31, 33). Bile acid solubilities and pK′a values were calculated following the method described by Back and Steenberg (34) with minor modifications (31).

Equilibrium bile acid solubility measurements. Solutions of non-radioactive UDCA or CDCA (Na salts) in various concentrations of NaCl (pH 10–11) were vortex-mixed with about 1.5 μCi of the respective ^{3}H-labeled bile acid to achieve a final bile salt concentration of 5 mM in 5 ml of solution. Three 10-μl samples were immediately taken for determination of radioactivity using a liquid scintillation spectrometer. Each solution was then titrated to an equilibrium pH of 2.4 with approximately 30 μl of 2M HCl. The two-phase system was then continuously shaken and kept overnight at either 20°C or 37°C to achieve equilibration. Solubility of the protonated bile acid was then determined by measuring radioactivity in portions (200 μl) of the clear supernatant phase after centrifugal separation of the crystalline precipitate. No chemical degradation of the bile acids or tritium exchange was observed. Each study was carried out in triplicate over a range of NaCl concentrations (0–1 M). For comparison, the solubilities of ^{14}C-labeled cholic, deoxycholic, and lithocholic acids at 20° and 37°C were studied in H_2O (no added NaCl) at pH 2.4 by the same method.

Capillary melting point measurements. Melting points of the anhydrous and solvent-free bile acids were determined in sealed glass capillaries of 1–1.2 mm I.D. affixed to the mercury bulb of an NBS certified thermometer (temperature range 0–300°C) in a thermostatted mineral oil bath. Corrections for the emergent thermometer stem were omitted since recrystallized anhydrous cholesterol (26) gave a sharp melting point of 148.5°C (35).

RESULTS

Single bile salt systems

Representative equilibrium titration curves (H_2O, 37°C) for UDCA and CDCA and their glycine conjugates are displayed in **Fig. 1** (A–D). A hypothetical titration curve obtained by the titration of an alkaline unconjugated or glycine-conjugated bile salt solution with HCl (31) is shown in the inset of Fig. 1 (D) and corresponding parts of the actual titration curves (A–D) are referred to according to the symbols employed (31). The initial part of each curve (Fig. 1 A–D) at high pH corresponding to the left of W (Fig. 1D inset) represents the titration of added excess NaOH with HCl (31, 33). At the first inflection point (W), the convex slope of each curve becomes concave, indicating the first equivalence point where the following chemical reaction between bile salt anions and the protons of hydrochloric acid begins:

$$Na^+A^- + HCl \rightarrow HA + Na^+Cl^- \qquad 1).$$

In this equation, A^- is the fully ionized bile salt anion

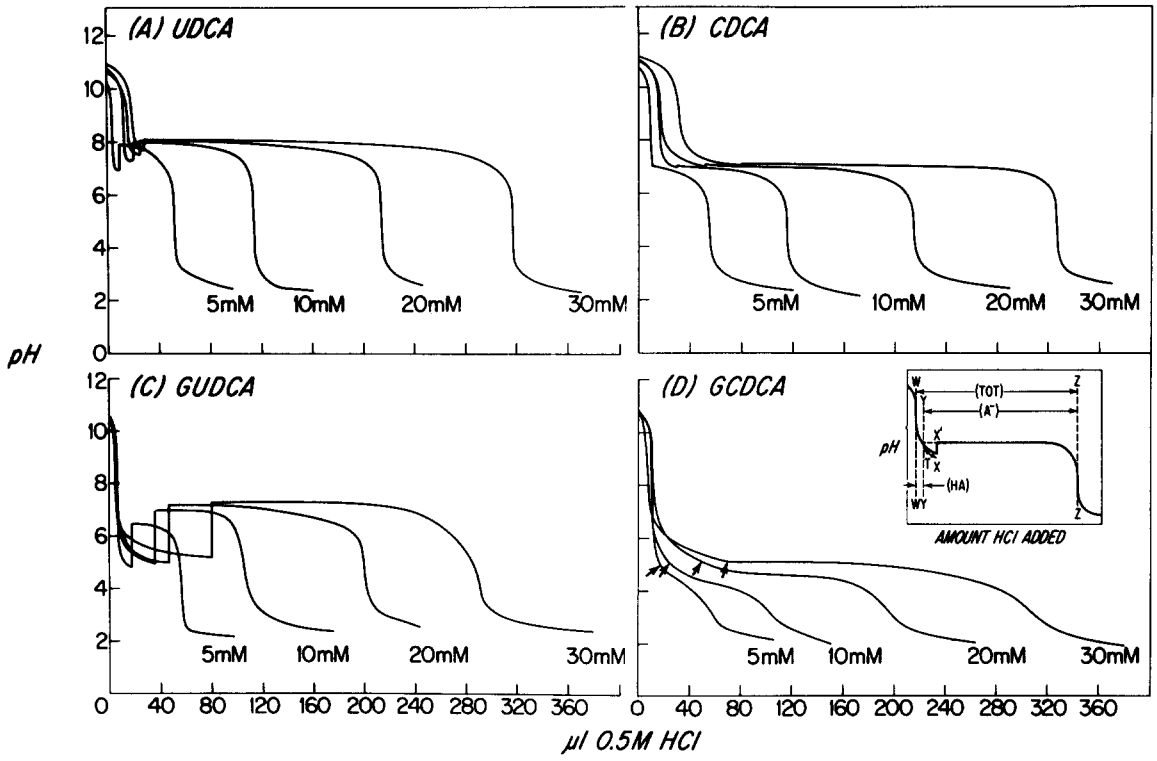

Fig. 1. Potentiometric titration curves of A) ursodeoxycholic acid (UDCA), B) chenodeoxycholic acid (CDCA), C) glycoursodeoxycholic acid (GUDCA), and D) glycochenodeoxycholic acid (GCDCA) in H_2O at 37°C. Each curve is labeled with the corresponding bile acid concentration. Inset in (D) represents a hypothetical titration curve for the titration of unconjugated or glycine conjugated bile salts with HCl (31). Explanation of the standard notation employed is described in the text.

and HA is the protonated bile acid. Even though the solubility of bile acid (HA) in an aqueous solution is small (see the results of direct determination below), the bile salt anion (A^-), being a detergent, can solubilize a certain amount of the sparingly soluble HA in "mixed micelles" of A^- + HA. Hence, if the initial concentration of A^- is above its critical micellar concentration (CMC) value, the solubility of HA represents the sum of its true aqueous solubility (i.e., intermicellar solubility) plus its solubility in micelles. If the initial concentration of A^- is below its CMC value, an estimate of the true aqueous solubility of HA is obtained. Since the solubility of HA can be derived from the titration curves for different A^- concentrations, the apparent pK (pK′a) of the bile acid can be calculated (34).

In the hypothetical bile acid titration curve (Fig. 1D inset), once the concave curve flattens out, a point X is reached where precipitation suddenly occurs and the pH rises sharply to an equilibrium value (X′) without further addition of HCl. This precipitation phenomenon is often heralded by the appearance of a Tyndall phenomenon (bluish opalescence) at some point in the vicinity of T and X. The sharp rise in pH (X → X′ transition) is clearly seen with titrations of UDCA and GUDCA (Fig. 1A, C), was slight with CDCA (Fig. 1B) and was not observed in the titrations of GCDCA (Fig. 1D). The arrows on the titration curves of GCDCA indicate the pH values where a Tyndall phenomenon was first noted and which heralded the onset of HA precipitation.

Once nucleation and precipitation occur, equivalent amounts of HA are precipitated with each successive addition of HCl. As all added HCl protons now go to form an insoluble crystalline HA phase, the H-ion activity detected by the glass electrode does not change appreciably, and a plateau region is observed in each curve (Fig. 1 A–D). Toward the end of the bile salt titration, this plateau portion changes to a convex slope and, finally, the curve shows a second inflection point Z (Fig. 1D inset) which is the final equivalence point. At this pH, the reaction given in Formula (*1*) is complete and the system is composed of two phases, a crystalline HA phase in equilibrium with a small aqueous concentration of HA molecules in solution as monomers (31). Because between points Y and X (Fig. 1D inset) the solution is supersaturated and, therefore, not in thermodynamic equilibrium, extrapolation of the plateau section of the curve to the initial concave slope, i.e., from point X′ to point Y, is

TABLE 1. Electrochemical properties of UDCA

[UDCA] mM	Equivalence %[a]	[HA] μM[b]	A^-/HA Ratio[c]	pK'a[d]	Precipitation pH[e]	Gross Viscosity[f]
			20°C, H_2O			
2.5	99.8	(51)	(49)	(5.60)	7.26	Liquid
5.0	99.6	(51)	(100)	(5.48)	7.44	Liquid
7.5	99.4	(51)	(150)	(5.53)	7.65	Liquid
10.0	99.2	24 (51)	404 (200)	5.24 (5.55)	7.79	Liquid
12.5	98.3	30 (51)	417 (250)	5.31 (5.53)	7.86	Liquid
15.0	96.1	40 (51)	367 (293)	5.41 (5.51)	7.90	Liquid
17.5	99.2	55	321	5.50	7.94	Liquid
20.0	100.0	70	291	5.59	7.97	Liquid
22.5	98.3	92	250	5.71	8.03	Liquid
25.0	100.2	120	212	5.83	8.07	Liquid
30.0	99.2	161	185	5.92	8.09	Liquid
40.0	101.1	277	142	6.04	8.10	Liquid
50.0	99.4	443	117	6.14	8.10	Liquid
75.0	98.4	754	99	6.24	8.11	Liquid
100.0	100.2	1098	90	6.26	8.11	Liquid
			37°C, H_2O			
5.0	103.3	15 (53)	324 (96)	5.51 (6.12)	7.98	Liquid
10.0	99.2	35 (53)	289 (167)	5.61 (5.85)	8.01	Liquid
15.0	98.6	60	247	5.72	8.04	Liquid
20.0	97.2	98	208	5.83	8.07	Liquid
30.0	100.0	191	156	6.00	8.10	Liquid
40.0	103.6	289	137	6.07	8.11	Liquid
100.0	100.0	1020	97	6.25	8.11	Gel
			50°C, H_2O			
20.0	98.6	100	201	5.89	8.11	Liquid
			60°C, H_2O			
20.0	95.0	130	160	5.95	8.08	Liquid
			37°C, 0.15 M NaCl			
5.0	100.3	25 (73)	205 (69)	5.29 (5.76)	7.56	Liquid
10.0	97.9	85	118	5.71	7.72	Liquid
15.0	96.8	130	114	5.86	7.84	Liquid
20.0	98.1	210	95	5.97	7.86	Liquid
40.0	99.9	380	105	5.98	7.90	Gel
100.0	99.0	840	118	5.99	7.91	Gel

Electrochemical properties of GUDCA

[GUDCA] mM	Equivalence %	[HA] μM	A^-/HA Ratio	pK'a	Precipitation pH	Gross Viscosity
			37°C, H_2O			
5.0	99.0	15	329	4.00	6.48	Liquid
7.5	97.8	31	244	4.36	6.71	Liquid
10.0	95.5	42	238	4.65	6.98	Liquid
20.0	98.0	87	230	4.89	7.19	Liquid
30.0	95.3	126	237	5.00	7.30	Liquid
40.0	96.3	177	225	5.09	7.36	Liquid
50.0	98.8	223	224	5.12	7.38	Liquid
100.0	98.6	426	234	5.16	7.41	Liquid

[a] Total moles of HCl used in titration/total moles of bile salt added × 100.

[b] Equilibrium solubility of undissociated bile acid as a single phase system. Values in parentheses were obtained by direct determination at pH 2.4.

[c] Ratio of number of moles of ionized bile salt to undissociated bile acid (rounded off) as a single phase system at equilibrium. Values in parentheses are corrected for HA solubility derived by the direct method.

[d] Apparent equilibrium pK. Values in parentheses are corrected for the direct determination of HA solubility described in [b].

[e] Extrapolated equilibrium precipitation pH of HA in the theoretical absence of supersaturation.

[f] Recorded qualitatively in the vicinity of the actual precipitation pH or at the Tyndall point.

necessary to obtain the pH at which precipitation of HA crystals would have occurred in the theoretical absence of supersaturation. Point Y, therefore, represents the maximum equilibrium solubility of HA in the A^- solution. As indicated earlier, the physical state of HA at this pH is predicated by whether the total bile salt concentration is above or below its CMC which, under these conditions, corresponds to the apparent CMC of the A^- solution *saturated* with HA. When the titration curves are compared (Fig. 1 A–D), it is obvious that UDCA and GUDCA show marked supersaturation with HA at all bile salt concentrations, whereas CDCA shows little supersaturation and GCDCA none. Further, for equimolar bile salt concentrations the plateau regions of the UDCA and GUDCA curves (≡precipitation pH values) are appreciably higher than the corresponding parts of the curves of CDCA and GCDCA.

Quantitative measurements from the experimental curves give a number of important characteristics for each bile salt with respect to bile salt concentration, temperature and variations in ionic strength (**Tables 1–3**).

The total moles of HCl (TOT) required to titrate a given amount of bile salt (point W to point Z, Fig. 1 A–D). This quantity is called the equivalence and is expressed in the tables as a percentage, i.e.:

$$\frac{\text{Total moles of HCl from W to Z}}{\text{Total moles of bile salt added}} \times 100 \quad 2).$$

The total moles of bile salt added is calculated from the gravimetric weight and anhydrous molecular weight of the bile salt. Provided the bile salt is pure and the titrant molarity is known accurately, equilibrium titrations should give an ideal equivalence value of 100%. As shown in Tables 1–3, the equivalences obtained are very close to the theoretical value, ranging from 95.0 to 103.6% in the titrations of UDCA and GUDCA (Table 1) and 90.0 to 104.2% in the case of CDCA and GCDCA (**Table 2**). This provides a satis-

TABLE 2. Electrochemical properties of CDCA[a]

[CDCA] mM	Equivalence %	[HA] μM	A^-/HA Ratio	pK'a	Equilibrium Precipitation pH	Gross Viscosity
			10°C, H_2O			
20.0	96.6	2901	6	6.40	7.10	Liquid
			20°C, H_2O			
20.0	98.5	2843	6	6.38	7.09	Liquid
			37°C, H_2O			
5.0	90.0	200 (256)	25 (20)	5.67 (5.78)	7.02	Liquid
10.0	98.4	823	11	6.05	7.05	Liquid
20.0	97.4	2886	6	6.38	7.09	Liquid
30.0		4520	6	6.44	7.09	Gel
40.0		6220	5	6.47	7.10	Gel
			50°C, H_2O			
20.0						Gel

Electrochemical properties of GCDCA[a]

[GCDCA] mM	Equivalence %	[HA] μM	A^-/HA Ratio	pK'a	Equilibrium Precipitation pH	Gross Viscosity
			37°C, H_2O			
2.5	104.0	200	13	3.77	4.82	Liquid
5.0	100.0	690	7	4.21	4.96	Liquid
10.0	95.0	1430	7	4.35	5.05	Liquid
15.0	94.7	2760	5	4.53	5.09	Liquid
20.0	92.5	3900	5	4.61	5.11	Liquid
30.0	100.0	6400	5	4.61	5.12	Liquid
40.0		7300	5	4.54	5.10	Gel

[a] Footnotes for column headings as in Table 1.

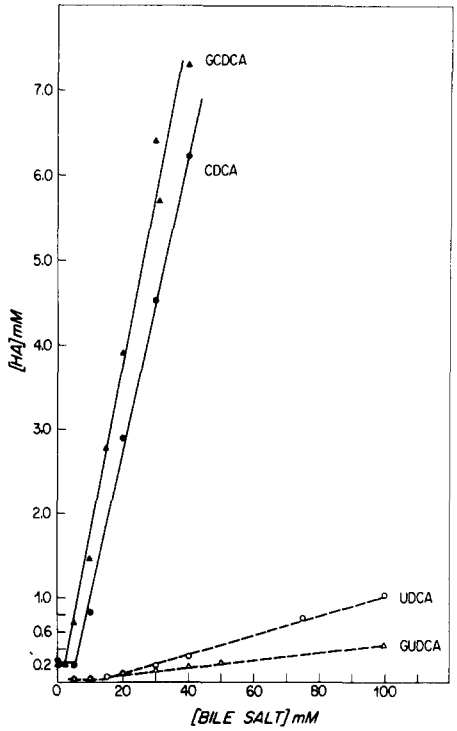

Fig. 2. Equilibrium bile acid (HA) solubilities (mM) (H_2O, 37°C) derived from the potentiometric titration curves plotted as a function of the total bile salt concentration (mM).

factory internal check of the purity of the bile salts and the accuracy of the measurements.

The equilibrium solubility of the protonated bile acid (HA) in the bile salt solution. All titrations were carried out with bile salt concentration in excess of the non-invasive CMC values (24). The bile acid concentration equivalent to the number of moles of HCl consumed between point W and point Y in each curve is equal to the solubility of HA (Fig. 1D). These values (μM) are quite low and in the case of UDCA and GUDCA (Table 1) are several fold smaller than CDCA and GCDCA (Table 2). In both series appreciable increases occur with increases in the total bile salt concentration. When the [HA] values are plotted (**Fig. 2**) against the corresponding total bile salt concentrations (H_2O, 37°C), the values are slight but constant at low bile salt concentrations and then increase sharply in the case of CDCA/GCDCA and gradually in the case of UDCA/GUDCA. It is apparent that the HA solubilities (aqueous + micellar) for equimolar bile salt concentrations above the breakpoints are markedly different. At high bile salt concentration the HA solubilities decrease in the order GCDCA > CDCA >>> UDCA > GUDCA. Extrapolation of the slopes of the curves to the short horizontal sections give concentrations on the bile salt axis which correspond to the apparent CMC values which are 2.5 mM (GCDCA), 5.5 mM (CDCA), 10 mM (GUDCA) and 14 mM (UDCA). Below these concentrations, the HA solubilities approximate the true solubilities in water (see below).

The saturation ratio of micelles with undissociated bile acid (HA). Ignoring the poor aqueous solubility of HA in water in the absence of micellar solubilization, we calculated the saturation ratio of the micelles with HA, employing the total HA value derived from the titration curves. The approximate ratio of the number of molecules of ionized bile salt (A^-) necessary to solubilize one molecule of HA at equilibrium in a mixed micellar solution (A^-/HA) may be obtained from

$$\frac{\text{Moles of HCl from Y to Z}}{\text{Moles of HCl from W to Y}} = \frac{A^-}{HA} \qquad 3).$$

This ratio is very large (90–404:1) in the case of UDCA (H_2O, 20°C) and decreases somewhat with increases in total bile salt concentration (Table 1). In contrast, in a 5.0 mM CDCA solution, 25 molecules of A^- are required to solubilize 1 HA, and in a 40 mM solution only 5 molecules of A^- are necessary, in agreement with previous deductions (31). It is apparent that GCDCA is an extremely good solubilizer and GUDCA an extremely poor solubilizer of their respective HA forms, and both show no significant concentration dependence once the apparent CMC is exceeded (Tables 1, 2). The slopes of the linear regressions in Fig. 2 give the *mean* A^- + HA/HA saturation ratios in H_2O at 37°C, which are 235:1 (GUDCA), 90:1 (UDCA), 6:1 (CDCA) and 5:1 (GCDCA). However, since marked supersaturation can occur in the UDCA and GUDCA systems (Fig. 1A, C), the metastable A^-/HA supersaturation ratios are significantly less. Thus, at pH 7.0 the metastable A^-/HA ratio for UDCA is 6:1 and the corresponding ratio for GUDCA at pH 5.0 is 5:1.

Actual and equilibrium pH values at which the bile acid (HA) precipitates (pH at X and X', Fig. 1D inset). The actual precipitation pH values give an idea of how much supersaturation can be tolerated in each bile salt system and are equivalent to the pH values at point X (Fig. 1 A–C). It is interesting that UDCA and GUDCA can reach, in the metastable state, *actual* precipitation pH values identical to those found at equilibrium for CDCA and GCDCA (Fig. 1). The equilibrium pH values at the point of precipitation, i.e., the pH at which the first HA molecules would have precipitated had there been no supersaturation (pH at X'), are also derived from the curves and are listed in Tables 1 and 2. As the equilibrium values are of most interest physiologically, since UDCA or CDCA molecules go into solution only above these pH's, we plot selected values for each system (H_2O, 37°C) in **Fig. 3.** These curves show that the equilibrium precipitation pH (pH ppt) values increase with the bile salt concentra-

tion between 5 and 40 mM and then reach constant values. The values for UDCA fall between 7.98 (5 mM) and 8.1 (100 mM), whereas those of CDCA fall between 7.0 (5 mM) and 7.1 (100 mM), exactly 1 pH unit lower. In the case of GUDCA, the precipitation pH values increase from 6.5 (5 mM) to 7.4 (100 mM), a range which is about 2 pH units higher than the values for GCDCA (4.8–5.1). Thus, glycine conjugation lowers the precipitation pH values of UDCA by only ≃1 pH unit compared with ≃2 pH units in the case of CDCA. The precipitation pH values of GUDCA are thus slightly higher than the values for *unconjugated* CDCA (Fig. 3) and other unconjugated bile acids (31).

The apparent pK (pK′a) values of the bile acids (Tables 1 and 2 and Fig. 3). The pK′a values were calculated from the W–Y portion of the titration curves (Fig. 1A–D) utilizing a simplified version of the formula proposed by Back and Steenberg (34), which omits a small correction for the concentrations of OH^- and H_3O^+ ions present at point Y (Fig. 1D inset) (31). Employing the correction for the ionic product of water, only the third significant figure in the pK′a calculations is changed and hence the present procedure is well within the experimental error of the method. The formula used is

$$pK'a = pHy + \log \frac{HA}{TOT\text{-}HA} + \frac{0.5\sqrt{\mu}}{1+\sqrt{\mu}} \qquad 4).$$

where pHy is the pH at point Y, TOT is equivalent to the total amount of acid (in μmoles) required to carry the reaction (Eq. 1) to completion (i.e., from W → Z), HA is equivalent to the amount of acid added from the first equivalence point (W) to the point Y, and μ is the total ionic strength at point Y. As this formula requires a knowledge of the value of HA with great precision, the accuracy of the calculated pK′a values will depend on the accuracy with which HA values can

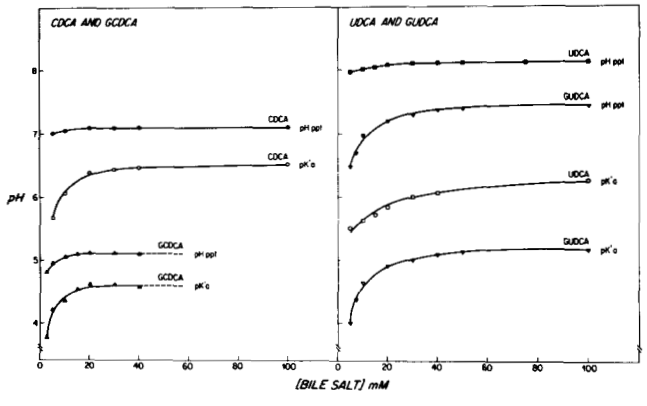

Fig. 3. The equilibrium precipitation pH (pH ppt) and apparent pK (pK′a) values of CDCA and GCDCA (left panel) and UDCA and GUDCA (right panel) as a function of bile salt concentration (mM) in H_2O at 37°C.

TABLE 3. Influence of added NaCl on the electrochemical properties of UDCA, GUDCA, CDCA, and GCDCA[a]

Conc. of NaCl (M)	Equivalence (%)	[HA] μM	A^-HA Ratio	pK′a	Precipitation pH	Gross Viscosity
			UDCA, 20 mM, 37°C			
0.00	97.2	98	207	5.83	8.07	Liquid
0.05	98.5	117	170	5.90	8.02	Liquid
0.10	98.3	173	115	5.99	7.92	Liquid
0.15	98.1	210	95	5.97	7.86	Liquid
0.30						Gel
			GUDCA, 20 mM, 37°C			
0.00	98.0	87	230	4.89	7.19	Liquid
0.15	98.5	86	231	4.92	7.13	Liquid
			CDCA, 20 mM, 37°C			
0.00	97.4	2886	6	6.38	7.09	Liquid
0.05	98.3	2698	7	6.35	7.05	Liquid
0.10	98.5	3097	6	6.41	7.02	Liquid
0.15	96.5	3316	5	6.44	6.99	Liquid
			GCDCA, 20 mM, 37°C			
0.00	92.5	3900	5	4.61	5.11	Liquid
0.15	95.5	4100	5	4.60	5.01	Liquid

[a] Footnotes for column headings as in Table 1.

be derived from the curves. As indicated in Table 1, it was not possible to estimate the HA values of UDCA and GUDCA from the titration curves below 5–7.5 mM, i.e., below the apparent CMC values (Fig. 2) owing to these experimental uncertainties. In fact, since the HA solubility derived from the curves of 10–15 mM UDCA was less than the correct HA solubility in H_2O (Table 1), our pK′a values for these concentrations are also corrected (see next section). Selected pK′a values for the bile acids in H_2O at 37°C are plotted in a similar fashion to the equilibrium precipitation pH values in Fig. 3. These values increase appreciably with bile salt concentration to reach constant values at about 20–40 mM. The pK′a values of UDCA (5.5–6.25) are in general slightly lower than those of CDCA (5.7–6.5), whereas the pK′a values of GUDCA (4–5.2) are in general slightly higher than GCDCA (3.8–4.6). It is apparent from the combined data in Fig. 3 that the equilibrium precipitation pH values of CDCA and GCDCA are ≃0.5–1 pH unit higher than the respective pK′a values, whereas the precipitation pH values of UDCA and GUDCA are about 2–2.5 pH units higher than their pK′a values.

Influence of variations in temperature and ionic strength on the electrochemical properties of UDCA and CDCA. These influences were studied systematically, employing 20 mM bile salt solutions in water (10–60°C, Tables 1 and 2) and at 37°C (0–0.15 M NaCl, **Table 3**) respectively. To illustrate these results, we plot the

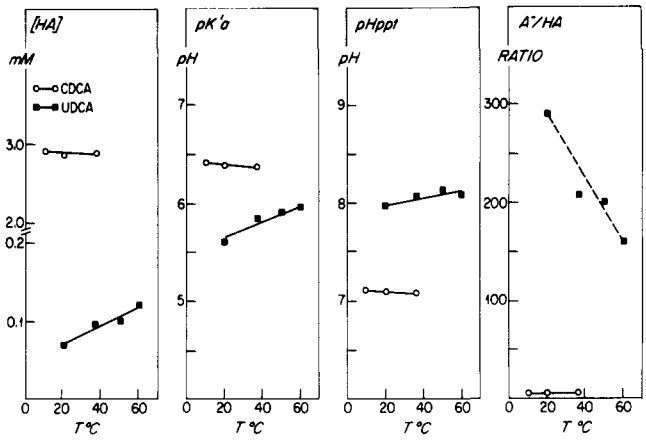

Fig. 4. Influence of temperature on HA solubility [HA], pK'a, precipitation pH (pH ppt) and saturation ratio (A^-/HA) of 20 mM CDCA and UDCA in water. In the vicinity of the pH ppt, UDCA gelled at 10°C and CDCA gelled at 60°C.

variation of HA solubility, pK'a, precipitation pH and A^-/HA ratio for both bile acids as a function of temperature in **Fig. 4** and as a function of ionic strength in **Fig. 5.** With increases in temperature (Fig. 4), all values for CDCA remain essentially constant, whereas in the case of UDCA, [HA], pK'a and precipitation pH increase appreciably and concomitantly A^-/HA ratios fall. With increases in ionic strength at constant temperature (Fig. 5), [HA] and pK'a values of both bile acids increase and the precipitation pH values fall slightly. The A^-/HA ratio for CDCA shows little change, whereas the ratio for UDCA falls dramatically.

Viscosity of the solutions near the precipitation pH. The gross (qualitative) viscosity of the solutions was documented during each titration and this observation was recorded near the precipitation pH, usually at the first appearance of the Tyndall phenomenon, in

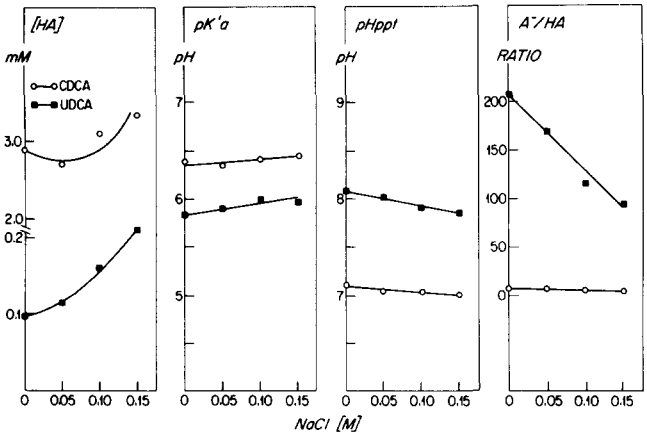

Fig. 5. Influence of added NaCl on HA solubility [HA], pK'a, precipitation pH (pH ppt) and saturation ratio (A^-/HA) of 20 mM CDCA and UDCA at 37°C. Both UDCA and CDCA formed gels in the vicinity of the precipitation pH values with NaCl concentrations of 0.3 M.

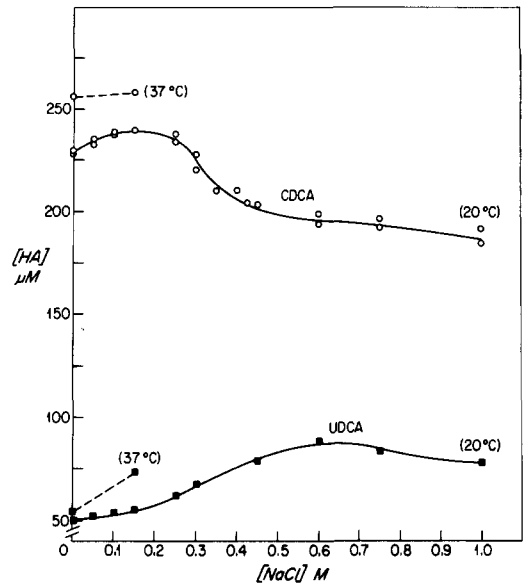

Fig. 6. Absolute aqueous solubilities of undissociated CDCA and UDCA at 20°C and 37°C as a function of NaCl concentration (M) at pH 2.4, employing radiolabeled bile acids. Each data point represents the mean of 3 determinations and the size of the symbols encompasses the standard error of the measurements.

Tables 1–3. In general, all systems were liquid at the Tyndall point when both the bile acid concentration and ionic strength were low. Gel formation occurred with both UDCA and CDCA at high bile acid concentrations and in high ionic strength (>0.3 M NaCl). Variations in temperature produced paradoxical effects in that 20 mM CDCA in H_2O (no added NaCl) gelled at 50°C but was liquid at 10°C, whereas 20 mM UDCA gelled at 10°C but was liquid at 60°C.

Direct measurement of bile acid (HA) solubility. Because the HA solubility of UDCA at low bile salt concentrations could not be obtained reliably from the potentiometric titration curves (Tables 1 and 2), it was necessary to obtain the aqueous (non-micellar) HA solubilities directly (see Methods). In both cases the [11,12-^{3}H]-labeled compounds were chosen to essentially rule out tritium exchange with the solvent protons. These results (**Fig. 6**) show that the aqueous solubility of the HA (CDCA) is fivefold greater than the aqueous solubility of HA (UDCA). Second, the shape of the two curves in response to the addition of NaCl is dissimilar. With added NaCl, HA (CDCA) is "salted in" and then "salted out" at lower ionic strengths than HA (UDCA). With increases in temperature from 20°C to 37°C, a slight increase in the HA solubility of both species occurs. The [HA] (CDCA) obtained by this method is in excellent agreement with that derived from the potentiometric titration curves (Table 2) in the vicinity of the apparent CMC (Fig. 2). However, the [HA] (UDCA) values obtained from the potentiometric titration curves for dilute bile salt con-

centrations (Table 1) are somewhat less than those directly measured. Hence, the corrected HA solubilities are also plotted in Tables 1 and 2 and the A^-/HA and pK'a values obtained by substitution of the more accurate HA (UDCA) and HA (CDCA) values in Formula *4* are shown in parentheses.

To explore further the reasons for the differences between HA (UDCA) and HA (CDCA) solubilities, we measured the aqueous solubilities of the undissociated form of the other common unconjugated bile acids of man (**Table 4**). The anhydrous melting point values which provide an estimate of the crystal energy (Table 4) together with the literature values (35, 36–46) are also tabulated. No clearcut inverse correlation between melting point and HA solubility was found. For example, cholic acid, with the highest HA solubility, has a melting point similar to UDCA which has the second lowest HA solubility. When the HA solubilities are plotted as a function of the number of hydroxyl functions on each bile acid (**Fig. 7**), a linear increase from lithocholic acid through CDCA to cholic acid is observed. When the dihydroxy bile acids are compared it is apparent that the aqueous solubility promoted by a 12α-OH group (deoxycholic acid) is only about half as strong as that of a 7α-OH group (CDCA), whereas a 7β-OH group (UDCA) is only about half as strong as a 12α group (deoxycholic acid).

TABLE 4. Absolute aqueous solubilities and capillary melting points of undissociated bile acids

Bile Acid (H_2O, pH 2.4)	Temperature (°C)	Value (μM)	Melting Point (Uncorrected)
Cholic	20	428[a]	201–202[b]
	37	460	
Chenodeoxycholic	20	229	166–168[d]
	37	256[c]	
Deoxycholic	20	111[e]	176–178[f]
	37	114	
Ursodeoxycholic	20	51[g]	201–203[h]
	37	53	
Lithocholic	20	1	187–189[i]
	37	1	

[a] Literature (36, 37) values at 20°C are 190 μM (by potentiometry) and 225 μM (by dry weight), and 685 μM (by dry weight) at 15°C.
[b] The literature (35, 38–40) mp values are 198, 199, 198–199, 198–200°C.
[c] Potentiometric value from the present work is 200 μM.
[d] The literature (38, 40–43) mp values are 119, 142–143, 140–144, 168–172°C.
[e] The literature (36, 37) value at 20°C is 110 μM (by potentiometry) and at 15°C is 611 μM (by dry weight).
[f] The literature (35, 38, 40, 44) mp values are 172–173, 175–177, 176–177, 177°C.
[g] Potentiometric values from the present work are 15–40 μM.
[h] The literature (45–48) mp values are 194, 198–200, 201–202 and 203°C.
[i] The literature (35, 38, 40, 49) mp values are 182–183, 184–185, 184–186 and 188°C.

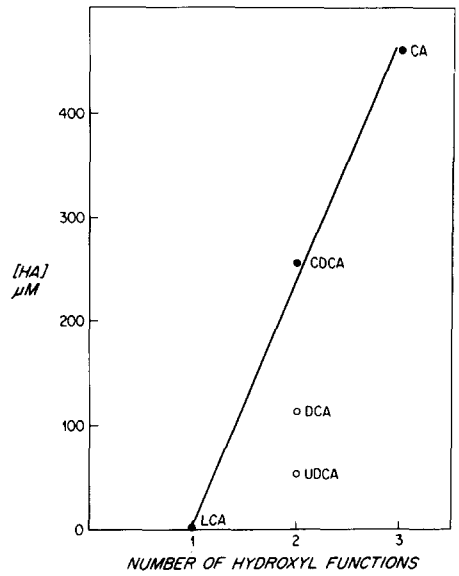

Fig. 7. Absolute aqueous solubilities (H_2O, 37°C) of the major bile acids (HA species) of man (pH 2.4) as a function of the number of hydroxyl functions on each molecule using radiolabeled bile acids. The strength of hydroxyl functions in promoting solubility decreases in the following order: 3α,7α,12α (cholic acid, CA) > 3α,7α (CDCA) > 3α,12α (deoxycholic acid, DCA) > 3α,7β (UDCA) > 3α (lithocholic acid, LCA).

Mixed bile salt systems

The potentiometric titration curves of binary UDCA/CDCA and GUDCA/GCDCA mixtures were of similar configuration to those of the single bile salt systems and were analyzed in the same fashion. The electrochemical constants for various molar ratios of the unconjugates and glycine conjugates as 20 mM solutions in 0.15 M NaCl at 37°C are listed in **Table 5** and the pH of precipitation and pK'a values are illustrated in **Fig. 8.** In both cases, the pK'a and precipitation pH values fall close to an additivity line between the values for the pure components. The changes in HA values and A^-/HA ratios are inversely related (Table 5) and demonstrate the low solubility of HA of either species of UDCA and GUDCA micelles, but the excellent solubility of both HA species in CDCA and GCDCA micelles. CDCA and its glycine conjugate share a similar maximum capacity to solubilize the HA species of either acid (A^-/HA = 5), but UDCA is approximately threefold better than its glycine conjugate (A^-/HA = 95 versus 231).

To test how biliary bile acid composition during established urso-therapy (7, 9, 18) might influence the electrochemical properties of unconjugated UDCA in 0.15 M NaCl at 37°C, we employed equimolar proportions of TUDC/TCDC (1:1) and GUDC/GCDC (1:1) as 10 mM solutions to determine how varia-

TABLE 5. Electrochemical constants for binary bile salt mixtures in 0.15 M NaCl (37°C)[a]

	Equivalence (%)	[HA] μM	A^-/HA Ratio	pK'a	Precipitation pH	Gross Viscosity
20 mM UDCA	98.1	210	95	5.97	7.86	Liquid
15 mM UDCA + 5 mM CDCA	97.6	380	51	6.09	7.64	Liquid
10 mM UDCA + 10 mM CDCA	97.2	620	31	6.12	7.45	Liquid
5 mM UDCA + 15 mM CDCA	93.5	1540	12	6.26	7.16	Liquid
20 mM CDCA	96.5	3316	5	6.44	6.99	Liquid
20 mM GUDCA	98.5	86	231	4.92	7.13	Liquid
15 mM GUDCA + 5 mM GCDCA	94.5	130	145	4.81	6.82	Liquid
10 mM GUDCA + 10 mM GCDCA	97.4	718	27	4.86	6.15	Liquid
5 mM GUDCA + 15 mM GCDCA	94.4	2380	8	4.66	5.35	Liquid
20 mM GCDCA	95.5	4100	5	4.60	5.01	Liquid

[a] Footnotes for column headings as in Table 1.

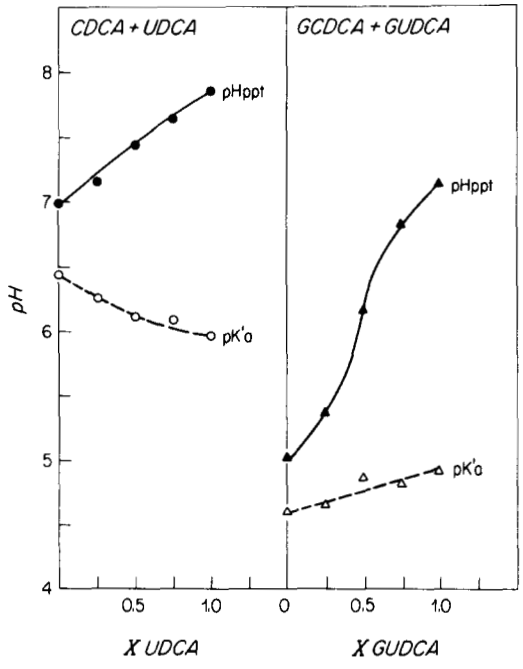

Fig. 8. Equilibrium precipitation pH (pHppt) values and apparent pK (pK'a) values of binary CDCA and UDCA (left panel) and GCDCA and GUDCA (right panel) mixtures plotted as a function of the mole fraction (X) of UDCA or GUDCA in each mixture (20 mM, 0.15 M NaCl, 37°C).

tions in the *G:T* conjugated bile salt ratio might influence the titration characteristics of 10 mM UDCA (**Fig. 9**). Since taurine conjugates are stronger acids than the HCl employed, they were not titrated in these experiments, so that the electrolytic constants of UDCA were obtained directly. With added glycine conjugates, the predicted equivalences for the amounts added were obtained from the measured equivalences of the total UDCA, GCDCA and GUDCA titrated. As shown in Fig. 9, the percent equivalence of the glycine conjugates increases linearly from zero to ≃90%, indicating good agreement between the amount of glycine conjugates added and those titrated. In the absence of the glycine conjugates, the electrochemical properties of UDCA were influenced appreciably by the presence of the taurine conjugates. Thus the precipitation pH value fell from 7.7 (pure UDCA, Table 1) to 6.8 in the presence of 10 mM TUDC/TCDC (1:1) and the pK'a value rose from 5.7 (Table 1) to nearly 6.3. Thus, UDCA in a mixture of taurine conjugates behaves very much like CDCA in water (Table 2) and, further, the A^-/HA ratio was 5:1. With the progressive substitution of the GUDC/GCDC mixture, the precipitation pH values increase and the pK'a values fall slightly (Fig. 9), but even in

100% of glycine conjugates, the precipitation pH of UDCA is lower and the pK′a higher when compared with pure UDCA under identical conditions (Table 1). The widening of the difference between pK′a and precipitation pH is a reflection of the diminished capacity of the glycine conjugates to solubilize the protonated UDCA species (A^-/HA = 10:1) (Fig. 9). With mixtures containing >50% glycine conjugates in the vicinity of physiological pH, the physical state of the mixtures changed to that of a clear gel.

To test the gelation phenomenon further, we studied the influence of different mixtures of conjugated bile salts together with other amphiphiles on the physical state and titration characteristics of UDCA. The effects of addition of 5 mM lecithin and 5 mM monoolein to equimolar amounts of 5 mM GUDCA/GCDCA (1:1) plus 5 mM TUDCA/TCDCA (1:1) in the presence of 10 mM UDCA are shown in **Fig. 10.** In Fig. 10A, the conjugated bile salt mixture (no UDCA) remained a clear liquid without gel formation over the course of the titration and a Tyndall phenomenon was first noted at pH 3.8 without metastable supersaturation. The addition of UDCA to the conjugated bile salt mixture (Fig. 10B) resulted in the appearance of a Tyndall phenomenon at pH 6.8 and clear gel formation at pH 6.7. The gel did not precipitate until pH 4.5, at which point the physical state reverted to that of a cloudy solution. In Fig. 10C and D, the addition of lecithin and monoolein did not influence the physical state found in 10B significantly since clear gel formation occurred at pH ≃ 7.0 and a Tyndall phenomenon did not appear until pH 5.5 with lecithin

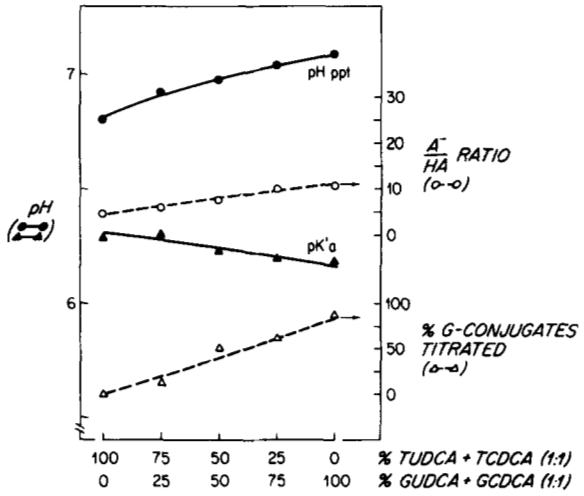

Fig. 9. Electrochemical constants derived from the titration of 10 mM UDCA in 10 mM mixtures of TUDCA/TCDCA (1:1) plus GUDCA/GCDCA (1:1) as a function of variation in the G:T conjugated bile salt ratio (0.15 M NaCl, 37°C). From the top are displayed, the equilibrium precipitation pH (pH ppt) values, the HA solubility in micelles of the mixed conjugates, i.e., A^-/HA ratios, the apparent pK (pK′a) values and the percent of glycine conjugates titrated.

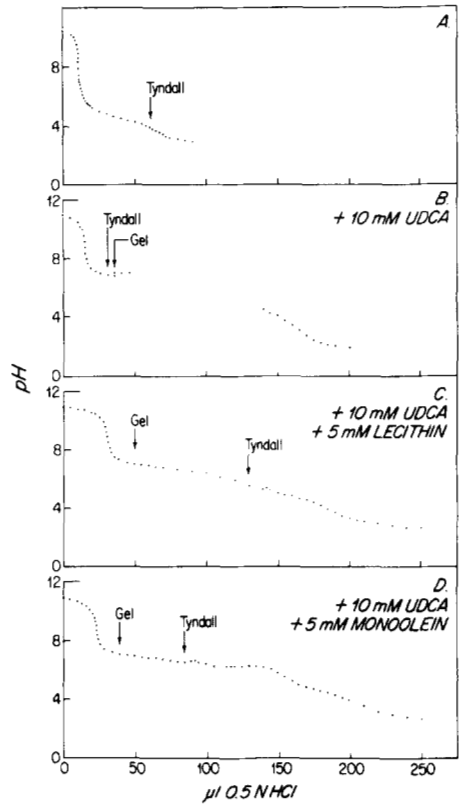

Fig. 10. Potentiometric titration curves (0.15 M NaCl, 37°C) of A) 5 mM GUDCA/GCDCA (1:1) plus 5 mM TUDCA/TCDCA (1:1), B) the conjugate composition in (A) plus 10 mM UDCA, C) the conjugate composition in (A) plus 10 mM UDCA plus 5 mM lecithin and D) the conjugate composition in A plus 10 mM UDCA plus 5 mM monoolein. See text for details.

and 6.5 with monoolein. However, in both cases, precipitation with the formation of cloudy solutions occurred ≃0.3 of a pH unit lower.

To test whether gelation was a unique finding with equimolar UDCA and CDCA conjugates, we titrated 10 mM solutions of the glycine and taurine conjugates of cholic, chenodeoxycholic and deoxycholic acids (2:2:1) with and without equimolar amounts of CDCA and UDCA. The results displayed in **Fig. 11A** show that in the absence of CDCA or UDCA the glycine conjugates could be titrated in the taurine conjugates to pH 2.5 before a Tyndall phenomenon was produced. With the addition of 10 mM UDCA, gel formation occurred at pH 6.6–6.8 and a Tyndall phenomenon did not appear until the pH was lowered to pH 3.5 (Fig. 11B). With the titration of 10 mM CDCA in the same mixture (Fig. 11C), no gel formation occurred and a Tyndall was first noted at pH 6.1–6.2.

DISCUSSION

Single bile salt systems

Since the hydrophobic–hydrophilic balance of bile salt molecules and thus their solubility in aqueous

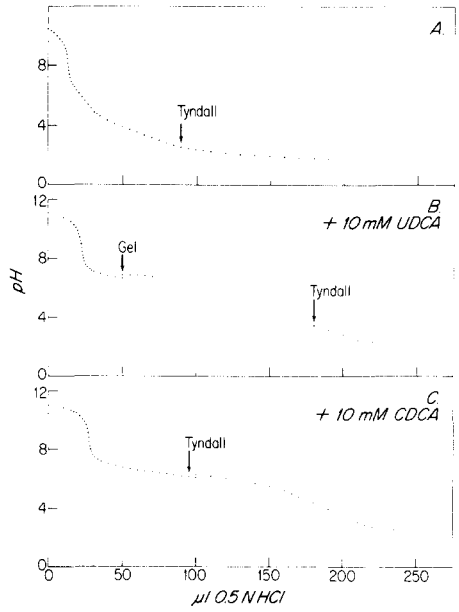

Fig. 11. Potentiometric titration curves (0.15 M NaCl, 37°C) of A) 5 mM glycocholic, glycochenodeoxycholic, and glycodeoxycholic (2:2:1) acids plus 5 mM of the respective taurine conjugates (2:2:1); B) the conjugate composition in (A) plus 10 mM UDCA; C) the conjugate composition in (A) plus 10 mM CDCA. See text for details.

systems is primarily influenced by the dissociation of the ionizable polar groups, a knowledge of the electrochemical properties of bile salts is fundamental to our understanding of the physical state and membrane interactions of these important biological detergents. The protonated or undissociated species (HA) of all the common bile acids are sparingly soluble in water (Table 4) and thus cannot form micelles.[2] On account of their nonionic nature they are more lipophilic than the ionized bile salt species, and can therefore partition into a lipid environment such as biological membranes and into the hydrophobic core of micelles including bile salt (A^-) micelles.

Since nonionized bile acids possess one or more hydroxyl functions, their aqueous solubilities are appreciably higher than undissociated long chain fatty acids (50, 51), notwithstanding their larger molecular weights. Under conditions of quite low pH, bile acids precipitate as crystalline solids and leave only a small finite concentration of HA in the aqueous phase in equilibrium as monomers (Table 4), whereas at high pH, bile salt molecules ionize, forming monomeric solutions below their CMC values, and micellar solutions above this concentration. At intermediate pH values, the physical state of bile salt solutions is much more complex, as shown systematically in this work.

[2] This discussion only considers the unconjugated and glycine conjugated bile salts. The taurine conjugates are very strong acids with pK'a values <1.8 (31) and thus cannot be protonated by titration with the common mineral acids.

Protonation begins about ≃pH 9.0 in the case of the unconjugates and ≃pH 8.0 in the case of the glycine conjugates (Fig. 1), but even though the true aqueous solubility of the HA form is quickly exceeded, molecules of HA only begin to precipitate when the pH is lowered further because the otherwise insoluble HA molecules are solubilized as "mixed" micelles of A^- and HA. Thus, the equilibrium pH of precipitation depends on the solubilizing capacity of the micelles for HA and the extent of HA aqueous solubility as monomers, whereas the actual pH of precipitation also depends on the degree of metastable supersaturation of the micelles with HA. Therefore, owing to the weak solubility of HA (UDCA/GUDCA) in water and in UDCA/GUDCA micelles, the equilibrium precipitation pH values of these acids are ≃1–2 pH units higher than those of CDCA and GCDCA.

In the case of the latter, the equilibrium precipitation pH values are kept low, due to the moderate HA solubility in water and the strong capacity of the micelles to solubilize HA. In the metastable state UDCA and GUDCA attain [HA] values, A^-/HA saturation ratios and *actual* precipitation pH values similar to the range observed at equilibrium for CDCA and GCDCA which show little or no supersaturation (Fig. 1). This suggests that an A^-/HA ratio of 5:1 is the highest saturation limit for micellar solubilization of the protonated species of the dihydroxy bile salts and apparently cannot be exceeded. Since no acid salts (="acid soaps") have been detected during the titration of bile salt solutions (36, 52), the aqueous bile salt system along the plateau region of the curves (Fig. 1) is composed of the following molecular species in equilibrium

$$[HA]_{cryst.} \rightleftharpoons [HA]_{aq} \rightleftharpoons [HA]_{micellar} \rightleftharpoons [A^-]_{micellar} \rightleftharpoons [A^-]_{monomeric} \quad 5).$$

all in varying proportions depending on the amount of bile salt anion which has been converted to the protonated form. This plateau region encompasses the physiologic pH of bile, small bowel and colon with important pathophysiological implications (see below). At the completion of the titration at quite low pH, the equilibrium is represented by

$$[HA]_{cryst.} \rightleftharpoons [HA]_{aq} \quad 6).$$

When the equilibrium solubilities of HA were measured directly in water, it was found that HA (CDCA) is nearly five times more soluble than HA (UDCA) under identical physical–chemical conditions (Table 4, Fig. 6). Thus, HA solubility of UDCA is strikingly smaller than observed for all dihydroxy bile acids but is comparable to the value observed potentiometrically (30 μM) for the solubility of the acid form of a 3α,7β

dihydroxy fusidane derivative (33), whereas the solubility of the analogous 3α, 11α dihydroxy fusidane derivative was $\simeq$100 μM (33), similar to the solubility of deoxycholic acid (Table 4). It is therefore apparent that the 7β orientation of the OH function is of critical importance in reducing solubility.

The variations observed in the solubility values of the other bile acids (Table 4, Fig. 7) are consistent with the hypothesis (53) that the water solubilizing efficiency of hydroxyl functions is potentiated when these substituents are situated in close proximity on a molecule, due to the cooperative nature of hydroxyl group–water interactions. Hence more water molecules are bound when two or more hydroxyl functions are situated on the same face and in close proximity on a molecule, than the sum of the number of individual OH-water hydrogen bonds they can form would predict. With both CDCA and deoxycholic acid, two OH groups are α-oriented lying about 5 Å apart on the concave undersurface of the molecule (53). Since the C7 hydroxyl function is β-oriented in UDCA, the two OH groups lie $\simeq$8 Å apart with the 7 OH function oriented towards the opposite side of the hydrocarbon ring system. This arrangement allows for only local hydration of these functions in UDCA but cooperative hydration in the case of CDCA. Cooperative formation of hydrogen bonds within water also occurs when the bulky hydrocarbon parts of bile acid molecules interact with water. The extent of this "hydration shell" is related to the size of the uninterrupted hydrophobic areas of these molecules (54). Hence the different responses of HA aqueous solubility in response to added NaCl (Fig. 7) reflects the quantitative differences in both types of hydration.[3] The cooperative structuring of water is impaired by the addition of a chaotropic (water-breaking) agent such as NaCl, and thus the aqueous solubility of CDCA is decreased at low ionic strengths. The progressive increase in aqueous UDCA solubility between 0 and 0.6 M NaCl is consistent with the hypothesis that no cooperative hydration interactions between the two stereochemically different OH groups can occur and thus the only effect apparent is a decrease in the "hydrophobic hydration" of the amphiphile with a result that aqueous solubility is doubled. The decrease in solubility above 0.6 M NaCl is a result of salting out of both the hydrocarbon and OH group-H_2O interactions, since this only becomes apparent at an ionic strength where dielectric saturation ($\simeq$0.7 M NaCl) of the medium commences. However, since aqueous solubility of HA represents a balance between the chemical potential of the crystalline bile acid and the affinity of the HA monomers for water, we considered that differing crystal energies might play a role in determining HA solubility.

The melting point values which give an estimate of the crystal energy show (Table 4) that UDCA requires a higher energy to break the lattice (mp 201–203°C) than CDCA (mp 166–168°C), which in the absence of other evidence might explain the different aqueous solubilities of the HA monomer. However, the observation that the solubilities of lithocholic, deoxycholic, or cholic acids do not correlate inversely with their melting point values suggests that the aqueous solubilities of bile acids correlate better with the number and position of hydroxyl functions as outlined above, rather than with their crystal energies. In addition, the low aqueous solubility of HA (UDCA) may also, in part, explain the marked differences in equilibrium HA saturation of UDCA compared to CDCA micelles. Mazer, Benedek, and Carey (55) concluded from an analysis of hydrodynamic radii and of cholesterol solubilities in simple and mixed bile salt micelles that the intermicellar solubility of the solubilizate, and not the saturation of the micellar binding sites, limited cholesterol solubility. Thus, the low micellar solubility of HA (UDCA) may also be related to the intermicellar monomeric concentration of HA, since it can be significantly increased by metastable supersaturation (Fig. 1), by increasing bile salt concentration (Tables 1, 2), increasing temperature (Fig. 4), and increasing ionic strength (Fig. 5), all of which result in an increase in aqueous HA solubility, suggesting that the number of micellar binding sites is not the limiting factor, whereas this appears to be the case with CDCA (Table 2, Fig. 4, 5).

The apparent CMC values of the bile salts derived in Fig. 2 record an estimate of the bile salt concentration when the first HA molecules are solubilized. They are appreciably larger than precise estimates for these bile salts by less invasive methods (24) and reflect the fact that when large hydrophobic solubilizates are solubilized by bile salts, the solubilizate molecules are solubilized only when the concentration of micelles is well above the true CMC values. For reasons identical to those outlined above, a large hydrophobic solubilizate, which has a low aqueous solubility, will result in a high solubilizer to solubilizate saturation ratio in micelles and hence binding sites are not occupied until well above the true CMC values. The apparent CMC values can be an order of magnitude larger than the true values, as shown in the case of UDCA/GUDCA in this work, and similar to what was found for cholesterol solubility in pure bile salt systems (26).

[3] The influence of added NaCl on the crystal energy of bile acid precipitates is likely to be minimal and is thus ignored in this discussion.

In spite of the high precipitation pH values, it was not altogether unexpected that the pK′a values of UDCA are lower than CDCA. We previously recognized in 7β hydroxylated fusidane derivatives (33) that the carboxyl group of steroid amphiphiles can exhibit stronger acidic properties secondary to electronic delocalization induced by a 7β-oriented hydroxyl function. However, since GUDCA is a weaker acid than both GCDCA and other glycine conjugated bile salts (31), it is possible that electronic delocalization influenced the peptide linkage, which in turn weakens the acidic properties of the terminal glycine carboxyl group. The increases in pK′a values with increases in bile salt concentration above the CMC values (Fig. 2) are well known effects in steroid amphiphile systems and have been commented on extensively (31, 33, 52) as resulting from the close proximity of charged groups at the micelle–water interface that weaken the acidic properties of the constituent molecules. Once micelle formation is completed, the nearly constant ratio of the activities of HA and A^- are reflected in the fairly constant pK′a values. Similarly shaped curves have been obtained by Ekwall, Rosendahl, and Löfman (52) and Small (31) for the dissociation constants of cholic, deoxycholic and chenodeoxycholic acids and their glycine conjugates utilizing potentiometric methods (34). Our results for CDCA and GCDCA are similar to the results for equivalent bile salt concentrations studied by Small (31). In general, the effects of added ionic strength and increasing temperature produce little effect on the pK′a values of CDCA, an observation noted previously (31), but produce a pronounced effect on UDCA consistent with alternations in aqueous HA solubility and the occupancy of potential micellar binding sites.

Mixed bile salt systems

Since the CMC values and micellar sizes of UDCA and CDCA are virtually identical[4], these bile acids and their glycine conjugates, when mixed in various molar proportions, should result in mutually mixed systems. The observation in Fig. 8 that the pK′a and preciptation pH values fall on an additivity line connecting the values of the pure components is therefore expected. Further, since the solubility of HA increases linearly between UDCA and CDCA, and between GUDCA and GCDCA, and since A^-/HA decreases inversely (Table 5), micelles of UDCA/GUDCA can solubilize the HA species of CDCA/GCDCA and vice versa. This furnishes additional proof that micellar solubilization in UDCA micelles is not limited by the geometric configuration of the HA (UDCA) molecule. The marked differences in precipitation pH values between GCDCA and GUDCA (2.1 pH units) compared with the unconjugates (1 pH unit) reflects the extremely poor solubility of HA (of either species) in GUDCA micelles and suggests that GUDCA solubility under physiological conditions is for all intents and purposes the same as unconjugated CDCA, even though its pK′a value is 1.5 pH units lower. Thus, GUDCA had the same potential as *unconjugated* CDCA to precipitate from biological fluids if it becomes the predominant bile acid. However, the electrochemical properties of UDCA can be appreciably modified in the presence of taurine and glycine conjugates (Fig. 9). In the presence of conjugated UDCA and CDCA, the pK′a values of 10 mM UDCA are elevated when compared to pure UDCA in water, reflecting the fact that UDCA becomes a weaker acid in a micellar environment (see Fig. 3). Simultaneously the precipitation pH values fall compared with UDCA alone, reflecting the fact that HA (UDCA) is more soluble in micelles of the conjugates than in ionized UDCA. The slight elevation in precipitation pH, as the glycine conjugates are substituted, correlates with the fact that GUDC-GCDC mixtures are poorer solubilizers of HA (UDCA) than TUDC/TCDC mixtures under all conditions.

The results in Fig. 10 and 11 provide insight into how the physical state of gut luminal contents might be altered by UDCA. In the absence of UDCA, the solubility of GUDCA/GCDCA in their respective taurine conjugates is less than a mixture of glycocholic, glyco-chenodeoxycholic and glyco-deoxycholic acids in their taurine conjugates, since a Tyndall phenomenon was first noted at pH 3.8 in the former, but not until pH 2.5 in the latter. All 10 mM UDCA/CDCA conjugated mixtures containing 10 mM UDCA formed a clear viscous gel between pH 6.5–7.0, conditions which are typical of intraluminal gut contents during urso-therapy. With the common conjugated bile salts, 10 mM UDCA also induced gel formation over the same pH range (Fig. 11), whereas CDCA titrated under the same conditions did *not* form a gel and a Tyndall phenomenon did not appear until pH 6.1–6.2 was reached. Additional studies are required to investigate the physical nature of these gels and to explore whether they have properties in common with the gels observed in the titration of pure UDCA at concentrations $\simeq$100 mM. Whether gel formation is induced with therapeutic concentrations of UDCA in the presence of conjugated bile salts in the duodenum in vivo needs experimental verification.

[4] Carey, M. C., J. C. Montet, and M. C. Phillips. Unpublished observations.

Pathophysiological implications

It is clear from these studies that as a result of their unusual electrochemical properties, UDCA and GUDCA at physiological pH values are the most insoluble of the common bile acids. These observations relate essentially to the extraordinarily low solubility of the protonated form HA in aqueous UDCA and GUDCA solutions and not to the intrinsic acid strength of the carboxyl groups. Even though equilibrium precipitation pH values of UDCA and GUDCA represent the pH points where the micelles are just saturated with HA, it is important to consider that protonation commences at pH $\simeq$ 9.0 in the case of UDCA and pH $\simeq$ 8 in the case of GUDCA. Thus, to ensure complete ionization these bile salts must be solubilized in buffers to achieve bulk pH value of at least 10.0.

Second, it is now well established (56, 57) that CDCA at physiological pH is a potent intestinal secretogogue and for this reason diarrhea has been a major problem with patient acceptance of CDCA as a gallstone dissolving agent (25). However, oral therapy with UDCA does not suffer from this drawback (4–7) and in in vivo colonic perfusion studies, UDCA was not found to reduce the capacity of the human colon to handle a saline load (58) nor does it induce secretion, increased mucosal permeability, or mucosal damage in the rabbit colon (59). In the case of the secretogenic bile acids, an aqueous concentration of 3–7 mM must be reached for the effect to be unmasked, and only bile salts with 2α-oriented OH groups appear to be active (59). Unfortunately, no investigators have paid attention to the actual physical species of the bile salt (A^- and/or HA) responsible for intestinal secretion and diarrhea or whether this species must be absorbed to induce secretion.[5] Because deconjugation of bile salts in the colon is virtually complete, we will consider, for the purpose of the present discussion, that the HA species of dihydroxy bile salt is responsible for inducing secretion and that it must interact with membranes and/or be absorbed for activity. Since this species is the most lipophilic it will be the most rapidly absorbed by large intestinal membranes by passive nonionic diffusion mechanisms. However, for the HA species to induce secretion, a sufficient amount must be in solution at physiological pH so that diffusion to the enterocyte absorptive membranes can occur. CDCA satisfies all these criteria. At a pH of 7.00–7.1 (Table 2) over a bile salt range of 5–40 mM, 0.2–6 mM of the HA form is present in micellar *plus* true solution. This considerable solubility is facilitated by *A*) the high intrinsic solubility of HA (CDCA) in H_2O in the absence of micelles, *B*) the high capacity of CDCA (A^-) micelles to solubilize HA, and *C*) the fact that at pH 7.0 the micelles are just saturated with HA at *all* CDCA concentrations (Table 2), i.e., the precipitation pH displays no appreciable concentration dependence (Fig. 3). In contrast, not only is the true aqueous solubility of the HA (UDCA) fivefold smaller (Fig. 6), but its micellar solubility is also less by an order of magnitude, i.e., the solubility of HA over a 5–50 mM range of UDCA is 0.01–0.3 mM in both micelles and intermicellar solution. However, these solubility considerations only apply to UDCA solutions at pH 8–8.1.

Inspection of the titration curves (Fig. 1) shows that at pH 7.0 greater than 95% of intraluminal UDCA would be precipitated as the crystalline acid and thus, under physiological conditions, the maximum solubility of HA (UDCA) will never exceed its true aqueous solubility ($\simeq$.05 mM). Even if this concentration were depleted by absorption, crystalline HA could not equilibrate rapidly enough to maintain the saturated aqueous solubility of HA. Thus, at pH 7.0, HA (UDCA) cannot serve as a source of supply for absorbed aqueous HA, owing to the slow dissolution of kinetics of the crystalline form. In contrast, a reservoir for aqueous HA (CDCA) is constantly provided by the "mixed" $A^- + HA$ micelles of the bile acid. This hypothesis, which is shown schematically in **Fig. 12,** also suggests that the microclimate pH at the membrane aqueous interface may be appreciably lower than the bulk pH (64, 65) and would facilitate further formation of the HA species from A^-. It is also apparent that if A^- were the active secretogenic species, similar principles hold, since in contrast to CDCA, sufficient A^- (UDCA) would not be present in the aqueous phase at physiological pH to induce secretion.[6]

Last, since it is desirable that absorption of CDCA and UDCA should be efficient from the conjugated bile

[5] It has been both claimed (56, 57, 60, 61) and refuted (62, 63) that the taurine and glycine conjugates of di(α)hydroxy bile salts are also active secretogogues in the colon. However, since bacterial deconjugation has never been unequivocally outruled, it is not yet certain whether A^- and/or HA bile salt species are secretogenic. Studies in germ-free animals would be of considerable interest in this regard.

[6] The hypothesis that the aqueous HA species is secretogenic gains added support from two observations: *1*) deoxycholic acid which is also secretogenic has similar electrochemical properties to CDCA (pH ppt = 6.92, A^-/HA = 6(31)), which at neutral pH would allow for continuous replenishment of the intermicellar aqueous HA concentration (114 μM, Table 4) absorbed by passive nonionic diffusion; and *2*) cholic acid which is not secretogenic is the strongest of the unconjugated bile acids (pK'a, 5–5.5, pH ppt 6.5, (31)) and would be mainly soluble as the A^- species at colonic pH. The poor permeability of ionized bile salts by passive diffusion should thus limit cholic acid absorption. For these reasons the bulk and surface pH of the colon may play a key role in the secretory diarrhea produced by different bile acids.

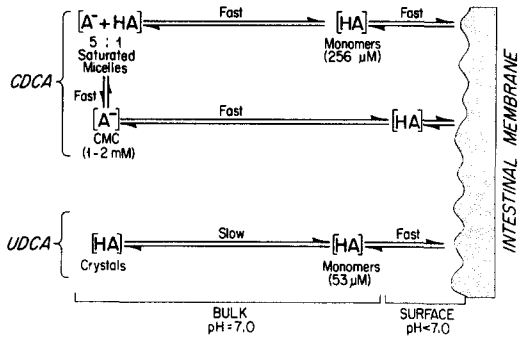

Fig. 12. Hypothetical physical states of UDCA and CDCA in the absence of other bile salts in the lumen of the colon at a bulk aqueous pH of 7.0 (0.15 M NaCl, 37°C) as interpreted from the potentiometric titration curves, aqueous solubilities of the bile acids (HA) and the critical micellar concentration of CDCA.[4] The microscopic surface pH, which is probably 1–2 units more acidic than the bulk aqueous pH (64, 65), is also suggested in this diagram. The electrochemical properties of CDCA facilitate a continuous and rapid flow of HA from the bulk aqueous phase into the absorptive membranes, whereas in the same environment UDCA is for all intents insoluble. Hence, only CDCA should be capable of exhibiting secretogenic properties in the colon. (See discussion for further details.)

salt milieu of the upper small intestine, it is necessary that orally administered unconjugated bile acids be solubilized. On account of its high precipitation pH values and tendency to form gels, it is apparent that with physiological conjugated bile salt concentrations in the duodenum–jejunum, UDCA would be poorly solubilized and absorbed, whereas CDCA should be solubilized and absorbed better. The results of recent studies (66–68) in bile-fistula dogs and rats provide some confirmation of this hypothesis, since the cumulative absorption and biliary secretion of equivalent doses of UDCA was significantly less than CDCA after oral administration. Since 90% of equivalent CDCA and UDCA doses were recovered in bile by 8 hr and 24 hr, respectively, the possibility exists that most ingested UDCA is only being absorbed by the ileum where an alkaline pH will facilitate its self-ionization, solubilization, and active transport.

We suggested from our phase equilibrium studies (28) that dietary taurine supplements, by altering the glycine–taurine ratio, would improve the cholesterol solubilizing capacity of UDCA conjugates in bile; likewise our results here suggest that a greater proportion of taurine conjugates in the duodenal-jejunal lumen would facilitate UDCA absorption by depressing its precipitation pH values and by eliminating gel formation. Further, dietary taurine should minimize the risk of precipitating GUDCA in bile or in gut luminal contents by offsetting the altered glycine–taurine ratio secondary to bile acid feeding (29). The fact that the bile of patients on urso-therapy apparently cannot be enriched with UCDA conjugates to the same degree as CDCA conjugates suggests that this may be related to the GUDCA content, since our observations suggest that when this conjugate reaches ≃70% of the total bile acids, its precipitation pH approximates the pH of bile.

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