Thermodynamic and Molecular Basis for Dissimilar Cholesterol-Solubilizing Capacities by Micellar Solutions of Bile Salts: Cases of Sodium Chenodeoxycholate and Sodium Ursodeoxycholate and Their Glycine and Taurine Conjugates†

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ABSTRACT: The bile salts chenodeoxycholate (CDC) and its 7β-hydroxy epimer ursodeoxycholate (UDC) are administered therapeutically (as acids) to dissolve cholesterol gallstones in man. Since their micellar solutions and those of their physiological conjugates differ strikingly in their capacities to solubilize cholesterol, we studied the interfacial and micellar properties of the epimers by a number of complimentary physical–chemical methods and correlated these with their solubilizing capacities. The critical micellar concentrations (cmc) estimated by surface tension, dye titration, and turbidimetry were similar (1–5 mM), varying slightly with the bile salt species, the method employed, NaCl concentration (0–1 M), and temperature (10–50 °C). The weight-average aggregation number (number of monomers per micelle, $\bar{n}_w$) at the cmc, derived from Debye plots of conventional light-scattering data and from the mean hydrodynamic radii of the micelles obtained by quasi-elastic light-scattering spectroscopy, revealed no appreciable differences between the UDC–CDC epimers or between their conjugates. From the mean hydrodynamic radii, the taurine conjugates were found to form larger micelles ($\bar{n}_w$ = 15–17) than the glycine conjugates ($\bar{n}_w$ = 13) which in turn were larger than the free species ($\bar{n}_w$ = 5), respectively. Consistent with previous experimental deductions, free and conjugated CDC micelles grew slightly in size with increases in total lipid concentration, but UDC micelles did not. With solubilization of cholesterol monohydrate, the mean sizes of UDC (13.4 Å) and of CDC (13 Å) micelles in 10 g/dL solutions did not change appreciably, even as the cholesterol saturation limit was reached. At the air–5 M NaCl (pH 2) interface, the glycine conjugates formed more expanded monomolecular films than the free acids, and both UDC and its glycine conjugate collapsed at surface pressures that were 10–20 mN m^{-1} lower than the collapse pressures of monolayers of CDC and its glycine conjugate. Similarly, adsorbed monolayers of ionized UDC and its taurine conjugate lowered the surface tension of water ~5 mN m^{-1} less than equimolar concentrations of CDC and its taurine conjugate. By employing high-performance reversed-phase liquid chromatography (HPLC), we measured the relative hydrophilic–hydrophobic properties of the bile salts and found a close correlation between HPLC mobility and cholesterol-solubilizing capacity. Assuming a single cholesterol binding site per micelle, we estimated from the $\bar{n}_w$ values and bile salt/cholesterol saturation ratios that the magnitude of the cholesterol binding constant (K) was 5.7 × 10^6 L/mol for unconjugated CDC and 2.5 × 10^5 L/mol for unconjugated UDC at 30 °C. These results suggest that the differences in cholesterol-solubilizing capacities of CDC and UDC and their conjugates are due to subtle differences in micellar structure, resulting from the axial or equatorial orientation of the 7-hydroxyl function and the various conjugating groups. The differences most likely occur at the micellar surface where the hydroxyl and conjugating groups would project into the aqueous phase and hence would imply a strong interaction between the solubilized cholesterol and the micellar surface. The data further suggest that the more hydrophilic bile salts (taurine > glycine conjugates > free species and UDC > CDC, as inferred by HPLC mobility) solubilize cholesterol less well, presumably because the solubilized molecules interfere with hydrophilic interactions at the micellar surface. The possible molecular origins of this interaction are discussed.

The dihydroxy bile acids, chenodeoxycholic (3α,7α-dihydroxy-5β-cholanoic acid, CDC)[1] and ursodeoxycholic (3α,7β-dihydroxy-5β-cholanoic acid, UDC), are of exceptional medical as well as scientific interest since in contrast to the other common bile acids of man (Thistle & Hofmann, 1973; LaRusso et al., 1977) they induce dissolution of cholesterol gallstones after oral administration (Danzinger et al., 1972; Makino et al., 1975). CDC and UDC enrich the bile salt pool with their glycine and taurine conjugates, decrease the secretion rates of cholesterol into bile, and in many cases desaturate bile with cholesterol (Stiehl et al., 1978). The molecular basis for this metabolic effect is uncertain (Anderson, 1979), but suppression of hepatic cholesterol synthesis (Coyne et al., 1976) and a subtle alteration in the physical–chemical coupling between cholesterol and phospholipid (lecithin) secretion rates (Lindblad et al., 1977; Einarsson & Grundy,

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[1] Abbreviations used: cmc, critical micellar concentration; CDC, chenodeoxycholic acid (3α,7α-dihydroxy-5β-cholanoic acid); UDC, ursodeoxycholic acid (3α,7β-dihydroxy-5β-cholanoic acid); prefixes T and G represent taurine and glycine conjugates, respectively; HPLC, high-performance liquid chromatography; $\bar{n}_w$, weight-average aggregation number (number of monomers per micelle); QLS, quasi-elastic light-scattering spectroscopy; R_h, mean hydrodynamic radius.

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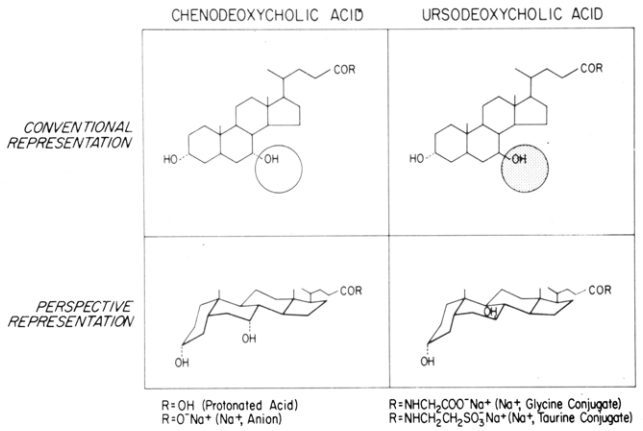

FIGURE 1: Conventional and perspective chemical representations of chenodeoxycholic (3α,7α-dihydroxy-5β-cholanoic) and ursodeoxycholic (3α,7β-dihydroxy-5β-cholanoic) acids. The bile acids are related structurally by epimeric inversion of the hydroxyl group at the C-7 position (open and stippled circles). R represents the side chain modifications which give the protonated acid, the sodium salt, and the two physiological conjugates studied in this work.

1980) have been suggested. Despite the therapeutic efficacy of these agents, the cholesterol-solubilizing capacity of UDC and its conjugates is far less than the capacity of CDC and its conjugates even in the presence of physiological lecithin contents (Carey, 1978; Carey & Ko, 1979). Until recently (Mazer et al., 1979), only sparse and, with a single exception (Small, 1968), unsystematic information was available on the micellar properties of some of these bile salts (Yoshimuta, 1960; Miyake et al., 1962; Norman, 1960; Hofmann, 1963; Oh et al., 1977). Mazer et al. (1979), using quasi-elastic light-scattering spectroscopy, found that under physiologic conditions (bile salt concentration 10 g/dL, temperature 37 °C, 0.15 M NaCl) the mean micellar size of the taurine (T) conjugate of CDC (TCDC, 18 Å) was similar to that for TUDC micelles (16.5 Å), whereas at high NaCl concentration (0.6 M NaCl) and low temperature (20 °C) the size of TCDC micelles (50 Å) had grown substantially larger than that of TUDC micelles (30 Å). These and other data were shown to be quantitatively compatible with primary and secondary structures for the bile salt micelles, whose relative populations were dependent on temperature, bile salt and NaCl concentration, and bile salt species (Small, 1968; Mazer et al., 1979).

We have now investigated the solution properties of CDC and UDC both as free salts and as glycine and taurine conjugates as functions of a number of additional physical–chemical variables, including those of physiological relevance and, in addition, have examined their interfacial and hydrophilic properties employing both surface balance, surface tension, and high-performance liquid chromatography (HPLC) methods. We quantitatively analyze our results to show that an understanding of the striking differences in cholesterol-solubilizing capacities of the epimers is forthcoming from a thermodynamic and molecular evaluation of their micellar and interfacial properties. The study allows us to evaluate the influence of the orientation of the C-7 hydroxyl function (Carey & Small, 1970, 1972) as well as the influence of the nature of the ionic side chain (conjugating group) on the physical–chemical properties of the bile salt solutions and suggest an important role of the micellar surface in the cholesterol-solubilizing process.

Experimental Procedures

Materials. Samples of free bile acids (Figure 1) were generously supplied by Dr. Herbert Falk (GmbH Co., Freiburg, West Germany) and by Dr. W. O. Godfredson (Leo Pharmaceutical Products, Ballerup, Denmark). Taurine and glycine conjugates (Na^+ salts, Figure 1) were obtained from Calbiochem (San Diego, CA), the Tokyo Tanabe Pharmaceutical Co. (Tokyo, Japan), and the Eisai Co. Ltd. (Tokyo, Japan). Only bile acids and salts which gave a single spot (purity > 98%) by thin-layer chromatography (200-μg application) were studied further. For detection of volatile acidic impurities, 1 g/dL solutions were titrated from approximately pH 11.0 to 2.0 with 2 N HCl, and, when a correct titration curve was not obtained (Small, 1971; Igimi & Carey, 1980), the bile salt was usually washed with diethyl ether and recrystallized from dilute ethanol or ether–ethanol mixtures (Igimi & Carey, 1980). Free bile acids were found to be >99% pure by gas–liquid chromatography employing standard methods and by titration with HCl (Igimi & Carey, 1980). Anhydrous cholesterol was obtained from Nu-Chek Prep (Austin, MN) and recrystallized several times from hot ethanol to achieve a >99.5% purity by thin-layer chromatography (mp 150 °C, uncorr). [1,2-$^3H_2$]Cholesterol (New England Nuclear Co., Boston, MA) was of specific activity (0.5 mCi/mmol) identical with that described elsewhere (Igimi & Carey, 1981) and was chemically and radiochemically pure (>99.5%) by thin-layer and gas–liquid chromatography. The radiolabel was stable under the experimental conditions (Igimi & Carey, 1981). A highly purified dye-laser grade of Rhodamine 6G (Matheson, Coleman and Bell, Norwood, OH) was employed for estimating the critical micellar concentrations (Carey & Small, 1969). Baker (Phillipsburg, NJ) reagent grade NaCl was roasted in a muffle furnace at 600 °C for 2–6 h before use to remove organic contaminants. Hexane was a "Lipopure" product supplied by Applied Science (State College, PA). Filtered deionized and glass-distilled water (Corning Glass Works, Corning, NY) was used throughout. Prior to surface balance measurements, the distilled water was filtered through a column of activated charcoal. Pyrex glassware was alkali–acid washed by steeping overnight in tanks of EtOH–2 N KOH (50:50 by volume) and in 1 N HNO_3 followed by thorough rinsing in running distilled water.

Methods. (*a*) *Solutions.* The sodium salts of CDC and UDC were prepared by dissolving a quantity of the acids in MeOH, adding a stoichiometric amount of 2 N NaOH to give a final solution pH of 10.0, and evaporating the solvent under reduced pressure to achieve constant weight. Bile salt solutions were composed on a w/v basis with the appropriate aqueous solvents. With unconjugated bile salts and glycine conjugates the solvent was 0.01 M carbonate–bicarbonate buffer (pH 10 ± 0.2), adjusted to the desired ionic strength with NaCl. The pH of TCDC and TUDC solutions was simply adjusted to pH 7.0 ± 0.5 with 2 N NaOH. Since these bile salts are rendered insensitive to variations in pH (Matschiner, 1973) due to their very low pK_a values (<1.8), no buffer was employed. Unless otherwise specified, all studies were carried out at 23 ± 0.5 °C.

(*b*) *Dye Titration.* Critical micellar concentrations (cmc) were estimated from the λ_{max} of 2.5×10^{-6} Rhodamine 6G (Cary-Varian 118C recording spectrophotometer, Varian Associates, Palo Alto, CA) plotted as a function of added bile salt concentration as described (Carey & Small, 1969). The cmc values were obtained for a range of added Na^+ concentrations (0.01 to ~1.5 M Na^+) and in the case of TCDC and TUDC for a number of temperatures between 10 and 50 °C in 0.15 M NaCl. During these experiments, water at the required temperature (±0.05 °C) was circulated continuously around the thermostated cuvettes by utilizing a Haake Model

FE water bath (Haake, Inc., Saddlebrook, NJ) to which was coupled a Neslab PBC-4 bath cooler (Neslab Instruments, Inc., Portsmouth, NH).

(*c*) *Surface Tension.* Equilibrium surface tensions were measured with an automatic Dognon-Abribat tensiometer (Prolabo, Paris, France) employing a platinum Wilhelmy (2 × 1 cm) blade (Carey & Small, 1971; Carey et al., 1975). The Wilhelmy blade was washed thoroughly in distilled water and heated to incandescence between each measurement, and the precise dimensions were checked with calipers (to ±0.1 mm) between each series of studies. The surface of the solutions was first marked with a few grains of roasted talc and then aspirated with a suction line and air jet. Surface tensions were recorded as a function of time until stable and reproducible values were obtained (~10–60 min). In general, 12 bile salt concentrations (0.125–15 mM) were studied at each concentration of added NaCl. The sharp break points in the curves provided estimates of the cmc of the bile salts, and the interfacial areas of the bile salt molecules were calculated from the steep portions of the curves by using the modified form of the Gibbs adsorption isotherm (Kratohvil & DelliColli, 1968; Carey & Small, 1971).

(*d*) *Conventional Light Scattering.* A Brice-Phoenix light-scattering apparatus (Model 2000) with an incandescent light source (wavelength 546 nm) was employed (Carey et al., 1975). A total of 16 solutions of each bile salt (0.0125–2 g/dL) were prepared with 0.14 M NaCl in 0.01 M carbonate–bicarbonate buffer (pH 10.0 ± 0.2). The solutions were filtered through prewetted Sartorius membranes (pore diameter ~0.2 μm) under low N_2 pressure into octagonal cells of 30 mL capacity, and the mean scattered light intensity was measured at angles of 0° and 90°. The refractive index increment (dn/dc) for the same series of solutions was determined with a Brice-Phoenix differential refractometer (Model BP 2000 V) at a wavelength of 546 nm as described (Carey et al., 1975). In these experiments the solution turbidity (τ_{soln}) was plotted as a function of bile salt concentration (mg/mL), and the interpolated concentrations where the turbidity began to increase steeply were taken as estimates of the cmc values. Estimates of the weight-average micellar weights and anhydrous aggregation numbers (number of monomers per micelle, $\bar{n}_w$) were derived by linear extrapolation of Debye plots of the light-scattering data as described (Kratohvil & DelliColli, 1968).

(*e*) *Quasi-Elastic Light Scattering Spectroscopy (QLS).* The apparatus, methods, and calculations employed have been described by us in detail previously (Mazer et al., 1976, 1979). The scattering angle was kept at 90°, and the cell temperature was 25 ± 0.1 °C. The micellar solutions [0.15 M Na^+, pH 10.0 (free and glycine conjugates) or pH 7.0 (taurine conjugates)] were pipetted directly into cylindrical glass scattering cells of 1-mL capacity and first centrifuged at 15000*g* for 30 min to sediment dust. The mean translation diffusion coefficients of the micelles were measured at four or five bile salt concentrations (0.63–10 g/dL) by utilizing the refractive indices measured directly as described under (*d*), and from these values the mean hydrodynamic radii ($\bar{R}_h$) of the micelles were derived. Estimates of the weight-average aggregation number ($\bar{n}_w$) were calculated from the $\bar{R}_h$ values as described by Mazer et al. (1979).

(*f*) *Surface Balance.* A Langmuir–Pockels surface balance with a Teflon trough and horizontal float was employed (Phillips & Chapman, 1968). The subphase was 5 M NaCl adjusted to pH 2.0 with HCl at 22 ± 2 °C. Free and glycine-conjugated bile acids were spread from 3:1 (v/v) hexane–ethanol, and after 5 min was allowed for solvent evaporation, the film was compressed manually over a period of 10 min (15 Å molecule^{-1} min^{-1}). Due to very slight bulk solubility of the bile acids (Igimi & Carey, 1980), the isotherms were sensitive, especially at high film pressures, to the rate off compression and time of spreading. These effects caused some irreproducibility so that, at a given film pressure, the molecular areas can only be considered accurate to within ±10 Å.

(*g*) *Equilibrium Spreading Pressure.* Equilibrium spreading pressures (π_e) were measured by first positioning the horizontal float and barrier of the surface balance so that the total surface area of the trough was exactly 50 cm^2 [subphase as described under (*f*)]. After a portion of the bile acid [crystallized as described earlier (see Methods)], was sprinkled on the interface, the surface pressure was monitored as a function of time until no further increase could be detected. Repeat additions of bile acid crystals were carried out at frequent intervals until, in the presence of excess crystals, the surface pressure had reached a constant value. This final pressure was taken as the equilibrium spreading pressure (Kellner & Cadenhead, 1978).

(*h*) *High-Performance Liquid Chromatography.* High-performance liquid chromatography (HPLC) was performed on a 250 × 4.6 mm reversed-phase octadecylsilane column (Altex Ultrasphere ODS) by employing an Isocratic liquid chromatograph Model 330 (Beckman Instruments, Inc., Fullerton, CA), with a Model 110A pump and Model 210 injection valve. The detection system was a variable wavelength UV–vis spectrophotometer Model 100-40 (Hitachi Ltd., Tokyo, Japan), set at 210 nm. Peak retention times were recorded and then integrated with a computing integrator Model CRI-A (Shimadzu Corp., Kyoto, Japan). The modile phase consisted of 75% aqueous methanol (by volume) buffered to pH 5.0 with 0.005 M KH_2PO_4–H_3PO_4. Injected bile salt concentrations were 100 mg/dL. All retention times were normalized to the retention of the solvent front as described (Bloch & Watkins, 1978).

(*i*) *Cholesterol Monohydrate Solubility in Bile Salt Solutions.* In pilot phase equilibria studies, an estimate of this value was obtained by composing multiple mixtures of bile salts and cholesterol with small increments of cholesterol between mixtures (Carey & Small, 1978). Then a series of 10 g/dL (0.15 M Na^+, pH 10) mixtures were prepared which contained anhydrous cholesterol in a concentration slightly in excess of the maximum cholesterol monohydrate solubility. The mixtures were equilibrated for at least 8–10 days in a water bath at 37 °C with continuous shaking. The excess cholesterol (monohydrate) was then removed by filtration (0.2-μm Millipore membranes, Millipore Corp., Lexington, MA), and the saturated mixed micellar solutions were assayed for both cholesterol and bile salts as described (Carey & Small, 1978). Since chemical analysis was not sufficiently sensitive for use with the UDC series, powder dissolution of radiolabeled [1,2-^{3}H]cholesterol monohydrate in 100 mM bile salt solutions was carried out to equilibrium as described (Igimi & Carey, 1981). The results are expressed in millimoles of cholesterol/liter of solution and as the bile salt/cholesterol molar ratio at saturation.

Results

Micelle Formation. (1) cmc Values. Preliminary investigations revealed that the critical micellar temperatures (Krafft points) of all the bile salts studied were <0 °C and thus were freely soluble as anions (Igimi & Carey, 1980) at the pH, ionic strength, and temperature studied. The cmc's estimated by dye titration and surface tension are plotted as

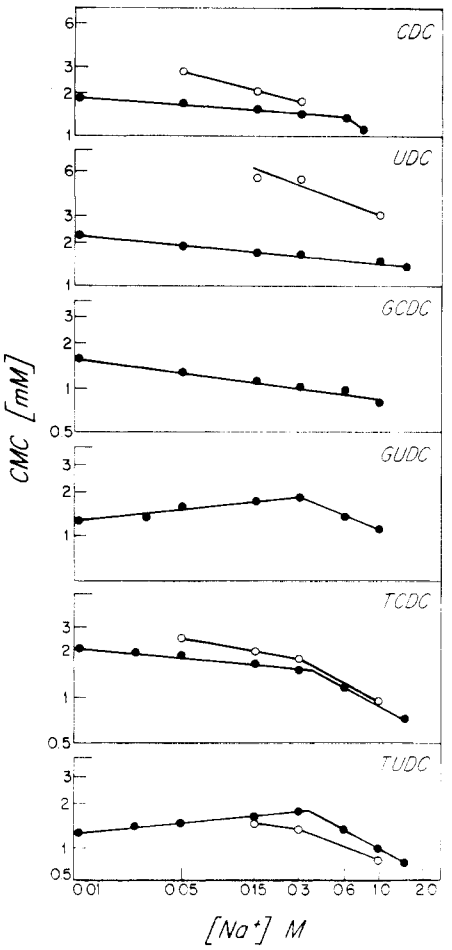

FIGURE 2: Double-logarithmic plots of the critical micellar concentrations (cmc, millimolar) of the bile salts vs. the added counterion (Na^+) concentration (molar) at 23 ± 1 °C. The cmc values (○) were estimated by surface tension (Figure 7) and by the spectral shift of 2.5×10^{-6} M Rhodamine 6G (●) at pH 10.0 (free and glycine conjugates) and at pH 7.0 (taurine conjugates).

a function of added Na^+ concentration as double-logarithmic plots in Figure 2. In Table I, the values by both methods (in 0.15 M Na^+) are listed for comparison with the cmc's derived by conventional light scattering. In general, the values (Table I) fall between 1 and 5 mM depending slightly on the bile salt species and the method employed. However, the dye titration method tends to give slightly lower values for steroid detergents than do the noninvasive (surface tension and turbidimetry) methods, as noted before (Carey et al., 1975). Up to the Na^+ concentraton where salting out commences (break points in the curves, Figure 2), the cmc values by both surface tension and dye titration can be fitted by straight lines and, in general, decrease slightly as the Na^+ concentration is increased. The apparent increase in the cmc's of GUDC and TUDC by dye titration is likely to be an artifact of this method since the spectral shift is dependent on the solubilization of a dye–detergent ion-pair complex (Mukerjee & Mysels, 1970). Further, this pattern was not reproduced with unconjugated UDC nor in the surface tension studies of TUDC (Figure 2).

The influence of variations in temperature (10–50 °C) on the cmc's (in 0.15 M NaCl) of TCDC and TUDC are compared to the cmc's of the other common taurine-conjugated bile salts by the dye titration method (Carey & Small, 1969; Carey et al., 1979) in Figure 3. The cmc values display a possible minimum in the vicinity of 10–25 °C, and at higher temperatures some increase in cmc occurs. At 37 °C (arrow) taurocholate (TC) exhibits the highest cmc and taurolithocholate sulfate ($TLCSO_4$) the lowest. A thermodynamic

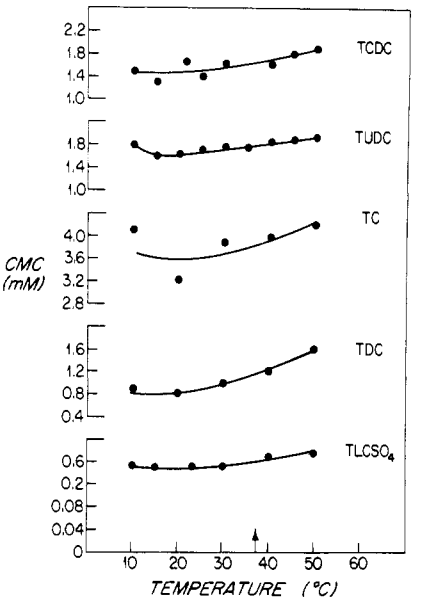

FIGURE 3: Temperature dependence of the critical micellar concentrations (cmc, millimolar) of TCDC and TUDC compared with the cmc values of the other common taurine-conjugated bile salts of man, all estimated by the spectral shift of 2.5×10^{-6} M Rhodamine 6G (0.15 M NaCl, pH 7.0). The values for taurocholate (TC) and taurodeoxycholate (TDC) are from Carey & Small (1969), and the values for taurolithocholate sulfate ($TLCSO_4$) are from Carey et al. (1979). At 37 °C (vertical arrow) the ordering of cmc values is TC > TUDC = TCDC > TDC > $TLCSO_4$.

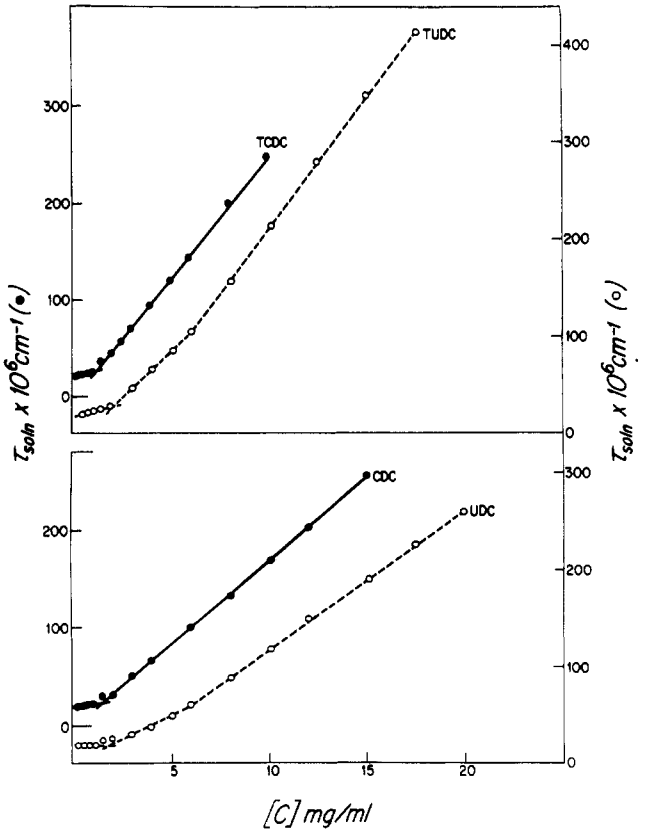

FIGURE 4: Plots of solution turbidity ($\tau_{soln} \times 10^6$ cm^{-1}) as a function of bile salt concentration (C) for TCDC and TUDC at pH 7.0 and CDC and UDC at pH 10.0 (23 °C, 0.15 M Na^+). The lowest bile salt concentrations corresponding to the intersections of the curves were taken as estimates of the cmc (Table I).

analysis of such data has been given previously (Carey & Small, 1969).

The cmc values of UDC and CDC and of TUDC and TCDC were also obtained from the intersections of the gradual

Table I: Micellar and Molecular Data for Chenodeoxycholic and Ursodeoxycholic Acids[a]

Na salt	cmc (mM) dye titration	cmc (mM) surface tension	cmc (mM) light scattering	solubility (mM) of cholesterol (37 °C) as a function of total lipid concn: 192–240 mM (10 g/dL) ChM	100 mM[b] (~5 g/dL) ChM	100 mM[b] (~5 g/dL) ChA	pK_a (20 mM, 37 °C)[c]	solubility of 20 mM in NaCl (M)	molecular area[d] (Å²)	$\bar{R}_h$ at cmc[e] (Å)	$\bar{n}^f$ QLS	$\bar{n}^f$ CLS
CDC	1.5	2.0	3.2	12.1 (18:1)[g]	16:1[g]	13:1[g]	6.44	sol 0.8 M, insol 1.0 M	88 (87)	12.0	5	10
UDC	1.7	5.5	4.3	0.81 (298:1)	303:1	265:1	5.97	sol 1.5 M, insol 2.0 M	85 (93)	11.5	5	7
GCDC	1.1	nd[h]	nd[h]	5.43 (39:1)	45:1	33:1	4.60	sol 1.0 M, insol 1.5 M	nd[h] (97)	16.5	13	nd[h]
GUDC	1.7	nd	nd	0.65 (326:1)	526:1	278:1	4.92	sol 1.0 M, insol 1.5 M	nd (105)	16.5	13	nd
TCDC	1.8	2.0	2.5	3.32 (54:1)	68:1	50:1	<2.0	sol 1.5 M, insol 2.0 M	89 (nd)	17.5	15	16
TUDC	1.6	1.3	3.8	0.37 (518:1)	770:1	357:1	<2.0	sol 1.5 M, insol 2.0 M	79 (nd)	18.0	17	15

[a] Conditions unless otherwise specified are pH 10.0 (free and glycine conjugates) and pH 7.0 (taurine conjugates) at 23 °C in 0.15 M Na^+. [b] From Igimi & Carey (1981); ChM, cholesterol monohydrate; ChA, anhydrous cholesterol. [c] From Igimi & Carey (1980). [d] By surface tension in 0.3 or 1.0 M Na^+; values in parentheses are derived from the monolayer collapse point of the π–A isotherms (Figure 8). [e] Mean hydrodynamic radius extrapolated to the cmc (Figure 6). [f] $\bar{n}_w$ rounded off mean aggregation numbers at the cmc from $\bar{R}_h$ values (by quasi-elastic light-scattering spectroscopy QLS) and conventional light scattering (CLS). [g] Bile salt to cholesterol molar ratios at saturation; values from the present work and from Igimi & Carey (1981). [h] nd, not determined.

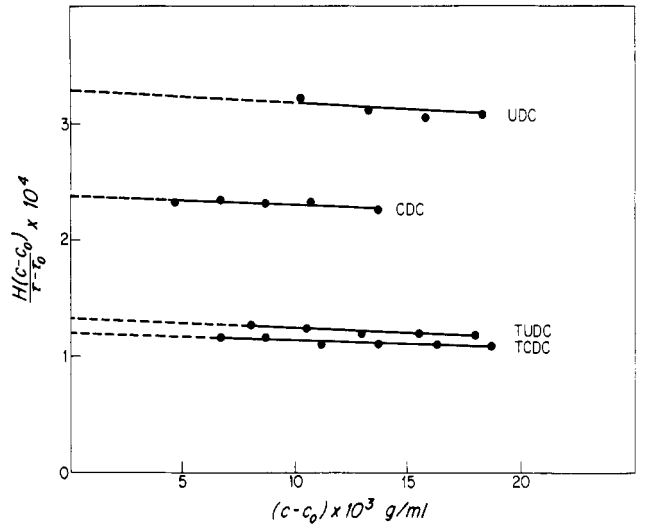

FIGURE 5: Debye plots of conventional light-scattering data (0.15 M Na^+, 23 °C) for UDC and CDC (pH 10.0) and TUDC and TCDC (pH 7.0) where $\tau - \tau_0$ represents the turbidity of the bile salt solution (τ) minus the turbidity at the cmc (τ_0) (from Figure 4) and $c - c_0$ represents the bulk bile salt concentration (c) minus the cmc (c_0) (from Figure 4). H represents the Einstein optical constant listed in Table II.

and steep straight lines of each set of solution turbidity data as shown in Figure 4 (values listed in Table I). In general, the cmc values for the UDC and CDC conjugates found here are comparable to careful estimates, 1.5 mM (TCDC), 1.0 and 1.5 mM (TUDC), and 1.5 and 2.0 mM (GUDC), obtained by noninvasive techniques (surface tension, conductivity) in the literature (Miyake et al., 1962; Yoshimuta, 1960). However, these values are generally less than the "apparent" cmc values obtained in solubilization studies (Norman, 1960; Hofmann, 1963).

(2) *Micellar Aggregation Numbers* ($\bar{n}_w$) *and Size* ($\bar{R}_h$). Conventional light-scattering measurements were plotted according to the method of Debye (Figure 5). In these plots the cmc (c_0) values were those obtained in the turbidity (light-scattering) measurements, i.e., 2.5–4.3 mM (Figure 4; Table I).[2] Following accepted procedure (Prins & Hermans, 1956; Kratohvil & DelliColli, 1968), the extrapolated intercepts (I values in Table II) of the plots are equal to the reciprocal of the weight-average micellar mass ($1/M$) at the cmc, and slope S (Table II) is a function of the micellar charge, micellar interactions, and polydispersity, in addition to the aggregation number ($\bar{n}_w$). Aggregation numbers were obtained by dividing the micellar mass by the anhydrous molecular weight of each bile salt. No corrections were made for the so-called thermodynamic "preferential interactions" (Kratohvil & DelliColli, 1970). These $\bar{n}_w$ values are given in Table II and are rounded off the nearest whole number for comparison with $\bar{n}_w$ values derived by quasi-elastic light-scattering spectroscopy (QLS) in Table I.

The mean hydrodynamic radii ($\bar{R}_h$) of the bile salt micelles as deduced by QLS are plotted vs. bile salt concentration (0.625–10 g/dL, 0.15 M NaCl, 25 °C) for UDC and CDC and their respective glycine and taurine conjugates in Figure 6. The dependence of $\bar{R}_h$ on concentration can in all cases be described by straight lines and allows us to extrapolate micellar size and estimate aggregation number ($\bar{n}_w$; Mazer et

[2] It should be noted that the Debye plots are quite sensitive to the choice of cmc values. The $\bar{n}_w$ values tabulated in Table I are ~1 monomer less when the cmc values estimated by dye titration (1.5–1.8 mM) are employed in the calculations.

Table II: Conventional Light-Scattering Results[a]

bile salt (Na^+)	dn/dc[b] (mL/g)	$H^c \times 10^6$ (cm^2/g^2)	$I^d \times 10^4$ (mol/g)	$S^e \times 10^4$ (mol mL/g^2)	micellar wt (g/mol)	mean aggregation no. ($\bar{n}_w$)[f]
CDC	0.191	3.99	2.38	−8.5	4201.7	10.1 (10)
UDC	0.191	3.99	3.28	−11.3	3048.8	7.4 (7)
TCDC	0.166	3.00	1.20	−5.4	8333.3	15.9 (16)
TUDC	0.165	2.97	1.32	−8.3	7575.8	14.5 (15)

[a] Conditions: pH 10.0 (free bile salts, CDC and UDC), pH 7.0 (taurine conjugates, TCDC and TUDC) 0.15 M Na^+, 23 °C. [b] Refractive index increment. [c] Einstein optical constant. [d] Intercept of Debye plots (Figure 5). [e] Slope of Debye plots (Figure 5). [f] Number of monomers per micelle at the cmc (rounded off values in parentheses).

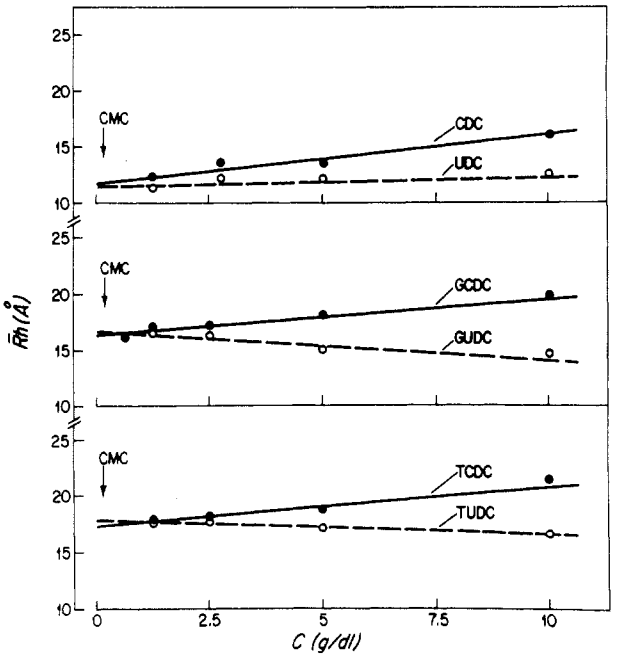

FIGURE 6: Mean hydrodynamic radii ($\bar{R}_h$) of the bile salt micelles as a function of bile salt concentration as deduced by quasi-elastic light-scattering spectroscopy (25 °C, 0.15 M NaCl, pH 10.0 free and glycine conjugates, pH 7.0, taurine conjugates). The $\bar{R}_h$ values of each epimer are identical at the cmc values (arrows, from dye titration estimates; Table I).

al., 1979) at the cmc. In this way we find that the $\bar{R}_h$ values for UDC and CDC are both approximately equal to 11.5–12.0 Å at the cmc and that the sizes of the glycine and taurine conjugates do not differ between epimers but are slightly larger, 16.5 and 17.5–18.0 Å, respectively. In contrast, the slopes describing the concentration dependence of $\bar{R}_h$ are different for the two epimeric species, having a zero or negative value for the 7β species and a positive value for the 7α species. Although these slopes reflect both the intermicellar interactions and the possible concentration dependence of micellar size (Mazer et al., 1979), the comparison between epimers would suggest that the micelles formed by the 7α species would have a greater tendency to form the large secondary micelles than the 7β species, consistent with previous studies (Mazer et al., 1979). In contrast, Oh et al. (1977), using QLS, have obtained $\bar{R}_h$ data for GCDC (21.9 Å) and TCDC (32.2 Å) micelles that are in extremely poor agreement with both the present and previous data (Small, 1968).

The influence of solubilized cholesterol on the $\bar{R}_h$ values measured in 10 g/dL micellar solutions (0.15 M NaCl, 25 °C) was also studied. With bile salt/cholesterol molar ratios corresponding to the saturation limits of ~20:1 (CDC) and 300:1 (UDC), the $\bar{R}_h$ values were found to be 13.0 and 13.4 Å, respectively. These were little changed from the values deduced at 10 g/dL for the pure bile salts, 16 (CDC) and 12.5 (UDC) Å, and suggest that cholesterol incorporation does not markedly alter mean micellar size.

Cholesterol Solubilization. The results for equilibrium cholesterol monohydrate solubilities in 10 g/dL mixtures at 0.15 M Na^+ and 37 °C reveal a number of striking differences between UDC and its conjugates compared with CDC and its conjugates (Table I). First, CDC micelles whether free or conjugated solubilize more than 10 times more cholesterol than free or conjugated UDC micelles. Second, the free salts of either species solubilize significantly more cholesterol than the glycine conjugates, which in turn solubilize more cholesterol than the taurine conjugates (Table I). On a molar basis, 298, 326, and 518 molecules of UDC and its glycine and taurine conjugates are required to solubilize 1 molecule of cholesterol monohydrate in 10 g/dL solutions, compared with 18, 39, and 54 molecules in the case of CDC and its glycine and taurine conjugates. The molar ratios obtained for CDC, GCDC, and TCDC in the present work have an ordering and magnitude similar to the values obtained earlier by Igimi et al. (1977) and Hegardt & Dam (1971) (17:1, 34:1, and 47:1, respectively) in more dilute (100 mM bile salt) solutions and were recently verified in our laboratory (Igimi & Carey, 1981) by dissolution of cholesterol monohydrate and anhydrous cholesterol to equilibrium in 100 mM bile salt solutions (data tabulated for comparison in Table I). However, our results for equilibrium cholesterol solubility in UDC, GUDC, and TUDC are at variance with two previous estimates; Yoshimuta (1960) obtained low saturation ratios of 35–42:1 for UDC and its conjugates, whereas Igimi et al. (1977) obtained a spuriously high value of 1842:1 for UDC. The ordering and magnitude of our results for the UDC series are, however, in close agreement with recent estimates (Igimi & Carey, 1981) obtained by dissolution of radiolabeled cholesterol in 100 mM bile salt solutions (data also tabulated in Table I).

Surface Properties. The equilibrium surface tensions of free and taurine-conjugated CDC and UDC in ionic strengths of 0.05–1.0 M Na^+ are plotted semilogarithmically against bile salt concentration (mM) in Figure 7. Each set of data is well fitted by two straight lines which intersect at a bile salt concentration customarily taken as the cmc of the detergents (values plotted in Figure 2 and Table I). Note that the sharp break points found in all cases are similar to those found with classical detergents (Shinoda et al., 1973). The equilibrium surface tensions of equimolar CDC and TCDC in the same ionic strength are essentially identical. However, these values are as much as 5 mN m^{-1} lower than the surface tensions generated by equimolar concentrations of UDC and TUDC under the same conditions. From the slopes of these curves just below the break points, the interfacial molecular areas of the bile salts were derived by means of the modified Gibbs adsorption isotherm equation. For solutions containing a swamping excess of NaCl, this equation can be written in the form

$$\Gamma = -\frac{1}{RT}\frac{d\gamma}{d \ln c} \quad (1)$$

where Γ represents the surface excess concentration (g/cm^2),

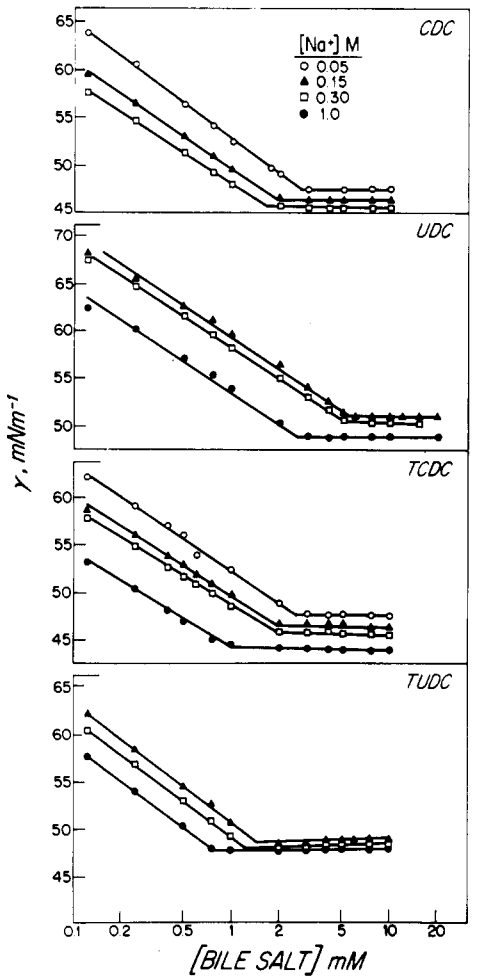

FIGURE 7: Dependence of equilibrium (Wilhelmy) surface tensions (mN m^{-1}) at 23 °C (pH 10.0, CDC and UDC; pH 7.0, TCDC and TUDC) on bile salt concentration plotted logarithmically (millimolar). The supporting electrolyte (Na^+) concentrations are given in the inset in *M*. The bile salt concentrations corresponding to the break points in the curves provide an estimate of the cmc of each bile salt (Figure 2; Table I). The pre-cmc slopes were utilized to calculate the interfacial area per molecule (Table I) by means of the modified Gibbs adsorption isotherm (eq 1).

γ is the surface tension (mN m^{-1}), c is the bulk bile salt concentration (g/dL), and R and T are the gas constant and absolute temperature, respectively. This form of the Gibbs equation assumes that the activity coefficients are independent of the bile salt concentration and approach unity (Kratohvil & DelliColli, 1968). The molecular areas (A) are then obtained from

$$A = 1/(\Gamma N_A) \quad (2)$$

where N_A is Avogadro's number. The calculated A values in $Å^2$ were 78–88 (CDC), 79–85 (UDC), 85–89 (TCDC), and 75–79 (TUDC), depending slightly upon the ionic strength. The "best" values obtained with the highest ionic strengths (0.3–1 M Na^+) are listed in Table I. No significant differences in molecular areas are observed with either of the epimers or with their respective conjugates.

In Figure 8, the pressure (π)–molecular area ($Å^2$) isotherms for undissociated (protonated) UDC and CDC and their glycine conjugates are displayed. The isotherms indicate that all four bile acids form coherent films at ~200 $Å^2$/molecule and have similar shapes when π is <30 mN m^{-1}, except that the glycine conjugates form slightly more expanded films [100–110 $Å^2$ (CDC and UDC) and 120–130 $Å^2$ (GCDC and GUDC)]. Upon further compression, significant differences

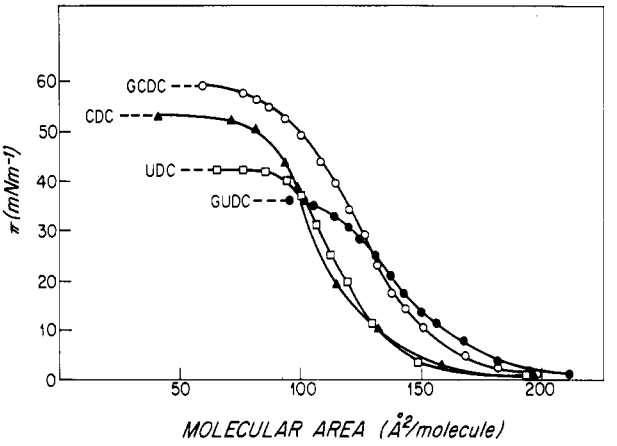

FIGURE 8: Surface pressure (π, mN m^{-1}) – molecular area (A, $Å^2$/molecule) isotherms (22 ± 2 °C) of free and glycine-conjugated bile acids on a subphase of 5 M NaCl (pH 2.0); (▲) CDC (chenodeoxycholic acid), (□) UDC (ursodeoxycholic acid), (○) GCDC (glycochenodeoxycholic acid), (●) GUDC (glycoursodeoxycholic acid).

are detectable: CDC and GCDC monolayers begin to collapse at ~40–50 mN m^{-1}, whereas UDC and GUDC monolayers collapse at ~30–40 mN m^{-1}. Presumably because of differences in substrate and compression rate, these collapse pressures are somewhat higher than those reported earlier for deoxycholic (Small, 1971) and chenodeoxycholic (Ekwall & Ekholm, 1957) acids spread on 3 M NaCl. Despite these absolute variations, the relative differences in the collapse pressures are real because the four bile acids were handled identically throughout. The relative differences in collapse pressure correlate with the equilibrium spreading pressures (π_e) (which are obtained at zero compression rate; see Methods) of CDC–GCDC monolayers and of UDC–GUDC monolayers which are 40 ± 5 and 30 ± 5 mN m^{-1}, respectively. Different preparations of the bile acids were apparently crystal polymorphs, since π_e for a given chromatographically pure material depended to some extent on how it was crystallized prior to study. The data in Figure 8 show that the molecular areas of the bile acids at values of π equal to the above π_e values were 93–102 (CDC), 108–121 (GCDC), 104–114 (UDC), and 106–131 (GUDC) $Å^2$, respectively. Estimates of the molecular areas of the close-packed films from intersections of tangents to the π–A curves in the vicinity of the collapse region were 87 (CDC), 97 (GCDC), 93 (UDC), and 105 (GUDC) $Å^2$, respectively. Further compression of the surface films to 20 $Å^2$/molecule did not lead to a further abrupt increase in π. All molecular areas at the collapse pressure as derived in these experiments are similar to those of the anions obtained by the Gibbs adsorption isotherm (Table I). Further, they are in essential agreement with values (87–95 $Å^2$) obtained from surface tension plots of taurodeoxycholate and glycodeoxycholate (Kratohvil & DelliColli, 1968) and from surface balance studies (80–90 $Å^2$) on deoxycholic, chenodeoxycholic, lithocholic, and cholic acids (Ekwall & Ekholm, 1957; Leal Lõpez et al., 1966; De Moerloose, 1960; Miñones-Trillo et al., 1968; Small, 1971). Hence free and conjugated UDC give, within experimental error, identical surface monolayer areas as do the other common bile salts and their acids and confirm that all di- and trihydroxy bile salts lie flat at interfaces with the polar groups projecting into the aqueous phase.

HPLC Properties. In Figure 9 the normalized retention times $(\text{mobility})^{-1}$ of CDC and UDC and their respective glycine and taurine conjugates are plotted vs. the means ± 2 SD of the cholesterol-solubilizing capacities (S^{-1}) where S is defined as the ratio of moles of bile salt per mole of cholesterol

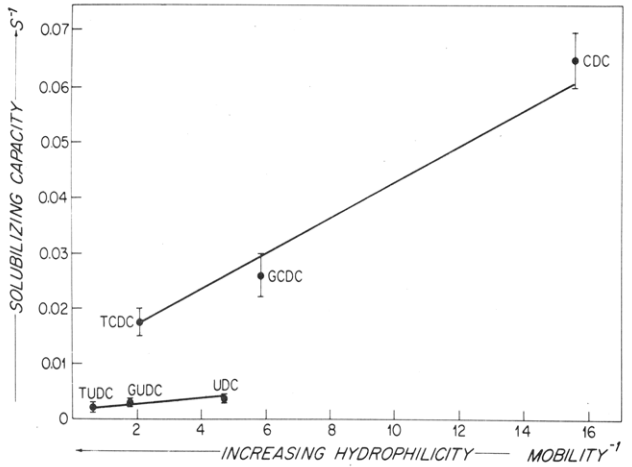

FIGURE 9: Cholesterol solubilizing capacity (S^{-1}), where S is the ratio of moles of bile salt per mole of cholesterol, vs. the normalized retention times (mobility^{-1}) as measured by reversed-phase high-performance liquid chromatography (HPLC). (See list of abbreviations.)

(means of the cholesterol monohydrate and anhydrate data; Table I). The HPLC mobilities of the 7β-hydroxy epimers are considerably greater than the corresponding 7α epimers. In addition, the HPLC mobility of each epimer is dependent upon conjugation as well as 7-OH group orientation in that the rank order of decreasing mobilities is T conjugates > G conjugates $\gg$ free species. The correlation between cholesterol-solubilizing capacity and HPLC mobility is striking, in that the faster the bile salt mobility by HPLC, e.g., TUDC, the less the cholesterol-solubilizing capacity. In contrast, the slower the mobility (or the greater the retention) by HPLC, e.g., CDC, the larger the cholesterol-solubilizing capacity. This important relationship is preserved when S^{-1} and (HPLC mobility)$^{-1}$ of the other common bile salts of man are plotted in similar fashion (M. J. Armstrong and M. C. Carey, unpublished experiments).

Discussion

Micelle Properties. The cmc values determined by all three methods (dye titration, surface tension, light scattering) were in general agreement, falling within the range of 1–5 mM typical of the common di- and trihydroxy bile salts when estimated by noninvasive methods (Carey & Small, 1969; Kratohvil & DelliColli, 1968; Small, 1971). Since in the dye titration experiments the spectral shift is attributed to the solubilization of a dye–detergent complex (Mukerjee & Mysels, 1970), this method tends to give slightly lower estimates of the cmc's of steroid detergents than surface tension or turbidity methods (Carey & Small, 1971; Carey et al., 1975). Considering the experimental uncertainties involved, it can reasonably be assumed that both epimers, whether free or conjugated, self-associate at about the same concentration. In spite of the apparent encroachment of the equatorial 7β-OH group in the so-called hydrophobic side of the bile salt monomer in UDC (Figure 10), the similarities between the cmc's of CDC and UDC and their conjugates (Table I) strongly suggest that similar areas of hydrocarbon are removed from contact with water upon micellization. While it has been suggested by some investigators that bile salt molecules do not exhibit a true cmc (Djavanbakht et al., 1977; Mukerjee & Cardinal, 1976), it should be noted that such conclusions were based on studies of sodium cholate in the presence of little or no added electrolyte. In the present case, the dihydroxy species clearly show a well-defined break point in the surface tension plots (Figure 7) as expected for self-associating systems ex-

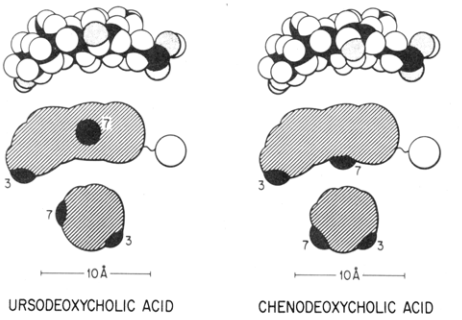

FIGURE 10: Stuart–Briegleb molecular models of unconjugated ursodeoxycholic (UDC) and chenodeoxycholic (CDC) acids with schematic longitudinal and cross-sectional views to show the position and orientation of the 3α- and 7β-hydroxyl functions in the former and the 3α- and 7α-hydroxyl functions in the latter. The 7β-hydroxyl function in ursodeoxycholic acid is equatorial (see Discussion).

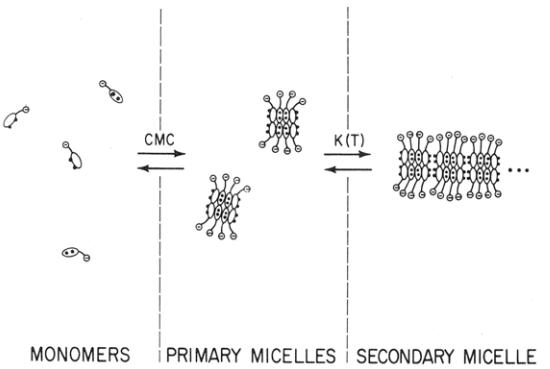

FIGURE 11: Schematic molecular models of bile salt micelles showing primary–secondary micelle hypothesis [from Mazer et al. (1979)]. The ovals represent the steroid body of the bile salts, the closed circles represent hydroxyl functions, and the inscribed negative signs represent the ionized aliphatic side chains.

hibiting cooperative interactions. The use of the cmc concept for these systems is thus appropriate (Ruckenstein & Nagarajan, 1976; Kratohvil et al., 1980).

The present deductions of cmc, micellar size ($\bar{R}_h$), and aggregation number ($\bar{n}_w$) provide important insights into the structural and thermodynamic aspects of bile salt micelles and, in particular, emphasize the important role of bile salt species in the aggregation process. As discussed in previous work (Carey & Small, 1972; Mazer et al., 1979), the available data suggest that in the initial stages of association (i.e., at the cmc), bile salts form roughly globular aggregates ($\bar{R}_h$ 10–15 Å) containing 5–10 molecules. These aggregates, termed primary micelles (Small, 1968), have the constituent bile salts oriented with their so-called "hydrophilic" surface facing the aqueous phase and the "hydrophobic" surfaces interacting in a back-to-back manner (see Figure 11). The structure of the larger bile salt aggregates ($\bar{R}_h$ 15–60 Å and larger) was shown by Mazer et al. (1979) to involve rodlike structures in which some of the constituent molecules contacted one another via both their hydrophobic and hydrophilic surfaces. Whether such secondary micelles form by a linear polymerization of primary micelles (as suggested by Mazer et al., 1979) or via the successive addition of monomers to the primary micelle has not been resolved. However, it is clear that the hydrophilic surface of the bile salt molecule must interact with other bile salts in these larger structures. Only in this way can one account for the fact that different bile salt species have different tendencies to form the secondary micelles. In this connection, the micelles formed by the 7α species have been shown to "grow" more readily (at high NaCl contents) than the 7β species (Mazer et al., 1979). In the present case, the $\bar{R}_h$ and $\bar{n}_w$ values

measured at physiologic NaCl concentrations suggest that both the 7α and 7β species form micelles whose size remains close to that of the primary micelles. At the cmc, both species appear to have nearly the same $\bar{R}_h$ and $\bar{n}_w$ values (Table I), although the conjugated species form slightly larger micelles than the unconjugated. This suggests that the formation of the primary micellar structure is not substantially affected by the orientation of the C-7 hydroxyl group. This view is further consistent with the previous finding that the cmc values were essentially the same for both epimers. Thus, the free energy change for the back-to-back association of the molecules in the primary micelles is relatively insensitive to the orientation of the 7-OH group which necessarily lies on the hydrophilic surface of the monomer and sees essentially the same water environment in the primary micelles.

Above the cmc, the dependence of $\bar{R}_h$ on bile salt concentration does suggest some systematic differences between epimers. While the actual variation of $\bar{R}_h$ with concentration is relatively small in magnitude (±25%) over the range ~1–10 g/dL, the 7α species uniformly show a positive slope for $\bar{R}_h$ vs. C, while the 7β species show a zero or negative slope. These slopes will, in general, reflect both thermodynamic and frictional interactions between micelles, as well as the variation of the micelle size distribution with concentration (Mazer et al., 1979; Missel et al., 1980; Corti & Degiorgio, 1978; Kratohvil, 1979). Quantitative analysis of such effects has been undertaken in other systems where micellar growth was presumed to be negligible (Kratohvil, 1979; Corti & Degiorgio, 1975) or where the micellar interactions were ignored and micellar growth was considered to be the major effect (Mazer et al., 1976; Missel et al., 1980). In the present case, both effects appear to be influencing the dependence of $\bar{R}_h$ on concentration comparably, and thus a rigorous quantitative analysis of the data is considerably more difficult. For this reason, we shall comment only on the qualitative implications of these results. In the case of the 7β species, the zero or negative slope is indicative of repulsive micellar interactions that reduce the apparent $\bar{R}_h$ with increasing concentration. Such effects have been commonly seen in QLS studies of other charged macromolecular systems, at low ionic strengths (Doherty & Benedek, 1974; Rhode & Sackman, 1979; Corti & Degiorgio, 1978). Under these conditions, it is difficult to assess whether the micelles grow to any degree above the cmc. In contrast, the 7α species show a positive slope in these studies, indicating that some degree of micellar growth is occurring. In fact, the actual degree of growth, while probably small, may still be underestimated by the apparent increase in $\bar{R}_h$ values because these are also affected by the repulsive interactions between the micelles. Nevertheless, the greater tendency for micellar growth to occur for 7α species than for 7β species inferred here is consistent with earlier studies (Mazer et al., 1979) in which the polymerization constant for secondary micelle formation, K, was shown to be ~5-fold greater for TCDC than for TUDC in 0.6 M NaCl. This marked difference was attributed to the different amounts of hydrocarbon–water contact area on the surface of the primary micelles. The reduction of this area through secondary micelle formation (i.e., a hydrophobic interaction between micelles) was concluded to be the driving force for micellar growth (Mazer et al., 1979) rather than via intermicellar hydrogen bonds, as had been suggested by Small (1968). Within this framework, the TUDC primary micelles were found to have a smaller hydrocarbon–water interfacial area than TCDC micelles. These apparent differences between the hydrophilic surfaces of the 7α and 7β epimers are further reflected in the other properties of the bile salt molecules we have measured (i.e., surface activity and HPLC mobility) and ultimately in the cholesterol-solubilizing capacities of the micelles.

Surface Properties. The experimental molecular areas derived from π–A curves and surface tension data (Table I) indicate that, at the air–water interface, bile acid molecules are oriented coplanar with the interface, since measurement from space-filling models on a flat surface indicate that the close-packed projection areas should be 85–95 Å^2/molecule. The degree to which the 7β epimers reduced γ, the surface tension of water was significantly less at any bulk concentration relative to the 7α bile salts. The degree to which γ is reduced depends principally on the driving force to being molecules from the bulk, up to the surface. Hence, it can reflect bulk interactions between monomer and solvent *and* surface interactions. Surface interactions may well be different, since the 7β epimers in the protonated form were forced out of the surface at lower pressures than the 7α epimers, suggesting a weaker interfacial affinity of the 7β-OH function compared with the 7α-OH function. However, it seems more likely to us that the reduced surface activity is simply due to the fact that UDC monomers are more hydrophilic (see HPLC data, Figure 9) and presumably would rather remain in the aqueous bulk phase. In a similar way the markedly different π values of the close-packed monomolecular films could be explained by the likelihood that the surface monolayers of UDC and GUDC were compressed more easily into the aqueous subphase. Thus, the surface properties, taken together, appear also to be consistent with the greater hydrophilic tendency of the 7β species. Since significant differences in molecular areas were found by the surface balance method between the free acids and their glycine conjugates (Figure 8), we can assume that the side chains of the protonated conjugating groups contribute significantly to the interfacial areas. However, since no differences in interfacial areas were evident in the adsorbed (ionized) monolayers (Table I), this suggests that the charged side chains project into the aqueous subphase under these conditions. On this basis we can infer that a similar orientation of the conjugating groups would take place on the surface of a primary micelle.

HPLC Properties. As is now well-known, HPLC mobility in a reversed-phase system is a parameter reflecting the tendency of a solute, in this case a bile salt monomer, to partition between a moving polar phase and a stationary hydrocarbon phase. In the present experiments the bile salt concentration in the moving phase is below its cmc, but, also, micelle formation is precluded by the high content of MeOH. Thus, HPLC mobility reflects the relative time spent by the monomer in the two (polar and hydrocarbon) domains. Since both bile salt epimers have the same hydrophobic surface (see cmc, $\bar{R}_h$, and $\bar{n}_w$ data), the mobilities thus reflect differences in the tendencies of the less hydrophobic ("hydrophilic") side of the monomers to partition between a hydrophobic and polar environment, i.e., high mobility can be equated with high hydrophilicity. Here we see that the 7β-OH group strongly favors partitioning into the polar phase rather than the hydrocarbon phase. We do not know if this is due to a strong bile salt 7β-OH–H_2O (solvent) interaction, but the qualitative results is that the 7β epimers are more hydrophilic than the 7α epimers.[3] Similarly, the effect of conjugation is also

[3] Mass spectrometry measurements of the molecular ions formed by the loss of water from the hydroxyl functions of hydrated UDC and CDC methyl esters suggest that a water molecule is much more tightly bound to the 7β-OH group of UDC than to the 7α-OH group of CDC (I. A. MacDonald, personal communication.)

reflected in the mobilities, unconjugated < glycine conjugates < taurine conjugates. The taurine conjugates are most polar and thus spend more time in the mobile phase; therefore, their mobility is the highest. Hence, relative hydrophilicity is proportional to mobility. In the same context, we have found that the relative hydrophilic tendency of the bile salt monomers correlates inversely with their ability to solubilize cholesterol in the primary micelles. Not only is this inverse relationship marked with respect to the 7α and 7β epimers but it also is a function of the state of conjugation. We will discuss the possible reasons for this in the final section.

Cholesterol Solubilization. We confirmed in this work that not only is the capacity of bile salt micelles to solubilize cholesterol small but it also varies appreciably depending on whether the bile salt is the free species or taurine or glycine conjugated. While the same ordering of cholesterol solubility between free salts and conjugates is observed when CDC and UDC are compared, the capacity of micelles of the latter to solubilize cholesterol monohydrate is one or two orders of magnitude less than that found with CDC and its conjugates. Even though both bile salts demonstrate a similar capacity to solubilize lecithin, the capacity of conjugated UDC–lecithin micelles to solubilize cholesterol is also much less than that of conjugated CDC–lecithin micelles (Carey, 1978; M. C. Carey and G. Ko, unpublished observations), and this is of considerable pathophysiological and clinical importance.

Since the mean hydrodynamic radii of UDC and CDC micelles changed only slightly when cholesterol monohydrate molecules were solubilized, a finding also observed with other common bile salts (Small, 1971; Mazer, 1978), our results suggest that the incorporation of cholesterol molecules into the bile salt micelles does not markedly alter their preexisting structure.[4] From this finding we can quantitatively interpret the solubilization data by using the same analysis employed earlier by Mazer (1978) in the case of taurocholate–cholesterol systems. We assume that each bile salt micelle effectively has a single cholesterol binding site characterized by a temperature-dependent binding constant $K_i(T)$. The ratio of bile salt to cholesterol molecules corresponding to the maximum degree of solubilization, denoted S, is thus

$$S = \bar{n}\left[\frac{1 + K_i(T)S_0(T)}{K_i(T)S_0(T)}\right] \quad (3)$$

where $\bar{n}$ is the aggregation number of the bile salt micelle, $K_i(T)$ is the cholesterol binding constant, and $S_0(T)$ is the monomeric solubility of cholesterol (also dependent on temperature). Only in the limit where the product $K_i(T)S_0(T)$ is $\gg 1$ does S approach the limiting value of $\bar{n}$. Conversely, if $K_i(T)S_0(T)$ is $\ll 1$, S will be $\gg n_0$, as appears to be the case in the present studies. Using the experimental values of $\bar{n}$ (QLS results) and S (from Table I) and Saad and Higuchi's (Saad & Higuchi, 1965) value for the solubility of cholesterol monohydrate in water at 30 °C (6.7×10^{-8} M), we deduce values for K_i(30 °C) equal to 5.7×10^6 L/mol for unconjugated CDC and 2.5×10^5 L/mol for unconjugated UDC. These values "bracket" the value of K_i(30 °C) deduced for the TC micelle of 1.34×10^6 L/mol (Mazer, 1978). Since the K_i value for the UDC micelle is roughly 20 times smaller than for CDC we can see why the former micelles are poorer solubilizers of cholesterol. By converting these binding constants to dimensionless units (i.e., by expressing $S_0(T)$ in mole fraction units), we can deduce the standard free energy change ΔG_s associated with the solubilization of cholesterol in the primary micelles. For CDC and UDC the ΔG_s values correspond to −11.7 and −9.87 kcal/mol, respectively, at 30 °C. Thus, the binding of cholesterol to the CDC micelle is more favorable by ~2 kcal/mol than for the UDC micelle. To provide a molecular interpretation for this finding, we make use of our previous deductions concerning the structure and thermodynamics of the pure CDC and UDC micelles. We earlier concluded that under physiologic conditions both epimers formed small primary micelles, whose interiors were the same but whose surfaces reflected the different 7-OH orientations. The fact that the ΔG_s values differ markedly between the two micelles suggests that cholesterol binding to the micelles must involve a strong interaction with the micellar surfaces, rather than with the micelle interior. The simplest picture to explain such an interaction is to suppose that the cholesterol molecule binds directly to the surface of the micelle, presumably by a hydrophobic interaction (Figure 12). In this case, we estimate that roughly half of the cholesterol surface[5] will be eliminated from water contact (~400 Å^2) and a portion of the hydrocarbon exposed on the primary micelle surface (~200 Å^2) will also be covered. With these estimates the free energy change associated with the hydrophobic bonding is approximately

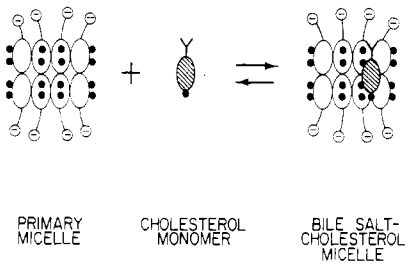

FIGURE 12: Highly schematicized picture of a cholesterol molecule binding to the surface of a primary micelle (see Discussion; symbols are as described in Figure 11).

$$(\Delta G_s)_{\text{estimate}} = [-20 \text{ cal}/(\text{mol}\cdot\text{Å}^2)](400 + 200 \text{ Å}^2) = -12.0 \text{ kcal/mol} \quad (4)$$

the factor 20 cal/(mol·Å^2) represents the estimate of Reynolds and co-workers (Reynolds et al., 1974) of the free energy of hydrophobic interaction and is also consistent with recent deductions based on the thermodynamics of $NaDodSO_4$ (sodium dodecyl sulfate) and bile salt micelles (Mazer et al., 1977; Missel et al., 1980). Interestingly, this relatively simple estimate gives a value for ΔG_s that is in good agreement with the value experimentally deduced for the CDC micelle.[6] Conceivably, the 3β-OH group on the cholesterol molecule can interact with the hydroxyl groups on the surface of the micelle and thereby modify the actual value of ΔG_s. Similar hydrophilic interactions have been shown to be important in the interactions of saponins, e.g., digitonin with cholesterol (Akiyama et al., 1980). In addition, differences in the amount of hydrocarbon exposed on the primary micelle surface which

[4] If added cholesterol had induced the formation of even a small population of large mixed micelles ($\bar{R}_h > 20$ Å), this would have been evident since they would have made the dominant contribution to the measured $\bar{R}_h$ values.

[5] Gilbert et al. (1975) estimate the cavity surface area of the hydrophobic component of the cholesterol molecule to be 700–800 Å^2, depending upon the extent of folding or the extension of the aliphatic side chain.

[6] Gilbert & Reynolds (1976) have experimentally deduced estimates of the free energy of transferring cholesterol to $NaDodSO_4$ micelles (~10.3 kcal/mol) that are similar in magnitude to this value but propose a different solubilization mechanism.

depends upon the bile salt species and conjugates can also influence the magnitude of ΔG_s. It is by these mechanisms that the different bile salt species can manifest different solubilizing capacities for cholesterol. In the case of the 7α and 7β epimers, the latter species having a more hydrophilic surface on the primary micelle, would be less able to interact hydrophobically with the cholesterol molecule. For similar reasons the more hydrophilic taurine conjugates interact hydrophobically with cholesterol less well than the glycine conjugates which in turn are less effective than the free species. In this way we can see quantitatively why the more hydrophilic bile salt species are poorer solubilizers of cholesterol. Thus, the empirical correlation illustrated in Figure 9 is consistent with a theoretical understanding based on the molecular and thermodynamic aspects of cholesterol binding to the surface of the primary micelle.

In summary, this paper principally shows that the two "sides" of a bile salt molecule each play important but distinct roles in its physical–chemical properties. Micelle formation, specifically the primary micelles, reflects the desire of the more hydrophobic (hydrophobic) side of the bile salt monomer to eliminate its contact with the aqueous solvent and thus shows little variation between the conjugates of CDC and UDC in so far as cmc, micellar weight ($\bar{M}_w$), $\bar{n}_w$, and $\bar{R}_h$ (at the cmc) are concerned. Conversely, the less hydrophobic (hydrophilic) side, found on the external surface of the primary micelle, governs secondary micelle growth, surface properties (surface tension lowering, collapse pressure, equilibrium spreading pressure), and HPLC mobilities, and all of these characteristics appear to correlate more or less with the cholesterol-solubilizing properties of the bile salt. This suggests to us that the less hydrophobic or "hydrophilic" surface of the bile salt molecule (and hence the surface of the primary micelle) is involved importantly in the micellar binding of cholesterol. While the precise geometry of this interaction cannot be elucidated from the present data, we have nevertheless suggested in Figure 12 the simplest possible molecular scheme. In this model, surface adsorption of cholesterol occurs by hydrocarbon–hydrocarbon contact onto the hydrophilic face of the bile salt micelle. This interaction may also involve OH–OH group interactions between cholesterol and the bile salt. It is also possible, however, that the cholesterol molecule may be inserted between the bile salt monomers in a micelle analogous to the idea of a "palisade layer" insertion in classic detergent systems (Shinoda et al., 1963). Irrespective of the molecular details of the "attachment" site, it is the surface (hydrophilic) part of the bile salt micelles which is involved intimately in the cholesterol binding interaction.

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Modification of Myosin Subfragment 1 by Carbodiimide in the Presence of a Nucleophile. Effect on Adenosinetriphosphatase Activities†

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ABSTRACT: The modification of myosin subfragment 1 by *N*-cyclohexyl-*N'*-[2-(4-morpholinyl)ethyl]carbodiimide methyl *p*-toluenesulfonate in the presence of the nucleophile nitrotyrosine ethyl ester was investigated. For elimination of interference of the thiol groups, the two most reactive thiols were protected by cyanylation with 2-nitro-5-(thiocyanato)benzoic acid. The ATPase activity of the cyanylated myosin subfragment 1 was not lost, but had changed. At pH 5.9, carbodiimide in the presence of the nucleophile rapidly inactivated the cyanylated enzyme. The inactivation followed first-order kinetics. The K^+(EDTA)–, Ca^{2+}–, and Mg^{2+}–ATPase activities decreased at the same rate. Inactivation and incorporation of nucleophile occurred simultaneously. A full loss of activity resulted from the incorporation of 1 mol of nitrotyrosine per mol of myosin subfragment 1. Pyrophosphate, ITP, ADP, and ATP protected against inactivation, and the efficiency of the protection was parallel to the ligand binding strength. These results suggested that one carboxyl group was essential for the active conformation of myosin.

The cleavage of ATP[1] catalyzed by myosin in the presence of actin is the direct source of energy for muscle contraction. Kinetic features of this biological reaction have already been studied (Lymn & Taylor, 1970; Bagshaw & Trentham, 1974). In contrast, structural information on the active site of myosin is scarce. Data regarding the amino acid residues involved in the hydrolytic role of myosin are rare and sometimes controversial. For example, the cysteinyl side-chain residues SH1 and SH2 (Sekine & Kielley, 1964) have been implicated in the mechanism of the ATP hydrolysis (Reisler et al., 1974). This now appears very unlikely, since the blockage of the thiols only affected the conformation of the active site (Wiedner et al., 1978; Botts et al., 1979). Other amino acids have been proposed, including lysine, histidine, and tryptophan.

The modification of lysine (Fabian & Mühlrad, 1968), histidine (Hegyi & Mühlrad, 1968), and tryptophan (Yoshino, 1976) did not promote a parallel inactivation of the ATPase activities. Chantler & Szent-Gyorgyi (1978) reported that the well-known tryptophan fluorescence enhancement following the nucleotide binding to skeletal myosin was absent in invertebrate myosins. Their research suggests that tryptophan does not seem to be involved in the active site (Chantler, 1980). A concomitant loss of Mg^{2+}– and Ca^{2+}–ATPase activities was observed by blocking tyrosine with diazonium-1*H*-tetrazole, but the reagent was not specific. In addition to tyrosine, some modified histidine residue was detected (Shimada, 1970). It is therefore difficult to attribute a definite role to these residues in the mechanism of catalysis. We suggest that the most likely residue involved in the active site seems to be arginine. The labeling of arginine by phenylglyoxal affected the K^+(EDTA)–, Ca^{2+}–, and actin-activated ATPase activities, and the nucleotide substrates afforded efficient protection against

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[1] Abbreviations used: S-1, α-chymotryptic myosin subfragment 1; CMC, *N*-cyclohexyl-*N'*-[2-(4-morpholinyl)ethyl]carbodiimide methyl *p*-toluenesulfonate; NTEE, 3-nitro-L-tyrosine ethyl ester; EDTA, ethylenediaminetetraacetic acid; ATP, adenosine triphosphate; ADP, adenosine diphosphate; ITP, inosine triphosphate; Tris, tris(hydroxymethyl)-aminomethane.

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