Aggregation patterns of bile salts: crystal structure of calcium cholate chloride heptahydrate[1]

Arthur Hogan,*† S. E. Ealick,*† C. E. Bugg,*† and S. Barnes*,**

Departments of Biochemistry* and Pharmacology,** Institute of Dental Research,† and the Comprehensive Cancer Center,* University of Alabama in Birmingham, Birmingham, AL 35294

Abstract Crystals of calcium cholate chloride heptahydrate, $CaC_{24}H_{39}O_7Cl \cdot 7H_2O$, are monoclinic, space group $P2_1$, with a = 11.918(2), b = 8.636(1), c = 15.302(3) Å, β = 97.93(3)°, V = 1559.9(8) Å^3, and Z = 2. A trial structure was obtained by Patterson and Fourier techniques and was refined by full-matrix least-squares calculations using absorption corrected CuK$\bar{\alpha}$ diffractometer data. The final R index is 0.047. The crystal structure contains bilayer-type arrangements, with hydrophobic portions of cholate rings sandwiched between layers of polar groups that are interacting with calcium ions and water molecules. The calcium ion is coordinated to five water molecules and to the two carboxylate oxygen atoms of the cholate residue. Two additional water molecules are involved only in crystal packing through the formation of hydrogen bonds. Cholate-cholate hydrophobic interactions involve contacts between the hydrocarbon portions of the carboxylate sidechains and the A and B rings. This results in a staggered packing pattern that is nearly identical to that found in crystals of sodium cholate and rubidium deoxycholate. Similar bilayer aggregation patterns may also be involved in the formation of bile salt micelles in aqueous media. The characteristic bilayer packing arrangement can accommodate a variety of cation-binding patterns, as evidenced by the finding that calcium, sodium, and rubidium ions interact with the polar faces of the bilayers in different ways. The carboxylate sidechain displays two different conformations in the crystal structure of calcium cholate chloride heptahydrate. Variation in sidechain conformation may be of importance in the adjustment required to accommodate different cation coordination schemes.—**Hogan, A., S. E. Ealick, C. E. Bugg, and S. Barnes.** Aggregation patterns of bile salts: crystal structure of calcium cholate chloride heptahydrate. *J. Lipid Res.* 1984. **25:** 791–798.

Supplementary key words X-ray diffraction • cholate bilayers • micelle interactions

Bile salts are physiological detergents that emulsify lipids in the biliary tree and in the intestine. In vivo, bile salts form mixed micelles with phospholipid and cholesterol. In the intestine, the mixed micelles consist of fatty acids and monoacylglycerols in combination with bile salts. Cholesterol and fat-soluble vitamins (A, D, E, and K) are dispersed in the micelles through the aqueous phase which leads to their absorption by the intestine. Clinically, cholanoate derivatives are used in the treatment of cholesterol gallstones (1). Various models for bile salt micelles have been proposed (2). A general feature of these models is the role played by interactions between hydrophobic parts of the bile salts, but little is actually known about the nature of these interactions or about the geometries of bile salt aggregates.

Sodium, being the predominant cation in bile, has been assumed to be the cation involved in the formation of bile salt micelles. However, recent studies indicate that calcium ions may be of particular importance in the interactions that are involved in both hepatic and gall bladder bile. Calcium is a normal component in bile; however, unlike sodium, calcium undergoes a process of concentration which parallels increasing concentrations of bile salts in the gallbladder (3). Williamson and Percy-Robb (4) demonstrated that calcium binds to glycocholate, the predominant cholanoate derivative in bile. It has also been demonstrated that calcium cannot be dialyzed out of gallbladder bile until the concentration of bile acids inside the dialysis bag falls below the critical micellar concentration (5). These observations suggest that calcium is involved in interactions with bile salt micelles. Recent studies using Ca^{2+}-specific electrodes have confirmed the importance of calcium-bile salt interactions (6, 7). It has been suggested that bile salt micelles may act as a chelating system for calcium, thus preventing calcium precipitation as inorganic or bilirubinate salts (7). Pigment gallstones rich in calcium are a major problem in gallbladder disease (8).

We determined the crystal structure of a calcium salt of cholic acid in order to learn more about the types of interactions that may be of importance in the formation of bile salt micelles and the possible roles that calcium ions might play in these interactions.

Abbreviations: cholate, 3α,7α,12α-trihydroxy-5β-cholan-24-oate; chenodeoxycholate, 3α,7α-dihydroxy-5β-cholan-24-oate; ursodeoxycholate, 3α,7β-dihydroxy-5β-cholan-24-oate; deoxycholate, 3α,12α-dihydroxy-5β-cholan-24-oate.

[1] The material herein was presented at the Annual Meeting of the American Association for the Study of Liver Diseases, Chicago, Illinois, November 3, 1982, and was published in abstract form in *Hepatology*, 1982, **2:** 716.

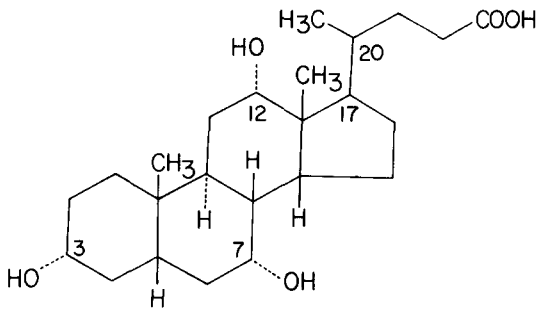

Fig. 1. Chemical formula of cholic acid.

EXPERIMENTAL

Cholic acid (**Fig. 1**) (Sigma Chemical Co., St. Louis, MO) was recrystallized three times from ethanol–benzene. It was exhaustively dried in vacuo to remove the solvents. A solution of sodium cholate was prepared by mixing equimolar amounts of cholic acid and aqueous sodium hydroxide. The solution was then lyophilized.

The calcium salt of cholic acid was prepared by mixing a saturated solution of sodium cholate with a saturated solution of calcium chloride and adjusting the pH with

TABLE 1. Nonhydrogen atom parameters and their estimated standard deviations

Name	x	y	z	Beq (Å^2)
CA	−6462 (1)	−3274 (1)	389 (1)	3.1
CL	−9545 (1)	−489 (3)	8995 (1)	7.6
C1	4683 (4)	1345 (6)	4052 (3)	3.6
C2	5088 (3)	1552 (6)	3160 (3)	3.4
C3	5334 (4)	3231 (6)	3015 (3)	3.3
C4	4272 (3)	4194 (5)	3056 (3)	3.0
C5	3819 (3)	4006 (6)	3939 (3)	3.1
C6	2776 (4)	5034 (6)	3988 (3)	3.7
C7	1688 (3)	4398 (6)	3486 (3)	3.5
C8	1490 (3)	2701 (5)	3703 (3)	2.8
C9	2541 (3)	1702 (6)	3581 (2)	2.7
C10	3621 (3)	2307 (6)	4174 (2)	3.0
C11	2318 (3)	−35 (6)	3696 (3)	3.4
C12	1220 (3)	−639 (5)	3179 (3)	3.1
C13	195 (3)	332 (5)	3391 (2)	2.7
C14	431 (3)	2034 (5)	3174 (2)	2.7
C15	−697 (4)	2857 (6)	3217 (3)	3.6
C16	−1585 (3)	1631 (6)	2895 (3)	3.8
C17	−957 (3)	68 (5)	2801 (3)	2.7
C18	58 (4)	121 (6)	4376 (3)	3.6
C19	3499 (4)	2180 (8)	5172 (3)	4.7
C20	−1688 (4)	−1323 (6)	2960 (3)	3.4
C21	−1058 (4)	−2868 (6)	3051 (4)	4.5
C22	−2663 (5)	−1377 (7)	2150 (3)	3.2
C22'	−287 (2)	−173 (3)	258 (1)	3.1
C23	−3606 (6)	−2447 (8)	2320 (4)	4.4
C23'	−290 (2)	−212 (3)	163 (1)	3.0
C24	−4482 (5)	−2705 (7)	1523 (3)	3.2
C24'	−417 (2)	−251 (3)	125 (1)	2.8
O1	5756 (3)	3375 (4)	2191 (2)	4.2
O2	1717 (3)	4641 (4)	2553 (2)	4.1
O3	1345 (2)	−596 (4)	2259 (2)	3.8
O4	−4681 (3)	−4056 (5)	1241 (3)	3.6
O4'	−442 (1)	−387 (2)	113 (1)	4.3
O5	−4978 (3)	−1562 (4)	1142 (3)	4.4
O6	−6611 (3)	−6054 (5)	503 (5)	5.6
O7	−8312 (4)	−3705 (6)	−415 (3)	6.4
O8	−6966 (3)	−781 (5)	−250 (3)	5.2
O9	−5429 (3)	−3553 (4)	−813 (2)	4.6
O10	−7273 (3)	−2967 (4)	1705 (2)	4.4
O11	−249 (4)	−1630 (8)	809 (3)	9.1
O12	−8197 (8)	−8219 (12)	932 (4)	7.1
O12'	−722 (1)	−872 (2)	126 (1)	6.9

Beq = $8\pi^2(U_{11}U_{22}U_{33})^{1/3}$, where $U_{11,22,33}$ are the mean square displacements along the principal axes of the thermal ellipsoids. The atom-numbering scheme for the cholate anion is given in Fig. 2. The water-oxygen atoms are O6–O12. Minor sites for disordered atoms are labeled with apostrophes. Coordinates have been multiplied by 10^4 for major sites and by 10^3 for minor sites. Estimated standard deviations for Beq range from 0.1 to 0.3 for C and O atoms.

hydrochloric acid to 5. Crystals suitable for X-ray diffraction experiments were grown by slowly evaporating this solution. The crystals are long needles; the one selected for data collection had dimensions 0.04 × 0.11 × 0.78 mm. Weissenberg photographs show that the crystals are monoclinic, space group $P2_1$. The unit cell parameters were determined by least-squares refinement of the angular settings for the Friedel mates of 12 reflections measured with a Nicolet P3F diffractometer. Three-dimensional X-ray intensity data were collected at room temperature for the 3459 symmetry independent reflections with $2\theta < 120°$. Intensity measurements were made by use of Ni-filtered CuK$\bar{\alpha}$ radiation (λ = 1.5418 Å). A variable scan-speed method was used; we attempted to measure a total of about 10,000 counts for each reflection, but had a minimum cutoff scan speed of 2°/min. If the intensity exceeded 60,000 counts/sec, the reflection was rescanned using an attenuator with attenuation factor = 30.8(5). Dispersion of X-rays was accounted for by using a 2.6° scan width plus a correction factor. Background measurements were made by counting for half the scan time on each side of reflections [intensity = scan speed × {peak − (the sum of the backgrounds)}]. Reflections with $I < 0.0$ were omitted from subsequent analyses. Six standard reflections, (3,1,3), (−3,0,5), (7,0,11), (4,2,4), (−6,0,0), (−2,0,8), were monitored every 100 reflections. The intensities of these reflections declined during data collection by 21%, 13%, 6%, 16%, 5%, and 12%, respectively. Intensities were corrected for decay by an equation based on the decay rate of the standards as modeled by least-squares analysis. All subsequent calculations were made using the Enraf-Nonius SDP set of computer programs. The data were corrected for Lorentz and polarization effects. Analytical absorption corrections were applied. Each observation was assigned errors based on counting statistics plus an additional variance of $(0.02 \times \text{scan speed} \times \text{peak})^2$, which was introduced to account for systematic error from instrumental instability.

The structure was determined using heavy-atom and difference-Fourier techniques. The absolute configuration of the molecule was chosen to be consistent with the known absolute configuration of the starting material and was not determined crystallographically. Coordinates, anisotropic temperature factors and Zachariasen's (9) isotropic extinction parameter were refined. The trial structure was refined by full-matrix least-squares. The quantity minimized was $\Sigma w(Fo - Fc/k)^2$, where k is a scale factor and w is equal to $1/\sigma^2(Fo)$. Scattering factors and anomalous dispersion coefficients were obtained from the International Tables for X-Ray Crystallography (10). A difference Fourier map revealed the position of an additional water molecule and suggested that the carboxylate side chain (atoms C22, C23, C24, O4, O5) and one of the water molecules (O12) are disordered. Two conformations were assigned to the carboxylate sidechain. Based on peak heights in the difference Fourier maps, these two conformations were assigned occupancies of 0.8 and

TABLE 2. Hydrogen atom positional parameters and their estimated standard deviations (×10^5)

Name	X	Y	Z
H(C1)A	534 (3)	163 (5)	449 (2)
H(C1)B	457 (3)	24 (5)	414 (2)
H(C2)A	574 (3)	94 (6)	305 (3)
H(C2)B	453 (3)	118 (5)	270 (2)
H(C3)	602 (3)	354 (5)	352 (2)
H(C4)A	451 (3)	524 (6)	298 (3)
H(C4)B	366 (3)	385 (5)	254 (2)
H(C5)	450 (3)	439 (5)	436 (2)
H(C6)A	257 (4)	489 (6)	467 (3)
H(C6)B	286 (3)	602 (5)	381 (2)
H(C7)	111 (4)	500 (6)	367 (3)
H(C8)	136 (3)	282 (5)	435 (2)
H(C9)	262 (3)	185 (5)	297 (2)
H(C11)A	235 (3)	−32 (5)	430 (2)
H(C11)B	287 (3)	−52 (6)	349 (3)
H(C12)	109 (2)	−172 (4)	330 (2)
H(C14)	47 (3)	205 (5)	259 (2)
H(C15)A	−75 (4)	316 (6)	385 (3)
H(C15)B	−78 (4)	364 (7)	280 (3)
H(C16)A	−197 (4)	194 (7)	231 (3)
H(C16)B	−215 (4)	149 (7)	332 (3)
H(C17)	−78 (4)	−11 (6)	218 (3)
H(C18)A	−1 (4)	−88 (6)	456 (3)
H(C18)B	68 (4)	60 (6)	483 (3)
H(C18)C	−71 (3)	66 (6)	451 (3)
H(C19)A	331 (5)	99 (9)	548 (4)
H(C19)B	409 (3)	259 (5)	553 (2)
H(C19)C	285 (4)	279 (6)	535 (3)
H(C20)	−193 (4)	−107 (6)	348 (3)
H(C21)A	−144 (3)	−365 (5)	317 (3)
H(C21)B	−69 (4)	−306 (8)	256 (3)
H(C21)C	−47 (4)	−281 (6)	359 (3)
H(C22)A	−222 (4)	−162 (7)	162 (3)
H(C22)B	−303 (4)	−40 (6)	200 (3)
H(C23)A	−367 (8)	−205 (16)	271 (6)
H(C23)B	−326 (5)	−332 (10)	239 (4)
H(O1)	572 (4)	426 (8)	201 (3)
H(O2)	96 (3)	458 (6)	226 (3)
H(O3)	71 (3)	−85 (6)	199 (3)
H(O6)A	−695 (3)	−678 (6)	82 (4)
H(O6)B	−668 (4)	−593 (5)	0 (2)
H(O7)A	−855 (4)	−438 (7)	−31 (3)
H(O7)B	−861 (9)	−254 (20)	7 (7)
H(O8)A	−650 (4)	−10 (7)	−66 (3)
H(O8)B	−764 (5)	−46 (10)	−42 (4)
H(O9)A	−530 (4)	−454 (6)	−105 (3)
H(O9)B	−598 (3)	−385 (6)	−103 (3)
H(O10)A	−764 (4)	−209 (7)	176 (3)
H(O10)B	−755 (3)	−379 (5)	192 (2)
H(O11)A	−27 (4)	−312 (7)	−84 (4)
H(O11)B	−100 (4)	−137 (6)	−21 (3)
H(O12)A	−750 (13)	−844 (7)	104 (3)
H(O12)B	−861 (4)	−834 (5)	41 (4)

The labels in parentheses indicate the atoms to which hydrogen atoms are covalently bounded. Letters are used to distinguish individual hydrogen atoms that are bonded to a given atom. Only hydrogen atoms that are bonded to the major sites of disordered atoms are given. The geometry for water O(11) is unsatisfactory and the positions for H(O11)A and H(O11)B should be considered unreliable.

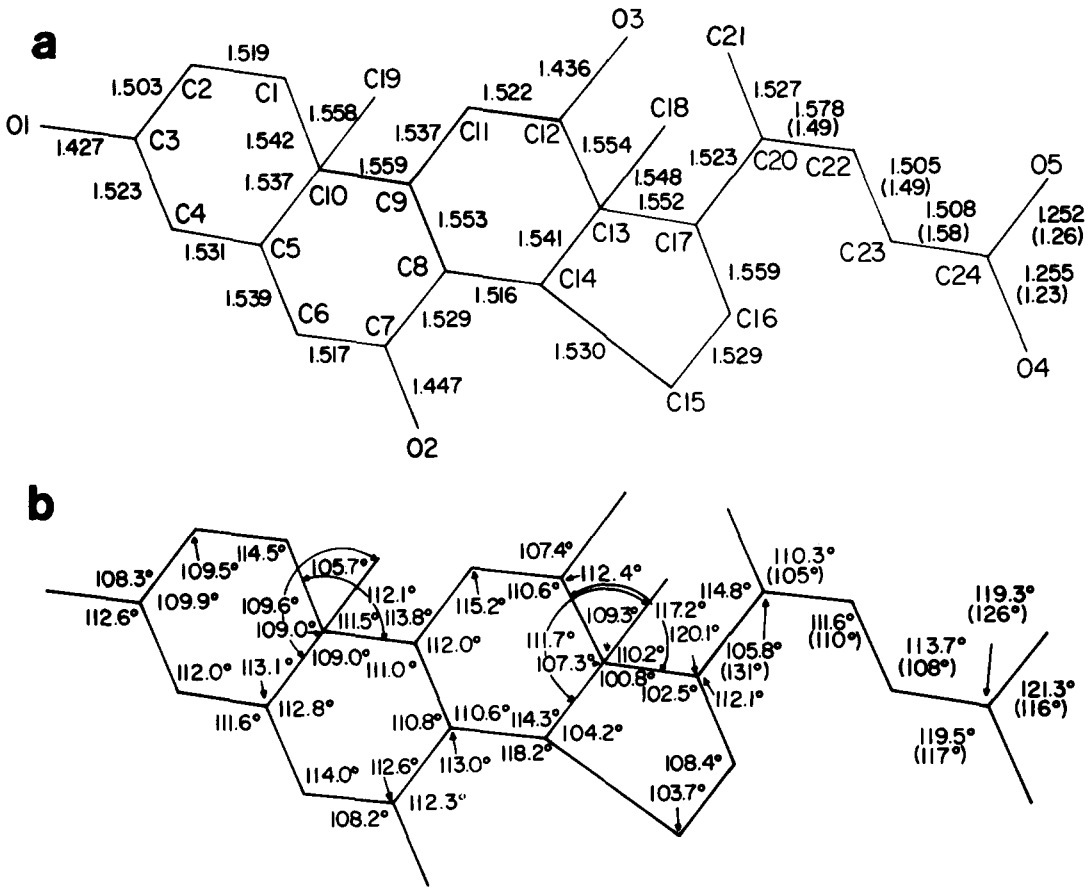

Fig. 2. Bond distances (Å) and angles (°) within the cholate anion. Estimated errors in bond distances and angles are 0.008 Å and 0.3°, respectively. Distances and angles in parentheses give values that correspond to the minor conformation of the carboxylate sidechain; these distances and angles have estimated errors of 0.02 Å and 1°, respectively.

0.2, respectively. The major and minor sites for the disordered water molecule were also given occupancies of 0.8 and 0.2, respectively. Occupancies were not refined. Presumably, atom C21 is also disordered; however, difference Fourier maps provided no evidence of a minor site for C21, so no second site for this atom was introduced. After further refinement, a difference Fourier map revealed positions for hydrogen atoms for the major conformation. Hydrogen atoms were assigned the isotropic temperature factors of the atoms to which they are bonded, and the hydrogen-atom positional parameters were included in the refinement. The final R index is 0.047 and the goodness-of-fit $\{[\Sigma w(Fo - Fc)^2/(m - s)]^{1/2}$, where m is the number of reflections used and s is the number of parameters refined$\}$ is 3.58.

RESULTS

Nonhydrogen atom coordinates, temperature factors, and their estimated standard deviations are listed in **Table 1.** Hydrogen atom coordinates and their estimated standard deviations are given in **Table 2.** The average estimated errors in atomic positions are about 0.005 Å, except for the minor sites for disordered atoms, which have estimated errors of 0.02–0.04 Å. Bond distances and angles are given in **Fig. 2.** These distances and angles are similar to those in other cholanoate structures (11–20). The conformation of the cholate moiety is shown in **Fig. 3,** which includes the two orientations found for the carboxylate sidechain. The crystal packing scheme is shown in **Fig. 4.** Hydrogen bond distances and angles are given in **Table 3.** The calcium ion is coordinated to five water molecules, and to the pair of oxygen atoms from the carboxylate group. These seven ligands form a distorted pentagonal bipyramid around the calcium ion, as depicted in Fig. 4. The calcium-oxygen coordination distances are listed in **Table 4.** The A/B rings have a *cis* junction, while the B/C and C/D junctions are *trans.* Torsion angles within the carboxylate sidechain are given in **Table 5.**

DISCUSSION

The crystal-packing scheme involves a bilayer arrangement of cholate moieties. As can be seen in Fig. 3, the

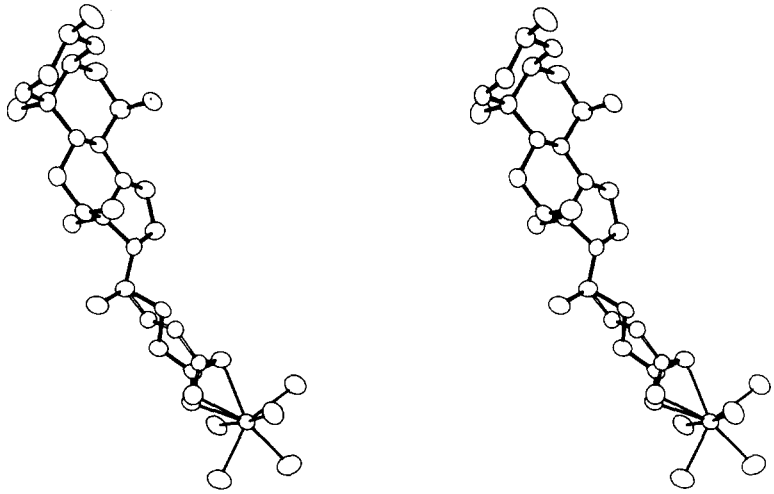

Fig. 3. Stereodrawing of the calcium cholate complex. The five water molecules that complete the calcium coordination shell are included. This drawing and those in Figs. 4 and 5 were prepared by the use of the computer program ORTEP (23). Thermal ellipsoids are plotted at 50% probability.

conformation of the cholate anion is such that one face is hydrophobic and contains the three methyl groups, while the other face is hydrophilic with the carboxylate group and the hydroxyl groups forming a cluster on that side of the cholate anion. As shown in Fig. 4. and **Fig. 5a,** the crystal-packing scheme takes advantage of the amphipathic conformation of the cholate anion. Cholate moieties form a bilayer arrangement in which sheets of cholate anions are stacked together with their hydrophobic surfaces facing in toward the core of the bilayer and the hydrophilic surfaces facing outwards. The crystal structure consists of extended bilayers of the hydrophobic surfaces of cholate moieties sandwiched between polar surfaces that are interacting with calcium cations and water molecules.

As shown in Fig. 5, similar packing schemes are also found in the crystal structures of sodium cholate monohydrate (19) and rubidium deoxycholate monohydrate (20). In both of these crystal structures, amphipathic bilayers are formed, consisting of hydrophobic interiors sandwiched between hydrophilic surfaces that are interacting with water molecules and cations. The hydrophobic contacts that stabilize the bilayer arrangement show similarities in the three crystal structures, despite the fact that different cations are involved in different coordination schemes and the hydrogen bonding schemes at the hydrophilic faces are different. It is noteworthy that in these crystal structures the sodium ion is coordinated to only one of the carboxylate oxygen atoms, whereas the calcium ion is bound to both oxygen atoms in a bidentate chelation pattern; perhaps similar bidentate chelation of calcium by carboxylate groups in bile salt micelles is a factor that contributes to the preferential accumulation of calcium in gallbladder bile (3).

Although the interactions between cholate moieties are similar in the three crystal structures described in Fig. 5, there are significant differences in the observed interactions of the calcium, sodium, and rubidium cations in these structures. In all three structures, the cations are coordinated to the carboxylate groups. The sodium and

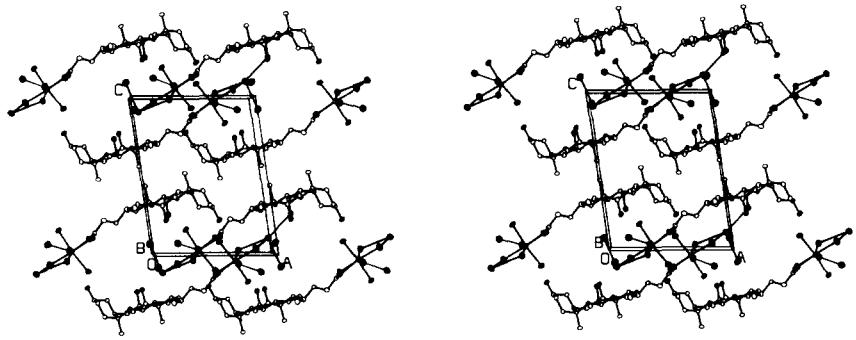

Fig. 4. Crystal packing scheme of calcium cholate chloride heptahydrate. Filled in circles are the oxygen atoms.

TABLE 3. Hydrogen-bond distances and angles

Donor	Hydrogen	Donor-Hydrogen Distance	Hydrogen-Acceptor Distance	Acceptor	Donor-Hydrogen-Acceptor Angle
O1	H(O1)	0.81 Å	1.89 Å	O4	158°
O1	H(O1)	0.81	2.09	O4'	159
O3	H(O3)	0.84	2.11	O11	148
O6	H(O6)A	0.92	1.85	O12	157
O6	H(O6)A	0.92	1.96	O12	151
O7	H(O7)A	0.68	2.47	O11	148
O8	H(O8)A	1.07	1.98	O4	166
O8	H(O8)A	1.07	1.75	O4'	168
O9	H(O9)A	0.95	1.78	O5	163
O10	H(O10)A	0.88	1.99	O3	158
O10	H(O10)B	0.86	1.95	O2	169
O2	H(O2)	0.94	2.35	CL	154
O8	H(O8)B	0.86	2.36	CL	160

Estimated errors are 0.05 Å and 5°, respectively. Based on donor-acceptor distances, water O(11) probably forms hydrogen bonds with water O(12) and two different chloride ions.

rubidium ions also interact with the hydroxyl groups from the cholanoate moieties. On the other hand, the calcium ion interacts only with the oxygen atoms of the carboxylate group, and the calcium coordination shell is then completed by water molecules. It is clear that the arrangement of hydroxyl and carboxylate oxygen atoms on the hydrophilic surface might permit a variety of different coordination patterns for cation interactions. The flexible conformation of the carboxylate sidechain (21), which is seen in the disordered structure of the calcium complex, may be an important factor in accommodating different types of cations in different coordination patterns.

The major interactions that appear to be involved in the common hydrophobic packing patterns found in the crystal structure of calcium cholate chloride heptahydrate, sodium cholate monohydrate (19), and rubidium deoxycholate monohydrate (20), are the contacts between the hydrocarbon portion of the carboxylate side chain (C20–C23) from one bile salt molecule and the A and B rings of the steroid nucleus from a neighboring molecule. The cholate residues are staggered within the bilayers resulting in patterns that maximize hydrophobic interactions between carboxylate sidechains and steroid rings. Since this is the predominant feature of the hydrophobic core, the carboxylate sidechain appears to play a key role in the hydrophobic interactions between cholate residues. The importance of similar interactions within micelles has been suggested by recent solution studies showing that the concentrations at which micellar association occurs (so-called uncritical multimer concentration) are dependent on sidechain length (22). In a series of 3α,7α,12α-trihydroxy-5β-cholanoates with a common steroid nucleus, the C27 (cholestanoate), C24 (cholate), C23 (norcholate), and C22 (bisnorcholate) derivatives display uncritical multimer concentrations of 0.5 mM, 13 mM, 30 mM, and 58 mM, respectively (22). Examination of Fig. 5 indicates that hydrophobic interactions between carboxylate sidechains and steroid rings within the hydrophobic bilayers should be enhanced as the lengths of the carboxylate sidechains are increased until they reach a length that is too great to permit full overlap with adjacent steroid

TABLE 4. Calcium-oxygen distances

Oxygen distance from calcium ion	
O4	2.429 Å
O4'	2.59
O5	2.466
O6	2.415
O7	2.401
O8	2.406
O9	2.363
O10	2.366

Standard deviations are approximately 0.005 Å, except for O4' which is 0.05 Å.

TABLE 5. Torsion angles with the carboxylate sidechain

Atom 1	Atom 2	Atom 3	Atom 4	Angle
C17	C20	C22	C23	−166.2°
C20	C22	C23	C24	−172.3
C22	C23	C24	O5	−56.2
C22	C23	C24	O4	122.1
C17	C20	C22'	C23'	69
C20	C22'	C23'	C24'	−179
C22'	C23'	C24'	O5	69
C22'	C23'	C24'	O4'	−103

Angles for the major conformation are given first, followed by angles for the minor conformation; the estimated errors in these angles are 0.4° and 2°, respectively.

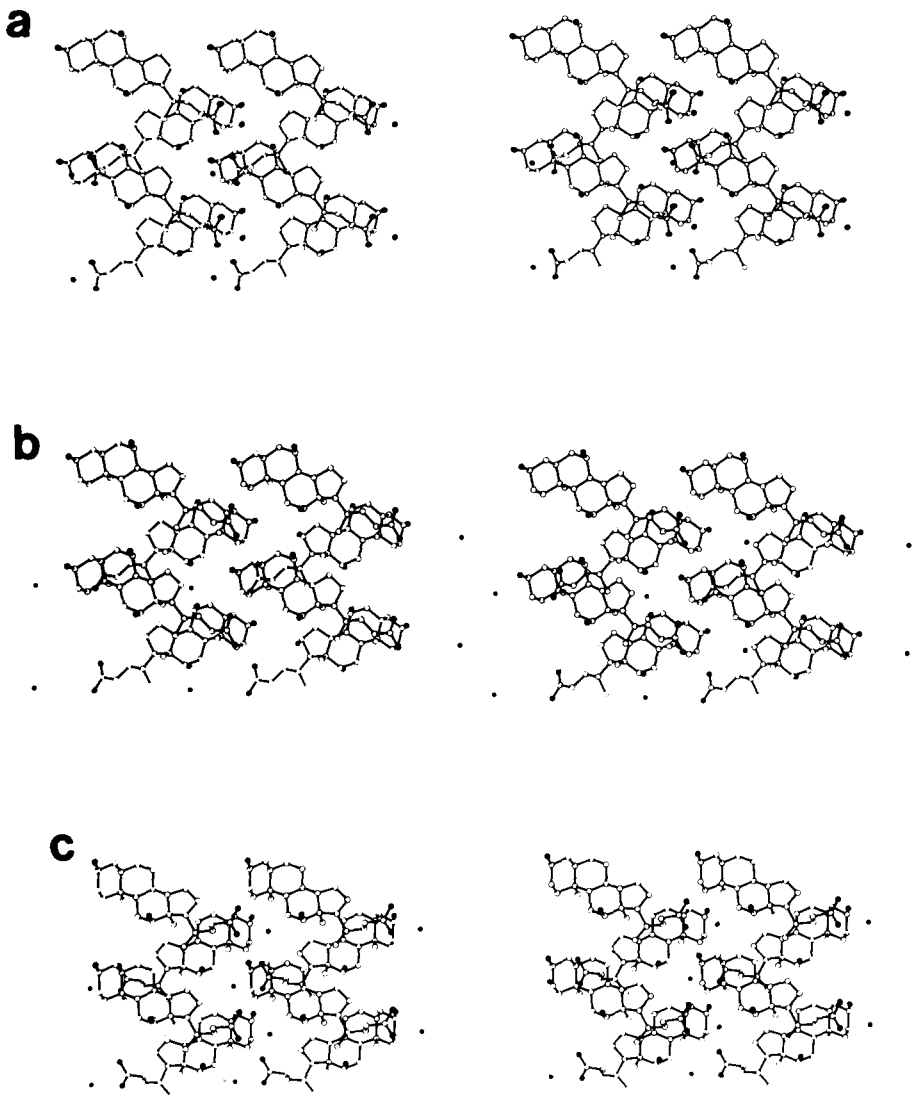

Fig. 5. Hydrophobic bilayer packing patterns in cholanoate crystal structures: (a) calcium cholate chloride heptahydrate; (b) sodium cholate monohydrate; (c) rubidium deoxycholate monohydrate. Filled in circles are the oxygen and calcium atoms.

rings. Therefore, it seems probable that the cholate packing pattern depicted in Fig. 5 may provide a useful model for the interactions that are involved in the formation of bile salt micelles in solution.

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