ACTA CHEMICA SCANDINAVICA 11 (1957) 590—598

Studies on Bile Acid Salt Solutions

I. The Dissociation Constants of the Cholic and Desoxycholic Acids

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The dissociation constants of cholic and desoxycholic acids have been evaluated from data obtained by potentiometric titration of aqueous solutions of their sodium salts with hydrochloric acid at 20 °C. The values obtained are: cholic acid, $pK_A = 4.98 \pm 0.05$, $K_A = 1.05 \times 10^{-5}$; desoxycholic acid $pK_A = 5.17 \pm 0.04$; $K_A = 6.76 \times 10^{-6}$.

Sodium cholate behaves as a normal uni-univalent electrolyte up to a concentration of 0.014—0.015 M; for sodium desoxycholate, the corresponding concentration limit is 0.0045—0.0055 M. Above these concentration limits, deviations occur which are due to micelle formation. After a transition region which for sodium cholate extends to a concentration of approximately 0.05 M and for sodium desoxycholate to about 0.01 M, a concentration range is reached in which the micelle formation is fully developed and which is characterized by a high, nearly constant ratio of the activities of the bile acid and its anion.

Many attempts have been made to determine the dissociation constants of the bile acids. Bondi [1] used conductance values to calculate the dissociation constant of glycocholic acid, Hammarsten [2] determined the constants of tauro- and glycocholic acids by potentiometric titration, whereas Henriques [3] utilized colorimetric pH-determinations to obtain the dissociation constants of several bile acids. Josephson [4] calculated the dissociation constants of a number of bile acids from potentiometrically determined pH values of aqueous solutions of the sodium salts to which different amounts of hydrogen chloride had been added. With the aid of glass electrode measurements, Kumler and Halverstadt [5] determined the dissociation constants of six bile acids in 50—67 % ethanol-water solutions and from these values they calculated the approximate values of the constants in water by assuming that the constants of all the acids in pure water are 17 times greater than in 50 % ethanol-water. They emphasized, however, that the difference is not constant for different acids and hence "an exact calculation of K_A values in one solvent cannot be made from those in another solvent" [5].

The values reported by the above-mentioned authors are collected in Table 1. The values are seen to deviate considerably from each other.

Table 1. The dissociation constants of bile acids.

Acid	Bondi	Hammarsten	Henriques	Josephson	Kumler and Halverstadt
Glycocholic acid	$1.32 \cdot 10^{-4}$	$4.0 \cdot 10^{-5}$	$1.66 \cdot 10^{-3}$	$3.55 \cdot 10^{-5}$	
Taurocholic »		$6.77 \cdot 10^{-2}$	$5.89 \cdot 10^{-4}$	$2.75 \cdot 10^{-2}$	
Glycodesoxycholic »			$3.47 \cdot 10^{-3}$	$10.49 \cdot 10^{-5}$	
Taurodesoxycholic »			$1.18 \cdot 10^{-3}$	$1.23 \cdot 10^{-2}$	
Cholic »				$6.46 \cdot 10^{-6}$	$1.12 \cdot 10^{-5}$
Desoxycholic »				$3.8 \cdot 10^{-7}$	$1.08 \cdot 10^{-5}$
Apocholic »					$1.09 \cdot 10^{-5}$
Lithocholic »					$1.02 \cdot 10^{-5}$
Dehydrocholic »					$1.22 \cdot 10^{-5}$
Dehydrodesoxycholic »					$1.24 \cdot 10^{-5}$

At the time these determinations were carried out, very little was known about the colloidal properties of the bile acid salts; these properties may have affected many of the results. It occurred to us that the values of the dissociation constants should be checked. We have therefore carried out a redetermination of the dissociation constants of cholic and desoxycholic acids [11–13].

EXPERIMENTAL

Cholic acid (Hoffman-La Roche, Basel) was recrystallized several times from 96 % ethanol. The sample used in the measurements had after drying a m. p. of 197 °C.

Desoxycholic acid (Hoffman-La Roche, Basel) was recrystallized three times from glacial acetic acid, dissolved in ethanol and precipitated with ether two times, and boiled in water for 3 h. After drying the sample had a m. p. of 172—173 °C.

The sodium salts of both acids were prepared by dissolving the corresponding acids in absolute ethanol and neutralizing the warmed solutions with equivalent amounts of sodium ethylate in absolute ethanol. The salts crystallizing from the solutions on cooling were dried *in vacuo* over phosphorus pentoxide at an elevated temperature.

The determination of the dissociation constants involved potentiometric titration of aqueous solutions of the bile acid sodium salts with dilute hydrochloric acid. The pH values were determined with a valve potentiometer (Radiometer PHM3, Copenhagen) fitted with a glass electrode and a saturated calomel electrode as reference electrode. The titrations were conducted in a nitrogen atmosphere and were performed so that the method of Back and Steenberg [6] for the determination of the solubilities and solubility products of the acids could be utilized. Before the titration a slight excess of sodium hydroxide solution was added to the bile acid salt solution and the titration was continued over the equilibrium point until the bile acid began to precipitate.

RESULTS

Figs. 1 and 2 show two typical titration curves. The first parts of the curves are quite normal. The liberated acid, particularly cholic acid, has, however, a tendency to remain in solution, whereupon a supersaturated solution is produced. When precipitation finally occurs, the pH of the solution increases abruptly to the equilibrium value of the solution saturated with acid.

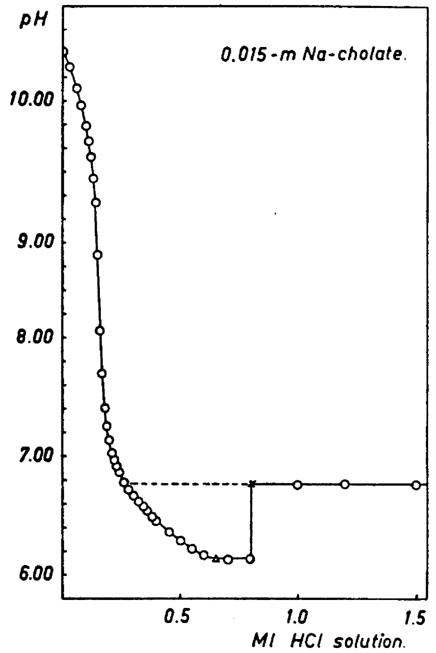

Fig. 1. Potentiometric titration of sodium cholate with HCl. 20 ml 0.0150 M sodium cholate solution + NaOH. Titrant: 0.0500 M HCl. △ The solution became turbid. × Precipitation occurred.

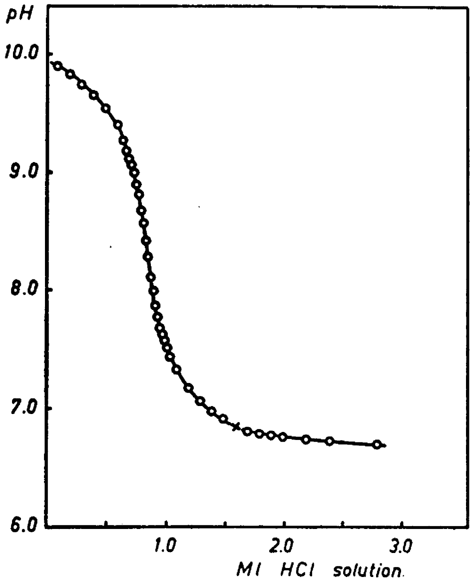

Fig. 2. Potentiometric titration of sodium desoxycholate with HCl. 40 ml 0.0050 M sodium desoxycholate solution + NaOH. Titrant: 0.0050 M HCl. × Precipitation occurred.

The calculation of the dissociation constants was based on the assumption that the ordinary dissociation equilibrium of weak acids is valid:

$$1. \qquad \frac{a_{\mathrm{H_3O^+}} \cdot a_{\mathrm{A^-}}}{a_{\mathrm{HA}}} = K_{\mathrm{A}}$$

Only the first parts of the titration curves which were unaffected by precipitation of acid or supersaturation were used in the calculations which were based on eqn. 2 [6].

Table 2. The dissociation constant of cholic acid at 20° C.

Initial sodium cholate concentration Moles per litre	pK_A	Mean value of pK_A
0.00300	5.03 4.99 5.00 4.94	4.98 ± 0.05
0.00600	4.96 4.94	
0.00800	4.95 4.96	
0.0100	4.98 4.96 5.01	
0.0120	4.95	
0.0140	4.99 5.02	
0.0150	5.07	5.07
0.0160	5.07	5.07
0.0180	5.13 5.14	5.13
0.0200	5.17 5.22	5.19
0.0220	5.23	5.23
0.0250	5.25 5.27	5.26
0.0300	5.35	5.35
0.0400	5.44	5.44
0.0500	5.48	5.48 ± 0.01
0.0700	5.49	
0.100	5.49	
0.125	5.48	
0.150	5.49	
0.175	5.48	
0.200	5.48	

Table 3. The dissociation constant of desoxycholic acid at 20° C.

Initial sodium desoxycholate concentration Moles per litre	pK_A	Mean value of pK_A
0.00100	5.21 5.17	5.15 ± 0.04
0.00200	5.15 5.15	
0.00300	5.16 5.20 5.17	
0.00400	5.20 5.17 5.16	
0.00500	5.17 5.15 5.14 5.15	
0.00550	5.24	5.24
0.00600	5.30 5.28 5.30	5.30
0.00650	5.45	5.45
0.00700	5.44 5.48	5.46
0.00800	5.58 5.56	5.57
0.00900	5.82 5.71	5.76
0.0100	5.79	5.79
0.0110	5.89	5.89
0.0120	5.89	5.89
0.0130	5.96	5.96
0.0150	6.04 6.12	6.08
0.0170	6.18	6.18
0.0200	6.17	6.17
0.0300	6.23	6.23
0.0500	6.35	6.35
0.0750	(6.21)	

2.
$$\mathrm{p}K_A = \mathrm{pH}_x + \log \frac{C_s + C_{OH^-} - C_{H_3O^+}}{C_{tot} - C_s - C_{OH^-} + C_{H_3O^+}} + \frac{0.5\sqrt{\mu}}{1+\sqrt{\mu}}$$

where

$$(C_s + C_{OH^-} - C_{H_3O^+}) \cdot f_{HA} = a_{HA}$$

and

$$(C_{tot} - C_s - C_{OH^-} + C_{H_3O^+}) \cdot f_{A^-} = a_{A^-}$$

(pH_x is a recorded pH value; C_s is the corresponding concentration of hydrogen chloride added after the equivalence point and C_{OH^-} and $C_{H_3O^+}$ the corresponding hydroxyl and hydroxonium ion concentrations; C_{tot} is the original concentration of bile acid salt recalculated to the conditions at the point x; a_{HA} and a_{A^-} are the activities of the undissociated bile acid and its anion, respectively, and f_{HA} and f_{A^-} the corresponding activity coefficients; μ is the ionic strength of the solution).

In the calculation of pK_A, we have used 10—20 titration points after the equivalence point which yielded conforming values for the constant. The results of the calculations are given in Tables 2 and 3.

The titrations of dilute cholate solutions up to 0.014 M give a constant value of the dissociation constant of cholic acid: $pK_A = 4.98 \pm 0.05$ (Fig. 3). In the range from 0.015 to 0.040 M, the pK_A values increase with cholate concentration. Beginning with the 0.05 M and up to 0.20 M cholate solution, the

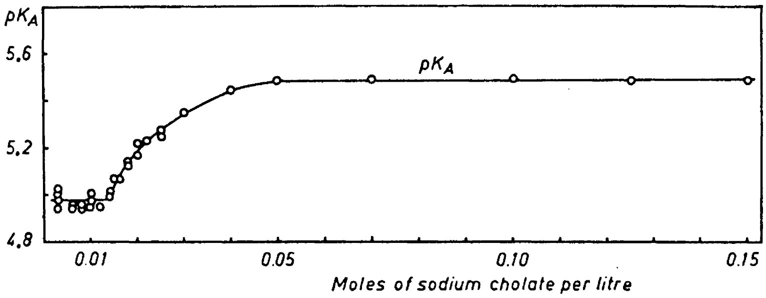

Fig. 3. The dissociation constant of cholic acid determined by potentiometric titration of sodium cholate solutions of different concentration with HCl.

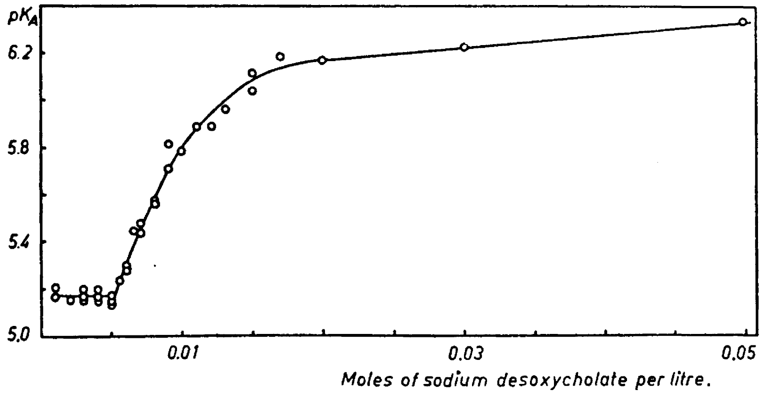

Fig. 4. The dissociation constant of desoxycholic acid determined by potentiometric titration of sodium desoxycholate solutions of different concentration with HCl.

titrations again give a constant pK_A value, pK_A = 5.48 ± 0.01, which is considerably higher than the value obtained for the dilute cholate solutions.

The results for desoxycholic acid are similar (Fig. 4). At high desoxycholate dilutions, up to 0.005 M, the dissociation constant is constant, pK_A = 5.17 ± 0.04. The value of the constant then increases rapidly in the concentration range from 0.006 to 0.010 M, but more slowly above the latter concentration. The highest value of pK_A obtained was 6.35. Values relating to concentrations higher than 0.05 M are uncertain.

DISCUSSION

Many earlier studies have shown that sodium cholate and desoxycholate behave as normal uni-univalent electrolytes only in relatively dilute solutions [7–13]. The deviations from the normal behaviour that occur at higher concentrations are due to micelle formation. One of us has previously established that the power to solubilize hydrocarbons is first observed when the concentration of the solution exceeds 0.015 M in the case of cholate and 0.005 M in the case of the desoxycholate [9–13]. This indicates that micelle formation sets in approximately at these concentrations. The micelle formation influences many of the other properties of the solutions.

The method we have used to calculate values of dissociation constants assumes that micelle formation does not take place and gives correct values only if this condition is fulfilled. Consequently only those values are reliable that apply to dilute solutions whose concentrations are below the limits given above. Accordingly, the following values are presented for the dissociation constants of the two bile acids:

Cholic acid: pK_A = 4.98 ± 0.05; $K_A = 1.05 \times 10^{-5}$ (20 °C)
Desoxycholic acid: pK_A = 5.17 ± 0.04; $K_A = 6.76 \times 10^{-6}$ (20 °C)

These dissociation constants are definitely higher than those reported by Josephson [4]. The value for cholic acid agrees very well with that calculated by Kumler and Halverstadt [5], but our value for desoxycholic acid is lower than their value.

Kumler and Halverstadt [5] remarked that the dissociation constants of the unconjugated bile acids should be of the same order as the constants of the higher aliphatic carboxylic acids, *i. e.* 1.0—1.4 × 10^{-5}, since the bile acids can be considered derivatives of valeric acid with a sterol group in the γ position. They presumed that the introduction of an unsubstituted sterol nucleus should lead to a decrease in the acid strength; hydroxyl and ketonic groups substituted on the sterol nucleus should strengthen the acid properties, but owing to the distance separating them from the carboxyl group, their effect can only be very slight. Kumler and Halverstadt thus came to the conclusion that the dissociation constants of cholic and desoxycholic acids should be almost equal, since the only structural difference between them is the presence of a hydroxyl group in position 7 in cholic acid which is separated from the carboxyl group by seven carbon atoms and that both acids should be approximately equal in strength to caproic acid ($K_A = 1.32 \times 10^{-5}$). Their own measurements of the dissociation constants in ethanol-water solutions and also the calculated values for water solutions seemed to verify these assumptions. Josephson's value for the dissociation constant of cholic acid was, however, seventeen times as great as the value obtained for desoxycholic acid, and both acids, especially the latter, seemed to be much weaker than caproic acid. Our results verify the assumption of Kumler and Halverstadt that the dissociation constants of both acids are of the same magnitude as but slightly lower than that of caproic acid. The presence of one hydroxyl group more in cholic acid than in desoxycholic acid molecule seems, however, to influence the acid strength in water more than Kumler and Halverstadt presumed.

The reason for the very low value of desoxycholic acid obtained by Josephson is evident from our study. When the calculation of the value of the dissociation constant is based on pH measurements in 0.01—0.015 M solutions and the presence of micelles in these solutions is not taken into account, the values obtained are approximately $16—8 \times 10^{-7}$. Josephson's calculations were based on measurements of solutions whose concentrations were 0.01 M. Furthermore, many of the measurements were carried out on solutions supersaturated with bile acid.

From the data we have presented, also the concentrations at which the properties of the solutions begin to deviate from those typical of solutions of uni-univalent electrolytes become clearly evident. These deviations are indicated by the increased values of the dissociation constant when the concentration exceeds 0.013—0.015 M for sodium cholate and 0.0045—0.0055 M for sodium desoxycholate. At these concentrations, micelle formation by bile acid anions evidently begins, which is in accordance with the results of other measurements carried out in this laboratory [9-13].

If it is assumed that the equilibrium is correctly represented by eqn. 1 in all concentration ranges and that the values of K_A are those given above, it is obviously possible to determine from the experimental data the ratio of the activities of the bile acid and its anion at different concentrations and at different stages of the titrations. We shall only draw attention to the variation of this activity ratio with the concentration of the bile acid salt. The pH values at the equivalence points have been employed to determine this relationship. It is seen from Fig. 5 that the activity ratio remains constant in cholate solutions above 0.05 M and nearly constant in desoxycholate solutions above 0.010 M. This provides an explanation for the observation that the apparent dissociation constants calculated from the experimental data for the more concentrated bile acid salt solutions remain practically constant at values higher than the correct value. In solutions above 0.015 M in cholate and above 0.005 M in desoxycholate the bile acid anions associate to micelles in increasing proportion [9,11-13], as a result of which the activities of the free anions are lowered. On the other hand, solubility measurements conducted in this laboratory have shown that a part of the bile acid molecules are solubilized by the micelles [11,14] and as a consequence the activity of the bile acid molecules is lower than would otherwise be the case. Micelle formation and solubilization thus tend to maintain the ratio of the activities of bile acid molecules and bile acid anions nearly constant at higher concentrations.

Our study shows that sodium cholate behaves as a normal uni-univalent electrolyte only up to a concentration of about 0.014 M and that micelle formation obviously is fully developed when the concentration exceeds 0.05 M. In

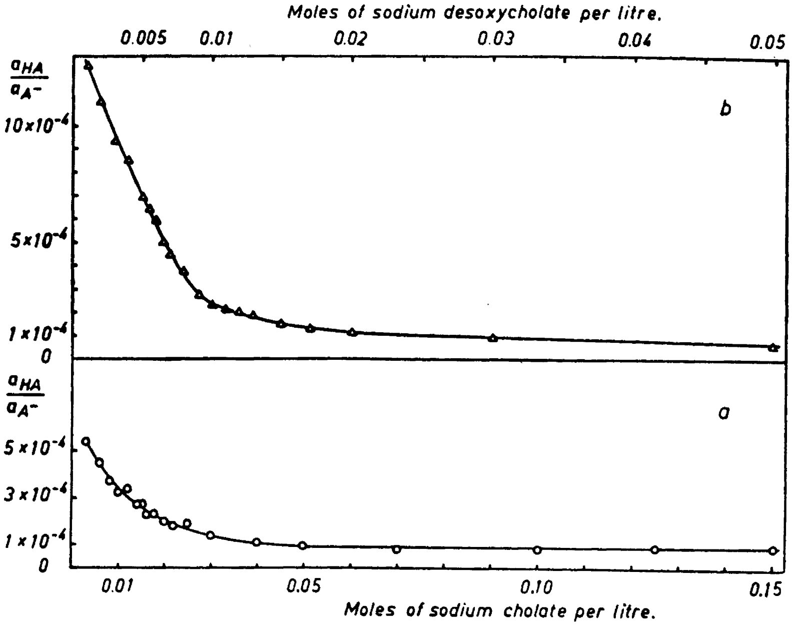

Fig. 5. The ratio of the activities of bile acid and its anions in sodium cholate and desoxycholate solutions.
a) Sodium cholate solutions.
b) Sodium desoxycholate solutions.

the concentration range between these two limits, evidently only a part of the cholate anions have undergone micelle formation. — Sodium desoxycholate behaves normally up to a concentration of about 0.0045 M and micelle formation is obviously fully developed in solutions above 0.01 M; between these two limits there is a transition region.

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Received December 14, 1956.