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On the protonation of glycocholate and glycodeoxycholate ions

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ABSTRACT

The protonation of glycocholate (GC^-) and glycodeoxycholate (GDC^-) ions was studied at 25°C and in $N(CH_3)_4Cl$ as an ionic medium at concentrations 0.100, 0.500 and 0.800 mol L^{-1} by measuring the electromotive force of a galvanic cell containing a glass electrode, as a function of acidity of solution. Also the solubility of glycocholic and glycodeoxycholic acids was determined in the same experimental conditions. The protonation constants of GC^- and GDC^- were determined and it was found that a further protonation of HGC occurs by increasing acidity. Its protonation constant was also determined.

Keywords: Anions of bile acids, protonation constants, solubility.

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INTRODUCTION

Sodium salts of cholic and deoxycholic acids and their conjugates with glycine and taurine are present in human bile (Small, 1971). Many such compounds play an important role from the biological point of view because they are able to form micellar aggregates in aqueous solutions, even at low concentrations and to solubilize many compounds, like cholesterole and lecitine (Carey, 1985) that are slightly soluble in water.

The behaviour of anions of some salts of dihydroxycholanic bile acids, like deoxycholate (DC^-) (Bottari *et al*. 1985; 1989), glycodeoxycholate (GDC^-) (Bottari and Festa, 1993) and taurodeoxycholate (TDC^-) (Bottari and Festa, 1996) was studied previously.

More recently the trihydroxycholanic ions, in particular the taurine and glycine conjugated cholic acids, have been a subject of two papers, the first treating the composition (Bottari *et al*., 1999) and the second correlating structure and composition (Bottari *et al.*, 1999) of micellar aggregates (micelles).

To study the behaviour of these ions in solution able to give micelles, composition and existence range of the formed species, it is necessary to know their protolytic properties and the solubility of the corresponding acids.

Taurocholic and taurodeoxycholic acids are water soluble and are strongly dissociated in aqueous solutions. Dihydroxy- and trihydroxy-cholanic acids and their conjugates with glycine are slightly soluble in water. Only a few data have been reported in the literature on the properties of these compounds (Small, 1971). This scarcity of information on bile acids is due to their lack of solubility, while the fully ionized forms self-associate over a narrow concentration range to form micelles. As a consequence of this self-association, the apparent dissociation constant values were found to vary markedly as a function of the concentration of the ionized species (Ekwall *et al.*, 1957). This subject was further studied against similar difficulties for obtaining protonation constants of cholate and deoxycholate species (Ekwall *et al.*, 1957; 1958).

Other researchers (Fini *et al.*, 1987), by studying chemical properties of bile acids, obtained dissociation constants for carboxylic group of a series of glycine(N-acyl) conjugated and not conjugated bile acids, in mixture of solvents. Different ratios of dimethylsulfoxide(DMSO)–water or methanol–water were used. Constant values were obtained by extrapolation.

At 25°C and 0.5 mol L^{-1} $N(CH_3)_4Cl$ the solubility of deoxycholic acid in water and its dissociation constant were determined previously (Bottari *et al.*, 1985).

In this paper the investigation of glycocholate and glycodeoxycholate protonation and solubility of the corresponding bile acids are reported. Their protonation constants and solubility are determined.

The constant ionic medium method (Biedermann *et al.*, 1953) was adopted in order to minimize the change of activity coefficients of the reagents in spite of the variation of the concentrations.

Experiments were carried out at three different concentrations of $N(CH_3)_4Cl$ used as ionic medium (W = 0.100; 0.500 and 0.800 mol L^{-1}) and at 25°C.

EXPERIMENTAL

Apparatus

Electromotive force (e.m.f.) measurements were carried out by using Radiometer PHM64 or Metrohm model 654 potentiometers. The potentiometric salt bridge and the reference electrode (RE = Ag, AgCl/W mol L^{-1} $N(CH_3)_4Cl$, saturated with AgCl) were similar to those previously described (Forsling *et al.*, 1952). The Ag, AgCl electrode was prepared as previously described (Brown, 1934). The e.m.f. measurements were reproducible within ±0.2 mV. The measured values were constant a few minutes after each addition of HCl in the absence of solid bile acids, whereas, in the presence of solid, they reached a constant value after 1–2 hours. In both case e.m.f. values remained constant within ±0.2 mV, overnight.

A model 6186 Hewlett-Packard DC source was used to deliver a current of constant intensity (1 or 0.5×10^{-3} A). The time of current generation was measured automatically by means of a digital chronometer with an accuracy of 0.01s. The current intensity was checked by using a standard resistance Norma model 80 No. 1702676 and a Keithly model 199 or a Leeds and Northrupp model K5 apparatus.

During the flow of current, solution S was magnetically stirred and a stream of purified N_2 was bubbled through it.

Reagents

Hydrochloric acid and tetramethylammonium chloride were prepared and analysed as previously described (Bottari *et al.*, 1986; 1988).

Glycocholic acid, HGC, Sigma, was used without further purification. Glycodeoxycholic acid, HGDC, was prepared from NaGDC, a Sigma product, by slow addition of a slight excess of HCl 1:1 to a diluted and warm solution of NaGDC whilst stirring. The formed solid was washed with distilled water until the excess HCl was eliminated. HGC and HGDC were successively analysed by thin layer chromatography(TLC) and the absence of the appreciable amount of impurity (≤1%) was checked.

Method

The dissociation of HGDC and HGC, respectively, at 25°C and W constant of $N(CH_3)_4Cl$ (where W = 0.100, 0.500 and 0.800 mol L^{-1}), can be expressed by the following equilibria:

$$\text{HGDC} \Leftrightarrow H^+ + GDC^- \qquad K_1$$
$$\text{HGC} \Leftrightarrow H^+ + GC^- \qquad K_2$$

the constants K_1 and K_2 are defined by the following expressions:

$$K_1 = c_H \cdot c_{GDC} \, (c_{HGDC})^{-1} \quad (1) \text{ and}$$
$$K_2 = c_H \cdot c_{GC} \, (c_{HGC})^{-1} \quad (2)$$

where c_x indicates the free concentration of the ion x and charges are omitted.

In a solution where solid HGDC and HGC, respectively are present, it is possible to assume that c_{HGDC} and c_{HGC} respectively are constant and equations (1) and (2) can be written, as follows:

$$c_{HGDC} \, K_1 = K'_1 = c_H \cdot c_{GDC} \quad (3) \text{ and}$$
$$c_{HGC} \, K_2 = K'_2 = c_H \cdot c_{GC} \quad (4)$$

From equations (3) and (4), it can be seen that K'_1 and K'_2 are constant, when c_{HGDC} or c_{HGC} are constant, *i.e.* in the presence of solid bile acids. The values of K'_1 and K'_2 can be calculated when the values of respective solubility are known. They ($s_{HGDC} = c_{HGDC}$) and ($s_{HGC} = c_{HGC}$) can be determined for saturated solutions after filtration. K'_1 and K'_2 can be obtained experimentally by measuring c_H and c_{GDC} or c_{GC}, respectively.

The free concentration of hydrogen ions, c_H, was obtained by measuring the e.m.f. of the following galvanic cell:

$$\text{RE / solutions S/ GE} \qquad \text{(I)}$$

where RE is the reference electrode, GE is the glass electrode and solution S the ionic medium at W concentration.

At 25°C and in mV units, the e.m.f. of the cell (I) can be written, as follows:

$$E_I = E^0_I + 59.16 \log c_H + E_j \qquad (5)$$

where E^0_I, a constant, and E_j the liquid junction potential, are determined in the first part of each measurement, in the absence of bile acid, by adding known amounts of HCl in the same ionic medium, so that $c_H = C_H$, the analytical excess of hydrogen ions.

After the determination of E^0_I and E_j, the excess of HGDC or HGC is added to the solution S until the solid is obtained. All measurements are performed in the presence of solid bile acid (HGDC or HGC). The solution

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is gradually alkalinized so that known amounts of GDC^- or GC^- are formed, and, for each point E_I is measured, to obtain c_H.

The alkalinization of the saturated solution is carried out electrochemically. A platinum net electrode used as a cathode is dipped in the measurement vessel, while an external silver electrode is used as anode. The Ag electrode is dipped in the solution of ionic medium and a salt bridge (W mol L^{-1} $N(CH_3)_4Cl$) connects the test solution and Ag electrode.

In such a way, little and known quantities of OH^- can be produced coulometrically by using the Bloch apparatus of Figure 1.

The knowledge of the quantity of the generated current and the e.m.f. measurement of cell(I) allow each point K'_1 and K'_2 to be calculated for HGDC and HGC, respectively.

As described previously, the solubility of the two bile acids also has to be determined.

At 25°C, different series of $N(CH_3)_4Cl$ solutions at W concentration are equilibrated with an excess of solid HGDC or HGC overnigth. From preliminary measurements it was inferred that 6 hours were sufficient to reach solubility equilibrium.

A measured volume of filtered solution is introduced into the measurement vessel and s_{HGDC} (or s_{HGC}) is determined potentiometrically, by producing coulometrically OH^- by using the apparatus described in Figure 1. The equivalence point is appreciated as previously described (Gran, 1952). From K'_1, s_{HGDC} and K'_2, s_{HGC} the values of K_1 and K_2 can be obtained. By increasing C_H in solution S, the solubility of HGDC and HGC shows a different trend.

The former decreases by increasing C_H, while the latter (HGC solubility) increases by increasing C_H. This effect was explained by supposing a further protonation of HGC at higher C_H, according to the following equilibrium:

$$HGC + H^+ \Leftrightarrow H_2GC^+ \qquad (6)$$

Equation (6) can be defined by the constant $K_b = c_{H2GC}\,(c_H\, c_{HGC})^{-1}$ and it can be investigated with a procedure similar to that described for K_1 and K_2.

In solutions, where solid HGC is present, $c_{HGC} = s_{HGC}$ can be assumed as a constant, so that $K'_b = K_b \cdot c_{HGC} = c_{H2GC} \cdot c_H^{-1}$ remains constant.

To determine K'_b, e.m.f. measurements of cell (I) were carried out. In solution S, in the presence of solid

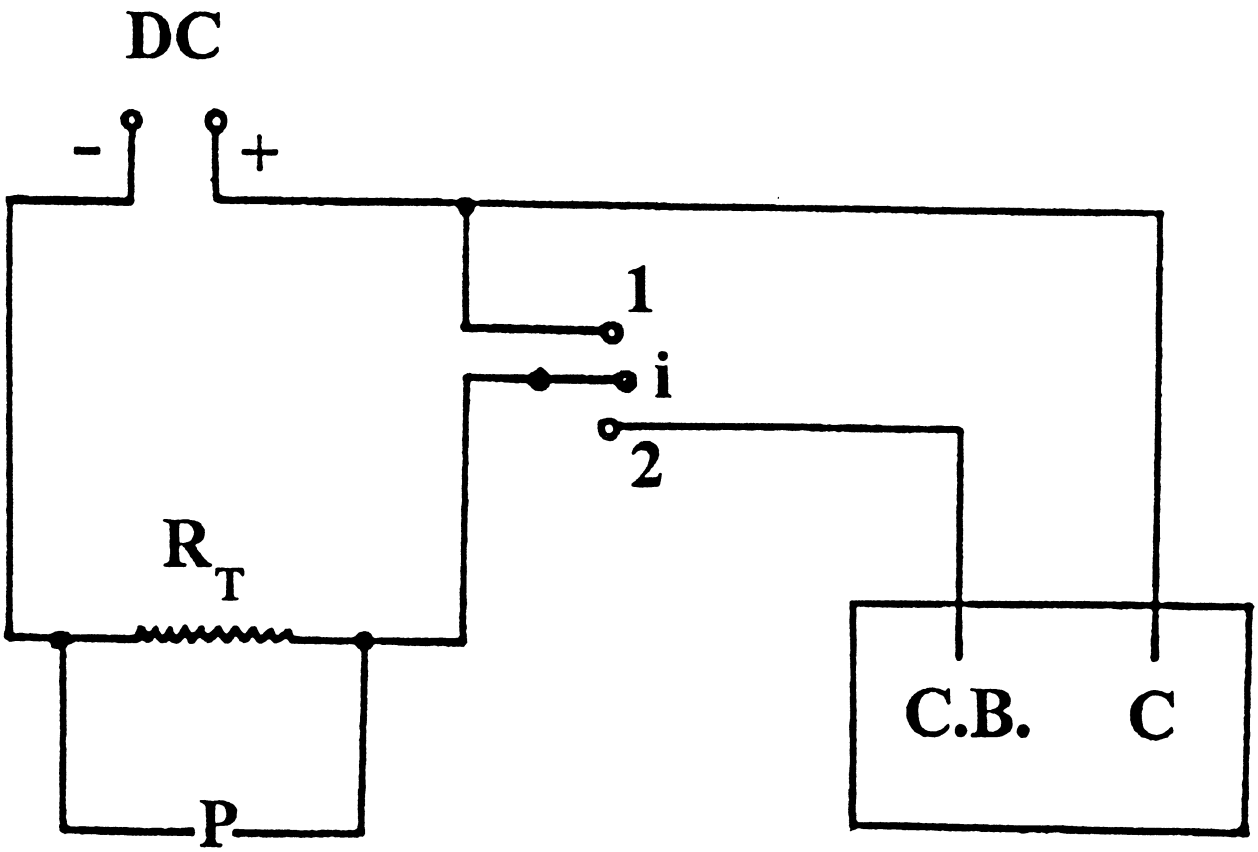

Figure 1 *Source of constant corrent, DC. R_T is a standard resistance, P is a potentiometer, C. B. (Coulometer Bridge), is a block, representing the external anode electrode connected through the salt bridge to the Solution S, where a Pt net (as a cathode) is dipped. C indicates the galvanic cell(I).*

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HGC, C_H was gradually increased and c_H was obtained from e.m.f. measurements.

It could be seen that for all points $C_H > c_H$. A similar procedure applied to HGDC, provided $C_H = c_H$. In the first case protonation of HGC occurs.

RESULTS AND DISCUSSION

Analytical values, C_H, and e.m.f. measurements, c_H, together with the knowledge of s_{HGDC} and s_{HGC}, respectively, are treated in order to obtain the values of the constants K_1, K_2 and K_b.

On the basis of equation (5), c_H can be obtained from E_I when E_j is known. In a constant ionic medium, E_j depends only on c_H, according to the equation $E_j = -j\ c_H$ (Biedermann *et al.*, 1953).

The j values, depending on W, are determined by measuring the values of E_I as a function of $C_H = c_H$ in the absence of other equilibria. From equation (5), it follows that at 25°C and in mV units:

$$E^{0'}{}_I = E^0{}_I + E_j = E_I - 59.16 \log c_H = E^0{}_I - j\ c_H$$

The plot $E^{0'}{}_I$ *versus* c_H can be well approximated with a straight line. Extrapolation on the ordinate gives $E^0{}_I$ and slope provides j. As an example, in Figure 2, the determination of j for W = 0.500 mol L^{-1} is shown. A value $j = -110 \pm 5$ mV L mol^{-1} was obtained. The values $j = -63 \pm 3$ mV L mol^{-1} and $j = -490 \pm 10$ mV L mol^{-1} are obtained at $W = 0.800$ and $W = 0.100$ mol L^{-1}, respectively.

The trend of j with W agrees with literature data, because the liquid junction potential depends on the composition of the ionic medium. It was proposed (Dyrssen, 1952) $j = -440$ mV L mol^{-1} for 0.100 mol L^{-1} $NaClO_4$, while it was found that $j = -16.5$ and -63 mV L mol^{-1} for 3.00 and 1.00 mol L^{-1} $NaClO_4$, respectively (Rossotti *et al.*, 1956).

As explained above to obtain K_1 and K_2, it is necessary to determine in a first step K'_1, K'_2, s_{HGDC} and s_{HGC}. In Table 1 series of K'_1 and K'_2 are collected as examples are different values of W.

It is evident that K'_1 and K'_2 remain constant, so that formation of aggregates in appreciable concentration can be excluded. The production of small amounts of OH^- (*via* coulometry) and the presence of the solid bile acid allows to e.m.f. measurements to be made at total concentration of GDC^-, GC^-, respectively, not higher than 10^{-5} mol L^{-1}.

This concentration is much less than c.m.c. of both salts of glycodeoxycholic and glycocholic acids.

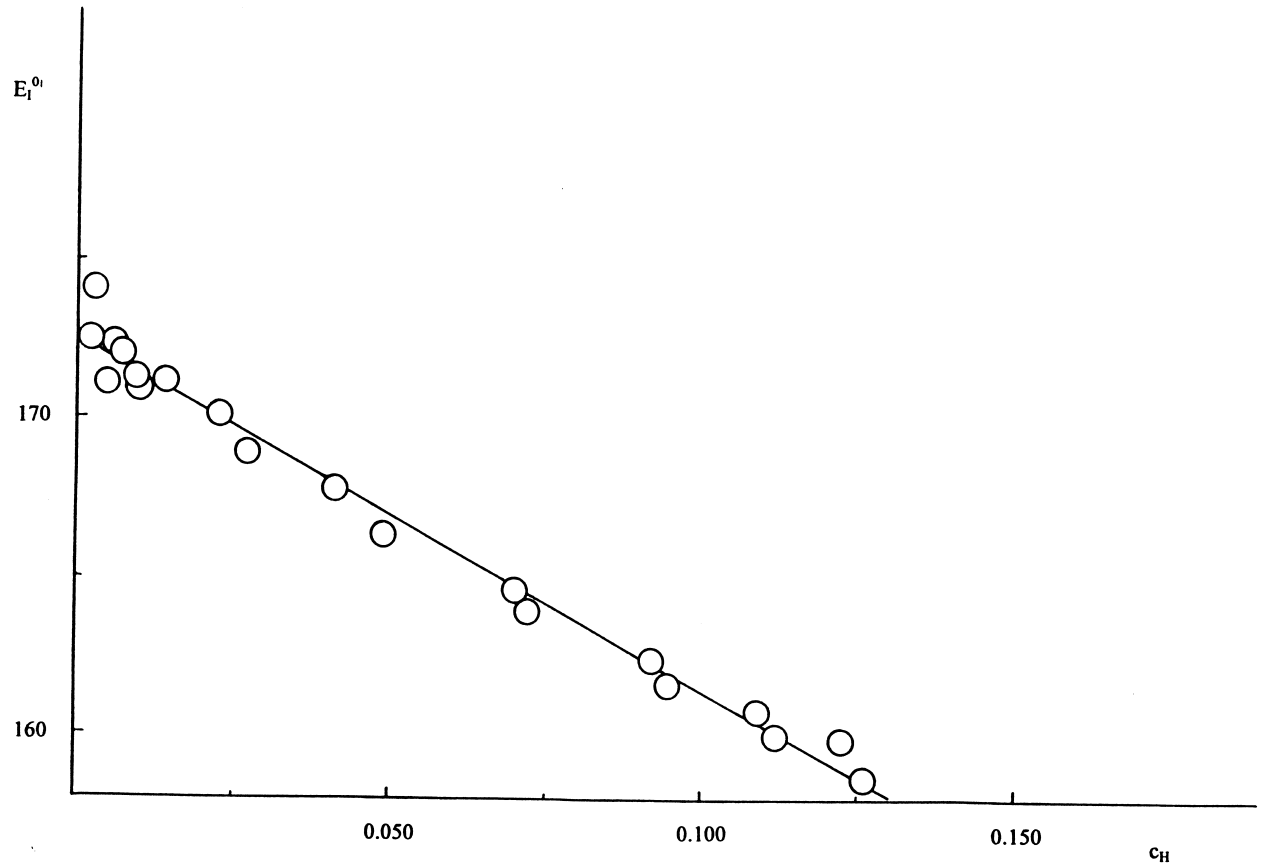

Figure 2 *An example of the determination of j, the liquid junction potential. Experimental data, obtained at 25°C and* W = *0.500 mol* L^{-1}*, can be well approximated with a straight line with slope* j = *–110 mV L* mol^{-1}.

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Table 1 *Examples of obtained values of K_1' and K_2' for HGDC and HGC, respectively at 25°C and W mol L^{-1} $N(CH_3)_4Cl$ as ionic medium*

W	$-\log K_1'$	$-\log K_2'$
0.100	8.62, 8.61, 8.63, 8.66, 8.64, 8.65, 8.64, 8.66 (average value 8.64)	7.10, 7.12, 7.13, 7.12, 7.13, 7.11, 7.11, 7.09. (average value 7.10)
0.500	8.80, 8.89, 8.86, 8.82, 8.89, 8.81, 8.88, 8.87. (average value 8.84)	7.14, 7.13, 7.14, 7.12, 7.15, 7.14 7.16, 7.14. (average value 7.14)
0.800	9.04, 9.02, 9.01, 9.03, 9.07, 9.05, 9.04, 9.03. (average value 9.03)	7.15, 7.14, 7.18, 7.16, 7.16, 7.15, 7.14, 7.18. (average value 7.16)

Table 2 gives the values of the solubility of HGDC (s_{HGDC}) and of HGC (s_{HGC}) determined as described above for all three W; log K_1 and log K_2 are also reported. The error limits indicate the upper and the lower values obtained in the determinations.

The solubility of both HGDC and HGC increases by increasing W. The dependence of K_1 and K_2 on W is more evident. Both the constants decrease by increasing W. A similar dependence on the concentration and the quality of the ionic medium was found for acetic acid (Ellilä, 1953).

A comparison between HDC, HGDC and HGC shows that the latter is the most soluble and the strongest of the three acids. HDC is the least soluble and the weakest.

The trend of solubility agrees with that proposed by others (Fini *et al.*, 1987). They found that the solubility of coniugated bile acids was higher than that of non-conjugated ones and the solubility of the trihydroxycholanic was higher than that of dihydroxycholanic acids. The greater solubility of the former could be expected because of the higher number of hydrophilic groups present in their molecule.

Only a partial agreement exists between our results and those of earlier researchers (Fini *et al.*, 1987) for the constants of the three bile acids. In both cases, conjugated bile acids are stronger than non-conjugated ones. On the other hand, a substantial difference exists between the constants of dihydroxycholanic and trihydroxycholanic bile acids. Fini *et al.* 1987 found the same values for both, while our results show a remarkable difference between them.

The differences can be partially attributed to different experimental conditions. Our data have been obtained in a constant ionic medium, in the absence of any organic solvent and at much lower reagent concentrations. Our concentration of HGDC and HGC is

Table 2 *Solubility of HGDC and HGC, respectively and log K_1 and log K_2, at 25°C and W mol L^{-1} $N(CH_3)_4Cl$ as ionic medium*

W	$-\log s_{HGDC}$	$-\log s_{HGC}$	$-\log K_1$	$-\log K_2$
0.100	4.22 ± 0.03	3.62 ± 0.03	4.42 ± 0.05	3.48 ± 0.05
0.500	4.20 ± 0.03	3.60 ± 0.03	4.64 ± 0.05	3.54 ± 0.05
0.800	4.14 ± 0.03	3.55 ± 0.03	4.89 ± 0.05	3.61 ± 0.05

100 times lower than that of the other Authors, using 2×10^{-3}mol L^{-1} concentration for both bile acids. This concentration is close to the c.m.c. of NaGDC. It is not clear which ionic medium or ionic strength was used (Fini *et al.*, 1987). It seems that ionic strength was only determined by the solubility of the bile acids and the values were extrapolated from data obtained in mixtures of water and organic solvents (DMSO and CH_3OH) at different percentage.

The smallest percentage of organic solvent was 10%. In the case of DMSO, the extrapolation shown by Fini *et al.* above fit only approximately the experimental data.

The behaviour of HGDC and HGC appears very different by increasing C_H. As explained above, for HGDC in all the cases $C_H = c_H$ at $-\log c_H \leq 3$.

In the case of HGC, by increasing C_H, the solubility of HGC increases and we find for all the points $C_H > c_H$. To explain this evidence, a further protonation of HGC was assumed, according to the equilibrium (6). The material balance of hydrogen ion, by taking into account the mass action law, can be written as follows:

$$C_H = c_H + \Sigma_n K_{bn} c_H^n c_{HGC}$$

If it is assumed $n = 1$, *i.e.* only one protonation, the protonation constant can be defined $K_b = c_{H2GC} (c_H \cdot c_{HGC})^{-1}$, where charges are omitted.

As described above, in the presence of solid HGC, c_{HGC} = constant, and $K'_b = c_{H2GC}/c_H$. As an example, some K'_b values are collected in Table 3 for different W. As they are constant, the formulated hypothesis is confirmed and from knowledge of s_{HGC}, the value of K_b can be obtained.

Table 3 *Examples of obtained values of log K_b' and log K_b, at 25°C and W mol L^{-1} $N(CH_3)_4Cl$ as ionic medium*

W	$-\log K_b'$	$\log K_b$
0.100	1.07, 1.05, 1.09, 1.06, 1.08, 1.07, 1.07, 1.08 (average value 1.07)	2.55 ± 0.10
0.500	1.05, 1.09, 1.06, 1.07, 1.08, 1.05, 1.07, 1.07 (average value 1.07)	2.53 ± 0.10
0.800	1.01, 1.05, 1.04, 1.03, 1.06, 1.02, 1.01, 1.04. (average value 1.03)	2.52 ± 0.10

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Table 3 shows the dependence of K_b on W is shown. The trend is similar to the effect of the solubility on W.

Increasing shows the solubility of HGC by increasing C_H is a surprising effect and it stresses the fundamental role played by the protons in the intermolecular bonds that occur in the case of trihydroxycholanic bile acids. This effect is absent in the case of dihydroxycholanic bile acids. The different behaviour between trihydroxycholanic and dihydroxycholanic bile sodium salts has already been stressed with regard to the micelles formation (Bottari *et al.*, 1999; 1999).

Since glycocholic acid can be present in solution as GC^-, HGC and H_2GC^+, the dependence of the solubility on c_H can be expressed according to the following equation:

$$S = c_{HGC} + c_{GC} + c_{H2GC} = s_{HGC} (1 + K_1 c_H + K_b c_H^{-1})$$

Figure 3 shows the dependence of HGC solubility on c_H, for 0.500 mol L^{-1} $N(CH_3)_4Cl$. It can be seen that solubility is a minimum at –log c_H = 3, while it increases at lower and higher –log c_H.

By increasing –log c_H, solubility of HGC increases with a greater slope than that of the increasing S with decreasing –log c_H (left side of the plot).

The study of glycocholate aqueous premicellar and micellar solutions in the same ionic medium seems to be important to confirm the differences between dihydroxycholanic and trihydroxycholanic bile salt. It will be the subject of another paper.

CONCLUSION

The main results of this paper are represented by the values of the constants K_1, K_2 and K_b and of the solubility of HGDC and HGC. The comparison of conjugated (HGDC and HGC) and unconjugated (HDC) bile acids shows that the former are more soluble and stronger than the latter.

It is hard to compare the results of this paper with the properties of the taurine conjugated bile acids because of their very high solubility and very high dissociation constants.

Our results show that the behaviour of dihydroxycholanic and trihydroxycholanic acids towards protonation in acid solutions is different.

These differences confirm those previously found in investigations on the aggregates of deoxycholate and its conjugates with glycine and taurine (trimers as building units) and taurocholate (dimer or octamer as

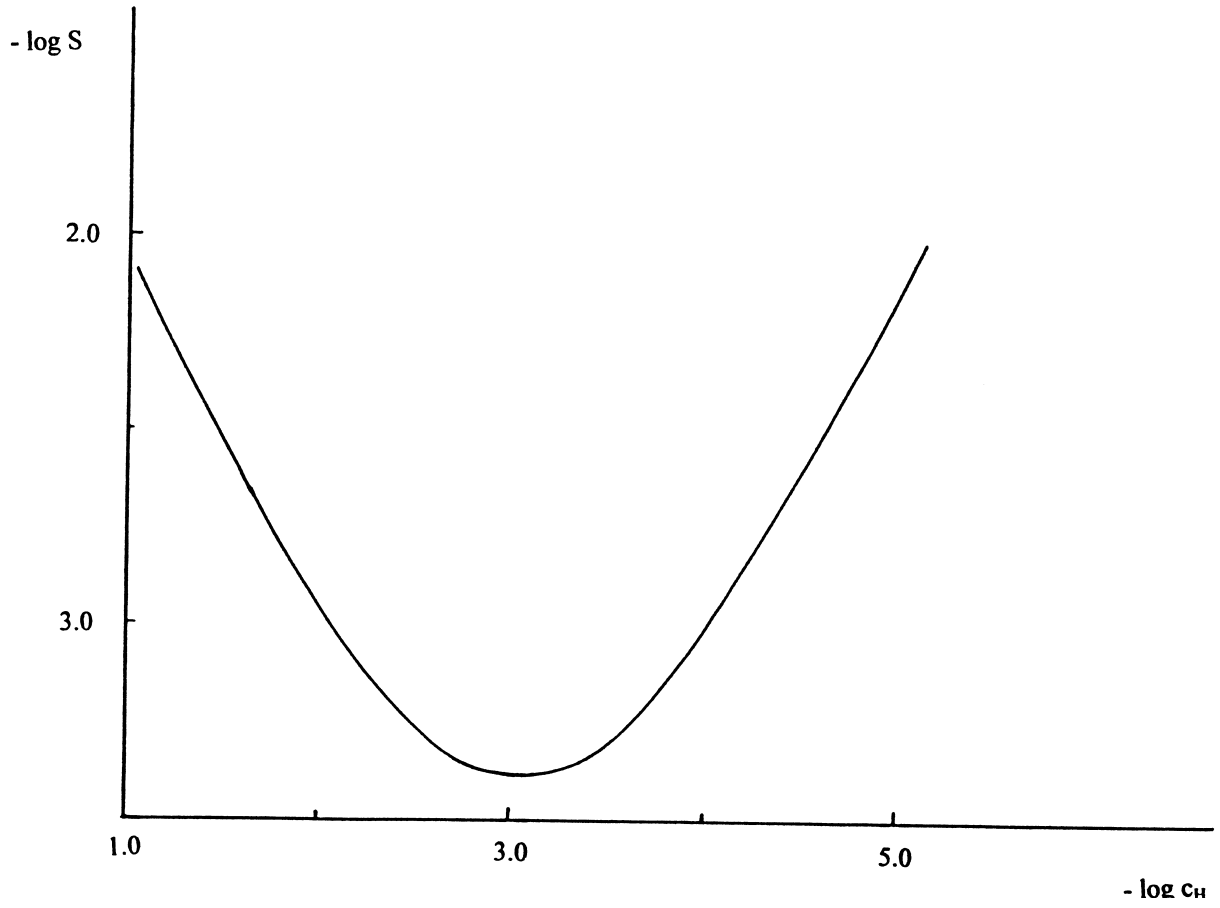

Figure 3 *The dependence of solubility, S, of glycocholic acid on –log c_H at 25°C and in 0.500 mol L^{-1}.*

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building units) (Bottari *et al.*, 1999; 1999).

Finally, the increasing solubility of HGC at high acidity also seems to play an important role from a physiological point of view, because of the presence of excess HCl in the human stomach. From Figure 3, it can be calculated that in solutions where C_H ~0.1 mol L^{-1}, several grams per litre of HGC can be dissolved.

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