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Micellar electrokinetic capillary chromatography analysis of the behavior of bilirubin in micellar solutions

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ABSTRACT

The capacity factor of bilirubin is determined by micellar electrokinetic capillary chromatography (MECC) techniques in three different surfactant systems. The capacity factor of bilirubin in cholic acid, taurocholic acid, and taurochenodeoxycholic acid solutions are compared to each other as a function of pH. The pH range studied is 6.5 to 9.5 which includes the pH range of bile, and includes the most likely pK_a values of bilirubin carboxyl groups. MECC techniques are used to estimate these apparent pK_a values for bilirubin as well as to determine the capacity factors for the separate ionization states of bilirubin in the three different surfactants. Due to the complexity of the bilirubin–bile salt system, it appears as though it is not possible to use MECC to accurately determine the bilirubin apparent pK_a values. Separations are performed in 75 μm capillaries, typically 36 to 52 cm in length. UV detection, electrokinetic injection, and run voltages of 7 kV are typical. Solutions of 25 m*M* of each bile salt are prepared in a 20 m*M* phosphate–borate buffer system.

INTRODUCTION

Micellar electrokinetic capillary chromatography (MECC) was originally introduced in the middle 1980s as a way to utilize capillary electrophoresis (CE) systems for separations of neutral compounds [1,2]. It was soon realized, however, that MECC techniques could also offer enhancement of separations of charged compounds [3,4]. Indeed, since many samples contain components that are neutral as well as charged, it is important to study MECC separations of charged analytes. While some studies of charged analyte MECC separations have been performed, most studies of MECC systems have been separations of neutral analytes. What both types of studies have in common is that the vast majority of all MECC studies make use of sodium dodecyl sulfate (SDS) as the micelle phase [5]. This is to be expected as SDS is an extremely well behaved and well characterized surfactant.

There are other surfactants that can be used successfully in MECC applications. Some of the other pseudo-stationary phases that have been utilized include cyclodextrins [6,7], dodecyltrimethylammonium bromide (DTAB) [7] or cetyltrimethylammonium chloride (CTAB) [8], sodium decyl sulfate (STS) [8] and bile salts [9–13]. Most of the applications utilizing bile salts are separations of chiral compounds. Bile salts are synthesized in the liver from cholesterol and are concentrated in the gall bladder. They have a steroid backbone with various points of substitution. The positions marked R_1, R_2, and R_3 in Fig. 1 determine the general class of bile salts. Cholic acid, deoxycholic acid and chenodeoxycholic acid varieties are shown. In addition, bile salts can be unconjugated or conjugated with compounds such as taurine or glycine in the

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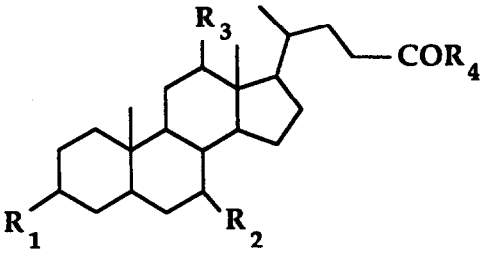

R1	R2	R3	BILE SALT CLASSIFICATION
-OH	-OH	-OH	Cholic Acid
-OH	-OH	-H	Chenodeoxycholic Acid
-OH	-H	-OH	Deoxycholic Acid

R4	CONJUGATES
-OH	Unconjugated
$-NHCH_2CH_2SO_3H$	Tauro-
$-NHCH_2CO_2H$	Glyco-

Fig. 1. Generalized bile salt structure, showing substitution positions for different classifications of bile salts as well as conjugated structures.

position marked R_4. Bile salts will form micelles, although the structure of these micelles is poorly characterized in comparison with more familiar surfactants such as SDS. In fact, all bile salts do not form the same type of micelle, the structure of the micelle can change as a function of bile salt concentration, and the critical micelle concentration (CMC) of various bile salts can change as a function of pH [14]. Nevertheless, bile salt micelles can be successfully used in MECC applications.

The primary biological function of bile salts and bile salt micelles is to solubilize dietary lipids and thereby aid in their elimination from the body. Perhaps the most important compound to be eliminated in this manner is bilirubin. Bilirubin is the breakdown product of haem. Normal human secretions of bile pigment are mainly composed of 65–85% bilirubin diconjugates with the remainder being monoconjugates and unconjugated bilirubin (UCB) [15]. UCB accounts for only 1–3% of the bilirubin forms found in

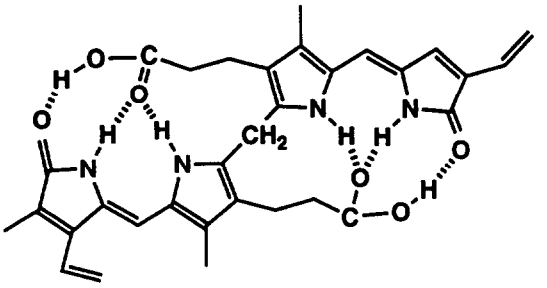

Fig. 2. Structure of fully protonated, unconjugated bilirubin, showing intramolecular hydrogen bonding.

bile [16]. The structure of UCB is shown in Fig. 2. While conjugates of bilirubin are water soluble, unconjugated bilirubin is relatively insoluble in aqueous solutions at pH 7.0 [17]. Yet, the concentrations of UCB in hepatic and gall bladder biles are 10 and 35 μM [18]. These high concentrations of UCB are stabilized in bile solutions through interactions with bile salt micelles and monomers. Bilirubin will begin to precipitate in bile fluids if the UCB concentration rises above the saturation level, if there is a decrease in the binding of UCB by biliary components, or through a combination of these two phenomena [19]. The precipitation of unconjugated bilirubin is thought to be an important step in pigment gallstone formation. Pigment gallstones contain precipitated UCB either in the form of calcium salts or polymers of pigment.

Any change in the bile fluid environment which influences either the concentration or solubility of UCB, or changes the binding of UCB to bile salt micelles, can thus be a precursor to pigment gallstone formation. For instance, the concentration or relative proportion of UCB in bile can change as a result of hemolysis, phototherapy, or hydrolysis of bilirubin conjugates [16]. There are numerous factors which are believed to be important in influencing the binding of UCB to bile salt micelles. Certainly a change in the total concentration of micelles present will affect how much bilirubin is solubilized. Also, if the various bile salts present in bile solutions can solubilize UCB to different degrees, then a change in the bile salt profile can influence the amount of solubilized bilirubin.

Changes in the concentration of calcium ion can change the CMC of bile salts [20]; a change in the CMC of a micelle can then in turn affect binding of solutes. Changes in the levels of lecithin and cholesterol may also affect the binding of bilirubin to bile salt micelles [21,22]. While these numerous factors are believed to influence the bile chemistry of bilrubin, analysis of these solutions is hampered in part by the extremely low solubility of bilirubin at physiological pH values as well as poor understanding of the bile salt micelle structures; thus, the interaction between the bile salt micelles and bilirubin remains incompletely understood.

The pK_a values for the two propionic groups on bilirubin have been the subject of controversy in the literature. Because of bilirubin's low solubility, these values can not be measured directly. Determination of these two pK_a values has been attempted in solvents other than water. Studies of bilirubin in solvents such as dimethyl sulfoxide (DMSO) probably yield inaccurate information as to bilirubin's behavior in aqueous solution. Reported values fall typically in the range of 5 to 9 [17,18]. This is considerably more basic than the pK_a value of typical carboxyl groups. This can be explained in part by the fact that these bilirubin carboxyl groups are known to be involved in intramolecular hydrogen bonding as depicted in Fig. 2. There is also some disagreement in the literature as to whether or not the two pK_a values are identical to each other. One study reported that the pK_a values were quite different from each other, with values of 6.5 and 9.0 [21]. This conclusion was later modified, and the pK_a values are reported to be 8.1 and 8.4 [23]. It is very interesting, and somewhat surprising, that this former work is the only work in the literature to date which attempts to determine the apparent pK_a values of bilirubin in the presence of bile salts.

MECC should be a very convenient method with which to probe the bilirubin–bile salt system. We have examined the behavior of bilirubin in a variety of bile salt solutions as a function of pH. The primary purposes of this study are to: first, determine if MECC systems can be used effectively in these studies; second, to determine the pK_a values of bilirubin; third, to determine the effects of pH on bilirubin's ability to associate with bile salts; fourth, to determine if bilirubin behaves differently in different bile salt solutions. We began these investigations by determining the capacity factor of bilirubin in cholic acid, taurocholic acid, and taurochenodeoxycholic acid solutions as a function of pH. The pH range chosen for study was pH 6.5 to 9.5. This includes the pH range of bile, which varies from pH 6.8 to 7.6, and thus represents the region of most interest in terms of physiological conditions. Our pH range also covers the pK_a range most often reported for bilirubin carboxyl groups. Lower pH values are difficult to study due to the insolubility of bilirubin and the instability of the micelle system. Higher pH values are of little interest physiologically and would slowly etch the fused silica surface of the capillaries.

EXPERIMENTAL

Apparatus and reagents

Separations were performed on fused-silica capillaries purchased from Polymicro Technologies (Phoenix, AZ, USA) with an inner diameter of 75 μm. The capillaries varied in length from 36 cm to 52 cm. A Linear Instruments UVIS 200 detector (Reno, NV, USA) fitted with a deuterium lamp was used for detection (225 nm) of peaks. The detector was placed 26 to 28 cm from the grounded end of the capillary. High voltage was supplied by a Spellman high-voltage d.c. unit (Plainview, NY, USA) with an output range of +/−30 kV. A Dell 316SX computer (Austin, TX, USA) was used for data acquisition. The computer was fitted with a multi-function data acquisition board from National Instruments (Austin, TX, USA). Software was developed in house with Microsoft QuickBasic (Redmond, WA, USA) and National Instrument's Lab Windows software. Distilled water was provided by a Barnstead NANO pure system purchased through Fisher Scientific (Austin, TX, USA). All reagents were purchased from Sigma (St. Louis, MO, USA) except for DMSO which was obtained from Fisher.

Separations performed with SDS systems were detected with a Jasco 2550 UV detector with a

100 μm slit in a microcell holder. Data were recorded directly on a chart recorder. The capillary lengths on this system were 62 cm for the entire capillary length and 33 cm to the detection window.

Bilirubin recrystallization

Bilirubin was purified according to the method of McDonagh and Assisi [24]. Approximately 400 mg of bilirubin IXα was stirred and heated in 460 ml of chloroform (analytical-reagent grade containing ethanol stabilizer) until the solution boiled. The mixture was cooled to room temperature and washed in a separatory funnel with 0.1 *M* $NaHCO_3$ (3 × 100 ml or until the washings were colorless). The solution was dried over 10 g of anhydrous Na_2SO_4 and filtered. The filtrate was heated until boiling and approximately one third of the chloroform was distilled off. Methanol was added to the boiling solution until the solution became perceptibly turbid. The mixture was cooled to room temperature, and after two hours the crystalline precipitate was collected by filtration. The precipitate was washed with chloroform–methanol (1:1) and dried under high vacuum for at least 12 h. The entire procedure was carried out under reduced lighting in order to prevent the isomerization of bilirubin. Typical yields were in the range of 60–70%. Bilirubin prepared in this manner was stored in the dark in a freezer.

Buffer preparation and sample preparation

Solutions of 20 m*M* monobasic sodium phosphate and 20 m*M* sodium tetraborate were prepared. The pH of the monobasic sodium phosphate was adjusted to 6.00 using phosphoric acid, and the pH of the sodium tetraborate was adjusted to 9.60 using sodium hydroxide. For the studies where pH was varied, these two solutions were mixed until the desired pH was obtained. For the SDS studies, equal volumes of the two buffers were mixed and the pH adjusted to 8.5 with phosphoric acid.

A 25-m*M* solution of the desired bile salt was prepared using the above buffer solution. The pH of this solution was recorded as the operating pH. This solution was then filtered using a 45-μm syringe filter (Gelman Sciences, Ann Arbor, MI, USA).

Bilirubin and Sudan III were dissolved separately in DMSO. The sample was then prepared by adding 20 μl of the bilirubin solution and 10 μl of the Sudan III to 1000 μl of the bile salt solution. This mixture was then filtered using a 45-μm syringe filter.

Capillary preparation and run conditions

A detection window was prepared on the capillary by holding a drop of hot sulfuric acid on the capillary surface. The length of the entire capillary and the length from the injection end of the capillary to the center of the window was then recorded for future calculations. The window was then washed with deionized water and then methanol and allowed to dry.

The capillary was rinsed every morning with 1 *M* KOH for 10 min and then with deionized water for 5 min. The rinsing was accomplished by aspirating the solution through the capillary using a Nalgene hand pump (Fisher Scientific) equipped with a solution trap. The bile salt solution was then aspirated into the capillary for 10 min and then the voltage was applied for 5 min. At this point the capillary was ready for a sample injection. If the bilirubin precipitated in the capillary, the capillary was treated again to remove the bilirubin. At the end of the day the capillary was rinsed with 1 *M* KOH for 5 min then deionized water for 5 min and stored in water overnight.

Run voltage was typically 7 kV. The sample was injected onto the capillary by placing the capillary tip and the electrode into sample vial and applying a voltage of 5 kV for 3 to 10 s. The capillary and the electrode were then placed back into the running buffer and the run was started.

RESULTS AND DISCUSSION

While MECC studies of neutral analytes are relatively straight forward, studies of ionizable compounds can be more involved. In a recent article, Khaledi *et al.* [25] developed mathematical models to deal with the behavior of anionic analytes in the presence of anionic micelles. In

that article, it was shown that the capacity factor of such a compound can be determined by the compound's mobility with (μ) and without micelles (μ_0) and the mobility of the micelle itself (μ_{mc}) as shown in eqn. 1.

$$k' = \frac{\mu - \mu_0}{\mu_{mc} - \mu} \quad (1)$$

The problem with analyses of bilirubin is that in the absence of micelles the insolubility of the compound makes determination of μ_0 impossible. In the same article, it was shown that the free solution mobility of an anionic analyte is related to the free solution mobility of the fully ionized species (μ_{A^-}), the ionization constant, and the hydrogen ion concentration as shown in eqn. 2.

$$\mu_0 = \frac{\mu_{A^-}(K_a/[H^+])}{1 + K_a/[H^+]} \quad (2)$$

But again the problem arises of applying this equation to an analysis of the bilirubin system in that the K_a is unknown. But we can make use of another equation found in this article that shows that the observed capacity factor of an anionic analyte is determined by the capacity factor of the protonated species (k'_{HA}), the capacity factor of the ionized species (k'_{A^-}), the ionization constant and the hydrogen ion concentration as shown in eqn. 3.

$$k' = \frac{k'_{HA} + k'_{A^-}(K_a/[H^+])}{1 + K_a/[H^+]} \quad (3)$$

If it is assumed that the capacity factor of the ionized species is significantly less than the capacity factor of the protonated species, then eqn. 3 can be simplified and rearranged to yield eqn. 4.

$$1/k' = 1/k'_{HA} + (K_a/k'_{HA})(1/[H^+]) \quad (4)$$

This equation shows that if the inverse of the observed capacity factor is plotted *versus* the inverse of the hydrogen ion concentration, then the slope and intercept of the resulting line will yield the capacity factor of the protonated species and the ionization constant of the anionic species under investigation.

In order to correct the apparent capacity factor of bilirubin for its mobility when ionized, an iteration process was used. The first step was to determine the mobility of the bilirubin anion in the absence of micelles. As this species is fairly soluble this number can be easily obtained. The next step was to calculate the capacity factor of bilirubin as if it were a neutral compound using the well known equations [1,25]. Using eqn. 4, a rough approximation of the ionization constant and fully protonated capacity factor were determined. The ionization constant and deprotonated, free solution mobility were used in eqn. 2 in order to calculate the mobility of bilirubin at the lower pH values. Finally, this mobility could be used in eqn. 1 to calculate the capacity factor of bilirubin taking into account it's ionization. This calculated capacity factor was then reintroduced into eqn. 4, and the entire process was repeated until the ionization constant and capacity factor each converged.

An example separation is shown in Fig. 3. Run conditions are provided in the figure caption. The resulting plots of calculated capacity factor *versus* pH for each of the three bile salt systems investigated are shown in Fig. 4. There

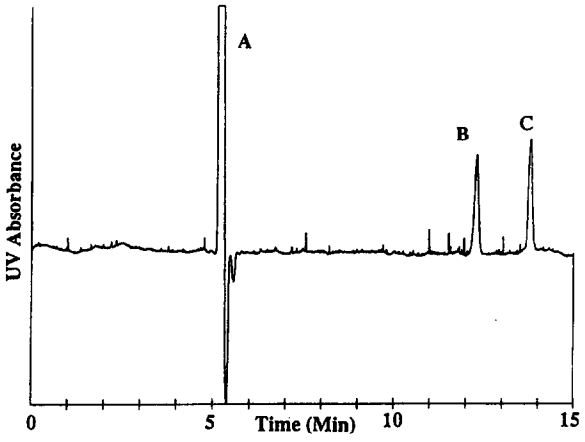

Fig. 3. Example separation. Peaks: A = DMSO; B = bilirubin; C = Sudan III. Run conditions were as follows: 20 m*M* phosphate–borate buffer with 25 m*M* taurochenodeoxycholic acid at pH 8.15; injection, 5 kV for 10 s; run voltage, 7 kV; capillary length 45 cm total, 27 cm from point of injection to detection window; UV detection at 225 nm; capillary inner diameter, 75 μm.

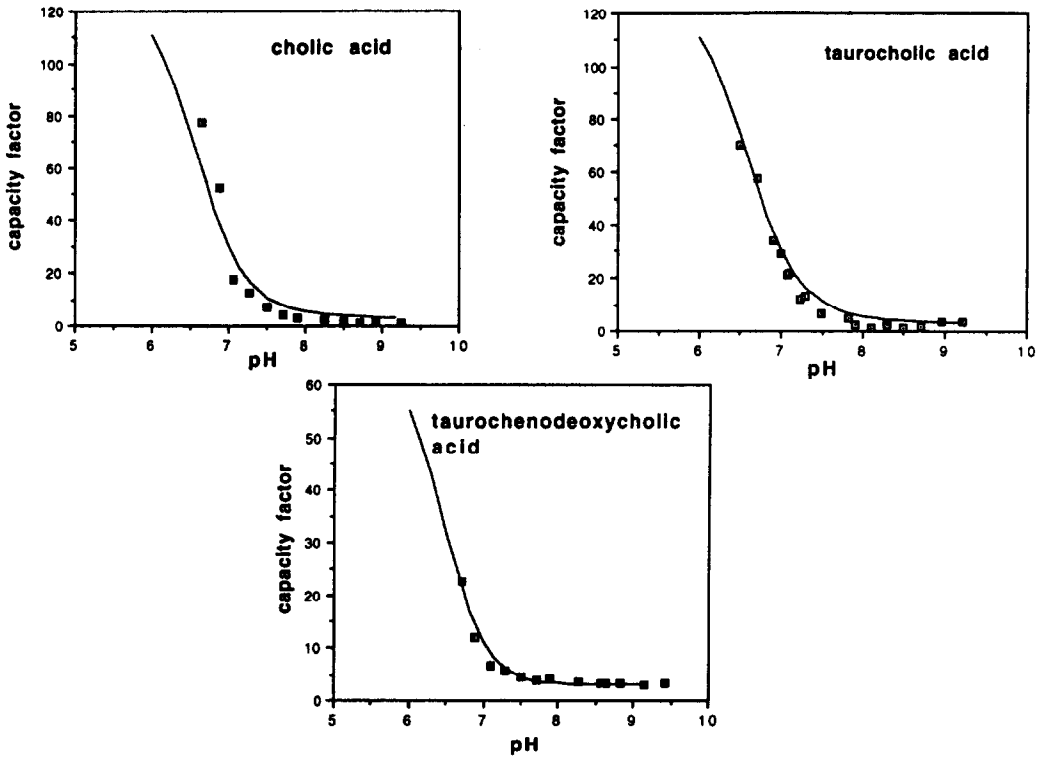

Fig. 4. Plots of capacity factor of bilirubin in cholic, taurocholic and taurochenodeoxycholic acid solutions as a function of pH. Symbols represent actual data, solid lines represent fitted equation five. See text for fitting parameters.

are several key features to these plots. First, the sharp decline in capacity factor occurring in the pH range of 6.5 to 7.5 is evidence of an ionization. It should be noted that this closely corresponds to the pH range of the biliary tract. This means that as bilirubin is transported in bile, it's state of ionization will vary. Therefore, at different points in the bile system, bilirubin should exhibit different capacities to interact with the bile salts, depending on the pH at these points. Second, there is no evidence for a second, distinct ionization of bilirubin. The two ionizations must both occur either at the same pH value or at pH values very close to each other. This assumes both ionizations occur over the pH range investigated which seems likely. Finally there are some apparent differences in the bilirubin capacity factor among the three different bile salts.

In order to visualize these differences more clearly, the solid line on each of the three graphs was generated using eqn. 5.

$$k' = \frac{k'_{H_2A} + k'_{HA^-}(K_{a1}/[H^+]) + k'_{A^{2-}}(K_{a1}K_{a2}/[H^+]^2)}{1 + K_{a1}/[H^+] + K_{a1}K_{a2}/[H^+]^2} \quad (5)$$

We are proposing eqn. 5 as an extension of eqn. 3 which was introduced by Khaledi *et al.* [25]. The difference is that where eqn. 3 applies to analytes which can undergo a single ionization, eqn. 5 applies to analytes which can undergo two ionizations in the pH range of study. Eqn. 5 shows that the observed capacity factor is related to the capacity factor of the fully protonated (k'_{H_2A}), intermediate (k'_{HA^-}), fully deprotonated species (k'_{A2^-}), the first (K_{a1}) and second (K_{a2}) ionization constants, and the hydrogen ion concentration. In order to determine the best fit,

values were chosen for eqn. 5 as follows: the three capacity factors were chosen initially according to values which seemed to fit visually with the three plots. Ionization constant values were then arbitrarily chosen, and eventually it was determined that the best fits were obtained if the ionization constants were chosen to be equal to each other, with pK_a values equal to 6.6. Since the pK_a values determined by this method are the pK_a values for the analyte in the buffer solution and not in the micelle [25], the same pK_a values were used for each of the three fits. The capacity factor values were then varied until a reasonable fit was achieved. For the cholic acid data capacity factors of 130, 30 and 1 were arrived at for the fully protonated, intermediate and fully deprotonated species respectively. For taurocholic acid the capacity factors were 130, 45 and 1, and for taurochenodeoxycholic acid the values were 80, 5 and 3. It should be noted that since the fitting was accomplished manually, these values must be viewed with caution.

It should also be noted that a slightly different data treatment would provide the ionization constants of bilirubin in the micelles themselves [25]. However, this data treatment requires that the mobility instead of capacity factor of bilirubin be plotted as a function of pH. It appears as though the electroosmotic flow value in a bile salt system is not as stable as an SDS system. This variation in electroosmotic flow imparts sufficient variation in mobility plots as to make this type of data analysis tenuous at best. These variations seem to be canceled out when capacity factors are used. Another explanation of our inability to determine intramicellar pK_a values is offered in the conclusions section.

While the apparent differences in capacity factor value for the fully deprotonated species in the different bile salts systems are probably within experimental error, there does appear to be a significant difference for the fully protonated species. It appears that protonated bilirubin does not interact as effectively with taurochenodeoxycholic acid as it does with cholic acid or taurocholic acid. This is important as the protonated species of bilirubin is quite prevalent at the pH range of bile if our apparent pK_a values are correct. If this is a real difference in the capacity factor, and not an artifact of the data treatment, it means that bilirubin interacts differently with different bile salts in the biliary tract. Should a patient show a change in his or her bile salt profile, that patient may be more or less susceptible to bilirubin precipitation. We are currently examining the behavior of bilirubin in other bile salt solutions to see if other differences in the capacity factor can be observed.

In order to determine if these differences in capacity factor among the different bile salts are real, we are currently conducting experiments to determine the capacity factor of bilirubin as a function of bile salt concentration at pH values of interest. Plots of capacity factor *versus* concentration will yield the partition coefficient of bilirubin once the plots have been corrected for the differing micelle volumes [2]. We have completed similar work in a buffer system containing 25 m*M* SDS at pH 8.5. The result is shown in Fig. 5. This plot shows that in comparison to some other test compounds, bilirubin interacts significantly with the SDS micelles at this pH. This is somewhat surprising since bilirubin is a dianion at this pH. On the same plot, the behavior of bilirubin is compared to two neutral compounds, caffeine and theobromine, as well as a monoanion, uric acid, and a partially charged compound, theophylline. The anions show little interaction with the micelles while the neutrals show limited interaction as compared to bilirubin. This might be explained by the fact that

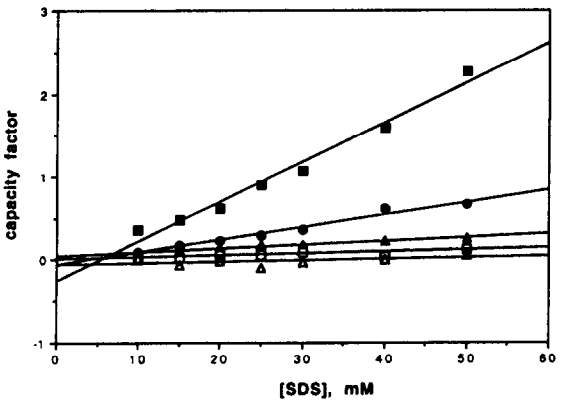

Fig. 5. Plot of capacity factor of test compounds in SDS solutions at pH 8.5. ■ = Bilirubin; ● = caffeine; ▲ = theobromine; □ = theophylline; △ = uric acid.

bilirubin is not a flat compound but rather has approximately a 90° bend across the center of the molecule. This structure may allow a significant portion of the molecule to interact with the SDS micelle while the charged groups, which are fairly well protected as compared to the comparison compounds shown in Fig. 5, are able to be held at some distance from the micelle surface.

CONCLUSIONS

There are several pieces of information to keep in mind when interpreting these results. Although it appears as though there are reasonable differences between the bilirubin capacity factor in the different bile salt solutions, these differences must be viewed with caution. Our procedures require an extensive data work up and these differences in capacity factor may be a result of data treatment procedures. The bile salt concentration studies currently underway in our laboratory will help to sort this out. Through MECC techniques, the apparent pK_a values for the bilirubin carboxyl groups appear to be similar, if not identical, to each other and fall within the range of 6.2 and 6.6. The value of 6.2 comes from the iteration procedure used to generate the corrected capacity factors, and the value of 6.6 comes from the fitted equation results. The value of 6.2 corresponds well to other data obtained at Trinity University by spectroscopic techniques [26]. Unfortunately, it appears as though MECC does not provide sufficiently conclusive data for the accurate determination of apparent pK_a values in this particular system. Perhaps the most important considerations to keep in mind when viewing this data is that the bilirubin–bile salt system is a very complicated system. The equations and theories we are using in our interpretation have been tested primarily on SDS based systems. SDS systems seem to fit these theories fairly well but several important assumptions were made in the development of these equations and theories which do not apply to the bilirubin–bile salt system. First is the assumption that the presence of an analyte does not appreciably change the structure or migration time of the micelle. While true for SDS, it is quite possible that this is not true for bilirubin and bile salts. Second is the assumption that the analyte does not interact appreciably with the surfactant monomer. Again, this is most likely true for bilirubin in SDS systems but probably is not true for bilirubin in bile salt systems [27]. Finally, we must also assume that the operating conditions chosen are those that will provide for a stable bile salt micelle. It is known that bile salt micelle structures, unlike SDS micelles, are a function of surfactant concentration. What is true at one concentration may not necessarily be true at another. Our inability to determine the intramicellar ionization constants may be an indication that this system does not comply with the criteria for interpretation of MECC systems as it it now developed. Exactly how much these considerations impact our results is unknown at this time. It appears as though we can apply these equations and theories to interpret our data but these interpretations must then be viewed with caution. However, it does seem clear that MECC techniques can yield valuable and interesting information about complicated systems such as the bilirubin–bile salt system.

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