Calcium binding by monosulfate esters of taurochenodeoxycholate

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Abstract The effect of sulfate esterification of the 3α- or 7α-hydroxyl groups of taurochenodeoxycholate on calcium binding was studied at 0.154 M NaCl in the presence and absence of phosphatidylcholine using a calcium electrode. For comparison, similar studies were made with taurochenodeoxycholate, taurodeoxycholate, and taurocholate. No high affinity calcium binding was demonstrable for any of these bile salts in pre-micellar solutions. Taurine-conjugated bile salts have greater affinity for calcium when in a micellar form. At elevated bile salt concentrations, the calcium binding of unsulfated dihydroxy taurine conjugates was similar to that of the monosulfate esters of taurochenodeoxycholate. The presence of phosphatidylcholine decreased calcium binding of the unsulfated dihydroxy bile salts and slightly increased calcium binding by taurocholate. However, the addition of phosphatidylcholine to monosulfate esters of taurochenodeoxycholate results in large increments in calcium binding. The results indicate that increased calcium binding due to the presence of phosphatidylcholine in bile salt solutions depends, in part, on the hydrophilicity of the bile salt and that the interaction of monosulfate esters of taurochenodeoxycholate with phosphatidylcholine leads to the formation of a high affinity calcium binding site. —**Stevens, R.D., L. Lack, and P. G. Killenberg.** Calcium binding by monosulfate esters of taurochenodeoxycholate. *J. Lipid Res.* 1991. **32:** 621-627.

Supplementary key words bile salts • phosphatidylcholine • cholestasis

Calcium in bile plays a central role in gallstone disease. Insoluble calcium salts are the major constituents of pigment stones (1) and are postulated to provide the nidus around which cholesterol gallstones are formed (2). In addition, free calcium ions influence the kinetics of cholesterol precipitation in supersaturated bile (3) and are instrumental in nucleation processes (4).

The formation of soluble calcium bile salt complexes is a principal factor controlling the activity of calcium in bile. It is generally concluded that, in micellar solutions, glycine conjugates bind calcium more effectively than taurine conjugates and dihydroxy bile salts more effectively than trihydroxy bile salts (5–9). General conclusions on the strength of metal ion binding in pre-micellar solutions are not easily made. Paramagnetic NMR studies indicate that the interaction of metal and anion primarily involves the side chain of the bile salt and is much stronger for the carboxylate group than the sulfonate group (7). Earlier studies using an ion selective electrode found little pre-micellar binding for glycocholate (2). More recent studies using similar methodology show strong pre-micellar binding which appears, under limiting conditions, to be independent of the anionic group and steroid nuclear hydroxylation (8). Previous studies (6, 9, 10) indicate that calcium binding is increased in mixed micellar solutions of trihydroxy bile salts and phosphatidylcholine. However, there is no consistent view of the effect of phosphatidylcholine on the calcium binding properties of dihydroxy bile salts (5, 6, 9).

We report here studies of calcium binding by monosulfate esters of taurochenodeoxycholate. These compounds occur naturally and result from the enzymatic esterification of either the 3α- or 7α-hydroxyl group. The monosulfate esters of taurochenodeoxycholate and other bile salts are particularly prevalent in cholestasis (11, 12). Previous studies in our laboratory (13) have shown that the monosulfate esters of taurochenodeoxycholate exhibit a high critical micellar concentration yet readily promote phospholipid and cholesterol excretion in bile. The study of calcium binding by the monosulfate esters of taurochenodeoxycholate, therefore, may elucidate more clearly the importance of substitution of the nuclear ring on calcium binding as well as the relative roles of pre-micellar and micellar bile salts for calcium complexation. The study of calcium binding by taurochenodeoxycholate monosulfate esters is also important to an understanding of the role which these compounds may play in the high frequency with which gallstones are seen in chronic cholestatic illnesses (14).

Abbreviations: TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TC, taurocholate; TCDC-3-SO_4, TCDC-7-SO_4, 3α- and 7α-sulfate esters of taurochenodeoxycholate; CMC, critical micellar concentrations; NMR, nuclear magnetic resonance; TLC, thin-layer chromatography; HPLC, high performance liquid chromatography.

EXPERIMENTAL PROCEDURES

Materials

The sodium salts of [^{14}C]taurine-labeled 3α- and 7α-monosulfate esters of taurochenodeoxycholate, (TCDC-3-SO_4 and TCDC-7-SO_4) were prepared as cited previously (13). The sodium salts of taurochenodeoxycholate (TCDC), taurodeoxycholate (TDC), and taurocholate (TC) were obtained from Calbiochem (San Diego, CA). Phosphatidylcholine (egg yolk lecithin-type V-E) and PIPES were obtained from Sigma (St. Louis, MO). Calcium chloride was obtained from Edlow Pharmaceuticals (Glendale, NY). Saline was from Abbott Laboratories (Chicago, IL) and sodium chloride from Mallinkrodt (Paris, KY). The sulfate esters of taurochenodeoxycholate migrated as one spot on TLC (15). The commercial bile salts were greater than 97% pure by HPLC (16). The impurities were mostly taurine-conjugated bile salts, and no free bile acids were demonstrable by TLC (17). Bile salts were lyophilized before use and quantitated enzymatically (18) with prior solvolysis where necessary. Sodium chloride contamination was monitored by passing an aqueous solution of the bile salt through a C_{18} μBondapak Sep Pak (Waters, MA) and measuring the sodium ion concentration in the eluate with a Beckman E2A Analyzer. The chromatographic purity of the lecithin was periodically checked by TLC (13) and quantitated enzymatically (Wako Pure Chemicals Industries, Osaka, Japan).

Ionized calcium measurements

Ionized calcium measurements were made using a Nova 7 Analyzer (Waltham, MA) which operates at 37°C in the 0.1–4.9 mM range and provides estimates to 10^{-5} M. The ion selective electrode characteristics are established with a two-point internal calibration every 2 h using protein-free calcium solutions (1 and 3 mM). Each unknown is automatically followed by a one-point calibration which is used by the microcomputer to compensate for the electrode drift. The electrode responses for standards and unknown are measured at a fixed time interval after exposure to the solutions. The performance of the electrode was also monitored with external standards supplied by the manufacturer. Stock solutions of calcium chloride were prepared in 0.154 M saline and calibrated against standards (Ricca Chemicals, Arlington, TX) using both flame photometry (Instrumentation Laboratories, Model IL 151, Andover, MA) and atomic absorption spectrometry (Perkin-Elmer, Model 5100, Norwalk, CT). The concentrations of the stock solutions determined by the Nova 7 Analyzer were within 1%. Thus, the values used for the total calcium ion concentration in the binding studies were those obtained from the Nova 7 Analyzer. The C.V. for repeat determinations was less than 1% and the maximum variation of the manufacturer's and stock standards that were assayed at the beginning and end of an experiment was less than 2%. The response of the electrode to a series of standards was linear in the range 0.3–5.5 mM calcium. The majority of the binding experiments were carried out at 1.6 mM calcium which is within the range of the internal standards of the analyzer.

As has been discussed elsewhere (19), sodium ion concentration and ionic strength are important considerations when measuring free calcium ion concentration and have opposing effects on the electrode response. The influence of sodium chloride (0.16–0.4 M) on the Nova electrode was determined. After a slight decrease (less than 1%) from 0.16–0.25 M, the measured value of a standard rose 3% at 0.4 M. For experimental simplicity, we elected to study calcium binding by bile salt at a constant sodium chloride concentration of 0.154 M. Thus, the calcium bound by the sulfate esters of taurochenodeoxycholate, assuming they behave as ordinary electrolytes, which is unlikely (20, 21), may be underestimated by 3% at the highest concentrations (80 mM) studied.

Sample preparation

Stock solutions of calcium chloride were prepared in 0.154 M saline. Mixed micellar solutions were prepared by the method of co-precipitation (22). Lyophilized bile salts and mixed lipids were rehydrated with appropriate concentrations of sodium chloride such that the final concentration of the solution was 0.154 M sodium chloride. The sodium chloride concentrations were based on the assumptions that the partial specific volumes were 0.75 (23) and 0.98 (24) ml/g for bile salts and phosphatidylcholine, respectively, and in the case of the lyophilized bile salts, that the impurity by weight was water. The final lipid concentrations were measured by lipid analyses and/or liquid scintillation counting. Bile salt/calcium chloride mixtures were prepared volumetrically. Equal volumes of concentrated bile salt solution and a calcium chloride solution containing twice the desired total calcium ion concentration were mixed. Further solutions were prepared by dilution with the desired total calcium ion concentration used for the binding study. The solutions were allowed to equilibriate for at least 1 h before measurement. The mixed micellar/calcium chloride solutions were prepared similarly; however, the initial addition of calcium chloride to the mixed micellar solutions was followed by 16 h equilibration. Further dilutions were followed by at least 4 h equilibration before measurements were made.

In general, we elected to study only taurine-conjugated bile acids in the absence of buffers, thus avoiding complications of prolonged equilibration times (25) and competitive interactions of calcium with buffer ions (9, 19). The pH of the solutions was in the range 6.3–7.9. Some measurements were made in the presence of 50 mM

PIPES, pH 7.0, to facilitate comparison with previously reported data.

Statistical analyses

The calcium binding data are expressed as means ± SD. The slopes of the lines were estimated by linear regression analysis.

RESULTS

Calcium binding by bile salts

In the presence of 50 mM bile salts, calcium binding was approximately proportional to total calcium concentration, as expected for the range of total calcium (0–5.2 mM) studied. The apparent ratio of free to total calcium was 0.46 ± 0.01 for TCDC; 0.48 ± 0.02 for TDC; 0.61 ± 0.02 for TCDC-3-SO_4; and 0.76 ± 0.01 for TC. The values for the unsulfated compounds compare well to data reported previously (6, 9, 26). The presence of 50 mM PIPES, pH 7.0, made no difference to these ratios after the binding of PIPES was accounted for. The ratio of free to total calcium in the presence of 50 mM PIPES was 0.83. The calcium binding isotherms for TCDC and TDC are shown in **Figs 1a and 1b** at different calcium concentrations. The families of curves of the Langmuir type are similar for both bile salts and appear to exhibit a positive intercept on the abscissa in the region of their critical micellar concentrations. The binding isotherms for the monosulfate esters, TCDC-3-SO_4 and TCDC-7-SO_4, at 1.6 mM total calcium are compared to those of more conventional taurine-conjugated bile salts in **Fig. 2**. The data confirm previous observations of greater calcium binding by micellar dihydroxy bile salts than by trihydroxy bile salts (5, 6, 8, 9). The calcium binding by TCDC-3-SO_4 was similar to TCDC at the highest concentration studied but TCDC-7-SO_4 was less than TCDC. At the lower bile salt concentrations, the sulfate esters always bound less calcium than the unsulfated dihydroxy bile salt. The isotherms for the bile salts in the 0–20 mM range are shown in **Fig. 3**. The overall appearance of the TCDC isotherm in this concentration range appears to be convex, whereas those of the sulfate esters and TC are initially concave but transform to a convex shape at higher concentrations (see Fig. 2). The computed apparent overall formation constants (assuming a 1:1 complex) are shown as a function of bile salt concentration in **Fig. 4**. In this concentration range, the overall trend is for the formation constant to increase with bile salt concentration. The formation constant for TCDC rapidly plateaus, however, TC reaches steady values at higher concentrations. On the other hand, the formation constants for the sulfate esters do not plateau in this concentration range and continue to increase over the entire concentration range studied.

Calcium binding by bile salt-phosphatidylcholine mixtures

Calcium binding by bile salt-phosphatidylcholine mixtures was studied at different bile salt concentrations and bile salt/phosphatidylcholine ratios from 6:1 to 1.5:1. The binding curves for the mixed micellar solutions at constant bile salt concentrations (10, 40, and 80 mM) are shown in **Fig. 5**. The addition of phosphatidylcholine to TCDC or TDC did not alter calcium binding at bile salt/phosphatidylcholine ratios greater than 3. In marked contrast, the addition of phosphatidylcholine to the sulfate esters resulted in large increments in calcium binding at each bile salt/phosphatidylcholine ratio. Mixed

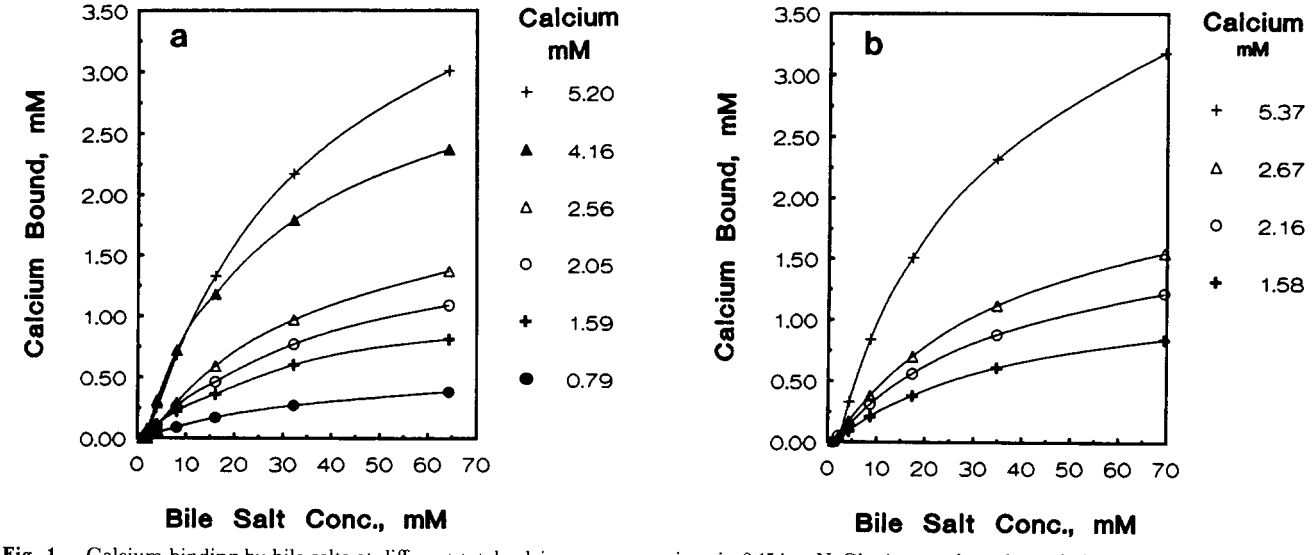

Fig. 1. Calcium binding by bile salts at different total calcium concentrations in 0.154 M NaCl; a) taurochenodeoxycholate; b) taurodeoxycholate.

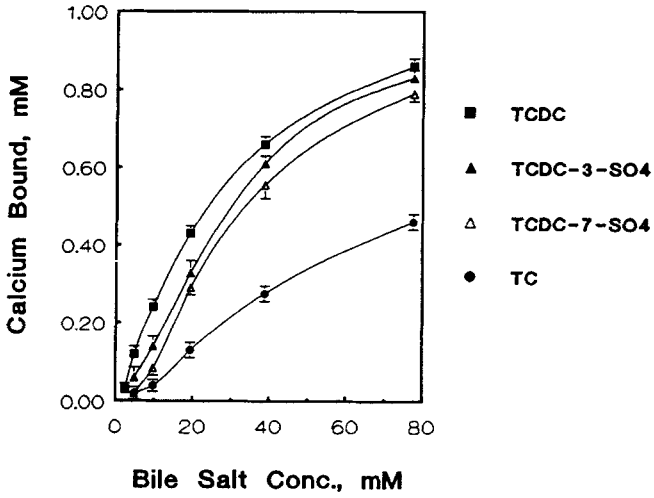

Fig. 2. Calcium binding by taurocholate, TC, taurochenodeoxycholate, TCDC, and the monosulfate esters of taurochenodeoxycholate, TCDC-3-SO_4 and TCDC-7-SO_4 at 1.6 mM total calcium concentration in 0.154 M NaCl. Taurodeoxycholate was similar to TCDC but has been omitted for clarity.

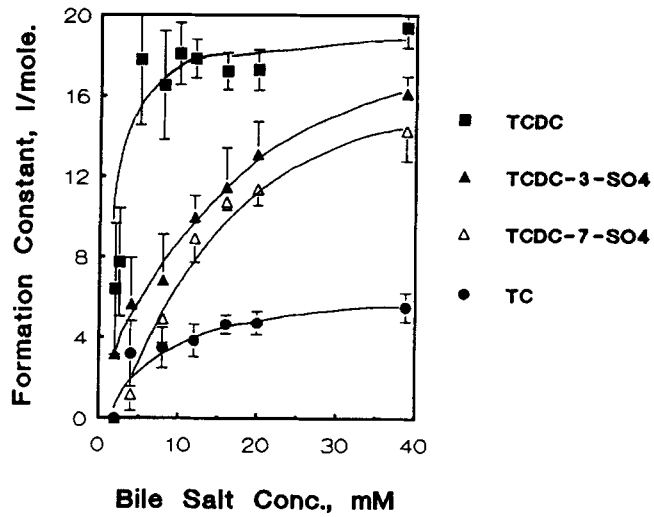

Fig. 4. Apparent formation constant K_f, for calcium bile salt complexes, Ca^+BS, of taurocholate, TC, taurochenodeoxycholate, TCDC, and the monosulfate esters of taurochenodeoxycholate, TCDC-3-SO_4 and TCDC-7-SO_4, at 1.6 mM total calcium concentration in 0.154 M NaCl. $K_f = [Ca^+BS]/([Ca^{2+}][BS])$.

micellar systems using TC, another hydrophilic bile salt, also exhibit increased calcium binding but to a lesser extent. The initial part of the binding curves was not as steep as those for the sulfate esters. The computed apparent overall formation constants for calcium bile salt complexes in mixed micellar solutions, assuming a 1:1 complex and no contribution from the phosphatidylcholine, are shown in **Fig. 6**. The addition of phosphatidylcholine to monosulfate esters of taurochenodeoxycholate results in a two- to threefold increase in the apparent formation constant.

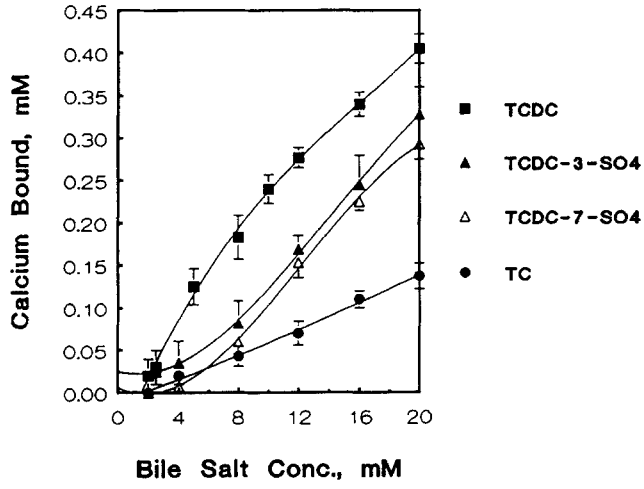

Fig. 3. Calcium binding at low bile salt concentrations for taurocholate TC, taurochenodeoxycholate, TCDC, and the monosulfate esters of taurochenodeoxycholate, TCDC-3-SO_4 and TCDC-7-SO_4, at 1.6 mM total calcium concentration in 0.154 M NaCl.

DISCUSSION

The present work documents the impact of sulfate esterification on calcium complex formation with TCDC in the presence and absence of phospholipids. The conversion of an OH group on the α-surface of the steroid to the charged sulfate moiety drastically modifies the hydrophilic/hydrophobic balance without altering the contiguous hydrophobic surface of the steroid nucleus. This increased hydrophilicity results in a tenfold increase in the critical micellar concentration (22) and, thus, permits investigation of pre-micellar calcium binding at higher bile salt concentrations. Recent ion selective electrode studies of calcium binding by unsulfated bile salts have suggested that pre-micellar bile salt solution have a high affinity for calcium (8, 19, 26). The present investigation fails to demonstrate comparable high affinity binding for any of the taurine conjugates studied. None of the binding curves shown in Figs. 1 and 3 exhibited a knee shape at low bile salt concentrations. To the contrary, the binding curves for the more hydrophilic bile salts were concave indicating a decreasing overall formation constant (Fig. 4) as bile salt concentrations are reduced below the CMCs. It was not possible in the present experiments to establish whether there are limiting values for the apparent formation constants as the bile salt concentration vanishes.

The absence of high apparent affinity for calcium by pre-micellar taurocholate has been noted in previous NMR studies (7). The intrinsic formation constant of the 17 M^{-1} was estimated for the pre-micellar calcium taurocholate complex. However, as pointed out by these authors and demonstrated by others (2), the apparent overall formation constant will be reduced since sodium

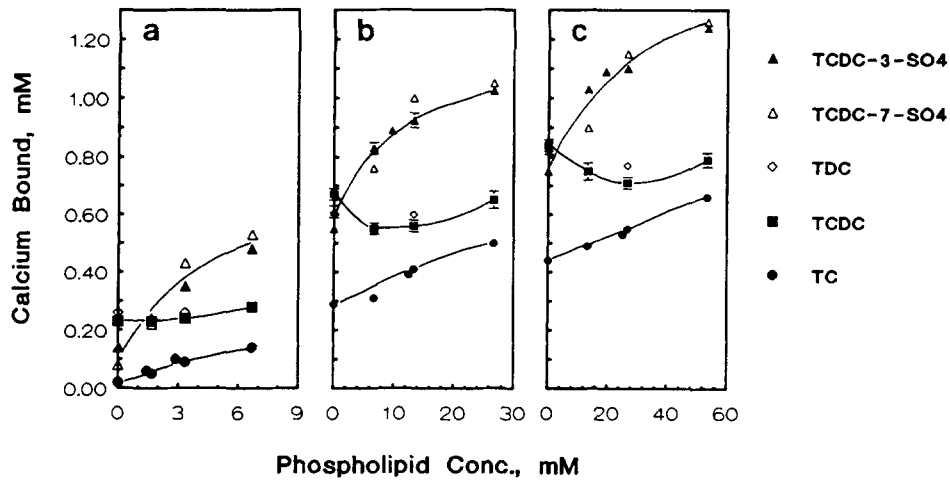

Fig. 5. Calcium binding by mixed micellar solutions of bile salts and phosphatidylcholine for taurocholate, TC, taurochenodeoxycholate, TCDC, taurodeoxycholate, TDC, and the monosulfate esters of taurochenodeoxycholate, TCDC-3-SO_4 and TCDC-7-SO_4, at 1.6 mM total calcium concentration in 0.154 M NaCl. Total bile salt concentrations: panel a, 10 mM; panel b, 39 mM; panel c, 78 mM.

competes for the ion binding site. This may explain our findings of low apparent affinity in pre-micellar bile salt solutions in the presence of sodium ions.

The results of the present study confirm the greater affinity of dihydroxy bile salts for calcium than that of trihydroxy bile salts at concentrations above the CMC and suggest that taurine-conjugated bile salts have a higher affinity for calcium when they are in micellar form. Mukidjam, Eglavish, and Barnes (27) have shown that the primary metal-bile salt interaction is at the end of the side chain and results in a conformation that is favorable for aggregate formation. We have recently demonstrated, using NMR, that monomeric TCDC and its monosulfate esters exhibit considerable motional freedom of the side chain in the presence of sodium ions (28). In micellar form, however, the C_{25} methylene protons are motionally constrained, which suggests conformational restrictions of the side chain. The increase in calcium affinity at high bile salt concentrations, therefore, may result from a favorable spatial arrangement or intramolecular conformation of the side chains in the micelle which leads to the formation of a higher affinity binding site involving one or more anionic groups. In turn, the differences in calcium binding by specific bile salts may be due to different conformations or spatial arrangements of the side chains in the micelle.

The effect of phosphatidylcholine on calcium binding by bile salts has been studied previously (5, 6, 9, 10). The

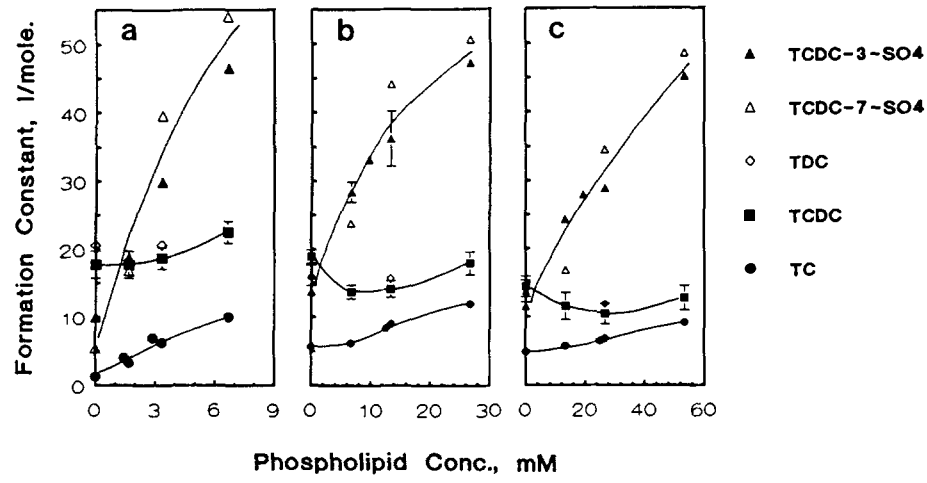

Fig. 6. Apparent formation constants, K_f, for calcium bile salt complexes, Ca^+BS, in the presence of phosphatidylcholine for taurocholate, TC, taurochenodeoxycholate, TCDC, taurodeoxycholate, TDC, and the monosulfate esters of taurochenodeoxycholate, TCDC-3-SO_4 and TCDC-7-SO_4, at 1.6 mM total calcium concentration in 0.154 M NaCl. $K_f = [Ca^+BS]/([Ca^{2+}][BS])$. Total bile salt concentrations: panel a, 10 mM; panel b, 39 mM; panel c, 78 mM.

present study supports the findings of Gleeson, Murphy, and Dowling (9) of no change or decreased calcium binding by dihydroxy bile salts in the presence of phosphatidylcholine and the small increase for taurocholate mixed micellar systems. However, this study demonstrates that the addition of phosphatidylcholine to monosulfate esters of taurochenodeoxycholate results in a large increase in calcium binding. Calcium has been shown to interact weakly with bilayers of egg yolk phosphatidylcholine (29, 30), with an estimated binding constant of 1–2 M^{-1} for a 1:1 complex. The present data suggest a correlation between the hydrophilicity of the α-surface of the bile salt and changes in calcium binding with the addition of phosphatidylcholine to bile salt solutions. It is generally accepted that the mixed micelle has a disc-like structure consisting of a phospholipid bilayer with bile salts at the periphery. Mazer, Benedek, and Carey (31) have proposed that bile salts are also incorporated as reversed aggregates within the interior of the disc. The degree of incorporation is proportional to the hydrophobicity of the bile salt. Thus, the decrease in calcium binding by dihydroxy bile salt mixed micellar solutions may reflect this partition due to inaccessibility of the binding site of the bile salt or alteration of the conformation or spatial arrangement of the side chains when the bile salts are hydrophilically associated. However, alteration in the affinity of the bile salt binding site at the periphery cannot be excluded. The magnitude of the increase in calcium binding by the mixed micellar solutions of the monosulfate esters of taurochenodeoxycholate is not easily accounted for on the basis of additional binding to the phospholipid bilayer and reduced partitioning of the hydrophilic bile salt within the bilayer. The increase in an apparent affinity must result from the favorable juxtaposition of the sulfate esters at the periphery of the mixed micelle, creating higher affinity or increased numbers of ion binding sites perhaps with involvement of the sulfate ester group per se.

Monosulfate esters of taurochenodeoxycholate and other bile salts occur naturally especially during periods of cholestasis (11, 12). This modification is presumed to be less hepatotoxic since these hydrophilic bile salts are less likely to disrupt hepato-cellular membranes (32). The present studies suggest that sulfation may have an additional role in reducing the likelihood of the precipitation of calcium salts in the extrahepatic space.

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