Calcium binding by bile acids: in vitro studies using a calcium ion electrode

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Abstract In this study, we compared in vitro calcium binding by the taurine and glycine conjugates of the major bile acids in human bile: cholic (CA), chenodeoxycholic (CDCA) and deoxycholic (DCA) acids, together with the cholelitholytic bile acids ursodeoxycholic (UDCA) and ursocholic (UCA) acids. At physiological total calcium (Ca_{TOT}) (1–15 mM) and bile acid (BA) (10–50 mM) concentrations, all the bile acids caused concentration-dependent falls in [Ca^{2+}], suggesting calcium binding. Except for glycine-conjugated CDCA, all the other calcium-bile acid complexes were soluble in 150 mM NaCl. The calcium binding affinities followed the pattern: dihydroxy (CDCA, UDCA and DCA) > trihydroxy (CA and UCA) bile acids, and glycine conjugates > taurine conjugates. The glycine conjugate of UDCA, which increases during UDCA treatment, had the highest calcium binding affinity. Ten–20 mM phospholipid modestly increased calcium binding by CA conjugates, but not by CDCA, UDCA, and DCA conjugates. Phospholipid also prevented the precipitation of glyco-CDCA in the presence of calcium. Bile acid–calcium binding was pH-independent over the range 6.5–8.5. The different calcium binding affinities of the major biliary bile acids may partly explain their varying effects on biliary calcium secretion. The results also suggest that neither precipitation of calcium-bile acid complexes nor impaired calcium binding by bile acids is important in the pathogenesis of human calcium gallstone formation. —**Gleeson, D., G. M. Murphy, and R. H. Dowling.** Calcium binding by bile acids: in vitro studies using a calcium ion electrode. *J. Lipid Res.* 1990. **31:** 781–791.

Supplementary key words phospholipids • biliary calcium • bile acid/phospholipid calcium binding • calcium in electrode

The calcium salts, calcium bilirubinate, carbonate, and phosphate are the major components of noncholesterol gallstones (1, 2). They are also found at the center of cholesterol-rich stones (3) and may play an important role in nucleating cholesterol crystals from bile which is supersaturated with cholesterol (4). Calcium salts precipitate out of solution only when the concentration product of their constituent ions exceeds their solubility product. Therefore, precipitation of calcium salts in bile should depend, in part, on the biliary free ionized calcium concentration ([Ca^{2+}]).

Bile acid-calcium binding in vitro has been the subject of several recent studies (4–8). However, there are still few quantitative data on the comparative calcium binding affinities of the major bile acids in human bile. We embarked on such a study for several reasons. First, only 20–30% of calcium in human hepatic bile and 10–15% in human gallbladder bile is in the free ionized form, the remaining 70–90% being bound (9–11). The results of equilibrium dialysis studies (10) suggest that up to 80% of calcium binding in hepatic bile and 40% of that in gallbladder bile is accounted for by bile acid micelles. Bile acids have been proposed as major calcium buffers which, by lowering [Ca^{2+}], minimize the risk of calcium precipitation in bile (4). The in vitro calcium binding properties of bile acids, therefore, have major implications for [Ca^{2+}] in bile.

Second, there are two situations in which changes in biliary bile acid composition are associated with a tendency to calcium salt precipitation in bile. *i*) Following terminal ileal resection in man, there is an increased risk of calcified gallstone formation (12) and bile is enriched with chenodeoxycholic acid (13) and with glycine-conjugated bile acids (14). *ii*) During ursodeoxycholic acid (UDCA) treatment, bile becomes enriched with the glycine conjugates of UDCA (15) and acquired gallstone calcification is common (16–20). The mechanism for this calcification may be increased carbonate ion concentration following UDCA-induced bicarbonate secretion by the bile ductular epithelium (21, 22), but the role of induced changes in biliary [Ca^{2+}] is still unclear. In the initial description of UDCA-associated gallstone calcification, it was suggested that calcium combined with glycoursodeoxycholic acid and that the resultant salt, being poorly soluble, precipitated onto the surface of the stone (16). We now know,

Abbreviations: CA, cholic acid; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; UCA, ursocholic acid; BA, bile acid; CMC, critical micellar concentration; TCA, taurocholic acid; GCA, glycocholic acid; TCDCA, taurochenodeoxycholic acid; TDCA, taurodeoxycholic acid.

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however, that calcium glycoursodeoxycholate is quite soluble in bile (23), suggesting that some other mechanism must be involved. If, for example, UDCA and its conjugates were poor calcium binding agents, the free ionized [Ca^{2+}] in bile would remain high, thereby risking calcium salt precipitation.

Third, bile acids increase biliary calcium output in dogs (24) and in humans (22, 25, 26). Different bile acids increase biliary calcium output to differing degrees (24, 26) which may be due to differences in their calcium binding affinities.

MATERIALS AND METHODS

Preparation of bile acids and phospholipid solutions

Sodium salts of the glycine (G) and taurine (T) conjugates of cholic (CA), chenodeoxycholic (CDCA), and deoxycholic (DCA) acids (approximately 98% pure) were obtained from Sigma Laboratories, Dorset, UK. The G and T conjugates of ursodeoxycholic acid (UDCA) were gifts from Roussel UCLAF, Paris, and the conjugates of ursocholic acid (UCA) were from Gipharmex SpA, Milan.

The sodium salts of the bile acids were made up in concentrations ranging from 0 to 100 mM in 150 mM NaCl and 100 mM Na-1,4-piperazinediethanesulfonic acid (PIPES) buffered at pH 7.0. Solutions of $CaCl_2$ (2–20 mM and, in some experiments, 4–30 mM) in 150 mM NaCl were also prepared. At every bile acid concentration, 1 ml of each $CaCl_2$ solution was added to 1 ml of bile acid solution in glass vials to give final total calcium concentrations of 1–10 mM (and in some experiments 2–15 mM), and final bile acid (BA) concentrations of 0–50 mM, in 50 mM Na-PIPES and 150 mM NaCl (saline–PIPES). These calcium concentrations encompass the range found in human gallbladder and hepatic biles. All solutions were made up in duplicate. After the addition of $CaCl_2$, the solutions were agitated gently for 30–60 min before measurement of free ionized calcium concentration [Ca^{2+}].

For the studies involving phospholipid, 2 g phosphatidylcholine dissolved in ethanol was obtained from Sigma Laboratories, Dorset, UK, and air-dried to a powder. This procedure was repeated twice after washing with deionized water. The powder was dispersed in 150 mM NaCl by sonication for 30 min which yielded a milky suspension, and the volume made up to 10 ml. Volumes of the suspension were then added to the bile acid solutions to give final concentrations of 50 mM BA and 0, 10, and 20 mM phospholipid, in saline–PIPES. After adding the phospholipid to the CA, CDCA, and DCA conjugates, the phospholipid dispersed immediately to give a clear solution. With G-UDCA and T-UDCA, however, dispersion of the phospholipid to a clear solution occurred only after gentle heating.

Measurement of free ionized calcium: the calcium selective electrode

The electrode membrane incorporated a highly calcium-selective neutral carrier ligand, ETH1001, dissolved in a polyvinyl chloride base, as previously described (27, 28). The electrode was filled with 10 mM $CaCl_2$ in saline–PIPES, the internal solution. The internal solution was connected via a silver chloride electrode to an AVO voltmeter. The circuit was completed by a calomel electrode containing concentrated KCl which was also in contact with the test solution.

Preliminary experiments indicated that, over the range of [Ca] studied in both bile acid and saline solutions, the electrode exhibited near-ideal Nernstian behavior, that is, the recorded potential ΔE was closely and linearly proportional to the log of the calcium concentration. Correlation coefficients between recorded potential and [Ca^{2+}] by linear regression analysis routinely exceeded 0.998. Furthermore, a ΔE of between 26.5 and 28.5 mV was seen for each 10-fold rise in [Ca]. At [$CaCl_2$] 1–10 mM, electrode readings were 11.0 $\pm$ 0.3 mV lower in 150 mM NaCl than in H_20, corresponding to a reduction in calcium ion activity (aCa^{2+}) of 61 $\pm$ 2%. This is as expected, because the calcium ion activity coefficient, γ, which relates (aCa^{2+}) to [Ca^{2+}], falls as total ionic strength increases. However, this fall seemed maximal at [NaCl] 150 mM, since the electrode readings were not affected by further increases in [NaCl] from 150 to 250 mM. Nor were the electrode readings affected by pH changes over the range 6.5–8.5, in unbuffered solutions.

All studies were carried out at room temperature. Each day, a standard curve was constructed using $CaCl_2$ (1–10 mM) or, in some experiments 2–15 mM, in saline-PIPES, without bile acid (or $CaCl_2$ in150 mM NaCl for the "unbuffered" studies). ΔE was plotted against log [Ca] and the slope of the line was estimated graphically. Electrode readings were then taken in bile acid $\pm$ phospholipid solutions; stable readings were attained with 2 min. Because of a small drift in baseline readings between repeated measurements in standard solutions (<3 mV in 1 day), each reading in bile acid solutions was bracketed between two readings in a standard solution (usually 1.0 mM $CaCl_2$). After completing the readings in the bile acid solutions, standard curves were again constructed. The slopes were identical to those generated initially.

At all [Ca_{TOT}] values, the electrode potentials were invariably lower in the bile acid solutions than in the corresponding standards, indicating lower calcium ion activity (aCa^{2+}). This was not due to precipitation of a calcium–bile acid complex because *a*), the solutions, with the exception of calcium–GCDCA (see Results section), remained clear for up to 6 months, and *b*), after centrifugation (3000 *g* for 20 min), supernatant [Ca_{TOT}] values, measured by atomic absorption spectrophotometry, corresponded closely with the expected stoichiometric values.

Neither was the fall in (a Ca^{2+}) in bile acid solutions likely to result from bile acid induced increases in total ionic strength, and subsequent falls in γ. **Fig. 1** illustrates calculated $[Ca^{2+}]$ values (see formula below) at $[Ca_{TOT}]$ 1–10 mM in 50 mM TCA dissolved in (a) saline–PIPES and (b) 50 mM PIPES in which the sodium concentration had been adjusted to keep total ionic strength ($1/2\ \Sigma\ cz^2$) constant. The differences in electrode readings, and therefore in calculated $[Ca^{2+}]$ between (a) and (b), are very small indicating that γ changes little in BA solutions over the range of calculated total ionic strength values encountered in these studies.

We concluded that the depression of (a Ca^{2+}) reflected a proportional fall in $[Ca^{2+}]$ consequent on BA binding of Ca in a soluble form. For all subsequent studies, therefore, γ was assumed to be identical in BA and standard solutions and $[Ca^{2+}]$ was calculated from the formula:

$$[Ca^{2+}]test = 10^{\frac{E_{test} = E_{1.0}}{S}}$$

where E_{test} = electrode reading in the test solution; $E_{1.0}$ = mean of two bracketed standard readings; and S = Δ E/10-fold increase in [Ca]; that is, the slope of the standard curve. All $[Ca^{2+}]$ measurements were carried out on solutions made up in duplicate. For most BA conjugates studied, experiments were repeated 1–6 times on separate days (see Results). Intra-batch variability of $[Ca^{2+}]$ estimations was < 1% and that from day to day < 5%. The slopes of the $[Ca^{2+}]$ versus $[Ca_{TOT}]$ relationships were then calculated by regression analysis.

K_f values for bile acids ± phospholipids at $[Ca_{TOT}]$ 2–15 mM, were calculated according to Moore, Celic, and Ostrow (7) from the formula:

$$K_f = \frac{[CaBA_2]}{[Ca^{2+}](1/2[BA] - [CaBA_2])} = \frac{[Ca_{TOT}] - [Ca^{2+}]}{[Ca^{2+}](1/2[BA] - [Ca_{TOT}] + [Ca^{2+}])}.$$

It was assumed that micellar bile acid-calcium binding was dimeric, that is, two bile acids bind one calcium ion. This appears to be the case for TCA and GCA (4, 7). In the event that bile acid-calcium binding is 1:1, the K_f values may be recalculated to give values almost exactly 50% of those presented. K_f values depend on the parameter ($[Ca_{TOT}] - [Ca^{2+}]$) which is usually small compared to $[Ca^{2+}]$. Thus, minor variations in $[Ca^{2+}]$ may cause considerable variations in K_f. This day-to-day variation in K_f estimates was, therefore, 10–20%.

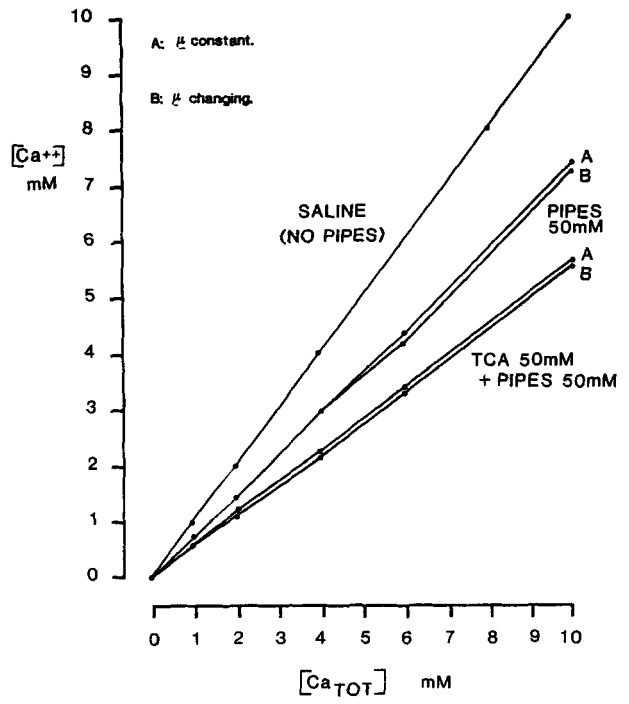

Fig. 1. Calculated free ionized calcium concentration $[Ca^{2+}]$ plotted against total calcium concentration $[Ca_{TOT}]$ in: *i*) saline, *ii*) 50 mM PIPES, and *iii*) 50 mM taurocholic acid (TCA) plus 50 mM PIPES. In each case, the results are shown when total ionic strength (μ or $1/2\ \Sigma\ cz^2$) was kept constant by manipulating sodium concentration (A), and when it changed as a result of increasing concentrations of total calcium and bile acid (B); see text. In the experiments designated A, [Na] was lowered by 1 mM for each mM [BA], and by 3 mM for each mM [$CaCl_2$]. (Since calcium is a divalent ion, for every increase in [$CaCl_2$], the corresponding increase in $1/2\ \Sigma cz^2$ will be three times that of the increase in [$CaCl_2$].)

Statistical methods

Results are expressed as means ± SD. The significance of differences was examined using Student's *t* test.

RESULTS

Concentration-dependent calcium binding by bile acids

Fig. 2 illustrates the effect of increasing concentrations of TCDCA on $[Ca^{2+}]$. $[Ca^{2+}]$ was always closely and linearly related to stoichiometric $[Ca_{TOT}]$ ($r > 0.998$). However, the slope of the $[Ca^{2+}]$ versus $[Ca_{TOT}]$ relationship fell progressively from unity as [BA] increased from 0 through 10 and 20 to 50 mM. Similar concentration-dependent decreases in slope were seen with TCA, GCA, TUDCA, GUDCA, TDCA, and GCDCA. The remaining bile acids (GDCA, GUCA, and TUCA) were studied at one concentration only (50 mM).

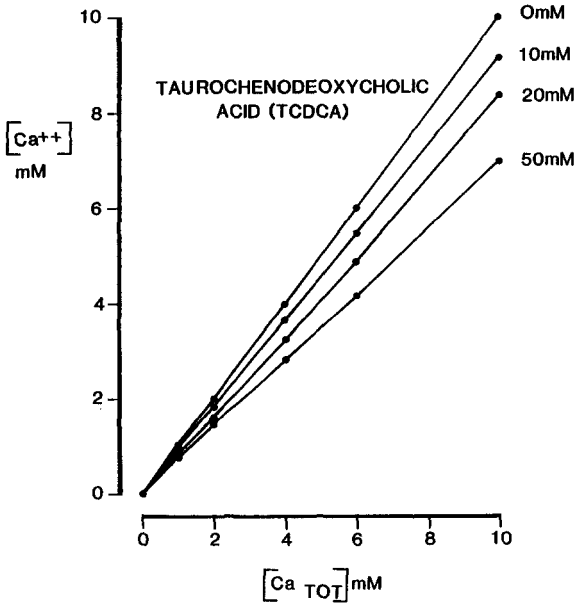

Fig. 2. Example of concentration-dependent calcium binding by bile acids; in this case taurochenodeoxycholic acid (TCDCA) at 0, 10, 20, and 50 mM concentrations.

Comparison of individual bile acid conjugates

Fig. 3 (a and b) shows the results of experiments comparing calcium binding by 50 mM solutions of five T- and five G- conjugated bile acids. The results of all studies in saline-PIPES are summarized in **Table 1**.

The slope of the $[Ca^{2+}]$ versus $[Ca_{TOT}]$ relationship ($\Delta[Ca^{2+}]/\Delta[Ca_{TOT}]$) was about 0.8 for the taurine conjugates of the trihydroxy bile acids, CA and UCA. However, the slopes for the taurine conjugates of the dihydroxy bile acids, CDCA, UDCA, and DCA, were all significantly lower (in the region of 0.7). This shows that the three dihydroxy bile acids bind calcium more avidly than the trihydroxy bile acids, CA and UCA.

A similar pattern was seen for the glycine-conjugated bile acids (Fig. 3b). The slopes were significantly lower for the three dihydroxy bile acids (about 0.5) than for the two trihydroxy (0.7-0.8) bile acids. However, for all the bile acids (except UCA), the slopes were significantly lower for the glycine than for the corresponding taurine conjugates, indicating that, in general, glycine-conjugated bile acids bind calcium more avidly than the taurine conjugates. A similar pattern was found (glycine > taurine; dihydroxy > trihydroxy) when calcium binding was compared at lower bile acid concentrations (10 and 20 mM) (Table 1).

After adding calcium, the behavior of GCDCA differed in two respects from that of the other bile acids. First, although not obvious in Fig. 3b, there was a trend towards nonlinearity with increasing $[Ca_{TOT}]$ with a smaller percentage of calcium in the free ionized form. Second, within 10-15 min of adding the calcium, the GCDCA solutions became viscous and at $[Ca_{TOT}] > 6$ mM, the gels became opalescent over a period of 5-7 days indicating formation of precipitates. The precipitates were not soluble in concentrated HCl and had a thick gel-like quality. We concluded that the lower $[Ca^{2+}]$ versus $[Ca_{TOT}]$ relationship slope in GCDCA solutions was due, at least in part, to precipitation of a calcium-GCDCA complex. Over a 6-month period of observation, none of

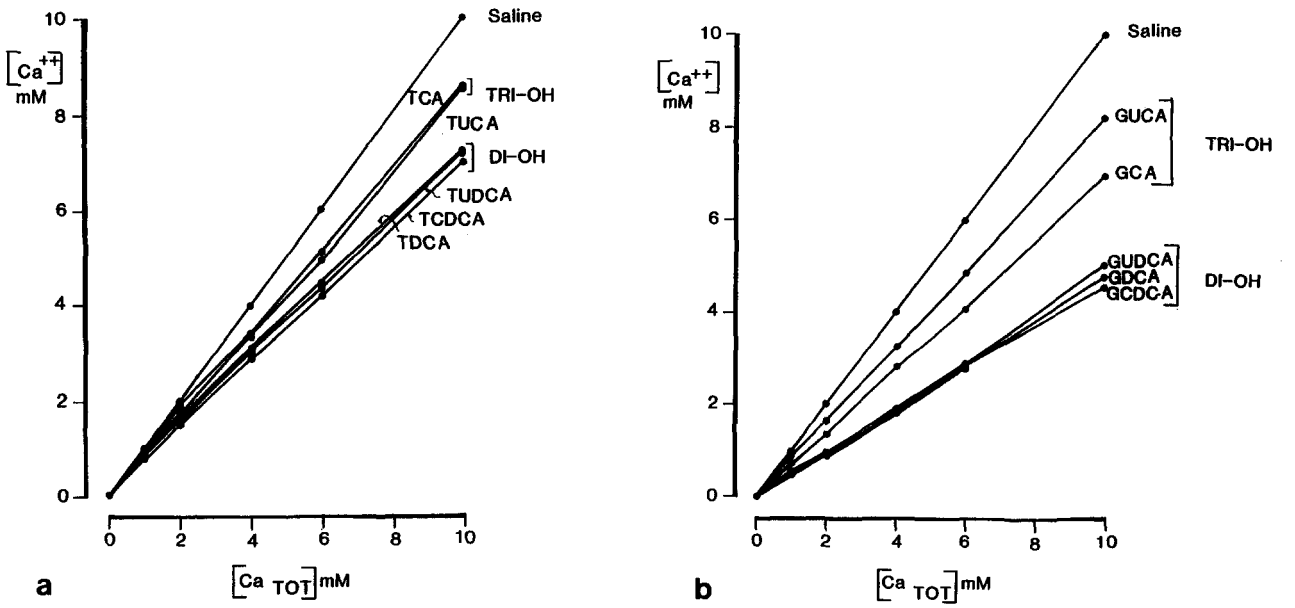

Fig. 3. Comparison of calcium binding by different tri-hydroxy (TRI-OH) and di-hydroxy (DI-OH) bile acids at 50 mM concentrations. As shown, the DI-OH bile acids bind calcium more avidly than the TRI-OH bile acids, and the glycine (G) conjugates (b, right panel) do so more than the taurine (T) conjugates (a, left panel). CA, cholic acid; UCA, ursocholic acid; UDCA, ursodeoxycholic acid; DCA, deoxycholic acid; and CDCA, chenodeoxycholic acid.

TABLE 1. Slopes of $[Ca^{2+}]/[Ca_{TOT}]$ relationships[a] and K_f values [b]

Bile Acid	Slope, $[Ca^{2+}]/(Ca_{TOT}]$ Bile Acid Concentration			Calcium Binding Affinity Constant Bile Acid Concentration		
	50 mM	20 mM	10 mM	50 mM	20 mM	10 mM
					K_f	
TCA (7)[c]	0.81 ± 0.03[d]	0.93	0.94	7.9 ± 1.00[d]	11.5	10.4
GCA (2)	0.71 ± 0.03	0.87	0.90	19.2 ± 1.40	20.8	24.5
TCDCA (3)	0.70 ± 0.02	0.82	0.90	17.1 ± 2.40	28.0	26.5
GCDCA (3)	0.41 ± 0.02	0.56	0.90	39.6 ± 4.69	46.9	39.9
TUDCA (2)	0.69 ± 0.03	0.86	0.93	13.4 ± 2.54	23.8	32.8
GUDCA (3)	0.51 ± 0.00	0.66	0.82	51.1 ± 1.04	43.9	30.1
TDCA (3)	0.70 ± 0.02	0.87	0.90	13.9 ± 1.30	20.8	24.5
GDCA (3)	0.50 ± 0.03			46.0 ± 5.50		
TUCA	0.85			8.96		
GUCA	0.81			8.71		

[a]Derived by linear regression analysis.
[b]Calculated according to Moore et al. (7).
[c]Number of experiments at 50 mM [BA] in parentheses.
[d]Mean ± SD; where SD is absent, only one experiment was performed. The following differences in slopes and K_f values were significant ($P < 0.01$; *t*-test); TCA versus all other conjugates except TUCA and GUCA; each of GCA, TCDCA, TUDCA, and TDCA versus each of GCDCA, GUDCA, and GDCA.

the other bile acid conjugates formed gels or precipitates in the presence of calcium.

Fig. 4 (upper panel) and Table 1 show the bile acid-calcium binding affinity constants (K_f) from all experiments performed in Na-PIPES. These calculated values show a close inverse correlation with the $[Ca^{2+}]$ versus $[Ca_{TOT}]$ slopes illustrated in the lower panel of Fig. 4 (n = 10; $r = 0.92$; $P < 0.001$). The only exception to this was for GCDCA, where the slope derived by linear regression analysis was artifactually lowered by the downward curva-

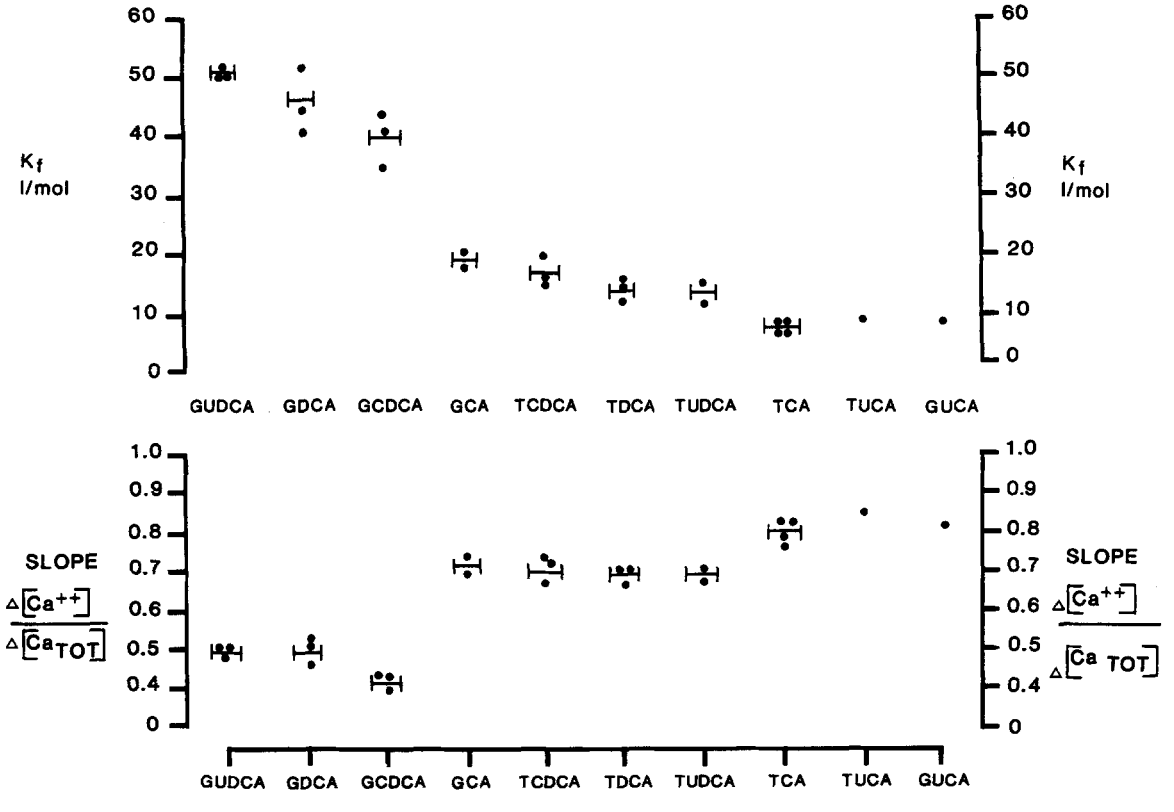

Fig. 4. Bile acid-calcium binding affinity constants (K_f) plotted in l/mol for 50 mM bile acids in saline-PIPES (upper panel) and slopes of the ionized calcium concentration/total calcium concentration relationships (lower panel). High K_f values and low $[Ca^{2+}]/[Ca_{TOT}]$ slopes indicate high binding affinities (see text). The short horizontal bars indicate mean values and the solid circles indicate data points for 1–4 studies per individual bile acid (for explanation of abbreviations, see legend to Fig. 3).

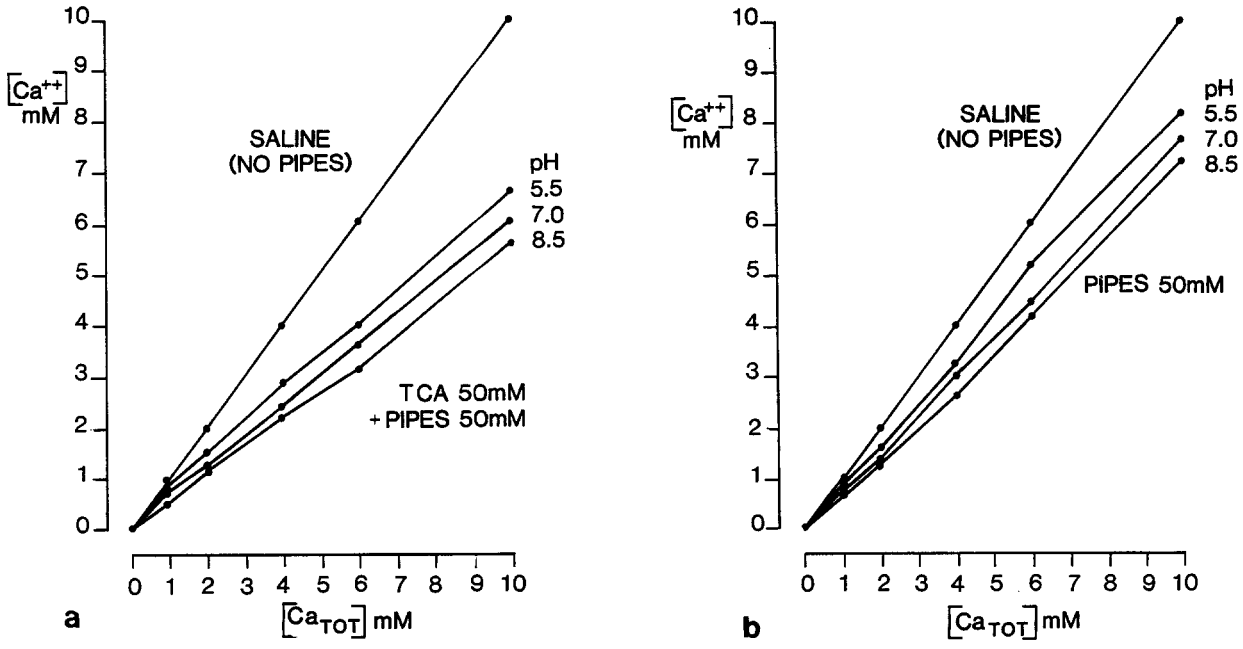

Fig. 5. The effect of pH on calcium binding in 50 mM saline-PIPES with (a, left panel) and without (b, right panel) 50 mM TCA. The small increase in binding as pH increases from 5.5 to 8.5 is comparable whether or not bile acid is present, suggesting either that the change in pH affects calcium binding by saline-PIPES or that it affects the calcium ion electrode directly.

ture of the $[Ca^{2+}]/[Ca_{TOT}]$ relationship. Furthermore, K_f values derived by linear extrapolation from Scatchard plots were virtually identical to those obtained by the method illustrated (r = 0.98; y = 0.002 + 0.993x).

As noted by Moore et al. (4, 7) for TCA and GCA, in the present study there was a tendency for K_f to increase as [BA] fell from 50 to 20 and 10 mM (Table 1). This finding is consistent with the observation that monomeric bile acids bind calcium more avidly than those in micellar form (12). The pattern of results for the individual bile acids that was found at 50 mM was also seen at 10 and 20 mM.

Studies were also performed with 50 mM taurine and 50 mM glycine in unbuffered 150 mM NaCl. There was no detectable depression of $[Ca^{2+}]$ compared to that found in 150 mM NaCl at $[Ca_{TOT}]$ 1–10 mM, indicating that the conjugating amino acids on their own did not bind calcium.

The effect of pH on calcium binding by bile acids

Fig. 5a illustrates a progressive fall in the slope of the $[Ca^{2+}]$ versus $[Ca_{TOT}]$ relationship for 50 mM TCA plus saline-PIPES as pH increases from 5.5 to 7.0 and 8.5. A similar phenomenon was found in saline-PIPES without TCA (Fig. 5b). In comparison to values obtained in unbuffered saline standards, $[Ca^{2+}]$ was depressed in a similar pH-dependent manner. This suggests that 50 mM PIPES binds significant amounts of calcium ion and that binding increases with increasing pH over the range 5.5–8.5. An alternative explanation is that hydrogen ion has a direct stimulatory effect on the electrode, so that $[Ca^{2+}]$ is artifactually lowered as $[H^+]$ falls, that is, as pH increases. Such a pH interference effect was excluded by performing the $[Ca^{2+}]$ measurements in unbuffered solutions; there were no differences in $[Ca^{2+}]$ at pH 6.0 and at pH 8.0. Finally, when the calcium binding by TCA and

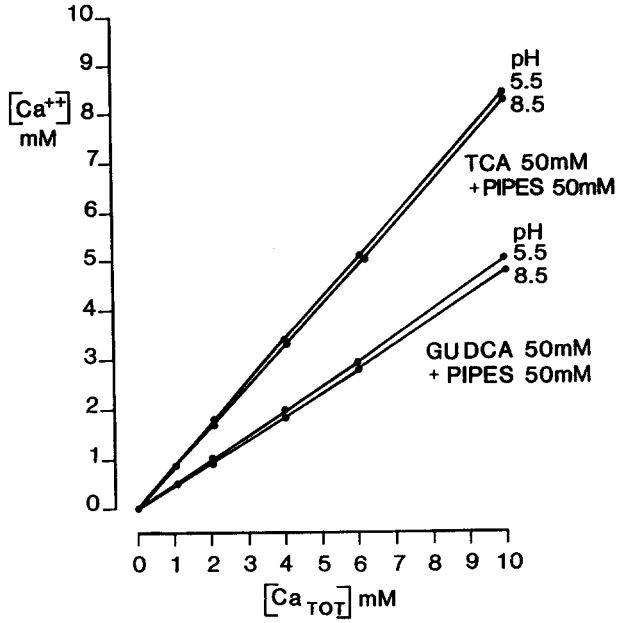

Fig. 6. Absence of effect of changes in pH (from 5.5 to 8.5) in calcium binding by saline-PIPES plus 50 mM solutions of two representative bile acids (TCA and GUDCA, see legend to Fig. 3).

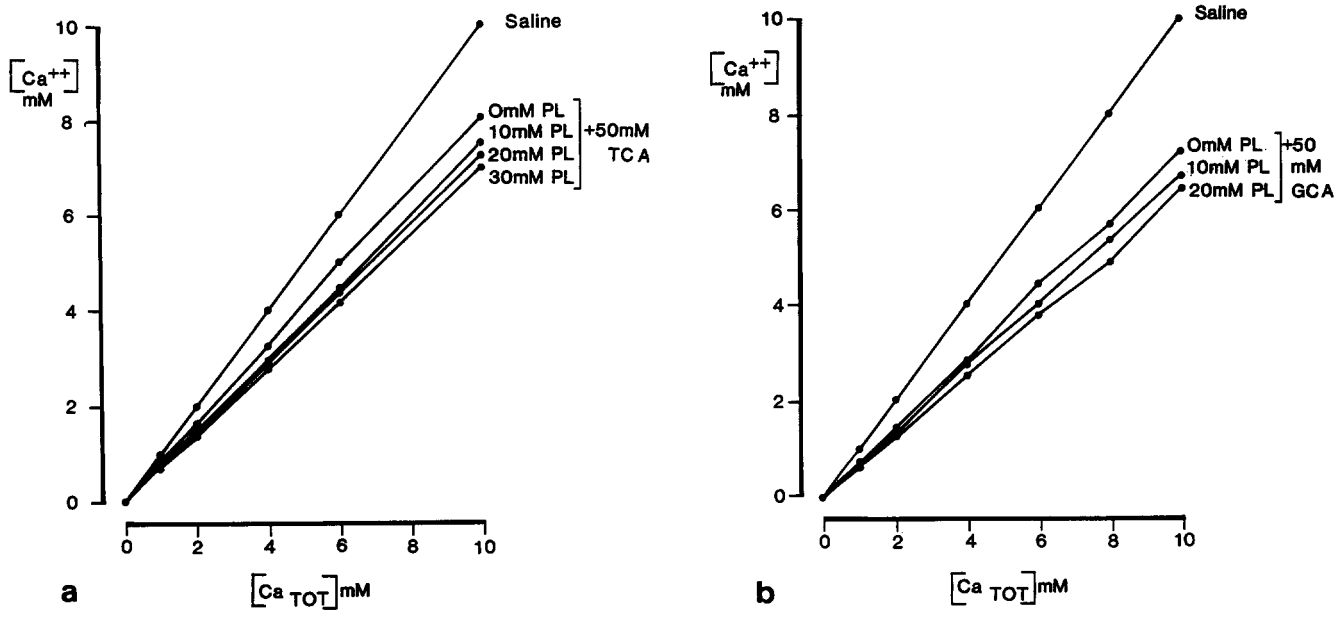

Fig. 7. Effects of increasing concentrations of phosphatidylcholine (PL) on calcium binding by 50 mM solutions of (a) TCA and (b) GCA.

GUDCA in 50 mM PIPES was studied at pH 5.5 and pH 8.5, respectively, using 50 M PIPES-saline standards buffered at the same pH (**Fig. 6**), the slopes of the $[Ca^{2+}]$ versus $[Ca_{TOT}]$ relationships were virtually identical.

Thus, the apparent pH-dependence of $[Ca^{2+}]$ binding in PIPES-buffered bile acid solutions seems to be due to pH-dependent calcium binding to the PIPES buffer. Calcium-bile acid binding, at least for TCA and GUDCA, shows no pH-dependence over the pH range studied.

Effect of added phospholipid on bile acid-calcium binding

The addition of phospholipid to the bile acid solutions had a modest, dose-dependent enhancing effect on calcium binding by both TCA and GCA (**Fig. 7a and b** and **Fig. 8**). However, there was no such effect with the taurine or glycine conjugates of DCA (Fig. 8 and **Fig. 9a**), UDCA (Figs. 8 and 9) or CDCA (Fig. 8). Indeed, with TDCA, GDCA, and GCDCA, increasing [PL] was associated with decreases in apparent calcium binding. In contrast to what was seen when calcium was added to GCDCA in the absence of PL (see above), in the presence of PL a gel or precipitate no longer formed, and the slope of the $[Ca^{2+}]$ versus $[Ca_{TOT}]$ relationship was linear. This reinforces the view that, in the presence of GCDCA alone, the marked depression of $[Ca^{2+}]$ (Fig. 3b) represents, in part, precipitation of a calcium-GCDCA complex that can be prevented by adding PL.

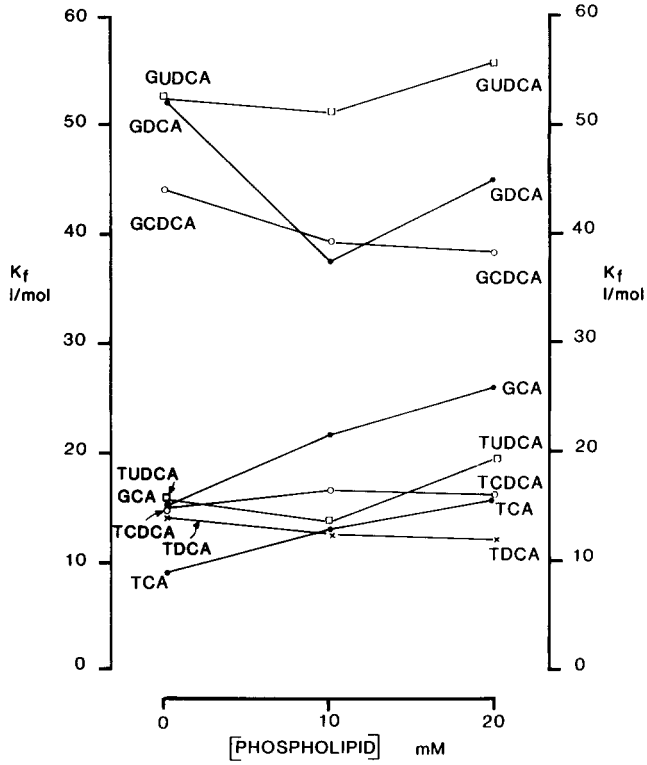

Fig. 8. Effect of increasing concentrations of phospholipid on bile acid-calcium binding affinities (K_f) for 50 mM solutions of eight different bile acid conjugates in saline-PIPES.

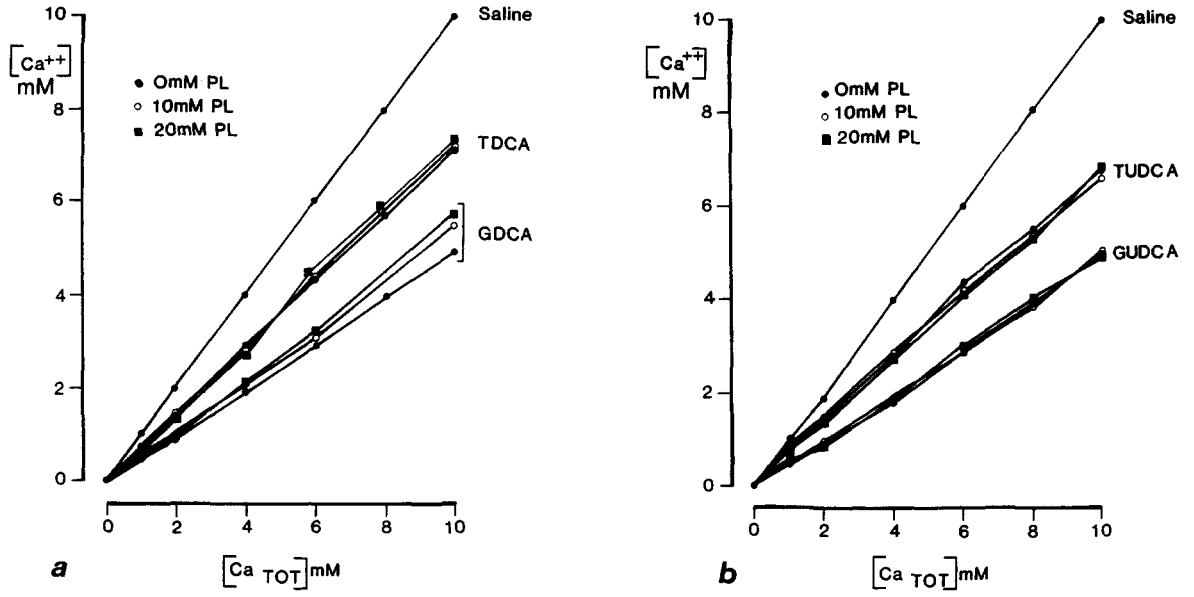

Fig. 9. Effect of increasing concentrations of phosphatidylcholine (PL) on calcium binding by 50 mM solutions of the taurine (T) and glycine (G) conjugates of deoxycholic acid (a, left panel) and ursodeoxycholic acid (b, right panel). In contrast to the small but definite effect seen with the trihydroxy bile acid, cholic acid (Fig. 7), there was no appreciable effect of increasing phospholipid (PL) concentration on these two dihydroxy bile acids.

DISCUSSION

Relationship between bile acid structure and calcium binding affinity

The present work represents the first quantitative comparative study of the calcium-binding properties of all the major biliary bile acids, together with those of the cholelitholytic bile acids, ursodeoxycholic and ursocholic acids. Our findings, therefore, substantially extend the results of previous studies of calcium-bile acid interactions which have either been semiquantitative (6) or quantitative, but limited in the number of bile acid conjugates studied (4, 7, 8, 23, 29). Furthermore, the effects of pH and of added phospholipids have not previously been examined systematically.

The present results clearly show that the calcium-binding affinity of a bile acid varies both with the structure of its steroid nucleus and with its conjugating amino acid. Thus, the dihydroxy bile acids CDCA, DCA, and UDCA have binding affinities that are very similar to one another, but 2- to 3-fold higher than those of the trihydroxy bile acids UCA and CA. A recent study indicates that the monohydroxy bile acid, lithocholic acid, has an even higher calcium-binding affinity (29). Furthermore, with the exception of UCA, the glycine-conjugated bile acids have binding affinities that are 2–3 times higher than those of the taurine conjugates, even though neither taurine nor glycine on their own appear to bind calcium. Moore and colleagues (4, 7, 30) have calculated K_f values for micellar concentrations of TCA (7.9 l/mol), GCA (19.2 l/mol), TCDCA (12.5 l/mol), and TDCA (16.8 l/mol) that agree closely with the present results. As these authors noted previously for TCA, GCA, and TCDCA, we also found a tendency for K_f to increase as bile acid concentration was lowered from 50 to 20 and 10 mM. This is consistent with Moore's proposal (4, 7) that bile acid monomers, which tend to predominate at lower [BA], have much higher calcium-binding affinities than bile acid micelles. As already noted, however, the general order of binding seen at 50 mM (di-OH>tri-OH; G>T) was preserved at 10 and 20 mM.

The K_f values do not appear to correlate well with other physico-chemical properties of the bile acids, such as their critical micellar concentrations (31, 32), pKa values (31) or hydrophobic–hydrophilic balance (33). Moore (4) has proposed that "high affinity" monomeric binding involves interposition of a calcium ion between the terminal COOH (glycine-conjugated) or SO_3^- (taurine-conjugated) group, and one or more OH groups on the steroid nucleus. However, a recent study of the crystalline salt of calcium taurocholate using nuclear magnetic resonance does not support such a configuration (34). Furthermore, the considerably lower binding affinities at bile acid concentrations above the CMC suggest that, even if correct, the binding arrangement is compromised by micelle formation. This could be partly, but not entirely, due to bile acid aggregation with a consequent reduction in the number of calcium binding sites: dihydroxy bile acids aggregate to a greater degree than

trihydroxy bile acids but, despite this, bind more calcium at micellar concentrations. Thus, the structural basis for the marked differences in bile acid-calcium binding affinity, remain unclear.

Effect of pH and PIPES

Previous studies (5, 6) using the hydrogen ion buffer Tris-HCl have suggested that the bile acid binding of calcium is pH-dependent. However, Tris is an unphysiological buffer in that its protonated form is positively charged. Furthermore, the pKa in Tris is 8.0. Therefore, at pH 7.0, the positively charged form should predominante and might compete for the bile acid-calcium binding site (6). In contrast, PIPES (pK 6.8) is uncharged in its protonated form and should not compete for calcium binding sites. Indeed, PIPES itself appeared to bind calcium (Fig. 5b) and the apparent increase in binding with increasing pH suggests that the calcium binds to the nonprotonated, negatively charged form of the buffer. It is likely, therefore, that the apparent pH-dependence of bile acid-calcium binding in the presence of PIPES is due to pH-dependent calcium binding to PIPES, and not calcium to bile acid. Since all conjugated bile acids have pKas < 5.0, they are already mainly ionized at pH 5.5. Increasing the pH to 8.5 would have little further effect on the degree of ionizaton. For this reason, the pH-independence of bile acid–calcium binding over this pH range (Fig. 7b) is hardly surprising.

The presence of PIPES in the bile acid solutions and the saline standards would be expected to depress bile acid-calcium binding by competing for calcium ion. The effect of 50 mM PIPES used in the present study should, however, be very similar to that of the physiological buffer, bicarbonate, which in human bile is present in concentrations of 5–30 mM, and binds calcium with an affinity about twice that of PIPES (34).

Effects of added phospholipid

In bile, bile acids combine with phospholipids to form mixed micelles. Despite this, previous studies of bile acid-calcium binding have been concerned mainly with bile acids alone; the effects of adding phospholipid have not been evaluated systematically. However, the finding that calcium binding by GCA is enhanced by phospholipid does confirm the results of a previous study (5); a similar effect of phospholipid on calcium binding by TCA has not been demonstrated previously. On the other hand, phospholipid has been claimed to enhance calcium binding by GCDCA and GUDCA (8), a claim that we could not confirm. Indeed, with the six dihydroxy bile acid conjugates studied, the addition of phospholipid either had no effect or actually diminished calcium binding. The reason for the discrepancy between the present and previous findings is not apparent. However, where present, the effect of phospholipid was modest and the overall order of bile acid-calcium binding affinities was not changed.

The presence of phospholipid prevented the gel and precipitate formation that would otherwise have occurred on the addition of calcium to GCDCA. Similar findings have been reported recently by Jones et al. (23). With all the other bile acid conjugates studied, precipitate formed neither in the presence nor in the absence of phospholipid. In vivo, when phospholipids are present, it is likely that the calcium complexes of all of the bile acids studied would be soluble.

Implications for $[Ca^{2+}]$ in bile

The demonstration that, in the presence of calcium, bile acids remain in solution for up to 6 months provides a physico-chemical basis for the observation that the calcium salts of bile acids are rarely found in human gallstones (35). In particular, it also challenges the hypothesis (16) that UDCA-associated gallstone calcification is due to precipitation of the calcium salts of GUDCA. Indeed, structural analyses of gallstones with acquired calcification during UDCA treatment (ref. 20; and P. M. Bills, D. Gleeson, and R. H. Dowling, unpublished observations) show that the material deposited on the surface of the stone consists of different crystalline forms of calcium carbonate and not a calcium–bile acid complex.

The finding that all ten bile acid conjugates studied bind calcium is consistent with the hypothesis proposed by Moore et al. (4, 7) that, in vivo, bile acids function as calcium buffers, that is, by lowering $[Ca^{2+}]$ they tend to lessen the risk of calcium salt precipitation in bile. The related hypothesis, that diminished bile acid binding of calcium is associated with calcium salt precipitation, is not supported by the present results. When patients take UDCA, the predominant biliary bile acids become the conjugates of UDCA and there is a reciprocal fall in the percentage of biliary CA conjugates. Moreover, the percentage of bile acids that are conjugated with glycine rises from around 70% to over 90%. From the present results, and assuming that the total bile acid-calcium binding activity of a bile sample is the sum of those of its constituent conjugates, such patients would be expected to have an increased rather than a decreased ability to bind calcium. Thus, their apparent tendency to calcium salt precipitation (16–20) cannot be explained by diminished bile acid calcium binding. Similarly, in patients with ileal dysfunction, the bile acid composition changes in such a manner (increased glycine:taurine ratio; increased CDCA:CA acid ratio) as to increase calcium binding.

It is possible that bile acids have other effects on biliary $[Ca^{2+}]$ which counterbalance their theoretically beneficial buffering effects. For instance, if the biliary epithelium were permeable to calcium ions, then bile acid binding of calcium might, be creating a chemical gradient, cause more calcium to diffuse into bile thus maintaining biliary

[Ca^{2+}]. If so, bile acid binding of calcium might be an important determinant of biliary concentrations of bound and total calcium. However, [Ca^{2+}] might be independent of binding. Indeed, this seems to be the case in human bile. Recent studies (25) have shown that [Ca_{TOT}] is closely correlated with [BA] but [Ca^{2+}] is independent of [BA]. Moreover, in both humans (26) and the dog (27), the increases in biliary total calcium output induced by different bile acids correlate (CDCA and UDCA > CA) with the calcium-binding affinities determined in the present study. This further reinforces the hypothesis that bile acid-induced biliary calcium secretion depends, in large part, on bile acid-calcium binding.

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