Characterization of the adsorption of conjugated and unconjugated bile acids to insoluble, amorphous calcium phosphate

Mirjam J. A. P. Govers,* Denise S. M. L. Termont,* George A. Van Aken,† and Roelof Van der Meer[1,*]

Departments of Nutrition* and Biophysical Chemistry,† Netherlands Institute for Dairy Research (NIZO), P.O. Box 20, 6710 BA Ede, The Netherlands

Abstract Recently we showed that supplemental dietary calcium stimulates the intestinal formation of insoluble calcium phosphate and decreases the ratio of dihydroxy to trihydroxy bile acids in human duodenal bile. Because previous in vitro studies indicated that these effects could be due to differential adsorption of bile acids to amorphous calcium phosphate, we characterized the binding of bile acids to calcium phosphate. Freshly formed, amorphous, calcium phosphate bound and thus precipitated glycine-conjugated and unconjugated bile acids, whereas taurine-conjugated bile acids showed little binding. Glycochenodeoxycholic acid hardly adsorbed to other insoluble calcium phosphates, including hydroxyapatite. Adsorption studies using increasing amounts of glycine-conjugated and unconjugated bile acids showed that binding occurred above a bile acid-specific critical minimum concentration, dependent on bile acid hydrophobicity. The simultaneous use of a fluorescent hydrophobic probe indicated that this binding was due to ionic adsorption of monomers of bile acids, followed by their hydrophobic aggregation on the calcium phosphate surface, probably in the form of a bilayer. Finally, using human duodenal bile we found that amorphous calcium phosphate, but not Ca^{2+}, preferentially bound and thus precipitated dihydroxy bile acids. We conclude that freshly formed, amorphous, calcium phosphate is a prerequisite for adsorption of bile acids and that monomers of glycine-conjugated and unconjugated dihydroxy bile acids have a high binding affinity for amorphous calcium phosphate.—**Govers, M. J. A. P., D. S. M. L. Termont, G. A. Van Aken, and R. Van der Meer.** Characterization of the adsorption of conjugated and unconjugated bile acids to insoluble, amorphous calcium phosphate. *J. Lipid Res.* 1994. **35:** 741–748.

Supplementary key words glycine • taurine • calcium ion • CMC

Epidemiological studies indicate a negative association between dietary calcium intake and the risk of colorectal cancer (1). According to the hypothesis of Newmark et al. (2, 3), binding of bile acids by dietary calcium may play an important role in the mechanism of the protective effect of dietary calcium on colorectal carcinogenesis. Recently we have shown that dietary supplementation with calcium decreases the ratio of dihydroxy to trihydroxy bile acids in human duodenal bile almost twofold (4, 5). This suggests that the intestinal reabsorption of the dihydroxy, hydrophobic bile acids is inhibited by dietary calcium, probably by a selective luminal binding of these bile acids.

In the small intestine, the bulk of dietary calcium is precipitated by phosphate (4–7). Formation of insoluble calcium phosphate proceeds via an initial mineral-phase precipitation of an amorphous (noncrystalline) calcium phosphate (ACP) with a calcium:phosphate molar ratio of about 3:2 (8). Previous in vitro studies from our laboratory (9, 10) showed that this ACP can bind and thus precipitate glycine-conjugated bile acids, which are the predominant bile acids in human duodenal bile (11, 12). In contrast, taurine-conjugated bile acids are not bound by ACP (9, 10). This was recently supported by a study by Qiu et al. (13), who elegantly demonstrated that glycochenodeoxycholic acid, but not taurocholic acid, prevents the transformation of ACP to hydroxyapatite (HAP) by binding to the ACP.

Because in the small intestine ACP coexists with millimolar concentrations of bile acids (14), we have now characterized the preferential adsorption of glycine-conjugated and unconjugated bile acids to ACP. We also addressed the question whether this adsorption can explain the selective binding of dihydroxy bile acids, as observed in vivo, by using physiological mixtures of bile acids.

Abbreviations: C, cholic acid; DC, deoxycholic acid; CDC, chenodeoxycholic acid; UDC, ursodeoxycholic acid; TOC, 3,7,12-trioxocholanoic acid (prefixes G and T indicate glycine and taurine conjugates, respectively); ACP, amorphous calcium phosphate; HAP, hydroxyapatite; CBC, critical binding concentration; CMC, critical micellar concentration; DPH, 1,6-diphenyl-1,3,5-hexatriene.

[1]To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

Sodium salts of cholic acid (C), deoxycholic acid (DC), and chenodeoxycholic acid (CDC) and their glycine (G) and taurine (T) conjugates were acquired from Sigma (St. Louis, MO). Sodium salts of ursodeoxycholic acid (UDC), its glycine and taurine conjugates, and glyco-3,7,12-trioxocholanoic acid (GTOC) were obtained from Calbiochem (La Jolla, CA). Bile acids were of the highest purity commercially available. The enzymatically measured purity was at least 90% for GC and UDC and $\geq$ 96% for the other bile acids. HPLC analysis (15) of these bile acids showed that none of the conjugated bile acids was contaminated with its unconjugated counterpart. The critical micellar concentrations (CMC) of these bile acids were identical to values reported in the literature (10, 16). Recrystallization of GDC, GCDC, and GC from ethanolic solution with ether did not alter the CMC, or the adsorption characteristics of these bile acids. N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES) and 3α-hydroxysteroid-dehydrogenase (3α-HSD; EC 1.1.1.50) were obtained from Sigma and 1,6-diphenyl-1,3,5-hexatriene (DPH) was from Ega-Chemie (Steinheim/Albuch, Germany). Other chemicals were of analytical grade.

Four human duodenal bile samples of healthy volunteers, containing 40–91 mM bile acids, were obtained from Dr. A. K. Groen from the Academic Medical Center, Amsterdam, The Netherlands.

Incubations

$CaCl_2$ (final 0–40 mM), in the absence or presence of equimolar amounts of Na_2HPO_4 (adjusted to pH 7.0 with NaH_2PO_4), was added to Eppendorf tubes, containing HEPES (final 50 mM). The final pH in incubation was 7.0, unless mentioned otherwise. NaCl was added to maintain a constant physiological ionic strength of 0.15. After a preincubation of 10 min at 37°C, during which insoluble, amorphous calcium phosphate (ACP) was formed in the tubes containing calcium and phosphate, bile acids were added and incubated for 15 min at 37°C. The tubes were then centrifuged for 2 min at 10,000 *g* and supernatants were aspirated. It should be noted that varying the incubation time from 5 to 60 min did not affect the adsorption of bile acids to ACP, which is in line with earlier results (9). The insoluble calcium salts mentioned in Table 1 were suspended in 50 mM HEPES (final pH 7.0) and incubated analogously.

To study adsorption of purified bile acid mixtures to ACP, solutions of GDC and TDC were mixed in various ratios and preincubated for 15 min at 37°C. These solutions were added to the preformed ACP and incubated as described above. Analogously, in the experiments with human duodenal bile, diluted bile samples (final: about 4 mM bile acids) were added to the $CaCl_2$ solutions and to the preformed ACP.

Analyses of supernatants

Supernatants and stock solutions were assayed for bile acids using a fluorometric enzymatic assay (17). The amount of adsorbed bile acid was calculated by difference. GTOC was quantitated enzymatically by incubating the diluted supernatants (maximum 0.1 mM GTOC) for 15 min at 37°C with 100 mM phosphate buffer (pH 7.0), 0.1 mM NADH, and 0.02 U/ml 3α-HSD. Formation of the 3α-OH group was determined by monitoring the decrease in NADH absorption at 340 nm.

Supernatants of the incubations using mixed bile acids or human duodenal bile were assayed for the different conjugated bile acids using an HPLC procedure combined with sensitive amperometric detection (15).

To quantitate the ACP precipitate, supernatants and stock solutions of $CaCl_2$ and Na_2HPO_4 were acidified with trichloroacetic acid (final 5% w/v). Calcium was measured using an atomic absorption spectrophotometer (Model 1100, Perkin-Elmer Corp., Norwalk, CT); phosphate was measured as described by Fiske and SubbaRow (18). The amounts of precipitated calcium and phosphate were calculated by difference.

Fluorescence measurements

Formation of hydrophobic aggregates of bile acids was determined by simultaneously adding bile acids and 5 μM of the hydrophobic fluorescent probe DPH (19) to incubations with preformed ACP. Fluorescence was measured (at an excitation and emission wavelength of 366 and 430 nm, respectively) immediately after incubation as well as in the supernatants after centrifugation. Appropriate control experiments revealed that the ACP precipitate did not interfere with the measurement of fluorescence.

The apparent critical micellar concentration (CMC) was determined under the same conditions, but in absence of ACP, essentially as described (19). Briefly, increasing concentrations of bile acids were incubated for 15 min at 37°C in a medium containing 50 mM HEPES (pH 7.0), 5 μM DPH, and an ionic strength of 0.15. After incubation, fluorescence was measured as outlined above.

Statistics

Results are given as means of three or four experiments with their standard errors. For clarity, only a few error bars are given in some figures. After analysis of variance of data in the tables, differences between means were subjected to the least significant difference (LSD) test. The differences in bile acid concentrations and in the ratio of dihydroxy to trihydroxy bile acids in the experiment with duodenal bile (see Table 3) were tested using the Student's *t* test for paired samples. Differences were regarded as significant if $P < 0.05$.

RESULTS

Adsorption of bile acids to insoluble calcium phosphate

Fig. 1 shows the effects of increasing equimolar concentrations of calcium and phosphate on the aqueous concentration of conjugated CDC and UDC. An ACP with a calcium:phosphate ratio of 3:2 was formed. This ACP bound GCDC and GUDC, but not TCDC, in a dose-dependent manner, until a plateau value was reached. The adsorption of other bile acids to ACP is shown in **Fig. 2.** In contrast with dihydroxy bile acids, the trihydroxy bile acids (C, GC, and TC) were not bound by ACP under these conditions. The observation that glycine-conjugated and unconjugated bile acids adsorbed to ACP to the same extent, whereas taurine-conjugated bile acids showed almost no binding, suggests that a terminal carboxylic group is a minimum requirement for adsorption to ACP. To determine whether the ability to bind glycine-conjugated and unconjugated bile acids is a unique property of ACP, we studied the efficacy of other insoluble calcium salts in binding GCDC. Under the present conditions, at least 80% of the amount of calcium salts was insoluble (**Table 1**). GCDC was not bound by $CaCO_3$. It slightly adsorbed to $Ca_3(PO_4)_2$, but adsorption to the other insoluble calcium phosphates, including HAP, was negligible. Only ACP, freshly formed from $CaCl_2$ and Na_2HPO_4, was able to bind and thus precipitate significant amounts of GCDC.

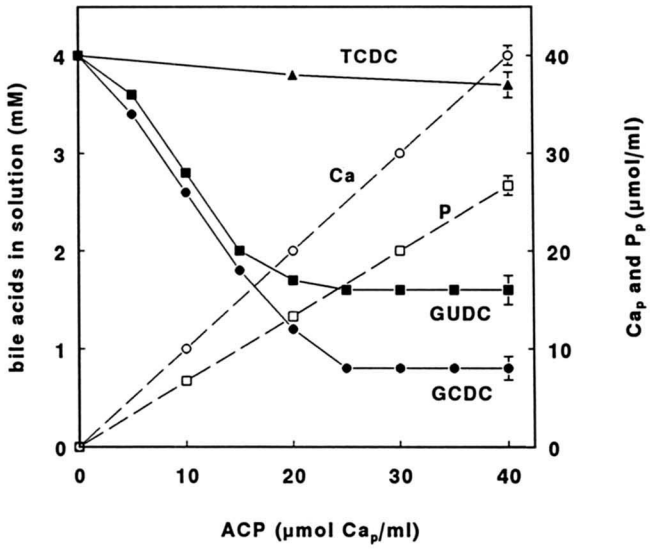

Fig. 1. Effects of increasing amounts of ACP on the aqueous concentration of conjugated bile acids. ACP was formed by incubating 0–40 mM $CaCl_2$ with equimolar amounts of Na_2HPO_4 in presence of 50 mM HEPES (pH 7.0, 37°C, I = 0.15). After 10 min, bile acids were added and incubated for 15 min. After centrifugation, bile acids were measured in the supernatants. Ca_p, precipitated calcium; P_P, precipitated phosphate.

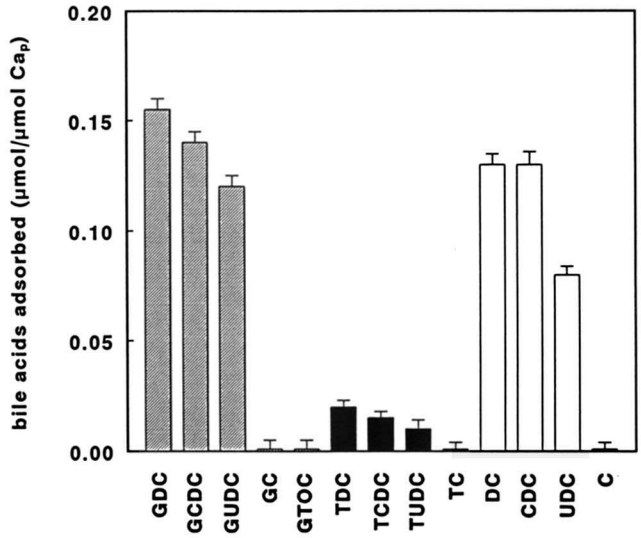

Fig. 2. Adsorption of glycine-conjugated, taurine-conjugated, and unconjugated bile acids (initial concentration 4 mM) to ACP (20 μmol Ca_p/ml). Incubations were performed as described in the legends of Fig. 1. Ca_p, precipitated calcium. Mean ± SE, n = 4 experiments.

Characteristics of the adsorption of glycine-conjugated and unconjugated bile acids to ACP

The binding characteristics of ACP were determined using increasing concentrations of glycine-conjugated and unconjugated bile acids. Some typical examples of the adsorption isotherms are given in **Fig. 3.** We found that concentrations up to 20 mM of GTOC did not bind to ACP (not shown). Fig. 3 shows that significant binding starts at a bile acid-specific critical minimum concentration, which we call the critical binding concentration (CBC). The CBC was determined from the plot by extrapolating

TABLE 1. Adsorption of GCDC (initial concentration 4 mM) to various insoluble calcium salts (pH 7.0)

Calcium Salt[a]	Soluble Calcium[b]	GCDC Adsorbed
	% of total	
$CaCO_3$	20 ± 1[a]	2 ± 1[a]
$Ca_3(PO_4)_2$	2 ± 1[b,c]	19 ± 1[b]
$CaHPO_4 \cdot 2H_2O$	11 ± 1[d]	3 ± 1[a]
$Ca(H_2PO_4)_2 \cdot H_2O$	3 ± 0[e]	2 ± 1[a]
Hydroxyapatite	1 ± 0[b]	2 ± 1[a]
Amorphous calcium phosphate	5 ± 0[e]	71 ± 1[c]

Mean ± SE, n = 4 experiments. Values in the same column not sharing the same superscript are significantly different ($P < 0.05$).

[a] The insoluble calcium salts (final: 20 μmol Ca/ml) were suspended in HEPES buffer (final pH 7.0); HAP: hydroxyapatite ($Ca_{10}(PO_4)_6(OH)_2$); ACP: amorphous calcium phosphate, formed as described in the legends of Fig. 1.

[b] The concentration of calcium present in the supernatants of the incubations, thus indicating the amount of calcium salt that is soluble under the present conditions.

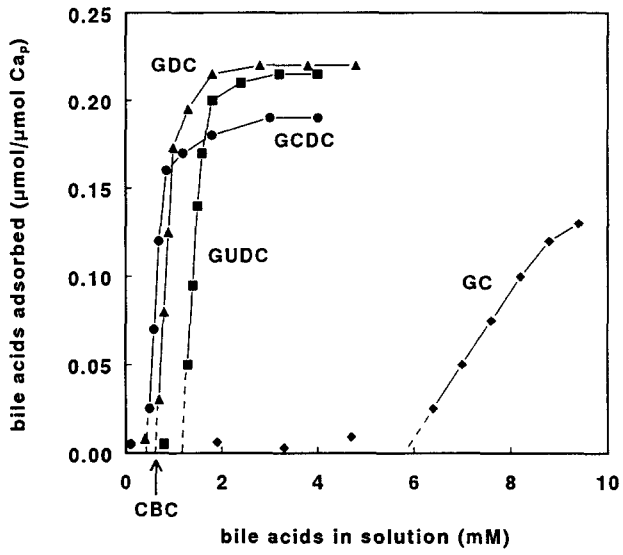

Fig. 3. Adsorption curves of the binding of glycine-conjugated bile acids to ACP (20 μmol Ca_p/ml). Incubations were performed as described in the legends of Fig. 1. The curves are representative for at least three different experiments. Ca_p, precipitated calcium; CBC, critical binding concentration, extrapolated from the curves.

the steep increase in the amount of bile acid adsorbed to zero value, as is indicated by the dotted line in Fig. 3. Furthermore, the curves are seen to level off at a maximum binding capacity, which is slightly different for the various bile acids. In addition, we determined the free bile acid concentration at which 50% of the maximum binding is reached (C_{50}). **Table 2** summarizes these adsorption parameters for the different bile acids, and comparison is made with the CMC values. From this table it is seen that the CMC and CBC of a bile acid are strongly correlated, the CMC being more than twofold higher than the CBC. Trihydroxy bile acids have much higher CBCs than dihydroxy bile acids. From the difference between CBC and C_{50} it can be seen that the conjugated bile acids show a steeper increase in adsorption than the unconjugated bile acids. The maximum binding capacity was comparable for the different dihydroxy bile acids, and the adsorption curves suggested a lower maximum binding capacity for the trihydroxy bile acids. The binding of trihydroxy bile acids occurred at much higher concentrations (Fig. 3, Table 2), and appeared to be fraught with large experimental errors. Therefore, the plateau values of these bile acids could only be estimated. The adsorption curve of UDC did not reach a plateau value at pH 7.0, probably because the critical micellization pH of this bile acid is above pH 7.0 (20). At this pH, the protonated species apparently interferes with the adsorption of the UDC anions. Indeed, increasing the pH to 7.5 resulted in an isotherm with a plateau value, the parameters of which are listed in Table 2.

The observed CBC values are lower than the values for CMC measured in the absence of ACP (Table 2; 10, 16), which indicates that the strong increase in the adsorption of bile acids to ACP occurs at submicellar concentrations. Because of the observed correlation between the CMC and CBC values, we may suspect that some kind of hydrophobic interaction between the bile acid molecules, perhaps aggregation at the interface, promotes the binding to ACP. This is supported by the sigmoidal shape of the adsorption curves, which indicates cooperative binding. In order to detect whether hydrophobic aggregation is involved in the binding mechanism, the binding was also studied in the presence of a fluorescent probe. The

TABLE 2. Critical micellar concentration and adsorption parameters of glycine-conjugated and unconjugated bile acids to ACP (20 μmol Ca_p/ml), pH 7.0

Bile Acid	CMC[a]	CBC[b]	C_{50}[c]	Max. Binding Capacity[d]
		mM		*μmol/μmol Ca_p*
GDC	2.1 ± 0.1[a]	0.5 ± 0.1[a,b]	0.7 ± 0.0[a]	0.22 ± 0.01[a,b]
GCDC	2.2 ± 0.1[a]	0.4 ± 0.1[a]	0.6 ± 0.1[a]	0.19 ± 0.01[c]
GUDC	4.4 ± 0.3[b]	1.2 ± 0.1[c]	1.6 ± 0.1[b]	0.21 ± 0.01[a,c]
GC	7.5 ± 0.4[c]	5.8 ± 0.6[d]	7.5 ± 0.5[c]	≈0.14
DC	3.0 ± 0.1[f]	0.7 ± 0.1[b,e]	1.1 ± 0.0[d]	0.23 ± 0.01[a,b]
CDC	2.8 ± 0.1[f]	0.8 ± 0.1[e]	1.2 ± 0.0[d]	0.24 ± 0.01[b]
UDC[e]	7.0 ± 0.6[c]	3.4 ± 0.2[f]	4.4 ± 0.4[e]	0.19 ± 0.01[c]
C	9.8 ± 0.6[f]	5.1 ± 0.3[d]	8.6 ± 0.8[c]	≈0.12

Mean ± SE, n = 4 experiments. Values in the same column not sharing the same superscript are significantly different ($P < 0.05$).

[a]CMC: critical micellar concentration, determined in absence of ACP.

[b]CBC: critical binding concentration, found from the adsorption curves by extrapolation, as indicated by the dotted lines in Fig. 3.

[c]C_{50}: the free bile acid concentration at which half of the maximum binding is reached.

[d]Maximum binding capacity, determined as the plateau value of the adsorption curves as shown in Fig. 3. The values for GC and C could only be estimated.

[e]Values of UDC are determined at pH 7.5 instead of 7.0 (see text).

results are similar for all glycine-conjugated and unconjugated bile acids, and as a typical example the result for GCDC is illustrated in **Fig. 4.** Fluorescence was observed at concentrations above the CBC, indicating the presence of aggregates in the system. Fluorescence appeared absent in the supernatants of these incubations, showing that hydrophobic aggregates were absent in the supernatants. Therefore, the aggregates must be bound to the surface of ACP. Only when ACP was saturated with bile acids, did fluorescence appear in the supernatants, indicating the presence of soluble bile acid micelles. The fluorescent curve for measurement of the CMC, which is performed in incubations without ACP, was similar to the curve shown for supernatants in Fig. 4. This indicates that the ACP surface is a prerequisite for formation of hydrophobic aggregates at submicellar concentrations.

Adsorption of mixed bile acids to ACP

Subsequently, we studied the adsorption of taurine-conjugated bile acids, which by themselves do not adsorb to ACP, in presence of glycine-conjugated bile acids. GDC concentration was kept constant at 6 mM, whereas the concentration of TDC was increased from 0 to 14 mM (**Fig. 5**). If only GDC was present, the ACP surface was saturated with GDC (maximum binding capacity of GDC: 0.22 μmol/μmol Ca_p, see Table 2). Increasing TDC concentrations gradually decreased the binding of GDC and increased the binding of TDC, indicating that TDC is incorporated in the hydrophobic aggregates on the ACP surface. Although the concentration of TDC increased up to a TDC:GDC ratio of 2.3:1, this resulted only in a 1:1 ratio bound to the ACP surface. Apparently, only 50% of the binding capacity is available for taurine-conjugated bile acids. Furthermore, we constructed an adsorption curve (similar to those shown in Fig. 3) for a bile acid mixture of GDC and TDC in a 1:1 ratio. The shape of the curve was comparable to that of GDC alone.

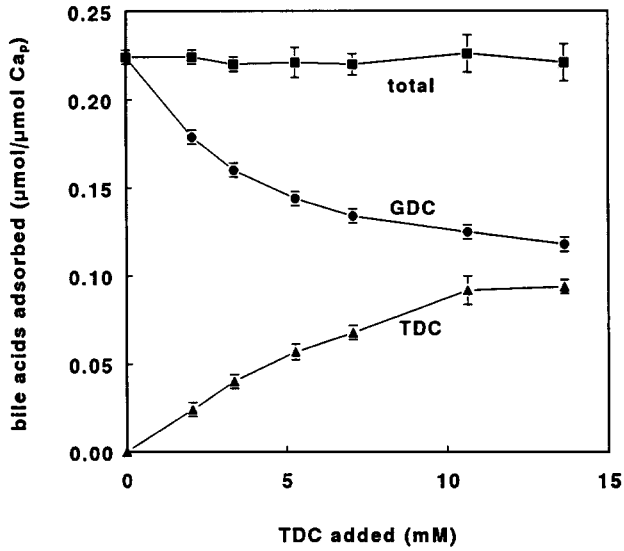

Fig. 5. Effects of constant GDC (6 mM) and increasing TDC (0–14 mM) concentrations on adsorption of GDC and TDC to ACP (20 μmol Ca_p/ml). Incubations were performed as described in the legends of Fig. 1. GDC and TDC were incubated together for 15 min at 37°C before adding to the preformed ACP. Ca_p, precipitated calcium. Mean ± SE, n = 3 experiments.

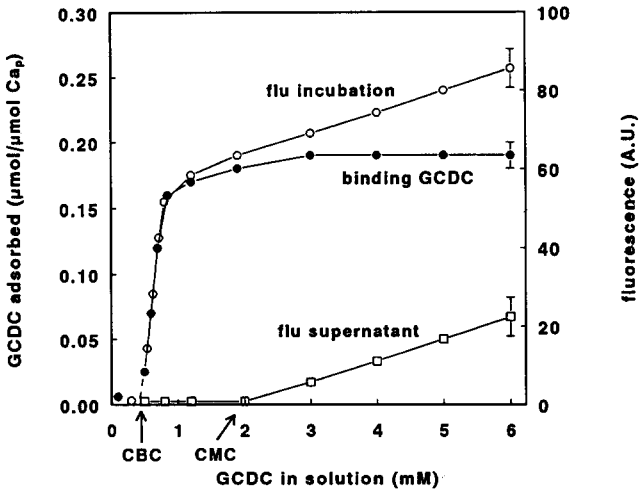

Fig. 4. Adsorption of GCDC to ACP (20 μmol Ca_p/ml) and its hydrophobic aggregation as measured by the fluorescence (5 μM DPH; A.U.: arbitrary units) in the total incubations and in the supernatants. Experiments were performed as described in the legends of Fig. 1. Ca_p, precipitated calcium; CBC, critical binding concentration, extrapolated from the curves; CMC, critical micellar concentration. Mean ± SE, n = 3 experiments.

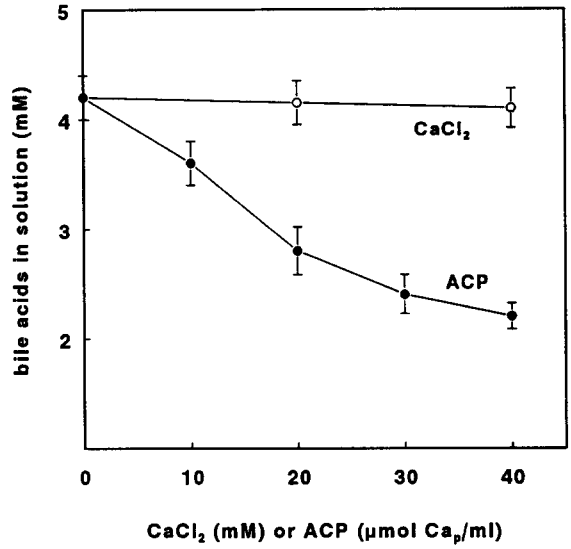

Fig. 6. Effects of increasing concentrations of either $CaCl_2$ or ACP on the aqueous concentration of bile acids in human duodenal bile. Incubations were performed as described in the legends of Fig. 1. Instead of purified bile acids, diluted human duodenal bile samples were added to the preformed ACP. Ca_p, precipitated calcium. Mean ± SE, n = 4 bile samples.

However, the CBC of the bile acid mixture appeared exactly twice as high as that of GDC alone, indicating that the concentration of carboxylic headgroups, and not the total concentration of bile acids, determines the CBC.

Finally, physiological bile acid mixtures were studied, by incubating duodenal bile samples with $CaCl_2$ and with freshly formed ACP (**Fig. 6**). The diluted bile samples themselves contained negligible concentrations of calcium and phosphate (final < 0.5 mM). No precipitation of bile acids by soluble Ca^{2+} ions up to 40 mM was observed, whereas ACP precipitated the mixed bile acids in a dose-dependent manner analogous to that observed for purified bile acids (Fig. 1). HPLC analysis of the supernatants (**Table 3**) revealed that about 70% of GDC and GCDC, 55% of TDC and TCDC, and 20% of GC and TC were precipitated by ACP. Consequently, the ratio of dihydroxy to trihydroxy bile acids in the supernatants was decreased from 1.40 to 0.63.

DISCUSSION

Binding capacity of insoluble calcium salts

Our experiments show that a freshly formed ACP is a prerequisite for binding of bile acids. This ACP is precipitated from $CaCl_2$ and Na_2HPO_4 in a molar calcium:phosphate ratio of 3:2 (Fig. 1), in accordance with previous studies (4, 8, 10). A crystalline calcium phosphate with a similar ratio as ACP, $Ca_3(PO_4)_2$, bound only small amounts of GCDC (Table 1). The absence of binding to $CaHPO_4$ supports the recent finding of Rémésy et al. (21), who reported hardly any binding of unconjugated bile acids to $CaHPO_4$ up to 1 M. Moreover, adsorption of GCDC to HAP was almost negligible. This seems to be in contrast with a recent publication of Qiu, Soloway, and Crowther (22), who reported significant binding of GCDC to HAP. However, they reported a maximum binding capacity of 0.23 μmol GCDC/mg HAP. Because 1 mg HAP contains 10 μmol calcium, this binding capacity corresponds to 0.023 μmol GCDC/μmol calcium, which is almost 10-fold lower than the binding of GCDC to ACP found in the present study. This indicates that adsorption of bile acids to ACP, which is the initially formed precipitate in the small intestine, is quantitatively more important than adsorption to HAP, possibly because the surface area of the amorphous precipitate is much larger than of the crystalline HAP.

TABLE 3. Bile acid concentrations and the ratio of dihydroxy to trihydroxy bile acids in supernatants from incubations of human duodenal bile without ACP and with ACP (40 μmol/ml Ca_p)

Bile Acid	Bile Acids in Solution		% Adsorbed
	Without ACP	With ACP	
	mM		
GDC	0.63 ± 0.20	0.16 ± 0.05[a]	73 ± 1[c]
GCDC	1.14 ± 0.11	0.33 ± 0.03[a]	71 ± 1[c]
GC	1.45 ± 0.27	1.01 ± 0.19[a]	30 ± 1[d]
TDC	0.17 ± 0.06	0.07 ± 0.03[a]	58 ± 1[e]
TCDC	0.48 ± 0.08	0.24 ± 0.04[a]	49 ± 2[f]
TC	0.49 ± 0.11	0.42 ± 0.09[a]	15 ± 0[g]
Total	4.34 ± 0.30	2.23 ± 0.23[a]	
Ratio $(OH)_2/(OH)_3$[b]	1.40 ± 0.32	0.63 ± 0.13[a]	

Mean ± SE, n = 4 bile samples. Values in the column "% Adsorbed" not sharing the same superscript are significantly different ($P < 0.05$).
[a] Significant effect of ACP ($P < 0.05$, paired t test).
[b] Ratio $(OH)_2/(OH)_3$: ratio of dihydroxy to trihydroxy bile acid concentration.

Mechanism of adsorption of bile acids to ACP

Apparently, the type of conjugation of bile acids is an important determinant for adsorption to ACP (Fig. 2). This confirms and extends the results of previous studies (9, 10), and indicates that binding of bile acids to ACP is mediated by the terminal carboxylic anion.

The adsorption curves (Fig. 3) show that significant binding to ACP occurs above a certain critical concentration (CBC) and obviously does not follow a Langmuir isotherm. The CBC was always below the CMC (Table 2; 10, 16), and its value appeared bile acid-specific. This refutes our earlier suggestion (9, 10), and also the assumption of Qiu et al. (13) that the presence of micelles is a prerequisite for adsorption to ACP. On the other hand, the fluorescence measurements (Fig. 4) indicate that some kind of hydrophobic aggregation facilitates the adsorption to the ACP surface. The occurrence of hydrophobic aggregation on the ACP surface explains why the adsorption cannot be described by a Langmuir isotherm, where interactions between adsorbed molecules, other than area constraints, are neglected (23). For monolayer adsorption, various theoretical isotherms exist that allow for lateral interaction, and these are usually developed to describe the adsorption of gases on solids (23, 24). A typical feature for adsorption with attractive lateral interactions between the adsorbed molecules is that the adsorption is cooperative. For large lateral attractive interaction this will lead to a steep increase of the adsorbed amount starting at a certain concentration, which is preceded by a region where the surface coverage remains low and is followed by a plateau region where the surface is completely covered, and the adsorption has reached a maximum value. This sigmoidal shape is clearly reflected in Fig. 3. Because the various models that describe adsorption with lateral interactions between the molecules yield very similar isotherms, it is at present not possible to judge solely from the shape of the isotherm of Fig. 3 which model applies, and this requires further investigation.

With regard to the mechanism of binding, we suppose that ionic adsorption of the negatively charged carboxylic

headgroup of the bile acid to the positively charged Ca^{2+} ions on the surface of the ACP initiates the binding, as suggested by Qiu et al. (22). This ionic adsorption leaves the hydrophobic tails of the bile acid exposed to the aqueous solution, which will attract other bile acids and thus stimulate the hydrophobic aggregation of bile acid monomers on the ACP surface. Consequently, this hydrophobic phase on the ACP surface facilitates the adsorption of other hydrophobic (including non-carboxylic) ligands. This is comparable to the principle of opposing forces, as described by Tanford (25), implying that the stability of aggregates of ionic amphiphiles is determined both by the attractive hydrophobic forces of the steroid skeleton and the repulsive forces of the charged headgroup: as soon as the negative charge of the headgroup is compensated, for instance by ionic binding, hydrophobic aggregation is promoted.

From this mechanism it can be explained why binding of taurine-conjugated bile acids per se is negligible, whereas substantial adsorption is found for taurine conjugates in mixtures of glycine- and taurine-conjugated bile acids. NMR studies have shown that the sulphonate anion has a much lower affinity for calcium than the carboxylic anion has (26, 27), and therefore will not adsorb. However, in the presence of glycine-conjugated bile acids, presumably a layer of the latter kind of bile acids will adsorb to the ACP surface by their carboxylic headgroups, their hydrophobic tails thus sticking out in the solution and rendering the hydrophobic adsorption sites available for taurine-conjugated bile acids. Therefore, in the presence of glycine-conjugated bile acids, half of the maximum binding capacity is available for taurine-conjugated bile acids (Fig. 5). In our opinion this indicates that the bile acids form a bilayer on the ACP surface. Because taurine-conjugated bile acids do not bind to the ACP surface, our results indicate that these bile acids are only incorporated in the outer layer, favored by a cooperative, hydrophobic effect. Assuming a cross-sectional area of GDC of about 38 $Å^2$ (28), a total surface of 0.025 m^2 per μmol calcium in ACP can be calculated.

The question may arise whether dimers would exist at concentrations below the CMC, and whether adsorption takes place through these dimers. Even if they would exist, these dimers could not be detected by our hydrophobic fluorescent probe. However, as suggested by the adsorption curve of the GDC:TDC mixture, it is the concentration of carboxylic headgroups that determines the CBC. Assuming that GDC and TDC form mixed dimers under similar conditions as GDC alone, this experiment therefore indicates that the formation of dimers is not a prerequisite for adsorption to ACP.

Consistent with our proposed mechanism, we found that dihydroxy hydrophobic bile acids have lower CBCs than trihydroxy hydrophilic bile acids (Table 2). In accordance with this, we observed no binding up to 20 mM of the very hydrophilic bile acid GTOC to ACP. This is in accordance with the findings of Gleeson, Murphy, and Dowling (29), showing that the hydrophobicity of bile acids is a main determinant of its interaction with calcium. The maximum binding capacity of ACP for dihydroxy bile acids is higher than the estimated value for trihydroxy bile acids (Table 2), which may indicate that dihydroxy bile acids are more closely packed on the ACP surface than trihydroxy bile acids. Analogously, the observed small difference in maximum binding capacity between unconjugated and conjugated dihydroxy bile acids (Table 2) may indicate that unconjugated bile acids occupy slightly less space on the ACP surface than their conjugated counterparts.

Adsorption of physiological mixtures of bile acids to ACP

Human duodenal bile was used to determine whether the effects of conjugation and hydrophobicity on binding to ACP, as observed with purified bile acids, are of relevance to physiological bile acid mixtures. Indeed, predominantly glycine- and taurine-conjugated dihydroxy bile acids were precipitated by ACP, whereas trihydroxy bile acids were precipitated to a lesser extent (Table 3). The ACP-induced decrease in the $(OH)_2/(OH)_3$ ratio corresponds well with the results of our in vivo study (4), in which the $(OH)_2/(OH)_3$ ratio in human duodenal bile decreased from 1.44 ± 0.22 to 0.76 ± 0.07 (mean ± SE, n = 7) after dietary calcium supplementation. This selective precipitation of dihydroxy bile acids in the intestinal lumen after dietary calcium supplementation might be of relevance in the prevention of bile acid-related diseases. In patients with colorectal neoplasia and with adenomatous polyps, higher relative concentrations of dihydroxy bile acids in duodenal bile (30, 31) and in serum (32) were found when compared with controls. Moreover, Castleden, Detchon, and Misso (33) reported that patients with gallstone disease also had higher biliary DC levels.

We previously demonstrated that purified bile acids are not rapidly precipitated by soluble Ca^{2+} (9, 10), which was recently substantiated by Hofmann and Mysels (20). We have now also shown that in physiologically more relevant systems, such as human duodenal bile, soluble Ca^{2+} ions do not precipitate bile acids (Fig. 6).

In conclusion, the present results demonstrate that under physiological conditions freshly formed ACP is able to bind and thus precipitate bile acids, dependent on their conjugation and hydrophobicity. This binding is apparently mediated by ionic adsorption of bile acid monomers to the charged surface of ACP and subsequent formation of hydrophobic aggregates on the ACP surface.

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REFERENCES

1. Sorenson, A. W., M. L. Slattery, and M. H. Ford. 1988. Calcium and colon cancer: a review. *Nutr. Cancer.* **11:** 135–145.
2. Newmark, H. L., M. J. Wargovich, and W. R. Bruce. 1984. Colon cancer and dietary fat, phosphate, and calcium: a hypothesis. *J. Natl. Cancer Inst.* **72:** 1323–1325.
3. Newmark, H. L., M. J. Wargovich, W. R. Bruce, A. L. Boynton, L. P. Kleine, J. F. Whitfield, B. Jansson, and I. L. Cameron. 1985. Ions and neoplastic development. *In* Large Bowel Cancer. Praeger Press, New York. 102–130.
4. Van der Meer, R., J. W. M. Welberg, F. Kuipers, J. H. Kleibeuker, N. H. Mulder, D. S. M. L. Termont, R. J. Vonk, H. T. De Vries, and E. G. E. De Vries. 1990. Effects of supplemental dietary calcium on the intestinal association of calcium, phosphate, and bile acids. *Gastroenterology.* **99:** 1653–1659.
5. Welberg, J. W. M., J. H. Kleibeuker, R. Van der Meer, F. Kuipers, A. Cats, H. Van Rijsbergen, D. S. M. L. Termont, W. Boersma-Van Ek, R. J. Vonk, N. H. Mulder, and E. G. E. De Vries. 1993. Effects of oral calcium supplementation on intestinal bile acids and cytolytic activity of fecal water in patients with adenomatous polyps of the colon. *Eur. J. Clin. Invest.* **23:** 63–68.
6. Shiga, A., T. Sasaki, and N. Horii. 1987. Correlations among pH and Mg, Ca, P, Na, K, Cl^- and HCO_3^- contents of digesta in the gastrointestinal tract of rats. *Jpn. J. Vet. Sci.* **49:** 973–979.
7. Govers, M. J. A. P., and R. Van der Meer. 1993. Effects of dietary calcium and phosphate on the intestinal interactions between calcium, phosphate, fatty acids and bile acids. *Gut.* **34:** 365–370.
8. Termine, J. D., and A. S. Posner. 1970. Calcium phosphate formation in vitro. I. Factors affecting initial phase separation. *Arch. Biochem. Biophys.* **140:** 307–317.
9. Van der Meer, R., and H. T. De Vries. 1985. Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate. *Biochem. J.* **229:** 265–268.
10. Van der Meer, R., D. S. M. L. Termont, and H. T. De Vries. 1991. Differential effects of calcium ions and calcium phosphate on cytotoxicity of bile acids. *Am. J. Physiol.* **260:** G142–G147.
11. Rossi, S. S., J. L. Converse, and A. F. Hofmann. 1987. High pressure liquid chromatographic analysis of conjugated bile acids in human bile: simultaneous resolution of sulfated and unsulfated lithocholyl amidates and the common conjugated bile acids. *J. Lipid Res.* **28:** 589–595.
12. Spigelman, A. D., R. W. Owen, M. J. Hill, and R. K. S. Philips. 1991. Biliary bile acid profiles in familial adenomatous polyposis. *Br. J. Surg.* **78:** 321–325.
13. Qiu, S. M., G. Wen, N. Hirakawa, R. D. Soloway, N. K. Hong, and R. S. Crowther. 1991. Glycochenodeoxycholic acid inhibits calcium phosphate precipitation in vitro by preventing the transformation of amorphous calcium phosphate to calcium hydroxyapatite. *J. Clin. Invest.* **88:** 1265–1271.
14. Fordtran, J. S., and T. W. Locklear. 1966. Ionic constituents and osmolality of gastric and small-intestinal fluids after eating. *Am. J. Dig. Dis.* **11:** 503–521.
15. Dekker, P. R., R. Van der Meer, and C. Olieman. 1991. Sensitive pulsed amperometric detection of free and conjugated bile acids in combination with gradient reversed-phase HPLC. *Chromatographia.* **31:** 549–553.
16. Roda, A., A. F. Hofmann, and K. J. Mysels. 1983. The influence of bile salt structure on self-association in aqueous solutions. *J. Biol. Chem.* **258:** 6362–6370.
17. Mashige, F., I. Kazuhiro, and T. Osuga. 1976. A simple and sensitive assay of total serum bile acids. *Clin. Chim. Acta.* **70:** 79–86.
18. Fiske, C. H., and Y. SubbaRow. 1925. The colorimetric determination of phosphorus. *J. Biol. Chem.* **66:** 375–400.
19. Chattopadhyay, A., and E. London. 1984. Fluorimetric determination of critical micelle concentration avoiding interference from detergent charge. *Anal. Biochem.* **139:** 408–412.
20. Hofmann, A. F., and K. J. Mysels. 1992. Bile acid solubility and precipitation in vitro and in vivo: the role of conjugation, pH and Ca^{2+} ions. *J. Lipid Res.* **33:** 617–626.
21. Rémésy, C., M. A. Levrat, L. Gamet, and C. Demigné. 1993. Cecal fermentations in rats fed oligosaccharides (inulin) are modulated by dietary calcium level. *Am. J. Physiol.* **264:** G855–G862.
22. Qiu, S. M., R. D. Soloway, and R. S. Crowther. 1992. Interaction of bile salts with calcium hydroxyapatite: inhibitors of apatite formation exhibit high-affinity premicellar binding. *Hepatology.* **16:** 1280–1289.
23. Pierotti, R. A., and H. E. Thomas. 1971. Surface and Colloid Science. Vol. 4. Wiley, New York, NY. 93–259.
24. Parfitt, G. D., and C. H. Rochester. 1983. Adsorption from Solution at the Solid/Liquid Interface. Academic Press, London, UK.
25. Tanford, C. 1980. The Hydrophobic Effect: Formation of Micelles and Biological Membranes. John Wiley & Sons, New York. 60–78.
26. Irving, C. S., B. E. Hammer, S. S. Danyluk, and P. D. Klein. 1981. Coordination and binding of taurine as determined by nuclear magnetic resonance measurements on ^{13}C-labeled taurine. *Adv. Exp. Med. Biol.* **139:** 5–17.
27. Mukidjam, E., S. Barnes, and G. A. Elgavish. 1986. NMR studies of the binding of sodium and calcium ions to the bile salts glycocholate and taurocholate in dilute solution, as probed by the paramagnetic lanthanide dysprosium. *J. Am. Chem. Soc.* **108:** 7082–7089.
28. Balducci, R., A. Roda, and R. S. Pearlman. 1989. A theoretical model for the critical micelle concentration of bile salts. *J. Solution Chem.* **18:** 355–368.
29. Gleeson, D., G. M. Murphy, and R. H. Dowling. 1990. Calcium binding by bile acids: in vitro studies using a calcium ion electrode. *J. Lipid Res.* **31:** 781–791.
30. Moorehead, R. J., G. R. Campbell, J. D. Donaldson, and S. T. D. McKelvey. 1987. Relationship between duodenal bile acids and colorectal neoplasia. *Gut.* **28:** 1454–1459.
31. Van Der Werf, S. D. J., F. M. Nagengast, G. P. Van Berge Henegouwen, A. W. M. Huybregts, and J. H. M. van Tongeren. 1982. Colonic absorption of secondary bile acids in patients with adenomatous polyps and in matched controls. *Lancet.* **1:** 759–762.
32. Bayerdörffer, E., G. A. Mannes, W. O. Richter, T. Ochsenkühn, B. Wiebecke, W. Köpcke, and G. Paumgartner. 1993. Increased serum deoxycholic acid levels in men with colorectal adenomas. *Gastroenterology.* **104:** 145–151.
33. Castleden, W. M., P. Detchon, and N. L. A. Misso. 1989. Biliary bile acids in cholelithiasis and colon cancer. *Gut.* **30:** 860–865.