Rapid association of unconjugated bilirubin with amorphous calcium phosphate[1]

Christa N. van der Veere,[2,*] Berry Schoemaker,* Roelof van der Meer,† Albert K. Groen,* Peter L. M. Jansen, and Ronald P. J. Oude Elferink***

Department of Gastroenterology,* Academic Medical Center, Amsterdam; Department of Nutrition,† Netherlands Institute for Dairy Research (NIZO), Ede; and Department of Hepatology and Gastroenterology,** University Hospital Groningen, The Netherlands

Abstract The association of unconjugated bilirubin (UCB) with amorphous calcium phosphate was studied in vitro. To this end UCB, solubilized in different micellar bile salt solutions, was incubated with freshly prepared calcium phosphate precipitate. It was demonstrated that amorphous calcium phosphate (ACP) rapidly binds and precipitates UCB in a dose-dependent way. The results indicate that binding of UCB to ACP is specific: binding to barium phosphate was negligible and addition of low amounts of Mg^{2+} before formation of the calcium phosphate precipitate (Ca:Mg = 5:1) inhibited binding by 80%. Free Ca^{2+} stimulated binding, whereas free phosphate ions inhibited binding of UCB in taurocholate solutions and to a lesser extent in glycocholate solutions. The apparent affinity of UCB for amorphous calcium phosphate was different in the various bile salt solutions. Binding of UCB decreased at pH > 8.5 in taurocholate solutions, but not in glycocholate solutions where binding of UCB was constant from pH 7.5–10.5. We propose a model in which UCB directly binds to amorphous calcium phosphate in the presence of bile salts that weakly interact with ACP, like taurocholate. In the presence of bile salts that strongly interact with ACP, such as glycochenodeoxycholate, binding of UCB may also occur via the bile salt. In conditions of unconjugated hyperbilirubinemia, such as the Crigler-Najjar syndrome, neonatal jaundice, and in the Gunn rat, considerable amounts of UCB diffuse across the intestinal mucosa. Binding of UCB to calcium phosphate in the intestine may stimulate its excretion and thereby constitute a relevant mechanism of excretion.—**van der Veere, C. N., B. Schoemaker, R. van der Meer, A. K. Groen, P. L. M. Jansen, and R. P. J. Oude Elferink.** Rapid association of unconjugated bilirubin with amorphous calcium phosphate. *J. Lipid Res.* 1995. **36:** 1697–1707.

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Crigler-Najjar patients suffer from unconjugated hyperbilirubinemia, due to an inherited deficiency in bilirubin-UDP-glucuronosyltransferase. The liver is not able to glucuronidate unconjugated bilirubin and therefore UCB cannot be excreted in bile (1, 2). Bilirubin accumulates in blood and in tissues, which can lead to neurological damage, kernicterus, and death. Gunn rats have the same enzyme deficiency and are used as an animal model for Crigler-Najjar disease. Although in this disease bilirubin is not excreted in the normal way, serum bilirubin levels of both Gunn rats and Crigler-Najjar patients are relatively constant. This indicates that an equilibrium between production and excretion of bilirubin exists. Therefore, one or more alternative pathways for the removal of UCB must be present. A possible alternative metabolic route has been postulated (3, 4). In short, intact UCB is transferred directly across the mucosa into the intestinal lumen. The exact mechanism of this process is unknown but it could involve simple diffusion of the nonpolar lipid UCB. However, UCB is also efficiently reabsorbed by the intestine (5–8). Thus in this model the gut contains a bilirubin pool that equilibrates via the intestinal mucosa with the plasma pool. In line with this model is the observation that considerable amounts of unconjugated bilirubin are present in the intestine and feces of Gunn rats and Crigler-Najjar patients (9, 10). Increased binding of UCB in the lumen of the intestine would prevent reabsorption and might therefore reduce the plasma UCB pool and constitute a therapy for Crigler-Najjar patients.

Abbreviations: ACP, amorphous calcium phosphate; $[Ca]_p$, concentration of Ca^{2+} ions present as precipitate; HAP, calcium hydroxylapatite; UCB, unconjugated bilirubin; TC, taurocholate; TCDC, taurochenodeoxycholate; GC, glycocholate; GCDC, glycochenodeoxycholate; $B^{=}$, dianion of bilirubin; HB^{-}, monoanion of bilirubin; H_2B^0, protonated form of bilirubin; DMSO, dimethylsulfoxide; Tris, 2-amino-2-(hydroxymethyl)-1,3-propandiol; MOPS, 3-[N-morpholino]propanesulfonic acid; HEPES, 2-[4-(2-hydroxyethyl)-1-piperazyl]-ethanesulfonic acid; CMC, critical micellar concentration.

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[2]To whom correspondence should be addressed.

It is possible to decrease bilirubin levels in neonates (11) and in Gunn rats (12) by oral administration of activated charcoal. Because of its aspecific binding, which includes binding of essential food elements, activated charcoal is not suitable for prolonged administration. Binding of UCB to agar (11, 13) and to cholestyramine (14, 15), in vivo and in vitro, has been studied also, but conflicting conclusions regarding the effectiveness of those resins were obtained.

It has been shown that amphipathic anions like some bile salts and fatty acids are able to bind to insoluble calcium phosphate (16–19). Furthermore, UCB is present in pigment, as well as in cholesterol gallstones, and is often associated with ionized calcium, calcium carbonate, and calcium phosphate (20, 21).

In view of these considerations, we studied the association of UCB with calcium salts in different bile salt solutions. Our findings may explain the existence of the alternative, extrahepatic excretion pathway in hereditary unconjugated hyperbilirubinemia and may lead to a new therapy for Crigler-Najjar patients.

MATERIALS AND METHODS

Materials

Unconjugated bilirubin from bovine gallstones, taurochenodeoxycholate (TCDC; sodium salt), glycochenodeoxycholate (GCDC; sodium salt), and activated charcoal were purchased from Sigma Chemical Co. (St. Louis, MO). Taurocholate (TC; sodium salt) and glycocholate (GC) were obtained from Fluka Chemie AG (Buchs, Switzerland). Calcium hydroxylapatite was from Bio-Rad (Richmond, CA).

UCB was analyzed by HPLC (22) using a detection wavelength of 450 nm, and contained 89% bilirubin IXα and 11% isomers (IIIα and XIIIα). It was used without further purification. Bile acids were of the highest purity commercially available. 3α-Hydroxysteroid dehydrogenase was obtained from Worthington. Other chemicals were of analytical grade.

Methods

All procedures were performed under dim light. Micellar bile salt solutions were freshly prepared before each experiment in the following concentrations: TC and GC 40 mM, GCDC and TCDC 10 mM in bidistilled water; pH was adjusted to 7.4. UCB was dissolved in 0.1 M NaOH to a concentration of 0.01 M, and was subsequently added to a bile salt solution to a final concentration of 20 μM. Because of possible oxidation of UCB when solubilized in NaOH, the results obtained in this way were compared to results obtained when UCB was solubilized in DMSO. Spectrum analysis and λ_{max} of UCB in bile salt solutions was similar and binding of UCB to calcium phosphate was the same in both procedures. In both TC and GC solutions the spectrum showed a λ_{max} of 450 nm with a shoulder at 426 nm, which was similar to the spectra of 26 μM UCB in 57 mM TC as presented by Carey and Spivak (23). The shoulder at 426 nm was somewhat higher in GC than in TC solutions, which indicates that in GC solution more UCB is present as monomeric bilirubin and less as monomers in/on bile salt micelles as compared to TC solutions (23), although this is in contrast with the recent finding that binding of $B^{=}$ is greater to glyco- than to tauro-amidated bile salts (24).

Preformed washed calcium phosphate precipitates were prepared in the following way. Equimolar amounts of $CaCl_2$ and Na_2HPO_4 were mixed and washed twice with bidistilled water. In this way, an amorphous calcium phosphate is formed with an apparent stoichiometry of 3:2 (25) and excessive phosphate is removed.

One ml of the UCB/bile salt solution was added to a mixture containing Tris/MOPS (pH 7.4 except where stated otherwise), calcium phosphate precipitate, and NaCl which had been preincubated for 10 min. Final incubation volume was 2 ml. Final incubation concentrations were: 10 μM UCB, 100 mM Tris/MOPS, different amounts of calcium phosphate precipitate, and sufficient NaCl to maintain a constant ionic strength of 150 mM. Tubes were incubated for 15 min (unless stated otherwise) at 37°C and subsequently centrifuged for 2 min at 10,000 *g*. Association of UCB with calcium phosphate was determined by measuring the absorption of the supernatant at 450 nm. As a control, decrease of A_{450} was measured in identical experiments without phosphate and was less than 3%. Only in the binding experiments where the effect of pH was determined was the decrease of A_{450} higher at pH 7. Values were corrected for spontaneous decrease of absorbance which was probably due to precipitation of the diacid of UCB from the solution. Binding of UCB to calcium phosphate was calculated using the difference in A_{450} from solutions that had been incubated with and without calcium phosphate. The same results were obtained when the Tris/MOPS buffer was replaced by HEPES.

In some binding experiments incubation time or preincubation time was varied. Bile acids were measured according to Palmer (26). Calcium was determined using a colorimetric assay (27) or flame spectrophotometry. Phosphate was measured as described by Böttcher, Van Gent, and Pries (28).

RESULTS

Time-dependent association of UCB with calcium phosphate

Binding of unconjugated bilirubin (UCB) to calcium phosphate precipitate was tested by addition of a mixture of UCB solubilized in TC or GC to a solution containing precipitated calcium phosphate. Fresh precipitates of calcium and phosphate are amorphous (noncrystalline) and are further designated as ACP (amorphous calcium phosphate). The precipitates we used had a stoichiometry of 2.8:2 (Ca:P). The amount of added ACP is expressed as the concentration of Ca^{2+} ions present as precipitate and is designated as $[Ca]_p$. In both TC and GC solutions the absorption of the supernatant at 450 nm decreased immediately, indicating a rapid binding of UCB to calcium phosphate. Maximal binding was 90% (18 nmol) in GC and 65% (13 nmol) in TC solutions. In control experiments (without calcium phosphate) decrease in A_{450} was 2% in 5 min and this remained constant throughout the experiment (**Fig. 1A**).

This method to measure association of UCB to calcium phosphate was compared with HPLC analysis of the supernatant (22). HPLC analysis showed that during the experiments isomers of bilirubin IXα are formed (± 7% of total bilirubin) and these isomers (IIIα and XIIIα) associate with calcium phosphate more quantitatively than IXα. Results in terms of total bilirubin binding were the same with both methods, but 79 ± 1% of total binding is due to binding of bilirubin IXα per se, whereas 21 ± 1% is due to binding of isomers (not shown).

According to Qiu et al. (25) transformation of ACP [$Ca_3(PO_4)_2 \cdot XH_2O$] to calcium hydroxylapatite [HAP; $Ca_{10}(OH)_2(PO_4)_6$] starts approximately 1 h after mixing at 37°C. In the experiment of Fig. 1B, 5 mM $[Ca]_p$ was preincubated at 37°C for 15 min and 24 h, respectively. UCB in bile salt solution was added followed by another 15-min incubation at 37°C. Binding of UCB to 1-day old calcium phosphate precipitate was decreased from 13 to 6.8 nmol in GC and from 7.8 to 4 nmol in TC solutions.

Association of UCB with mineral phases

To assess the specificity of the association of UCB with calcium phosphate, binding of UCB to various preformed precipitates was measured by addition of 1 ml 20 μM UCB solubilized in 40 mM TC to 1 ml of a solution containing one of the following precipitates: ACP (20 mM $[Ca]_p$), calcium hydroxylapatite [$Ca_{10}(OH)_2(PO_4)_6$]; 20 mM $[Ca]_p$, barium phosphate (20 mM $BaHPO_4$, freshly precipitated from $BaCl_2$ and Na_2HPO_4), or a mixture of calcium and magnesium phosphate (20 mM $CaCl_2$, 5.6 mM $MgCl_2$, and 8 mM Na_2HPO_4). As shown

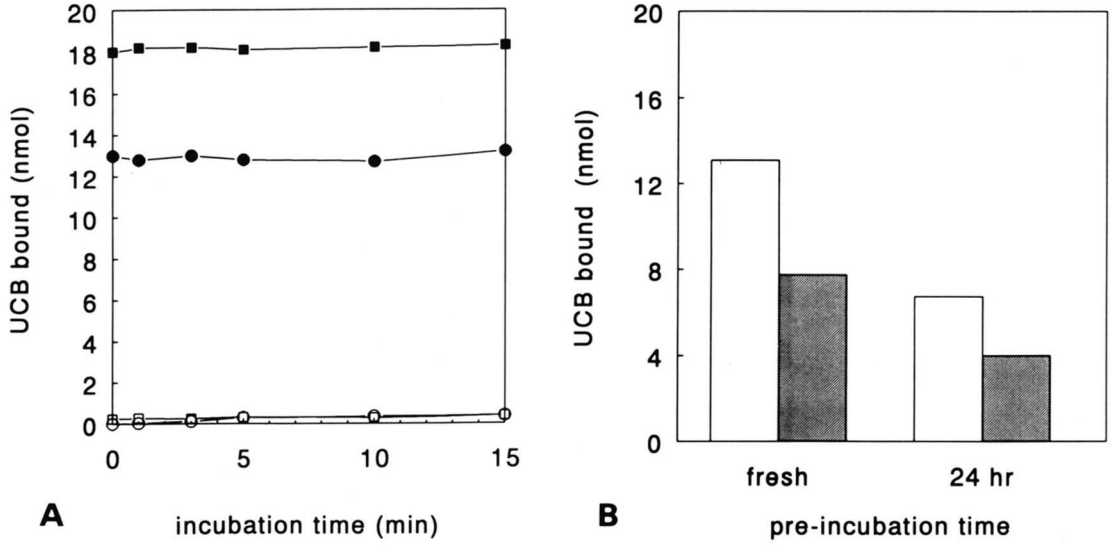

Fig. 1. Time dependent association of UCB to calcium phosphate. A: Effect of incubation time on binding. UCB was added to a solution that contained preformed, washed ACP precipitate. Final incubation concentrations were: 10 μM UCB, 20 mM bile salt (glycocholate; GC or taurocholate; TC), 100 mM Tris/MOPS (pH 7.4), 20 mM $[Ca]_p$ and NaCl to maintain an ionic strength of 150 mM. Tubes were incubated for different time spans at 37°C and subsequently centrifuged for 2 min at 10,000 *g*. Association of UCB with calcium phosphate was determined by measuring the absorption of the supernatant at 450 nm. ■, □: UCB/GC; ●, ○: UCB/TC. Solid: with calcium phosphate. Open: without calcium phosphate (control). Values are means ± SD, n = 3. SDs are smaller than the size of the symbols. B: Effect of preincubation time on binding. $CaCl_2$ and Na_2HPO_4 were mixed, washed, and preincubated at 37°C for 15 min or 24 h. After addition of UCB/bile salt solutions to final concentrations of 10 μM and 20 mM, respectively, tubes were incubated for 15 min at 37°C and subsequently centrifuged for 2 min at 10,000 *g*. Final concentration of $[Ca]_p$ was 5 mM. Open bars: UCB/GC. Filled bars: UCB/TC.

in **Fig. 2A,** substantial binding of UCB only occurred to amorphous calcium phosphate. When Ca^{2+} was replaced by another divalent cation (Ba^{2+}) or when calcium phosphate was formed in the presence of $MgCl_2$, binding of UCB was hardly observed. Addition of Mg^{2+} to the incubation after formation of the calcium phosphate precipitate had no effect on the binding of UCB. Binding to commercial calcium hydroxylapatite was only 3.2 nmol (16%).

To elucidate the mechanism by which magnesium inhibits the binding of bilirubin to the calcium phosphate precipitate, a titration experiment was performed in which 0, 2, 4, or 6 mM $MgCl_2$ was present before 20 mM $CaCl_2$ and 8 mM Na_2HPO_4 were added to the tubes; subsequently the buffers and UCB/TC solution were added. Calcium, phosphate, and magnesium were analyzed in the supernatants as well as in the precipitate at the end of the binding experiment. Two mM $MgCl_2$ was enough to inhibit binding of bilirubin by 60% and both 4 and 6 mM $MgCl_2$ inhibited binding by 80% (Fig. 2B). Analysis of the precipitates and supernatants revealed that magnesium was hardly incorporated into the precipitate, but the presence of magnesium changed the stoichiometry of calcium and phosphate in the mineral phase. Without Mg^{2+}, the Ca:P ratio of the precipitate was 2.7:2, so ACP was formed. In the presence of 2 mM Mg^{2+}, Ca:P:Mg was 3.4:2:0.14, whereas in the presence of 4 or 6 mM Mg^{2+}, Ca:P was 2:2 and Mg was 0.13 and 0.17, respectively. Binding of UCB was not related to concentrations of calcium, phosphate, or magnesium in the supernatant. Thus, in the presence of a limited amount of magnesium (Ca to Mg ratio 5:1) instead of amorphous calcium phosphate, another precipitate is formed (probably $CaHPO_4$) and as a consequence binding of UCB is inhibited. Therefore binding of UCB seems to be specific for ACP.

Ionized calcium is known to induce auto-oxidation of bilirubin (29), which also leads to a decrease in A_{450} and therefore could influence the results. This was examined by omitting the phosphate while 20 mM $CaCl_2$ was present in the incubations. Decrease in A_{450} was less than 3% under these circumstances, indicating that within the time frame of these experiments no oxidation or precipitation of UCB had occurred (Fig. 2A, last bar). Also, spectrum analyses of UCB in TC or GC solutions were performed in the presence of 0 or 5 mM Ca^{2+}. Spectra taken at the end of the incubation period were identical in solutions with and without Ca^{2+} (not shown).

Influence of the type of bile salt on binding of UCB to calcium phosphate

We studied the association of UCB with calcium phosphate in different bile salt solutions, because it is known that some bile salts also bind to insoluble calcium phosphate (16) and this may facilitate as well as interfere with the binding of UCB to calcium phosphate. In these experiments, besides TC and GC, micellar solutions of TCDC and GCDC were used to solubilize UCB. For TCDC and GCDC lower concentrations were used because these bile salts have lower CMCs. More UCB was

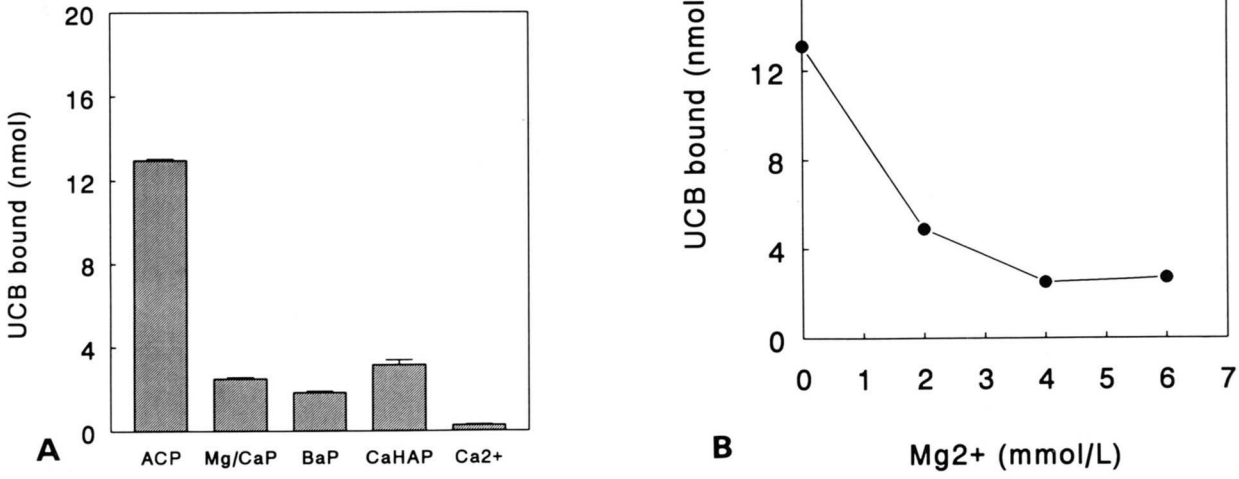

Fig. 2. A: Association of UCB with various mineral phases. Ten μM UCB and 20 mM TC (final) were incubated for 15 min with the following precipitates (from left to right): amorphous calcium phosphate (20 mM), magnesium/calcium phosphate (20 mM Ca^{2+}, 5.6 mM Mg^{2+}), barium phosphate (20 mM), calcium hydroxylapatite (20 mM). Control: 20 mM $CaCl_2$ (no precipitate formed). B: Influence of Mg^{2+} on the binding of UCB to calcium phosphate. Twenty mM $CaCl_2$ and 8 mM Na_2HPO_4 were added to tubes containing 0, 2, 4, or 6 mM $MgCl_2$; subsequently 10 μM UCB, 20 mM TC (final concentrations) were added. Total volume 2 ml, pH 7.4.

bound with increasing amounts of calcium phosphate, but the affinity varied in the different bile salt solutions. Under the given conditions (TC and GC: 20 mM; TCDC and GCDC: 5 mM) half-maximal binding of UCB was reached at 4, 3, 2, and 4 mM $[Ca]_p$ for incubations in TC, GC, TCDC, and GCDC, respectively (**Fig. 3A**). For purposes of comparison, binding of UCB solubilized in 20 mM TCDC and GCDC is also shown (Fig. 3A). It appears that when UCB is solubilized in TC, a fraction of the UCB does not bind to ACP, even though more binding sites are added. To further analyze this phenomenon, titration experiments were performed using 20 mM TC or GC and increasing amounts of UCB. In GC solutions, virtually all UCB binds over a concentration range of 0–20 μM UCB to 20 mM $[Ca]_p$. In TC solutions, binding of UCB is linear but submaximal over the whole concentration range. Furthermore, when 5 mM instead of 20 mM $[Ca]_p$ is used in the UCB/GC solution, binding of UCB is also submaximal over the whole concentration range (Fig. 3B). Thus the absolute amount of bound bilirubin increases when more bilirubin is added, but a balance exists between the affinity of the bile salt for UCB and the affinity of calcium phosphate for UCB.

Binding of bile salts to calcium phosphate was also measured. The binding affinity of bile salts for calcium phosphate had the following order: GCDC > GC > TCDC > TC (Fig. 3A). The amount of bound TC shown is around the limit of detection. Evidently much more bile salt than UCB is bound to ACP. Therefore an additional experiment was performed. UCB was solubilized in DMSO in the absence of bile salts. Binding of UCB to ACP was analyzed in two ways. A: one ml 20 μM UCB in DMSO was added to a 1-ml solution containing different amounts of ACP, Tris/MOPS (final: 100 mM) and sufficient NaCl to obtain a final ionic strength of 0.15. In these experiments the DMSO to water ratio was 1:1. B: one ml 20 μM UCB in DMSO was added to a solution containing different amounts of ACP. NaCl and buffer were replaced by DMSO to reach a DMSO to water ratio of 4:1. In both experiments binding of UCB to ACP was present and dose-dependent. Binding of UCB was higher in the experiments with a DMSO to water ratio of 4:1 (**Fig. 4**).

It has been shown that bilirubin levels in neonates (11) and in Gunn rats (12) decrease by oral administration of activated charcoal. Therefore the in vitro association of UCB/TC to activated charcoal was measured to determine whether the substantial binding of bilirubin by calcium phosphate may play a possible physiological role.

Half-maximal binding of UCB was reached at 0.6 mg/ml (**Fig. 5**). Thus, since 4 mM $[Ca]_p$ is equivalent to 1.24 mg/ml, under these conditions the affinity of UCB for activated charcoal and for calcium phosphate is in the same order of magnitude when calculated on weight basis.

Effects of ionized calcium and phosphate on the association of UCB with calcium phosphate

The effect of ionized calcium was investigated by adding $CaCl_2$ in a concentration range of 0–5 mM to preformed calcium phosphate precipitate. In order to assess the effect of free Ca^{2+} in this experiment, in GC solutions 5 mM $[Ca]_p$ was used whereas in TC solutions 20 mM $[Ca]_p$ was used and mixed with 10 μM UCB (final) which leads to submaximal binding of UCB. Free Ca^{2+} stimulated the binding of UCB to calcium phosphate in a dose-dependent way (**Fig. 6**). As mentioned before, the concentrations of free calcium used in these experiments did not cause measurable decrease in A_{450} in control experiments, which indicates that auto-oxidation of bilirubin did not occur within the experimental time interval.

Second, the effect of phosphate was examined by keeping the $CaCl_2$ concentration at 20 mM, while Na_2HPO_4 was added in concentrations ranging from 0–30 mM. Mixtures of $CaCl_2$, Na_2HPO_4, NaCl, and buffer were allowed to form precipitates for 10 min before the UCB/bile salt solution was added. UCB did not precipitate when the phosphate concentration was zero, i.e., in the absence of a mineral phase. Binding of UCB occurred as soon as phosphate had been added to the incubation and increased until a phosphate concentration of 15 mM (in the presence of GC) or 10 mM (in the presence of TC) was reached (**Fig. 7** panel A). At these concentrations, the free phosphate concentration in the supernatant starts to rise because the ACP has a stoichiometry of Ca:P = 3:2. In panel B (Fig. 7) binding of UCB is plotted against the measured free phosphate concentration in the supernatant. Higher phosphate concentrations inhibited binding of UCB markedly in incubations with TC, but hardly in GC (Fig. 7).

Effect of pH on binding of UCB to calcium phosphate

pH-dependent binding was investigated in order to gain more insight into the mechanism by which bilirubin binds to calcium phosphate. In this experiment 5 mM $[Ca]_p$ was used in order to obtain submaximal binding of UCB.

In GC solutions, binding of UCB was present over a pH range of 7.5–10.5 whereas in TC solutions, binding was maximal in the pH range 7.5–8.5 and was inhibited at a pH > 9 (**Fig. 8**). At a pH < 7.5, binding decreased in both solutions but nonspecific decrease in A_{450} started to occur. We performed spectrum analysis of supernatants from solutions with and without calcium phosphate. The decrease in absorbance of UCB (A_{450}) at

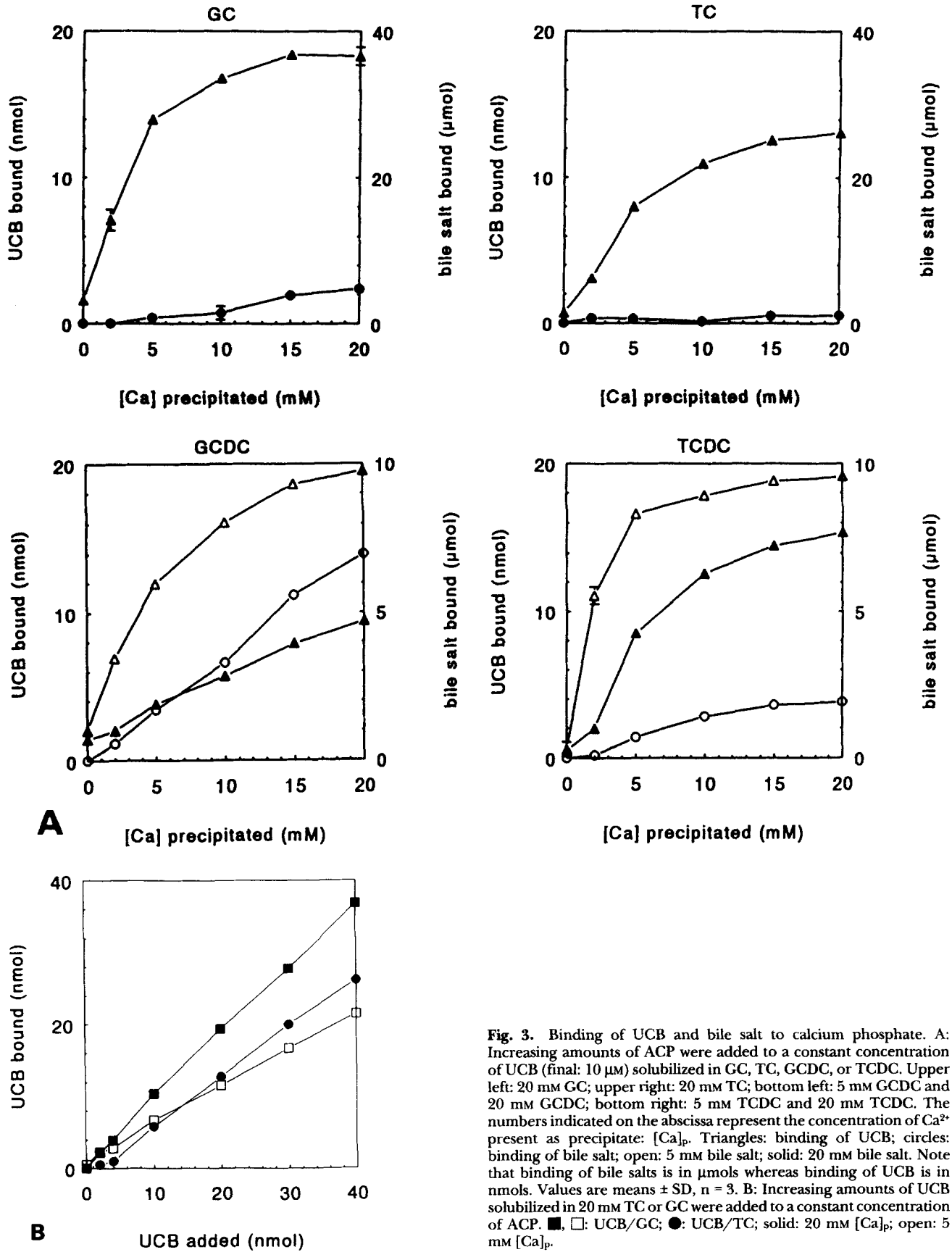

Fig. 3. Binding of UCB and bile salt to calcium phosphate. A: Increasing amounts of ACP were added to a constant concentration of UCB (final: 10 μM) solubilized in GC, TC, GCDC, or TCDC. Upper left: 20 mM GC; upper right: 20 mM TC; bottom left: 5 mM GCDC and 20 mM GCDC; bottom right: 5 mM TCDC and 20 mM TCDC. The numbers indicated on the abscissa represent the concentration of Ca^{2+} present as precipitate: $[Ca]_p$. Triangles: binding of UCB; circles: binding of bile salt; open: 5 mM bile salt; solid: 20 mM bile salt. Note that binding of bile salts is in μmols whereas binding of UCB is in nmols. Values are means ± SD, n = 3. B: Increasing amounts of UCB solubilized in 20 mM TC or GC were added to a constant concentration of ACP. ■, □: UCB/GC; ●: UCB/TC; solid: 20 mM $[Ca]_p$; open: 5 mM $[Ca]_p$.

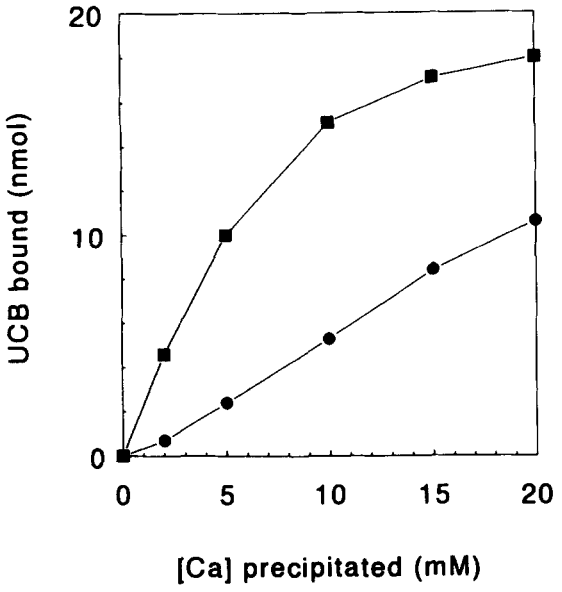

Fig. 4. Binding of 20 μM UCB/DMSO to different amounts of ACP. ●: DMSO–water 1:1. One ml of 20 μM UCB in DMSO was added to a 1 ml solution containing different amounts of ACP, Tris/MOPS (final: 100 mM) and sufficient NaCl to obtain a final ionic strength of 0.15. pH 7.4. ■: DMSO–water 4:1. One ml 20 μM UCB in DMSO was added to a solution containing different amounts of ACP. NaCl and buffer were replaced by DMSO to reach a DMSO–water ratio of 4:1.

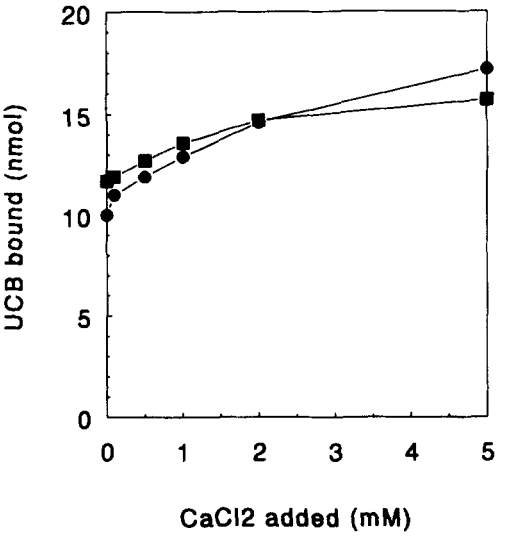

Fig. 6. Effect of free Ca^{2+} on binding of UCB to calcium phosphate. Additional $CaCl_2$ was added to washed calcium phosphate precipitate (ACP) in a concentration range of 0–5 mM. UCB/bile salt solutions were added and binding of UCB was determined, pH 7.4. ● Final: 10 μM UCB, 20 mM TC, 20 mM $[Ca]_p$; ■ final: 10 μM UCB, 20 mM GC, 5 mM $[Ca]_p$.

lower pH (6.5 and 7.0) was not accompanied by an increase at lower wavelength. Instead absorption was decreased over the whole range 380–500 nm. Therefore self-aggregation of UCB could not be demonstrated and the decrease of A_{450} was probably due to precipitation of the bilirubin diacid from the solution.

Calcium and phosphate concentrations in the supernatants were measured and decreased with increasing pH as expected (not shown).

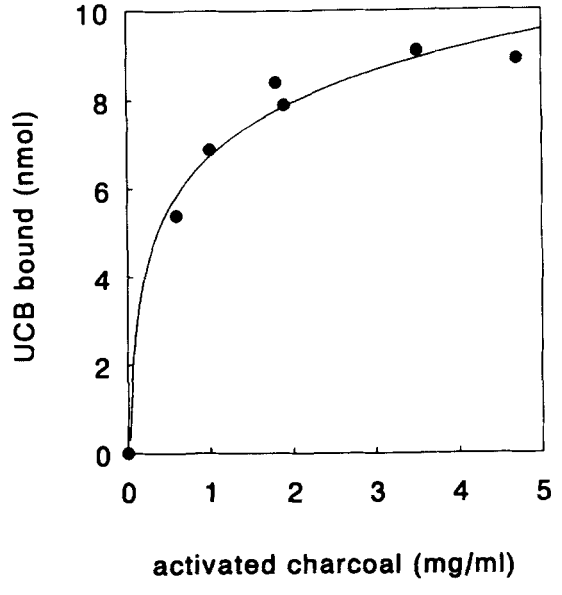

Fig. 5. Binding of UCB to activated charcoal. One ml 10 μM UCB solubilized in 40 mM TC was added to different amounts of activated charcoal.

DISCUSSION

Unconjugated bilirubin rapidly binds to amorphous calcium phosphate (ACP). The observed precipitation of UCB was not due to precipitation of UCB with free calcium ions; in control incubations where phosphate was omitted, free calcium did not induce UCB precipitation. Although precipitation of UCB with Ca^{2+} is a well-known phenomenon, the time frame in which this occurs is much slower than what we observed here. In addition, in the presence of free calcium the decrease in A_{450} was less than 3%, which proves that oxidation of UCB to non-absorbing products was negligible within the experimental time frame.

When calcium was replaced by another divalent cation (Ba^{2+}) binding did not occur. This experiment represents an important control because, similar to Ca^{2+}, Ba^{2+} forms an insoluble salt with UCB. The absence of a decrease in A_{450} in the presence of the barium salt is strong evidence against the possibility that UCB precipi-

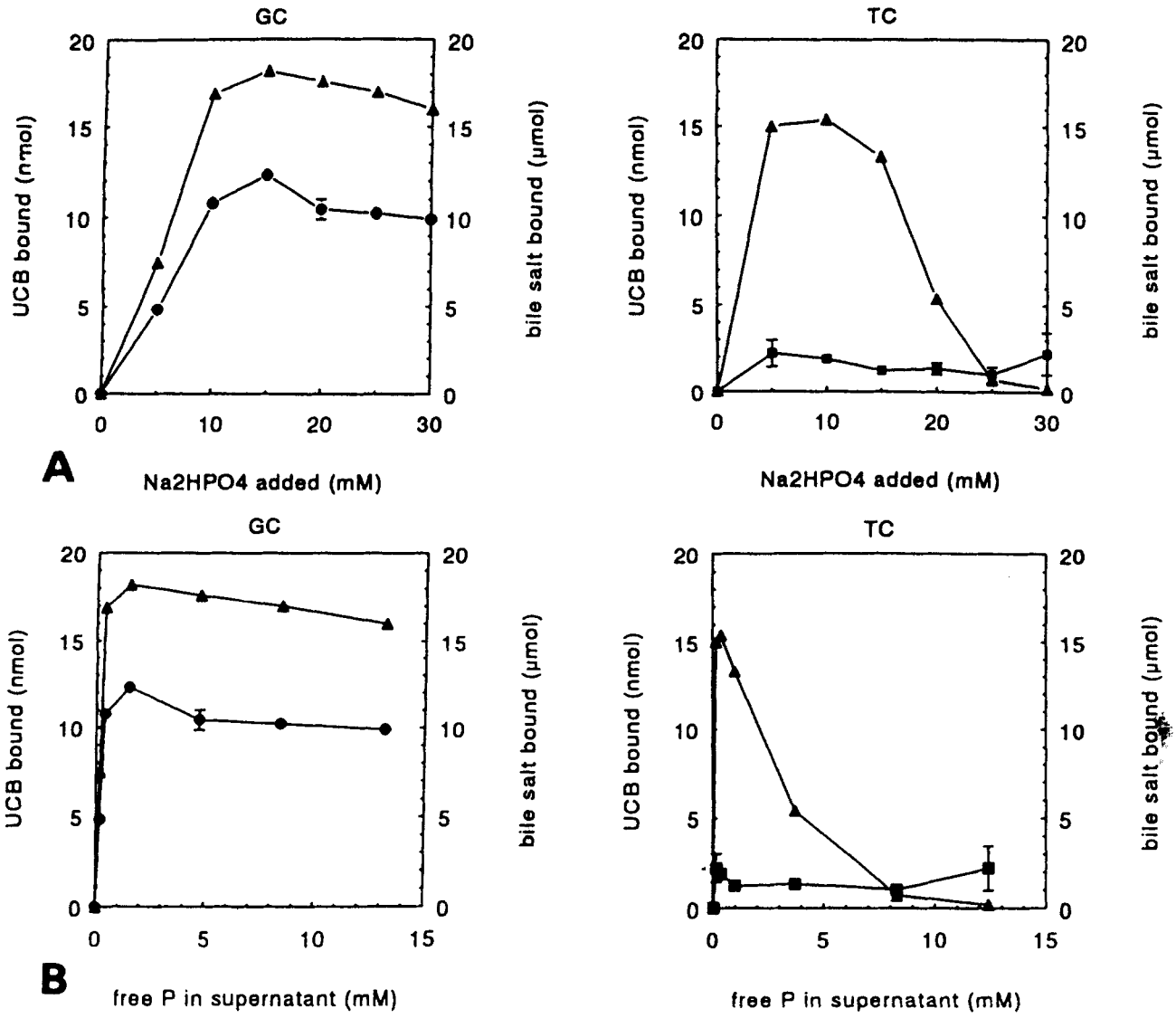

Fig. 7. Effect of free HPO_4^{2-} on binding of UCB to calcium phosphate. Na_2HPO_4 was added in concentrations ranging from 0–30 mM to a solution in which the $CaCl_2$ concentration was constant (20 mM) at pH 7.4. After preincubation for 10 min UCB/bile salt solution was added and binding of UCB to the precipitate was measured. Left: 10 μM UCB/20 mM GC; right: 10 μM UCB/20 mM TC; ▲: binding of UCB; ●, ■: binding of bile salt. Note that binding of bile salts is in μmols whereas binding of UCB is in nmols. Panel A: binding of UCB plotted against *added* Na_2HPO_4 (i.e., total P_i). Panel B: binding of UCB plotted against *measured* phosphate concentration in supernatant (i.e., free P_i). Values are means ± SD, n = 3.

tates with either of these cations within the experimental time frame. This experiment also demonstrates that seeding the solution with a mineral phase other than ACP does not lead to rapid precipitation of UCB from the metastable solution. The latter possibility is also excluded by the fact that binding of UCB to ACP also occurred in DMSO, which does not form a supersaturated solution with UCB.

Addition of Mg^{2+} before formation of the calcium phosphate precipitate (Ca:Mg = 5:1) inhibited binding of UCB by 80%. Analysis of the precipitate revealed that in this circumstance no amorphous calcium phosphate is formed, but rather a calcium phosphate precipitate with a stoichiometry of 1:1. Amorphous calcium phosphate probably forms a lattice structure to which bilirubin can bind. The structure of the calcium phosphate precipitate strongly influences bilirubin binding. One-day-old calcium phosphate precipitate, which consists mainly of calcium hydroxylapatite (25), binds UCB much less, and commercially available hydroxylapatite also shows little affinity for UCB. The difference in binding capacity between ACP and hydroxylapatite has

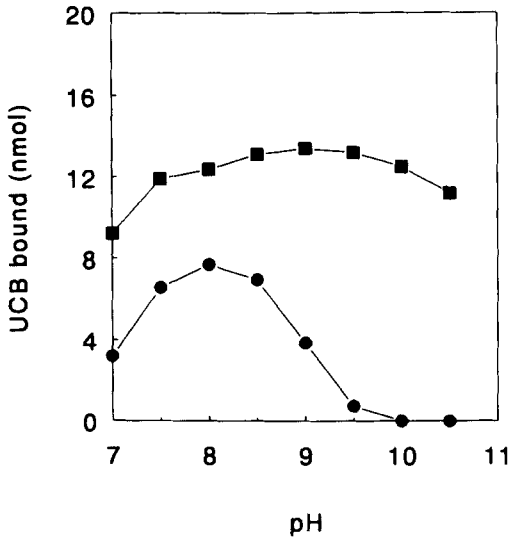

Fig. 8. pH-dependent binding of UCB to calcium phosphate. UCB was added to a solution that contained preformed, washed ACP precipitate. Final incubation concentrations were: 10 μM UCB, 20 mM bile salt, 100 mM Tris/MOPS (pH range from 7 to 10.5), 5 mM $[Ca]_p$ and NaCl to maintain an ionic strength of 150 mM. Tubes were incubated at 37°C and binding of UCB with calcium phosphate was measured. ■: UCB/GC; ●: UCB/TC. Values are means ± SD, n = 3.

also been found for bile salts (30) and may reflect differences in the surface to volume ratio or differences in the spacing of Ca^{2+} and HPO_4^{2-} on the surface of these two materials (31).

The exact mechanism by which unconjugated bilirubin associates with calcium phosphate is unclear. Several models can be formulated. *1)* Precipitation of UCB is secondary to binding of bile salts to calcium phosphate. As discussed above, bile salts are able to bind to amorphous calcium phosphate. Possibly UCB precipitates from the bile salt solution as a result of the decreased bile salt concentration in solution. In this case UCB precipitates due to self-aggregation.

2) UCB and bile salts bind together. UCB is solubilized in micellar bile salt solutions in two ways: part of it is present as monomer between bile salt micelles and part of it is associated as monomer in or on the bile salt micelle (23). In an elegant study, it was recently demonstrated that binding of bile salt to calcium phosphate does not occur via micelles, as was previously proposed (25, 32), but as monomers (30). The proposed mechanism of binding involves ionic adsorption of the negatively charged carboxylic headgroup of the bile acid to calcium ions on the surface of the crystal, which leads to exposure of the hydrophobic side of the steroid nucleus of the bile acid. Subsequently, other hydrophobic ligands such as additional bile salt molecules or UCB can bind ("piggy-back binding"). In this model binding of bile salt is a prerequisite for binding of UCB.

3) Direct binding of UCB to ACP by ionic adsorption of monomers or dimers of UCB. The hypothesis about the mechanism by which bile salts bind to calcium phosphate may also hold for UCB: the target for binding may be calcium ions incorporated in a crystal. Maybe the anionic groups of UCB are optimally aligned to interact preferentially with the calcium ions in this specific calcium phosphate precipitate.

As we demonstrated that UCB can bind to ACP in the absence of bile salts (Fig. 4), we believe that the third mechanism is certainly possible. Furthermore, binding of UCB did not correlate with bile salt binding: binding of the bile salts strongly depend on their hydrophobicity and conjugation whereas binding of UCB varies less than twofold in the various bile salt solutions. The binding affinity of the bile salts for calcium phosphate had the following order: GCDC > GC > TCDC > TC, which is in accordance with the findings of van der Meer and De Vries (16) who demonstrated that glycine-conjugated bile salts bind better to calcium phosphate than taurine-conjugates and hydrophobic bile salts bind better than hydrophilic bile salts (18, 33). This indicates that binding of UCB to calcium phosphate is not necessarily dependent on, or secondary to, binding of bile salts to calcium phosphate. The apparent affinity of calcium phosphate for UCB in the various bile salt solutions probably reflects the balance between the affinity of the bile salt for UCB and the affinity of calcium phosphate for UCB. Therefore, the first model is excluded.

Interestingly, in incubations with taurocholate, binding of UCB was negatively influenced by increasing free phosphate concentrations, which suggests that UCB binding is electrostatic. This was, however, not observed when UCB was dissolved in glycocholate. Similarly, UCB binding strongly decreased at pH > 8.5 in taurocholate, but not in glycocholate solutions. These discrepancies suggest that binding of UCB in the presence of glycocholate involves another mechanism than that in the presence of taurocholate. Govers et al. (30) showed that under the conditions we used, in experiments with glycocholate, the ACP is fully saturated with this bile salt. It may, therefore, be that the glycocholate-covered precipitate represents an amphipathic surface to which UCB can bind. This differs from the situation with taurocholate where the ACP surface is not fully covered by bile salt and UCB can bind via electrostatic interaction. These distinct binding mechanisms may explain the different effects of free phosphate and pH on binding of bilirubin to ACP in the two bile salt solutions.

Studies on the binding of unconjugated bilirubin to other compounds are complicated by the relative insolubility of UCB in water. Controversy exists about the pKs

of bilirubin, and therefore about the solubility of UCB. According to multiple studies (23) pK_1 (H_2O) is ± 4.3–4.5 and pK_2 (H_2O) is ± 5.3–5.9, and the first pK may even be more acidic. On the other hand it was postulated recently that the pKs of UCB in 150 mM NaCl are 8.0 and 8.4, respectively (34), whereas the mean apparent overall pK_a value for UCB in 50 mM TC was found to be about 7.0 (35). In the pH-dependent binding experiments it was shown that UCB solubilized in GC was able to bind over a pH range of 7.5–10.5, possibly due to "piggy-back" binding as discussed above. Binding of UCB in TC solutions was inhibited at pH > 9. This may be caused by the fact that the surface of the ACP will change as the pH increases. It will become more negatively charged and will tend to repel UCB. Another possibility is that calcium phosphate might have little affinity for the dianion since at pH > 9 all UCB is present as $B^{=}$. However, when bilirubin is solubilized in DMSO, the internal hydrogen bonds are broken (36) and then both pK_a values for UCB are around 5. We have shown that in these circumstances bilirubin does bind to ACP. Therefore it remains unclear whether the monoanion or the dianion of bilirubin is preferentially bound to calcium phosphate. Furthermore, the affinity of bile salts for each ionic species of UCB differs so that UCB-bile salt interactions may vary with pH (35, 37) and this might influence the binding of UCB to ACP.

Equilibrium pH values for bilirubin precipitation are 7.9 in 28.6 mM TC and 7.5 in 57.2 mM TC. At the equilibrium precipitation pH values, bilirubin solubilities are about 60 μM in 57.2 mM TC (23). We used solutions of 10 μM UCB in 20 mM TC or GC, and a pH of 7.4. These solutions are supersaturated with bilirubin and calcium bilirubinate, and therefore capable of precipitating. However, in view of the above and the fact that within the experimental time interval decrease of A_{450} was less than 3%, we conclude that the solutions we used were metastable and decrease in A_{450} was not due to precipitation or selfaggregation of UCB.

Although commercially available bilirubin was used which contained 89% bilirubin IXα and 11% isomers (IIIα and XIIIα), in our opinion this does not hamper the results. As was mentioned in the Result section, isomers of bilirubin IXα are also formed during the experiment which will influence the data even when purified bilirubin is used. However, it should be noted that 20% of total binding of UCB to calcium phosphate is due to binding of isomers, whereas 80% is due to binding of bilirubin IXα. We have not chosen to further purify the commercial bilirubin. Therefore the preparation may contain traces of surface active degradation products that do not absorb at 450 nm. The amounts of these compounds are, however, much too small to explain the characteristics of bulk binding of UCB to ACP.

In conclusion, unconjugated bilirubin rapidly associates with calcium phosphate precipitate in vitro. The affinity of UCB for activated charcoal is found to be in the same order of magnitude as the affinity for calcium phosphate. Oral administration of activated charcoal has been used to decrease bilirubin levels in neonates (11) and in Gunn rats (12). Binding of UCB to precipitated calcium phosphate in the intestine might explain the mechanism of extrahepatic disposition of UCB in unconjugated hyperbilirubinemia since the normal diet also contains calcium phosphate. Oral calcium phosphate supplementation may provide a new therapy for Crigler-Najjar patients and other conditions of unconjugated hyperbilirubinemia such as neonatal jaundice. In order to test this hypothesis we are currently conducting in vivo studies.

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