Differential binding of glycine- and taurine-conjugated bile acids to insoluble calcium phosphate

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It is demonstrated that bile acids bind to insoluble calcium phosphate at pH values beyond 5.5. Significant binding occurs with glycine-conjugated dihydroxy bile acids. Results indicate that these bile acids are bound in a micellar mode. Taurine conjugation almost completely inhibits the binding of these bile acids to insoluble calcium phosphate. Since glycine-conjugated dihydroxy bile acids are predominant in the rabbit, but not in the rat, our results suggest an explanation for the intriguing species-dependence of casein-induced hypercholesterolaemia, which is high in the rabbit but absent in the rat.

In several animal species the serum cholesterol concentration can be controlled by the enterohepatic cycle of bile acids [see Packard & Shepherd (1982) for review]. It has been hypothesized (Van der Meer, 1983) that dietary casein interferes with this cycle by means of its phosphorylation state. The hypothesis is that, in the small-intestinal lumen, bile acids can bind to insoluble calcium phosphate, and that this binding is competitively inhibited by dietary casein and phosphopeptides derived from it. Thus dietary casein would increase the free concentration of bile acids and consequently stimulate the re-absorption of sterols from the gut, resulting in an increase in serum cholesterol concentration. Studies *in vitro* using casein and dephosphorylated casein have shown that this mechanism is indeed dependent on the phosphorylation state of casein (Van der Meer, 1983). Moreover, feeding experiments with rabbits have shown that casein-induced hypercholesterolaemia is Ca^{2+} -dependent, as predicted by this hypothesis (Van der Meer *et al.*, 1985). However, whether this hypothesis is compatible with the intriguing species-dependence of the casein response remains to be established. As reviewed by West *et al.* (1983), dietary casein very significantly increases the serum cholesterol concentration in rabbits, but not in rats. The question arises whether this difference in response is due to the well-known species-dependent difference in glycine/taurine

conjugation of bile acids (see, e.g., Coleman *et al.*, 1979; Kempen *et al.*, 1982). In the present study we deal with this question by studying, under near-physiological conditions, the binding of glycine- and taurine-conjugated bile acid to insoluble calcium phosphate.

Experimental

Binding of bile acids to insoluble calcium phosphate was studied as follows. To equimolar solutions (pH 2.0) of calcium and phosphate in Eppendorf tubes, buffer solutions (final concn. 100 mM) were added to reach the desired pH values. Mes was used as a buffer for pH values below 6.5, whereas Mops was used in the pH range 6–8. NaCl was added in order to maintain a constant physiological ionic strength at 150 mM. After 5 min preincubation at 37°C, during which insoluble calcium phosphate was formed, bile acids were added at concentrations indicated in the Figures. The final incubation volume was 1.0 ml, and the pH was measured. After 10 min incubation the tubes were centrifuged for 2 min at 10000g and the supernatants aspirated. It should be pointed out that increasing the preincubation time to 20 min and varying the incubation time between 15 s and 20 min did not affect the results.

Samples of the supernatants from solutions that had been incubated with and without calcium phosphate were analysed for bile acid concentration by an enzymic method (Turkey & Dietschy, 1978), and binding to the sediment was calculated

Abbreviation used: CMC, critical micellar concentration.

by difference. In control experiments, bile acids were also measured in the pellet fraction after solubilization with EDTA. This procedure yielded binding data identical with those obtained by the difference method, which is used routinely. After both pellets and supernatants had been acidified with trichloroacetic acid (final concn. 5%, w/v), calcium was measured in an atomic-absorption spectrophotometer (Perkin-Elmer 503) and phosphate was measured as described by Fiske & SubbaRow (1925).

3 α -Hydroxysteroid dehydrogenase was obtained from Worthington (cat. STDHMP) or from Sigma (cat. H 1506). Bile acids were acquired from Calbiochem and from Sigma and were of the highest purity commercially available. Other chemicals were of analytical grade.

Results

In order to simulate gastro-intestinal conditions, calcium phosphate solutions (pH 2.0) were adjusted to pH values covering the range 5.5–8, which is that reported for the small-intestinal lumen (Carey *et al.*, 1983). Fig 1(a) shows that calcium phosphate starts precipitating at pH 5.6, in accordance with the known pH-dependence of the solubility product of calcium and phosphate (Moreno *et al.*, 1966). Up to pH 7, the amounts of calcium and P_i in the pellet increase, but at higher pH they practically remain constant. In Fig. 1(b) the pH-dependence of the binding of glycodeoxycholate and of the binding of taurodeoxycholate is given. Binding occurs at pH values higher than 5.5, and has an optimum at about pH 6.1. Comparison of Fig. 1(b) with Fig. 1(a) shows that the binding coincides with the formation of insoluble calcium phosphate and cannot therefore be attributed to precipitation of bile acids by Ca^{2+} (see also the Discussion section). Because of the great difference in binding between the glycine and taurine conjugates of deoxycholate, as given in Fig. 1(b), the structural requirements for the binding of bile acids were examined in more detail. As shown in Fig. 2(a), very significant binding occurs with glycine-conjugated dihydroxy bile acids, whereas glycocholate binds to a much smaller extent. In contrast, taurine conjugation (Fig. 2b) almost completely prevents the binding of bile acids to the sediment. No difference is observed in this respect between dihydroxy and trihydroxy bile acids. The difference in binding between glycine and taurine conjugates is not due to the presence of sulphonic acid buffers, since a similar difference was observed with imidazole as a buffer (results not shown).

The difference in binding between the glycine-conjugated dihydroxy and trihydroxy bile acids is

Fig. 1. pH-dependence of the binding of bile acids to insoluble calcium phosphate

In (a) the concentrations of insoluble calcium (■) and phosphate (□) are given; the total calcium and P_i concentration was 20 mM. In (b) the binding of 3.4 mM-glycodeoxycholate (●) and of 3.3 mM-taurodeoxycholate (○) is shown. The means for three different experiments are given; the bars indicate S.D.

probably due to these dihydroxy bile acids having a lower apparent CMC than the trihydroxy bile acids (Small, 1971; Roda *et al.*, 1983). This would imply that micelles of dihydroxy bile acids bind to the calcium phosphate sediment, whereas monomolecular bile acids remain in the supernatant. To examine this point further, binding was studied at increasing concentrations of calcium phosphate; a typical experiment is shown in Fig. 3. On introduction of increasing amounts of binding sites, bile acid disappears from the supernatant until a minimum concentration is reached. As shown in Fig. 3, this minimum is independent of the total concentration of glycodeoxycholate, which indicates that micelles are bound and that

Fig. 2. Concentration-dependence of the binding of bile acids to insoluble calcium phosphate. The binding of glycine (a) and taurine (b) conjugates of deoxycholate (●), chenodeoxycholate (○) and cholate (△) was measured under standard conditions at a total concentration of calcium and P_i of 20 mM and pH 6.9–7.0. Means for three to five experiments are given; the bars indicate S.D.

Fig. 3. Effect of calcium phosphate on the concentration of glycodeoxycholate in the supernatant

Results are given of a representative experiment at pH 7.0 and with two total concentrations of glycodeoxycholate, 1.65 mM (○) and 3.3 mM (●) respectively.

no binding occurs below the apparent CMC. These results suggest an apparent CMC of 0.84 ± 0.02 mM ($n = 3$), which is of the same order of magnitude as that reported elsewhere (see, e.g., Small, 1971; Roda *et al.*, 1983). Similar curves were obtained for glycine-conjugated chenodeoxycholate (results not shown).

Discussion

The results presented here show that bile acids bind to insoluble calcium phosphate. As shown in Fig. 1, binding, at neutral pH, occurs at free Ca^{2+} concentrations of less than 0.5 mM. Therefore this binding should not be confused with a Ca^{2+} -dependent precipitation of bile acids, which occurs

at free Ca^{2+} concentrations exceeding 12 mM (Williamson & Percy-Robb, 1979). Since a detailed study of the molecular mechanism is beyond the scope of the present paper, a few tentative conclusions may be drawn. First, the difference in binding between glycine and taurine conjugates is reminiscent of the reported difference in their affinity for Ca^{2+} (Williamson & Percy-Robb, 1979; Rajagopalan & Lindenbaum, 1982). The latter is attributed to the SO_3^- group in taurine being in a more hydrated state than the carboxylate group in glycine (Rajagopalan & Lindenbaum, 1982). Preliminary experiments with unconjugated deoxycholate showed that its binding to insoluble calcium phosphate is analogous to that of glycodeoxycholate. Thus these results suggest that the presence of a terminal carboxylate group in the side chain is a minimum requirement for this type of binding. Secondly, the difference in binding between dihydroxy and trihydroxy bile acids is probably due to the difference in CMC (see, e.g., Small, 1971; Roda *et al.*, 1983). Experiments such as that whose results are given in Fig. 3 indicate that significant binding to the sediment occurs with bile acid micelles only. Whether the slight decrease in binding observed at pH values above 6.1 (Fig. 1) is due to pH-dependent changes in CMC or aggregation number of micelles (Small, 1971), or to pH-dependent changes in the surface potential of the sediment, is not known at present and requires further investigation.

The physiological relevance of the results of our experiments is supported by the following data *in vivo* for the small-intestinal lumen: a pH range of 5.5–8 and a bile acid concentration range of 2.5–10 mM are reported (Carey *et al.*, 1983). As shown in Figs. 1 and 2, these are the conditions for the very significant binding of glycine-conjugated

dihydroxy bile acids. Furthermore, several studies indicate the formation of insoluble calcium phosphate in the small intestine (see Holdsworth, 1975, for review). This is in accordance with the finding that the concentration product for intestinal Ca^{2+} and P_i is in the range from 3×10^{-4} to at least $50 \times 10^{-4} \text{ M}^2$ (R. Van der Meer, unpublished work; see also Carey *et al.*, 1983). These values are at least two orders of magnitude higher than the apparent solubility product of about $3 \times 10^{-6} \text{ M}^2$ at neutral pH (see Fig. 1a). A consequence is that free Ca^{2+} concentrations as high as 12 mM, required to precipitate bile acids (Williamson & Percy-Robb, 1979), are not likely to occur under these intestinal conditions.

The structural requirements (glycine conjugation of dihydroxy bile acids) could especially be relevant to the enterohepatic cycling of bile acids. For instance, they could explain why the efflux from the enterohepatic cycle of glycine conjugates is greater than that of the taurine conjugates (Danielson & Sjövall, 1975). They also suggest a species-dependence with regard to binding of bile acids to insoluble calcium phosphate. Such a binding will hardly occur in the rat, because of the predominant taurine conjugation of bile acids in this animal (Kempen *et al.*, 1982). However, significant binding of the bile acids may be expected in the rabbit, since these acids are almost exclusively glycine-conjugated and dihydroxylated (Coleman *et al.*, 1979). It is worth mentioning that this proposed species-dependence of the binding of bile acids parallels the species-dependence of the response to dietary casein: a low increase, if any at all, of the serum cholesterol concentration in the rat, but a high response in the rabbit (West *et al.*, 1983). This proposed species-dependence of the binding of bile acids and the interaction with protein binding now require investigation.

In conclusion, the results presented here seem to justify the suggestion that insoluble calcium phosphate in the small intestine can contribute to the

efflux of glycine-conjugated dihydroxy bile acids from the enterohepatic cycle.

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