Bile acid inhibition of vitamin B₁₂ binding by intrinsic factor *in vitro*

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SUMMARY The effect of conjugated and unconjugated bile acids on the binding of vitamin B₁₂ to intrinsic factor was investigated. The dihydroxy bile acids (deoxycholic, glycodeoxycholic, taurodeoxycholic, glycochenodeoxycholic, and taurochenodeoxycholic) inhibit the binding of intrinsic factor to vitamin B₁₂ at physiological concentrations. On the other hand, the trihydroxy bile acids (cholic, glycocholic, and taurocholic) are not effective in this respect. The inhibition is dependent both on concentration and time, and its pattern is similar to that previously reported for duodenal juice. On column chromatography, there is a close correlation between the degree of intrinsic factor inhibition and the total acid concentration in the duodenal juice. The binding of vitamin B₁₂ by R protein in saliva is not affected by bile acids. The results show that bile acids at concentrations found in duodenal juice inhibit intrinsic factor vitamin B₁₂ binding. It is suggested that this observation may have physiological significance for vitamin B₁₂ absorption.

Vitamin B₁₂ (B₁₂) has recently been shown to bind to both intrinsic factor (IF) and R protein in almost equal proportions in the stomach.¹ The R protein subsequently releases its B₁₂ after its degradation by the pancreatic enzymes at neutral pH in the duodenum.² In pancreatic insufficiency this mechanism is disturbed and the B₁₂ becomes sequestered by R protein, a glycoprotein that is widely distributed in the gastrointestinal secretions but which does not promote the absorption of B₁₂.³ In the physiological state, the B₁₂ that is released by pancreatic enzymes binds to free IF in the duodenum and proximal jejunum. Interference of this latter phase of IF B₁₂ binding may affect the normal absorption of B₁₂.

It has been suggested that bile interferes with the binding process.^{4,5} Inhibition of IF binding occurs when gastric juice is incubated with duodenal juice, and is related to bile concentration. The inhibitor appears to be heat stable, and has two molecular weights, the first over 50 000 and the second around 1000. These characteristics are consistent with bile acids: the first molecular weight may represent bile acids in mixed micelles and the second in pure micelles.⁶

In this study we have investigated the effect of different bile acids on the binding of B₁₂ to IF.

Methods

CHEMICALS

⁵⁷Co cyanocobalamin, of specific activity 1.1 µCi per µg, was purchased from the Radiochemical Centre, Amersham. Bovine serum albumin was supplied by Armour Pharmaceuticals Co. Ltd., and Norit A activated charcoal by the Sigma Chemical Co. Ltd. Sodium salts of cholic, deoxycholic, glycocholic, taurocholic, glycodeoxycholic, taurodeoxycholic, glycochenodeoxycholic, and taurochenodeoxycholic acids were obtained from Sigma Chemical Co. Ltd. Sephadex G-50 (fine grade) chromatography resin was purchased from Pharmacia Fine Chemicals.

COLLECTION OF GASTRIC JUICE, DUODENAL JUICE, AND SALIVA

Gastric juice was collected from patients undergoing pentagastrin stimulation tests and the first 20 minute sample after pentagastrin injection was collected. This was depepsinised by adding 1 N NaOH to pH 10 for 20 minutes, then neutralising with 1 N HCl to pH 7, and centrifuged for 20 minutes at 3000 g. The supernatant was collected and stored at -20°C.

Duodenal juice was obtained from patients undergoing pancreozymin stimulation test. A double-

barrelled tube was positioned in the stomach and the duodenum. The proximal tip of the tube was in the most dependent portion of the stomach and the gastric juice was collected from there by continuous aspiration and discarded. The distal tip was in the second part of the duodenum and the secretion thereof was collected for 30 minutes after pancreozymin injection, and subsequently centrifuged for 20 minutes at 3000 g. The supernatant was stored at -20°C.

Saliva was collected over 15 minutes from a normal volunteer after an overnight fast. The collection was centrifuged for 20 minutes at 3000 g, and the supernatant, free of mucus, was stored at -20°C.

Total B₁₂ binders and IF in the gastric juice and the saliva were estimated by a modification of the method of Gottlieb *et al.*⁷ In our assay ⁵⁷Co cyanocobalamin (25 ng/ml) and Tris HCl buffer pH 7 were substituted for ⁶⁰Co cyanocobalamin (7.5 ng/ml) and 0.9% saline as incubation medium in the original method. Three samples of gastric juice were used in the experiments. Ninety per cent of the binders in each gastric juice was IF. The IF assay was performed at pH 7, which may explain the low percentage of R protein in the samples because the affinity of R protein is substantially less at neutral pH.² The level of IF present is expressed as unit/ml which is equivalent to ng/ml.

Total bile acid concentration of the duodenal juice fractions was assayed using the enzyme 3 alpha-hydroxy dehydrogenase.⁸

The bile acids investigated in the experiments were dissolved in deionised water, and were incubated with gastric juice or saliva for two hours at 37°C and pH 7.2-7.4 unless specified. The following experiments were performed.

EFFECT OF BILE ACIDS ON BINDING OF VITAMIN B₁₂ TO INTRINSIC FACTOR BY MODIFIED METHOD OF GOTTLIEB *et al.*

Bile acid (0.05 ml) at concentrations of 2.5, 5, 10, and 15 mmol/l was incubated with 0.05 ml of gastric juice. As control, 0.05 ml of gastric juice was incubated with 0.05 ml of normal saline. The concentration of active IF at the end of incubation was estimated. The experiments were performed with two different samples of gastric juice and similar results were obtained.

In the time dependent experiment, 0.05 ml of bile acid at a concentration of 10 mmol/l was incubated with 0.05 ml of gastric juice for periods of five, 10, 15, 30, minutes, and one, two, and four hours. The IF was estimated and compared with control samples of the gastric juice which were incubated for the corresponding periods of time.

EFFECT OF BILE ACIDS ON INTRINSIC FACTOR VITAMIN B₁₂ BINDING BY COLUMN CHROMATOGRAPHY

A Sephadex G50 gel column, 30 cm by 1 cm, was used to separate free B₁₂ from IF-bound B₁₂. The column was precalibrated with blue dextran (which marked the eluted fractions that contained IF B₁₂ complex) free ⁵⁷Co B₁₂, and riboflavin. Gastric juice (0.2 ml) was incubated with 0.2 ml of bile acid at concentrations of 1, 2, 4, and 8 mmol/l for two hours. One millilitre of ⁵⁷Co B₁₂, containing 20 ng/ml, was then added to the above and to a sample of control gastric juice. The mixture was subsequently applied on the column and eluted with Tris/HCL buffer at pH 7. The eluents were collected in 1 ml fractions and the radioactivity counted in a Packard gamma counter.

EFFECT OF BILE ACIDS ON SALIVARY R PROTEIN

Saliva (0.05 ml), which contains R protein, was incubated with 0.05 ml of bile acid at 10 mmol/l for periods of two and four hours. The level of R protein, a non-IF B₁₂ binder, was estimated at the end of the incubation and compared with that of the control saliva incubated for the same periods, by the modified method of Gottlieb *et al.*

CORRELATION OF ENDOGENOUS BILE ACIDS IN DUODENAL JUICE WITH THE INHIBITION Duodenal juice (2 ml) was eluted with 0.1 M phosphate buffer at pH 7.2 through Sephadex G-50 gel

Fig. 1 Effect of bile acids on the vitamin B₁₂ binding activity of intrinsic factor by the modified method of Gottlieb *et al.* ●—● GC, ○—○ TC, ▲—▲ GDC, △—△ TDC, ■—■ GCDC, □—□ TCDC, ◇—◇ C, ◆—◆ DC.

column which separated the duodenal juice components into fractions that had molecular weight greater than 50 000 and smaller molecular weight fractions which eluted in the included volume. A sample (0.05 ml) of each fraction was incubated with 0.05 ml of gastric juice for two hours. Control samples of the fractions were also incubated for the same length of time. At the end of the incubation the IF level of the samples of gastric juice treated with the duodenal juice fractions was measured by the method of Gottlieb *et al.* and compared with that of the untreated gastric juice and the result expressed as percentage of inhibition. The radioactivity count of the control samples of the duodenal juice fractions when assayed was not different from the background radioactivity count. Total bile acid concentration was also measured in each fraction.

Results

EFFECT OF BILE ACIDS ON INTRINSIC FACTOR VITAMIN B₁₂ BINDING

The results by the modified method of Gottlieb *et al.* are shown in Fig. 1. Glycocholate (GC) and taurocholate (TC), both conjugated trihydroxy bile acids, did not inhibit IF B₁₂ binding at concentrations normally present in intestinal juice. In contrast, glycodeoxycholate (GDC), taurodeoxycholate (TDC), glycochenodeoxycholate (GCDL), and taurochenodeoxycholate (TCDC), all conjugated dihydroxy bile acids, caused profound inhibition. Complete inhibition was present at bile acid concentrations of 5 mmol/l. A similar pattern of interaction was observed with unconjugated bile acids. Cholate (C), an unconjugated trihydroxy bile acid,

Fig. 2 Effect of trihydroxy bile acids on the vitamin B₁₂ binding activity of intrinsic factor using Sephadex G50 gel column to separate free from IF-bound ⁵⁷Co B₁₂. ○—○ control GJ. ●—● GJ preincubated with GC. ●---● GJ preincubated with TC. Both GC and TC are of 8 mmol/l concentration.

Fig. 3 Effect of dihydroxy bile acids on the vitamin B₁₂ binding activity of intrinsic factor using Sephadex G50 gel column. GDC, TDC, GCDC, and TCDC at the following concentrations were preincubated with gastric juice before elution: $\triangle$ — $\triangle$ 1 mmol/l. $\blacksquare$ — $\blacksquare$ 2 mmol/l. $\circ$ — $\circ$ 4 mmol/l. $\bullet$ — $\bullet$ 8 mmol/l.

Fig. 4 Effect of duration of incubation on the vitamin B₁₂ binding activity of intrinsic factor by dihydroxy bile acids at 10 mmol/l. $\circ$ — $\circ$ control GJ. $\triangle$ — $\triangle$ incubated with GDC. $\blacksquare$ — $\blacksquare$ GJ incubated with GCDC. $\bullet$ — $\bullet$ GJ incubated with TCDC.

caused slight inhibition, whereas deoxycholate (DC) an unconjugated dihydroxy bile acid, very significantly inhibited binding.

To preclude the possibility that these results were related to the assay method used, similar experiments were carried out using column chromatography. Inhibition of IF B₁₂ binding by dihydroxy bile acids, in contrast with trihydroxy bile acids, was again demonstrated using Sephadex G-50 gel filtration. Thus, GC and TC did not significantly alter the distribution of ⁵⁷Co B₁₂ (Fig. 2), while GDC, TDC, GCDC, and TCDC inhibited intrinsic factor, and as a result the majority of ⁵⁷Co B₁₂ was not bound to IF and consequently eluted as free ⁵⁷Co B₁₂ (Fig. 3).

The inhibition was time dependent. GDC and GCDC at 10 mmol/l concentration caused progressive inhibition of the binding over four hours, which was evident as early as 10 minutes and reached a plateau at two hours (Fig. 4).

EFFECT OF BILE ACIDS ON SALIVARY R PROTEIN

The results of incubation of saliva that contains R protein, a non-IF B₁₂ binder, with GC, GDC, and GCDC are shown in Fig. 5. Bile acids did not affect the activity of R protein over four hours.

Fig. 5 Effect of bile acids on the vitamin B_{12} activity of salivary R protein. ●...● control saliva. ○---○ saliva with GC. △---△ saliva with GDC. ■---■ saliva with GCDC, at concentration of 10 mmol/l.

CORRELATION OF ENDOGENOUS BILE ACIDS IN DUODENAL JUICE WITH INHIBITION

Two peaks of inhibition were observed in duodenal juice that was fractionated by Sephadex G-50 gel column (Fig. 6). The first peak was related to macromolecular fractions of duodenal juice and the second peak smaller molecular weight fractions.

Fig. 6 Inhibition of vitamin B_{12} binding activity of intrinsic factor by fractions of duodenal juice separated by Sephadex G50 gel column.

Both peaks of inhibition were closely related to total bile acid concentration. The results suggest that bile acids in mixed micelle with molecular weight around 50 000 and bile acid pure micelle of small molecular weight may be the factor in duodenal juice that inhibits IF.

Discussion

Bile acids are capable of dissociating IF- B_{12} complex *in vitro*⁹ but the effect of bile acids on intrinsic factor itself has not been investigated before. We have shown by two different methods that both conjugated and unconjugated dihydroxy bile acids (Figs. 1 and 3) but not trihydroxy bile acids (Figs. 1 and 2) inhibit intrinsic factor from binding B_{12} . Interference of the binding of free B_{12} to albumin coated charcoal, which is used in the method of Gottlieb *et al.*, has been shown previously but this occurs at concentrations much higher than those used in our investigations.¹⁰ It is unlikely therefore that the results we have obtained are an artefact. This *in vitro* inhibition occurs under conditions that are

normally present in the small intestine. The concentrations of the conjugated bile acids, the length of incubation, and the neutral pH are consistent with that normally present in intestinal juice and thus suggest that the inhibition is likely to occur in the intestinal lumen, especially in the distal intestine where virtually all the bile acids present are monomers or pure bile acid micelles.¹¹

The pattern of inhibition demonstrated with bile acids is very similar to that caused by duodenal juice as reported previously.⁴ The close correlation between the inhibitory activity of the fractions of the duodenal juice that has been eluted through the Sephadex gel column and the total bile acid concentration provides additional evidence that bile acids are the factor in the duodenal juice that inhibits IF (Fig. 6). Furthermore, the apparent molecular weights of the inhibitor would be consistent with bile acids in mixed micelles or in pure micelles.

The exact mechanism of the inhibition remains unknown. The finding that unconjugated bile acids have the same effect as the conjugated forms suggests that it may be the steroid group and not the amino acid group of the bile acids that is responsible for the inhibition. It seems that the addition of an extra hydroxyl group reverses the inhibitory effect of the dihydroxy bile acids and that the relative positions of the two hydroxyl groups in the dihydroxy bile acids are not important. The observation that the glycine and the taurine conjugates, which have very different pK values,¹² inhibit IF to the same degree indicates that the basis of the interaction is not primarily due to ionic interaction between IF and bile acid. It is possible that the two hydroxyl groups facilitate the binding of bile acid to the hydrophobic position of IF which subsequently becomes inactivated; such a mechanism would be analogous to interactions between certain proteins and bile acids and other detergents.^{13,14}

The inhibition seems to be specific for IF. In identical *in vitro* conditions bile acids did not inhibit the vitamin B₁₂ binding activity of R protein which is present in the saliva.

The physiological significance of this *in vitro* effect of bile acid is also unclear but there is evidence which suggests that bile may influence the absorption of B₁₂. The presence of bile affects the absorption of free and IF-bound B₁₂ in rat,¹⁵ and, in patients with obstructive jaundice, the absorption of B₁₂ is abnormal.¹⁶ It has been suggested that contamination of gastric juice by bile might be a cause of malabsorption of B₁₂ in patients with partial gastrectomy.¹⁷ We have recently demonstrated a more definite association between bile and the absorption of vitamin B₁₂.¹⁸ Ligation of the bile duct in rat impaired the absorption of B₁₂, and the malabsorption

was reversed when bile was replaced in the intestinal lumen. Also, the absorption of B₁₂ was impaired in man when the bile flow was diverted by T tube drainage of the common bile duct after operation for cholelithiasis. The absorption improved significantly when normal bile flow was re-established after removal of the T tube.

Normally IF is secreted many times in excess of that which is required to bind the dietary B₁₂.¹⁹ Whether this excess amount of IF inhibits the attachment of the IF-B₁₂ complex to the specific receptors in the ileum remains controversial. One study showed that the uptake of B₁₂ from the IF-B₁₂ complex by the guinea-pig ileal microvillous membranes was impaired when excess free IF was present.²⁰ However, this was not confirmed by experiments using human ileal receptor preparation.²¹ Bile acids may exert their effect by inhibiting the excess free intrinsic factor in the intestinal lumen, or by affecting the B₁₂ absorption at the receptor sites in the ileum.

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