Intestinal alkaline phosphatase in bile: evidence for an enterohepatic circulation

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SUMMARY The isoenzyme characteristics of alkaline phosphatase in human bile obtained from patients with gallstones have been determined by means of electrophoresis on polyacrylamide gel, both before and after preincubation with neuraminidase, and also by means of inhibition tests with heat, urea, and L-phenylalanine. Bile alkaline phosphatase is shown to be partly secreted by the liver cell, but partly derived from the small intestine. The presence of small-intestinal alkaline phosphatase in bile implies an enterohepatic circulation of this high molecular weight glycoprotein. The significance of this finding is discussed in relation to the mechanism whereby large protein molecules may pass across the plasma membrane of the liver cell.

There are two main theories concerning the origin of alkaline phosphatase in bile. The first is that serum alkaline phosphatase, primarily derived from bone, is excreted into bile; the other postulates that the alkaline phosphatase in bile is formed by the liver cells.¹ Most of the experimental evidence suggests that the biliary tree is not an important excretory route for serum alkaline phosphatase and supports the concept of 'over-production' by the liver as the most important cause of the increased serum alkaline phosphatase in biliary obstruction.^{2,3} The most convincing evidence has been provided by the many experiments in which infused homologous alkaline phosphatase has failed to accumulate in the liver or be excreted in the bile in the experimental animal.⁴⁻⁷ Furthermore, infused placental alkaline phosphatase is not excreted into bile in the human.⁸ However, in the experimental animal, there is some evidence that the intestinal enzyme, unlike alkaline phosphatase derived from other organs, may be secreted into bile.^{9,10} Serum alkaline phosphatase exists in the form of isoenzymes which can be differentiated by immunological means, by inhibition tests with heat, urea and L-phenylalanine, and by electrophoresis.

A number of immunological studies of alkaline phosphatase isoenzymes have been made, but immunological cross-reactivity of the isoenzymes has created problems. Boyer¹¹ showed cross-reactivity between intestinal and placental alkaline phosphatase and also between both of these and a minor kidney component. More recently it has been shown that intestinal alkaline phosphatase consists of two isoenzymes, one of which shows cross-reactivity with placental alkaline phosphatase and the other with liver alkaline phosphatase.¹² Mulivor *et al.*¹³ have shown that at least three genetic loci are involved in determining the various forms of human alkaline phosphatase, one for the placental form, at least one for the intestinal form, and at least one or more for the liver, bone, and kidney isoenzymes. Lehmann¹⁴ has shown, however, that it is possible to prepare a monospecific antiserum against intestinal alkaline phosphatase by absorbing out other components and, therefore, immunological methods may have an important role to play in the identification of intestinal alkaline phosphatase.

The amount of small-intestinal alkaline phosphatase in serum is known to be genetically determined, individuals who are blood group O of B having higher levels than those of blood group A.¹ In addition, there is an effect of secretor status. Thus, within any given blood group, individuals who possess the glycoprotein secretor substances in saliva show higher levels

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of intestinal alkaline phosphatase in serum.

On electrophoresis of serum, liver, bone, and intestinal bands may be found and in serum from 78% of patients with liver disease an origin band may be observed. This may represent a complex of the liver isoenzyme with lipoprotein.¹⁵ The intestinal band can be easily distinguished on electrophoresis, as it is inhibited by L-phenylalanine¹⁶ and its electrophoretic mobility is unaltered after treatment with neuraminidase.¹⁷

Electrophoretic separation of the bile alkaline phosphatase isoenzymes has largely made use of the older techniques of paper or starch gel electrophoresis and conflicting results have been obtained regarding the number and origin of the bands so obtained.¹⁷⁻²¹ Keiding,²² after electrophoresis of human bile on starch gel, noted the presence of a zone of alkaline phosphatase activity which appeared to be identical with that found in intestinal fluid and lymph. Rosenberg¹⁹ had previously made a similar observation, although he did not specifically suggest that it represented intestinal alkaline phosphatase. The present study of the alkaline phosphatase isoenzymes in human bile uses polyacrylamide gel electrophoresis (PAGE), which is known to give a superior resolution of alkaline phosphatase isoenzymes,¹ to confirm and extend these earlier observations.

Methods

Twenty-four samples of bile were obtained from 17 patients either from the gall-bladder (10), hepatic duct (five), or common bile duct (one) at the time of operation, or from the common bile duct by means of a T-tube drain after operation (eight). One patient with carcinoma of the pancreas and one with chronic relapsing pancreatitis were found to have no gallstones at operation, while one patient with acute relapsing pancreatitis and one with chronic relapsing pancreatitis were found to have gallstones. The remaining 20 samples of bile were obtained at the time of routine cholecystectomy in patients with gall-stones.

Alkaline phosphatase was measured by a modification of the King-Armstrong method²³ and the isoenzyme characteristics determined by inactivation with heat (56°C for 15 minutes) and 1M urea, and by inhibition with 0.005M L-phenylalanine.²⁴ Electrophoretic separation of the bile isoenzymes was performed by PAGE and the enzyme bands located by staining with beta-naphthyl phosphate and Fast Blue BB.¹⁵ In

some experiments, L-phenylalanine was incorporated into the staining mixture. Samples were also treated with neuraminidase (type IV *Cl. perfringens*, Sigma Chemical Co.) before electrophoresis, using a modification of the method of Robinson and Pierce.¹⁷ The enzyme was extracted from human liver obtained at necropsy, and from small-intestinal mucosa obtained at operation, by a modification of the butanol method of Morton.²⁵

The results were analysed statistically by Schiffé's method of adjusted significance levels. Blood-groups in serum were determined by standard serological techniques.

Results

Bile alkaline phosphatase was significantly different from small-intestinal alkaline phosphatase in its sensitivity to heat ($P < 0.001$), urea ($P < 0.001$), and L-phenylalanine ($P < 0.001$) and also from the liver isoenzyme after heat ($P < 0.001$), urea ($P < 0.001$), and L-phenylalanine inhibition ($P < 0.01$) (Table 1). Gall-bladder bile differed from T-tube bile on heat inactivation ($P < 0.001$), urea inactivation ($P < 0.005$), and L-phenylalanine inhibition ($P < 0.005$) and also from hepatic duct bile after heat inactivation ($P < 0.005$) and L-phenylalanine inhibition ($P < 0.02$), though not after urea inactivation. Small-intestinal alkaline phosphatase was significantly different from liver alkaline phosphatase after heat inactivation ($P < 0.001$), urea inactivation ($P < 0.001$), and L-phenylalanine inhibition ($P < 0.001$).

After PAGE the main alkaline phosphatase bands found in the extracts of liver and small intestine corresponded to the positions of the liver and small-intestinal bands found in serum. In 12 of the 19 samples of bile that were electrophoresed a fast-running liver-type band (L) was

Table 1 Isoenzyme characteristics of bile, liver, and small-intestinal alkaline phosphatase

	Heat % remaining	Urea % remaining	L-phenylalanine % inhibition
Hepatic duct (5)	24.5 ± 8.6	29.8 ± 17.3	9.6 ± 5.4
Gall bladder (10)	44.6 ± 19.8	42.6 ± 15.0	18.2 ± 16.5
T-tube (8)	26.1 ± 8.3	26.6 ± 14.9	9.2 ± 10.2
Common bile duct (1)	25.9	11.4	18.6
All bile (24)	34.0 ± 16.9	33.2 ± 16.7	13.3 ± 13.0
Liver extract (3)	13.2 ± 10.8	8.2 ± 6.7	8.3 ± 1.4
Small-intestinal extract (5)	78.3 ± 12.9	44.2 ± 7.6	78.9 ± 6.6

Samples were heated at 56°C for 15 minutes, inactivated with 1M urea and inhibited with 0.005M L-phenylalanine. Results are given ± S.D.

Fig. 1 Polyacrylamide gel disc electrophoresis showing the liver band (L), origin band (O), and intestinal band (I). A. Serum from a patient with liver disease. B. Gall-bladder bile. C. Small-intestinal extract. D. Liver extract.

Fig. 3 The effect of phenylalanine on the ALP isoenzymes of bile. A. T-tube bile—normal stain. B. T-tube bile—stain incorporating L-phenylalanine.

Fig. 2 The effect of neuraminidase on electrophoretic mobility. A. Liver extract. B. Small-intestinal extract. C. Hepatic-duct bile. The right-hand gel of each pair, superscript N, has been preincubated with neuraminidase.

Table 2 Electrophoretic bands observed in bile, liver and small-intestinal alkaline phosphatase

	Liver band	Intestinal band	Origin band	Other
Hepatic duct (4)	2	2	4	—
Gall bladder (9)	8	7	6	—
T-tube (5)	2	3	5	2
Common bile duct (1)	—	1	1	—
All bile (19)	12	13	16	2
Liver extract (3)	3	—	—	—
Small-intestinal extract (5)	2*	5	2	1

*The liver band in small-intestinal extract was very faint.

seen, and 16 had an origin band (O) identical with that previously described¹⁵ in the serum of patients with liver disease (Fig. 1). In 13 samples of bile a slower band (I) was found which was identical in position with both the main band of small-intestinal extract and with the small-intestinal band in serum. When samples of bile demonstrating band I were incubated with neuraminidase before electrophoresis the electrophoretic mobility of this slower band, like that of small-intestinal extract, was unaltered, while the main band L of bile was slowed in a similar manner to that of liver extract alkaline phosphatase (Fig. 2). When L-phenylalanine was incorporated into the staining mixture the slower band I decreased in intensity, while both the liver-type band (L) and the origin band (O) were unaltered (Fig. 3). The small-intestinal band was seen in the majority of samples of bile, whether the sample was obtained from gall-bladder, hepatic duct, common bile duct, or by T-tube drainage (Table 2). In two samples of bile an additional minor band was found running ahead of the liver band.

Blood group data were available for 14 patients; bile samples from 11 of these were electrophoresed and the incidence of intestinal bands in these samples from patients of known blood group is shown in Table 3.

Table 3 *The incidence of intestinal bands in bile from patients of known blood group*

Blood group	No.	Intestinal band	
		Present	Absent
O	2	1	1
A	6	4	2
B	2	2	0
AB	1	1	0

There was no significant difference in incidence of intestinal bands between individuals in blood group A and those in blood groups O and B combined ($p = 1$, Fischer's exact test).

Discussion

Substances secreted by the liver, absorbed by the intestine, and resecreted by the liver have an enterohepatic circulation, which may be portal as with bile salts or extraportal as in the case of lipids. Apart from phospholipids and cholesterol, a number of other substances such as bile pigments, folate, and certain steroid hormones and antibiotics undergo this latter form of enterohepatic circulation,²⁶ but there is no concrete evidence that high molecular weight substances

such as proteins undergo an enterohepatic circulation.

The isoenzyme characteristics of bile alkaline phosphatase as determined in the present study are similar to those previously reported by Horne *et al.*²⁷ As these lie intermediate between those of liver and small intestinal alkaline phosphatases, they are compatible with the thesis that bile alkaline phosphatase represents a mixture of the other two isoenzymes. The inhibition data by themselves are, however, not conclusive.

We have shown that on electrophoresis two main isoenzymes of alkaline phosphatase are found in bile. One is identical with that of liver extract and with the liver main band in serum, and this finding is in agreement with the report of previous workers.²⁸ The other appears to be small-intestinal alkaline phosphatase and, like the latter, is unaltered in electrophoretic mobility by neuraminidase. The decrease in intensity of this band when L-phenylalanine is incorporated into the staining mixture (Fig. 3) confirms its small-intestinal origin.

The higher levels of intestinal alkaline phosphatase in the serum of individuals in blood groups O or B are thought to be mainly due to an increased synthesis or release of the isoenzyme into the serum. If this were the overriding factor, then one would expect also to find higher levels of intestinal alkaline phosphatase in the bile of these patients. However, if the higher levels were due to a slower rate of elimination of the isoenzyme then one might expect to find lower levels in the bile of individuals in blood groups O or B, and correspondingly higher levels in the bile of individuals in blood group A. In the present study we found no significant difference in the incidence of intestinal bands in bile between patients of blood group A and those of blood groups O and B. However, in serum the differences are known to be quantitative rather than qualitative, and this may well also be the case with bile. If this is so, then an immunological method for measuring intestinal alkaline phosphatase in bile may clarify this point, providing the problems of cross-reactivity can be overcome.

Three possible explanations for the presence of small-intestinal alkaline phosphatase in bile have to be considered. The first is that it is due to direct reflux from duodenal juice into bile. However, the 'small-intestinal' isoenzyme has been identified in samples taken as high as the hepatic ducts and radiological observations taken at the time of endoscopic retrograde cholangiography suggest that free reflux of con-

trast into the common bile duct does not occur under the conditions obtaining—that is, in the absence of a previous sphincterotomy or of concomitant administration of CCK-pancreozymin. The second possibility is that the 'small-intestinal' alkaline phosphatase found in bile is, in fact, derived from the gall-bladder epithelium. This is extremely unlikely in view of the fact that the isoenzyme was identified in three out of five specimens of T-tube bile obtained after removal of the gall-bladder; in these samples the small-intestinal band was still present one week after cholecystectomy. The third, and, in our opinion most likely, possibility is that the intestinal isoenzyme arises in bile after direct passage through the hepatocyte. Thus, alkaline phosphatase in the bile of these patients appears to consist largely of an isoenzyme secreted by the hepatocyte, and another derived from the small-intestinal brush borders which has traversed the liver cell and been excreted into bile. Small-intestinal alkaline phosphatase is released into duodenal juice by the hormones secretin and CCK-pancreozymin.²⁹ Histological studies have shown³⁰ that, after feeding of long chain triglycerides, the isoenzyme passes from the brush border across the small intestinal epithelium and into the lymphatic system.¹ Thus, in demonstrating the presence of small intestinal alkaline phosphatase in bile, the present study implies that alkaline phosphatase undergoes an enterohepatic circulation.

Recent studies have elucidated the mechanism whereby glycoproteins are able to traverse the plasma membrane of the hepatocyte. Ashwell and Morell³¹ have shown that sialic acid, which occurs only in the terminal portion of glycoprotein carbohydrate side chains, plays an important role in determining the circulating life of serum glycoproteins. Thus, removal of the terminal sialic acid groups and exposure of the penultimate galactosyl group of the carbohydrate side chain of a glycoprotein results in rapid clearance from the circulation of many serum glycoproteins which can then be recovered in the liver.^{32, 33} It has also been demonstrated that isolated hepatic cell plasma membranes contain specific saturable binding sites for the desialylated glycoproteins.³⁴ It is therefore relevant that small-intestinal alkaline phosphatase is unique among the alkaline phosphatase isoenzymes in lacking terminal sialic acid residues, as shown electrophoretically by its resistance to neuraminidase. Our study is consistent with previous work in both man and the experimental animal in that, apart from the liver isoenzyme

which is presumably secreted into bile by the hepatocyte, no sialic acid-containing isoenzymes of alkaline phosphatase are present in bile.

Until very recently, no definite evidence was available that galactosyl receptors specific for small-intestinal alkaline phosphatase existed on the plasma membrane of the hepatocyte, although our findings suggested that they should exist and might play a part in the uptake of small-intestinal alkaline phosphatase into the liver. However, work has now been published by Scholtens *et al.*³⁵ which demonstrates conclusively that, in the experimental animal, a galactosyl receptor for small-intestinal alkaline phosphatase exists on the hepatocyte plasma membrane, strongly supporting the theory that small-intestinal alkaline phosphatase does undergo an enterohepatic circulation.

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