γ -Glutamyl transferase isoenzymes in human bile

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SUMMARY The γ -glutamyl transferase isoenzymes of bile were studied using electrophoretic, gel filtration, and ultracentrifugation techniques. In view of the known association of other biliary enzymes with lipids the effects of butanol extraction were investigated. The results show the presence of four isoenzymes of γ -glutamyl transferase in bile, differing in electrophoretic mobilities, molecular size, and density. The correlation between the properties of biliary γ -glutamyl transferase and of alkaline phosphatase is discussed.

It has been recognised that there are several isoenzymes of γ -glutamyl transferase (γ GT) (E.C. 2.3.2.2.) present in the serum, although few attempts have been made to characterise them. In contrast, little attention has been paid to the presence of γ GT in the bile, and the origin of the biliary isoenzymes has not been established. A study of γ GT in the bile seems worthwhile in view of the fact that the enzyme level in the serum is grossly elevated in biliary obstruction and that a study of another enzyme, alkaline phosphatase (also present in increased amounts in the circulation of patients with biliary obstruction), has shown a relationship between the isoenzymes present in the bile and those present in the serum (Price and Sammons, 1974).

The presence of γ GT activity in the bile has been reported (Orlowski, 1963), with levels higher than those found in the serum. It has been supposed that biliary γ GT arises from the cells of the liver and biliary tract by a process of wear and tear (Szczeklik *et al.*, 1961; Orlowski, 1963; Rutenburg *et al.*, 1963).

This study was undertaken in an attempt to characterise the γ GT present in the bile. The techniques employed reflect experience gained in previous studies of serum and biliary enzymes.

Material and methods

Specimens of hepatic bile were obtained from

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Received for publication 24 November 1977

patients with T-tube drainage of the common bile duct after cholecystectomy.

Agar gel electrophoresis was carried out using the Multiphor electrophoresis equipment (LKB Produkter, Bromma, Sweden). Separation employed 1% agar (Difco Noble) in barbitone buffer, pH 8.6, on rectangular glass plates, 8.4 × 9.4 cm. Electrophoresis was performed for one hour at 250 volts. A sample of pooled serum was run in parallel with bile samples and then stained for protein using naphthalene black. In this way zones of γ GT activity were related to the serum protein fractions.

Polyacrylamide gel disc electrophoresis was carried out using the Shandon electrophoresis equipment and the method of Azzopardi and Jayle (1973). Before application of the sample, bromophenol blue and albumin were added to the bile in order that the mobilities of bands of enzyme activity could be related to albumin. Mobilities were expressed as a fraction of the mobility of albumin.

Using both electrophoresis procedures, the γ GT isoenzymes were visualised by means of incubation with γ -L-glutamyl- α -naphthylamide, coupling the liberated α -naphthylamine with Fast Blue B according to the optimised procedure of Wenham *et al.* (1978). More accurate quantitation of the isoenzyme fractions after polyacrylamide gel electrophoresis was accomplished by cutting the gel into 0.5-cm segments and incubating the homogenised gel segments in buffered substrate according to the method of Rosalki *et al.* (1970).

Gel filtration chromatography was performed using Sephadex G200 (Pharmacia, Uppsala, Sweden) by a method previously described (Price and Sammons, 1974) using 0.02 M Tris-HCl, pH 8.0, containing 0.05 M sodium chloride. A 60 × 2 cm column was used with a flow rate of 5.2 ml buffer per hour.

This technique was complemented by buoyant density ultracentrifugation studies using a method previously described (Price and Sammons, 1974). A stepwise density gradient was formed from 10–40% (w/v) sucrose. After centrifugation for 18 hours at 36 000 rpm 15 fractions were obtained by dropwise release of the contents of the centrifuge tube. In both techniques the protein fractionation patterns were monitored using a Uvicord II absorptiometer at 280 nm. A comparison of isoenzyme characteristics was made with serum protein patterns. Enzyme activity in all fractions was determined by the method of Rosalki and Tarlow (1974).

The effect of butanol extraction on bile enzyme activity was observed by vigorously mixing equal volumes of hepatic bile and *n*-butanol for two minutes on a vortex mixer. The aqueous and solvent phases were then separated by centrifugation at 3000 rpm for 10 minutes in an MSE Super Minor centrifuge. The γ GT activity before and after extraction was measured by the kinetic method of Rosalki and Tarlow (1974).

Results

ELECTROPHORETIC STUDIES

In nine of the 10 samples of hepatic bile studied, three zones of enzyme activity were observed after agar gel electrophoresis. Activity was found in the (albumin- α_1 -), α_2 -, and β -globulin fractions. In the remaining sample only one fraction was observed, in the α_2 -globulin region. The predominant fractions were the (albumin- α_1 -) and α_2 - fractions.

Using polyacrylamide gel electrophoresis, nine of the bile samples were studied and in seven cases two bands of activity were observed with mobility 0 and 77% of albumin (R_{alb} values of 0 and 77). One sample showed activity only at the origin, and the remaining sample showed only one band with a mobility of 77% of albumin. The predominant band in every other case was the origin fraction, and this was confirmed using the quantitative localisation technique.

Table 1 γ GT activity in different fractions obtained by gel filtration and ultracentrifugation of hepatic bile expressed as a percentage of total activity recovered

Sample	Gel filtration		Ultracentrifugation	
	Void volume	7S-4S	19S	4S
FS	100	—	72	38
PM	86	14	59	41
MB	99	1	—	—
CG	53	47	53	47
JB	99	1	34	66
MS	98	2	48	52
MD	100	—	—	—

GEL FILTRATION STUDIES

Fractionation of serum proteins yielded three main peaks, as observed by Flodin and Killander (1962). In five of the seven bile samples studied, two peaks of γ GT activity were observed, one eluting in the so-called 19S protein fraction and the other between the 7S and 4S protein fractions. The remaining two samples showed only the 19S fraction. The mean recovery of enzyme activity after gel filtration was 84%.

ULTRACENTRIFUGATION STUDIES

In the six samples of hepatic bile studied using this technique two fractions of enzyme activity were identified. One of the fractions was found with the density similar to the 19S protein fraction and the other was found with the 4S protein fraction. The proportion in each fraction varied from 40 to 70%. The mean recovery of enzyme activity was 87%.

COMPARISON OF ISOENZYME CHARACTERISTICS OBTAINED FROM GEL FILTRATION AND ULTRACENTRIFUGATION OF BILE

Quantitation of the fractions of activity after gel filtration and ultracentrifugation (Table 1) showed that there was no clear relationship between recovery of isoenzymes and the nature of the fractions obtained. Therefore fractions constituting bands of activity after gel filtration were pooled and concentrated using Lyphogel (Hawksley and Sons Ltd, Lancing, Sussex, UK) and then subjected to buoyant density ultracentrifugation. Fractions obtained by ultracentrifugation were studied without prior concentration and subjected to both agar and polyacrylamide gel electrophoresis followed by qualitative localisation.

The results of these comparative studies are summarised in Table 2 and suggest the presence of

Table 2 Physicochemical characteristics of the γ GT isoenzymes of hepatic bile

Iso-enzyme	Elution from G200	Buoyant density ultracentrifugation	Electrophoretic mobility		Relative contribution toward total activity
			Agar	Polyacrylamide	
I	Void volume	4S	Alb- α_1	0	+++++
II	7S – 4S	4S	α_2	77	+
III	Void volume	19S	α_2	0	+++++
IV	Void volume	19S	β	0	+

up to four isoenzyme fractions in hepatic bile. The numbering system adopted is given solely for convenience of discussion.

EFFECT OF BUTANOL ON BILIARY γ GT ACTIVITY

After extraction, with butanol, of samples of hepatic

bile from nine subjects there was a mean loss of γ GT activity of 59% (range 25-91%). No activity was observed in either the butanol phase or the re-suspended interface material. Sufficient material was available from five of the treated samples to allow further study of the extracted material.

Polyacrylamide gel electrophoresis of the aqueous phase resulted in a decrease in the fraction previously found at the origin with the appearance of a fraction with a mobility of 55% that of albumin. This was quite clearly shown using the quantitative localisation technique, in which 79% of the origin activity was lost with the appearance of a new isoenzyme with 72% of the total activity (Fig. 1). The overall loss of activity of this sample was 25%.

Fig. 1 Quantitative localisation of γ GT isoenzymes after polyacrylamide gel electrophoresis of hepatic bile before and after butanol extraction: (a) protein pattern; (b) enzyme activity expressed as absorbance at 405 nm — before butanol — — after butanol.

Gel filtration of hepatic bile after extraction with *n*-butanol showed a loss of activity in the 19S fraction with the appearance of a new peak of activity between the 19S and 7S protein fractions. The small 7S-4S peak of activity was unaltered by the solvent extraction (Fig. 2). Concentration of the new fraction of activity and electrophoresis in polyacrylamide gel revealed a mobility 55% that of albumin; in agar gel its electrophoretic mobility was similar to that of the α_1 -globulins.

Ultracentrifugation of three samples of bile after butanol extraction showed a loss of activity in the 19S fraction with the appearance of more activity

Fig. 2 Gel filtration pattern of γ GT isoenzymes of hepatic bile and the effect of butanol extraction: serum protein pattern, — enzyme activity before butanol, — — — enzyme activity after butanol.

Fig. 3 Ultracentrifugation pattern of γ GT isoenzymes of hepatic bile and the effect of butanol extraction: serum protein pattern, ● — ● enzyme activity before butanol, ○ — — — ○ enzyme activity after butanol.

in the 4S fraction (Fig. 3). Electrophoresis, in polyacrylamide gel, of the increased 4S fraction after butanol showed a loss of activity at the origin and the appearance of a fraction with a mobility 55% that of albumin.

The results of this study show that on extracting bile with butanol an isoenzyme present in the bile is transformed into an isoenzyme not previously observed.

Discussion

Although the presence of γ GT in the bile has been known for some years, much uncertainty exists as to its origin. Since biliary obstruction often produces the largest increases in serum γ GT activity (Szczeklik *et al.*, 1961; Rutenburg *et al.*, 1963; Łukasik and

Richterich, 1965; Whitfield *et al.*, 1972) it is generally believed that the biliary enzyme is hepatic in origin. However, Kryszewski *et al.* (1973), having studied the effects of bile duct ligation, also considered the possibility of the bile being an excretory route for γ GT produced from non-hepatic sources.

In the present study, the γ GT activity of the bile has been shown to be heterogeneous and to consist of four isoenzyme fractions (Table 2). The study has involved the use of several protein fractionation techniques depicting various physical characteristics of protein molecules. Previous workers have described various numbers of isoenzymes which can in part be explained by the use of fewer separation techniques than we have used, although this does not settle all of the controversy. Hetland *et al.* (1975) were able to detect only one band of enzyme activity after agarose gel electrophoresis of hepatic bile; Kuska (1965), on the other hand, was able to demonstrate γ GT activity in all of the protein fractions after paper electrophoresis of hepatic bile.

In the presence of heterogeneity of enzyme activity it is quite common to relate isoenzyme activity to some physical characteristic, for example, electrophoretic mobility, molecular size, or density. In the case of biliary γ GT, however, no one physical separation technique merits choice as the basic fractionation technique, and consequently the isoenzymes are numbered somewhat arbitrarily and their properties are defined in Table 2. The isoenzymes I and III contribute equally towards the majority of the biliary γ GT activity. In the case of biliary γ GT isoenzymes it is interesting to note that no single fractionation technique demonstrates the full heterogeneity of the enzyme activity.

It has been shown by gel filtration that three of the isoenzymes (I, III, and IV) are of large molecular size; furthermore, they are excluded from a 7% polyacrylamide gel. Of the predominant two isoenzymes, ultracentrifugation studies suggest that isoenzyme III is a high molecular weight protein but isoenzyme I, while having a large molecular size, is less dense than isoenzyme III.

There are several possible explanations for the existence of isoenzymes, and in this context it is now well established that enzymes may be attached to lipid or lipoprotein components (Moss, 1962; Dunne *et al.*, 1967; Jennings *et al.*, 1970). Such an association has been demonstrated for biliary alkaline phosphatase (Price *et al.*, 1972); in this study of biliary alkaline phosphatase the effect of butanol extraction was to produce another isoenzyme with lower molecular weight and altered electrophoretic mobility, resulting from the removal of a phospholipid moiety from the biliary isoenzyme. Treatment of the biliary γ GT with *n*-butanol led to a loss of

activity, varying between 25 and 90%. This loss of activity may be due to the removal of an essential lipid component or rearrangement of the enzyme molecule on the loss of a lipid component. The loss of activity was accompanied by a change in physicochemical properties of the isoenzyme I, producing a smaller molecule, which migrated into the polyacrylamide gel on electrophoresis as in the case of alkaline phosphatase. In a subsequent study of γ GT isoenzymes in serum of patients with biliary obstruction it was noted that an isoenzyme band similar to this fraction (obtained by butanol extraction of biliary γ GT) was present.

While there are similarities in the behaviour of biliary γ GT isoenzyme I and biliary alkaline phosphatase on extraction with *n*-butanol, it is unlikely that the two enzyme molecules are derived from a single complex as the extracted isoenzymes differ in their properties. After extraction with *n*-butanol γ GT appears as a 4S protein, while alkaline phosphatase is a 7S protein. Furthermore, the biliary isoenzymes themselves are not identical, demonstrating different buoyant density ultracentrifugation characteristics, alkaline phosphatase being more dense than γ GT isoenzyme I.

There is a second major γ GT isoenzyme in bile which has the characteristics of a high molecular weight protein. Further work is required to determine the detailed nature and origin of this fraction, and the possibility that it represents an isoenzyme bound to a fragment of membrane must be considered. It has recently been shown that some biliary enzymes may be associated with membranous vesicles, and this may be the situation as far as high molecular weight biliary γ GT is concerned (De Broe *et al.*, 1975).

The results of this study show a certain degree of similarity in physicochemical properties between biliary γ GT and alkaline phosphatase; however, there are also important differences. It remains for these observations on the γ GT isoenzymes of bile to be related to isoenzymes present in the liver and in the serum of patients with biliary obstruction. This information may then lead to a better understanding of the mechanisms involved in the increased γ GT activity in the serum of patients with liver disease.

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