Serum γ -glutamyltransferase isoenzymes in extrahepatic biliary obstruction

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SUMMARY The γ -glutamyltransferase isoenzymes in the sera of patients with extrahepatic biliary obstruction have been studied, using electrophoretic, gel filtration, and ultracentrifugation techniques, and compared with those present in normal sera. Five isoenzymes were shown to exist in patients' sera, three of which were not demonstrated in normal sera. The observations are discussed in relation to the influence of biliary regurgitation and the possible solubilisation of membrane-bound enzymes. The results are compared with those of previous studies on alkaline phosphatase.

The elevation of γ -glutamyltransferase (γ GT) (E.C.2.3.2.2) activity in the sera of patients with obstructive liver disease is well documented, but the reason for the change is unknown. It has been suggested that the liver normally eliminates the enzyme into the bile, but when the bile duct is obstructed this process results in the accumulation of the enzyme in the blood (Szczechlik *et al.*, 1961; Orłowski, 1963; Rutenburg *et al.*, 1963; Lukasik and Richterich, 1965; Whitfield *et al.*, 1972). The possibility of the bile being an excretory route for γ GT produced from non-hepatic sources has also been considered (Kryszewski *et al.*, 1973).

Previous work has shown that the rise in serum alkaline phosphatase (E.C.3.1.3.1) observed in biliary obstruction may in part arise by regurgitation of the biliary enzyme (Price and Sammons, 1974). A study of the isoenzymes of γ GT in the sera of patients with extrahepatic biliary obstruction was therefore undertaken, in relation to those isoenzymes already described in human bile (Wenham *et al.*, 1978a), with a view to finding an explanation of the elevation in serum γ GT activity observed in this group of patients.

Material and methods

Sera were obtained from 10 patients with extrahepatic biliary obstruction, diagnosed at laparotomy or necropsy, and from nine laboratory staff who acted as normal controls.

Agar gel electrophoresis was performed on the Multiphor electrophoresis equipment (LKB Produkter, Bromma, Sweden) by a method already described (Wenham *et al.*, 1978a).

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

The γ GT isoenzymes fractionated by these two techniques were visualised by means of incubation with γ -L-glutamyl α -naphthylamide, coupling the liberated α -naphthylamine with Fast Blue B, by the optimised method of Wenham *et al.* (1978b). The isoenzymes were also quantitated after polyacrylamide gel electrophoresis 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 using Sephadex G200 (Pharmacia, Uppsala, Sweden) and buoyant density ultracentrifugation were both performed by methods previously described (Wenham *et al.*, 1978a), and enzyme activity in the fractions obtained was determined by the method of Rosalki and Tarlow (1974).

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Results

ELECTROPHORETIC STUDIES

Each of the patient's sera showed three zones of enzyme activity after agar gel electrophoresis, in the α_1 -, α_2 -, and β -globulin fractions. In normal sera, only two bands of activity were obtained, in the α_1 - and α_2 -globulin fractions.

Polyacrylamide gel disc electrophoresis of normal sera also resulted in two zones of activity with mobilities of 23% and 77% of albumin (R_{alb} values of 23 and 77). Sera from patients with extrahepatic biliary obstruction gave rise to four zones of activity after polyacrylamide gel electrophoresis, with R_{alb} values of 0, 23, 55, and 77. The origin band and the band with an R_{alb} value of 55 appeared to contribute the most towards enzyme activity, and this was confirmed using the quantitative technique (Fig. 1).

Fig. 1 Quantitative localisation of γ GT isoenzymes after polyacrylamide gel electrophoresis of sera from two patients with extrahepatic biliary obstruction. Enzyme activity expressed as absorbance 405 nm: --- patient CC; — patient PM.

GEL FILTRATION STUDIES

Fractionation of serum yielded three main protein peaks, as described by Flodin and Killander (1962). The normal sera each yielded two peaks of γ GT activity, eluting between the 19S and 7S and between the 7S and 4S proteins, respectively (Fig. 2). The mean recovery of enzyme activity was 95%. Three fractions were obtained from each of the sera from patients with extrahepatic biliary obstruction, one eluting with the void volume, one between the 19S and 7S, and the other between the 7S and 4S proteins (Fig. 3). In all samples studied, the first two peaks always possessed much more activity than the third, whose activity did not differ significantly from the level observed in the normal sera (Table 1). The mean recovery of enzyme activity was 98%.

Fig. 2 Gel filtration characteristics of γ GT isoenzymes in normal serum: --- serum proteins; — enzyme activity (IU/l).

Fig. 3 Gel filtration characteristics of γ GT isoenzymes in serum from a patient (PM) with extrahepatic biliary obstruction: --- serum proteins; — enzyme activity (IU/l).

Table 1 γ GT activity in different fractions obtained by gel filtration and ultracentrifugation of sera from patients with extrahepatic biliary obstruction, expressed as a percentage of total activity recovered

Sample	Gel filtration			Ultracentrifugation	
	Void volume	19S-7S	7S-4S	4S	Low density fraction
PM	48	49	3	60	40
CC	57	41	2	60	40
NS	—	—	—	36	64
MW	81	16	3	24	76
FG	82	15	3	41	59
MG	—	—	—	31	69
AM	86	9	5	14	86
AB	54	43	3	—	—
Normal sera	Zero	57-65	35-43	100	Zero

ULTRACENTRIFUGATION STUDIES

Buoyant density ultracentrifugation of normal sera showed only one peak of γ GT activity, sedimenting with the 4S proteins (Fig. 4). Enzyme recovery

Fig. 4 Ultracentrifugation characteristics of γ GT isoenzymes in normal serum: --- serum proteins; ●—● enzyme activity (IU/l).

Fig. 5 Ultracentrifugation characteristics of γ GT isoenzymes in serum from a patient (PM) with extrahepatic biliary obstruction: --- serum proteins; ●—● enzyme activity (IU/l).

averaged 95%. All of the patients' samples studied showed two peaks of γ GT activity, one sedimenting with the 4S proteins and the other floating at the top of the gradient (Fig. 5). The mean recovery of enzyme activity was 79%. The relative contributions of the 4S and the low density peaks towards total activity were not always constant (Table 1), but the activity of the 4S peak in the patients' samples was always higher than the 4S peak observed in the normal sera.

COMPARISON OF ISOENZYME CHARACTERISTICS OBTAINED BY FRACTIONATION TECHNIQUES
Fractions representing peaks of activity, obtained by gel filtration, were pooled, concentrated using Lyphogel (Hawksley and Sons Ltd, Lancing, Sussex, UK), then subjected to buoyant density ultracentrifugation, and agar and polyacrylamide gel electrophoresis. Pooled fractions obtained after buoyant density ultracentrifugation were electrophoresed without prior concentration.

The results of these investigations in patients' sera are shown in Table 2 and indicate the presence of up to five isoenzymes, of which two are present in

normal sera. They are arbitrarily numbered in Table 2 solely for ease of discussion.

Discussion

Many methods have been used for the fractionation of serum γ GT isoenzymes in hepatobiliary diseases, but little comparative work has been undertaken on the techniques employed. Our results using agar gel electrophoresis show that, in extrahepatic biliary obstruction, zones of enzyme activity are present in the α_1 -, α_2 - and β -globulin fractions. These results are in agreement with those of Miyazaki and Okumura (1972) using agar, and also with Hetland *et al.* (1975) using agarose, another medium that does not exert a molecular sieving effect.

Polyacrylamide gel electrophoresis of patients' sera produced four zones of enzyme activity, two of which were not present in normal sera (Table 2) and which together constituted almost all of the total activity. A high activity origin band was also demonstrated by Azzopardi and Jayle (1973) using polyacrylamide, and by Orłowski and Szczeklik (1967) and Kokot and Kuska (1968) using starch gel. Sephadex gel filtration of patients' sera produced

Table 2 Physicochemical characteristics of the γ GT isoenzymes in patients' sera with extrahepatic biliary obstruction

Isoenzyme	Elution from G200	Buoyant density ultracentrifugation	Electrophoretic mobility		Relative contribution towards total activity
			Agar	Polyacrylamide	
S1	Void volume	Floats	β	0	++
S11	Void volume	Floats	α_2	0	+++++
S111	19S-7S	4S	α_1	55	+++++
S1V*	19S-7S	4S	α_1	23	+
SV*	7S-4S	4S	α_2	77	+

*Present also in normal sera

three γ GT fractions, eluting with the void volume, 19S-7S, and 7S-4S protein fractions. The last two fractions were also obtained from normal sera. These results agree with the work of Orłowski *et al.* (1965), Orłowski and Szczeklik (1967), and Kokot and Kuska (1968), except that these authors claimed to have shown the 19S fraction to be present in normal sera.

After buoyant density ultracentrifugation of normal sera, all of the enzyme activity sedimented with the 4S protein fraction (Fig. 4) in agreement with Szewczuk (1966). Patients' sera, on the other hand, showed the presence of an additional low density fraction (Fig. 5).

From the results of the comparison study it was concluded that five isoenzymes could be identified in the sera of each patient, and that the increase in the total serum γ GT activity observed in these patients was due to the appearance of three isoenzymes, S1, S11, and S111 (Table 2), not normally present. Isoenzymes S11 and S111 appear to contribute equally towards most of the activity but are very different in their chromatographic characteristics. Isoenzyme S11 is a low density molecule of high molecular size, whereas isoenzyme S111 is a smaller, denser molecule. The remaining abnormal isoenzyme, isoenzyme S1, is identical with S11 in every respect except for its electrophoretic mobility on agar gel (Table 2). It may be that these isoenzymes differ only in a component conferring a different electrical charge at pH 8.6.

The high molecular size of isoenzymes S1 and S11 may arise from polymerisation of the normal liver enzyme, although this is unlikely owing to their very low buoyant density. It is much more likely that they are the result of the association of γ GT with a very low density component such as lipid or lipoprotein. Such an association has been demonstrated for alkaline phosphatase (Moss, 1962; Dunne *et al.*, 1967; Jennings *et al.*, 1970; Price and Sammons, 1974). In the case of γ GT, it has been suggested that the biliary enzyme may be associated with lipid (Wenham *et al.*, 1978a); furthermore, Beck (1978) suggested that a serum isoenzyme might be associated with a lipid component.

In a study resembling our own, Huseby (1978) concluded that the heterogeneity of γ GT found after gel filtration and electrophoresis was due to aggregates of an amphiphilic form of γ GT with lipids and other proteins. This author, studying γ GT in liver extract, bile, and a normal serum pool, showed that papain digestion and incubation with detergent each produced a smaller molecule, corresponding to isoenzymes SV and S111, respectively, in this study. The detergent effect upon biliary γ GT confirmed our previous demonstration (Wenham *et al.*, 1978a) that

extraction of biliary γ GT with *n*-butanol resulted in the production of a smaller molecule possessing identical physicochemical characteristics to isoenzyme S111 (Table 2), a process similar to that already proposed for biliary alkaline phosphatase (Price *et al.*, 1972).

We propose that isoenzyme S111 is the main liver isoenzyme, and in response to the obstruction its rate of release into the circulation is increased. It is normally present in the bile but attached to a lipid component, resulting in a larger molecule with slower electrophoretic mobility. Whether this increased rate of release into the circulation is due to an increased rate of production remains to be determined. Such a process has been ruled out in the guinea-pig (Huseby and Vik, 1978). The origin of the isoenzyme SV remains obscure. Huseby (1978) was able to produce it by incubating liver homogenate at pH 7.2 and 37°C as well as by the action of papain digestion. This enzyme therefore may well arise by *in vivo* proteolytic activity on isoenzyme S111.

Isoenzymes S1 and S11 may arise by either:

(i) regurgitation of biliary γ GT back into the circulation due to obstruction of normal bile flow. During regurgitation the biliary enzyme may become associated with a lipoprotein carrier, conferring the low buoyant densities of isoenzymes S1 and S11, similar to that proposed for alkaline phosphatase (Price and Sammons, 1974);

or:

(ii) solubilisation of liver membrane-bound enzyme into membranous vesicles by the action of accumulated bile salts. Such a process has been suggested in the case of alkaline phosphatase (De Broe *et al.*, 1975; 1978) and also γ GT during experimental obstruction in the guinea-pig (Huseby and Vik, 1978).

Present evidence cannot prove which, if not both, processes occur. Nevertheless this study has demonstrated that in obstructive liver disease a similar process occurs with respect to biliary γ GT as occurs with biliary alkaline phosphatase, the biliary enzyme appearing in the circulation in modified form. In the case of alkaline phosphatase, fewer isoenzymes have been demonstrated in the sera of patients with obstructive liver disease, and therefore it would be unwise to extend the similarities too far. However, it is well known that changes in serum γ GT and alkaline phosphatase are not necessarily the same in all patients with liver disease, and clearly other factors may contribute to the elevation of serum γ GT activity.

As the factors that affect levels of enzymes in the blood are determined, the importance of the isoenzymes of γ GT in serum will be better understood. Then the measurement of the isoenzymes of γ GT

and alkaline phosphatase may prove to be of value in the differential diagnosis of liver disease.

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