

The nature of the serum alkaline phosphatases in liver diseases

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SYNOPSIS The two main alkaline phosphatase isoenzymes in serum from patients with extrahepatic biliary obstruction have been purified. Properties of these isoenzymes, such as electrophoretic mobility, separation on gel filtration, ultracentrifugation characteristics, Michaelis constants, and sensitivity to neuraminidase have been studied and compared with the isoenzymes of liver and bile. The results show that there is an abnormal isoenzyme in the serum from these patients and that this isoenzyme is similar but not identical with the main bile isoenzyme. It is suggested that it may be associated with a lipoprotein carrier.

The estimation of serum alkaline phosphatase has been employed as a liver function test for many years although its value has often been questioned. Its specificity has, however, been improved as a result of techniques available for the differentiation of alkaline phosphatase isoenzymes.

Electrophoretic studies of serum alkaline phosphatase isoenzymes from patients with obstructive liver disease have indicated the presence of an abnormal isoenzyme that differs from that found in liver tissue (Hodson, Latner, and Raine, 1962; Chiandussi, Green, and Sherlock, 1962; Gordon, 1965; Hill and Sammons, 1967; Wieme and Demeulenaere, 1971).

The present study was undertaken to determine the nature of the serum alkaline phosphatase isoenzymes in patients with obstructive jaundice in relation to the work on bile alkaline phosphatase previously described (Price, Hill, and Sammons, 1972).

Materials and Methods

Sera were obtained from 58 patients with extrahepatic biliary obstruction diagnosed at laparotomy or necropsy, and from 20 laboratory staff who acted as normal controls. Specimens of hepatic bile were obtained from 12 patients with T-tube drainage of the common bile duct after cholecystectomy. Samples of liver and intestine were obtained from necropsy material; crude homogenates were made from these samples for use as tissue controls. Serum

was obtained from a patient with Paget's disease for use as a bone alkaline phosphatase control.

Electrophoretic studies were performed in polyacrylamide gel discs using the Shandon electrophoresis equipment. The procedure was similar to that of Smith, Lightstone, and Perry (1968), modified to include a 3.5% (w/v) polyacrylamide 'topping gel' above the 5.0% (w/v) running gel. This topping gel was employed so that the isoenzyme that remained at the origin could be separated from a nonspecific staining reaction at the top of the gel. This isoenzyme was able to penetrate the 3.5% (w/v) gel.

Cellulose acetate electrophoresis was employed using the Sepratek system (Gelman Instruments Limited, Michigan, USA) and a barbitone buffer pH 8.6 (Kohn and Feinberg, 1965).

Visualization of the alkaline phosphatase isoenzymes was attained by means of incubation with β -naphthyl phosphate and Fast Blue RR according to Smith *et al* (1968). In the case of the cellulose acetate technique the strips were incubated with substrate only and the reaction products visualized separately, to avoid heavy background staining. Quantitative localization of enzyme activity after disc electrophoresis was accomplished by cutting the gels into 0.5 cm segments and estimating the activity in the fraction homogenized in buffered substrate by the method of Kind and King (1954); 10 mM Mg^{++} was included in the reaction mixture.

Gel filtration studies were carried out with Sephadex G200 (Pharmacia, Uppsala, Sweden). The gel was prepared and equilibrated in a 2×60 cm column with 0.01 M Tris-HCl pH 7.4 buffer containing 1.0

M sodium chloride. Samples (2.0 ml) were applied to the base of the column and the proteins fractionated by upward displacement at a flow rate of 5 ml per hour. The eluate was scanned at 280 nm using a Uvicord II absorptiometer (LKB Produkter, Sweden) and fractions were collected over 20-minute periods and assayed for alkaline phosphatase activity.

Ultracentrifugation studies were carried out by a method similar to that of Pope and Cooperband (1966). A gradient ranging from 10 to 40% (w/v) sucrose in distilled water was constructed in the Spinco SW50 swinging bucket head and 0.5 ml of sample layered on top. The tubes were then spun for 18 hours at 32 000 rpm. The tubes were fractionated by upward displacement with 50% (w/v) sucrose. The absorption at 280 nm was monitored and 15 × 0.3 ml fractions were collected. The positions of the 7S and 19S proteins were detected by assaying the levels of IgG and IgM respectively in each fraction of a serum control sample. Alkaline phosphatase activity was determined on each fraction.

To purify the alkaline phosphatase isoenzymes, 10 ml aliquots of serum (mean activity 50 Kind-King units per 100 ml) were first treated with dextran sulphate according to the method of Stokes (1965). To the supernatant was added anhydrous sodium sulphate to a concentration of 17% (w/v). The resulting precipitate was separated by centrifugation. The concentration of sodium sulphate was increased in 2% (w/v) stages by the addition of more anhydrous sodium sulphate, removing the resulting precipitate at each stage by centrifugation. Alkaline phosphatase activity was found in two fractions: one in the precipitate between 19 and 25% sodium sulphate and the second in the supernatant at 25%. The precipitates containing activity were redissolved in 0.1 M Tris-HCl buffer pH 7.4 and both alkaline phosphatase fractions concentrated by reduced pressure ultrafiltration in Visking tubing at 4°C. Each fraction was separately purified by gel filtration as described above and by ion exchange chromatography (Price *et al*, 1972).

In an effort to characterize the isoenzyme fractions further, the effect of neuraminidase was investigated (Hill and Sammons, 1967). The Michaelis constants of the two fractions were also obtained and compared with that of bile isoenzymes, at a constant pH 9.8 over the range 0.5 mM disodium phenyl phosphate.

The effect of butanol extraction on the electrophoretic mobility of the isoenzyme fractions was assessed using polyacrylamide gel electrophoresis. Because of the reported findings with the bile alkaline phosphatases, thin-layer chromatographic procedures (Price *et al*, 1972) were carried out on the butanol extract from each isoenzyme fraction to identify any lipid components present.

Results

ELECTROPHORESIS

The serum alkaline phosphatase isoenzyme patterns were studied in 58 patients with extrahepatic biliary obstruction (fig 1). The main serum alkaline phosphatase isoenzyme migrated in polyacrylamide gel to a position equivalent to the liver tissue isoenzyme and in front of the bone isoenzyme. A further isoenzyme was found to be present at the origin, contributing at least 20% to the total activity. Three further isoenzyme bands were detected in much smaller quantities, contributing to less than 10% of the total activity. Two patients (g) showed a faint band of activity that migrated in a position equivalent to the intestinal alkaline phosphatase. Four patients (e) showed a faint band of activity that migrated a short distance into the 5% gel, and four patients (f) showed a faint band of activity at the junction of the 3.5 and 5% gels. This band could be abolished by heating so was assumed not to be an artefact. The presence of these minor bands bore no relation to enzyme activity or type of biliary obstruction. As previously found, the main bile isoenzyme remained at the origin (Price *et al*, 1972).

The main serum isoenzyme on cellulose acetate electrophoresis was in the α_2 -globulin region with a

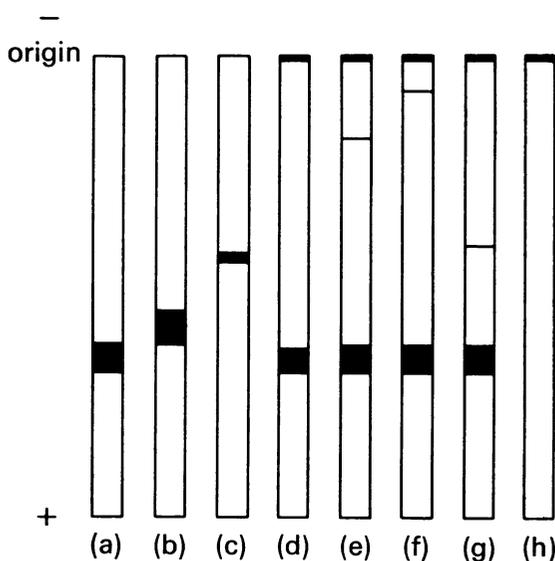


Fig 1 Separation of serum alkaline phosphatase isoenzymes in patients with extrahepatic biliary obstruction following polyacrylamide gel electrophoresis: (a) liver tissue preparation; (b) serum from patients with Paget's disease; (c) intestinal tissue preparation; (d) 48 patients; (e) four patients; (f) four patients; (g) two patients; (h) hepatic bile.

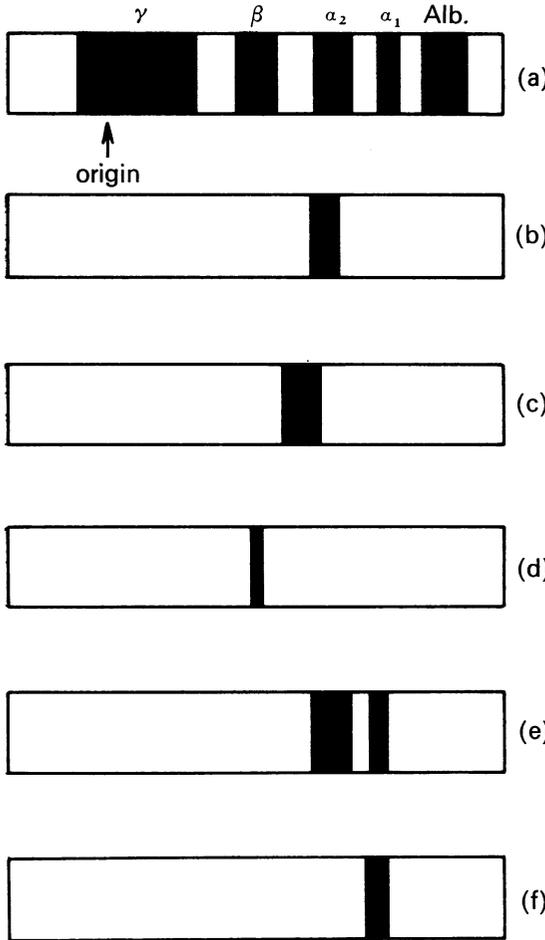


Fig 2 Serum alkaline phosphatase isoenzymes after cellulose acetate electrophoresis: (a) serum proteins; (b) liver tissue preparation; (c) serum from patients with Paget's disease; (d) intestinal tissue preparation; (e) patients with extrahepatic obstruction; (f) hepatic bile.

less prominent band in the α_1 -globulin region (fig 2). This α_1 -globulin band was found to be equivalent to the 'origin' band on disc electrophoresis. The bile samples also showed one major band of activity in the α_1 -globulin region.

GEL FILTRATION

Gel filtration fractionation of serum proteins showed three main peaks (fig 3), as described by Flodin and Killander (1962). In 20 normal people alkaline phosphatase activity was observed in the second (7S) protein peak. Studies of the control samples indicated that the liver and bone alkaline phosphatases were found solely in the 7S protein fraction. The intestinal

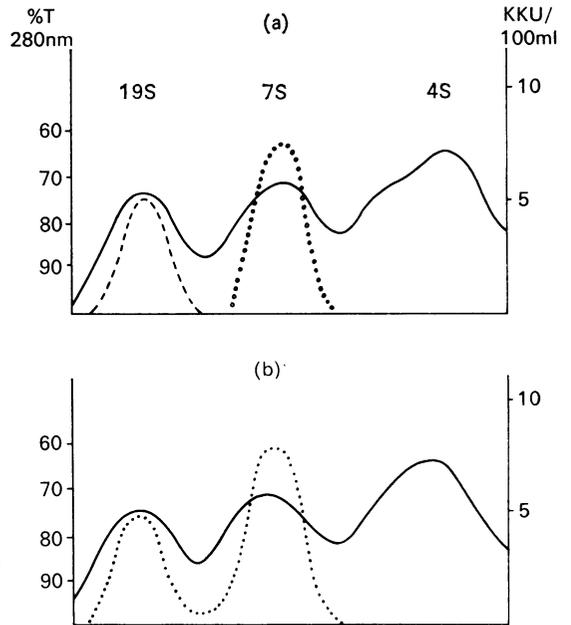


Fig 3 Gel filtration characteristics of various alkaline phosphatase isoenzymes: — Serum proteins (a); liver tissue - - - - -; hepatic bile; (b) sera from patients with extrahepatic obstruction.

alkaline phosphatase was found in the 7S peak with a shoulder on the leading edge of the peak.

In eight samples of hepatic bile the main fraction of alkaline phosphatase was found in the first protein fraction; this was found to correspond with the 'origin' isoenzyme on electrophoresis. Where the bile showed a proportion of 'liver' alkaline phosphatase a second fraction was found on gel filtration in the 7S protein fraction.

Sera from patients with extrahepatic obstruction showed two peaks of alkaline phosphatase activity equivalent to the 19S and 7S protein fractions (fig 3b). In the eight samples studied there was a good relationship between the contribution of the 'origin' isoenzymes on electrophoresis and the 19S fraction, which ranged between 12 and 42% of the total activity (table I).

ULTRACENTRIFUGATION

Ultracentrifugation of four samples of hepatic bile was performed. Only one fraction of alkaline phosphatase activity was found, in the position of the 7S proteins. In 18 samples of sera from patients with extrahepatic obstruction two fractions of alkaline phosphatase activity were observed, one in the 7S protein fraction and one floating at the top of the

Patient No.	Electrophoresis	Gel Filtration	Ultracentrifugation
1	10.4	—	17.0
2	12.9	—	15.2
3	30.9	—	31.9
4	10.8	12.4	10.2
5	33.6	32.2	34.2
6	34.2	30.4	34.8
7	23.0	31.0	32.0
8	43.6	41.4	45.5
9	40.5	37.8	35.5
10	18.3	—	18.6
11	17.4	—	16.2
12	10.4	—	9.4
13	15.2	15.0	13.6
14	9.4	—	8.0
15	24.1	—	23.0

Table I Comparison of quantitative results for the origin isoenzyme on electrophoresis, 19S fraction on gel filtration, and upper fraction on ultracentrifugation, of sera from patients with hepatobiliary disorders¹

¹The enzyme activity has been expressed as a percentage of the total serum activity.

sucrose gradient. The floating fraction after ultracentrifugation on electrophoresis was found to correspond with the 'origin' isoenzymes previously observed (table I). Quantitative distribution showed that this fraction contributed 12-15% to the total alkaline phosphatase activity (fig 4).

COMPARISON OF SEPARATION TECHNIQUES

Several samples were studied by the electrophoretic technique with quantitative localization, the gel

filtration and ultracentrifugation techniques. The quantitative distribution of the main isoenzyme fractions is compared in table I and shows that there is close agreement between the estimates of the abnormal serum isoenzyme activity separated by the three techniques described.

PURIFICATION AND PROPERTIES OF ISOENZYMES

In view of the association of the bile alkaline phosphatase isoenzymes with a lipid component (Price *et al*, 1972) attempts to purify the serum isoenzymes initially involved a delipidation stage. It was found that treatment of serum with dextran sulphate resulted in no loss of activity or change in electrophoretic mobility. This technique was followed by salt fractionation and ion exchange and gel filtration chromatography. These procedures resulted in a 56% recovery of enzyme with a 123-fold increase in specific activity of the 'origin' isoenzyme and a 162-fold increase in specific activity of the 'liver' isoenzyme.

Ultracentrifugation and electrophoretic studies were carried out at each stage of the purification; after sodium sulphate precipitation these studies were confined to the fraction precipitating between 19 and 25% sodium sulphate. The results shown in table II indicate a change in the density of the molecule without change in mobility after DEAE cellulose chromatography.

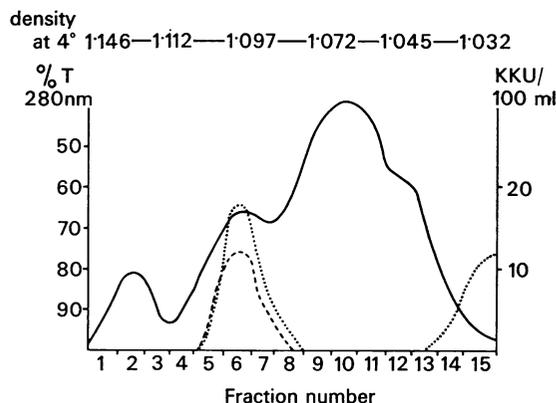


Fig 4 Ultracentrifugation characteristics of serum and bile alkaline phosphatase isoenzymes: — serum proteins, - - - - - hepatic bile, serum from patients with extrahepatic obstruction.

Stage of Purification	Electrophoretic Mobility	Ultracentrifugation Characteristics
After sodium sulphate 19-25%	'Origin'	Top of gradient
After DEAE cellulose chromatography	'Origin'	7S Fraction
After gel filtration	'Origin'	7S Fraction

Table II Electrophoretic mobility and ultracentrifugation characteristics of the serum alkaline phosphatase isoenzyme found at the origin after electrophoresis

Extraction of the purified isoenzyme preparations with butanol caused no change in the liver isoenzyme. There was no change in activity of the 'origin' isoenzymes but a change in mobility was noted to a position equivalent to that of the liver isoenzyme. Extraction of the bile 'origin' isoenzyme, as previously reported, resulted in not only a similar change in electrophoretic mobility but also an overall increase in activity.

Incubation with neuraminidase caused a decrease in the electrophoretic mobility of the faster serum isoenzyme; this corresponded to the result found using the liver tissue alkaline phosphatase. The mobility of the alkaline phosphatase remaining at the origin was not altered by neuraminidase. However, when this origin fraction was extracted with butanol and then incubated with neuraminidase it showed a decrease in mobility identical to that of the liver alkaline phosphatase. These results are shown in table III and are expressed as the ratio of the distance moved by the test (incubated with neuraminidase) over the distance moved by the control (incubated with inactivated neuraminidase). Treatment of the 'bone' alkaline phosphatase from the serum of a patient with Paget's disease showed a much greater decrease in electrophoretic mobility.

The apparent K_m values were calculated at a pH of 9.8. The results for various isoenzyme fractions are shown in table IV. These data represent the mean of results on at least six isoenzyme preparations in each case. The K_m values were determined from Lineweaver Burke plots by the method of least squares regression. It can be seen that the K_m of the faster bands of serum and bile and the 'origin' isoenzyme

of serum differ markedly from the 'origin' isoenzyme of bile. Butanol extraction of the bile 'origin' isoenzyme resulted in a decrease in K_m value but there was no change in the K_m value of the extracted serum 'origin' isoenzyme.

The thin-layer chromatography carried out on the butanol extracts of the two serum isoenzyme preparations indicated the presence of a phospholipid in the 'origin' isoenzyme extraction solvent. Further chromatography in a second solvent system showed this component to be a phosphatidylcholine. The other isoenzyme solvent extract yielded no detectable lipid component. In order to ascertain whether the phosphatidylcholine was a normal finding, a sample of normal serum was fractionated in a similar manner to the abnormal samples and the resulting preparation, containing no alkaline phosphatase, was extracted with butanol. The solvent phase did not contain any detectable lipid component.

Discussion

Few attempts have been made to correlate the data obtained by different techniques for the differentiation of serum alkaline phosphatase isoenzymes in liver disease. Our results using cellulose acetate electrophoresis show that in obstructive liver disease the main enzyme activity is found in the α_2 -globulin fraction with lesser activity in the α_1 -globulin fraction; the latter fraction is not found in normal control serum. These results are in agreement with previous results employing electrophoretic techniques relying mainly on the net charge on the protein molecule, and using, for example, paper (Baker and

Samples	Mobility (cm)		
	Control	Test	Ratio T/C
Main serum isoenzyme	9.4	6.9	0.74
'Origin' serum isoenzyme	0.1	0.1	1.00
Liver tissue isoenzyme	9.7	7.2	0.74
Serum			
Paget's disease isoenzyme	9.5	5.0	0.53
'Origin' isoenzyme after butanol treatment	9.4	6.8	0.72

Table III Effect of neuraminidase on the electrophoretic mobility of various alkaline phosphatase isoenzymes

	Mean Apparent K_m Value (mM)			
	Bile		Serum	
	'Fast' Band	'Origin' Band	'Fast' Band	'Origin' Band
Constant pH 9.8	2.04	2.63	2.00	1.94
Constant pH 9.8 after butanol extraction	2.02	2.04	2.02	1.94

Table IV Apparent K_m values of purified bile and serum alkaline phosphatase isoenzymes determined at a constant pH

Pellegrino, 1954), starch block (Rosenberg, 1959; Keiding, 1969), cellulose acetate (Korner, 1962), and agar gel (Wieme and Demeulenaere, 1971).

Using polyacrylamide gel electrophoresis we found that in patients with obstructive liver disease the serum alkaline phosphatase showed a gross increase in the fast liver isoenzyme fraction with the appearance of several other isoenzyme fractions, the major contribution remaining at the origin. Our results are in agreement with those of other workers who employed similar techniques which included molecular sieving superimposed on the true electrophoretic mobility, for example, starch gel (Kowlessar, Pert, Haeflner, and Slesinger, 1959; Moss, Campbell, Anagnostou-Karakas, and King, 1961; Chiandussi *et al*, 1962; Hodson *et al*, 1962; Taswell and Jeffers, 1963; and Hill and Sammons, 1967) and polyacrylamide gel (Jennings, Brocklehurst, and Hirst, 1970; Walker and Pollard, 1971).

Our studies suggest that the isoenzyme migrating with the 'liver' fraction on polyacrylamide gel is equivalent to the β -globulin fraction on starch gel and the α_2 -globulin fraction on cellulose acetate. Furthermore, the results suggest that the isoenzyme remaining at the origin on acrylamide gel is identical with the 'origin' and α_1 -globulin fractions of starch gel and cellulose acetate respectively. The main bile isoenzyme on cellulose acetate electrophoresis was found in the α_1 -globulin fraction and at the origin using polyacrylamide gel electrophoresis. We conclude from these findings that the abnormal serum isoenzyme differs significantly from the main 'liver' alkaline phosphatase isoenzyme not only in terms of net charge but also in terms of size. However, it appears to behave similarly with respect to electrophoretic mobility to the main bile isoenzyme.

Using gel filtration, the liver tissue isoenzyme as well as the bone and intestinal isoenzymes were found in the 7S protein fraction. In the majority of bile samples the alkaline phosphatase activity was found mainly in the 19S protein fraction although a second fraction was occasionally found with the 7S proteins. When all of the alkaline phosphatase activity was found in the 19S protein fraction it was observed that only the 'origin' isoenzyme was found after polyacrylamide gel electrophoresis; the 7S protein fraction containing enzyme activity had the mobility of 'liver' alkaline phosphatase on electrophoresis.

Our gel filtration studies of sera from patients with liver diseases confirmed those of previous workers by showing that in general there are two alkaline phosphatase isoenzyme fractions, the major peak of activity being present in the 7S protein fraction with small amounts in the 19S fraction (Dunne, Fennelly, and McGeeney, 1967; Jennings

et al, 1970). On polyacrylamide gel, electrophoresis of concentrated 7S and 19S alkaline phosphatase fractions showed electrophoretic mobility identical with the 'liver' and 'origin' isoenzymes. It can be seen from table I that the contributions of the 'origin' isoenzyme by quantitative localization following electrophoresis agreed well with the contributions of the 19S alkaline phosphatase fractions to the total activity.

The present work confirms the findings of Pope and Cooperband (1966) who, using an ultracentrifugation technique, observed that the bile alkaline phosphatase isoenzymes and normal serum isoenzyme float with the 7S proteins. These authors further suggested that the abnormal serum isoenzyme was also found with the 7S proteins. Our results, however, show that the abnormal serum isoenzyme has a much lower density than the 'liver' isoenzyme as it was found to float to the top of the sucrose gradient. The quantitative distribution studies, shown in table I, indicate that the contribution of this fraction to the total activity is very similar to that of the 'origin' isoenzyme on electrophoresis.

Hill (1967) first reported that extraction of bile with n-butanol resulted in an overall increase in alkaline phosphatase activity. This was accompanied by a change in electrophoretic mobility of the 'origin' isoenzyme to the β -globulin position on starch gel. It was subsequently found that extraction of sera from patients with liver disease did not result in an overall increase in enzyme activity but did result in a change in electrophoretic mobility of one isoenzyme from the origin to the β -globulin position. Jennings *et al* (1970) and Walker and Pollard (1971) also found that butanol extraction of an abnormal serum isoenzyme resulted in a change in its electrophoretic mobility. No form of quantitation was, however, included in these studies.

The work presented shows that there are many similarities between the main bile isoenzyme and the abnormal serum 'origin' isoenzyme. However, certain fundamental differences do exist which are apparent from the ultracentrifugation data. Results show that the abnormal serum 'origin' isoenzyme is less dense than the main bile isoenzyme (fig 4) but the density increases during purification by ion exchange chromatography (table II). Butanol extraction of the two slow moving isoenzymes results in identical changes in electrophoretic mobility, the resulting isoenzymes being identical in every respect studied. However, there appears to be a decrease in the K_m value at pH 9.8 of the bile isoenzyme to a value similar to that of the serum 'origin' isoenzyme, which is unaltered by extraction.

The changes in ultracentrifugation characteristics

during purification suggest that a 'light' component is split from the enzyme molecule during ion exchange chromatography. The enzyme molecule so produced is then virtually identical with the main bile isoenzyme in its electrophoretic mobility and behaviour with butanol. The differences in Km value of the two 'origin' isoenzymes may reflect the combination of the serum isoenzyme with another component, possibly lipoprotein.

There are several possible theories to explain the presence of this abnormal isoenzyme in the serum. In the first place, this isoenzyme could represent a polymerization of the normal isoenzyme, but this would seem unlikely in view of the low buoyant density of the molecule relative to the normal enzyme. By the same reasoning it is unlikely that the abnormal enzyme reflects linkage of the normal enzyme to another protein. Secondly, the possibility of an enzyme-carbohydrate complex is probably ruled out by the neuraminidase experiments and the insensitivity of the isoenzyme to this treatment.

The concept of lipoprotein carriage of an enzyme has been suggested by many workers (Moss, 1962; Latner, 1966; Dunne *et al*, 1967; Jennings *et al*, 1970). The low buoyant density of the abnormal serum isoenzyme would support the idea of lipoprotein carriage; ion exchange chromatography may remove this carrier. This is the first time that the nature of the carried enzyme has been suggested. Our work has shown that a lipid component has been extracted from the purified abnormal serum isoenzyme which is identical to the lipid component of the main bile isoenzyme. Furthermore, the extracted serum isoenzyme shows identical characteristics to the extracted bile isoenzyme.

Taking all these facts into account, therefore, our results indicate that in obstructive liver disease the following situation exists with respect to the raised serum alkaline phosphatase activity. There are two main isoenzymes present: one is an α_2 -globulin derived directly from the liver cells, which contributes the major fraction. The second isoenzyme is present in smaller proportions and represents the regurgitation of bile alkaline phosphatase as a result of obstruction to the normal flow of bile. The abnormal serum isoenzyme probably represents the 'bile' isoenzyme, a 'lecithin-liver isoenzyme complex' (Price *et al*, 1972) associated with a lipoprotein carrier thus increasing its effective size and affecting its electrophoretic mobility in certain media. The source, site, and method of addition of this lipoprotein carrier remain unknown. These observations have nevertheless allowed a pattern of isoenzyme changes to be determined and followed in

liver diseases. These results will be the subject of a further communication.

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