Polyacrylamide gel disc electrophoresis of alkaline phosphatase isoenzymes in bone and liver disease

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SYNOPSIS Acrylamide gel disc electrophoresis provides a reliable and reasonably rapid method of differentiating the raised serum alkaline phosphatase (AP) of bone origin from that of liver origin. The technique has been placed for the first time on a semiquantitative basis. Measurement of both band width and band position effectively distinguishes the bone from the liver isoenzyme, but band width provides superior discrimination.

An origin band was seen in none of the normal subjects and in only 7% of patients with bone disease but was present in 78% of patients with liver disease, a highly significant increase. Fifty percent of normal individuals had a small-intestinal band in serum taken two hours after a meal, as did 35% of patients with liver disease, but the incidence of intestinal bands in bone disease was only 11%, significantly less than in the other two groups. The genetic control of small-intestinal AP in serum has been confirmed, but it has been demonstrated that the decrease of intestinal AP in bone disorders is not genetically determined.

Following the first demonstration that serum alkaline phosphatase (EC 3.1.3.1) exists in more than one form, many attempts have been made to determine the organ source of this enzyme in health and disease by means of electrophoresis on a variety of media including paper, agar gel, cellulose acetate, starch gel, Pevikon C-870, and, more recently, acrylamide gel (Warnes, 1972). Most of these electrophoretic techniques reveal bone and liver components which are thought to comprise the main band, together with a third isoenzyme which is present in some individuals and which is identical with intestinal alkaline phosphatase, while a fourth isoenzyme which remains at the origin is frequently found in patients with liver disease. The results obtained by different techniques have often been conflicting. Thus some workers have claimed that the differentiation between bone and liver alkaline phosphatase can be made on the basis of the minor bands (Chiandussi *et al*, 1962). In contrast, other workers have claimed to make the distinction between bone and liver enzymes by the position of the major bands, the liver band running slightly ahead of the bone band (Newton, 1967). The recent advent of polyacrylamide gel electrophoresis has been claimed to produce a clear and constant difference in both mobility and shape between the bone and liver

bands (Smith *et al*, 1968; Walker and Pollard, 1971). However, even the use of this technique has yielded conflicting results. Kaplan and Rogers (1969) reported that, in their series, four patients with bone disease alone had a liver type main band, and in two of these there was no reason to suspect liver disease, while Sussman *et al* (1968) found that the AP isoenzymes of liver, bone, kidney, and intestine all moved with indistinguishable mobilities. More recently, Afonja and Baron (1974) found that normal adult serum always contained a slow 'liver' band and sometimes a bone band. Many of the discrepancies are accounted for by the fact that interpretation of the results obtained by electrophoretic separation of the alkaline phosphatase isoenzymes in serum has until now been largely subjective.

We have therefore placed the technique of acrylamide gel disc electrophoresis of serum alkaline phosphatase isoenzymes for the first time on a semiquantitative basis. This has permitted the results obtained to be analysed statistically. Although intestinal alkaline phosphatase has been identified in serum electrophoretically, the incidence of gut bands in health and disease is a subject of controversy. Kaplan and Rogers (1969) found an intestinal band in fasting serum in only 2 out of 135 patients with a variety of bone and liver disorders, while Langman *et al* (1966) reported that up to 60% of normal

human sera contained a small-intestinal band on electrophoresis. The finding of raised levels of intestinal alkaline phosphatase in serum has been reported in cirrhosis of the liver (Fishman *et al*,1965), but estimation of this isoenzyme in osteoblastic bone disease has not previously been reported.

Material and methods

The normal group comprised 28 healthy medical students with a mean AP of 4·8 King-Armstrong (KA) units/dl (table I). The liver disease group, which comprised 68 patients with proven liver disease and a raised serum AP, had a mean AP of 35·5 KA units/dl. The diagnosis of liver disease was based on clinical and laboratory findings, supplemented in most cases by liver biopsy, laparotomy or necropsy findings. The bone disease group consisted of 27 patients with a variety of osteoblastic bone disorders who had a mean AP of 35·2 KA units/dl. Several months after the electrophoresis had been completed, the hospital records were reviewed and the definitive diagnoses were recorded.

All blood samples were taken two hours after a hospital meal containing between 20 and 40 g fat. The assay procedure for alkaline phosphatase was the Kind-King modification of the King-Armstrong method (Kind and King, 1954). For a single estimation the 95% confidence limits were activity ± 2-3%.

Samples of serum were subjected to disc electrophoresis in a Shandon electrophoresis tank (Shandon Scientific Co Ltd, London) using a 5% polyacrylamide gel and a continuous buffer system (tris-borate buffer, pH 9·5), the method being basically that of Smith *et al* (1968). A 100 μl aliquot of serum was mixed with 200 μl of 20% sucrose, and 20 μl of this mixture was applied to the gel. Electrophoresis was

continued until the bromphenol blue-albumin complex of a reference serum had reached the end of the index gel, the length of which was approximately 65 mm. The enzyme bands were located by a diazo-coupling technique, using β-naphthyl phosphate and Fast Blue BB. Band width was measured on serum diluted to give an AP value of 10-15 KA units/dl since preliminary observations showed that for any given serum an increased AP level resulted in an increase in band width. Each serum was also run undiluted, and the position of the bands on the gel was measured in millimetres from the origin. All measurements were made with a ruler to the nearest 0·5 mm. Band width and position were then expressed as a percentage of the length of the index gel, so producing modified 'R_f' values which could then be subjected to statistical analysis. The coefficient of variation for duplicates within a run was 1·6%, and between runs was 2·0%. All observations on intestinal AP were made on the undiluted sera.

The nature of the origin band was further investigated by gel filtration using Sephadex G200. Two-millilitre samples of serum obtained from a patient with obstructive jaundice were applied to a column 2·5 cm × 40 cm which was equilibrated with a tris-saline buffer, pH 8·0 (Tris 50 mmol/l, NaCl 100 mmol/l, adjusted with HCl 1 mol/l). Two-millilitre fractions were collected at a flow rate of 30 ml per hour, and the fractions were first assayed for alkaline phosphatase activity and then subjected to electrophoresis.

The ABO blood group and secretor status of each individual were determined by standard techniques (Dunsford and Bowley, 1967; Dacie and Lewis, 1968).

Results

On acrylamide gel disc electrophoresis intestinal and origin bands were seen in addition to the main band (fig 1). The main band seen in bone disease was diffuse and ran slightly behind the more compact liver band, while two main bands were always found in normal serum.

The main liver band was significantly faster than the bone band (table II); the faster band of normal serum (band I) did not differ significantly in position from the main liver band, and the slower band (band II) did not differ significantly in position from the bone band. The difference in width between the bone and liver bands was highly significant. Although there was a significant difference in both mean position and mean width, there was considerable overlap as regards position between the bone and liver bands (fig 2). Band width was a better dis-

Group	No.	Mean AP ± 1 SD
Normal	28	4·8 ± 1·4
Liver[1]	68	35·5 ± 28·2
Bone[2]	27	35·2 ± 22·3

Table I *Composition of the normal, liver, and bone disease groups*

AP results are in King-Armstrong units/dl

[1]Cirrhosis of the liver (6), hepatitis (8), stones in the common bile duct (4), carcinoma of the extrahepatic bile ducts (2), primary carcinoma of the liver (3), secondary carcinoma of the liver (10), carcinoma of the gall bladder (1), carcinoma of the head of the pancreas (8), primary biliary cirrhosis (9), infiltrations of the liver (3), intrahepatic cholestasis (8), cholangiohepatitis (4), and gallstones (2)

[2]Composed of 14 with Paget's disease of bone diagnosed on *x*-ray, 7 with osteomalacia diagnosed by *x*-ray and bone biopsy, 3 with rickets diagnosed clinically, biochemically and radiologically, 2 with hyperparathyroidism confirmed at operation, 1 with renal osteodystrophy
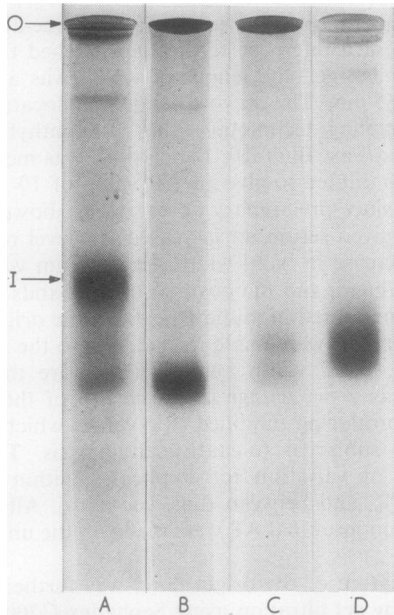
Fig 1 *Electrophoresis of serum alkaline phosphatase:* (A) *liver disease, main band with intestinal band* (I); (B) *liver disese, main band with origin band* (O); (C) *normal serum;* (D) *bone disease. The band at the origin for normal serum* (C) *is a non-specific protein band and not AP.*

	Position	Width
Liver disease	60·3 ± 3·4	3·3 ± 0·9
Normal (band I)	61·4 ± 3·0	
Normal (band II)	56·7 ± 2·6	
Bone disease	57·1 ± 2·5	8·0 ± 1·9
Student's t test		
Liver v bone	$p < 0.001$	$p < 0.001$
Liver v normal I	NS	
Bone v normal II	NS	
Liver v normal II	$p < 0.001$	
Bone v normal I	$p < 0.001$	

Table II *Band width and position for normal, bone, and liver groups*

Results are given as mean ± 1 SD

criminant since there was virtually no overlap between bone and liver.

INTESTINAL BANDS

Fifty per cent of normal individuals were found to have a small-intestinal band in serum taken two hours after a meal, as also did 35 % of patients with liver disease (table III). However, in the bone disease group the incidence of gut bands was only 11 %, which is significantly less than in the other two groups.

ORIGIN BANDS

Table III also shows the incidence of origin bands in the three groups. The origin band was absent in all normal subjects, and was found in only 7 % of patients with osteoblastic bone disease. It was, however, present in 78 % of the patients with liver disease, a highly significant increase compared with the other two groups.

GEL FILTRATION

When serum from patients with liver disease was subjected to gel filtration, two peaks of AP activity were obtained. One eluted in the same peak as the 19S proteins and the other in the same peak as the 7S proteins. Subsequent electrophoresis of the two

peaks of activity showed the 19S protein-like peak to contain the origin band while the 7S protein-like peak contained the main liver band (fig 3).

BLOOD GROUPS

The influence of the ABO blood group of a normal individual in determining the level of AP in his serum is seen in table IV. Normal subjects of blood group O or B have higher total levels of AP than those of blood group A, while within a given blood group there was a tendency for individuals who were secretor positive to have higher levels of AP than those who were secretor negative, the mean for group O secretor positive being 5·7 and for group O secretor negative 4·4 KA units/dl. On electrophoresis the higher levels of AP were associated with a higher incidence of intestinal bands, all seven subjects of blood group O who were secretor positive having an intestinal band. In five of these the band was of medium intensity, and in two it was strong. In contrast, of the 10 normals who were group A secretor positive, only three showed an intestinal band.

In the bone group, however, genetic control appeared to be lost, since patients with blood group O had a *lower* mean AP than those of blood group A, and even when groups O and B were combined there was no significant difference between the mean AP of the combined group and that of patients with bone disease who were blood group A (table IV).

The distribution of blood groups observed in the normal, bone, and liver groups is summarized in table V. These figures are compared with the numbers which would be expected for the UK population (Kopec, 1970). It can be seen that in each group there is no significant difference between the observed and the expected values.

Discussion

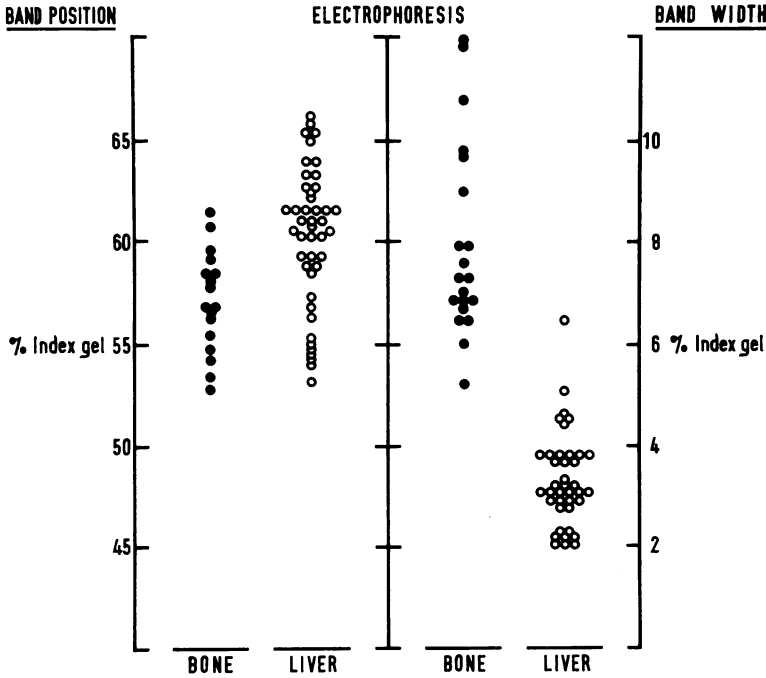This work helps to explain the discrepancies in the

BAND POSITION ELECTROPHORESIS BAND WIDTH

Fig 2 *Band width and position on electrophoresis.*

Group	No.	Intestinal Bands		Origin Bands	
		No. Obs.	Percent	No. Obs.	Percent
Normal	28	14	50	0	0
Bone	27	3	11	2	7
Liver	68	24	35	53	78
Student's t *test*					
Normal v bone		P < 0·01		NS	
Normal v liver		NS		P < 0·001	
Bone v liver		P < 0·05		P < 0·001	

Table III *Incidence of intestinal and origin bands in the normal, liver, and bone groups*

results reported by different workers in that we have shown that both the main band and the origin band are of value in differentiating the elevated serum AP of osteoblastic bone disease from that of hepatic origin. Both the width and the position of the main band effectively separate the bone and liver iso-enzymes, but band width appears to provide superior discrimination. Our results also suggest that in normal serum both bone and liver AP are present, the fast band (I) arising from liver and the slow band (II) from bone.

Our gel filtration results confirm the previous finding of Dunne *et al* (1967) that the origin band consists of a large 19S protein-like molecule which is fairly specific for liver disease. This macromolecule may be a complex formed by the linkage of an AP
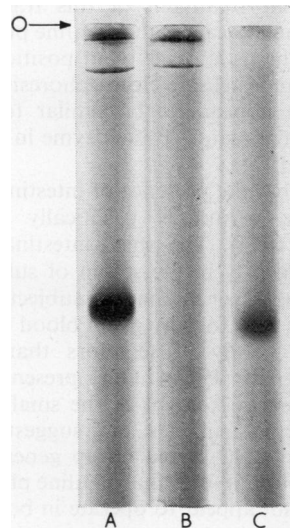
isoenzyme to lipoprotein (Moss, 1962), or it may represent a polymer, since it has been shown that precipitation of the lipoproteins from serum using

Fig 3 *Electrophoresis of the two peaks of alkaline phosphatase activity obtained by gel filtration in liver disease:* (A) *before gel filtration;* (B) *after gel filtration: 19S peak;* (C) *after gel filtration: 7S peak* (O *marks the origin*).

Blood Group	Normal		Bone	
	No.	Mean AP	No.	Mean AP
O	11	5·37	8	21·79
B	4	5·70	2	41·15
AB	2	4·50	1	38·00
A	14	4·18	6	32·57
t test	O v A	P < 0·05	O v A not significant	
			(O + B) v A not significant	

Table IV   Influence of blood group on AP levels in normal and bone groups

(Three normals are included here whose sera were not electrophoresed) AP results are in King-Armstrong units/dl.

	Group					
	O	A	B	AB	Total	x^2
Normals						
Observed	11	14	4	2	31	
Expected	14·5	12·9	2·7	0·9	31·0	2·909 NS
Bone						
Observed	8	6	2	1	17	
Expected	7·9	7·1	1·5	0·5	17·0	0.8384 NS
Liver						
Observed	32	22	5	4	63	
Expected	29·4	26·3	5·4	1·9	63·0	3·2837 NS

Table V   Comparison of observed and expected distribution of blood groups in the normal, bone, and liver groups

dextran sulphate has little effect on the activity of this fraction (Dunne et al, 1967). Other workers have shown that treatment of this fraction with n-butanol frequently causes the alkaline phosphatase to migrate to the usual liver band position on subsequent acrylamide gel slab electrophoresis (Jennings et al, 1970). It appears to be similar to, but not identical with, the main AP isoenzyme in bile (Price and Sammons, 1974).

It is known that the presence of intestinal alkaline phosphatase in serum is genetically controlled (Arfors et al, 1963). The small-intestinal band is seen more frequently in the serum of subjects who are blood group O or B than in subjects who are blood group A, and for any given blood group it is seen more frequently in secretors than in non-secretors (Warnes, 1972). Our present findings illustrate the genetic control of the small-intestinal enzyme in normal subjects but suggest that the influence of the ABO blood group genes in determining the amount of intestinal alkaline phosphatase in serum does not appear to operate in bone disease (table IV). Since, in contrast to normal subjects, patients of blood group A did not have significantly lower levels of alkaline phosphatase than did patients with blood groups O and B, the mechanism of the reduction in intestinal AP in osteoblastic bone

disease is obscure; it may be that an increased secretion of an isoenzyme from one body source can 'switch off' another source of the enzyme. This hypothesis is not unreasonable since it has been shown that disease may alter the rate of synthesis of AP at sites remote from the primary disease process (Sussman, 1970). It is noteworthy in this context, however, that there was no significant difference in the mean AP of the bone and liver disease groups, and that no corresponding decrease in the incidence of intestinal bands was found in liver disease. Furthermore, the decreased incidence of intestinal bands in bone disease is unlikely to be genetically determined since the distribution of blood groups in the patients with bone disease was the same as in the normal subjects and patients with liver disease, and the observed distribution of blood groups within the bone disease group did not differ from the predicted distribution (table V).

Small-intestinal alkaline phosphatase passes into the blood stream after a fatty meal, and blood samples in all three groups were therefore taken at a standard time of two hours after a meal. Consequently it is unlikely that the decreased incidence of intestinal bands in bone disease is of dietary origin. Since intestinal alkaline phosphatase is known to play an active role in the absorption of oleic acid (Linscheer et al, 1971) it would be of interest to know whether the absorption of long chain fatty acids is reduced in patients with osteoblastic bone disorders. The finding of decreased amounts of small-intestinal alkaline phosphatase in patients with osteoblastic bone disorders is of great interest in view of the recent discovery that the intestinal isoenzyme is concerned in calcium absorption (Haussler et al, 1970). The intestinal tract of many species is known to contain a phytate-splitting enzyme, which is probably non-specific alkaline phosphatase, the activity of which depends on cholecalciferol status (Wills, 1973). Since the presence of phytic acid in the diet reduces the availability of calcium for absorption by the precipitation of insoluble calcium salts within the lumen of the intestine, and phytic acid binds calcium as a complex (Søgnen, 1964), our finding of low levels of intestinal alkaline phosphatase in serum in osteoblastic bone disease may imply an associated malabsorption of calcium in these patients due to inability to split phytate.

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