ALKALINE PHOSPHATASE ISOENZYMES IN SERUM AND TISSUE

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Alkaline phosphatase was one of the first enzymes to be explored in human disease. Although its considerable clinical significance is well known, most fundamental questions concerning the enzyme in normal and pathological states are still unanswered. During the the past few years newer techniques of separation have shown that so-called alkaline phosphatase actually consists of multiple enzymes that have similar functions though they can be separated because of differences in molecular charge. Workers have investigated these isoenzymes (or isozymes) in different states of health and disease, but there is no unanimity on their presence or significance. The present study was undertaken to explore the different patterns in serum and tissue with the technique of starch-gel electrophoresis.

Method

The method used allows accurate qualitative separation and a crude quantitative assessment of the isoenzymes of alkaline phosphatase. Vertical starch gel electrophoresis[1] was performed using a gel buffer of 0·076 M tri (hydroxy-methyl)-amino-methane and 0·005 M citric acid (pH 8·65) and an electrode buffer of 0·3 M boric acid and 0·05 M sodium hydroxide.[2] After electrophoresis for 16 hours at 4 v./cm. gel length the gel was sliced and one-half stained with papers impregnated with α naphthyl phosphate sodium and diazo-O-dianisidine.[3] Monophosphatase isoenzymes released α naphthol, which coupled with the diazo salt of O-dianisidine to form purple bands. The other half of the gel was stained with amidoschwarz to give a protein marker system. After 4 hours' incubation at room temperature the position and approximate intensity of the different bands were recorded.

Preparation of specimens. Serum was used freshly separated or after storage at 4°C. It was found that the enzyme was stable and no new bands appeared when the specimens were kept for more than 3 months at 4°C. Haemolysis and jaundice did not interfere with demonstration of enzyme activity.

Tissue extracts were prepared after washing postmortem material, usually taken on the day after death, till free of blood. Mechanical homogenization of 4 G of tissue in 4 ml. of $NaHCO_3$ - Na_2CO_3 buffer (pH 9·7) and separation of the supernatant fraction gave extracts with satisfactory

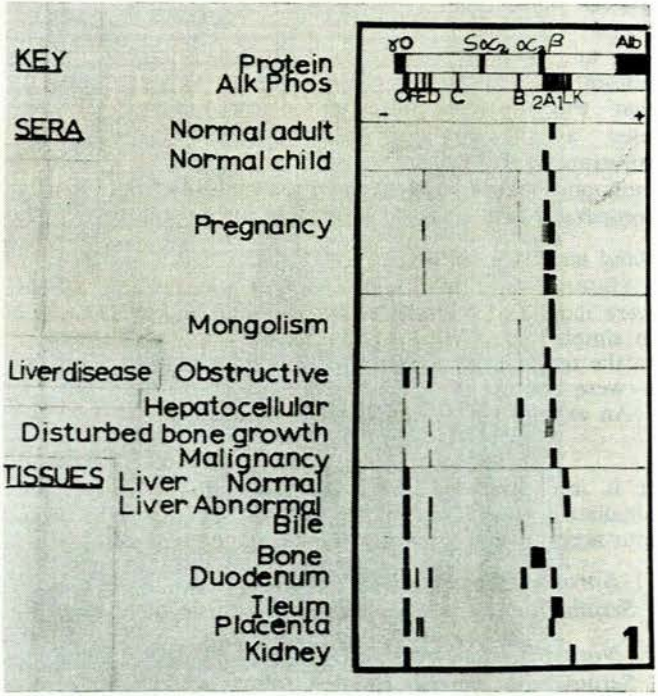

Fig. 1. Alkaline-phosphatase isozymes. The fainter lines represent zones that are variably present.

activity. Estimation of activity allowed specimens to be prepared with activities in the range of serum specimens. One-fifteenth of a millilitre of 22% bovine albumin (pH 7·5) was added to about 2 ml. of extract to facilitate electrophoresis. Controls with serum and with tissue extract alone showed no artefact owing to the use of the albumin.

Comments on Experience

Staining papers more than 3 - 5 days old was not found satisfactory. No benefit in enzyme demonstration was gained by the addition of Zn^{++} or Mg^{++}.

Alkaline phosphatase activity was quantitatively measured by the auto-analyser with a modification of one of the King-Armstrong techniques. Phenyl disodium phosphate was hydrolized by the enzyme at 37°C and the liberated phenol condensed with 4-amino - antipyrine. Alkaline potassium ferricyanide oxidized this to give a red product measured colorimetrically at 505 mμ. The results were quoted as King-Armstrong units/100 ml. It is important to note that this method uses a different substrate for the monophosphatase activity and is therefore not strictly comparable with that demonstrated on the gel.

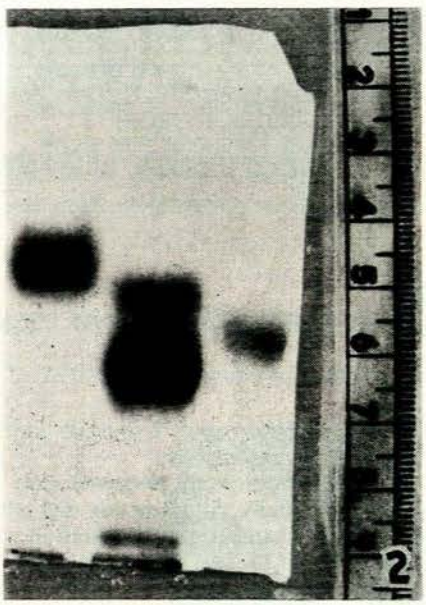

Fig. 2. An example of part of a stained gel. The specimens show (starting from the right) adult bone extract, broad zone extending from A_2 region up to B; duodenum showing A B D F O; ileum showing A and O activity.

Band and Nomenclature

Unfortunately there is no standard nomenclature. Zones were named alphabetically as shown in Fig. 1. In order to simplify matters, two rare zones — A_3 (slightly closer to the origin O than A_2) and ante-D (between C and D) — were omitted from the diagram.

An example of a stained gel is shown in Fig. 2.

RESULTS

It is important to appreciate that different results are obtained with different buffer systems. This must be taken into account in comparing results of different authors.

1. *Normal Adult Serum*

Serum from normal adults shows a single zone, A_1.

2. *Normal Child Serum*

Serum from normal children shows a single zone, A_2, clearly distinct from A_1. This zone was present in the cord blood of all 14 neonates examined. All normal children up to the age of 14 also showed this zone. In children over 14, and in 4 patients under 14 with abnormalities of bone growth, the A_1 zone was present.

The A_2 zone is therefore regarded as the alkaline phosphatase of normal youth. In the neonates, direct comparison with placental extract in 4 cases showed a distinct difference in the A zone. The placental zone was A_3, whereas the maternal serum showed A_1 or A_2 in different combinations, and the neonate A_2.

3. *Bone*

Extraction of foetal and child bone showed mostly strong $\underline{O}$* activity, but no clear picture of any A component could be obtained. Adult bone, however, showed a broad zone extending from the A_2 region almost up to the B zone. Dilution of the extract did not achieve resolution of this band into narrower zones. No other zone except for $\underline{O}$ activity was obtained.

It is possible that the alkaline phosphatase present in the neonate and the young child originates from bone.

4. *Pregnancy*

Sera from 27 women varying in age, duration and sex of gestation, and health, were electrophoresed. In 5 patients this was repeated after intervals of 1 - 6 weeks. The general pattern can be summarized as $\pm$ A_1 $\pm$ A_2 $\pm$ B $\pm$ E $\pm$ O ($\pm$ represents variable presence).

(*a*) *Changes in the A zone* are seen with pregnancy. There are 5 possibilities, viz. A_1, A_2, A_1A_2, $\underline{A_1}A_2$, and $A_1\underline{A_2}$. Of these, A_1 was the commonest, occurring in 10 of the 27 patients; A_2 occurred in 3 patients, aged 26, 30 and 25 years; A_1A_2 was rare, being present in only one patient; $\underline{A_1}A_2$ was also rare, being present in only one patient; and $A_1\underline{A_2}$ was found in 3 patients. The remaining 9 patients were more difficult to assess accurately but were regarded as $A_1?A_2$ in 4 patients, $?A_1A_2$ in 3 patients, $?A_2$ in 1 patient, and $A_2?A_3$ in 1 patient.

Comparison with placental extract did not show any relationship between the different A zones and that present in placenta.

(*b*) *Zone E*, a unique zone found only in pregnancy, was demonstrated in 14 of the 27 patients. It was seen as early as at 16 weeks' gestation. Some patients near term did not show this zone. By term, and during the two days after delivery, all patients tested showed this zone; but from the 3rd day after delivery some patients did not show it. It was demonstrated as late as 1 week after delivery. All placental extracts showed a double zone in this region, regarded as 'E' and F. The 'E' zone was slightly nearer the origin than the serum E. It was thought that the E zone in pregnancy originates from the placenta.

(*c*) *Zone B* was demonstrated in 8 of the 27 patients. Two patients showed an increase in activity over one week's interval (the day of delivery and 1 week later). This zone was not present in placental extract. It was not related to the duration of gestation nor to the height of serum-alkaline-phosphatase activity.

(*d*) *Zone O* was weak or absent except in 2 patients with associated liver disease.

*Strong staining of a zone is represented by underlining of the letter concerned, e.g. $\underline{A_1}$, $\underline{A_2}$, $\underline{B}$, $\underline{O}$.

(*e*) *Serum alkaline phosphatase* showed an average of 10·8 King-Armstrong units/100 ml. (extremes 6·2 - 19·7). None of the zones appeared only with increased total serum enzyme activity, but zone E was often absent at low levels. However, several of the higher levels did not show an E zone either.

(*f*) *Race*. Of the 27 patients 15 were White, 11 Coloured, and 1 African. Zones E and B showed no significant racial difference, but occurred in the expected ratio. Of 10 patients in whom the A_1 zone alone was present, 7 were White and 3 Coloured; this may suggest an increased incidence in Whites. The A_1A_2 changes occurred in too few patients for racial differences to be assessed.

(*g*) *Duration of gestation*. The earliest case seen was 16 weeks pregnant, the most advanced 9 days postnatal. The relative E-zone pattern is mentioned in paragraph (b). The A zone showed no pattern of change relative to duration of gestation. Follow-up of 5 patients showed an increase in B-zone activity in 2 (both 1 week later) but no change in the other 3 (all 6 weeks later).

(*h*) *Maternal age* varied from 18 to 41. No significant features emerged from comparison of this with E-zone activity.

(*i*) Parity was known in 11 cases. Of 4 primiparae 3 showed E zones. Two patients, gravida II and III, both showed E zones. Of 4 patients pregnant for the 5th time only 1 showed an E zone. One patient, gravida IX, showed no E zone. The number of cases is small, but it may be that E-zone activity is less common in the multipara.

(*j*) *Toxaemia of pregnancy*. Of 8 patients with toxaemia of pregnancy only 2 did not show E-zone activity. Of the 19 patients without toxaemia of pregnancy 10 showed E zones and 9 did not. Again the significance of this difference is doubtful.

(*k*) *Twins*. Two sets of dissimilar twins were included. In one case the mother showed an E zone, in the other not.

(*l*) *Diagnosis of pregnancy*. In 21 patients pregnancy could be suggested from E- and A-zone changes in the serum. Two others, with A_2 present, were 30 and 26 years old and would not be expected to have this zone normally. In the remaining 4 patients, with only A_1 present and where pregnancy could therefore not be suggested, one patient was less than 16 weeks pregnant (exact duration not known), one was 3 days postnatal, one was 7 days postnatal, and the last one had obstructive jaundice at 26 weeks. The accuracy of pregnancy estimation during the period 16 - 40 weeks would therefore be very high.

5. *Mongolism*

It has been suggested that the chromosomal abnormality in mongolism is associated with an increase in leucocyte alkaline phosphatase.[4] The relationship between normal serum alkaline phosphatase and leucocyte alkaline phosphatase is not well documented, although it is held by some that there is no relationship at all. It was decided to investigate a group of mongols for serum alkaline phosphatase isoenzymes.

The 24 mongol patients investigated ranged in age from 8 to 49 years. The average serum alkaline phosphatase was 9·8 King-Armstrong units/100 ml., with a range from 5 to 21·5.

11 patients showed A_1, 2 showed A_2, 2 showed A_1A_2, 5 showed A_1B, and 4 showed A_2B.

The A_2 zone could be attributed to age. The B zone, present in 9 out of 24 patients, was not attributable to age. This (B) zone was also seen in pregnancy, in children with bone-growth disturbance, and in hepatocellular disease. It was never seen in normal adults. The signficance of its presence in mongols is not clear. It is possible that it reflects the increased genetic information stored in the excess chromosomal material. Chromosomal studies were performed in 2 of the 9 mongol patients with zone B — in 1 case trisomy of the 21st chromosome was found; in the other translocation from chromosome 23 to 14.

6(a). *Liver Disease*

The 34 patients with liver disease whose sera were investigated were grouped into 4 main groups, viz. hepatocellular (H), obstructive (O), infiltrative/neoplastic (I/N), indeterminate (I). This subdivision was based on clinical and biochemical features, and in several cases on histological follow-up. The general pattern (serum) can be summarized as A ±B ±C ± ante-D ±F ±O.

The zone findings (sera) for each of the 4 groups were as follows:

Zone	*Group and number of cases*			
	13 H	*13 O*	*5 I/N*	*3 I*
A	All	All	All	All
B	1	3	2	0
strong $\underline{B}$	3	0	0	0
C	1	2	1	0
ante-D	1	1	0	0
D	Nil	10	2	2
F	0	1	0	0
strong $\underline{O}$	1	12	3	2
weak O	12	1	2	1

The following points emerge:

(*a*) Strong $\underline{O}$ and D suggest obstruction.

(*b*) Strong $\underline{B}$ was present in hepatocellular disease only. Of the 3 hepatocellular cases, 2 had chronic active hepatitis and one had cirrhosis. Acute infective hepatitis and inactive cirrhosis did not show such a strong B zone.

(*c*) C, ante-D, and F were non-specific.

(*d*) Steroids: 3 patients on steroids showed patterns that were atypical according to the above scheme. One with obstructive jaundice did not show a strong $\underline{O}$ but only O, and 2 patients with hepatitis showed a strong $\underline{O}$ and a weak O respectively. Theoretically a case of hepatocellular disease could show obstructive features. Of the cases on steroids, the patient with subacute hepatitis was the only one with such obstructive features.

(*e*) Of the infiltrative/neoplastic group, 3 patients show the obstructive picture and 2 do not.

(*f*) A child with hepatic failure showed A_1 A_2 C O. The combination of A_1 and A_2 here can be readily explained, and confirms that the A_1 zone of liver disease has a source different from the A_2 normally present in children.

6(b). *Liver Extracts*

Extracts from 17 liver specimens were analysed.

(*a*) All specimens except 2 (technically poor) showed a pre-A_1 zone (L in Fig. 1).

(*b*) All showed a strong $\underline{O}$ zone, except one that showed only O.

(*c*) None showed a B zone.

(*d*) *A D Zone* was present in 10 out of the 17 liver-extract specimens, being especially prominent in a patient dying from acute yellow atrophy. The D-zone distribution was as follows: 7 were found in 9 specimens from abnormal livers (3 in 5 specimens from hepatocellular cases, 2 in 2 from obstructive cases, and 2 in 2 from infiltrative/neoplastic cases), and 2 were found in 8 specimens from normal livers (1 in 5 specimens from adults, 1 in 1 from a 2-year-old child, none from 1 from a foetus, and none from a specimen of indeterminate origin). Therefore 7 out of 9 abnormal livers showed D, including all types of hepatic disease, and 2 out of 8 normal livers showed D. This suggests that abnormal livers of all types contain D but that normal livers do not.

6(c). *Comparison of Liver Extract and Serum*

In 6 patients the liver extract was compared with serum from the same patient taken shortly before death. On the whole correlation was poor:

(*a*) The A zone was different.

(*b*) The D zone was seen in 3 sera but only 1 of these patients showed a D zone in his liver extract. This patient was the only one with obstructive jaundice. One other patient had a D zone in his liver extract but not in his serum.

(*c*) Strong $\underline{O}$ was only seen in two sera, although all livers showed this. These results suggest that the D zone originates outside the liver.

7. *Bile*

This was found to vary. The general pattern was A (pre-A) $\pm$B $\pm$D $\pm\underline{O}$.

(*a*) In 1 patient with carcinoma of the common bile duct and marked obstruction (proved at laparotomy), bile was obtained from an indwelling T tube on the 3rd day of drainage and compared with the serum. They were identical—A_1 D $\underline{O}$. Adult bone extract from a different patient run simultaneously was clearly different — a broad zone extending from A_2 up to B.

(*b*) In another patient with confirmed carcinoma of the gallbladder, bile obtained from the T tube on the 3rd day showed a pattern different from the patient's own serum. The serum showed A$\underline{O}$ (note $\underline{O}$ but no D) whereas the bile showed a faint B zone and $\underline{O}$ but no A zone at all.

(*c*) Normal bile from a baby showed only a pre-A zone.

(*d*) Normal bile from an adult showed only a faint A and an O zone.

8(a). *Disturbance of Bone Growth: Children*

Serum specimens from 25 patients varying in age from 33 days to 21 years were electrophoresed. Of these, 21 were Coloured, 3 White, and 1 African. The sexes were equally represented. Growth disturbance was present in 9 of the patients; the rest were normal or suffered from unrelated disease, e.g. nephrotic syndrome. There was very great variation but all could be summed up as A_1 or A_2 $\pm$ B $\pm$C $\pm$ D $\pm$ O.

(*a*) A_2 indicated youth (discussed above).

(*b*) Of the 9 children with bone-growth disturbances, 8 showed D zones and 5 showed B zones. The 9 patients consisted of 4 with rickets, 2 with metabolic bone disease, and 3 hypothyroid children receiving thyroxine and showing a good growth response.

(*c*) Children with unrelated disease or normal: faint D zones were present in 2 non-thriving premature infants. Another patient (a Coloured female of 3 years with the nephrotic syndrome and receiving cortisone) showed a B zone.

(*d*) One patient with liver disease showed A_1 A_2 C O.

(*e*) O was present in 13 patients but was strong in only one patient with rickets.

8(b). *Disturbance of Bone Growth: Adults*

Five patients were suspected cases of metabolic or neoplastic bone disease. They showed A and a faint O only. In one other patient with a D zone the available data were inadequate for a firm diagnosis.

9. *Malignant Disease*

Seven patients were analysed in whom no liver involvement was found clinically or at postmortem. Of these, 4 showed a D zone, of whom 2 had bony metastases, and in the 2 patients without bone involvement specimens of liver obtained at postmortem were compared with serum, when in both instances a D zone was found in the serum, but not in the liver extract. It is possible that the D zone originated in the tumour itself.

An extract of a gastric carcinoma showed a faint D zone in addition to A and O zones.

10. *Small Bowel*

The small intestine was found to be a very rich source of enzyme. The duodenum and ileum have different isoenzyme patterns. The duodenum (washed free of bile and blood) showed $\underline{A}$ $\underline{B}$ D F $\underline{O}$, with B the dominant zone. The ileum showed an overwhelming $\underline{A}$ zone with a faint trace of B, but no other zones except for O. One specimen taken midway along the jejunum showed a pattern similar to the duodenum.

11. *Kidney*

A zone was present in kidney (K in Fig. 1) faster than any in liver. This was also found in a urinary specimen from a patient with Wilson's disease, in whom there was presumably renal tubular damage.

12. *Analysis of B Zone Findings*

Sera

Normal adults	Nil	
Children	9/25	5 with growth disturbance and 4 without growth disturbance
Pregnancy	8/27	1 liver damage
Mongolism	9/24	
Liver disease	9/34	4 hepatocellular (in 3 the dominant zone), 3 obstructive and 2 infiltrative

Tissues

Liver	Nil
Duodenum and jejunum	++
Bone	Nil
Bile	$\pm$
Kidney	Nil
Placenta	Nil

Since the B zone does not occur in any tissue except the small bowel it is possible that this is the source of enzyme in hepatocellular liver damage. This may be due to failure of the abnormal cell to alter an enzyme that arises from the small bowel and has an interohepatic circulation. This isoenzyme was demonstrated in bile on one occasion. In those cases with obstruction where B was present the zone was not prominent nor was obstruction complete.

The origin of the B zone in children with or without bony disturbance, and its absence from bone extract, are against a bony origin. The source in these children and in pregnancy is a mystery.

14. *Analysis of D Zone Findings*

Sera

Normal adults — Nil
Obstructive liver disease — 10/13
Bone-growth disturbances in children — 8/9
Malignant disease — D zone present in 7 cases, 3 involving liver, 2 involving bone, and 2 involving neither
Indeterminate, D zone present in 6 cases

Tissues

Liver abnormal	7/9
normal	2/8
Bone	Nil
Small bowel, proximal	++
distal	Nil
Bile	±
Kidney	Nil
Placenta	Nil

The composition of the sera is consistent with a bony origin of D zone in children and possibly in obstructive liver disease. The liver extracts also suggest an extrahepatic source. The absence of D zone from any bone extract is unaccountable.

Malignant tissue may sometimes be the source.

CONCLUSIONS

1. Different isoenzymes occur in different tissues, but some are shared.
2. Normal adults have A_1 only.
3. Normal children have A_2.
4. In pregnancy an E zone arising from the placenta appears in the serum. Changes in the A zone may have differing racial incidences.
5. Some mongols have a B zone in their sera. This may reflect the genetic abnormality present in this condition.
6. Obstructive liver disease can be recognized by the presence of D and strong $\underline{O}$.
7. Hepatocellular disease of the persistent hepatitis type may show a strong $\underline{B}$ zone. This does not arise from liver tissue itself. The small bowel is rich in this isoenzyme.
8. Children with bone-growth disturbance show a D zone.
9. Bile in patients with obstructive jaundice may or may not correlate with serum isoenzymes.
10. Malignant tissue may produce isoenzymes. This compares well with clinical suggestion.

SUMMARY

The pattern of alkaline-phosphatase isoenzymes as shown by starch-gel electrophoresis of different sera and tissues is described. Where possible, tissues and serum from the same patient were electrophoresed together to try to give a clear picture of the origin of the serum enzyme.

I wish to express my grateful thanks to Prof. J. E. Kench, Dr. M. C. Berman and Dr. G. M. Potgieter, of the Department of Chemical Pathology, University of Cape Town, for their interest and encouragement; the Department of Obstetrics for allowing investigation of patients and for providing placental tissue; Dr. S. Weinberg, and the matron and staff of the Alexandra Home, Cape Town, for their cooperation in obtaining sera from mongol patients; and Dr. G. Selzer, Department of Pathology, University of Cape Town, for performing the chromosome studies.

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