SEPARATION AND CHARACTERIZATION OF ALKALINE PHOSPHATASE ISOENZYMES FROM BLOOD AND OTHER TISSUES

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INTRODUCTION

Alkaline phosphatases may be contributed to the blood by a number of organs; thus the serum alkaline phosphatase may consist of a mixture of phosphatase isoenzymes. Whether or not these isoenzymes differ other than in their tissues of origin has long been debated, some evidence indicating that they are identical, some that they are different. Attempts to characterize alkaline phosphatase by a study of its properties as it occurs in serum have yielded inconclusive results, because of uncontrolled factors introduced into the experiments by the presence of indeterminate amounts of inhibitors (*e.g.* amino-acids, bile acids or albumin) and activators (metal cations, amino-acids in low concentrations) in the serum itself. Therefore, if characteristics of tissue phosphatases, such as their Michaelis constants, are to be compared with the corresponding characteristics of the serum enzyme, at least some purification of the latter is necessary.

This paper gives a comparison of the Michaelis constants of certain tissue alkaline phosphatases with those of serum alkaline phosphatase from a number of pathological states, the enzymes from both tissues and pathological sera being partially purified by starch-gel electrophoresis. From these comparisons, certain conclusions have been drawn concerning the tissues of origin of serum phosphatase in these diseases.

METHODS

The methods of electrophoretic purification of the enzymes and determination of their Michaelis constants (K_m) were described by Moss *et al.*[1], and are briefly recapitulated here. Starch-gel electrophoresis was carried out on sera and extracts of human bone, liver, kidney and small intestine essentially by the method of Smithies[2]. Since electrophoresis on horizontal gels is limited to the use of small samples of protein, the amount of enzyme recovered from each gel was small; therefore, for the determination of K_m values, a highly sensitive assay was used, in which β-naphthol enzymically liberated from β-naphthyl phosphate was determined spectrofluorimetrically[3].

The velocity at optimum pH for each substrate concentration was used in determining K_m, since Motzok[4] has shown that, under these conditions, K_m

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is independent of the phosphatase concentration used. pH-activity curves for the range of substrate concentrations employed are shown in *Figure 1*; because of the limited amount of enzyme solution recovered from the gel, only points sufficient to define each optimum were determined.

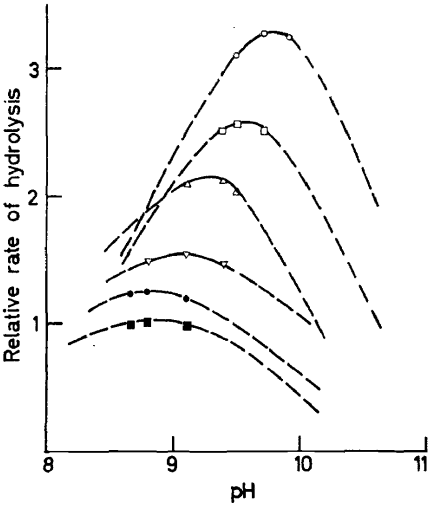

Figure 1. Variation of pH optimum for alkaline phosphatase with concentration of β-naphthyl phosphate. Broken lines show shape of full curves; points are those used to define optima for enzymes recovered from starch-gels. Substrate concentrations:
○, 1mM; □, 0·4mM; Δ, 0·2mM; ▽, 0·1mM; ●, 0·067mM; ■, 0·05mM.

Magnesium concentration was 5 mM throughout. For the phosphatase recovered from serum by electrophoresis, the increase in specific activity was of the order of 20- to 100-fold.

RESULTS

Bone, liver and intestinal phosphatases were found to migrate on starch-gel at a slightly lower rate than the transferrin-C serum globulin fraction, with kidney phosphatase moving somewhat more slowly than the others. These mobilities were unaltered by dissolving the tissue enzymes in normal

Table 1. Mean K_m values (mM β-naphthyl phosphate) for alkaline phosphatases partially purified from human tissues by starch-gel electrophoresis (Mg^{2+} concentration: 5 mM)

Tissue of origin	K_m (mM)
Bone	0·110
Liver	0·067
Kidney	0·103
Intestinal mucosa	0·090

human serum. Main bands of alkaline phosphatase activity were also found in this region in gels of the pathological sera which were studied, and all the K_m values reported in this paper refer to phosphatases recovered from this zone.

Table 1 gives the K_m values found for these four tissue phosphatases partially purified by electrophoresis. Although the differences between the values were small, they were demonstrated to be reproducible.

The Michaelis constants for phosphatases electrophoretically purified from pathological sera are collected, with other relevant biochemical data, in *Table 2*.

DISCUSSION

As mentioned above, various tissue phosphatases have similar mobilities on starch-gel; therefore, the phosphatase fraction from an electropherogram

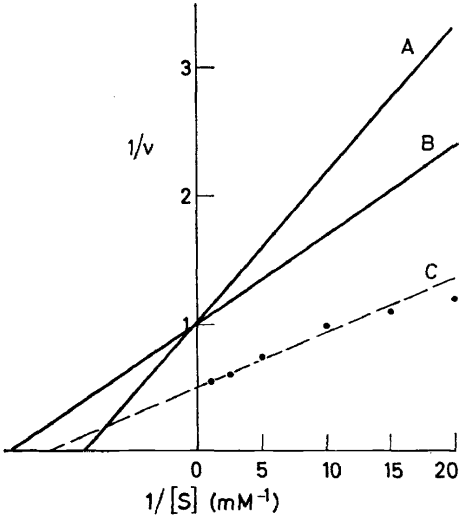

Figure 2. Reciprocal plots (1/v against 1/[S], where v is the initial velocity of reaction and [S] is the substrate concentration) for partially purified bone (plot A) and liver (plot B) phosphatases and for a mixture of equal activities of the two enzymes (plot C). The calculated plot for the mixture is shown by the broken line (K_m = 0·087mM), and the experimental points for the mixture thus: ●

of serum will consist of a mixture of those tissue phosphatases which contribute to the serum enzyme. Hence the K_m value obtained will be that of a mixture of enzymes, the resultant depending on the respective K_m values of the individual enzymes in the mixture and their relative proportions. The largest difference in Michaelis constant was found to be between bone (0·11 mM) and liver (0·067 mM), and *Figure 2* shows reciprocal plots for these two enzymes (*A* and *B* respectively), with the calculated line (plot *C*) for a mixture of equal activities of the two over the range of substrate concentration used. Experimentally determined points for such a mixture are also shown in *Figure 2*.

For pathological sera, therefore, a value of 0·11 mM would be expected from sera in which the phosphatase was almost exclusively of bone origin, with lower values in cases in which substantial amounts of other phosphatases were present; values lower than the intestinal phosphatase K_m (0·09 mM) would suggest the presence of liver phosphatase. For this discussion, the possible effect of kidney phosphatase is ignored, since its K_m lies close to that of bone. It is probable, moreover, that kidney phosphatase does not contribute largely to the serum enzyme.

Table 2. K_m (mM β-naphthyl phosphate) of main alkaline phosphatase fractions recovered from several pathological sera by starch-gel electrophoresis

Group	*Case*	*Serum alkaline phosphatase*	K_m (mM)	*Diagnosis*	*Other biochemical evidence**
I Serum enzyme resembles that of bone (with possibly some intestinal enzyme)	C	53	0·11	Paget's disease	
	G	57	0·11	Cancer of breast with bone secondaries	No liver secondaries at *post mortem*; 5N normal; ICDH normal
	Cs	66	0·095	Cancer of breast with secondaries	5N raised; ICDH slightly raised
	Cn	96	0·094	Cancer of pancreas with biliary obstruction	Jaundiced; 5N raised; ICDH normal
II Serum enzyme resembles that of liver	I	200 310	0·067 0·061	Cancer of pancreas with secondaries	Jaundiced; 5N greatly raised; ICDH raised
	Ch	97	0·071	Biliary cirrhosis	Jaundiced; 5N raised; ICDH raised
	B	160	0·074	Cancer of stomach	Jaundiced; 5N raised; ICDH raised
III Serum enzyme resembles mixture of bone (and perhaps some intestinal) and liver enzymes	W	14	0·080	Uraemia, congestive heart failure	5N normal; ICDH greatly raised
	H	160	0·081	Lymphosarcoma	Jaundiced; 5N raised; ICDH slightly raised
	Cn	56	0·083	Cancer of pancreas with biliary obstruction	Jaundiced; 5N raised; ICDH raised

* 5N=serum 5-nucleotidase, ICDH=serum isocitric dehydrogenase.

These hypotheses have been applied to the K_m values obtained for the sera of *Table 2*, which have been classified into three groups.

In the first group, the K_m lies close to that of bone phosphatase, and this is the enzyme which presumably predominates. This is the expected result in the first case, of Paget's disease. In the second case, also, the indication that the raised enzyme is mainly from bone is confirmed by the *post mortem* finding of secondary growths only in the bone, and the absence of biochemical evidence of liver involvement such as a raised serum level of isocitric dehydrogenase (ICDH), which has been shown by Wolfson *et al.*[5]

to be a sensitive index of hepatocellular damage, or of 5-nucleotidase (5N), which is elevated when the liver is damaged or has an impaired excretory function (Dixon and Purdom[6]; Campbell[7]).

The third example shows some evidence of liver-cell damage in that ICDH is slightly raised and 5N elevated, but, since the K_m value lies fairly close to that of bone, it appears that skeletal metastases contribute the major part of the serum alkaline phosphatase.

The last case in this group also shows a K_m close to the bone phosphatase figure, and since 5N is raised and there is jaundice, with a normal ICDH, the raised serum phosphatase level appears to be due to obstruction of the normal flow of the bone enzyme through the liver into the bile. Any intestinal phosphatase entering the plasma is presumably excreted by the liver, as is the bone enzyme, and the accumulation of intestinal enzyme possibly accounts for the K_m value in this case being somewhat below that of the bone enzyme.

In the second group of sera, the finding of K_m values which indicate the presence of liver phosphatase is supported in each case by elevated ICDH levels, indicating liver damage.

Some liver enzyme is also presumably present in each case in the third group, though in smaller proportion than in the second group. In cases H and Cn, there is evidence of biliary obstruction (shown by jaundice and raised 5N levels), which would cause accumulation of bone and perhaps intestinal phosphatases, and also there is some evidence of liver damage. In the first case (W), there is no sign of obstruction (bilirubin and 5N are normal), but strong evidence of liver damage. However, since the serum phosphatase level is low, the total amount of liver enzyme is apparently relatively small—perhaps 50 per cent or less of this low serum level—giving a K_m midway between the liver and bone enzyme values.

The two results obtained in Case Cn, one of which fell in Group I and the other in Group III, are particularly interesting in that they show a change in K_m as the pathological state changed. In the first specimen (Group I), any small contribution of liver enzyme to the serum K_m of 0·094 mM was apparently somewhat overshadowed by accumulated bone and perhaps intestinal enzymes. In the second (Group III), obstruction had been relieved at operation, releasing the pent-up bone and intestinal enzymes, as is shown by the reduced serum alkaline phosphatase; the effect of the alkaline phosphatase released from the now-damaged liver (indicated by a raised ICDH) becoming apparent in the lowered K_m, now 0·083 mM.

To summarize these data, it appears that alkaline phosphatase isoenzymes from several human organs do show differences, though these differences are small, in their affinities for β-naphthyl phosphate. Furthermore, these differences can be demonstrated reproducibly on phosphatases which have been added to serum and recovered by starch-gel electrophoresis.

The results for comparison of the K_m values of phosphatases recovered from pathological sera by electrophoresis with those for tissue phosphatases partially purified in the same way seem to support the following conclusions.

When there is biliary obstruction, the serum enzyme resembles bone phosphatase in its affinity constant, with perhaps an indication of some accumulation of intestinal enzyme.

Damage to the liver-cells produces a change in the K_m of the serum enzyme towards the value characteristic of liver phosphatase, showing that in these cases the liver can make a significant contribution to the serum alkaline phosphatase.

Skeletal diseases, or metastases to bone, are associated, as would be expected, with a serum alkaline phosphatase which has the K_m of bone enzyme.

The results presented here also illustrate the usefulness in the study of serum enzymes of partial purification of isoenzymes from serum by starch-gel electrophoresis, when coupled with a sufficiently sensitive method of studying the enzyme fractions thus obtained.

Summary

Alkaline phosphatase isoenzymes have been partially purified by starch-gel electrophoresis from several human tissues, and from pathological human blood sera. The Michaelis constants of these preparations have been determined by a sensitive spectrofluorimetric technique (substrate: β-naphthyl phosphate).

Comparison of the K_m values of phosphatases from tissues with those from sera has permitted certain conclusions to be drawn as to the probable origin of serum alkaline phosphatase in some disease states.

References

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