EVIDENCE ON CERTAIN COMMON PROPERTIES OF ALKALINE PHOSPHATASE-LIKE ENZYMES DERIVED FROM HUMAN FOETAL MINERALIZING TISSUES

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1. Introduction

The substrate specificity of alkaline phosphatases derived from different types of tissue is still open to investigation. In this laboratory investigations have been carried out on the human foetal alkaline phosphatase activity in order to elucidate, above all, the substrate specificity of the enzymes obtained from cartilaginous epiphysis, parietal bone and liver. A preliminary report on the results obtained has been given earlier [1]. Various chromatographic experiments revealed that parietal bone contains an alkaline phosphatase which is not very active or is present only in small amounts in the other tissues. In spite of the location of the different enzymes, the alkaline phosphatases found were considered to be similar in many respects, displaying perhaps only one main type of enzyme. This was evidenced in this paper by 1) essentially similar kinetic behaviour of most of the enzymes identified under the test conditions employed, and 2) similar activating effect of various divalent metal cations tested.

2. Experimental

The parietal bones, cartilaginous epiphysis of long bones and the livers were obtained from human foetuses, 8–16 weeks old, which had, for practical purposes, been frozen to −20°C immediately after the abortion. After thawing, the hard tissue samples were homogenized first with scissors into as fine particles as possible and then treated for 60 sec in 1–2 g portions in 10 ml of cold (+4°C) 0.01 M tris-HCl buffer, pH 7.2, with an Ultra-Turrax top drive homogenizer (the liver samples were treated for 10 sec only). The mixtures were centrifuged at 43500 *g* (1900 rev/min) for 30 min (Sorvall model RC-2B, rotor SS-34). The pH of the somewhat turbid supernatant fluids resulting was changed to pH 5.2 by addition of cold 0.2 N HCl, while the mixture was being carefully stirred. After being left to stand appr. 1 hr at +4°C the mixtures were centrifuged otherwise as above, but now for 10 min. Only the liver preparations gave considerable precipitates at this step. The pH of the clear supernatant fluid was brought back to pH 7.2 by addition of 1.0 N NaOH. The occasional turbidity was spun down as above. Samples from all three tissues were fractionated on DEAE cellulose columns as indicated in the Results section.

The alkaline phosphatase activity was in principle tested at pH 9.2 with *p*-nitrophenylphosphate as suggested by Bessey, Lowry and Brock [2]. When 1-naphthylphosphate was used as the substrate, the estimation method used was principally the same as described elsewhere, i.e. by coupling the liberated 1-naphthol to diazotized 4-amino-1,3′-dimethylazobenzene [3]. In the determination of the enzyme activity with several sugar phosphates, the method used was that of Fiske and Subbarow [4]. In all estimations the reaction mixtures were composed of 0.3 ml buffer (usually 0.05 M glycine-NaOH, pH 9.2), 0.1 ml of substrate solution (usually 1 mM), of 0.1 ml water or solutions of compounds to be tested, and of 0.1 ml enzyme solution.

Table 1

Rate of the hydrolysis of various phosphatase substrates by crude unfractionated enzyme preparations from three foetal tissues expressed as the amount of liberated μgrams of phosphorus per mg protein and min ($\times 10^2$). The figures represent mean values obtained from five different foetuses. Tested in 0.025 M glycine-NaOH buffer, pH 9.2, in the presence of 1 mM $MgCl_2$.

Substrate	Parietal bone	Cartilaginous epiphysis	Liver
DL-α-Glycerophosphate	15.5	11.1	1.27
β-Glycerophosphate	17.5	9.6	1.36
Adenosine-5′-monophosphate	24.2	14.2	2.32
Adenosine-5′-diphosphate	23.7	13.9	3.09
Adenosine-5′-triphosphate	18.7	12.7	2.89
α-D-Mannopyranosyl-1-phosphate	20.5	10.3	1.45
Fructose-1,6-diphosphate	26.6	11.7	1.49
Fructose-1-phosphate	21.6	8.3	1.25
Glucose-1-phosphate	18.4	8.1	1.23
Glucuronic acid 1-phosphate	20.8	10.1	1.40
α-D-Galactopyranosyl-1-phosphate	17.8	11.0	1.09
6-Phosphogluconic acid	34.0	10.7	1.03
Phosphocholine chloride	18.5	9.9	1.26

3. Results and discussion

The parietal bones constantly displayed the highest alkaline phosphatase activity as is shown in table 1. The experiments of table 1 were performed in the presence of 1.0×10^{-3} M $MgCl_2$. When the same experiments were carried out without added $MgCl_2$, the rate of the hydrolysis of fructose-1,6-diphosphate, fructose-1-phosphate and glucose-1-phosphate was higher than in the presence of the salt. When the crude enzyme preparations were fractionated on DEAE cellulose columns, the parietal bone yielded a chromatogram different from that of the other two tissues (fig. 1). The fractions indicated in the legend to the figure were pooled to form several enzyme preparations. Some of their properties were studied with the following results.

The activating effect of various divalent metal cations in the hydrolysis of *p*-nitrophenyl phosphate and 1-naphthylphosphate by the partially purified enzymes increased in the following order: Mn^{++}, Ca^{++}, Sr^{++}, Ba^{++}, Mg^{++}. Zn^{++} ions were inhibitory (all tested as chlorides at concentrations ranging from 6.0×10^{-2} M to 0.03×10^{-2} M). The highest rate of

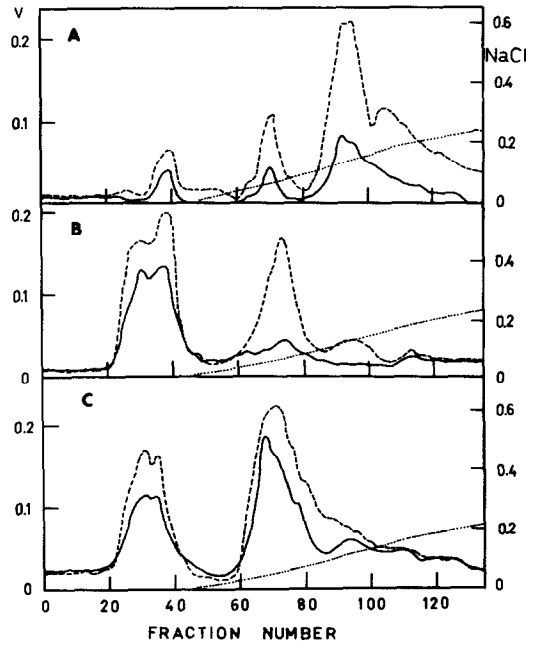

Fig. 1. Separation of alkaline phosphatase-like enzymes on DEAE cellulose (Schleicher and Schüll, 200–240 mesh) columns. A: parietal bone (PB); B: cartilaginous epiphysis (CE); C: liver (L); solid line: the enzyme activity (left hand scale) was tested without added $MgCl_2$; broken line: the enzyme activity was tested in the presence of 1 mM $MgCl_2$. Substrate: *p*-nitrophenyl phosphate. Column: 18 × 1.5 cm; elution buffer: 0.01 M tris-HCl, pH 7.2; NaCl gradient: 0 – 1.0 M; mixing volume: 150 + 150 ml; fraction volume: 1.5 ml; temperature: + 2°C; the samples (about 8 ml) were first passed through a Sephadex G-25 column with the above buffer. The sodium content of the fractions (dotted line, right-hand scale) was tested by atomic absorption spectrophotometer. The fractions were pooled and the pools were numbered as follows: A: 30–40 (= PB I); 62–78 (= PB II); 82–98 (= PB III); 102–120 (= PB IV); B: 20–40 (= CE I); 62–78 (= CE II); C: 20–40 (= L I); 62–78 (= L II).

the hydrolysis was usually obtained in the presence of 1 mM $MgCl_2$ with all enzyme preparations studied. The effect of pH on the action of the enzymes revealed was almost uniform in all cases, the pH optimum being 9.0–9.5. With the enzyme preparation termed L II (see fig. 1) the pH optimum was close to 7.0.

When the value of the apparent Michaelis constant, K_m, was estimated graphically by the method described by Hanes [5], most of the enzymes investigated displayed essentially similar kinetic behaviour under the test conditions employed, i.e. the value of

Table 2
Affinity of enzymes investigated in this study for some phosphatase substrates. The values of K_m were determined graphically by the method of Hanes. For abbreviations see the legend to fig. 1.

Substrate	K_m (in M X 10^5)					
	PB I	PB II	PB III	PB IV	CE I	CE II
1-Naphthyl phosphate	2.0	2.5	2.5	3.0	2.5	3.0
p-Nitrophenyl phosphate	2.5	3.0	4.0	5.0	3.0	4.5
Glucose-1-phosphate	10.5	10.0	12.5	17.0	15.5	11.0
β-Glycerophosphate	10.0	10.5	10.0	13.5	10.0	13.5
DL-α-Glycerophosphate	25.5	35.0	25.0	32.0	35.0	28.5
Adenosine-5′-triphosphate	4.0	4.5	5.0	5.5	5.0	5.5
Adenosine-5′-monophosphate	25.5	26.0	30.0	30.0	25.0	28.0

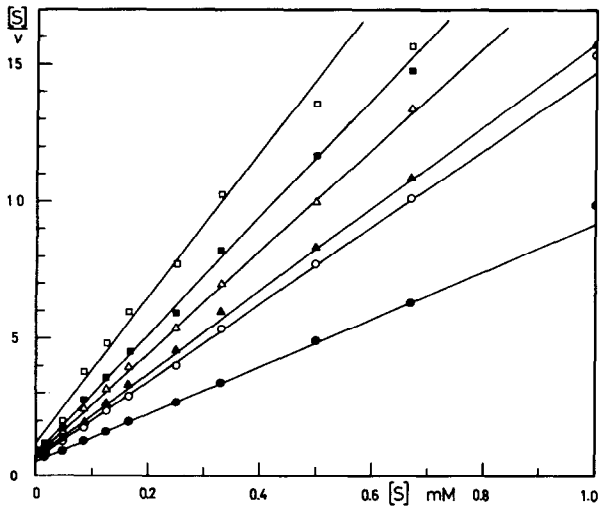

Fig. 2. Hanes' plot of the substrate concentration divided by the velocity (in 10^{-3} min) versus the substrate concentration of the hydrolysis of *p*-nitrophenyl phosphate catalyzed by enzymes obtained from the two tissues investigated. Legend: □, CE II; ■, PB III; △, CE I; ▲, PBI; ○, PB IV; ●, PB II.

K_m for a given substrate was essentially the same regardless of the origin of the enzyme preparation (the values were essentially the same if it is assumed that a 1.5 X 10^4 difference in K_m of different hydrolases is possible). An example of the results is given in fig. 2. In table 2 the value of K_m is given for a number of substrates and enzymes studied. Although the graphical method is subjective and no estimate of the errors could be determined, the values given do provide some support about the similarity in the affinity of the enzymes for their substrates. In most cases the kinetics was seen to be normal, i.e. the form of the Michaelis-Menten curves was parabolic, that of the Hanes curves linear. The liver enzyme preparations differed most from the other preparations in their kinetic properties.

The results obtained indicate that parietal bone possesses the highest alkaline phosphatase activity of the three tissues investigated and that the mineralized bones possess an alkaline phosphatase which is not very active or is present only in small amounts in the other tissues. This enzyme could therefore be considered an important factor in mineralization. The results suggest that the alkaline phosphatase-like enzymes identified are similar in several respect, displaying perhaps only one main type of enzyme.

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