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PHOSPHONIC AND ARSONIC ACIDS AS INHIBITORS OF HUMAN RED CELL ACID PHOSPHATASE AND THEIR USE IN AFFINITY CHROMATOGRAPHY *

JØRGEN DISSING [a,c,**], OTTO DAHL [b] and OLE SVENSMARK [a]

[a] *Institute of Biochemistry B, The Panum Institute and* [b] *Department of General and Organic Chemistry, The H.C. Ørsted Institute,* [c] *Institute of Forensic Medicine, Department of Serology, University of Copenhagen, Copenhagen (Denmark)*

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Summary

1. In order to obtain an effective ligand for affinity chromatography of the low molecular weight acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) from human red cells nine phosphonic and two arsonic acid substrate analogues were investigated as potential inhibitors. The two forms of acid phosphatase type B (b_1 and b_2) were isolated and partially purified using conventional methods and the inhibitory action of the substrate analogs investigated.

2. Four of the phosphonic acids were relatively effective competitive inhibitors. It appears that certain structural and electronic requirements have to be fulfilled by the phosphonic acids in order to exhibit significant affinity for the enzyme. A high affinity appears to require the presence of a bulky, hydrophobic moiety which has to be separated from the phosphorus atom by the distance of one atom.

3. *p*-Aminobenzylphosphonic acid exerted the highest affinity for acid phosphatase with a pH optimum at 6.5. K_i values of $4 \cdot 10^{-4}$ and $6 \cdot 10^{-4}$ M were found for the b_1 and b_2 forms, respectively.

4. Coupling of *p*-aminobenzylphosphonic acid to Agarose yielded an effective and specific affinity medium. By means of affinity chromatography using this medium, acid phosphatase was purified 500-fold in a single step.

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* A preliminary report of this work has been presented [26].

** Present address: Institute of Forensic Medicine, Department of Serology, Fr. Femtevej 11, DK-2100, Copenhagen, Denmark.

Introduction

The phosphatase activity in human red cells is mainly due to a low molecular weight acid phosphatase (orthophosphoric-monoester phosphohydrolase (acid optimum), EC 3.1.3.2) which occurs as several isoenzymes controlled by allelic genes at an autosomal locus [1—4]. Each allozyme occurs in two forms which may be conformational isomers [5]. The enzyme, once thought to be specific to the red cell, was later found in other tissues as well [6—8]. Neither the physiological function of acid phosphatase nor the significance of the polymorphism are so far obvious.

To elucidate on a molecular basis the differences between the multiple forms of the enzyme appreciable quantities of pure preparations are needed. Previous methods, based on conventional procedures, do not lead to pure products. Moreover, the yields are poor [4,9—16]. The introduction of affinity chromatography might therefore be an essential improvement. To this purpose a competitive inhibitor acting as a specific adsorbent would be advantageous because desorption of the enzyme might then be feasible under mild conditions. Furthermore, competitive inhibitors might be valuable tools in the study of the structural and electronic requirements which have to be fulfilled by molecules binding to the active site.

For these reasons a search was initiated for substrate analogues acting as competitive inhibitors of acid phosphatase.

The enzyme from red cells and placenta generally catalyzes the hydrolysis of aliphatic esters at low rates, whereas higher rates are obtained with esters containing an aromatic moiety: Phenylphosphate, *p*-nitrophenylphosphate, 17-β-estradiol-3-phosphate, 4-methylumbelliferylphosphate, phenolphthaleindiphosphate, riboflavin 5′-phosphate [4,11,17—20] and β-naphthylphosphate (Ref. 21 and unpublished data). Removal of the oxygen atom of the phosphate ester linkage yields phosphonic acids which are stable to cleavage by the enzyme. When the oxygen atom is replaced by a CH_2 group, the phosphonic acids obtained have about the same atomic dimensions as the corresponding ester [22]. This suggests that competitive inhibitors of acid phosphatase might be found among such compounds, especially those containing an aromatic moiety. Actually, phosphonic acids inhibit bacterial alkaline phosphatase [23], and the analogous arsenilic acid inhibits competitively alkaline phosphatase from calf intestine and has been used as a ligand in affinity chromatography [24]. Recently, phosphonic acids were shown to inhibit the latter enzyme competitively and to be usable as ligands in affinity chromatography [25].

This paper presents a study on the action of a series of phosphonic and arsonic acids on isolated and purified preparations of the two acid phosphatase forms b_1 and b_2 * which are determined by the commmon ACP_1^B-allele (gene nomenclature according to Ref. 7).

The results of chromatographic tests with substrate analogues coupled to Agarose are reported.

* In previous literature, b_2 referred to the enzyme form with the higher mobility towards the anode in electrophoresis [5]. In this paper the recommendations of The Commission on Biochemical Nomenclature on the Nomenclature and Classification of Enzymes are followed, i.e., b_1 refers to the enzyme form with the highest anodic mobility.

Materials and Methods

Reagents

Phenylphosphonic acid, phenylarsonic acid, *p*-aminophenylarsonic acid, *p*-aminophenylsulphonic acid, aniline and cyanogen bromide (Fluka), *p*-nitrophenylphosphate disodium salt (Merck), *p*-nitrophenol (British Drug House) and dithiothreitol (Boeringer) were all of analytical purity and were not purified further. Benzyl chloride (purum) and triethyl phosphite (purum) were from Fluka. DEAE-cellulose (DE23 and DE32) and CM-cellulose (CM32) were from Whatman; Sephadex G-75 and Sepharose-4B from Pharmacia and crystalline bovine albumin from Sigma. Active carbon (DARCO G60) was from Fluka.

Syntheses

The following compounds were synthesized according to published procedures unless otherwise specified.

p-Aminophenylphosphonic acid [27]. The crude product (m.p. 234—235° C) was dissolved in hot 6 M HCl, treated with active carbon and precipitated by adjusting pH to about 4 using 10 M NaOH and a small amount of glacial acetic acid. After repeated treatment as above and drying at 80° C in vacuo over NaOH pellets the purified compound melted at 248—251° C (decomp.). (m.p. 245° C [27], 254—256° C [23]).

Ethylphosphonic acid [28]. m.p. 58—61° C (from butanone). (m.p. 61—62° C [28]).

Benzylphosphonic acid. This compound was synthesized from benzyl chloride and triethyl phosphite followed by hydrolysis [23]. m.p. 172—172.5° C (from water). (m.p. 168—170° C [23]).

2-Phenylethylphosphonic acid. This was synthesized from 2-phenylethyl bromide and triethylphosphite by the same procedure as that used for the synthesis of benzylphosphonic acid. m.p. 137—139° C (from 1 : 1 ethanol/hexane). (m.p. 137—138° C [29]).

p-Nitrobenzylphosphonic acid. This was synthesized from benzylphosphonic acid by nitration [30]. m.p. 228—230° C (decomp.) (from 1 : 1 isopropyl alcohol/methylcyclohexane). (m.p. 232—234° C [30], 225—226° C (decomp.) [23]).

p-Aminobenzylphosphonic acid. This compound was synthesized from *p*-nitrobenzylphosphonic acid by reduction with sodium sulfide [31]. For purification, the buff coloured, crude product was dissolved in 2 M NaOH, filtered and pH brought to about 3.5 with glacial acetic acid. The precipitated product was isolated by filtration, washed with water and redissolved in 4 M HCl. The solution was treated with active carbon, filtered and the product precipitated by adjusting the pH to about 3.5 with sodium acetate. The pale yellow crystals were isolated by filtration, washed with water and dried in vacuo over NaOH pellets.

Found: C 44.40%; H 5.45%; N 7.40%;

Calc. from $C_7H_{10}NO_3P$:

C 44. 93%; H 5.39%; N 7.49%.

p-Chlorobenzylphosphonic acid [23]. m.p. 175.5—177° C (from ethyl acetate). (m.p. 168—171° C [23]).

Cyclohexylmethylphosphonic acid. This was synthesized by a Michaelis-Becker reaction from cyclohexylmethyl iodide [32] and $(EtO)_2PONa$ followed by hydrolysis. A mixture of $(EtO)_2PHO$ (6.9 g, 50 mmol), Na (1.15 g, 50 mmol) and tetrahydrofuran (50 ml) was heated to reflux for 4 h. After addition of cyclohexylmethyl iodide (11.2 g, 50 mmol), refluxing was continued for an additional 20 h. The reaction mixture was filtered, the filtrate was evaporated, and the residue was extracted with CH_2Cl_2 and H_2O. The organic phase was dried ($CaCl_2$) and the solvent was evaporated to give crude $C_6H_{11}CH_2PO(OEt)_2$ (10.0 g, 85%). The crude ester was refluxed with conc. HCl (25 ml) for 15 h and cooled, and the precipitated acid was isolated by filtration. Recrystallization from isopropyl alcohol yielded pure $C_6H_{11}CH_2PO_3H_2$ (4.4 g, 50%), m.p. 198.5—199.5° C. (m.p. 199.5—200.5° C [33]).

4-(4'-Amino-2'-methylphenylazo)benzylphosphonic acid. *p*-Aminobenzylphosphonic acid (7.5 g, 40 mmol) in 4 M HCl (30 ml) was cooled to 0° C and $NaNO_2$ (3.0 g, 45 mmol) in H_2O (7 ml) added with cooling. The resulting solution of diazonium salt was poured into a solution of *m*-toluidine (4.3 g, 40 mmol) in 2 M HCl (20 ml) at 0° C and the product precipitated by addition of 12 M NaOH to pH 2—3. The red-brown product (6.1 g, 50%) was used after washing with H_2O and drying in vacuo.

Determination of dissociation constants (pK_A) of phosphonic acids in aqueous solution

The pK_A values were determined graphically from pH titration curves obtained in water at 25° C. A solution of the phosphonic acid (1 mmol in 20 ml water) was titrated with 0.100 M NaOH and the pK_A values were set equal to pH at half-neutralization. *p*-Aminobenzylphosphonic acid (1 mmol) which is only slightly soluble in water was dissolved in 0.100 M NaOH (25.00 ml) and back-titrated with 0.100 M HCl.

Preparation of acid phosphatase

All operations were performed at 4° C unless otherwise stated. Outdated blood was obtained from the Blood Bank of the University Hospital (Rigshospitalet), Copenhagen and typed for acid phosphatase [34]. Blood of the homozygous type B was selected. Red cells were separated from plasma and white cells by repeated centrifugation at 7000 × *g* and washing with 0.16 M NaCl (four times). The buffy coat was removed by suction. Packed red cells were lysed by the addition of 2 vol. of 1 mM dithiothreitol and the lysates stored at −25° C until used. About 2.5 l of hemolysate were adjusted to pH 6.5 by the addition of 6 M HCl and dialyzed overnight against 10 vols. of 1 mM EDTA, pH 7. The precipitate was removed by centrifugation (30 min, 14 000 × *g*), the supernatant adjusted to pH 7.0 by the addition of 2 M NaOH and pumped on a column (5 × 25 cm) of DEAE-cellulose (DE23) equilibrated with 10 mM sodium phosphate, 1 mM EDTA, 0.25 mM dithiothreitol, pH 7.0. Hemoglobin was washed out with 400 ml of the phosphate buffer, and acid phosphatase eluted with 1 liter of 0.05 M sodium phosphate, 0.5 M NaCl, 1 mM EDTA, 0.25 mM dithiothreitol, pH 7.0. Fractions (25 ml) containing acid phosphatase were pooled and solid $(NH_4)_2SO_4$ added to give a final concentration of 2.5 M. The precipitate was collected by centrifugation (30 min,

30 000 × *g*) and dissolved in 0.05 M sodium phosphate, 1 mM EDTA, 3 mM NaN_3, 0.5 mM dithiothreitol, pH 6.5 (fraction I, final volume 75 ml). Insoluble matter was removed by centrifugation (20 min, 50 000 × *g*), and the clear supernatant pumped on a column (5 × 90 cm) of Sephadex G-75. The column was eluted with 0.05 M sodium phosphate, 1 mM EDTA, 3 mM NaN_3, 0.5 mM dithiothreitol, pH 6.5. Fractions (20 ml) containing the low molecular weight acid phosphatase were pooled and concentrated by ultrafiltration through a Diaflo UM 10 filter to about 10 ml. The concentrate was diluted 1 : 4 with 1 mM dithiothreitol and pumped on a column (2.5 × 35 cm) of DEAE-cellulose (DE32) equilibrated with 10 mM sodium phosphate, 1 mM EDTA, 1 mM dithiothreitol, pH 6.5. Elution was performed with a linear sodium phosphate-NaCl gradient from the 10 mM phosphate buffer (500 ml) to 0.05 M sodium phosphate, 0.05 M NaCl, 1 mM EDTA, 1 mM dithiothreitol, pH 6.5 (500 ml). The two molecular forms of the enzyme were separated by this step (Fig. 1). The two preparations were transferred to 20 mM sodium acetate, 20 mM sodium phosphate, 1 mM EDTA, 3 mM NaN_3, 1 mM dithiothreitol, pH 5.0, by repeated concentration by ultrafiltration and dilution with the buffer (final volume 50 ml). As a final step of purification each preparation was chromatographed on a column of CM-cellulose (2.5 × 20 cm) equilibrated with the acetate-phosphate buffer as specified above and eluted with a linear NaCl gradient from the starting buffer (500 ml) to the same buffer plus 0.1 M NaCl (500 ml). Both forms of the enzyme appeared in the chromatograms as a single peak coinciding with a protein peak as exemplified for the b_1-form (Fig. 2). Fractions (10 ml) containing phosphatase activity were pooled and transferred to 0.05 M sodium phosphate, 1 mM EDTA, 3 mM NaN_3, 5 mM dithiothreitol, pH 6.5 by repeated ultrafiltration and dilution with the buffer (final volume

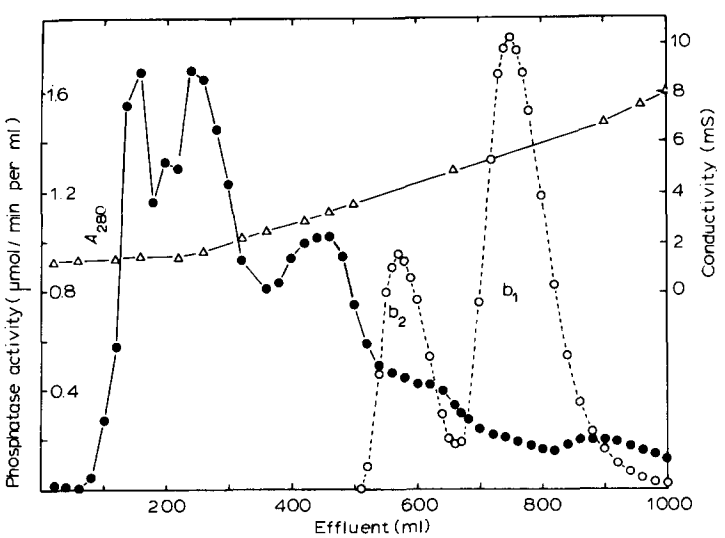


Fig. 1. Separation of the b_1 and b_2 forms of human red cell acid phosphatase type B by chromatography on a column (2.5 × 35 cm) of DEAE-cellulose (DE32) equilibrated with 10 mM sodium phosphate buffer, 1 mM dithiothreitol, 1 mM EDTA, pH 6.5. Elution was performed at a rate of 100 ml/h with a linear sodium phosphate-NaCl gradient. ○, Enzyme activity; ●, protein (A_{280}); △, conductivity at 25° C.

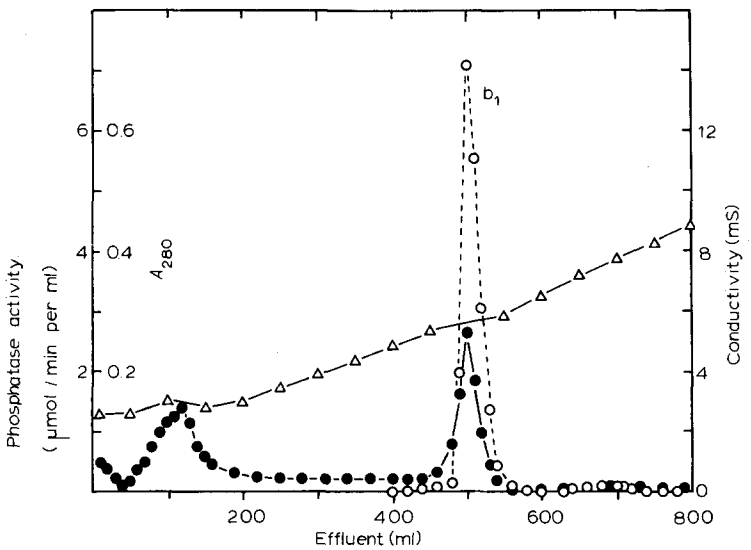


Fig. 2. Chromatography of partially purified human red cell acid phosphatase type B, b_1 form on a column of CM-cellulose (CM32) equilibrated with 20 mM sodium acetate, 20 mM sodium phosphate, 1 mM EDTA, 3 mM NaN_3, 1 mM dithiothreitol, pH 5.0. Elution was performed at a rate of 55 ml/h with a linear NaCl gradient. ○, Enzyme activity; ●, protein (A_{280}); △, conductivity at 25°C.

2—3 ml). The specific activities were about 30 μmol/min per mg, and the overall yield about 40% (Table I).

Electrophoresis in starch gel [34] showed that the b_1 and b_2 forms of the enzyme were completely separed. Interconversion of the two forms did not take place upon storage for one year at 4°C. This is in contrast with previous findings [5]. Electrophoresis in polyacrylamide and starch gels indicated a high degree of purity of the b_1 and b_2 enzyme. Also in contrast with other previous findings [15,16], our preparations did not show any decrease in activity for at least one year when stored at 4°C.

TABLE I

PURIFICATION OF HUMAN RED CELL ACID PHOSPHATASE TYPE B, b_1 AND b_2 FORMS

Purification step	Enzyme form	Volume (ml)	Total enzyme activity (μmol · min^{-1})	Protein (mg)	Specific enzyme activity (μmol · min^{-1} · mg^{-1})
Hemolysate (supernatant)	$b_1 + b_2$	2300	492	211 400 *	0.002
DEAE-cellulose chromatography	$b_1 + b_2$	660	430	4 800 **	0.09
Sephadex G-75 gel chromatography	$b_1 + b_2$	455	376	800	0.5
DEAE-cellulose chromatography	b_1	50	207	30	7.0
	b_2	54	78	50	1.6
CM-cellulose chromatography	b_1	3	150	5	30
	b_2	3	55	2	28

* Determined as hemoglobin.
** Including 1100 mg hemoglobin.

Protein was estimated by a modified biuret method [35] using crystalline bovine serum albumin as a standard and by measurement of A_{280nm}. Hemoglobin was determined by the hemoglobin cyanide method [36].

Determination of acid phosphatase activity

Acid phosphatase activity was determined at 30° C using *p*-nitrophenylphosphate disodium salt as substrate in a 0.2 M sodium citrate buffer containing 1 mg crystalline bovine serum albumin/ml and 1 mM dithiothreitol, pH 6.0.

Routine procedure. A solution of *p*-nitrophenylphosphate (11.11 mM) in the citrate buffer was freshly prepared. 0.1 ml of enzyme diluted in the citrate buffer was added to 0.9 ml of prewarmed substrate solution. The mixture was incubated in a thermostatically controlled water bath for 10—30 min. The reaction was stopped by the addition of 2 ml 0.6 M NaOH. A blank was made by adding NaOH before adding the enzyme. The concentration of *p*-nitrophenolate was determined by measuring A_{405nm} at 30° C in a thermostatically controlled spectrophotometer (Shimadzu 200-UV, 1 cm light path) with air in the reference beam. The absorbance varied with temperature, but did not change significantly at constant temperature for 1 h.

Calculation. The difference between the molar absorptivity of *p*-nitrophenolate and that of *p*-nitrophenylphosphate at 405 nm (pH 12) was 18 160 cm^2mol^{-1} (SD = 136, $n = 6$). The enzyme activity was calculated as $((A_t - A_b)/t) \times 1.652$ μmol/min per ml, where A_t is the absorbance at the incubation time t min and A_b the absorbance of the blank. Spontaneous hydrolysis of the substrate at pH 6.0 was insignificant. All determinations were

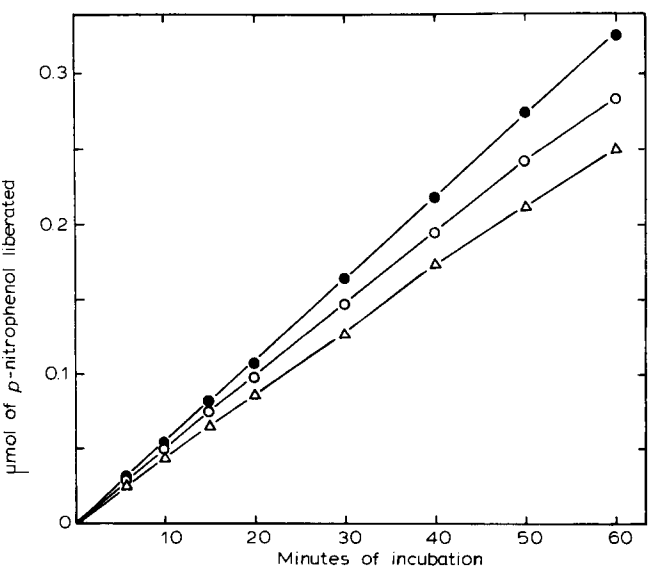


Fig. 3. Effect of the addition of albumin (1 mg/ml) and 1 mM dithiothreitol on the determination of the activity of human red cell acid phosphatase type B, b_1 form with *p*-nitrophenylphosphate as substrate (10 mM) in 0.2 M sodium citrate buffer, pH 6.0 at 30° C. The enzyme was diluted 2,000 times immediately before use. Liberation of *p*-nitrophenolate was determined as a function of time in the absence of albumin and dithiothreitol (△), in the presence of albumin (○) and in the presence of albumin and dithiothreitol (●).

performed in duplicate or more. The standard deviation as calculated from the differences found in 150 duplicate determinations was 0.45 nmol/min or 1.6% of the mean.

Addition of albumin prevented a fall in activity upon dilution of purified enzyme. A subsequent fall in activity during incubation with substrate at 30°C was prevented by the addition of the thiol reagent dithiothreitol. Albumin and dithiothreitol stabilized the enzyme in the pH range 4.5—7.5. An experiment performed at pH 6.0 is shown in Fig. 3.

Screening of inhibitors

The inhibitors were tested at the same concentration as that of *p*-nitrophenylphosphate (10 mM). Substrate and inhibitors were dissolved in 0.2 M citrate buffer pH 6.0. In a test tube 0.7 ml of buffer containing albumin (1 mg/ml) and dithiothreitol (1 mM) were mixed with 0.1 ml of 0.1 M *p*-nitrophenylphosphate, pH 6.0 and 0.1 ml of 0.1 M inhibitor. After prewarming of the mixture (30°C) 0.1 ml of enzyme solution was added. The enzyme solutions were prepared immediately before use by dilution of b_1 or b_2 preparations (400 and 100 times, respectively) in the citrate, albumin, dithiothreitol buffer. After incubation for 10 min at 30°C the reaction was stopped with NaOH as described above. In the controls the inhibitor was replaced by citrate buffer. Blanks were made by adding the NaOH before adding the enzyme. All determinations were performed in triplicate.

Determination of Michaelis constants (K_m)

Determination of acid phosphatase activity was performed as described above using different substrate concentrations (0.06—25 mM) at pH 4.5—7.5. Each determination of K_m was based on seven different substrate concentrations. The enzyme was incubated with substrate for 15 min. The concentration of the enzyme was adjusted so that the consumption of substrate did not exceed 10%. All enzyme activities were determined in triplicate, and each determination of K_m was performed on two or more sets of data. The mean concentration of substrate in the reaction mixtures during incubation was used in the calculation of the Michaelis parameters [37]. Two computerized methods of calculation were used: The method of Bannister et al. [38] using a Fibonacci search on the Michaelis function, and the non-parametric method of Eisenthal and Cornish-Bowden [39—41]. The two methods generally gave identical results, but since the latter method is less sensitive to outliers the K_m values given below were based on this method. The former method was used to control the goodness of fit to the Michaelis function. All computations were performed on a Univac-1100 system, RECKU, University of Copenhagen. The programs were written in UNIVAC FORTRAN V.

Determination of inhibitor constants (K_i)

The apparent K_m for *p*-nitrophenylphosphate was determined in the presence of inhibitor. Stock solutions of inhibitor (0.1 M) in water were diluted with 0.2 M citrate buffer of the appropriate pH containing albumin (1 mg/ml) and dithiothreitol (1 mM) to ten times the final concentration in the reaction mixture. A stock solution of *p*-nitrophenylphosphate (0.1 M) in the citrate

buffer was similarly diluted to ten times its final concentration. Substrate (0.1 ml), inhibitor (0.1 ml), buffer (0.7 ml) and enzyme (0.1 ml) were mixed and incubated for 15 min at 30°C. Controls and blanks were made as described above. Each determination was based on measurements at five different substrate concentrations in the presence of inhibitor at three fixed concentrations. All activities were determined in triplicate. As the inhibitors were competitive (see below), the K_i values were calculated from the expression $K_i = i/(K_m^a/K_m^{-1})$, where i is the concentration of the inhibitor and K_m^a is the apparent K_m. K_i values given below represent the mean of the estimates obtained at each of the three concentrations of inhibitor.

Preparation of affinity chromatography media

The aromatic amines *p*-aminobenzylphosphonic acid, 4-(4'-amino-2'-methylphenylazo)benzylphosphonic acid, aniline, *p*-aminophenylsulphonic acid, *p*-aminophenylphosphonic acid and *p*-aminophenylarsonic acid were coupled to Sepharose 4B. Solutions (0.2 M) were prepared in 0.1 M sodium borate buffer (pH 9.2) except for aniline, which was dissolved in the borate buffer mixed with 1.5 vol. of dimethylformamide.

Sepharose 4B was washed with large volumes of 0.16 M NaCl and finally with water. It was activated with CNBr (200 mg/ml Sepharose) [42]. Immediately after the activation the gel was washed on a glass filter funnel with several volumes of ice-cold water followed by several volumes of ice-cold 0.1 M sodium borate (pH 9.2), filtered to a moist cake and coupled to the ligand by mixing 1 vol. of the gel with 2 vols. of the solution of the ligand. The mixture was stirred gently at 4°C for 20 h, washed with large volumes of water, 0.1 M sodium hydrogencarbonate buffer (pH 9.0), 1 M sodium acetate buffer (pH 4.0) and 0.2 M sodium citrate buffer (pH 6.0). The gel was stored in the citrate buffer at 4°C. To determine the concentration of the ligand in the *p*-aminobenzylphosphonic acid-Sepharose, small columns (0.9 × 4 cm) were packed with this medium and washed with water. About 1.5 ml of the packed gel was transferred to a preweighed glass vial, weighed and dried in vacuo over concentrated H_2SO_4, and the content of phosphorus was determined by microanalysis. The concentration of phosphorus as determined in three batches varied between 8 and 11 mmol per l of moist, packed gel.

Screening for acid phosphatase binding activity

Columns (1.6 × 5—10 cm) were packed with the affinity chromatography medium in 0.2 M sodium citrate buffer (pH 6.0). Acid phosphatase (fraction I, 10—20 ml, 10—25 μmol/min) was dialyzed against the citrate buffer and pumped on the column followed by 50 ml of each of the following three buffers: 0.2 M sodium citrate (pH 6.0), 0.2 M sodium phosphate (pH 6.0) and 0.1 M sodium citrate, 2 M NaCl (pH 6.0). In experiments with *p*-aminobenzylphosphonic acid-Sepharose the sodium phosphate was applied as a linear gradient.

Results

Nine phosphonic and two arsonic acids were screened as potential inhibitors of acid phosphatase, type B at the same concentration as that of the substrate *p*-nitrophenylphosphate (10 mM) at pH 6.0. Phosphate, which is a competitive inhibitor of acid phosphatase [3,11,18] was also tested. Four of the compounds (benzyl-, *p*-aminobenzyl-, *p*-chlorobenzyl- and cyclohexylmethylphosphonic acid) and phosphate inhibited both enzyme forms (b_1 and b_2), whereas the other compounds were poor inhibitors (Table II). The inhibitory properties of these four phosphonic acids, and of *p*-nitrobenzylphosphonic acid and sodium phosphate were further investigated at pH 6.0 using varying substrate concentrations at three different concentrations of the inhibitor. All experiments fitted the Michaelis-Menten function within a standard error of estimate of less than 1% of V. The inhibition was competitive for all six inhibitors. The inhibition constants and the pK_A values of the relevant ionizable groups of the inhibitors are given in Table III. It is noted that at pH 6.0 the inhibitors exist predominantly in a monovalent anionic state.

The influence of pH (5.0—7.5) on the inhibitory effect of *p*-aminobenzylphosphonic acid was determined. All data sets showed excellent agreement with the Michaelis-Menten function, and competitive inhibition was observed over the entire pH range as exemplified by an Eadie-Hofstee plot of the effect on the b_1 enzyme at pH 6.5 (Fig. 4). The computed inhibitor constants for *p*-aminobenzylphosphonic acid were compared with the corresponding Michaelis constants for *p*-nitrophenylphosphate (Table IV). It is noted that the lowest

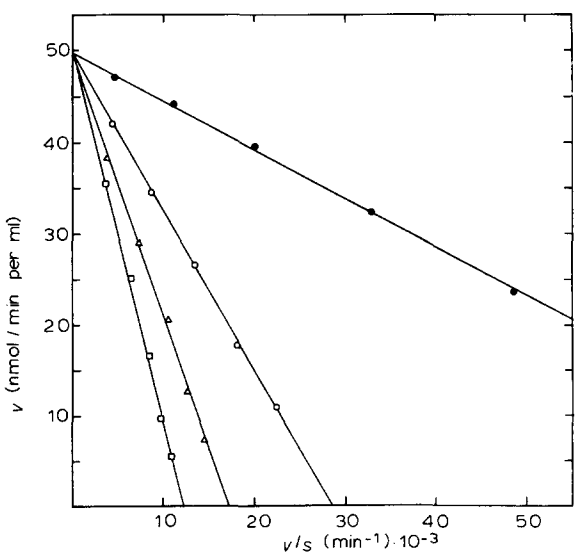


Fig. 4. Eadie-Hofstee plot for the b_1 form of acid phosphatase type B in the presence of *p*-aminobenzylphosphonic acid. The enzyme activity (v) was determined with *p*-nitrophenylphosphate as substrate in the concentrations (s) 0.5, 1, 2, 4 and 10 mM in 0.2 M sodium citrate buffer, pH 6.5 at 30°C. ●, Without *p*-aminobenzylphosphonic acid; ○, 1 mM *p*-aminobenzylphosphonic acid; △, 2 mM *p*-aminobenzylphosphonic acid; □, 3 mM *p*-aminobenzylphosphonic acid. The symbols represent the mean of triplicate determinations.

TABLE II

INHIBITION OF HUMAN RED CELL ACID PHOSPHATASE, TYPE B, BY PHOSPHONIC AND ARSONIC ACIDS IN 0.2 M SODIUM CITRATE, pH 6,0 AT 30°C

Concentration of inhibitors and the substrate (*p*-nitrophenylphosphate) was 10 mM

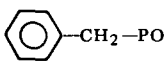
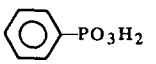
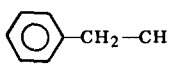
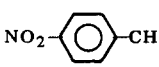
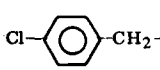
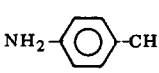
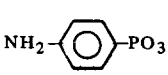
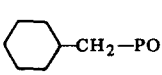
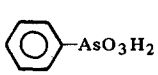
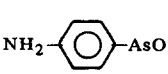
Compound	% Inhibition	
	b_1 enzyme	b_2 enzyme
	18	23
Benzylphosphonic acid		
	−1	0
Phenylphosphonic acid		
	4	3
2-Phenylethylphosphonic acid		
$CH_3—CH_2—PO_3H_2$	1	1
Ethylphosphonic acid		
	1	3
p-Nitrobenzylphosphonic acid		
	17	20
p-Chlorobenzylphosphonic acid		
	23	30
p-Aminobenzylphosphonic acid		
	0	−1
p-Aminophenylphosphonic acid		
	12	10
Cyclohexylmethylphosphonic acid		
	1	1
Phenylarsonic acid		
	3	3
p-Aminophenylarsonic acid		
H_3PO_4	12	35
Phosphoric acid		

TABLE III

HUMAN RED CELL ACID PHOSPHATASE TYPE B, b_1 AND b_2 FORMS. INHIBITOR CONSTANTS AND pK_A VALUES OF PHOSPHONIC ACIDS AND PHOSPHORIC ACID IN 0.2 M SODIUM CITRATE BUFFER, pH 6.0 AT 30°C

The pK_{A2} value of *p*-nitrophenylphosphate is 5.4 [46].

Inhibitor	K_i (mM)		pK_{A1}	pK_{A2}	pK_{A3}
	b_1	b_2			
p-Aminobenzylphosphonic acid	0.43	0.90	— **	5.2 *	7.5
Benzylphosphonic acid	0.55	1.8	3.1	7.3	
p-Chlorobenzylphosphonic acid	0.70	2.4	2.6	7.2	
p-Nitrobenzylphosphonic acid	3.6	13.5	2.9	7.0	
Cyclohexylmethylphosphonic acid	1.1	3.8	— **	— **	
Phosphoric acid	1.2	0.82	2.1	6.8	

* Protonation of the *p*-amino group. Due to the low solubility at this pH, the value is not accurate.
** Not determined.

K_i values fall in the higher pH range, and that the lowest K_m values fall in the lower range.

Substitution with the electron donating amino group in the aromatic nucleus of benzylphosphonic acid resulted in a decrease in K_i, whereas substitution with the electron withdrawing chloro and nitro groups yielded compounds with higher K_i values (Table III). A Hammet plot (Fig. 5) of $-\log K_i$ against the σ_m value of the substituents [43] gave acceptable linear correlations for both forms of the enzyme (b_1, $r = -0.922$; b_2, $r = -0.952$).

The strongest of the inhibitors, *p*-aminobenzylphosphonic acid, was coupled through its amino group to Sepharose and tested on a column for its ability to bind acid phosphatase. Control experiments were performed with Sepharose alone and with Sepharose coupled to *p*-aminophenylsulphonic acid, to aniline and to the weak inhibitors, *p*-aminophenylphosphonic acid and *p*-aminophenylarsonic acid. To reduce ionic binding forces, all experiments were performed at high ionic strength.

Acid phosphatase was retained only on *p*-aminobenzylphosphonic acid-Sepharose. It was eluted with sodium phosphate at low concentrations. Binding

TABLE IV

HUMAN RED CELL ACID PHOSPHATASE TYPE B. INHIBITOR CONSTANTS (K_i) OF *p*-AMINOBENZYLPHOSPHONIC ACID AND MICHAELIS CONSTANTS (K_m) OF *p*-NITROPHENYLPHOSPHATE AS A FUNCTION OF pH IN 0.2 M SODIUM CITRATE BUFFER AT 30°C

Values are quoted in mM.

Enzyme form		pH						
		4.5	5.0	5.5	6.0	6.5	7.0	7.5
b_1	K_m	0.035	0.036	0.054	0.13	0.46	1.45	
	K_i		1.9	0.84	0.43	0.43	0.59	
b_2	K_m	0.86	0.53	0.41	0.48	0.77	1.72	4.86
	K_i		11.7	2.3	0.90	0.64	0.60	0.87

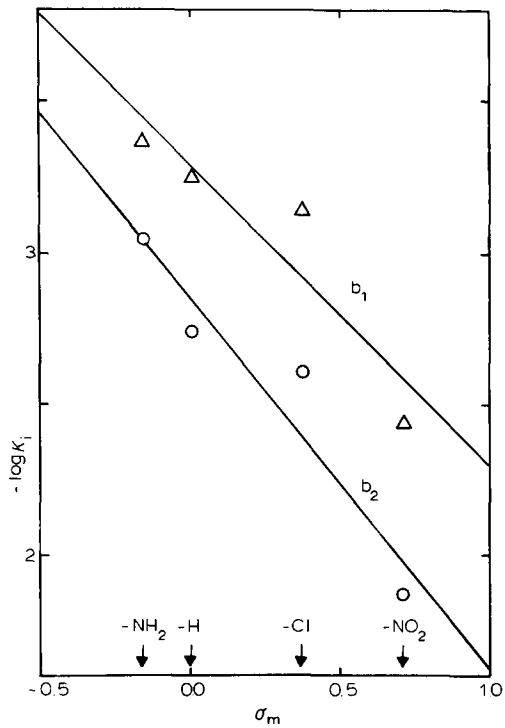


Fig. 5. Dependence on Hammet substitution constants (σ_m) of K_i for *para*-substituted benzylphosphonic acids. Inhibition constants were determined with *p*-nitrophenylphosphate as substrate at pH 6.0 and 30°C for human red cell acid phosphatase type B, form b_1 (△) and form b_2 (○). The lines represent least-square fits.

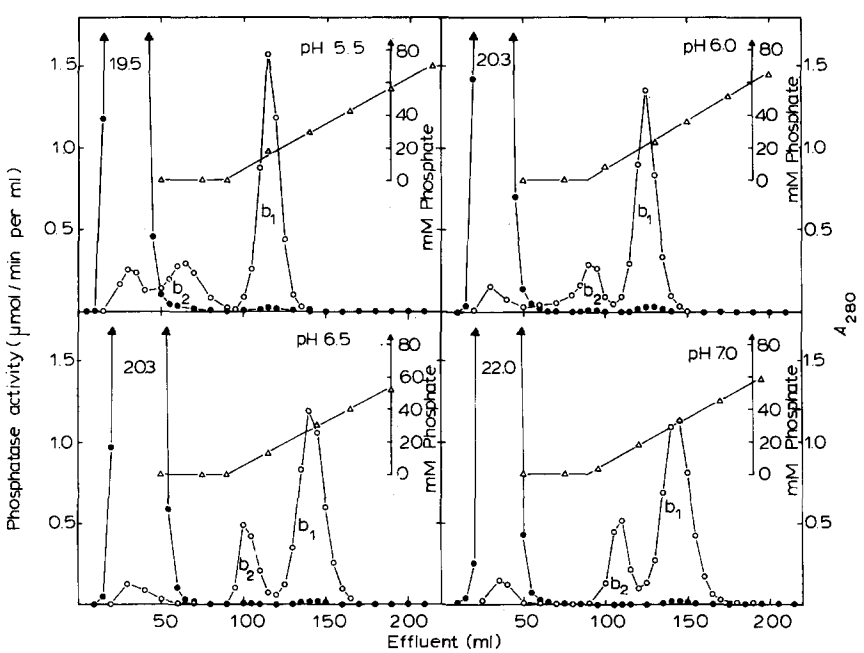


Fig. 6. Affinity chromatography of acid phosphatase on *p*-aminobenzylphosphonic acid-Sepharose at pH values from 5.5 to 7.0. A column (1.6 × 9 cm) was packed with *p*-aminobenzylphosphonic acid-Sepharose in 0.2 M sodium citrate buffer of the appropriate pH. 10 ml of acid phosphatase freed of hemoglobin (fraction I, 34 μmol/min) and dialyzed against the appropriate citrate buffer were pumped (50 ml/h) on the column followed by the citrate buffer (50 ml) and a linear gradient of sodium phosphate from the citrate buffer (100 ml) to 0.2 M sodium citrate, 0.1 M sodium phosphate buffer (100 ml) at the same pH. All buffers in contact with acid phosphatase contained dithiothreitol (1 mM). ○, Enzyme activity; ●, protein (A_{280}); △, sodium phosphate (mM).

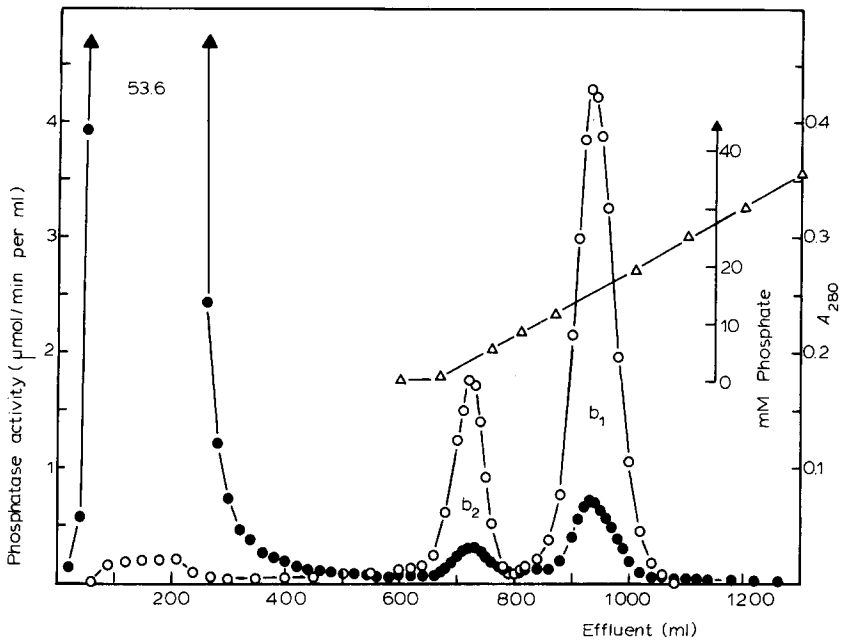


Fig. 7. Large scale affinity chromatography of acid phosphatase on *p*-aminobenzylphosphonic acid-Sepharose. Hemolysate from 2.5 l of blood (acid phosphatase type B) was freed of hemoglobin on DEAE-cellulose, concentrated to 140 ml (fraction I) and dialyzed against 0.2 M sodium citrate buffer, 1 mM dithiothreitol (pH 6.5). The dialysate was pumped (100 ml/h) on a column (2.5 × 17 cm) of *p*-aminobenzylphosphoric acid-Sepharose in the citrate buffer. Elution was carried out with 450 ml of 0.2 M sodium citrate, 1 mM dithiothreitol (pH 6.5) followed by a linear gradient of sodium phosphate from 0.2 M sodium citrate, 1 mM dithiothretol (500 ml, pH 6.5) to 0.2 M sodium citrate, 0.05 M sodium phosphate, 1 mM dithiothreitol (500 ml, pH 6.5). ○, Enzyme activity; ●, protein (A_{280}); △, phosphate (mM).

of other proteins was not detected for any of these media. The effect of introducing a spacer between *p*-aminobenzylphosphonic acid and Sepharose was studied with Sepharose coupled to 4-(4′-amino-2′-methylphenylazo)benzylphosphonic acid. Acid phosphatase was retained and eluted as for *p*-aminobenzylphosphonic acid-Sepharose, but the binding was less specific in that other proteins were adsorbed as well.

The chromatographic performance of *p*-aminobenzylphosphonic acid-Sepharose was investigated at pH values from 5.5 to 7.0 (Fig. 6). The most favorable chromatographic conditions were found at pH 6.5. Both enzyme forms (b_1 and b_2) were adsorbed and they were completely separated in the elution step. At higher pH values the separation of the two forms were poorer, and at lower pH values adsorption of the b_2 form was markedly decreased.

In larger scale affinity chromatography of hemolysate freed of hemoglobin (fraction I) at pH 6.5 clean separation of the two enzyme forms was still achieved (Fig. 7). A small amount of unidentified phosphatase was eluted with the peak of inert protein. The total yield of phosphatase was about 75%. The specific activity increased 500 times, which corresponded to a 25 000-fold purification as related to the centrifuged hemolysate. The protein patterns of Fig. 7 indicate a high degree in purity, and starch gel electrophoresis at pH 6.5 and 7.5 did not indicate contamination with other proteins. Interconversion of the two forms did not take place upon storage at 4°C. In the presence of

dithiothreitol the preparations were stable for months at 4° C, whereas freezing at −20° C inactivated the enzyme. The a_1 and a_2 forms of acid phosphatase type A were purified in the same manner.

Discussion

The structural resemblance between some aromatic phosphonic and arsonic acids and the substrates of acid phosphatase suggests that competitive inhibitors might be found among this type of compound. This is confirmed in the present study.

It appears that certain structural requirements have to be fulfilled by these substrate analogs for them to be effective inhibitors. Benzylphosphonic acid, which corresponds spatially to phenylphosphate, thus exhibits a high affinity for the enzyme, i.e. a low K_i value, whereas the absence of the CH_2 group resulted in an inactive compound (phenylphosphonic acid). The introduction of an additional CH_2 group between the phosphonic acid and the aromatic nucleus (2-phenylethylphosphonic acid) or the replacement of the benzene ring with a CH_3 group (ethylphosphonic acid) resulted in a large decrease in inhibitory power.

The effect of *para*-substitution in the aromatic ring was studied. Substitution with the electron-withdrawing nitro or chloro groups in benzylphosphonic acid caused a decrease in affinity, i.e. an increase in the K_i value, whereas substitution with the electron-donating amino group decreased K_i. This indicates that a high π-electron density of the aromatic ring is important for an effective binding to the enzyme. Actually, a Hammet relationship between K_i and σ_m seems to exist.

The finding that cyclohexylmethylphosphonic acid was a relatively effective inhibitor indicates that hydrophobic, and not necessarily aromatic, properties are of importance for the binding to the enzyme.

Although arsenate is an effective inhibitor of acid phosphatase [44], phenylarsonic acid and *p*-aminophenylarsonic acid were poor inhibitors. This may be due to the absence of a CH_2 group between the benzene ring and the arsenic atom which was essential for the phosphonic acid series.

Data on corresponding substrates are scant. Cyclohexylphosphate is reported to be a poor substrate [20]. This may be due to a low rate of hydrolysis and is therefore not inconsistent with a high affinity for the enzyme as indicated by the low K_i value of the corresponding inhibitor (cyclohexylmethylphosphonic acid). This assumption is supported by the finding of low K_m and low V values of another alicyclic compound, dehydroepiandrosterone 3-phosphate [20].

p-Nitrophenylphosphate exhibits a lower K_m than phenylphosphate [11], which apparently is in contrast with the K_i values of the corresponding phosphonic acids. This finding must await further studies to be solved.

In conclusion it seems that a high affinity for the enzyme requires the presence of a bulky, hydrophobic moiety separated from the phosphorus atom by the distance of one atom. With the exception of riboflavin 5′-phosphate, all substrates which are hydrolyzed at a high rate fulfil these requirements. The affinity of riboflavin 5′-phosphate for the enzyme can be attributed to a fold-back of the cyclic group into a proper position to the phosphate group

facilitated by the 5-carbon chain.

The variation of K_m and K_i values for *p*-nitrophenylphosphate and *p*-aminobenzylphosphonic acid as a function of pH suggests that acid phosphatase binds the substrate and its analog when they carry one negative charge on the acid group. Protonation of the amino group of *p*-aminobenzylphosphonic acid may explain the steep decrease in affinity for the enzyme below pH 6. For the b_2 species it is indicated that more than one ionizable group is involved, probably belonging to the enzyme. This assumption is supported by the finding that, below pH 6.5, the b_2 form exhibits a marked decrease in binding to *p*-aminobenzylphosphonic acid-Sepharose which does not carry a charged amino group. A more detailed study of these problems is in progress.

Although the two molecular forms of the enzyme showed the same trends in their reactions with the substrate analogues, differences were evident. The affinities for the analogs were consistently lower for the b_2 than for the b_1 form. This is in accordance with the present and other findings for *p*-nitrophenylphosphate obtained with the isolated enzyme forms [3—5]. Phosphate which cannot be considered a substrate analog deviated in showing a higher affinity for the b_2 form. Also, the b_1 and b_2 forms differed with respect to the pH-dependence of their reaction with *p*-aminobenzylphosphonic acid and *p*-nitrophenylphosphate. These differences stress the necessity of examining the two enzyme forms separately, and also emphasize risks involved in the interpretation of results obtained with preparations containing both forms of the enzyme.

The use of inhibitors as ligands in affinity chromatography has been treated theoretically by Graves and Wu [45]. They concluded that K_i values of less than $1 \cdot 10^{-3}$ M are required for proper action. The K_i values of less than $1 \cdot 10^{-3}$ M for *p*-aminobenzylphosphonic acid with respect to acid phosphatase therefore suggest its use as a suitable ligand for this enzyme.

Indeed, *p*-aminobenzylphosphonic acid-Sepharose was a highly effective affinity medium, allowing a 500-fold purification in a single step. The specificity of *p*-aminobenzylphosphonic acid-Sepharose for acid phosphatase was evidenced by the following findings. (1) Binding of acid phosphatase was not detected with Sepharose alone or with Sepharose coupled to ligands being hydrophobic (aniline) or negatively charged (*p*-aminophenylsulphonic acid) or to substrate analogs which were poor inhibitors (*p*-aminophenylphosphonic acid, *p*-aminophenylarsonic acid). (2) Elution of acid phosphatase could be performed with phosphate which competes with *p*-aminobenzylphosphonic acid for the active site. (3) Binding of other proteins was not observed. (4) The sequential elution of the b_1 and b_2 forms was in accordance with their different affinities for *p*-aminobenzylphosphonic acid and phosphate ions.
This sequence cannot be explained as an effect of simple cation exchange chromatography in which the sequence of elution would be reversed [14].

A spacer arm between *p*-aminobenzylphosphonic acid and Sepharose was not needed for proper binding of acid phosphatase, probably because of the small size of the enzyme. This is advantageous because the use of a spacer may lead to non-specific adsorption of undesired protein. Therefore, further efforts in this direction were not pursued.

Further investigations on the use of affinity chromatography in the purification of acid phosphatases are in progress.

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References

1 Hopkinson, D.A., Spencer, N. and Harris, H. (1964) Am. J. Hum. Genet. 16, 141—154
2 Fisher, R.A. and Harris, H. (1971) Ann. Hum. Genet. 34, 449—453
3 Fenton, M.R. and Richardson, K.E. (1971) Arch. Biochem. Biophys. 142, 13—21
4 White, I.N.H. and Butterworth, P.J. (1971) Biochim. Biophys. Acta 229, 193—201
5 Fisher, R.A. and Harris, H. (1971) Ann. Hum. Genet. 34, 439—448
6 Blake, N.M., Kirk, R.L., Barnes, K.R. and Thompson, J.M. (1973) Jap. J. Hum. Genet. 18, 10—23
7 Swallow, D.M., Povey, S. and Harris, H. (1973) Ann. Hum. Genet. 37, 31—38
8 Sensabaugh, G.F. (1975) in Isoenzymes, Vol. 1 (Markert, C.L., ed.), pp. 367—380, Academic Press, New York
9 Tsuboi, K.M. and Hudson, P.B. (1954) Arch. Biochem. Biophys. 53, 341—347
10 Angeletti, P.U. and Gayle, R. (1962) Blood 20, 51—55
11 Scott, E.M. (1966) J. Biol. Chem. 241, 3049—3052
12 Fenton, M.R. and Richardson, K.E. (1967) Arch. Biochem. Biophys. 120, 332—337
13 Shinoda, T. (1968) J. Biochem. 64, 733—741
14 Fisher, R.A. and Harris, H. (1971) Ann. Hum. Genet. 34, 431—438
15 DiPietro, D.L. (1971) Biochim. Biophys. Acta 235, 458—465
16 Rehkop, D.M. and van Etten, R.L. (1975) Z. Physiol. Chem. 356, 1775—1782
17 Luffman, J.E. and Harris, H. (1967) Ann. Hum. Genet. 30, 387—401
18 DiPietro, D.L. and Zengerle, S.F. (1967) J. Biol. Chem. 242, 3391—3396
19 Tsuboi, K.K. and Hudson, P.B. (1956) Arch. Biochem. Biophys. 61, 197—210
20 DiPietro, D.L. (1968) J. Biol. Chem. 243, 1303—1305
21 Sørensen, S.A. (1970) Clin. Genet. 1, 294—303
22 Engel, R. (1977) Chem. Rev. 77, 349—367
23 Williams, A., Naylor, R.A. and Collyer, S.G. (1973) J. Chem. Soc. Perkin II, 25—33
24 Brenna, O., Perrella, M., Pace, M. and Pietta, P.G. (1975) Biochem. J. 151, 291—296
25 Landt, M., Boltz, S.C. and Butler, L.G. (1978) Biochemistry 17, 915—919
26 Dissing, J., Dahl, O. and Svensmark, O. (1977) Proc. 7th Int. Congr. Soc. Forensic Haematogenet., Hamburg, Sept. 25—29th 1977 pp. 261—270, Gesellschaft für Forensische Blutgruppen Kunde e.V., Hamburg
27 Doak, G.O. and Freedman, L.D. (1952) J. Am. Chem. Soc. 74, 753—754
28 Kosolapoff, G.M. (1945) J. Am. Chem. Soc. 67, 1180—1182
29 Bergmann, E. and Bondi, A. (1930) Chem. Ber. 63, 1158—1173
30 Kagan, F., Birkenmeyer, R.D. and Strube, R.E. (1959) J. Am. Chem. Soc. 81, 3026—3031
31 Kosolapoff, G.M. (1947) J. Am. Chem. Soc. 69, 2112—2113
32 Laurent, A., Laurent, E. and Tardivel, R. (1974) Tetrahedron 30, 3423—3429
33 Seyfert, D. and Marmor, R.S. (1971) J. Org. Chem. 36, 128—136
34 Dissing, J. and Svensmark, O. (1976) Hum. Hered. 26, 43—58
35 Høring, N. and Svensmark, O. (1976) J. Neurochem. 27, 523—528
36 van Kampen, E.J. and Zijlstra, W.G. (1961) Clin. Chim. Acta 6, 538—544
37 Lee, H.-J. and Wilson, I.B. (1971) Biochim. Biophys. Acta 242, 519—522
38 Bannister, W.H., Anastasi, A. and Bannister, J.V. (1976) FEBS Lett. 64, 290—292
39 Eisenthal, R. and Cornish-Bowden, A. (1974) Biochem. J. 139, 715—720
40 Cornish-Bowden, A. and Eisenthal, R. (1974) Biochem. J. 139, 721—730

41 Cornish-Bowden, A. and Eisenthal, R. (1978) Biochim. Biophys. Acta 523, 268—272
42 March, S.C., Parikh, I. and Cuatrecasas, P. (1974) Anal. Biochem. 60, 149—152
43 Jaffé, H.H. (1953) Chem. Rev. 53, 191—261
44 Abul-Fadl, M.A.M. and King, E.J. (1949) Biochem. J. 45, 51—60
45 Graves, D.J. and Wu, Y.-T. (1974) in Methods in Enzymology, Vol. 34 (Jakoby, W.B. and Wilchek, M., eds.), pp. 140—162, Academic Press, New York
46 Sturtevant, J.M. (1955) J. Am. Chem. Soc. 77, 255—258