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Alkaline Phosphatase: Affinity Chromatography and Inhibition by Phosphonic Acids†

Michael Landt, Steven C. Boltz, and Larry G. Butler*

ABSTRACT: Five phosphonic acid derivatives were synthesized, coupled to agarose, and tested for affinity chromatographic binding of alkaline phosphatase from bovine intestine. Agarose coupled to L-histidyldiazobenzylphosphonic acid was found to be a highly effective adsorbent. In order to understand the large differences in binding capacity observed with derivatized agaroses, inhibition of alkaline phosphatase by phosphonic acid ligands, and related phosphonic acids, was measured. The results of affinity chromatography and inhibition studies were in good agreement, demonstrating that phosphonic acids with large aromatic/hydrophobic, carboxylate substituents bind strongly and competitively to the enzyme active site.

Alkaline phosphatase and 5′-nucleotide phosphodiesterase from bovine intestine are similar in many characteristics important in purification, causing these enzymes to purify together (Dardinger, 1974; Kelly et al., 1975). A persistent difficulty in purification of 5′-nucleotide phosphodiesterase has been removal of contaminating alkaline phosphatase. Previously a large fraction of the phosphodiesterase activity eluted from columns of DEAE-cellulose was sacrificed due to overlapping elution with alkaline phosphatase (Kelly et al., 1975).

The most promising avenue explored to effectively separate these enzymes was affinity chromatography. Because the amount of alkaline phosphatase protein present at the final stage of purification was considerably less than the amount of phosphodiesterase protein, the most efficient resolution was removal of alkaline phosphatase by affinity chromatography. Doellgast & Fishman (1974) employed phenylalanyl agarose in conjunction with high salt concentration to obtain threefold purification of human placental alkaline phosphatase. However, comparison of phenylalanyl agarose to agarose derivatized with other amino acids indicated that binding of alkaline phosphatase to these materials, including phenylalanyl agarose, was due to hydrophobic interactions.

Recently Brenna et al. (1975) have reported the synthesis and use of agarose derivatives incorporating arsonic acid moieties, which are analogues of phosphates, as affinity ligands for bovine intestinal alkaline phosphatase. Best results were achieved with tyraminyl agarose coupled via diazotization to 4-(4-aminophenylazo)phenylarsonic acid. Alkaline phosphatase from bovine intestine was purified 45-fold to apparent homogeneity. The capacity of tyraminyl-4-(4-diazophenylazo)phenylarsonic acid agarose for binding alkaline phosphatase is rather low. From the data of Brenna et al. (1975), approximately 20 mL of gel would be required to bind 1 mg of alkaline phosphatase. Our preliminary experiments with tyraminyl agarose and 4-(4-aminophenylazo)phenylarsonic acid indicated that poor solubility of the arsonic acid in conditions for derivatization and the considerable hydrophobicity of these materials which favors nonspecific protein binding (Shaltiel, 1974) made development of alternative affinity media desirable.

This report presents methods of synthesis and the results of affinity binding tests for a series of affinity chromatography

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media containing derivatives of phosphonic acids, which are analogues for substrates of alkaline phosphatase. The best affinity ligand, L-histidyldiazobenzylphosphonic acid, when coupled to agarose had a high capacity and good specificity for alkaline phosphatase. In order to better understand observed differences in effectiveness of various ligands on affinity chromatography, the inhibition of alkaline phosphatase by several of these analogues and other phosphonate compounds was measured. The results of affinity and inhibition tests are in good agreement and demonstrate that the substrate binding site of alkaline phosphatase has a high affinity for phosphonic acids which bear a hydrophobic/aromatic, carboxylate substituent.

Experimental Section

Materials. Aminomethylphosphonic acid, epoxy-activated Sepharose 6B, Sepharose 6B, *N*-acetyl-L-tyrosine, Triton X-100, DEAE-cellulose, Tris base, and 4-nitrophenyl phosphate were purchased from Sigma Chemical Co. Diethyl 4-nitrobenzylphosphonate and diethyl 4-aminobenzylphosphonate were obtained from Aldrich Chemical Co. Phenylphosphonic acid was purchased from Chemicals Procurement Laboratories. Phosphonoacetic acid was purchased from Richmond Organics. *N*-Acetyl-D-tyrosine ethyl ester was purchased from Vega-Fox Biochemicals. Ammonium sulfate (Ultrapure grade) was obtained from Schwarz/Mann. Sephacryl S-200 was a product of Pharmacia Co. and XAD-2 resin was obtained from Rohm and Haas. All other reagents were of analytical grade, obtained from conventional sources.

Assay of Enzymatic Activities and Protein. Alkaline phosphatase was assayed spectrophotometrically at 30 °C using 1.3 mM 4-nitrophenyl phosphate in 0.10 M Tris-HCl (pH 8.0). One unit of activity was defined as the amount of enzyme catalyzing production of 1.0 μmol of nitrophenol per min under standard conditions.

Protein concentration was measured spectrophotometrically at 280 nm using a mass extinction coefficient of 1.0 OD per mg of protein for alkaline phosphatase (Kelly et al., 1973).

Purification. Alkaline phosphatase and 5′-nucleotide phosphodiesterase were simultaneously purified from fresh bovine small intestine. During all purification manipulations the enzymes behaved as one molecular entity until separation by affinity chromatography as the final step. The purification procedure involved solubilization of the enzymes from mucosa and contents with Triton X-100, extraction with 1-butanol, ammonium sulfate precipitation, DEAE-cellulose chromatography, phenylalanyl agarose chromatography at high salt concentration, gel filtration on Sephacryl S-200, and resolution of the enzymes by affinity chromatography with L-histidyldiazobenzylphosphonic acid agarose. The method used was a modification of a procedure developed earlier to purify 5′-nucleotide phosphodiesterase (Kelly et al., 1975; Dardinger, 1974). The modified procedure will be described in detail elsewhere (Landt & Butler, 1978).

Gel Electrophoresis. Alkaline phosphatase was subjected to gel electrophoresis in 7% polyacrylamide gels at pH 8.3 using the method of Davis (1964), omitting the use of a stacking gel.

Syntheses of Phosphonic Acids. Criteria of Purity. Purity of synthetic phosphonic acid preparations was ascertained by elemental analysis, melting point determinations, and paper chromatography in 2-propanol:H_2O:NH_4OH (80:20:0.2, v/v). All of the preparations used in this study appeared to be homogeneous when chromatographed with this system.

N-Acetylaminomethylphosphonic Acid, Sodium Salt. Aminomethylphosphonic acid (0.12 g) was acetylated in a solution of 25 mL of acetic anhydride, 3 mL of H_2O, and 0.5 mL of concentrated NH_4OH, converted to the sodium form by ion exchange, and crystallized from methanol–ethanol mixtures. A satisfactory analysis of this compound was never obtained, apparently due to interference by sodium, mp 206–207 °C.

4-Nitrobenzylphosphonic Acid and 4-Aminobenzylphosphonic Acid. The diethyl esters of 4-nitrobenzylphosphonic acid and 4-aminobenzylphosphonic acid were hydrolyzed to the free acids under reflux in concentrated HCl:acetic acid (15:1 v/v) for 6 h (Myers et al., 1965). 4-Nitrobenzylphosphonic acid was crystallized from hot water, mp 221–223 °C (lit. value = 226 °C, Kosolapoff, 1951). Anal. Calcd for $C_7H_8NO_5P$ (fw[1] 217.1): C, 38.7; H, 3.7. Found: C, 38.9; H, 4.0.

4-Aminobenzylphosphonic acid was purified by raising the pH of an aqueous solution to >8 with triethylamine and washing the solution through a 2.5 × 15 cm column of XAD-2 with H_2O. The free acid was precipitated by addition of HCl or formic acid (pH ≤3), and collected by filtration, mp >300 °C (lit. value, mp 323–325 °C (dec), Kosolapoff, 1947). Anal. Calcd for $C_7H_{10}NO_3P$ (fw 187.1): C, 44.9; H, 5.3. Found: C, 44.1; H, 5.6.

N-(4-Aminobenzoyl)-4-aminobenzylphosphonic Acid. To 4-aminobenzylphosphonic acid, 0.75 g suspended in 30 mL of H_2O, triethylamine was added until the phosphonic acid was completely dissolved and the pH was approximately 8. Then 0.60 g of 4-nitrobenzoyl chloride was added with constant stirring; the pH was kept above 8 by addition of triethylamine. The reaction proceeded overnight with formation of a white precipitate. The filtered supernatant was reduced to near dryness and 30 mL of acetone was added. The pH was lowered slowly to 2.5 by addition of formic acid. Precipitates were removed by filtration. The solution was heated on a hot plate to drive off the acetone while the volume was maintained by addition of water. When crystals began to appear the solution was cooled on ice. The crystalline product, *N*-(4-nitrobenzoyl)-4-aminobenzylphosphonic acid, was faintly yellow in color and melted at 255–257 °C.

The above nitro compound was reduced to the amino compound by catalytic hydrogenation with 10% Pd/C. A 0.20-g sample of *N*-(4-nitrobenzoyl)-4-aminobenzylphosphonic acid was dissolved in H_2O; 0.05 g of catalyst was added and hydrogen was passed over the stirred solution for 4 h. A few drops of 0.10 M NaOH raised the pH to 8, the solution was filtered to remove catalyst, a few drops of HCl lowered the pH to approximately 4, and the resulting dense precipitate was collected by centrifugation to yield a faintly violet powder of *N*-(4-aminobenzoyl)-4-aminobenzylphosphonic acid, mp > 300 °C. Anal. Calcd for $C_{14}H_{15}N_2O_4P$ (fw 306.3): C, 54.9; H, 4.9; P, 10.1. Found: C, 54.6; H, 5.2; P, 10.3.

N-Acetyl-(D or L)-tyrosyldiazobenzylphosphonic Acid. Diazotized aminobenzylphosphonic acid was coupled to D or L isomers of *N*-acetyltyrosine and purified by the method of Tabachnick & Sobotka (1959): mp of L isomer 205–206 °C; mp of D isomer 240 °C (dec). Anal. Calcd for $C_{18}H_{20}N_3O_7P{\cdot}H_2O$ (fw 439.3): C, 49.2; H, 5.0; P, 7.0. Found: L isomer: C, 49.2; H, 5.1; P, 7.0. Found for D isomer: C, 49.1; H, 5.1; P, 7.1.

Coupling of Phosphonic Acids to Epoxy-Activated Sepharose 6B. Epoxy-activated Sepharose 6B was prepared for coupling by suspending 1.0 g in distilled H_2O and allowing the gel to hydrate for 30 to 60 min. The moist gel was transferred

[1] Abbreviation used: fw, formula weight.

to a 15-mL sintered-glass funnel and washed extensively with distilled water and finally one 15-mL wash of 0.10 M NaOH. A few milliliters of 0.10 M NaOH was used to dissolve 0.20 g of a phosphonic acid (aminomethylphosphonic acid, 4-aminobenzylphosphonic acid, or *N*-(4-aminobenzoyl)-4-aminobenzylphosphonic acid), and the pH was adjusted to 13. The moist gel was transferred to the phosphonic acid solution which was stirred or shaken overnight to react the aliphatic epoxy group of the agarose with the amino group of each ligand.

The derivatized agarose was transferred to a 0.7 × 9 cm column for washing with small volumes of 1.0 M NaCl in 100 mM acetic acid followed by 1.0 M NaCl in 100 mM ethanolamine (pH 9.0) and equilibration with 10 mM Tris-HCl (pH 8.0).

Preparation of Amino Acid Derivatives of Agarose. Procedures used to activate agarose with CNBr and to couple L-histidine, D,L-tyrosine, and L-phenylalanine were similar to that of Cuatrecasas (1970). In later coupling reactions, CNBr was dissolved in 10% *N*-methylpyrrolidone and pH was maintained by conducting the reaction in 2.0 M Na_2CO_3 (pH 11.0), instead of continuous titration with NaOH. The coupling buffer was 0.10 M $NaHCO_3$ (pH 9.5); 1 to 10 mmol of amino acid was present in the coupling solution containing 50 g of agarose and 50 to 100 mL coupling buffer.

Diazotization and Coupling of Aminobenzylphosphonic Acid to Amino Acid Agarose Derivatives. 4-Nitrobenzylphosphonic acid (0.5 g) was reduced to the amino compound by catalytic hydrogenation. The nitro compound was dissolved in 60 mL H_2O, 50 mg of 10% Pd/C catalyst added, and the suspension stirred at room temperature under a stream of hydrogen for 4 h. A few drops of NaOH were added until the pH was above 8, and the catalyst was removed by filtration.

4-Aminobenzylphosphonic acid was not isolated following reduction, but instead diazotized and coupled to either D,L-tyrosyl agarose or L-histidyl agarose. The phosphonic acid solution was made 1.5 M in HCl and 5.0 mL of 0.50 M $NaNO_2$ was added while the solution was cooled on ice. After 20 min, 50 mL of amino acid agarose and the diazotized 4-aminobenzylphosphonic acid solution were combined, while the pH was rapidly raised to 9.5 with 3 M NaOH. After stirring 4 h at 4 °C, the derivatized gel was washed in the same manner as the amino acid agarose derivatives.

Preliminary Screening of Phosphonic Acid Agarose Derivatives for Binding of Alkaline Phosphatase and Protein. Small columns of each phosphonic acid agarose derivative, 0.7 cm diameter and 3–7 cm in height, were equilibrated with 10 mM Tris-HCl (pH 8.0). Alkaline phosphatase from the DEAE-cellulose column (see Purification section above) was dialyzed against 10 mM Tris-HCl (pH 8.0). Small samples, 0.5 to 5.0 mL, were applied to the columns and elution with the equilibrating buffer was continued until sufficient volume had emerged from the column to remove unbound protein and alkaline phosphatase activity; then elution was switched to 10 mM Na_2HPO_4 in 10 mM Tris-HCl (pH 8.0) to elute specifically bound alkaline phosphatase. Fractions of 1–4 mL were collected and analyzed for absorption at 280 nm as well as alkaline phosphatase activity. Reduction of the anticipated 280 nm absorption indicated nonspecific binding of protein.

Quantitative Binding and Derivatization Measurements. Samples of the phosphonic acid agarose derivatives were prepared for analysis by washing a small portion of each gel on a 15-mL sintered-glass funnel with 15-mL volumes of 8 M urea, 1 M NaCl in 100 mM acetic acid, 1 M NaCl in 100 mM Na_2CO_3, and finally 75 mL of H_2O. Aliquots of 0.100 g (six samples per derivative) of moist gel were transferred to acid-cleaned tubes for analysis.

Derivatized gels (and control agarose) were ashed by the method of Ames & Dubin (1960) and analyzed for phosphate with the Josse (1966) procedure.

Binding of alkaline phosphatase was measured by adding a large excess of enzyme, 30 units in 0.25 mL, to each 100-mg agarose sample suspended in 5.00 mL of 10 mM Tris-HCl (pH 8.0). The enzyme used had a specific activity of 150 to 200. Each sample was mixed twice in 5 min and then the agarose was allowed to settle for 30 min. The difference in activity in the supernatant between nonbinding controls and agarose derivatives was taken as bound activity.

Inhibition Kinetics of Phosphonic Acids. Inhibition of alkaline phosphatase by phosphonic acids was studied at pH 8.0 in 100 mM Tris-HCl. 4-Nitrophenyl phosphate at concentrations of 33, 50, 100, and 200 μM was used as substrate. Continuous assays in 3-ml cuvettes were performed at 30 °C in a Beckman DB-G spectrophotometer at 400-nm wavelength. Changes in optical density were recorded on a Sargent SLRG recorder. Data were plotted as the least-squares fit on double-reciprocal graphs and values of K_i were determined from differences in slope.

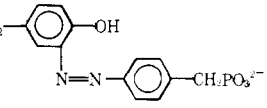
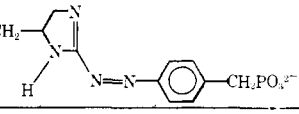
Results

Comparison of Phosphonic Acids as Ligands for Affinity Chromatography. Phosphonic acids prepared and coupled to agarose as described in Experimental Section were tested for alkaline phosphatase and protein binding in small column experiments. Samples of partially purified bovine intestinal alkaline phosphatase contained at least 90% of other proteins in order to estimate nonspecific protein binding. The results are summarized in Table I opposite representations of the structures of the phosphonic acid agarose derivatives. All of the derivatives except aminomethylphosphonic acid agarose exhibited specific binding of alkaline phosphatase. D,L-Tyrosyldiazobenzylphosphonic acid and L-histidyldiazobenzylphosphonic acid derivatives had the greatest binding capacity. Nonspecific binding of protein was detected only with the tyrosyl derivative. In each case, bound alkaline phosphatase was readily eluted by inclusion of 10 mM Na_2HPO_4, a strong competitive inhibitor (Fernley & Walker, 1967), in the eluting buffer.

Affinity media and unmodified agarose were tested for alkaline phosphatase binding quantitatively by a batchwise, rather than column procedure (see Experimental Section); binding was compared with ligand content, as shown in Table II. The ratio of units of alkaline phosphatase bound per μmol of phosphonic acid ligand is presented for each derivative as a comparative measure of binding efficiency. Despite a relatively high degree of substitution, the aminomethylphosphonic acid derivative of epoxy agarose bound no measurable alkaline phosphatase. With a much smaller degree of substitution the 4-aminobenzylphosphonic acid and *N*-(4-aminobenzoyl)-4-aminobenzylphosphonic acid derivatives of epoxy agarose bound significant amounts of enzyme. With the same moderate degree of substitution, D,L-tyrosyl and L-histidyl agarose coupled to diazotized 4-aminobenzylphosphonic acid bound large amounts of alkaline phosphatase (>200 units/mL). These derivatives had binding efficiencies which surpassed the other phosphonic acid affinity media and the arsonic acid derivatives reported by Brenna et al. (1975) by at least a factor of 4.

Inhibition Constants of Phosphonic Acid Affinity Chromatography Ligands and Related Compounds. Inhibition constants, K_i, were determined from double-reciprocal plots of continuous assays at pH 8.0 with four substrate concen-

TABLE I: Phosphonic Acid Derivatives of Agarose.

Derivative	Structure	Length (Å)[a]	Alkaline phosphatase binding[b]	Nonspecific binding[b]
Aminomethylphosphonic acid epoxy agarose	Agarose–$CH_2CHOHCH_2OCH_2CH_2CH_2CH_2OCH_2CHOHCH_2NHCH_2PO_3^{2-}$	21	–	–
4-Aminobenzylphosphonic acid epoxy agarose	Agarose–$CH_2CHOHCH_2OCH_2CH_2CH_2CH_2OCH_2CHOHCH_2NHC_6H_4CH_2PO_3^{2-}$	24	+	–
N-(4-Aminobenzoyl)4-aminobenzylphosphonic acid epoxy agarose	Agarose–$CH_2CHOHCH_2OCH_2CH_2CH_2CH_2OCH_2CHOHCH_2NHC_6H_4\overset{O}{\overset{\|}{C}}NHC_6H_4CH_2PO_3^{2-}$	31	+	–
D,L-Tyrosyldiazobenzylphosphonic acid agarose	Agarose–$NHCH(COO^-)CH_2$– 	18	+++	+
L-Histidyldiazobenzylphosphonic acid agarose	Agarose–$NHCH(COO^-)CH_2$– 	18	+++	–

[a] Length in angstroms of each phosphonic acid agarose affinity ligand was determined from molecular models and assumes the fully extended steric conformation. [b] Details of the small-column experiments summarized above are presented in the Experimental Section. A plus sign indicates that binding occurred; a minus sign denotes absence of detectable binding.

TABLE II: Measurements of Ligand Content and Binding Capacity of Affinity Chromatography Phosphonic Acid Derivatives.

Derivative	Phosphonic acid content (μmol g^{-1})[a]	Alkaline phosphatase bound (units g^{-1})[a]	Efficiency (units μmol^{-1})[b]
Unmodified agarose	0	0	0
Aminomethylphosphonic acid epoxy agarose	20	0	0
4-Aminobenzylphosphonic acid epoxy agarose	4–5	30	6
N-(4-Aminobenzoyl)4-aminobenzyl phosphonic acid epoxy agarose	3	40–50	15
D,L-Tyrosyl diazobenzylphosphonic acid agarose	3	210	70
L-Histidyldiazobenzylphosphonic acid agarose	3	220	70

[a] Analyses of ligand content and alkaline phosphatase binding were performed as described in Experimental Section on 100-mg aliquots of moist gel; data are expressed per g of moist gel. [b] Efficiency of alkaline phosphatase binding was computed as the ratio of units of enzyme bound g^{-1} to the phosphonic acid content g^{-1}.

TABLE III: Inhibition of Alkaline Phosphatase by Phosphonic Acids.[a]

Phosphonic acid inhibitor	K_i (mM)[b]	No. of determinations
Aminomethylphosphonic acid	27 (4)	5
N-Acetylaminomethylphosphonic acid	2 (1)	5
Phosphonoacetic acid	0.09 (0.03)	3
4-Aminobenzylphosphonic acid	1.1 (0.01)	3
4-Nitrobenzylphosphonic acid	0.075 (0.014)	5
Phenylphosphonic acid	3 (2)	9
N-(4-Aminobenzoyl)-4-aminobenzyl phosphonic acid	0.48 (0.03)	4
N-Acetyl-L-tyrosyldiazobenzylphosphonic acid	0.02 (0.006)	4
N-Acetyl-D-tyrosyldiazobenzylphosphonic acid	0.02 (0.008)	4

[a] Inhibition was measured at 30 °C in 100 mM Tris-HCl (pH 8.0) with 4-nitrophenyl phosphate as substrate. [b] Values of one standard deviation are given in parentheses.

trations. The K_m of the substrate, 4-nitrophenyl phosphate, calculated as the average of more than 20 determinations was 14 μM under the conditions used here. A value of 44 μM has been reported for this enzyme under similar conditions (Kelly et al., 1973). All of the phosphonic acids tested produced patterns of lines on double-reciprocal plots indicative of competitive inhibition.

The data presented in Table III indicate that phosphonic acid derivatives with small substituents were much less inhibitory than large, aromatic phosphonic acid substituents. Phosphonic acids with positively charged substituents were relatively poor inhibitors, whereas those with a negative charge on the carbon side chain were potent inhibitors. The optical configuration of the tyrosyl moiety of D or L isomers of *N*-acetyltyrosyldiazobenzylphosphonic acid had no effect on this compound's K_i, eliminating a stereospecific interaction of the amino acid portion of these compounds with alkaline phosphatase. The ability of the phosphonic acid agarose derivatives to specifically bind alkaline phosphatase closely paralleled the ability of these derivatives in free solution to inhibit this enzyme.

Use of L-Histidyldiazobenzylphosphonic Acid Agarose in Purification of Alkaline Phosphatase. Affinity chromatography of a partially purified sample of bovine intestinal alkaline phosphatase on L-histidyldiazobenzylphosphonic acid agarose produced the elution diagram shown in Figure 1. The enzyme was dialyzed against 10 mM Tris-HCl (pH 8.0) and applied to the column equilibrated with the same buffer. After washing out unbound materials with two column volumes of the same buffer, the enzyme was eluted by adding 10 mM Na_2HPO_4 to the buffer. The single sharp peak contained 85% of the original alkaline phosphatase activity. Specific activity had been improved 11-fold to 110 units mg^{-1}. Bound enzyme was not eluted on a 10-fold increase in buffer concentration, suggesting that binding was not due to ionic attraction.

When L-histidyldiazobenzylphosphonic acid agarose was

used in the final steps of the purification of 5′-nucleotide phosphodiesterase, relatively larger volumes of enzyme were applied. Alkaline phosphatase purified with 5′-nucleotide phosphodiesterase and resolved by affinity chromatography had a specific activity of 150–200 μmol of 4-nitrophenyl phosphate min^{-1} mg^{-1} at pH 8.0. These preparations produced a single sharp band of protein following polyacrylamide gel electrophoresis and contained only traces of phosphodiesterase activity.

Discussion

The agarose-coupled phosphonic acid derivatives described here exhibited a broad range of affinities for alkaline phosphatase. The most useful derivative for purification by affinity chromatography was L-histidyldiazobenzylphosphonic acid agarose; this derivative is inexpensive, stable, and easily prepared. The capacity for binding alkaline phosphatase is high and binding appears to be based on specific enzyme–ligand affinity; nonspecific protein binding was not detected, and 10 mM Na_2HPO_4, a strong competitive inhibitor (Fernley & Walker, 1967), completely eluted the enzyme. Homogeneous enzyme was obtained in a single step from highly impure preparations.

It is clear that substituents far removed from the phosphorus atom can have a profound influence on the affinity of the enzyme for the ligand. Our results do not support length of the spacer as a primary factor, because the shortest spacer arms (measured in the extended confirmation) gave the greatest binding capacity. Instead, our results strongly indicate that the extent of aromaticity/hydrophobicity of the ligand accounts for observed differences in binding; the inhibition studies also support this conclusion. Kochman et al. (1964) likewise reported greater inhibition by aromatic than by aliphatic phosphonic acids, under considerably different conditions.

The affinity of the enzyme for substrate analogues is also acutely influenced by the charge on the substituent; this is seen most clearly in the inhibitor studies. The presence of a positively charged amino group on the carbon to which the phosphorus atom is attached makes aminomethylphosphonic acid a much poorer inhibitor than the corresponding uncharged acetylated compound, whereas the presence of a negatively charged carboxylate group, as in phosphonoacetic acid, strongly enhances inhibition. Amino groups quite far removed from the phosphorus atom, as in 4-aminobenzylphosphonic acid and *N*-(4-aminobenzoyl)-4-aminobenzylphosphonic acid, apparently depress binding (compare with 2-nitrobenzylphosphonic acid). The most tightly bound ligands, as indicated by both enzyme inhibition and affinity chromatography, are phosphonic acids with substituents which are both aromatic and negatively charged.

Several phosphonic acids examined here are exceptionally good inhibitors of alkaline phosphatase. The only competitive inhibitors (nonsubstrates) of alkaline phosphatase with K_i values lower than that of *N*-acetyltyrosyldiazobenzylphosphonic acid (K_i = 20 μM) are inorganic phosphate (K_i = 15 μM) (Fernley & Walker, 1967) and arsenate (Harkness, 1968).

The stereospecific inhibition of this enzyme by aromatic L-amino acids (Cox & Griffin, 1967; Fishman et al., 1962) led us to examine the stereospecificity of inhibition by *N*-acetyl-(D or L)-tyrosyldiazobenzylphosphonic acid. The inhibition by this compound is nonstereospecific and competitive, whereas free aromatic amino acids are stereospecific uncompetitive inhibitors (Ghosh & Fishman, 1966); it is likely that these groups of compounds bind at different sites on the enzyme.

Our data allows some speculation concerning the nature of the alkaline phosphatase active site. Binding of phosphonic acid inhibitors appears to involve three domains: (1) a concentrated positive charge which interacts with the negative charges of phosphonic acids (inhibitors) and phosphates (substrates); (2) an adjacent hydrophobic pocket with a high affinity for large aromatic substituents; and (3) further removed, peripheral positive charges.

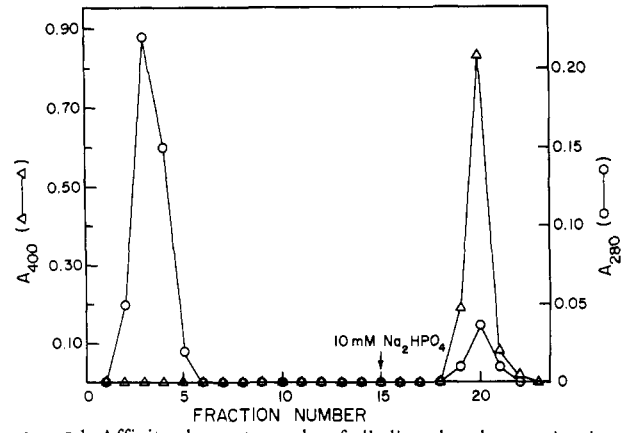


FIGURE 1: Affinity chromatography of alkaline phosphatase. A column of L-histidyldiazobenzylphosphonic acid agarose (0.7 × 17 cm) was equilibrated with 10 mM Tris-HCl (pH 8.0), and 2.0 mL of partially purified enzyme containing 8.0 unit of activity and approximately 0.8 mg of protein was applied in the equilibrating buffer to the column. After washing the column with 15 mL of the same buffer, 10 mM Na_2HPO_4 was added to the buffer at fraction 15. Specific activity was increased 11-fold to 110 units mg^{-1} and 85% of the activity was recovered in the purified fraction.

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