Activity of Epiphyseal Cartilage Membrane Alkaline Phosphatase and the Effects of Its Inhibitors at Physiological pH*

(Received for publication, November 9, 1981)

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The kinetics of epiphyseal cartilage membrane-bound alkaline phosphatase activity were studied at physiological pH using p-nitrophenylphosphate and pyrophosphate (PPi) as substrates. The effect of three general types of alkaline phosphatase inhibitors was studied on both purified and membrane-bound forms of the enzyme: 1) uncompetitive inhibitors (L-tetramisole and theophylline), 2) competitive inhibitors (phosphate, arsenate, and vanadate), and 3) metal ions (Mg\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\)). These studies were aimed at elucidating the physiological role of epiphyseal cartilage membrane alkaline phosphatase.

Hydrolytic activity of the enzyme at pH 7.5 toward both PPi and p-nitrophenylphosphate was only one-tenth and one-hundredth, respectively, of that observed at optimal pH. Arsenate (K\(_i\) = 1.0-2.7 \(\mu\)M) and vanadate (K\(_i\) = 0.7-1.3 \(\mu\)M) were powerful inhibitors of alkaline phosphatase; phosphate (K\(_i\) = 20-50 \(\mu\)M) was inhibitory at levels two orders of magnitude below the concentration in cartilage extracellular fluid. Neither Zn\(^{2+}\), Ca\(^{2+}\), or Mg\(^{2+}\) was inhibitory toward p-nitrophenylphosphate hydrolysis, whereas all three competitively inhibited PPi hydrolysis. The data suggest that formation of poorly hydrolyzable Ca\(^{2+}\)PPi, Mg\(^{2+}\)PPi, and Zn\(^{2+}\)PPi complexes was responsible. Inhibition of PPi hydrolysis by Ca\(^{2+}\) occurred at levels within the physiological range.

The close similarity in inhibition of both the purified and membrane-bound forms of alkaline phosphatase at pH 7.5 indicates that interaction with the membrane does not significantly alter conformation at the active site. The data obtained suggest that under physiological conditions, cartilage membrane alkaline phosphatase would be essentially inactive as a phosphohydrolase due to its intrinsically weak activity at pH 7.5 and the strong inhibitory effect of physiological levels of phosphate and Ca\(^{2+}\).

Several investigators have provided evidence that alkaline phosphatase may be involved in phosphate transport in vivo (1-3), although others have reported that inhibitors of alkaline phosphatase are without effect on this function in intestine and kidney (4-6). In calcifying cartilage, alkaline phosphatase is found both in chondrocyte membranes and in the membranes of extracellular matrix vesicles (7-8), structures believed to be the primary sites of initial endochondral calcification (9-10). Matrix vesicles isolated from homogenized or collagenase-digested tissue have been shown to accumulate \(^{40}\)Ca and/or \(^{32}\)P from metastable solutions (11-15), and this uptake can be suppressed by inhibitors of alkaline phosphatase (11, 14-15). While this suggests that the enzyme is involved, this interpretation is weakened by the lack of data on the effect of such inhibitors of alkaline phosphatase activity at physiological pH.

As part of our continuing investigation of the role of alkaline phosphatase in calcification, we recently reported on the isolation and characterization of a membrane-bound alkaline phosphatase from chicken epiphyseal cartilage (16). In that study the kinetic experiments were carried out at pH 10.3, the optimal pH for the substrate used (p-nitrophenylphosphate), although it was recognized that this was far from the pH (7.5) of the extracellular fluid of epiphyseal cartilage (17).

We now report on studies of the kinetics of cartilage membrane alkaline phosphatase carried out at physiological pH (7.5). We studied the effects of several inhibitors which have been used, or are potentially useful, in investigations of the role of the enzyme as a vector in the uptake of P\(_i\) by matrix vesicles. These studies were carried out with both pNPP\(^1\) (a common in vitro substrate for the enzyme) and PPi (a potentially important physiological substrate) in order to distinguish between direct effects of the inhibitors on the enzyme, and indirect effects caused by complexation with the substrate. We also studied the activity of the enzyme in its native environment, comparing inhibition of activity of the membrane-bound form with that of the purified enzyme. These experiments were designed to explore the nature and strength of inhibition of both purified and membrane-bound alkaline phosphatase at physiological pH using three general types of inhibitors: 1) heterocyclic uncompetitive inhibitors (L-tetramisole and theophylline), 2) the enzyme product and its analogues (phosphate, arsenate, and vanadate), and 3) several physiologically important divalent cations (Mg\(^{2+}\), Ca\(^{2+}\), and Zn\(^{2+}\)).

**EXPERIMENTAL PROCEDURES**

Purified alkaline phosphatase was prepared as previously described (16). For studies on the membrane-bound enzyme, partially purified matrix vesicles were prepared by a modification of the procedure of Watkins et al. (18). In the published procedure, a step gradient was used which was composed of 10, 50, 100, and 500 \(\mu\)M MgCl\(_2\) in the method used here, the step gradient was composed of 15 ml of 40% (w/v) sucrose and 20 ml of 10% (w/v) sucrose, both made in the TES-Mg\(_2\)Cl\(_2\) buffer. The material collected at the 10/40% interface was pooled, diluted with 2-3 volumes of the 10% sucrose buffer, and centrifuged at 85,000 \(\times\) g for 60 min. The pellet which corresponded approximately to a combination of the A and B fractions of the earlier procedure was resuspended in 10% sucrose made in the TES-Mg\(_2\)Cl\(_2\) buffer, and

\(^{1}\) The abbreviations used are: pNPP, p-nitrophenylphosphate; PPi, pyrophosphate; TES, \(N\)-tris(hydroxymethyl)-methyl-2-aminoethanesulfonate.
used without further purification. The protein concentration both of the purified enzyme and the partially purified matrix vesicles was determined by the method of Lowry et al. (19) using bovine serum albumin as a standard.

Hydrolysis Measurements—Hydrolysis of substrates was measured in 0.25 M TES buffer adjusted to pH 7.5 with NaOH. Appearance of p-nitrophenol was monitored at 402 nm and analyzed as previously described (16) using a molar extinction coefficient of 4500 cm$^{-1}$·mol$^{-1}$·L. Hydrolysis of PP$_i$ was followed in 35-min assays as previously described (16), the rate being linear for this length of time under the conditions used. The temperature of the assays was maintained at 37 °C either by circulating water through a jacketed cuvette holder (pNPP) or by a water bath (PP$_i$). The extent of hydrolysis was always limited to less than 10% of the available substrate, and the pH was monitored to ensure that it was not affected. All assays were initiated by the addition of substrate. Kinetic parameters ($K_m$ and $V_{max}$) were estimated by a nonlinear least squares fit (16, 20), and the inhibition constants were obtained by unweighted linear least squares fits to the appropriate secondary plots.

Transphosphorylation Measurements—The assay procedure was based on that of Arsenis et al. (21). A solution containing 5 mM glycerol, 0.25 M TES-NaOH buffer (pH 7.5), 2 mM NADH, and 2 units of glycerol-3-phosphate dehydrogenase in a total volume of 2.0 ml was equilibrated at 37 °C. Alkaline phosphatase (1.7 μg in 0.05 ml) was then added, and the reaction initiated by the addition of PP$_i$. Levamisole, when used, was added from a 1 mM stock solution immediately prior to the alkaline phosphatase. Absorbance was recorded at 340 nm for 10 min and the slope of this line was used for activity calculations.

Materials—The following were purchased from Sigma: p-nitrophenylphosphate (disodium hexahydrate), p-nitrophenol standard solution, TES, β-NADH (Grade III), glycerol-3-phosphate dehydrogenase (Type X), theophylline, and levamisole. The V$_{O_2}$ used to prepare the vanadate solutions was supplied by MCB (Cincinnati, OH). [V$^{35}$P]PP$_i$ was procured from New England Nuclear. All other chemicals were of reagent grade and were obtained from Fisher.

RESULTS

Table I presents comparative data on the $K_m$ and $V_{max}$ of the purified enzyme toward both pNPP and PP$_i$ as substrates, at both optimal and physiological pH. It is evident that both parameters are markedly decreased at pH 7.5. These data indicate that while binding to the enzyme is much tighter, hydrolytic activity toward both substrates is nevertheless greatly suppressed. Other data, not shown, reveal that the $K_m$ of the purified and membrane-bound forms of the enzyme for both substrates was essentially identical.

With pNPP as substrate at pH 7.5, levamisole (l-tetramisole) proved to be an uncompetitive inhibitor of both the purified and vesicular (membrane-bound) enzyme (Fig. 1). There was a small, but consistent difference between the $K_i$ values of the two enzyme forms (1.4 mM for purified, 0.6 mM for membrane-bound). Unfortunately, the effect of levamisole could not be studied with PP$_i$ as substrate because it severely interfered with the assay procedure.

Theophylline (Fig. 2) also proved to be an uncompetitive inhibitor of alkaline phosphatase at this pH. $K_i$ analysis from secondary plots indicated that, with pNPP as substrate, there was no difference between the vesicular and the pure enzyme. With PP$_i$ as substrate, however, there was a significant difference between the values for the purified enzyme ($K_i = 5.6$ mM) and the membrane-bound phosphatase ($K_i = 3.0$ mM). The direction of this difference was the same as that seen with levamisole inhibition of pNPP hydrolysis.

Phosphate (Fig. 3), arsenate (Fig. 4), and vanadate (Fig. 5) were all competitive inhibitors of alkaline phosphatase activity with both the purified and membrane-bound forms of the enzyme. While all three analogues were powerful inhibitors of hydrolysis activity toward both substrates, with $K_i$ values in the low micromolar range, both arsenate and vanadate were 10 to 20 times more effective than phosphate (Table II). Although slight differences between the $K_i$ values of the

![Table I](image)

**Table I** Kinetic parameters of purified epiphyseal cartilage membrane alkaline phosphatase at optimal and physiological pH

| Substrate | Optimal $^*$ | Physiological (7.5)$^+$ |
|-----------|-------------|-------------------------|
|           | $K_m$ μM    | $V_{max}$ μmol/min/mg   |
|           | $K_m$ μM    | $V_{max}$ μmol/min/mg   |
| PP$_i$    | 85          | 0.8                     | 10–20       | 0.2        |
| pNPP      | 700         | 220                     | 1–2         | 6.0        |

* pH 10.3 for pNPP in 0.25 M 2-amino-2-methyl-1-propanol buffer.

† pH 8.5 for PP$_i$ in 0.25 M Tris buffer.

‡ In 0.25 M Tris buffer.

Fig. 1. Inhibition of hydrolysis by levamisole (l-tetramisole). See "Experimental Procedures" for assay procedures. $V^0$ is $V_{max}$ in the absence of inhibitor, $V^{lev}$ is $V_{max}$ in the presence of inhibitor. Open symbols, membrane-bound (vesicular) phosphatase; filled symbols, purified epiphyseal cartilage membrane alkaline phosphatase. Bars represent the standard error of the parameter, as determined by Cleland's HYPER-fit program (20). Lines are drawn according to a linear least squares fit.

Fig. 2. Inhibition of hydrolysis by theophylline. See "Experimental Procedures" for assay procedures. $V^0$ and $V^{oph}$ are as defined in Fig. 1. Left, hydrolysis of pNPP; right, hydrolysis of PP$_i$. Open symbols, membrane-bound phosphatase; closed symbols, purified alkaline phosphatase. Bars represent the standard error of the parameter as defined in Fig. 1.
membrane-bound and purified enzyme activities were evident, no consistent pattern was discernible. In the case of phosphate, the purified enzyme was more strongly inhibited; with vanadate, the membrane-bound activity was more strongly affected. With arsenate, the effects depended on the substrate. These points will be discussed more fully later.

None of the metal ions tested (Zn\textsuperscript{2+}, Ca\textsuperscript{2+}, and Mg\textsuperscript{2+}) showed any effect on alkaline phosphatase activity at pH 7.5 when pNPP was used as substrate. In contrast, with PPi as substrate, all three divalent cations inhibited enzyme activity. As will be evident, the nature of the inhibitory species differed in each case.

Inhibition by ZnCl\textsubscript{2} was strictly competitive (Fig. 6) and was quite marked (\(K_i = 37-39\) \(\mu\)M). No apparent difference between the two forms of enzyme was evident. At the concentrations of Zn\textsuperscript{2+} studied, greater than 95% of the PPi would have been present as a Zn-PPi binary complex (\(K_d = 0.5\) \(\mu\)M), (22). Had this complex been inactive as a substrate, the hydrolytic activity should have been only 3–6% of that observed at low (5 \(\mu\)M) PPi concentration. The observed hydrolysis thus must have been of the binary Zn-PPi complex. Indeed, since the \(V_{max}\) seen at all Zn\textsuperscript{2+} concentrations tested was identical with that observed in the absence of Zn\textsuperscript{2+}, and since a plot of the \(K_{app}/V_{max}\) \textit{versus} Zn\textsuperscript{2+} concentration passed through the control value, the Zn-PPi complex must have been as active as a substrate as free PPi.

Inhibition by CaCl\textsubscript{2} (Fig. 7) was seen at concentrations (0.5–2.0 mM), and with \(K_i\) values (0.73–1.12 mM), similar but not identical to those expected for inhibition by substrate depletion. A \(K_d\) of \(3.4 \times 10^{-4}\) M for the Ca-PPi complex (22) was used to calculate the concentrations of free PPi at each concentration.

\begin{table}[h]
\centering
\caption{Summary of inhibition data for epiphyseal cartilage membrane alkaline phosphatase at physiological pH.}
\begin{tabular}{|c|c|c|c|}
\hline
Inhibitor & Enzyme substrate & Membrane-bound & Membrane-bound \\
& & pNPP & PPi \\
\hline
L-Tetramisoleb & & & \\
& & 620 & 1320 \\
& & 2850 & 2900 \\
& & 26 & 26.6 \\
& & 2.7 & 1.0 \\
& & 1.0 & 1.3 \\
& & 39 & 37 \\
& & 730 & 1120 \\
ZnCl\textsubscript{2} & & & \\
& & 350, 270 & 350, 280 \\
CaCl\textsubscript{2} & & & \\
& & 390 & 270 \\
MgCl\textsubscript{2}c & & & \\
& & 350 & 270 \\
\hline
\end{tabular}
\end{table}

a Membrane bound alkaline phosphatase was assayed in the matrix vesicle enriched fraction (see Methods).

b Inhibition of pyrophosphate activity by L-tetramisole (levamisole) could not be assayed because the drug interfered with extraction of phosphomolybic acid required for the assay method (18). d-tetramisole (dexamisole) exerted no inhibitory effect on pyrophosphate activity. No noticeable inhibition of pNPP hydrolysis was detected at pH 7.5 with concentrations of ZnCl\textsubscript{2} up to 1 mM, CaCl\textsubscript{2} up to 3 mM, and MgCl\textsubscript{2} up to 1.5 mM.

c No measurable inhibition of pNPP hydrolysis was detected at pH 7.5 with concentrations of ZnCl\textsubscript{2} up to 1 mM, CaCl\textsubscript{2} up to 3 mM, and MgCl\textsubscript{2} up to 1.5 mM.

d The first \(K_i\) value for MgCl\textsubscript{2} was as a competitive inhibitor, the second as an uncompetitive inhibitor.
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**Fig. 6.** Inhibition of hydrolysis by zinc. See “Experimental Procedures” for assay procedures. Only PP\textsubscript{i} hydrolysis is shown since there was no effect observed on pNPP hydrolysis with Zn\textsuperscript{2+} concentrations as high as 1.0 mM. Symbols and graphing methods are as shown in Figs. 1 and 3.

**Fig. 7.** Inhibition of hydrolysis by calcium. See “Experimental Procedures” for assay procedures. Only PP\textsubscript{i} hydrolysis is shown since there was no effect observed on pNPP hydrolysis with Ca\textsuperscript{2+} concentrations as high as 3.0 mM. Symbols and graphing methods are as shown in Figs. 1 and 3.

The concentration of Ca and PP\textsubscript{i}. When the expected rate of hydrolysis was calculated from these concentrations of free PP\textsubscript{i}, using the $K_{m}$ and $V_{max}$ values observed in the presence of added cations, it was noted that the calculated activity was consistently lower than that observed. When the reciprocal of the difference between the calculated (i.e., from free PP\textsubscript{i}) and observed activity was plotted against the reciprocal of the calculated Ca-PP\textsubscript{i} complex concentration, a linear relationship was observed. The $V_{max}^{app}$ so calculated was approximately one-tenth that observed in the absence of Ca\textsuperscript{2+}. Thus, it was evident that while the Ca-PP\textsubscript{i} complex was capable of acting as a substrate, it was only about 10% as active as free PP\textsubscript{i}.

With MgCl\textsubscript{2} as inhibitor, mixed-type inhibition was seen. As seen in Fig. 8A, Mg\textsuperscript{2+} stimulated vesicular phosphatase activity at low concentrations (<0.25 mM), as indicated by lower s/v values. At higher concentrations, however, it was inhibitory. The intersection of the lines of this plot (Fig. 8A) indicated an apparent $K'_i$ of 0.27 mM for the uncompetitive portion of the inhibition. Similar results were obtained with the purified enzyme ($K'_i = 0.29$ mM) (data not shown). Dixon plots of the inhibition data for both the vesicular (Fig. 8B) and purified phosphatase (not shown) gave a $K_i$ of 0.35 mM for the competitive portion.

The results of these various inhibition studies are summarized in Table II, where the apparent $K_i$ values for each inhibitor and enzyme form are shown. Although comparison of the vesicular and purified enzyme showed several differences, no clear pattern emerged. Levamisole and theophylline, which presumably share a common binding site (23), had no more than a 2-fold difference in $K_i$ value for the two substrates, or the two enzyme forms. This was also true for inhibitions by phosphate, arsenate, and vanadate. These results thus suggest that if binding of alkaline phosphatase to the membrane had an effect on enzyme conformation, it was not sufficiently large to markedly affect the kinetic parameters determined in this investigation.

Finally, to test the effectiveness of levamisole as an inhibitor of one of the other functions of alkaline phosphatase, transphosphorylation using PP\textsubscript{i} as the phosphate donor and glycerol as the acceptor was measured in the presence and absence of three concentrations of the drug. The results, shown as a plot of s/v versus concentration of levamisole (Fig. 9), indicated that levamisole was an uncompetitive inhibitor of this function.
that the buffer used. With an amino-alcohol buffer (Tris) the difference was 6-10-fold less than with a non-amino-alcohol buffer (carbonate). This difference is approximately the same change we observed in going from 2-amino-2-methyl-1-propanol at pH 10.3 to TES at pH 7.5. Our data are therefore basically consistent with the monovalent anion being the active inhibitor species.

Inhibition by vanadate has been hypothesized to be more potent in enzymes involving a stable covalent phosphoryl enzyme intermediate than in those in which the product is formed without covalent involvement of the enzyme (25). Thus, it is particularly active against Na+/K+-ATPase (26), Escherichia coli alkaline phosphatase (28), and less so against sarcoplasmic reticulum Ca2+-ATPase (29). The vanadate (from V2O5) used in this study is known to exist in the form of H3V04- and HVO42- in dilute neutral solutions (30). The Ki of vanadate (1.4-2.6 μM) is within the range expected for phosphoryl-enzyme mechanisms. Further, we have evidence that, in agreement with other alkaline phosphatases, a stable 3P-alanine phosphatase form of the cartilage enzyme exists at acid pH.

The presence of the membrane is known to affect the properties of several membrane-derived enzymes. The data presented here, however, do not show any consistent major change in the kinetic properties of the membrane-bound cartilage enzyme as a result of solubilization and purification. In several cases (levamisole versus pNPP; theophylline versus PPi; phosphate versus pNPP; and vanadate versus PPi) there was about a 2-fold difference between the Ki of the membrane-bound and the purified enzyme form. These rather small differences, and the lack of a consistent pattern, indicate that the membrane environment has little effect on the activity of the enzyme. A similar lack of effect of the membrane was also noted for the calf intestinal alkaline phosphatase (31).

Each of the three metal ions investigated showed different effects on pP hydrolysis. As noted before, Ca2+ seemed to act primarily by way of substrate depletion, with the Ca2-PP complex being only one-tenth as active as free PPi. By contrast, Zn2-PP complex appeared to bind to the enzyme and undergo hydrolysis much like free PPi. This interpretation is based on the fact that Vmax was unchanged, and plots of Km versus Vmax versus Zn2+ concentration extrapolated to the value seen in the absence of added cations, although essentially no free PPi should have been present under the conditions of these assays. Several workers have reported inhibition of mammalian alkaline phosphatases by excess Zn2+ (32-35). They proposed that the inhibition is caused by Zn2+ replacing Mg2+ at one site on the enzyme. This does not appear to be the mechanism involved here, since we observed no effect of ZnCl2 on pNPP hydrolysis at pH 7.5. In this regard, it is interesting to note that ZnCl2 was a powerful inhibitor of pNPP hydrolysis (Ki = 19 μM) at pH 10.3 (16). This suggests that the active form of zinc at alkaline pH is not Zn2+, but ZnOH+. It is known that Zn2+ forms a soluble Zn2-PP, complex (22) and it is likely that this ternary complex is the form directly involved in the competitive inhibitory process.

Finally, Mg-PPi may be a better substrate for the enzyme than is free PPi. This is evidenced by the observation of
stimulation of PPi hydrolysis by low concentrations of MgCl₂. We observed no effect of added Mg²⁺ on pNPP hydrolysis, suggesting that this stimulation is the result of Mg²⁺-PPi interactions rather than Mg²⁺-enzyme interactions. Since Mg²⁺ is also capable of forming soluble Mg₆-PP complexes (22), this may be the mechanism of competitive inhibition seen at higher MgCl₂ concentrations, much as was observed with Zn²⁺. The uncompetitive portion of the inhibition could be due to stabilization of the phosphoryl-enzyme complex by free Mg²⁺. Exact elucidation of the complexes and mechanisms involved must, however, await more detailed experiments using constant metal ion:PPi ratios, and competitive inhibitions of pNPP hydrolysis by metal-ion:PPi complexes.

In this study we chose conditions which would ensure extrapolation of the observed effects on the activity of alkaline phosphatase to the physiological situation. For instance, TES buffer was used in order to avoid the known stimulation by amino- and alcohol-containing buffers such as Tris and 2-amino-2-methyl-1-propanol (21, 36). Second, the activity was studied using phosphate monoester and anhydride substrates since both types are present in epiphyseal cartilage, and the metal ions might be expected to differ in their effects on the two types of substrates. Further, as discussed above, we determined the effect of the membrane environment on alkaline phosphatase activity. Finally, the effect of all of these parameters was studied at physiological pH. In terms of physiological function, these findings, in conjunction with previously published information on the weak activity of alkaline phosphatase at this pH (16), are not supportive of this enzyme acting as a hydrolase in vivo. Several observations support this conclusion. First, the specific activity of alkaline phosphatase at pH 7.5 was only one-tenth to one-hundredth (depending on substrate) of that seen at optimal pH. Thus, whereas the specific activity of pNPP hydrolysis at pH 10.3 in 2-amino-2-methyl-1-propanol buffer was approximately 200-220 μmol/min/mg of enzyme (16), the Vₘₐₓ observed at pH 7.5 was only about 0.5 μmol/min/mg. With PPi, the observed Vₘₐₓ was on the order of 0.2 μmol/min/mg. These activities are such that only very limited hydrolysis would occur, even in the absence of inhibitors. However, the concentration of phosphate in the extracellular fluid of epiphyseal cartilage (2.2-2.3 mM) (17), is approximately two orders of magnitude above the Kᵢ determined for both phosphate monoesters and pyrophosphate in this study. Since both nucleotide phosphates (37) and pyrophosphate (38) are present in the extracellular fluid in only micromolar amounts, it is unlikely that they could compete effectively for the enzyme in vivo.

Finally, it should be noted that extracellular cartilage fluid also contains 1.5-1.7 mM Ca²⁺ (17). This concentration, coupled with the fact that PPi is present in only micromolar concentrations as indicated above, leads to the conclusion that PPi would be largely complexed with Ca²⁺, a form which was shown above to be almost completely inactive as a substrate. These considerations thus lead to the conclusion that alkaline phosphatase in vivo would be almost entirely without hydrolytic activity toward any likely substrate. Of the proposed roles of the protein, that of phosphate transport across the membrane seems most likely. For example, the Kᵢ for phosphate indicates that alkaline phosphatase has a high affinity for this anion at physiological pH. Secondly, the role of alkaline phosphatase as a Pi vector has been recently investigated using vesicles isolated from non-protease-dependent methods (15). Those studies showed that levanositol strongly and selectively inhibited ³²P uptake, lending credence to this idea. The strong competitive inhibition by arsenate and vanadate observed in this study make it likely that these ions will prove useful in the study of phosphate transport, since they should be effective competitors of phosphate in the uptake system.

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*J. Biol. Chem.* 1982, 257:4141-4146.

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