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Inhibition of Human Alkaline Phosphatases by Vanadate

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Orthovanadate was shown to be a potent competitive inhibitor (K_i less than 1 μM) of purified alkaline phosphatase from human liver, intestine or kidney. Inhibition was reversed and full enzymic activity restored in the presence of 1 mM-adrenaline. Phosphate and vanadate competed for the same binding site on the enzyme.

During studies on the ATPase activity of purified human liver alkaline phosphatase we noted that this activity was inhibited at ATP concentrations greater than 0.5mM. Several reports have documented a similar effect on ($Na^+ + K^+$)-stimulated ATPase (Josephson & Cantley, 1977; Hudgins & Bond, 1977; Beaugé & Glynn, 1978), when the ATP used was from an extract of horse muscle. No inhibitor has been found in ATP from yeast. Cantley *et al.* (1977) and Quist & Hokin (1978) have now identified the inhibitor as orthovanadate (VO_4^{3-}). This compound has previously been shown to be an inhibitor of alkaline phosphatase from *Escherichia coli* (Lopez *et al.*, 1976) and acid phosphatase from human liver or wheat germ (Van Etten *et al.*, 1974).

The present report demonstrates that human liver alkaline phosphatase from several human tissues is inhibited by physiological concentrations of orthovanadate. In addition it is shown that orthovanadate and phosphate bind to human liver alkaline phosphatase in a mutually exclusive fashion.

Materials and Methods

Sodium orthovanadate (Na_3VO_4) was obtained from Fisher Scientific Co., Fair Lawn, NJ, U.S.A.; the vanadate concentration was determined indirectly by analysis for sodium by flame photometry. The following were from Sigma Chemical Co., St. Louis, MO, U.S.A.: *p*-nitrophenyl phosphate, ATP (horse muscle, no. 6144), vanadium-free ATP (horse muscle, no. 5394) and L-adrenaline. Alkaline phosphatase (EC 3.1.3.1) was purified from human liver, kidney or small-intestinal mucosa by a modification of the procedure previously described (Trépanier *et al.*, 1976; Seargeant & Stinson, 1979*a*). The enzyme preparations appeared to be free of contaminating protein, as judged by polyacrylamide-gel electrophoresis with and without sodium dodecyl sulphate. The specific activity of the enzyme from liver was 1300 μmol/min per mg. The procedures for polyacrylamide-gel electrophoresis with and without sodium dodecyl sulphate and staining for enzyme activity or protein were as described previously (Trépanier *et al.*, 1976).

Enzyme activity with *p*-nitrophenyl phosphate as substrate was measured as described (Trépanier *et al.*, 1976) in a buffer mixture that contained 25mM-2-methyl-2-aminopropan-1-ol, 25mM-Tes (2-{[2-hydroxy-1,1-bis(hydroxymethyl)ethyl]amino}ethanesulphonic acid), 25mM-Tris and 1.5mM-$MgCl_2$ (pH adjusted with HCl) at substrate concentrations that ranged from 5 to 100 μM (pH7.4) or 25 to 1000 μM (pH9.0). When the influence of L-adrenaline on vanadate inhibition was studied, activity was monitored by the release of phosphate (Anner & Moosmayer, 1975).

ATPase activity was measured at pH8.0 as described above by the amount of phosphate released (LeBel *et al.*, 1978). Since Mg^{2+} ions inhibited ATPase activity, the $MgCl_2$ concentration was kept at 5 μM.

Inhibition constants were determined from double-reciprocal plots (Lineweaver & Burk, 1934) or Dixon (1953) plots.

Results and Discussion

The results for the hydrolysis of ATP by human liver alkaline phosphatase when commercial ATP from horse muscle that contains 5–40p.p.m. of vanadium (molar ratio up to 1:2000) was used are shown in Fig. 1. The observed inhibitory effect was nearly absent when vanadium-free ATP was used, but could be partially restored upon addition of Na_3VO_4 (Fig. 1). Although the vanadium concentration was not determined in the ATP used for these experiments, the concentration was presumably in the range stated by the supplier (molar ratio up to 1:2000). Since less inhibition was observed when vanadate was added to vanadium-free ATP in a molar ratio of 1:1000, the inhibition cannot be attributed solely to the presence of vanadate. Quist & Hokin (1978) have found two ($Na^+ + K^+$)-stimulated ATPase inhibitors in horse muscle ATP: vanadate and a dithioerythritol-dependent inhibitor. It is thus

possible that the ATPase activity of alkaline phosphatase may also have been influenced by an inhibitor other than vanadate. In addition, the shape of the curves (Fig. 1) may be very dependent on small changes in the free Mg^{2+} concentration. Although very similar concentrations of free Mg^{2+} would be expected at each concentration of ATP, the absolute concentrations are low (total Mg^{2+} is 5μM). If vanadate binds Mg^{2+} appreciably (published results not available), the inhibitory effect could be due to further lowering of the free Mg^{2+} concentration by complexing to vanadate.

We have shown (Seargeant & Stinson, 1979*b*) that the maximal ATPase activity of human liver alkaline phosphatase was obtained at low Mg^{2+} concentration, since free ATP^{4-} ($V_{max.}$ was 50% of that for *p*-nitrophenyl phosphate) but not $MgATP^{2-}$ was hydrolysed by the enzyme. When sufficient $MgCl_2$ was added to complex all the ATP present, no ATPase activity could be detected. Thus an evaluation of the influence of vanadate on the ATPase activity under conditions where the free Mg^{2+} concentration was kept constant and the free ATP^{4-} concentration varied would be technically difficult. However, it is possible to evaluate the influence of vanadate by using substrates that do not bind Mg^{2+} appreciably, and therefore further studies were carried out with *p*-nitrophenyl phosphate as substrate.

Orthovanadate is a potent competitive inhibitor of the *p*-nitrophenyl phosphatase activity of alkaline phosphatases purified from human liver, intestine and kidney (Fig. 2, Table 1). The inhibition constants at pH7.4 and 9.0 are approx. 100-fold lower than the corresponding values for phosphate. This is in contrast with the enzyme from *E. coli*, in which the inhibitor constants are of similar magnitude. The series of parallel lines obtained from the Yonetani–Theorell (1964) plot shown in Fig. 3 for the human liver enzyme suggests that the two inhibitors, phosphate and orthovanadate, bind in a mutually exclusive fashion. Similar findings have been reported for *E. coli* alkaline phosphatase (Lopez *et al.*, 1976). Yonetani–Theorell plots were not done with the enzymes from kidney or intestine, but it is probable that results similar to the above would be obtained. The K_i values reported in Table 1 are of the same order as reported by Cantley *et al.* (1977) for the $(Na^+ + K^+)$-stimulated ATPase from striated muscle.

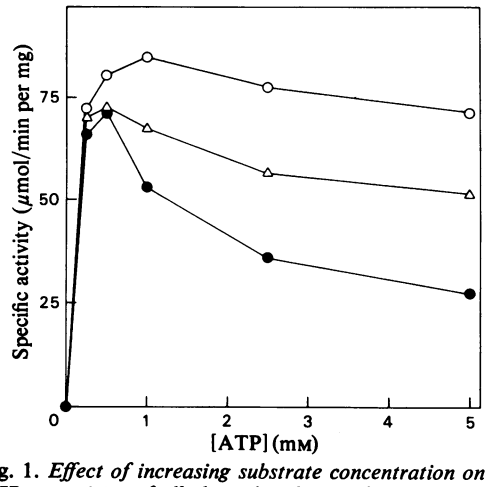

Fig. 1. *Effect of increasing substrate concentration on the ATPase activity of alkaline phosphatase from human liver*
Enzyme activity was measured at pH9.0 at an enzyme concentration of 40ng/ml as described in the Materials and Methods section. ●, ATP extracted from horse muscle; ○, ATP from horse muscle that had been treated to remove vanadium; △, vanadium-free ATP to which orthovanadate had been added in a molar ratio of 1:1000.

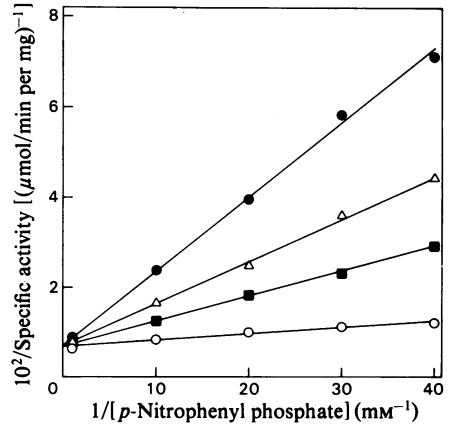

Fig. 2. *Inhibition of alkaline phosphatase activity by orthovanadate*
Double-reciprocal plot for human liver alkaline phosphatase (40ng/ml) at pH9.0. Orthovanadate concentrations were: ○, none; ■, 2.5μM; △, 5.0μM; ●, 10μM. Similar plots were obtained with the enzyme from human intestine or kidney.

Table 1. *Inhibition of alkaline phosphatase*
The assays contained *p*-nitrophenyl phosphate as substrate and the buffer mixture (see the Materials and Methods section).

		K_i (μM)	
Enzyme source	pH	Phosphate	Vanadate
Liver	7.4	40	0.6
Liver	9.0	90	0.9
Intestine	9.0	—	0.5
Kidney	9.0	—	0.6

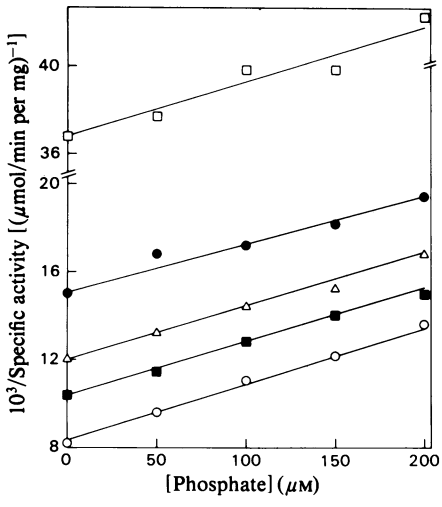

Fig. 3. *Yonetani–Theorell plot showing the effect of varying concentrations of orthovanadate and phosphate on human liver alkaline phosphatase activity*

Enzyme activity was measured at pH 9.0 at an enzyme concentration of 40ng/ml with 25μM-*p*-nitrophenyl phosphate as described in the Materials and Methods section. Orthovanadate concentrations were: ○, none; ■, 0.25μM; △, 0.5μM; ●, 1μM; □, 5μM.

Addition of adrenaline (1mM) to the reaction mixture abolished the inhibition by orthovanadate and restored *p*-nitrophenyl phosphatase activity. Similar findings have been reported for $(Na^+ + K^+)$-stimulated ATPase (Cantley *et al.*, 1977) and dynein ATPase (Kobayashi *et al.*, 1978; Gibbons *et al.*, 1978). Adrenaline is known to form complexes with vanadate (Kustin *et al.*, 1974), so it is probable that the reversal of inhibition is due to complexing of vanadate.

With the exception of alkaline phosphatase it appears that $(Na^+ + K^+)$-stimulated ATPase and dynein ATPase are the only ATPases that are inhibited by vanadate ions; Ca^{2+}-stimulated ATPase from sarcoplasmic reticulum, actomyosin ATPase and the F_1-ATPase from mitochondria are all relatively insensitive to the metal (Josephson & Cantley, 1977; Quist & Hokin, 1978). Intestinal alkaline phosphatase has been suggested to be a Ca^{2+}-stimulated ATPase (Haussler *et al.*, 1970; Russell *et al.*, 1972), but, since intestinal alkaline phosphatase is inhibited by vanadate and $Ca2^+$-stimulated ATPase is apparently not, it appears that the two enzyme activities are distinct. It is possible, however, that the intestinal enzyme acquired sensitivity to vanadate as a result of the process of purification.

Vanadate ions do not inhibit the ATPases in a strictly competitive manner (Josephson & Cantley, 1977; Kobayashi *et al.*, 1978) as observed for alkaline phosphatase (Fig. 2). Cantley *et al.* (1978) have found two vanadate-binding sites on the $(Na^+ + K^+)$-stimulated ATPase from dog kidney and suggest that the ion may be a physiological regulator of the enzyme. Tissue concentrations of the metal are in the near-micromolar range, with concentrations of 20–30μg/kg dry wt. in adult human liver, spleen, pancreas and prostate gland (Underwood, 1962). Since the K_i values reported herein are less than 1μM, it appears that the phosphohydrolytic activity of alkaline phosphatase *in vivo* could also be influenced by vanadate ions.

The more potent inhibition of alkaline phosphatase by orthovanadate than by phosphate suggests that the former compound through hydration or chelation can resemble a transition-state analogue of phosphate in the mechanism that involves a phosphoryl-enzyme intermediate. Lopez *et al.* (1976) point out that one of the transition states may be a trigonal bypyramidal species, and vanadate can resemble this type of structure (Lopez *et al.*, 1976; Van Etten *et al.*, 1974).

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