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Full Length Research Paper

Cobalt reverses vanadate inhibition of rat kidney alkaline phosphatase

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Vanadate ion (VO_4^{3-}) is an established inhibitor of alkaline phosphatase (ALP) and inorganic phosphate transport across biologic membranes. The concentration-dependent effects of Co^{2+} and VO_4^{3-} on rat kidney ALP activity were carried out over a range of 3.33 – 25.67 mM p–NPP concentrations. The investigation revealed Co^{2+} as an activator of rat kidney ALP, while vanadate inhibited the enzyme. The activatory effect of cobalt ion on ALP was compared with that of magnesium ion when vanadate-inhibited ALP was exposed to both cobalt and magnesium ions at both 2 and 4 mM concentrations. The results revealed that cobalt ion at these concentrations significantly reversed ($P < 0.05$) vanadate inhibition of rat kidney ALP activity while magnesium ion was significantly deficient ($P < 0.05$) in this regard. In the light of the foregoing, cobalt may be a better activator of rat kidney ALP in the presence of inhibitors of ALP. This reversal of ALP inhibition by Co^{2+} could be employed as a protector with a potential physiological relevance in cells.

Key words: Cobalt, vanadate inhibition, alkaline phosphatase.

INTRODUCTION

Alkaline phosphatase (EC 3.1.3.1) is an enzyme that catalyzes the hydrolysis of phosphate monoesters (e.g. β–glycerophosphate and para-nitrophenyl phosphate) with the release of inorganic phosphate and alcohol. Alkaline phosphatase (ALP) is a dimeric metalloenzyme that exists in almost all living forms, ranging from bacteria to man (Hung and Chang, 2001; Le Du et al., 2001). It can also function as a transferase when an appropriate acceptor is present (e.g. tris buffer and ethanolamine). It has wide substrate specificity and is said to be non-specific in its action (Hung and Chang, 2001; Le Du et al., 2001; Sowadski et al., 1981). Inhibitors of alkaline phosphatase include vanadate, arsenate, L-Phe and L-Trp. These inhibitors have been used in studies geared towards better understanding of the physiological role of alkaline phosphatase (Hiwada and Wachsmuth, 1974; Shirazi et al., 1981).

Vanadate in its monomeric form is a strong competitive inhibitor of wild- type ALP (Stankiewicz et al., 1995). The inhibitory effect of vanadate has been ascribed to its ability to mimic the anionic character of inorganic phosphate (Malomo et al., 2003). ALP has been implicated in phosphate transport across biological membranes and since vanadate could effectively inhibit both ALP and phosphate transport, it has been a useful probe of ALP's physiological function (Farley et al., 1980; Kempson and Dousa, 1979; Shirazi et al., 1981). The information obtained from studies with alternate substrates and inhibitors can be used to probe the mechanism of ligands-induced slow inactivation of enzymes.

This study therefore attempts to describe the effects of cobalt ion on vanadate- inhibited rat kidney alkaline phosphatase and compare the effects of cobalt and magnesium ions on vanadate-inhibited alkaline phosphatase. Arise et al. (2008) reported in their work that not only is Co^{2+} an activator of ALP but also a better activator than Mg^{2+}. The study will therefore provide information to ascertain which of these divalent ions stimulates the enzyme in the presence of this inhibitor better and whether their interaction with ALP in the presence of van-

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Table 1. Kinetic parameters of ALP in the presence of various concentrations of ligands.

Ligand (S)	V_{max} (nmol /min/mg Protein x 10^{-2})	K_M (mM)
0mM	18.18	6.41
2mM (Mg^{2+})	29.72	5.01
4mM (Mg^{2+})	30.00	5.77
2mM (Co^{2+})	37.73	4.00
4mM (Co^{2+})	45.00	5.00
2mM (VO_4^{3-})	4.00	10.47
4mM (VO_4^{3-})	4.00	14.69

adate is positively synergistic.

MATERIALS AND METHODS

Experimental animals

Albino rats (*Rattus novergicus*) were obtained from the Animal Holding Unit of the Department of Biochemistry, University of Ilorin, Kwara State, Nigeria.

Chemicals

Para-nitrophenyl phosphate (p-NPP), magnesium sulphate, cobalt chloride and sodium orthovanadate are products of Sigma Chemicals Company, Poole, England. All other reagents used were of analytical grade and were prepared in all glass distilled water.

Preparation of rat kidney alkaline phosphatase

Rat kidney crude homogenate was prepared using the procedure of Hung and Melnykovych (1977). Albino rats were sacrificed through cervical dislocation after which the rats were dissected and their kidneys collected. The kidneys were immediately placed in an ice-cold 0.25 M sucrose solution to preserve the cellular integrity of the tissue. The kidneys were then blotted with tissue paper, weighed and homogenized in sucrose solution at 4^oC. The crude kidney homogenate was then centrifuged at 5,000 rev/min for 20 min at the same temperature. To the supernatant fraction was added a 0.55 g/ml (4.17 M) solution of $(NH_4)_2SO_4$ gradually with stirring until 30% saturation was achieved. The precipitate was collected by centrifugation at 5,000 rev/min for 20 min and re-dissolved in 0.1 M carbonate-bicarbonate buffers, pH 10.1. The crude preparation was further fractionated on a sephadex G- 100 column to obtain a rich and highly active alkaline phosphatase fraction. The activities of the ALP prepared this way and used in this study were highly reproducible and gave linear results with a correlation level sufficient for kinetic work (Malomo et al., 2003).

Assay of alkaline phosphatase activity

The activity of alkaline phosphatase was assayed by monitoring the rate of hydrolysis of p-NPP at 25^oC in 0.1 M Na_2CO_3-$NaHCO_3$ (sodium carbonate – bicarbonate) buffer (pH 10.1) as described by Ahlers (1975). Alkaline phosphatase catalyzes the hydrolysis of p-NPP to yield para–nitrophenol, which is bright yellow (other reactants and products are colourless in aqueous solution), the intensity of which is proportional to the enzyme activity. The activity of the enzyme was spectrophotometrically determined at 400 nm wavelength in the absence and presence of Mg^{2+} and Co^{2+} respectively and ALP activity expressed in nmol/ min/mg protein. The reaction medium for determining concentration – dependent effects of each of Mg^{2+} and Co^{2+} on ALP activity was set up using the method of Wright and Plummer (1972) with concentrations of 2.0 and 4.0 mM and p–NPP concentration ranging between 3.33 – 25.67 mM. Protein concentration was determined using Biuret method (Henry et al., 1974) with Bovine Serum Albumin (BSA) as standard.

Effect of varying concentrations of Co^{2+} and VO_4^{3-} on ALP activity

To investigate the effect of concentrations of ligands ($Co^{2+}$and VO_4^3) on the kinetic parameters K_M and V_{max} of ALP, a substrate concentration–dependent study was carried out at 2 and 4 mM concentration of these ligands. The p-NPP concentration used ranged between 3.33 to 25.67 mM.

Effect of interaction of VO_4^{3-} with Mg^{2+} and Co^{2+} on ALP activity

The effect of Mg^{2+} and Co^{2+} at varying concentration of 0, 2, and 4 mM on the inhibition of ALP activity by vanadate ion was investigated at 5.17 mM p-NPP.

Kinetics of modulation of vanadate inhibition of ALP activity by Co^{2+}

A substrate concentration- dependent kinetic study was carried out in order to investigate the mechanism of reversibility of vanadate inhibition of ALP by Co^{2+} at 2 and 4 mM concentrations.

RESULTS

Inhibition of ALP activity by VO_4^{3-}

The kinetic parameters of independent effect of various concentrations of ligands on alkaline phosphatase activity are presented in table 1.

The Michaelis-Menten's curve and double reciprocal plot for the hydrolysis of p-NPP over a range of concentrations by alkaline phosphatase in the presence of varying concentrations of VO_4^{3-} (0, 2 and 4 mM) are shown in figures 1 and 2 respectively. The values of kine-

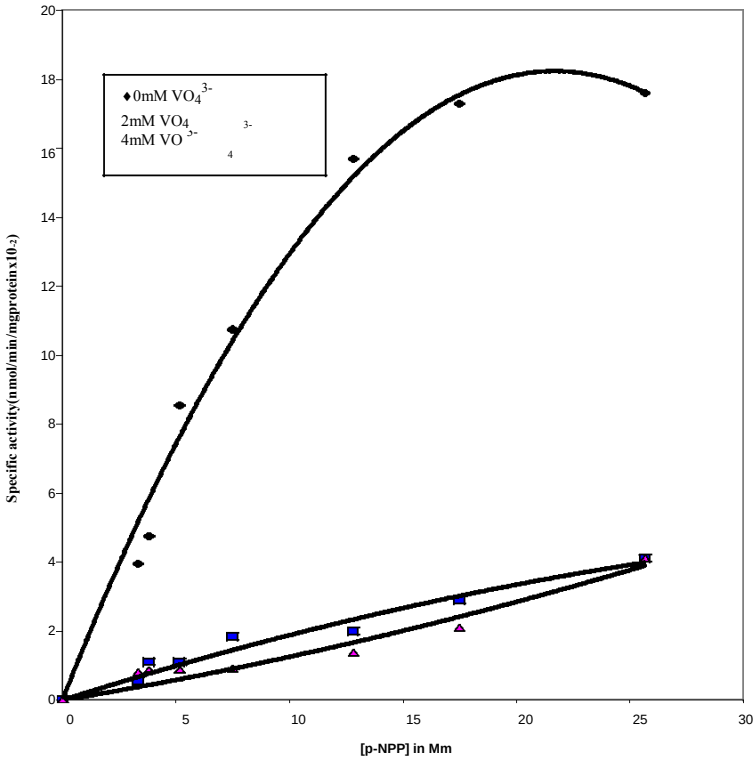

Figure 1. Michaelis-Menten's curve showing effect of VO_4^{3-} on rate of ALP-catalyzed hydrolysis of p-NPP.

Table 2. Effect of Co^{2+} on kinetic parameters of ALP in the presence of VO_4^{3-}.

Ligand (S)	V_{max} (nmol/min/mg Protein x 10^{-2})	K_M (mM)
4mM VO_4^{3-}	3.93	14.75
4mM VO_4^{3-} + 2mMCo^{2+}	6.30	6.33
4mM VO_4^{3-} + 4mMCo^{2+}	7.40	5.83

tic parameters (V_{max} and K_M) were extrapolated from Figure 1 (Table 2).

There was an evidence of inactivation of ALP when exposed to 2 mM vanadate as indicated by a sharp drop in V_{max} value which was still maintained at 4 mM vanadate concentration. There was a reduction in ALP activity by 78% in the presence of 2 and 4 mM vanadate. The inactivation of ALP by vanadate is also K_M–dependent as depicted by high K_M values at 2 and 4 mM VO_4^{3-}. This revealed that the binding affinity of ALP for p–NPP gradually declined as the concentration of VO_4^{3-} was increased from 2 to 4 mM.

Effect of Mg^{2+} and Co^{2+} on reversal of VO_4^{3-} inhibition of ALP

The effect of Mg^{2+} and Co^{2+} on vanadate inhibition of ALP catalyzed hydrolysis of p-NPP is shown in Figure 3. 4 mM vanadate inhibited the activity of ALP by 51.43%. The inhibition on ALP activity by vanadate was completely reversed as the concentration of Co^{2+} was increased from 2 to 4 mM. This was symbolized by a 56.57 and 286.29% rise in ALP activity in the presence of 2 and 4 mM Co^{2+} respectively. Mg^{2+} was unable to relieve the inhibition placed on ALP by vanadate. This was depicted by a 91.43 to 79.43% loss in ALP activity on moving from 2 to 4 mM Mg^{2+}.

Kinetics of modulation of vanadate inhibition of ALP activity by Co^{2+}

The Michaelis-Menten's curve and the double reciprocal plot for the hydrolysis of p-NPP over a range of concentrations (3.33 – 25.67 mM) by alkaline phosphatase in the presence of vanadate and Co^{2+} under the conditions in investigated are shown in Figures 4 and 5 respectively.

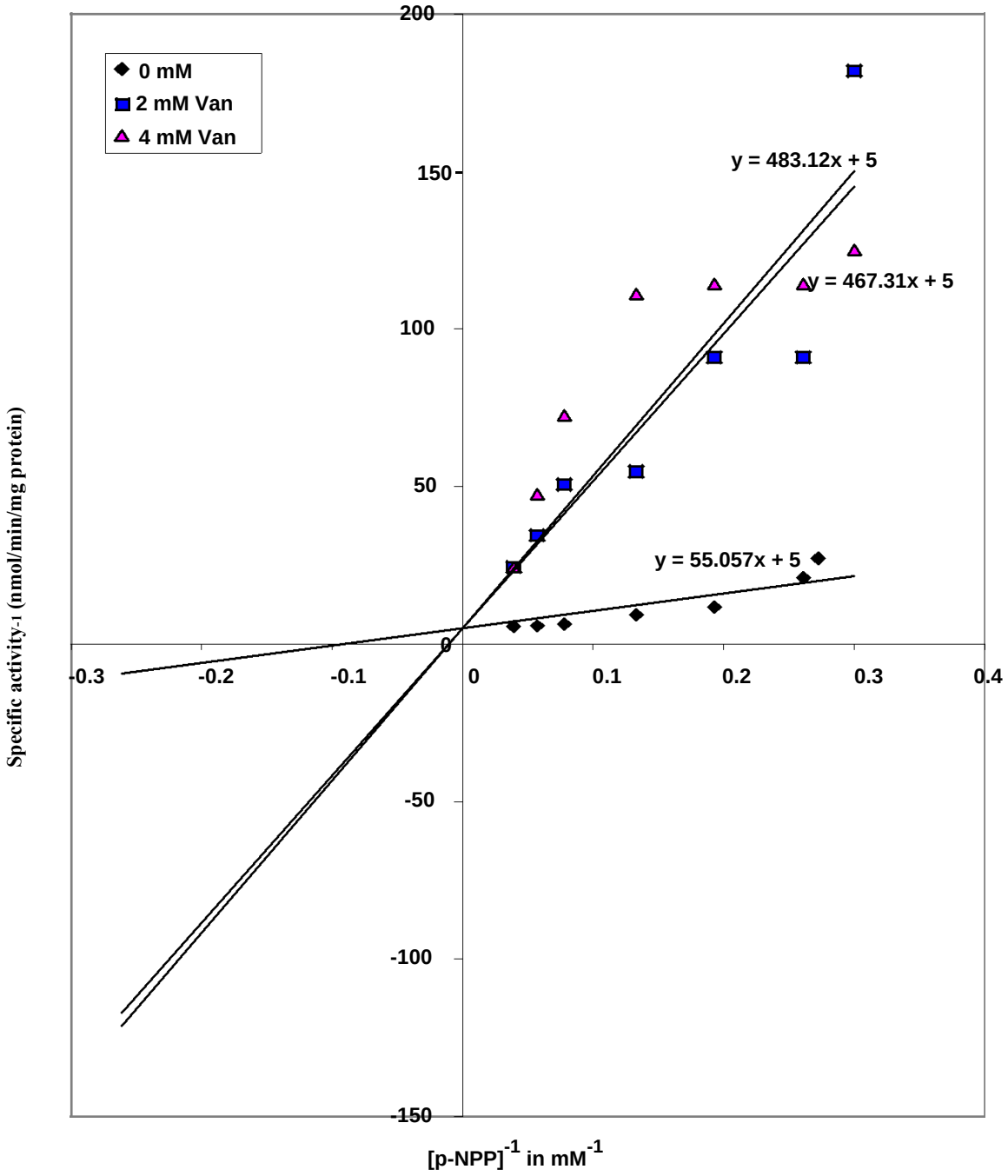

Figure 2. Double-reciprocal plot for effect of vanadate ion concentration on the kinetics of ALP-catalyzed hydrolysis of p-NPP.

The modulation of vanadate inhibition of ALP activity by Co^{2+} is both K_M - and V_{max}–dependent. This is evident by a progressive reactivation of ALP on moving from 2 to 4 mM Co^{2+} . Despite this noticed improvement in ALP activity, a subsequent decline in activity was observed at 4 mM Co^{2+} . There was a progressive decline in K_M values on moving from 2 to 4 mM Co^{2+}. This is suggestive of a significant improvement in affinity of ALP for p–NPP on moving from 2 to 4 mM Co^{2+}.

DISCUSSION

The inhibitory effect of vanadate on ALP activity mirrors a competitive mode of inhibition that is characteristic of VO_4^{3-} (Shirazi et al., 1981). Due to the structural and electronic similarity of vanadate with inorganic phosphate, vanadate may have distorted the configuration of the active site of ALP (Holtz et al., 1999) thereby resulting in a very slow rate of formation and breakdown of ES

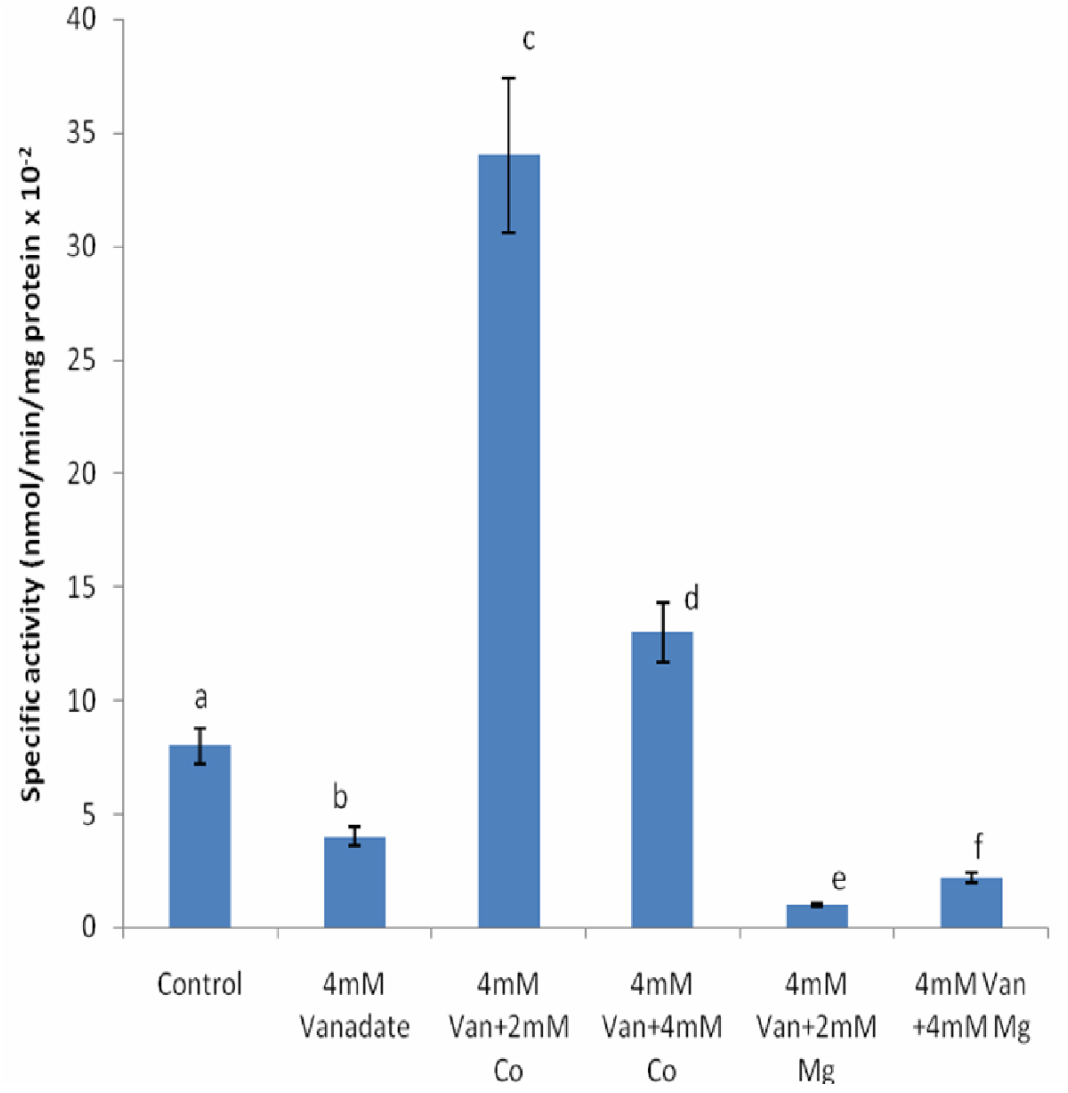

Figure 3. Effect of interaction of VO_4^{3-} with Mg^{2+} and Co^{2+} on ALP activity. Each value is an average of 3 determinations ± SEM. Values are significantly different in comparison with a, b, c, d, e and f at $P < 0.05$.

Complex.

Co^{2+} appeared to activate ALP in this study. The activation of ALP by Co^{2+} may be through formation of an activated Co^{2+}-ALP Complex where Co^{2+} occupies both catalytic and structural sites of ALP (Cathala et al., 1975). Co^{2+} may occupy more of the catalytic site at 2 mM, while occupying more of the structural site at 4 mM which may have favoured a rapid ES Complex formation and break-down to free enzyme and product. However, the decline in ALP activity noticed at 4 mM Co^{2+} may be as a result of substrate level inhibition (Hiwada and Wachsmuth, 1974). The ability of Co^{2+} to reverse vanadate inhibition on ALP further buttresses its activatory role as reported by Arise et al. (2008). Co^{2+} did not only relieve vanadate inhibition on ALP, but also reactivated the enzyme. On the other hand, Mg^{2+} appeared unable to reverse this inhibition. This may result from its inability to occupy distorted geometry created by vanadate which cobalt may have occupied (Simpson and Vallee, 1968). Another reason may result from slow binding nature of Mg^{2+} to its site that may lead to slow reaction rate (Hung and Chang, 2001) while the binding of Co^{2+} to the active site appears to generate a faster reaction rate.

The kinetics of modulation of vanadate inhibition on ALP by Co^{2+} suggests that Co^{2+} exerts its effect via both V_{max} and K_M. However, the reason for the subsequent in-activation of ALP noticed at 2 and 4 mM Co^{2+} is not clear. Can it be that the quantity of cobalt required to reactivate inactive ALP is supposed to be less than that required for normal activation of apoalkaline phosphatase, thereby leading one to suspect substrate level inhibition? Often, this type of inhibition occurs at elevated substrate level in

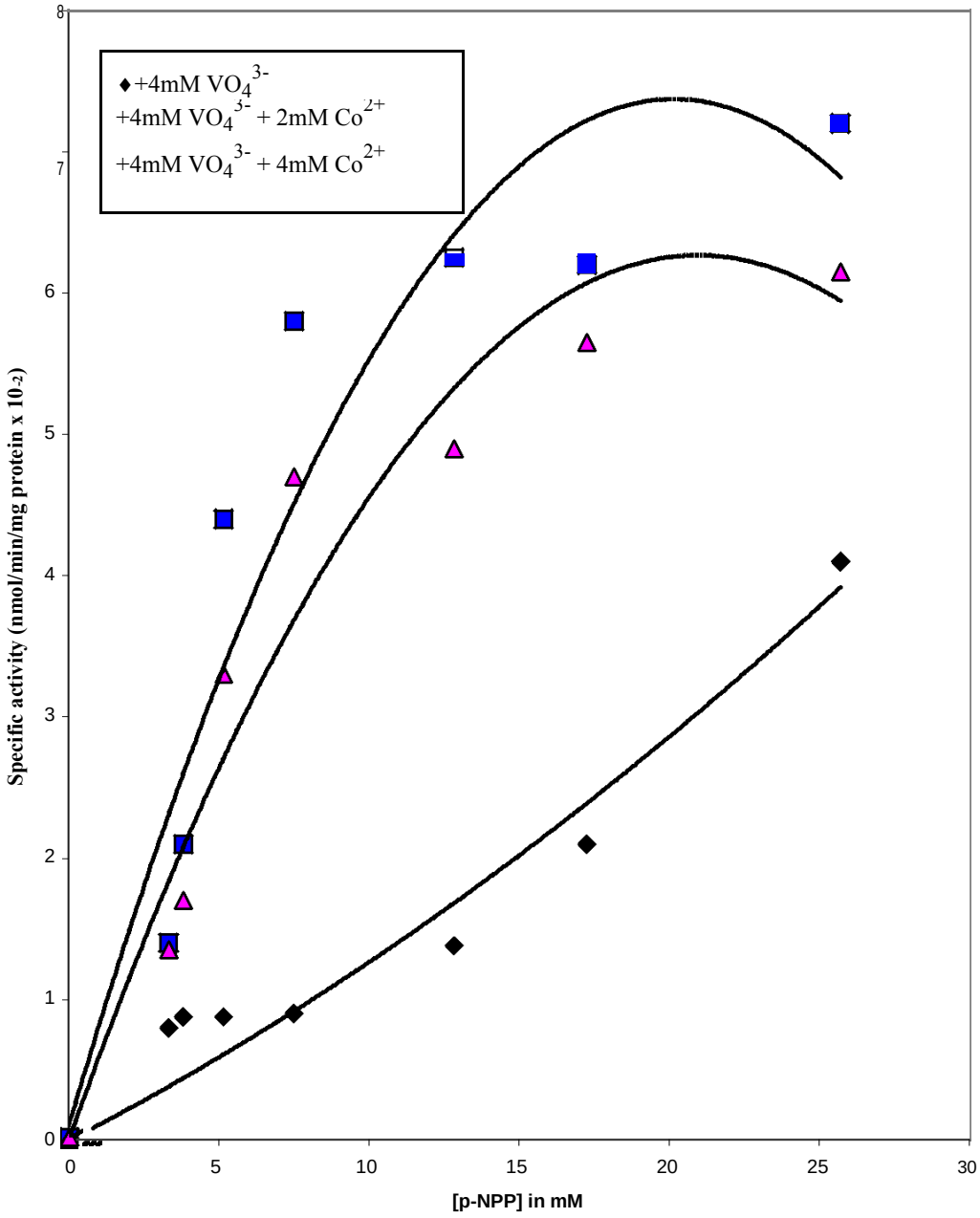

Figure 4. Michaelis-menten's curve showing kinetics of modulation of VO_4^{3-} inhibition of ALP by Co^{2+}.

which the substrate is binding to a second, non active site on the enzyme. Co^{2+} is an activator of alkaline phosphatase which was confirmed in this study. It may thus be that in the presence of 4 mM Co^{2+} and saturating substrate concentration, Co^{2+} may have displayed a mixed inhibition of kidney ALP. It therefore may be proposed that the first 2 mM concentration of Co^{2+} stabilized the structure of the protein as well as ensuring effective catalysis. While the additional 2 mM of Co^{2+} may have formed a Co^{2+}-p-NPP complex thus occupying a distorted geometry on the enzyme molecule.

Therefore it could be concluded that cobalt ion is capable of relieving vanadate inhibition of rat kidney alka-line phosphatase. Not only did cobalt ion relieve the inhi-bition on alkaline phosphatase but its performance was better than that of magnesium ion. The reversal of the usual role for Co^{2+} under different experimental cond-itions described here could have some physiological relevance. Hence, in the cell where enzymes, substrates, and products can exist together for an extended period, slow loss of enzyme activity could occur. However, de-pending upon which ligands are present, such mole-cular ligands could play an important protective function in the cell.

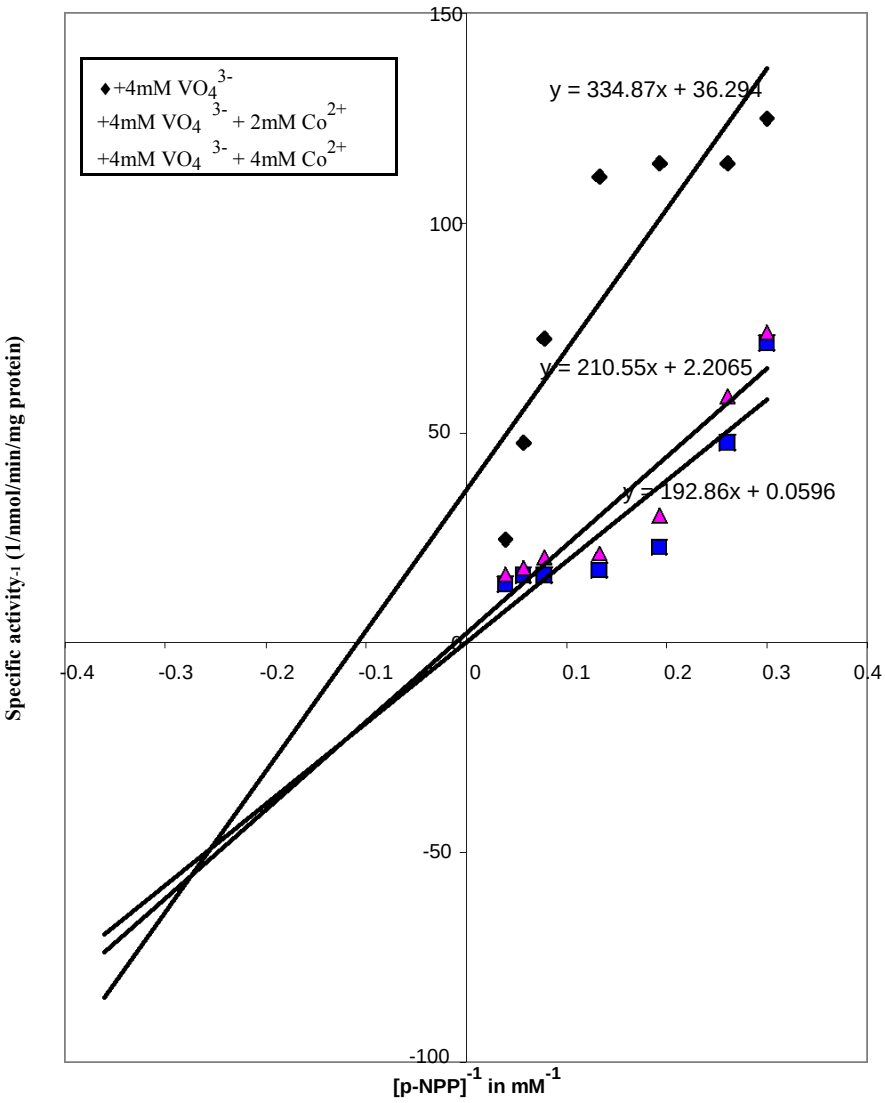

Figure 5. Double-reciprocal plot showing kinetics of modulation of VO_4^{3-} inhibition of ALP by Co^{2+}.

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