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Full Length Research Paper

Independent and interactive effects of Mg^{2+} and Co^{2+} on some kinetic parameters of rat kidney alkaline phosphatase

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It is a known fact that alkaline phosphatase (ALP) is a metalloenzyme that requires zinc ion (Zn^{2+}) and magnesium ion (Mg^{2+}) for activity. Cobalt ion (Co^{2+}) however has also been reported to stimulate its activity. The concentration-dependent effects of Mg^{2+} and Co^{2+} on rat kidney ALP activity was carried out over a range of 3.33 – 25.67 mM para-nitrophenyl phosphate (p-NPP) concentrations. The investigation showed that Mg^{2+} and Co^{2+} are activators of rat kidney ALP. Independent and interactive effects of Mg^{2+} and Co^{2+} on rat kidney ALP showed Co^{2+} to be a better activator than Mg^{2+}. The activity of rat kidney ALP was not significantly affected ($P < 0.05$) by 4 mM Co^{2+} in the presence of 4 mM Mg^{2+} when compared with ALP activity in the presence of 4 mM Co^{2+} alone. There was no synergism in the interactive effect of cobalt and magnesium ions on rat kidney ALP activity. The result may be of clinical and diagnostic importance in alkaline phosphatase assay procedure where cobalt ions may be employed as a better cofactor than magnesium ion used originally in the procedure. This will ensure sensitive detection of low ALP activity.

Key words: Independent, Interactive, Mg^{2+}, Co^{2+}, kidney, alkaline phosphatase.

INTRODUCTION

Alkaline phosphatase (ALP) is a metalloenzyme that requires both magnesium and cobalt ions for activity and is present in all living forms (Hung and Chang, 2001; Le Du et al., 2001). It catalyzes the hydrolysis of phosphomonoesters with the release of inorganic phosphate and alcohol. *Escherichia coli* ALP is a soluble protein in the periplasmic space while that of mammal is a membrane bound enzyme. There are four isoenzymes of ALP in mammals: placental, germ cell, intestinal ALPs which are tissue-specific, and tissue-nonspecific (e.g. liver, kidney and bone) ALPs (Huang et al., 1998). Divalent cations like nickel, manganese and cobalt have been reported as stimulators of alkaline phosphatase with cobalt ion been ranked to have an almost equal effect on prokaryotic alkaline phosphatase activity as magnesium and zinc ions (Cathala et al., 1975; Hiwada and Wachsmuth, 1974). Colbat is a necessary trace element for all cells and plays an essential role as cofactor in many biochemical processes but toxic at higher concentrations, a fact of considerable environmental importance (Battersby and Leeper, 1998). It is the central metal cofactor in the corrin ring of vitamin B_{12} and also plays crucial roles in biological functions. Methionyl aminopeptidase, which catalyzes the removal of the initiator methionine from nascent polypeptide chains, contain cobalt ions in both prokaryotes and eukaryotes (Arfin et al., 1995). Prokaryotic methylmalonylCoA carboxypeptidase, glucose isomerase and lysine 2,3-aminomutase are also cobalt containing (Arfin et al, 1995). It is a well known fact that alkaline phosphatase (ALP) is a zinc metalloenzyme which can be activated by magnesium ion (Curtis et al., 1986). In *E. coli* alkaline phosphatase, Mg^{2+} is thought to have a regulatory effect on the expression of catalytic activity and maintenance of structural integrity of the enzyme. However, the effects of Co^{2+} on the activatory role of Mg^{2+} on mammalian ALP have not been heretofore examined. This study therefore described an attempt to investigate the effect of cobalt ion and its activatory potency over magnesium ion on the hydrolysis of p-NPP by rat kidney ALP with a view to understanding the intera-

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tive mechanism of cobalt ion with mammalian ALP.

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 and cobalt chloride were 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

Crude homogenates of rat kidney 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°C. 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°C 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. All absorbance readings were taken using CamSpec M105 spectrophotometer. Significant differences between treatment means were determined at 5% confidence level, using Duncan's Multiple Range Test (Oyejola, 2003).

RESULTS

The hydrolysis of p–NPP over a range of concentrations (3.33 – 25.67 mM) by alkaline phosphatase in the presence of varying concentrations of Mg^{2+} (0, 2 and 4 mM) obeyed Michaelis-Menten kinetics as shown in Figure 1, while the double reciprocal plot is shown in Figure 2. This allows for the estimations of Michaelis constant and maximum rates. The good fit of the data to Michaelis–Menten equation affords a simple way of analyzing the data.

The kinetic analysis of the data revealed that 2 mM Mg^{2+} activated rat kidney ALP as depicted by a rise in V_{max} while there was no significant effect ($P > 0.05$) at 4 mM Mg^{2+}. The activation of ALP by Mg^{2+} also affected the binding affinity (K_m) of ALP for p–NPP. The affinity of ALP in the presence of 2 mM Mg^{2+} deteriorated while there was an improvement at 4 mM Mg^{2+} as depicted by the respective high and low Km values.

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 phosph-atase in the presence of varying concentrations of Co^{2+} (0, 2 and 4 mM) are shown in Figures 3 and 4 respectively.

The kinetic data suggests a progressive activation of ALP when exposed to increasing Co^{2+} concentrations (0, 2 and 4 mM) as depicted by elevated maximum reaction rate (V_{max}). However, a subsequent decline in activity despite the initial activation is also observed at 4 mM Co^{2+} (Figure 3). This observation reveals that ALP experienced 107.54% rise in activity in the presence of 2 mM Co^{2+} with a further 40% rise in activity at 4 mM Co^{2+}. The binding affinity of ALP for p-NPP also improved from 0 mM to 2mM Co^{2+}, while slightly reducing at 4 mM Co^{2+} (as represented by the respective low and high K_m values).

The effect of interaction of Mg^{2+} and/or Co^{2+} on ALP hydrolysis at a fixed concentration of 5.17 mM p–NPP is shown in Figure 5. Cobalt appeared to be a better activator of ALP than magnesium at the concentrations examined (2 and 4 mM). The interactive effect of Mg^{2+} and Co^{2+} on ALP activity was not synergistic. The interaction of 2 mM Co^{2+} with 2 and 4 mM Mg^{2+} resulted into activation of ALP by 208.93 and 139.29% respectively. The interaction of 4 mM Co^{2+} with 2 and 4 mM Mg^{2+} resulted into an activation of ALP by 305.36 and 373.21% respectively. The highest ALP activity was recorded at 4 mM Co^{2+} (376.78%) which is not significantly different ($P > 0.5$) from what was obtained when both 4 mM Co^{2+} and 4 mM Mg^{2+} were present in the assay medium.

DISCUSSION

The mechanisms by which metal ions exert their important biological roles in metal–activated enzyme systems are not clearly defined (Anderson et al., 1976), even though two mechanisms had earlier been proposed

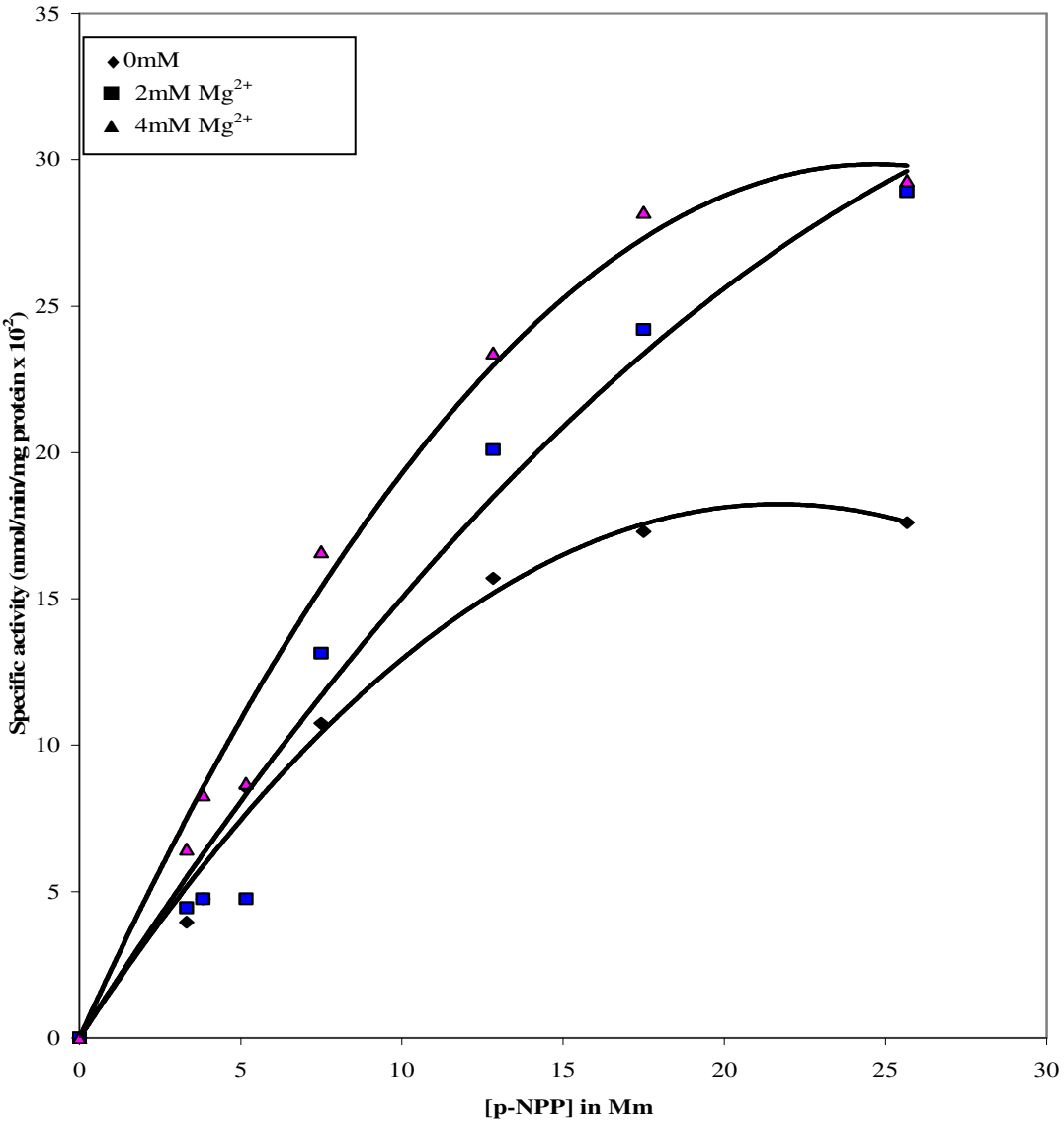

Figure 1. Michaelis-Menten's curve showing the effect of Mg^{2+} on rate of ALP-catalyzed hydrolysis of p-NPP

(Brunel and Cathala, 1973). They either act as bridges between enzymes and their substrates, or induce conformational changes that result in catalytically active enzymes.

The observed ALP activity in the absence of exogenous ligands is indicative of residual activity due to the patially purified kidney enzyme source (Brunel and Cathala, 1973). The activation of ALP activity by magnesium as observed is in conformity with earlier studies (Ahlers, 1974; Arise et al., 2005). Magnesium may have occupied the structural site on ALP that led to a subsequent conformational change from a less active form of ALP to a more activated form of the enzyme (i.e. activated ALP–Mg^{2+} Complex) to elicit this effect (Ahlers, 1974). At 2 mM, Mg^{2+} appeared to occupy more of the structural site of the enzyme which may have resulted in the enhancement of the breakdown of ES complex to free enzyme and product, while its occupation of more of the regulatory site may have led to a non significant increase in ALP activity. McCracken and Meighen (1981) established the existence of three classes of metal binding sites in ALP. These are designated "catalytic, structural and regulatory". Zn^{2+} occupies the catalytic and structural sites (Anderson et al., 1976). Brunel and Cathala (1973) in explaining the role of metal ions in metal-activated enzyme systems proposed that the metal ion can act as a bridge between the enzyme and its substrate or it can induce conformational changes and thereby convert an active or partially activated form of an enzyme into a catalytically active or more active form. Co^{2+} appeared to have activated ALP in this study. The activation of ALP by Co^{2+} may be through formation of an activated Co^{2+}

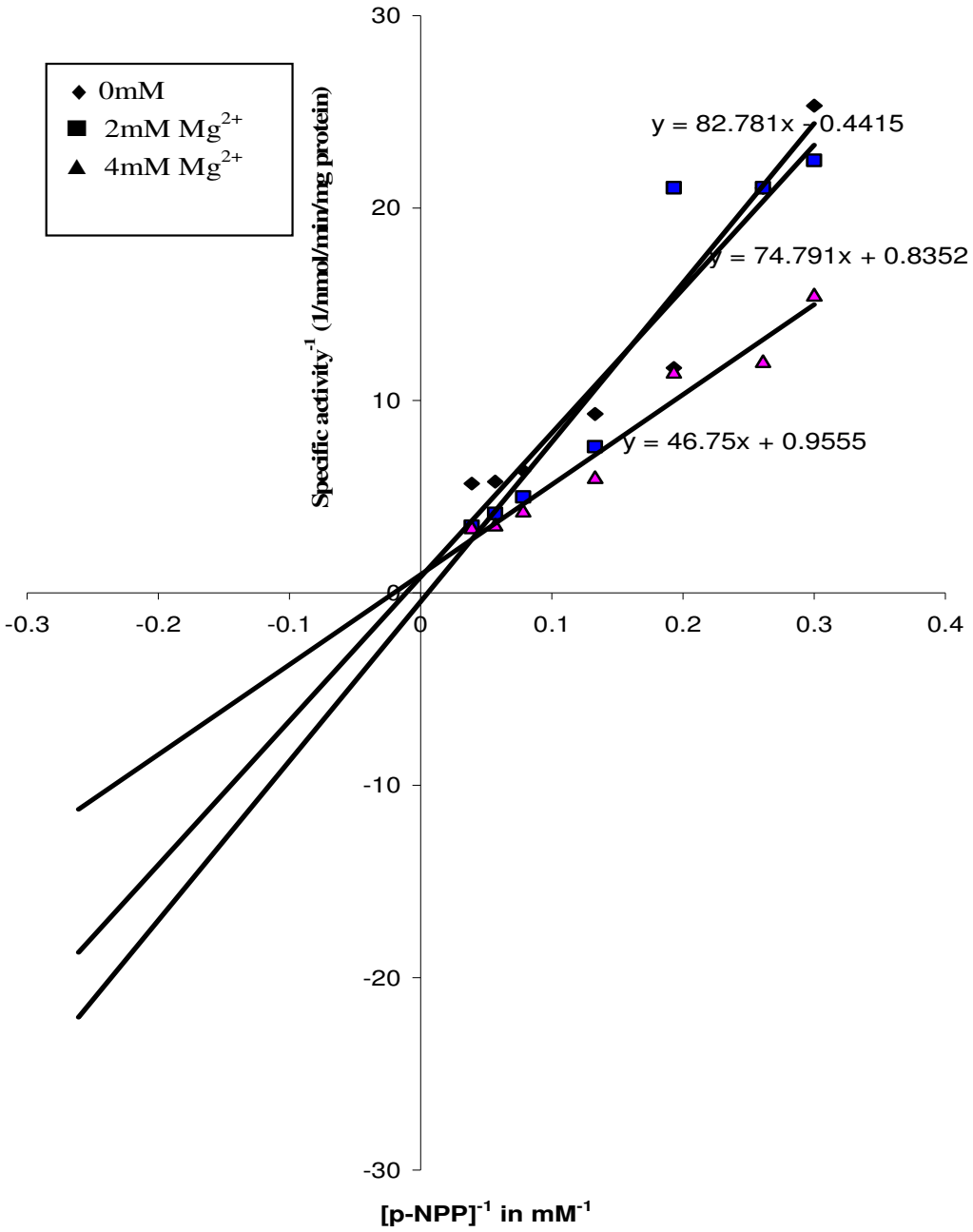

Figure 2. Double-reciprocal plot showing the effect of Mg^{2+} on rate of ALP-catalyzed hydrolysis of p-NPP.

- ALP complex where Co^{2+} occupies both catalytic and structural sites of ALP (Cathala et al., 1975) thus forming an octahedral geometry (Simpson et al., 1968). 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 breakdown 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). Often, this type of inhibition occurs at elevated substrate level in which the substrate is binding to a second, non active site on the enzyme. $Co^{2+}$is a co-substrate 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. The fact that Co^{2+} is capable of occupying both the catalytic and struc-

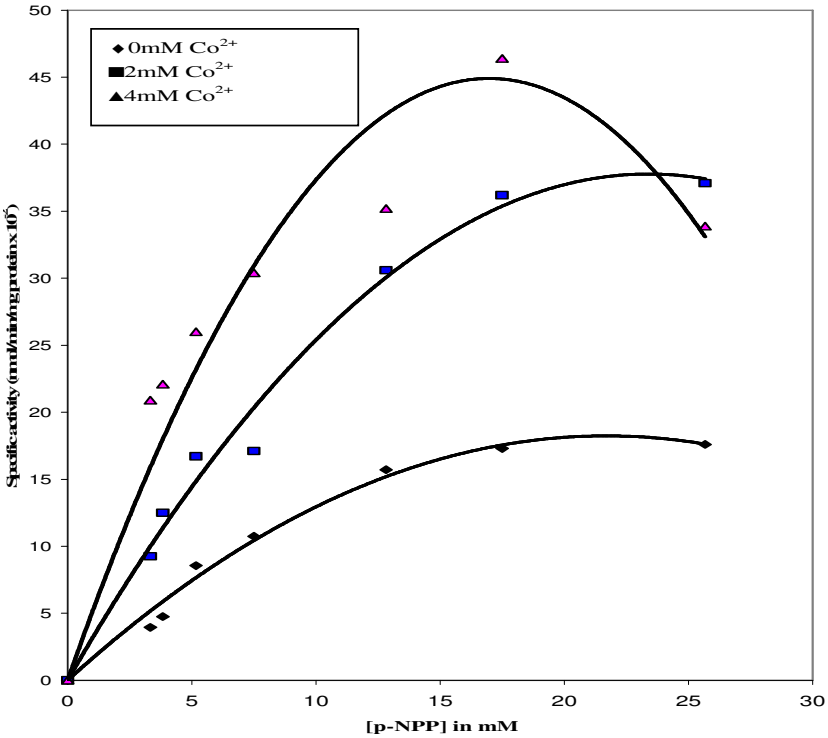

Figure 3. Michaelis-Menten's curve showing effect of Co^{2+} on rate of ALP-catalyzed hydrolysis of p-NPP.

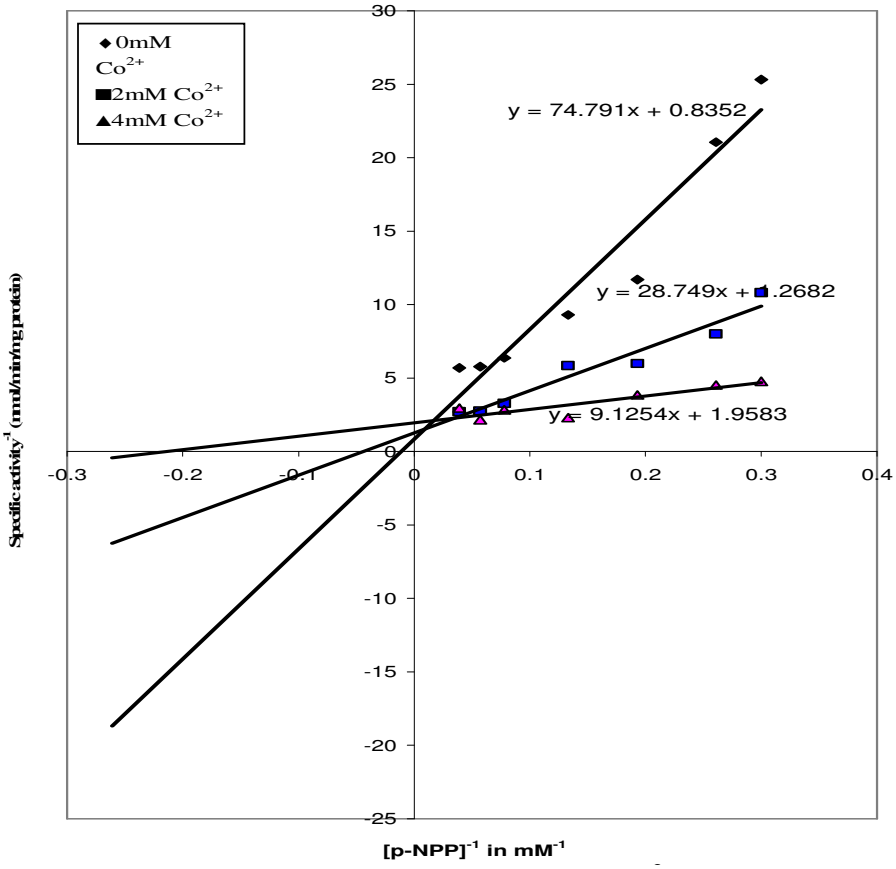

Figure 4. Double-reciprocal plot showing effect of Co^{2+} on rate of ALP-catalyzed hydrolysis of p-NPP.

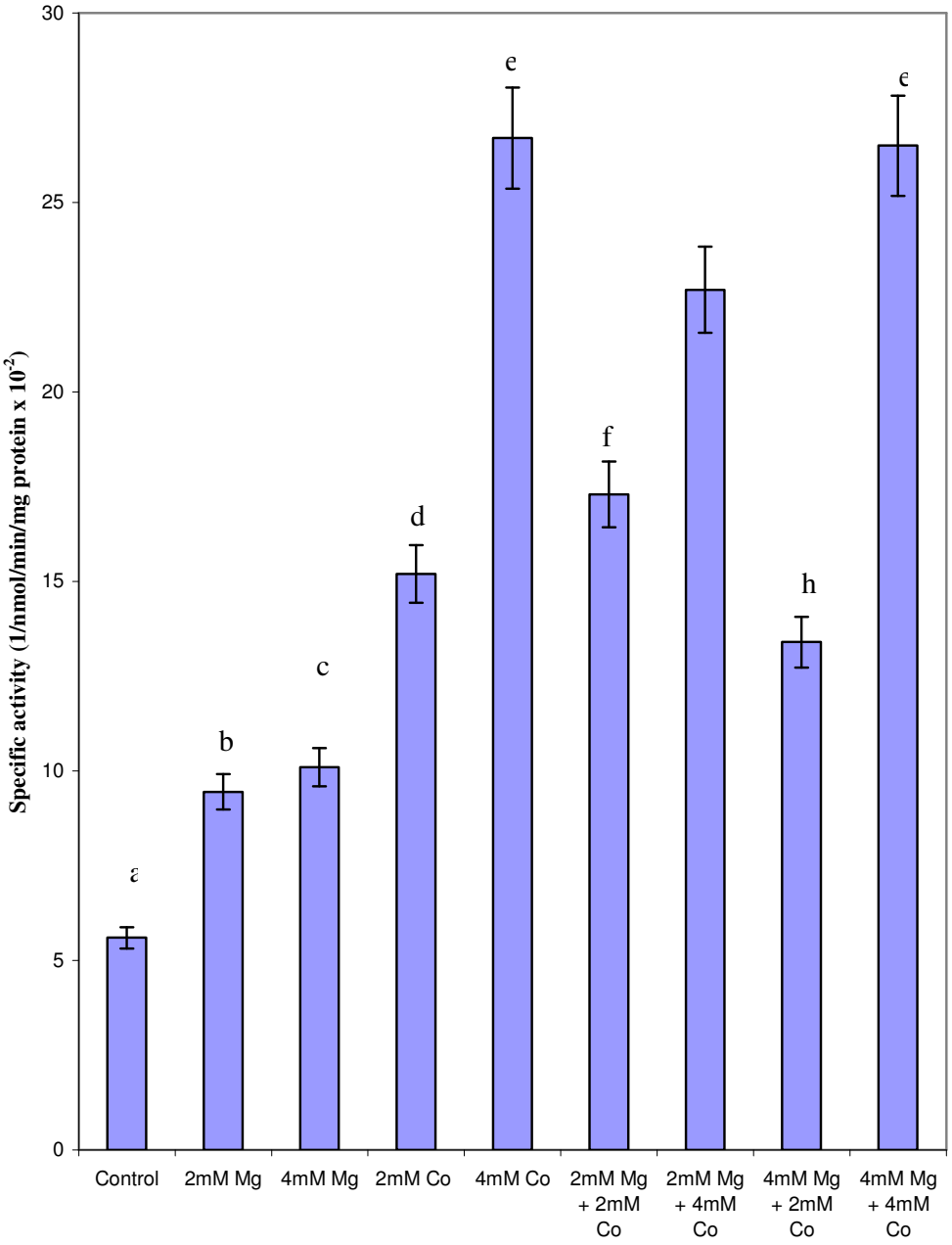

Figure 5. Bar-chart representation showing effect if interaction of Mg^{2+} and Co^{2+} ions on ALP activity at 5.17 mM p-NPP. Each value is an average of 3 determinations ± SEM. Values are significant different in comparism with a, b, c, d, f, g and h at $P < 0.05$.

tural sites of ALP (Cathala et al., 1975) may favour cobalt over magnesium as a better activator of ALP as seen in Figure 5. The non-significant difference in activity of ALP observed in the presence of 4 mM Co^{2+} when compared with that recorded in the presence of both 4 mM Co^{2+} and 4 mM Mg^{2+} may be suggesting insensitivity of ALP to magnesium at this concentration and condition (Brunel and Cathala, 1973).

In conclusion, this study has revealed that not only is Co^{2+} an activator of rat kidney ALP, but also a better activator than Mg^{2+}. Therefore, this activatory effect of cobalt on ALP may be channeled towards enhancing better responses obtained in routine assays that employ the method of Wright and Plummer (1974), where cobalt may replace magnesium that is originally used in these routine assays.

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