

## A Decrease in the Bovine Intestinal Mucosa Alkaline Phosphatase Activity in the Presence of Inorganic Phosphate

DON HOGARTH, DAVID JUNG, THOMAS MUROOKA, SHAWN PUN, AND MICHAEL SZETO

*Department of Microbiology and Immunology, UBC*

**The effect of varying  $Mg^{2+}$  levels on the enzymatic activity of bovine intestinal mucosa alkaline phosphatase in two different buffers was investigated. The maximum rates of bovine intestinal mucosa alkaline phosphatase enzymatic activity were determined by conducting a series of enzyme assays using p-nitrophenol phosphate as a substrate. Our observations determined that increasing the concentration of  $Mg^{2+}$  in Tris-HCl buffer initially increased enzyme activity until a plateau was reached; whereas  $V_{max}$  continued to increase in Tris-phosphate buffer within the range of  $Mg^{2+}$  levels tested. A corresponding decrease and leveling off of  $K_m$  was observed in Tris-HCl buffer. A continual decrease in  $K_m$  in Tris-phosphate buffer was observed, suggesting that an increase in concentration of  $Mg^{2+}$  increased the affinity of the enzyme for the pNPP substrate. A higher  $Mg^{2+}$  concentration in the buffer may have helped the ion overcome the complexing effects of  $P_i$  in the Tris-phosphate buffer and improved its access to the enzyme. As a result, the rate of the enzyme reaction increased and a gradual stabilization of the enzyme was observed.**

Alkaline phosphatase (AP) is a homodimeric metalloenzyme that catalyzes the non-specific hydrolysis of phosphate monoesters at alkaline pH to produce inorganic phosphate and an alcohol (1). AP contains three divalent cation binding sites within its active site that must be occupied for optimal enzyme catalytic function and stability (1). The metal binding sites for AP have been named M1, M2, and M3. It has been established that  $Zn^{2+}$  must occupy binding sites M1 and M2 of the active site of AP to ensure optimal enzymatic activity. Either  $Zn^{2+}$  or  $Mg^{2+}$  may occupy binding site M3, but it has been found that enzymatic activity is increased if  $Mg^{2+}$  occupies M3 (3).

Mammalian APs are glycoproteins that are present in many different tissue types including bone, intestine, kidney, and placenta (1). Despite extensive studies on *Escherichia coli* AP, mammalian APs have not been thoroughly investigated. The relatively small pool of research that exists for mammalian AP has yielded several distinct differences between prokaryotic AP and eukaryotic AP. These findings are understandable considering that there is only 25-30% homology between *E. coli* AP and the mammalian enzyme (3). It is therefore important to study specific behaviors of mammalian APs that have been previously well-documented for *E. coli* APs.

Experiment A3 (6) sought to investigate the differences in enzymatic activity of bovine intestinal mucosa alkaline phosphatase (BIMAP) as the reaction conditions were changed. It was observed that AP activity was approximately twenty times faster in Tris-HCl buffer at pH 7 than in sodium phosphate buffer at pH 7. It is hypothesized that the inorganic phosphate ( $P_i$ ) ions present in the sodium phosphate buffer served to chelate the  $Mg^{2+}$  ions that were present in solution thereby reducing enzyme activity by restricting magnesium availability to AP.

An enzyme kinetic assay was employed to investigate the observation that alkaline phosphatase activity was greater in Tris-HCl buffer relative to sodium phosphate buffer. The enzyme activity of bovine intestinal mucosa alkaline phosphatase was measured in Tris-HCl buffer and Tris-HCl buffer containing  $P_i$  at varying concentrations of p-nitrophenol phosphate and  $MgCl_2$ , then  $V_{max}$  was determined at each concentration of  $MgCl_2$ . A comparison of  $V_{max}$  against  $MgCl_2$  concentration was performed in order to determine if there was a positive correlation between  $MgCl_2$  concentration and  $V_{max}$ , and if the presence of  $P_i$  affected enzyme activity. If this was the case, the sequestration of  $Mg^{2+}$  by  $P_i$  could explain why enzyme activity was twenty times greater in Tris-HCl buffer than in sodium phosphate buffer.

### MATERIALS AND METHODS

#### Alkaline phosphatase, p-nitrophenol phosphate (pNPP) and buffer preparation

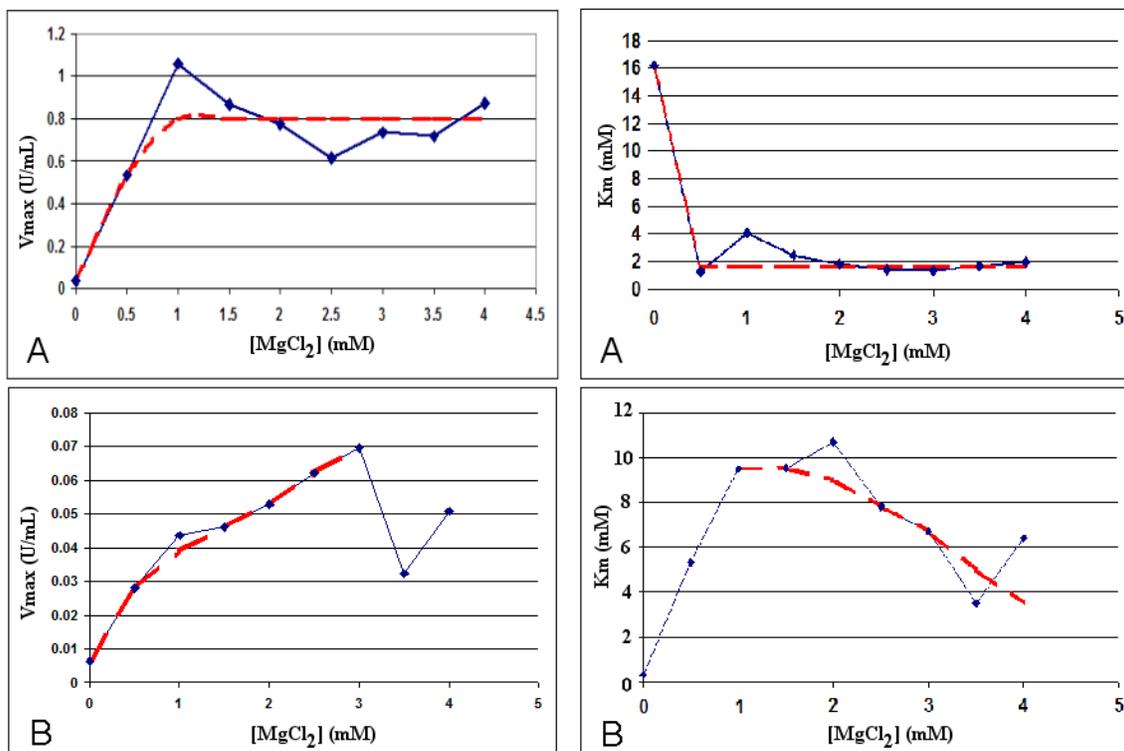
Appropriate amount of alkaline phosphatase (Calbiochem B-grade) was resuspended to 2.0mg/mL in de-ionized water. pNPP (Sigma 104 stock no. 104-407) was dissolved at 4.0mg/mL in de-ionized water. Both were covered and stored at 4°C when not in use. Appropriate amount of Tris (Gibco BRL) was added to de-ionized water to prepare a 1M Tris-Cl stock buffer, adjusted to pH 10.0 with 1N HCl. Sodium phosphate was added to the 1M Tris stock buffer for a final concentration of 0.1M to prepare the Tris-phosphate buffer, also adjusted to pH 10.0. The 10mM magnesium chloride solution was prepared by dissolving an appropriate amount of  $MgCl_2$  (Gibco BRL) in de-ionized water.

**Alkaline phosphatase Assay**

The protocol in *Exp A3: Alkaline Phosphatase Enzyme Kinetics* (6) was modified for these experiments. Since the amount of Mg in the buffers was to be varied, appropriate calculations were done to achieve the desired Mg concentration while maintaining a final volume of 3.1mL. The activity of alkaline phosphatase was relatively low, and therefore the stock concentration of the enzyme was increased from 15ug/mL to 2mg/mL. The substrate pNPP stock concentration was increased from 2.0mg/mL to 4.0mg/mL in order to maintain a final assay volume of 3.1mL. Also, pNPP volumes used for the assay were 0.025, 0.125, 0.250, 0.375, 0.500 and 0.625mL. In each case, an appropriate volume of distilled water was added to maintain the final volume at 3.1mL. Reaction rate of alkaline phosphatase in Tris-phosphate buffer was slow; therefore measurements were taken every minute over a 10-minute span to ensure that enough data points were considered for a proper analysis.

**RESULTS**

In order to determine whether varying the amount of  $MgCl_2$  in two different buffers would influence the  $V_{max}$  and  $K_m$  values of alkaline phosphatase, a series of experiments were set up. Enzyme activity at different  $Mg^{2+}$  concentrations was measured using varying amounts of substrate. The Lineweaver-Burk plot was then used to calculate the  $V_{max}$  and  $K_m$  of the enzyme.  $V_{max}$  values of alkaline phosphatase in Tris-Cl buffer and Tris-Cl-phosphate buffer, both pH 10.0, were plotted against  $Mg^{2+}$  concentration (Fig. 1). Similarly,  $K_m$  values of alkaline phosphatase in Tris-Cl buffer and Tris-phosphate buffer were plotted against Mg concentrations, shown in Fig. 2.



**Figure 1.**  $V_{max}$  values of alkaline phosphatase plotted against Mg concentration in two different buffers. (A) Tris-Cl buffer, pH 10.0 (B) Tris-phosphate buffer, pH 10.0 (n=1) The dashed line represents the most general trend.

**Figure 2.**  $K_m$  values of alkaline phosphatase plotted against Mg concentration in two different buffers. (A) Tris-Cl buffer, pH 10.0 (B) Tris-phosphate buffer, pH 10.0 (n=1) The dashed lines in Fig. A and B are the most general trend.

**DISCUSSION**

BIMAP activity was tested in both Tris-HCl buffer and Tris-phosphate buffer to investigate what effect  $P_i$  had on the concentration of  $Mg^{2+}$  in solution. Increasing the amount of  $Mg^{2+}$  available to BIMAP in solution caused an increase in enzyme activity. As has been previously determined,  $Mg^{2+}$  is primarily responsible for the maintenance of AP conformation that is crucial in order to attain the maximum enzyme velocity rate ( $V_{max}$ ) as measured in units

of product produced per unit of time (3). A deficiency in  $Mg^{2+}$  would therefore cause a change in AP conformation that leads to a decrease in the number of functional enzymes in a reaction mixture, which is effectively equivalent to decreasing the enzyme concentration ( $[E]$ ) within a reaction mixture. Consequently, the change in AP conformation due to the deficiency of  $Mg^{2+}$  can be measured by measuring  $V_{max}$  in units per milliliter because this  $V_{max}$  varies proportionally to  $[E]$  within a reaction mixture. Using this logic, it was possible to experimentally test the effect of  $P_i$  on the  $[Mg^{2+}]$  in solution by varying the concentration of  $MgCl_2$  added to the reaction mixture.

### **Influence of $MgCl_2$ and different buffers on BIMAP $V_{max}$**

For both buffering systems,  $V_{max}$  for BIMAP at different concentrations of  $MgCl_2$  were determined and plotted against the concentration of  $MgCl_2$  (Fig. 1). Consistent with the  $Mg^{2+}$  dependence of BIMAP enzymatic activity, there was a steady increase in  $V_{max}$  observed between 0 mM (the control) and 1 mM  $MgCl_2$  in both buffers. In Tris-HCl buffer, the  $V_{max}$  began to decrease between 1 mM  $MgCl_2$  and 3mM  $MgCl_2$ . It is possible that  $Mg^{2+}$  was inhibiting BIMAP at these concentrations, however, this observed fall in  $V_{max}$  between 1mM and 3mM  $MgCl_2$  was probably not absolute and reflect the fact that more trials were necessary to make any deductions with credible certainty. Therefore, the most logical trend is that  $V_{max}$  begins to plateau after 1mM  $MgCl_2$ . The leveling off of  $V_{max}$  after 1mM  $MgCl_2$  in Tris-HCl buffer (represented by a dashed line in Fig. 1A) was not observed in Tris-phosphate buffer (Fig. 1B) between 1mM and 3mM  $MgCl_2$ , instead  $V_{max}$  continued to rise in this region. The overall difference in the trends between the two buffer systems, i.e. lower  $V_{max}$  at any concentration of  $MgCl_2$ , suggests that  $P_i$  was responsible for inhibiting enzyme activity at these concentrations, since this was the key difference between the compositions of the two buffers. Although  $V_{max}$  values decreased suddenly at concentrations of  $MgCl_2$  higher than of 3mM in the Tris-phosphate buffer in Fig. 1B, we feel that more experimental trials must be attempted to confirm this observation.

The maximum theoretical  $V_{max}$  at a given concentration of AP is only possible when every AP enzyme in solution has  $Mg^{2+}$  in its active site was lower in Tris-phosphate buffer because of  $Mg^{2+}$  limitation by  $P_i$  sequestration.  $P_i$  anions act as metal-chelators, binding to  $Mg^{2+}$  and preventing  $Mg^{2+}$  binding to the AP active site, leading to a reduced enzyme efficiency and overall decrease in enzyme activity. In the Tris-phosphate buffer, the maximum  $V_{max}$  was not reached within the concentrations of  $MgCl_2$  tested but the general trend implied that  $V_{max}$  would continue to increase beyond 4 mM  $MgCl_2$ . This suggests that  $Mg^{2+}$  was not present in all of the active sites of AP. In Tris-HCl buffer, the maximum  $V_{max}$  was reached at 1mM  $MgCl_2$  (Fig. 1A). If  $P_i$  was not inhibiting the AP itself, maximum  $V_{max}$  would have been reached at the same  $MgCl_2$  concentration in both buffers. Therefore as  $MgCl_2$  was increased,  $Mg^{2+}$  ions were able to overcome the complexing effect of  $P_i$  in the Tris-phosphate buffer and gradually increase enzymatic activity. Another possibility is that  $P_i$  functions as a competitive inhibitor, competing with  $Mg^{2+}$  for placement within the active site of the AP. This situation is also consistent with our results, as a gradual increase in  $V_{max}$  may have been due to  $Mg^{2+}$  ions out-competing  $P_i$  for binding sites on the enzyme.

### **Influence of $MgCl_2$ and different buffers on BIMAP $K_m$**

In enzyme kinetics,  $K_m$  measures the binding affinity or the tendency at which the enzyme would bind the substrate. The  $K_m$  for BIMAP at the various concentrations of  $MgCl_2$  were calculated and plotted against  $MgCl_2$  concentration in both buffer systems tested (Fig. 2). At 0mM  $MgCl_2$  the predicted  $K_m$  should be high because substrate binding is inhibited by the absence of  $Mg^{2+}$  required for enzymatic efficiency. However, this observation was only seen in Tris-HCl buffer (Fig. 2A). The  $K_m$  for the Tris-phosphate buffer at 0mM  $MgCl_2$  was nearly zero. The correlation coefficient ( $R^2$ ) was determined during data analysis, for 0mM  $MgCl_2$  in the Tris-phosphate buffer  $R^2$  was 0.228, which is an unreliable correlation, mainly due to very slow reaction rates at these conditions and therefore the significance of experimental error. This data point does not truly represent the true  $K_m$  value at 0mM  $MgCl_2$  in the Tris-phosphate buffer and can be omitted from our analysis. The addition of  $MgCl_2$  drastically reduces the  $K_m$  in Tris-HCl buffer (Fig. 2A). The  $K_m$  in the Tris-HCl buffer begins to level after 1mM  $MgCl_2$ , which was expected because the maximum  $V_{max}$  was reached at this concentration. This leveling off of  $K_m$  further strengthens our interpretation of our data that  $V_{max}$  remains relatively constant after 1mM  $MgCl_2$ . In the Tris-phosphate buffer, there was a general trend of decreasing  $K_m$  with increasing amount of  $MgCl_2$  after discounting the data point corresponding to 0mM  $MgCl_2$ . The general trend found in the presence and absence of the  $P_i$  anion in the buffers suggests that the phosphate anion did interfere with the binding of the  $Mg^{2+}$  to the active site of the alkaline phosphatase. Again, the increase of  $MgCl_2$  slowly overcame the chelating effects of the  $P_i$  in the buffer and lead to a gradual stabilization of the enzyme, hence lowering of the  $K_m$ .

The results of our experiment were consistent with the results of experiment 3A (6). In our experiment, the buffers were set to pH 10 while in the previous experiment the buffers were set to pH 7. According to previous kinetics study on AP, the rate-determining step of the enzymatic reaction was pH-dependent (4). In addition, increasing the pH of the reaction mixture increases the activity of AP (3). It was previously documented that a 20 times difference in enzyme activity between the Tris-HCl buffer and sodium phosphate buffer, for reactions supplemented with 2mM MgCl<sub>2</sub> at pH 7. In our experiment, there was approximately a 15 times greater enzyme activity in Tris-HCl buffer than in Tris-phosphate supplemented with 2mM MgCl<sub>2</sub> at pH 10. Although the magnitude of the decrease in enzyme activity wasn't as pronounced as that in experiment 3A, the consistent trend of a significant decrease of enzyme activity in Tris-phosphate buffer was observed.

It has been clearly shown P<sub>i</sub> caused a decrease in the enzyme activity of BIMAP, since the difference between the two buffers was the presence of P<sub>i</sub>. Enzyme activity was slowly restored with increasing the amount of MgCl<sub>2</sub> in Tris-phosphate buffer, although V<sub>max</sub> was not approached within the tested MgCl<sub>2</sub> concentrations. Taking into account the pKa's of the oxygen molecules on P<sub>i</sub>, at pH 10 one mol P<sub>i</sub> could in theory complex with two mol of Mg<sup>2+</sup>. Therefore, increasing the range of MgCl<sub>2</sub> concentration up to 0.2M (i.e. twice the concentration of P<sub>i</sub>) should theoretically defeat the effect of P<sub>i</sub> on Mg<sup>2+</sup> availability by our hypothesis, provided that the increased ion concentration in solution wouldn't effect any other parameter of enzyme function. As a result, it is predicted that the Vmax of BIMAP in Tris-phosphate buffer would be equal to that of BIMAP in Tris-HCl buffer. The difference in K<sub>m</sub> between the Tris-HCl buffer and Tris-phosphate buffer suggests that P<sub>i</sub> was indeed responsible for the difference in stabilization of the enzyme. However, whether P<sub>i</sub> was affecting the enzyme indirectly by acting as a chelator, or the anion was actually directly influencing the activity of BIMAP cannot be determined from our observations.

#### ACKNOWLEDGEMENTS

We would like to thank Dr. William Ramey for his time and input on the design of the experiment and Nick Cheng for testing the alkaline phosphatase enzyme activity. We would like also to thank both Karen Smith and Andre Comeau for their assistance in the lab.

#### REFERENCES

1. **Bortolato M., F. Besson, and B. Boux.** 1999. Role of metal ions on the secondary and quaternary structure of alkaline phosphatase from bovine intestinal mucosa. *Proteins*. **37**:310-318.
2. **Bogluslaw S, K.M. Holtz, and E.R. Kantrowitz.** 2000. A revised mechanism for the alkaline phosphatase reaction involving three metal ions. *J. Mol. Biol.* **299**:1303-1311.
3. **Janeway C.M., Xu X., Murphy J.E., Chaidaroglou A., and Kantrowitz E.R.** 1993. Magnesium in the active site of *Escherichia coli* alkaline phosphatase is important for both structural, stabilization and catalysis. *Biochem.* **32**:1601-1609.
4. **Xu X. and Kantrowitz E.R.** 1993. Binding of magnesium in a *Mutant Escherichia coli* alkaline phosphatase changes the rate-determining step in the reaction mechanism. *Biochem.* **32**:10683-10691.
5. **Mornet E., Stura E., and Lia-Baldini A.S.** 2001. Structural evidence for a functional role of human tissue nonspecific alkaline phosphatase in bone mineralization. *J. Biol. Chem.* **276**: 31171-31178.
6. **Ramey W.D.** 2001. Experiment A3: Properties of alkaline phosphatase at different substrate concentrations, different enzyme concentrations and different reaction pH. *Microbiology 421: Manual of Experimental Microbiology.*

## APPENDIX A

**Table 1:  $K_m$ ,  $V_{max}$  and  $R^2$  values of alkaline phosphatase in 1M Tris-Cl buffer pH 10**

[MgCl <sub>2</sub> ] (mM)	$V_{max}$ (U/mL)	$K_m$ (mM)	$R^2$ of the trend line
0	0.038	16.2	0.9983
0.5	0.533	1.21	0.9733
1.0	1.060	4.04	0.9995
1.5	0.868	2.42	1.0000
2.0	0.772	1.81	0.9993
2.5	0.614	1.38	0.9974
3.0	0.736	1.32	0.9995
3.5	0.717	1.61	0.9967
4.0	0.873	1.95	0.9986

**Table 2:  $K_m$ ,  $V_{max}$  and  $R^2$  values of alkaline phosphatase in 1M Tris + 0.1M sodium phosphate buffer pH 10**

[MgCl <sub>2</sub> ] (mM)	$V_{max}$ (U/mL)	$K_m$ (mM)	$R^2$ of the trend line
0	0.006	0.31	0.2276
0.5	0.028	5.33	0.9998
1.0	0.044	9.50	0.9865
1.5	0.046	9.54	0.9984
2.0	0.053	10.72	0.9983
2.5	0.062	7.83	0.9966
3.0	0.070	6.72	0.9607
3.5	0.032	3.48	0.9705
4.0	0.051	6.42	0.9196

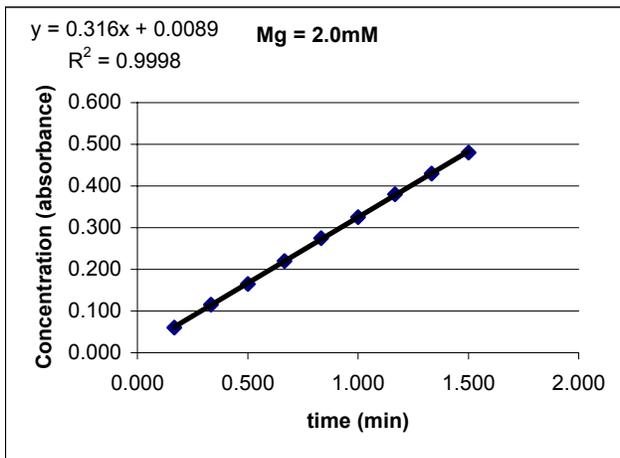
## APPENDIX B

### 1. *Reagent preparation calculations*

- 1M Tris-Cl buffer: 12.2g Tris added to 50mL dH<sub>2</sub>O,  
pH adjusted to 10.0 with 5N HCl, then topped to 100mL  
with water  
 $\text{Tris} = (121.14 \text{ g/mol})(0.1 \text{ mol}) = 12.2 \text{ g Tris}$
- 1M Tris + 0.1M phosphate buffer: 12.08g Tris and 1.2g sodium phosphate added to  
50mL dH<sub>2</sub>O, pH adjusted to 10, then topped with water  
to 100mL  
 $\text{Tris} = (121.14 \text{ g/mol})(0.1 \text{ mol}) = 12.2 \text{ g Tris NaH}_2\text{PO}_4 =$   
 $(120 \text{ g/mol})(0.01 \text{ mol})$   
 $= 1.2 \text{ g NaH}_2\text{PO}_4$
- 10mM MgCl<sub>2</sub> solution: 0.042g of MgCl<sub>2</sub>•6H<sub>2</sub>O added to 200mL dH<sub>2</sub>O  
 $\text{MgCl}_2 \cdot 6\text{H}_2\text{O} = 203.31 \text{ g/mol} \cdot 0.002 = 0.402 \text{ g}$
- 4.0mg/mL pNPP preparation: 198.0mg pNPP added to 49.5mL dH<sub>2</sub>O, mixed  
thoroughly
- 2.0mg/mL alkaline phosphatase: Added 20mg of AP to 10mL dH<sub>2</sub>O, mixed thoroughly

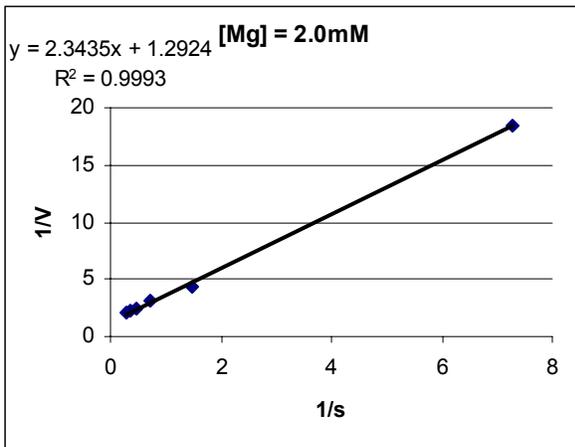
## 2. Sample Calculations

*Alkaline phosphatase activity in 1M Tris-Cl buffer, 2.0mM MgCl<sub>2</sub>:*



**Slope** = rise/run = 0.316 A/min  
**R<sup>2</sup> value** = 0.9998

$$\begin{aligned} \text{Enzyme activity} &= (A/t)(1000/20000)(N_v/E_v) \\ &= (0.316)(1000/20000)(3.1\text{mL}/0.1\text{mL}) \\ &= \mathbf{0.490 \text{ U/mL}} \end{aligned}$$



*Lineweaver-Burk Transformation*

$$\begin{aligned} \text{x-intercept} &= -1/K_m \\ 0 &= 7.265x + 1.2924 \\ \mathbf{K_m} &= \mathbf{1.81 \text{ mM}} \end{aligned}$$

$$\begin{aligned} \text{y-intercept} &= 1/V_{\text{max}} \\ \mathbf{V_{\text{max}}} &= \mathbf{0.772 \text{ U/mL}} \end{aligned}$$

**R<sup>2</sup> value** = 0.9993