Enzyme Kinetics of Alkaline Phosphatase

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Abstract

The purpose of this experiment was to study the enzyme kinetics of alkaline phosphatase. Specifically, we found the K_M, V_{Max}, and k_{cat} for alkaline phosphatase as well as the K_i with the competitive inhibitor inorganic phosphate. We used an enzyme assay with varying concentrations of PNPP as the substrate for the alkaline phosphatase enzyme. From two different assays we were able to calculate the K_m, V_{Max}, and K_i. Our Michaelis-Menten plot showed a V_{Max} of approximately 2 nmol/min and a K_M of approximately 20 µM. From our Lineweaver-Burk plot we found the V_{Max} to be 3.6179 nmol/min, the K_M to be 52.8437 µM, and the k_{cat} to be 0.882. From our Eadie-Hofstee plot we found the V_{Max} to be 2.9561 nmol/min, the K_M to be 35.506 µM, and the k_{cat} to be 0.721. Our Dixon plot showed the V_{Max} to be 1.922 nmol/min and the K_M to be 7.573 µM. We expected either the Lineweaver-Burk or Eadie-Hofstee plots to give us the best results, and our data suggests the Eadie-Hofstee plot as the most accurate and robust in light of possible experimental error.

Introduction

The purpose of this lab was to determine the K_m, V_{max}, and k_{cat} values of alkaline phosphatase through three graphical analyses.

Alkaline Phosphatase

Alkaline phosphatase is a phophatase enzyme that catalyzes the removal of phosphate monoesters by hydrolysis at a high pH (>7.0). The general reaction that this enzyme catalyzes is:

$$R\text{-}O\text{-}PO_3H^- + H_2O \longrightarrow R\text{-}OH + H_2PO_4^-$$

This enzyme is found in many eukaryotic and prokaryotic organisms. In *E. coli*, this enzyme can be found in the periplasmic space, which is the space between the outer membrane and the cytoplasmic membrane. Alkaline phosphatase functions to remove phosphate groups from proteins in the periplasmic space so that the inorganic phosphate can be taken up and used by the cell. The alkaline phosphatase enzyme is dimeric and is devoid of activity when its dimmers are separated. This enzyme contains two metal Zn atoms per molecule.

Alkaline Phosphatase Assay

An alkaline phosphatase assay is used to measure the activity of the enzyme. By adding a solution of p-nitrophenyl phosphate to a solution of alkaline phosphatase, the AP enzyme the will catalyze the removal of the phosphate group from colorless p-nitrophenyl phosphate producing p-nitrophenylate. P-nitrophenylate exhibits a yellow color of which the absorbance can be measured at 405nm and the activity of the AP enzyme can be measured in comparing known concentrations of the enzyme and substrate. Absorbance assays such as this are an essential and easy way to measure the activity of an enzyme especially when looking to calculate the enzyme kinetics.

First Week

During the first week of the experiment three graphical representations were used to illustrate and calculate the enzyme kinetics of alkaline phosphatase. Analysis of basal enzyme kinetics is an important aspect to researchers. Enzymologists commonly look to better understand the mechanisms and interactions of enzymes within their physiological role. As enzymes are essential in catalyzing reactions that allow an organism to maintain life, it is essential to understand how these reactions occur, specifically the speed of the reactions and how much reactants are needed to produce the desired amount of product. In this experiment we will estimate and calculate the maximum velocity at which the enzyme reacts under substrate saturation, V_{Max}, along with the amount of substrate concentration needed to produce a reaction rate at half the enzyme's V_{Max} and also the turnover number, k_{cat}, illustrating how much substrate is converted into product per second.

Through plotting and calculating the enzyme's speed of reaction along with the correlating substrate concentration, scientists can adjust the experimental conditions by adding possible inhibitors or other physiological factors and see how the kinetics of the reaction are changed. This method of analysis is essential in learning and understanding the role and mechanisms that enzymes employ in order to affect and control most every biological process. This experiment utilizes three well studied and used models of enzyme kinetics: the Michealis-Menten plot, the Lineweaver-Burk plot, and the Eadie-Hofstee plot. Each of these graphical representations offers a varied perspective and analysis of how to view and calculate the kinetics of an enzyme.

Michaelis-Menten Plot

The Michaelis-Menten graph plots the velocity of the reaction versus the substrate concentration. This graph provides a curved plot relating the substrate amount to the velocity of the reaction. This graph allows one to estimate the values of V_{Max}, K_M, and k_{cat} as the line approaches a vertical asymptote at the V_{Max}.

Lineweaver-Burk Plot

The Lineweaver-Burk graph plots the inverse of the velocity of the reaction versus the substrate concentration. This graph provides a linear plot with a positive slope relating the reaction velocity to the substrate concentration. Furthermore, this linear plot crosses the y-axis correlating to the V_{Max} and intersects the x-axis correlating to the negative inverse of the K_M, allowing us to more accurately calculate the V_{Max}, K_M, and k_{cat} values.

Eadie-Hofstee Plot

The Eadie-Hofstee graph plots the reaction velocity versus the velocity over the substrate concentration. This provides a linear plot with a negative slope relating to the K_M value. The V_{Max} is represented as the y-intercept. This graphical method allows a more adaptive analysis by

equally weighting the data points, regardless of the range used for substrate concentration and velocity. Though, an experimental error in the reaction velocity will be represented on both axes since both axes are dependent upon the reaction velocity. As a result, this negates the associability of the squared correlation coefficient, the R^2 value.

Second Week

On the second week of the experiment we utilize an inorganic phosphate to inhibit the alkaline phosphatase enzyme and further calculate the inhibitor dissociation value, k_i, by using the Dixon plot. Enzymologists commonly exploit the effects of various inhibitors on enzymes. Enzyme inhibitors play a major role in modern drug therapy and various treatment options for ameliorating disease. In order to understand how a specific inhibitor works on an enzyme it is necessary to know what type of inhibitor it is, competitive, non-competitive, or uncompetitive, and furthermore how the V_{Max} and K_M values are affected.

Dixon Plot

The Dixon graph plots the inverse of the reaction velocity versus the inhibitor concentration. This graph provides two linear lines detailing the enzyme with and without the inhibitor. The position at which the lines intersect relates to how the reaction is affected and thus what kind of inhibitor is begin used. Theoretically, lines that intersect at the x-axis illustrate a non-competitive. Theoretically, lines that intersect at the y-axis illustrate a competitive inhibitor. While lines that are perpendicular illustrate an uncompetitive inhibitor.

Methods

First week enzyme Assay

In our first enzyme assay, we set the spectrophotometer absorb at 405 nm. We blanked using buffer only. We added buffer and PNPP to 1.00 mL as shown in Table 1. We started each assay immediately after we added 20 µL of the alkaline phosphatase enzyme to each mixture in the cuvette. After mixing the contents of the cuvette, we assayed each mixture and determined the initial rate for each as shown in Table 1.

Second week enzyme Assay

For our second assay, we again set our spectrophotometer to absorb at 405 nm. We blanked again using buffer only. We added buffer, PNPP, and phosphate to 1.00 mL in each assay as shown in Table 4. As in the first assay, we started each assay upon the addition of 20 µL of alkaline phosphatase enzyme. We determined the initial rate for each assay as shown in Table 4.

Data

Table 1. *Data from the first assay.* The closest two values for the initial rate at each concentration were taken such that the difference between each value and their mean was less than 5%. The assay went to zero at 970 µL buffer and 30 µL PNPP, so the assay was stopped after this point.

Buffer, 0.2 M Tris, pH 8.0	PNPP, 0.400 M, in Buffer	v, initial rate	v in nmol/min	[S] (µM)	1/v	1/[S]	v/[S]
800 µL	200 µL	.030	2.000	80	.500	.0125	.0250
800 µL	200 µL	.031	2.067	80	.484	.0125	.0258
850 µL	150 µL	.028	1.867	60	.536	.0167	.0311
850 µL	150 µL	.027	1.800	60	.556	.0167	.0300
900 µL	100 µL	.026	1.733	40	.577	.0250	.0433
900 µL	100 µL	.027	1.800	40	.556	.0250	.0450
925 µL	75 µL	.020	1.333	30	.750	.0333	.0444
925 µL	75 µL	.020	1.333	30	.750	.0333	.0444
950 µL	50 µL	.014	.933	20	1.072	.0500	.0467
950 µL	50 µL	.015	1.000	20	1.000	.0500	.0500
970 µL	30 µL	.000	0.000	12	0.000	.0833	0.000

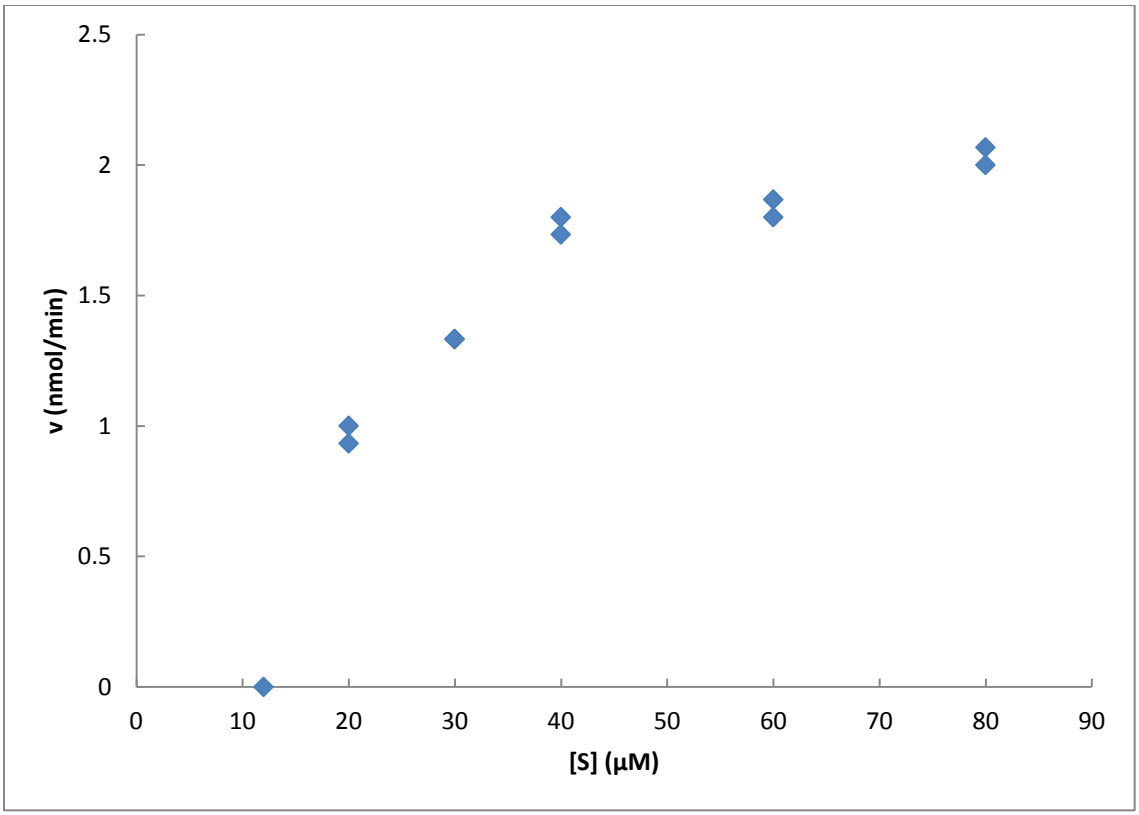

Figure 1. *Michaelis-Menten plot for the first assay.*

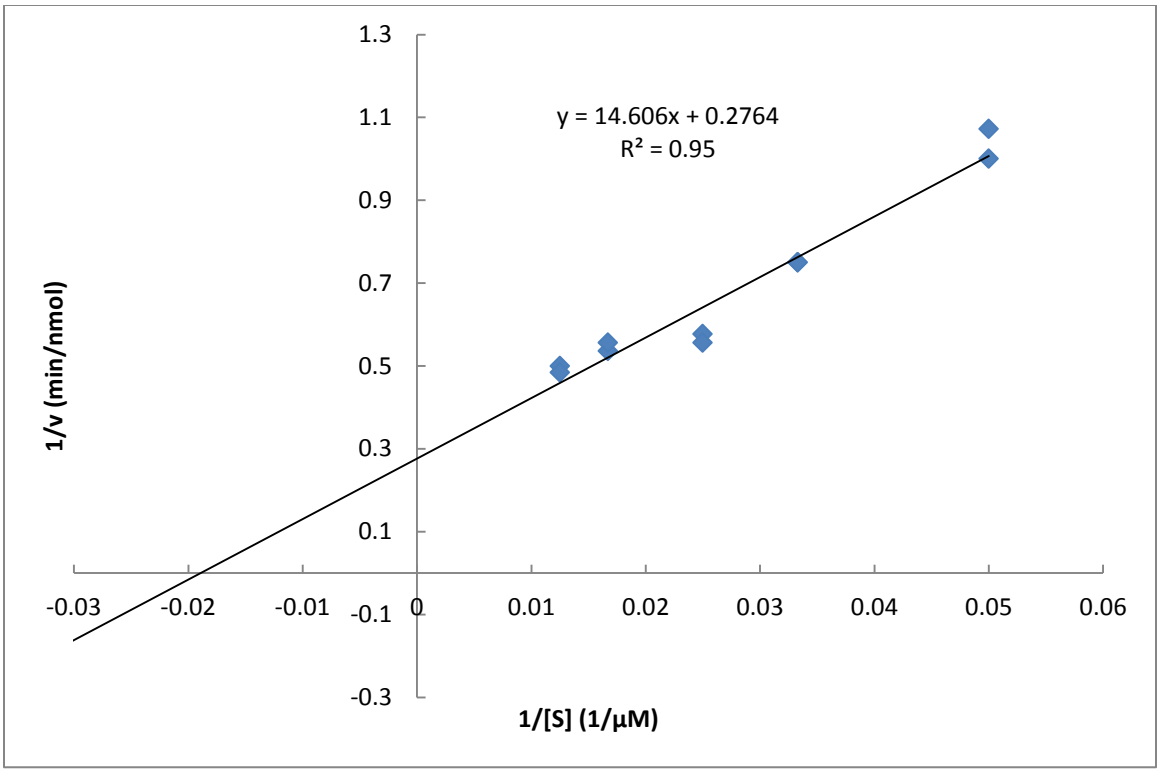

Figure 2. *Lineweaver-Burk plot for the first assay.*

Table 2. *Kinetics data from the obtained from analyzing the Lineweaver-Burk plot.*

Lineweaver-Burk Data
V_{Max} = 3.6179 nmol/min
K_M = 52.8437 µM
k_{cat} = 0.882

Calculations

$1/V_{Max} = 14.606(0) + 0.2764$

V_{Max} = 3.6179 nmol/min

$-1/K_M = -0.2764/14.606$

K_M = 52.8437 µM

$k_{cat} = 3.6179/4.1$

k_{cat} = 0.882

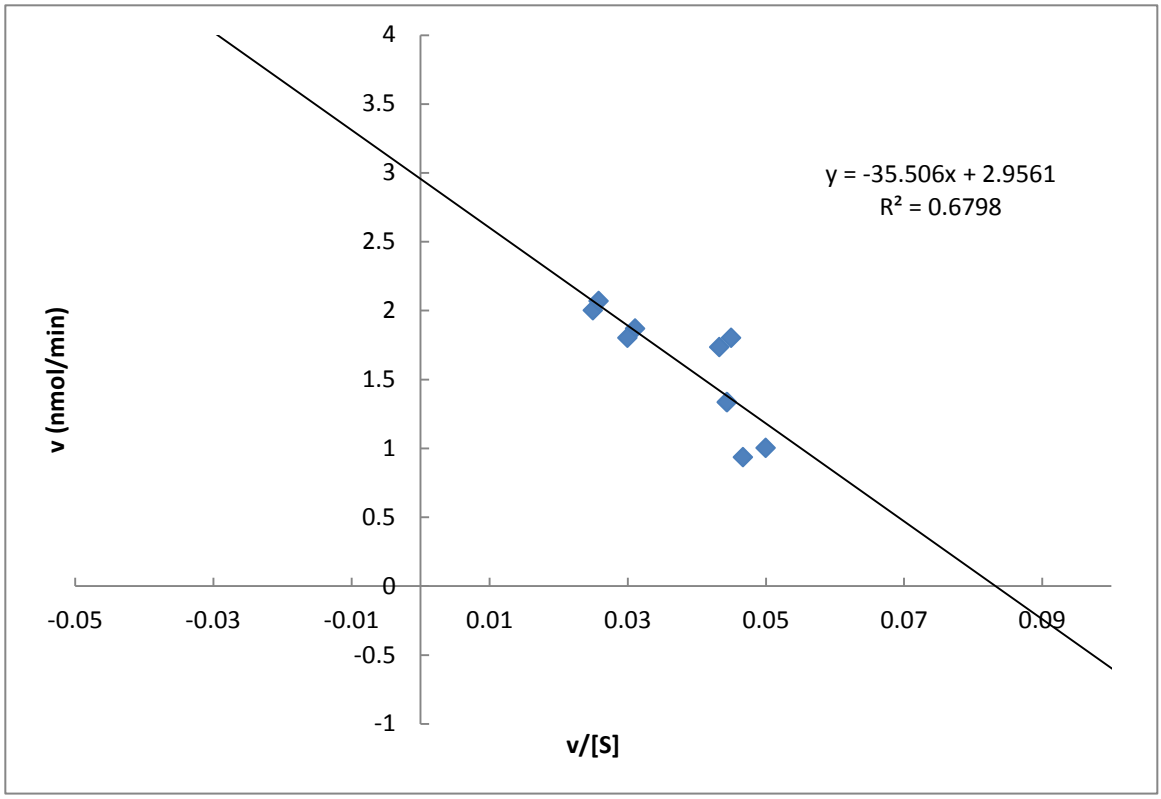

Figure 3. *Eadie-Hofstee plot for the first assay.*

Table 3. *Kinetics data obtained from analyzing the Eadie-Hofstee plot.*

Eadie-Hofstee Data
V_{Max} = 2.9561 nmol/min
K_M = 35.506 µM
k_{cat} = 0.721

Calculations

V_{Max} = -35.506(0) + 2.9561

V_{Max} = 2.9561 nmol/min

$-K_M$ = -35.506

K_M = 35.506 µM

k_{cat} = 2.9561/4.1

k_{cat} = 0.721

Table 4. *Data from the second assay.* The closest two values for the initial rate were taken such that the two values for each concentration were within 5% of the mean.

Buffer, 0.2 M Tris, pH 8.0	PNPP, 500 µM, in buffer	Phosphate, 1.50 mM, in buffer	v, initial rate	v in nmol/min	1/v	[I] (µM)
900 µL	100 µL	-	.028	1.867	.536	-
900 µL	100 µL	-	.029	1.933	.517	-
890 µL	100 µL	10 µL	.023	1.533	.652	15
890 µL	100 µL	10 µL	.025	1.667	.600	15
880 µL	100 µL	20 µL	.016	1.067	.937	30
880 µL	100 µL	20 µL	.017	1.133	.883	30
860 µL	100 µL	40 µL	.015	1.000	1.000	60
860 µL	100 µL	40 µL	.015	1.000	1.000	60
820 µL	100 µL	80 µL	.008	.533	1.876	120
820 µL	100 µL	80 µL	.007	.467	2.141	120
780 µL	100 µL	120 µL	.005	.333	3.003	180
780 µL	100 µL	120 µL	.005	.333	3.003	180
700 µL	300 µL	-	.030	2.000	.500	-
700 µL	300 µL	-	.033	2.200	.500	-
690 µL	300 µL	10 µL	.031	2.067	.484	15
690 µL	300 µL	10 µL	.030	2.000	.500	15
680 µL	300 µL	20 µL	.025	1.667	.600	30
680 µL	300 µL	20 µL	.026	1.733	.577	30
660 µL	300 µL	40 µL	.018	1.200	.833	60
660 µL	300 µL	40 µL	.020	1.333	.750	60
620 µL	300 µL	80 µL	.017	1.133	.883	120
620 µL	300 µL	80 µL	.017	1.133	.883	120
580 µL	300 µL	120 µL	.013	.867	1.153	180
580 µL	300 µL	120 µL	.014	.933	1.072	180

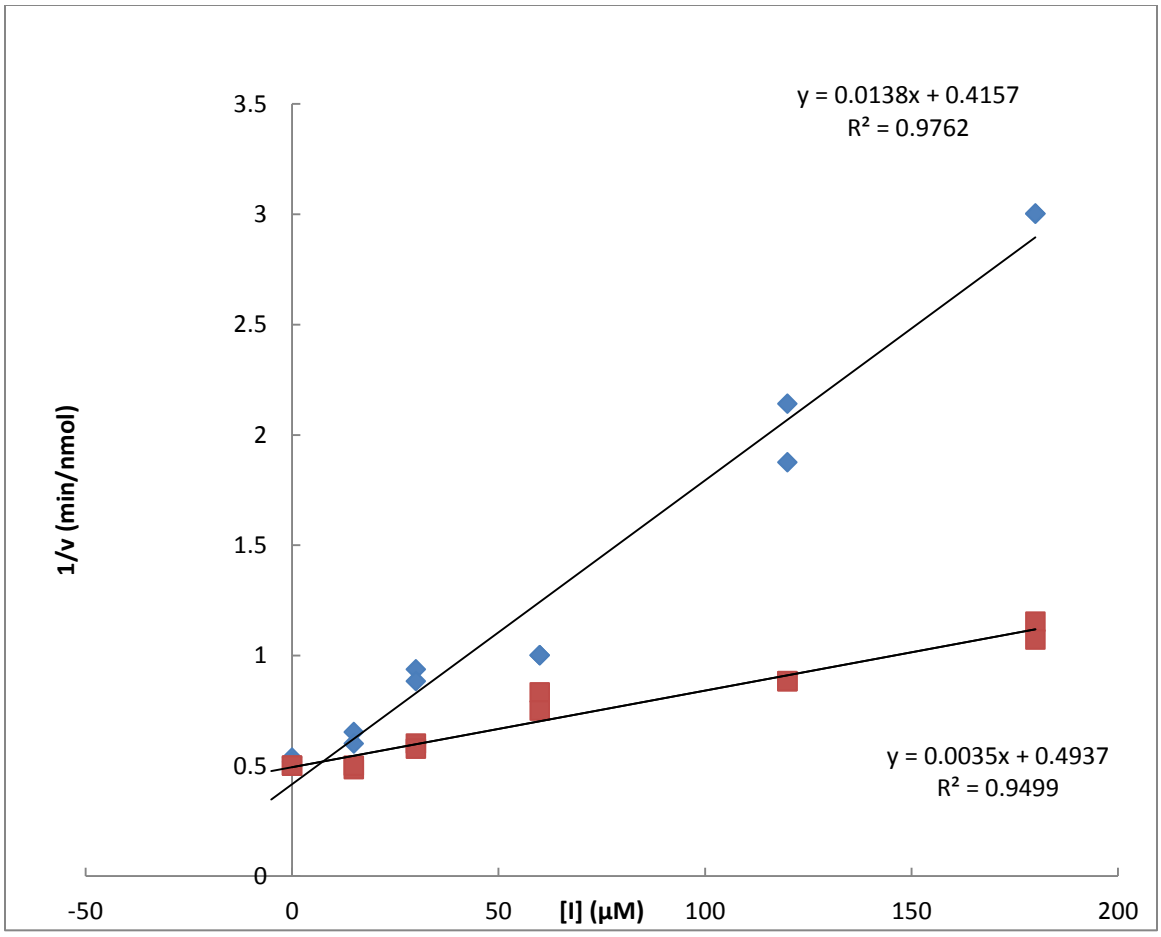

Figure 4. *Dixon plot from data from the second assay.*

Table 5. *Kinetics data obtained from analyzing the Dixon plot.* The inhibitor present was a competitive inhibitor.

Dixon Data
V_{Max} = 1.922 nmol/min
K_i = 7.573 µM

Calculations

$0.0138(K_i) + 0.4157 = 0.0035x + 0.4937$

$0.0103(K_i) = 0.078$

K_i = 7.573 µM

$1/V_{Max} = 0.0138(7.573) + 0.4157$

V_{Max} = 1.922 nmol/min

Discussion

First Week

The purpose of the first week was to measure and compare the enzyme kinetic values of K_M, V_{Max}, and k_{cat}, of alkaline phosphatase using three different graphical plots. We plotted the data from the absorbance assay of reacting alkaline phosphatase with p-nitrophenyl phosphate. In analyzing the plotted graphs of Michaelis-Menten, Lineweaver-Burk, and Eadie-Hofstee we found reasonable data that was to be expected in that the Michaelis-Menten plot showed a curved distribution whereas the Lineweaver-Burk and Eadie-Hofstee plots showed linear distributions that were relatively consistent.

In actual comparison of the K_M, V_{Max}, and k_{cat} values we noticed differences, though in understanding that measuring enzyme kinetics is largely and very sensitively dependent upon pipetting accuracy and that any variation can affect the data as a whole these slight variations can be attributed to pipetting error. The Michaelis-Menten plot appears to show a V_{Max} approaching a value slightly greater than 2 nmol/min and a rough K_M estimate of slightly greater than 20 μM. The Lineweaver-Burk plot provides a calculated V_{Max} of 3.6 nmol/min and a K_M of 52.8 μM . Lastly, calculation of the Eadie-Hofstee plot provides a V_{Max} of 2.9 nmol/min and a K_M of 35.5μM. Furthermore, since pipetting error most likely played the greatest role in distorting the data we find and prefer the Eadie-Hofstee plot to provide the most accurate results. The Eadie-Hofstee plot takes the reaction velocity into consideration on both the x and y axes, thus any pipetting error resulting in inconsistent data will be equally weighted and distributed among each point. This is also supported by the fact that the Eadie-Hofstee data provides values at almost the exact mean of the other two plots.

Second Week

The purpose of the second week was to analyze the inhibitor effects of inorganic phosphate on alkaline phosphatase. The Dixon plot provides an illustration and model in which to calculate the dissociation constant, K_i, of the inhibitor as well as determine the type of inhibitor that inorganic phosphate is. As seen by Figure 4 the lines cross to the left of the y-axis and thus correspond to the $-K_i$ and further illustrating that inorganic phosphate functions as a competitive inhibitor of alkaline phosphatase. These results match what we would expect, as the substrate for alkaline phosphatase must contain a phosphate, thus free inorganic phosphate will compete for the active site and consequently inhibit the enzyme. Had the lines intersected at the x-axis they would represent the effect of a non-competitive inhibitor. Using the Dixon plot to illustrate how specific molecules affect the kinetics and inhibition of an enzyme is extremely beneficial in understanding not only what type of inhibitor a molecule is, but also shows valuable information as to the active site and mechanism of reaction that the enzyme utilizes.

References

Handout 3. Enzyme Kinetics: Determination of K_M and V_{Max}. Enzyme Kinetics of Alkaline Phosphatase Experiment 3.

Handout 4. Enzyme Kinetics: Product Inhibition of Alkaline Phosphatase. Enzyme Kinetics of Alkaline Phosphatase. Experiment 4.

Ninfa, A.J. and Ballou, D.P. 2010. Fundamental Laboratory Approaches for Biochemistry and Biotechnology. Fitzgerald Science Publishing. 267-270.