

## 9 Kinetic Properties of Alkaline Phosphatase – Determining $K_M$ and $V_{max}$ Values

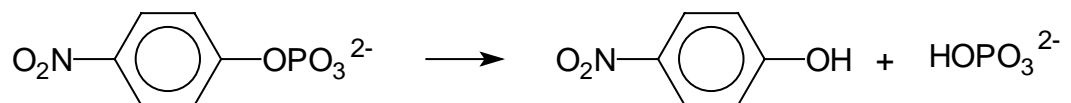


**Objectives:** A) To demonstrate the effect of substrate concentration upon the rate of the reaction catalyzed by alkaline phosphatase; B) to plot the velocity of the reaction versus the substrate concentration to produce the Michaelis-Menten curve; C) to plot the reciprocal of the velocity versus the reciprocal of the substrate concentration to generate the linear Lineweaver-Burk plot; and, D) to determine the  $K_M$ ,  $V_{max}$  and  $k_{cat}$  values for alkaline phosphatase from both of these plots.

**Introduction:** Alkaline phosphatase is made throughout the body. Its function is to remove phosphate groups from nucleotides and proteins, many enzymes have their activity controlled by the addition and removal of phosphate groups. The blood serum level of Alkaline Phosphatase is used as a marker for disease. Elevated levels (five-times higher than normal) are found in the blood serum of people suffering from various bone and liver diseases. Irritable bowel syndrome, germ-cell tumors and liver infections will raise serum levels of Alkaline Phosphatase to a lesser extent.

Different tissues in the body make slightly different versions of the enzyme, called isozymes. Recall Experiment IV, BSA and Casein did not precipitate under the same conditions. In the same manner different conditions can be used to inactivate specific isozymes of Alkaline Phosphatase, the activity of specific isozymes can be measured yielding even more diagnostic information.

However, the purpose of our experiment is not medical diagnosis but rather to test the kinetic parameters of bovine intestinal alkaline phosphatase. To do this we will use para-nitrophenyl phosphate (PNPP) as the substrate. We are using this substrate because one of the products (para-nitrophenol) has a very high molar extinction coefficient;  $\epsilon_{405} = 18,800 \text{ M}^{-1} \text{ cm}^{-1}$ . This allows us to measure very small quantities of the product easily. However, only the para-nitrophenolate ion absorbs.



Previously we used Acid Phosphatase for this experiment. As the name suggests this enzyme is active at acidic pH values. Because para-

nitrophenol has a pKa of about 7.2 we needed to add KOH to the reaction to generate the anionic form of the product. This addition of KOH also denatured the enzyme, therefore we could only generate one time-point per tube.

By using Alkaline Phosphatase (active at high pH values) we will generate the anionic form of the product directly and be able to take several time points for each well.

**Procedure:**

1. Prepare the stock concentrations of PNPP. Your instructor will give you a stock concentration of 200 mM PNPP. Make 100  $\mu$ L of the following concentrations:

Stock conc. mM	$\mu$ L of H <sub>2</sub> O	$\mu$ L of 200 mM PNPP
1		
2		
4		
6		
9		
15		
20		

As I mentioned for the Protein Quantitation Lab, I'm not giving you the amounts to add because you should be able to calculate this. If you are in one of Dr. Wolfgang's sections I guarantee that a dilution question similar to this will be on your final.

2. To wells A7 thru H7 add 175  $\mu$ L of Diethanolamine buffer.
3. To wells A8 thru H8 add 180  $\mu$ L of Diethanolamine buffer.
4. To wells A7 and A8 add 20  $\mu$ L of H<sub>2</sub>O.
5. To wells B7 and B8 add 20  $\mu$ L of 1 mM stock PNPP
6. To wells C7 and C8 add 20  $\mu$ L of 2 mM stock PNPP
7. To wells D7 and D8 add 20  $\mu$ L of 4 mM stock PNPP
8. Do you see the pattern? Continue until you have added 20  $\mu$ L of 20 mM stock PNPP to wells H7 and H8.

**YOU ARE NOW READY TO GO TO THE PLATE READER**

9. We want to preheat the buffer and substrate to 37° C to do this open the protocol called "preheat" (there is no section designation, all sections will use the same preheat protocol).
10. Place the plate in the plate reader and run the protocol. Do Not save the data from the preheat protocol.

11. Once the plate reader is finished it will open up, CLOSE THE PLATE READER AGAIN TO KEEP THE SAMPLES WARM, to do this **press the black button on the front of the plate reader.**
12. Now open protocol called “kinetics#” again the # is your section number.
13. Enter your Section#, name and experiment.
14. The machine will open up and the screen will show the window instructing you to “insert plate and press ok”.
15. Add 5  $\mu\text{L}$  enzyme **to wells A7-H7 ONLY.** Do not worry about mixing the sample, the plate reader will shake the whole plate. Press ok and let the plate reader carry out the protocol.
16. **DO NOT ADD ENZYME TO WELLS A8-H8,** these are control wells.
17. Save Data as before and e-mail it to yourself.

### Data Analysis:

The data for the #8 wells should not change as a function of time; as you read in the procedure these are controls. **Why are these controls important?** Answer that question in the report.

The data for the #7 wells will increase over time. You need to graph each of these out with  $A_{405}$  on the y-axis and time on the x-axis. Then do linear regression for each one.

These values are the rates that must be converted from change in  $A_{405}$  per unit time to amount of para-nitrophenolate produced per unit time. To do this correctly you will need to know three things 1) the path length (check back with experiment 1), 2) the molar extinction coefficient ( $\epsilon_{405}$ ) is  $18,800 \text{ M}^{-1} \text{ cm}^{-1}$ , and 3) the total volume of the reaction.

Now you are ready to make the Michaelis-Menten and Lineweaver-Burk plots. Make sure you use the correct concentrations of PNPP (substrate). What concentration of PNPP did the enzyme “see”?

Well #	PNPP Concentration (mMoles/L)	Velocity ( $\mu\text{moles}/\text{min}$ )	Reciprocal PNPP Concentration L/mMoles	Reciprocal Velocity ( $\text{min}/\mu\text{moles}$ )
A7	0	0	undefined	undefined
B7				
C7				
D7				
E7				
F7				
G7				
H7				

To make the Lineweaver-Burk plot calculate the reciprocals of the velocity and the PNPP concentration.

REMEMBER THAT THE LINEWEAVER-BURK PLOT SHOULD NOT GO THROUGH THE ORIGIN SO DON'T TELL THE LINEAR REGRESSION ANALYSIS TO FORCE IT THROUGH THE ORIGIN. This is a gross conceptual error and **will** result in many points taken off the lab report grade.

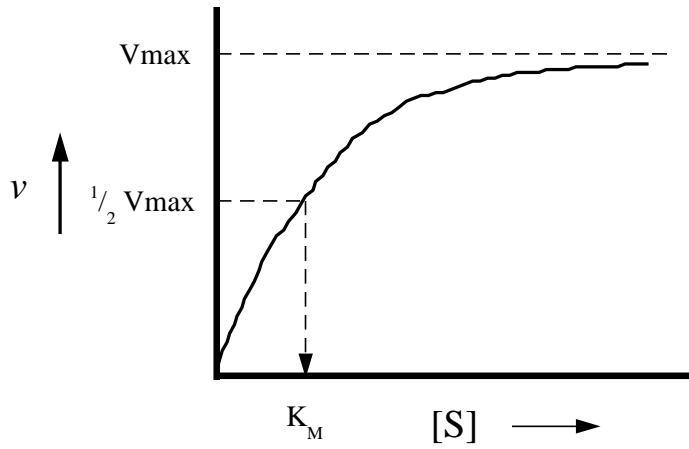
Estimate the  $K_M$  and  $V_{max}$  values from the Michaelis-Menten curve, CALCULATE the  $K_M$  and  $V_{max}$  values from the Lineweaver-Burk plot.

Finally calculate the turnover number or  $k_{cat}$ .

For this you will need to know that the molecular weight of Alkaline Phosphatase is 150,000, and you added 77ng of enzyme to wells A7-H7.

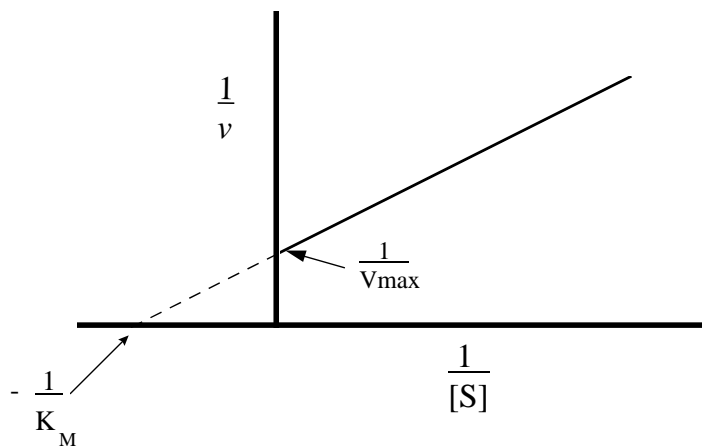
#### Checklist for report

1. Show the raw data.
2. Statement about the importance of the control wells (A8-H8).
3. Linear regression for wells A7-H7 (eight lines with regression data).
4. Sample calculation for conversion of velocity from  $A_{405}/\text{min}$  to  $\mu\text{moles}/\text{min}$ .
5. Fill in the above table.
6. Graph of Michaelis-Menten curve.



7. Estimate of  $K_M$  and  $V_{max}$  from Michaelis-Menten curve.

8. Graph of Lineweaver-Burk plot.



9. Calculation of  $K_M$  and  $V_{max}$  from the Lineweaver-Burk graph.

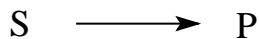
10. Comments about the values, if they match great if not some explanation as to why they don't, which do you trust more and why?

11. Calculation of  $k_{\text{cat}}$ .

**Record the raw data in the notebook:**

## Kinetic Properties of Acid Phosphatase - Determining $K_M$ and $V_{max}$ STUDY GUIDE

1. Alkaline phosphatase is a hydrolase. In general, what type of reaction do hydrolases catalyzed?
2. What are the functions of the  $MgCl_2$  and  $KOH$  in the acid phosphatase assay?
3. What is the quantity of enzyme present when 23  $\mu$ moles of p-nitrophenol are produced per minute?
  - a. 23  $\mu$ moles
  - b. 23 moles
  - c. 23 units
  - d. 23 mg/mL
4. The extinction coefficient of p-nitrophenol is  $18.8 \times 10^3$  liters/mol/cm. The  $A_{405}$  in a 1 cm cuvette was 0.68 for a  $2 \times 10^{-4}$  liter sample of solution assayed for acid phosphatase according to the procedure in the above experiment. What is the acid phosphatase activity in units/mL (U/mL)?
  - a. 2.71 U/mL
  - b. 0.271 U/mL
  - c. 13.56 U/mL
  - d. 1.36 U/mL
  - e. None of the above
5. From a Lineweaver-Burk plot of an enzyme-catalyzed reaction where  $1/v = 25$  hr/mole at  $1/[S] = 0$ , and  $1/[S] = 1.3 \times 10^2$  L/mol at  $1/v = 0$ , calculate  $V_{max}$  and  $K_m$ .
6. What information can be obtained from Lineweaver-Burk plots of enzyme kinetics for the reaction



under the following conditions?

- a.  $1/v$  is zero when  $1/[S]$  equals 40 L/mol.
- b.  $1/[S]$  is zero when  $1/v$  equals  $3.0 \times 10^5$  min/mol.

