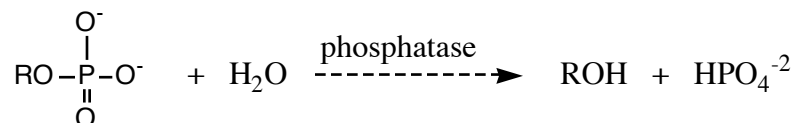


## BC 367 Experiment 4

### Kinetic Properties of Acid Phosphatase

#### Introduction

Phosphatases are enzymes that remove phosphate groups from substrates. Phosphorylated compounds are widely distributed in living systems. They serve as storage forms for energy (e.g., ATP and phosphocreatine), as components of informational macromolecules (i.e., nucleotides and deoxynucleotides), as allosteric effectors of certain enzymes (e.g., fructose-1-6-bisphosphate), and as second messengers (e.g., cAMP, cGMP, inositol phosphates). Phosphorylation-dephosphorylation reactions of proteins, mediated by protein kinases and protein phosphatases, modulate many enzyme activities (phosphorylase, pyruvate dehydrogenase, etc.). It is therefore not surprising that phosphatases of many kinds can be extracted from many tissues. In general, these enzymes catalyze the hydrolysis of phosphate monoesters as follows:



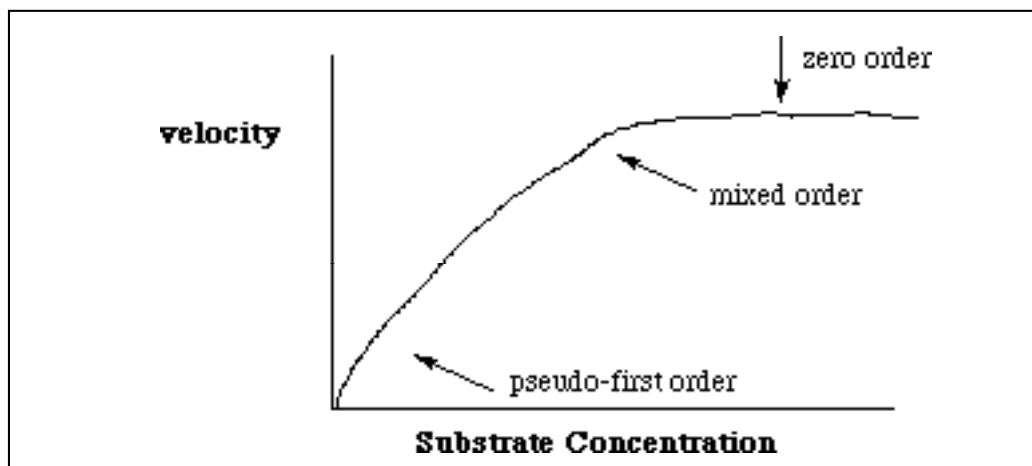
During this experiment, you will investigate the properties of an acid phosphatase from wheat germ. Acid phosphatases have optimal activity at pH values from 4 to 6, and are distinct from alkaline phosphatases with optimal activity in the pH range of 8-9. The function of the wheat germ phosphatase has not been definitively established, but it has been proposed that it is involved in the mobilization of the phosphate reserves of the seed. Most of the phosphate within a seed is esterified as inositol hexaphosphate (phytic acid). Since the free phosphate concentration in seeds is low enough to limit metabolic reactions, enzymes must be present to catalyze the hydrolysis of the phosphate reserve substances.

During the first week, you will perform a kinetic study on wheat germ acid phosphatase to determine its  $K_m$  and  $V_{max}$  with p-nitrophenylphosphate as the substrate. You will also determine the nature of the inhibition of acid phosphatase by inorganic phosphate. For the second week, you will examine the X-ray structure of acid phosphatase in the Schupf lab and meet with your instructor to discuss your plans for the following week. During the third week, you will carry out a set of your own kinetic experiments to investigate the effect of reaction conditions or a potential inhibitor of your choice on the kinetic properties of wheat germ acid phosphatase.

#### Approach

Kinetics concerns the study of reaction rates and the conditions that affect reaction rates. One of the primary concerns of kinetics deals with the study of the changes in the rate of a reaction as a function of the reactant (substrate) concentrations. Such measurements can lead to the calculation of rate constants and, where appropriate, of equilibrium constants. Kinetics can be of great value for predicting or eliminating possible reaction mechanisms under consideration, since an observed reaction rate must satisfy the proposed mechanism.

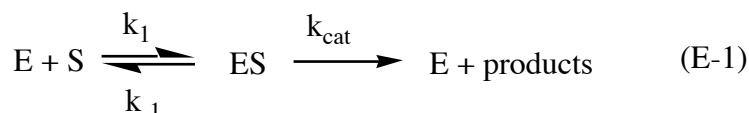
Maud Menten and Leonor Michaelis, two early 20th century biochemists, pioneered the study of the kinetics of enzyme-catalyzed reactions. They found that the dependence of the rate of enzymatic reactions on the substrate concentration was generally hyperbolic:



They were able to rationalize this finding by making several assumptions:

1. Enzyme and substrate must physically combine for catalysis to occur.
2. The surface of an enzyme has only a limited number of sites where catalysis actually takes place ("active sites").
3. The reversible binding of the substrate and enzyme occurs much more rapidly than subsequent bond breaking steps.

Schematically this may be represented as follows:



When the change in the rate of most enzyme-catalyzed reactions is measured as a function of increasing substrate concentration, pseudo-first order, mixed order, and zero order kinetics may be observed. At low substrate concentrations, not all of the active sites on the enzyme surface are occupied at all times, and the reaction rate will be proportional to the concentration of substrate; i.e., pseudo-first order kinetics will be observed. When the substrate concentration is very high, saturation conditions will exist; i.e., all active centers of the enzyme will be occupied by substrate, and the velocity will be independent of the substrate concentration. Under these conditions, the velocity is at a maximum, and zero order kinetics is observed.

Michaelis and Menten (with help from Briggs and Haldane) were able to derive an equation that describes quantitatively the relationship between the rate of an enzymic reaction and the substrate concentration. The final form of this equation is given below:

$$v = \frac{V_{\max} [S]}{K_m + [S]} \quad (\text{E-2})$$

$$\text{where } K_M = \frac{k_{-1} + k_{cat}}{k_1}$$

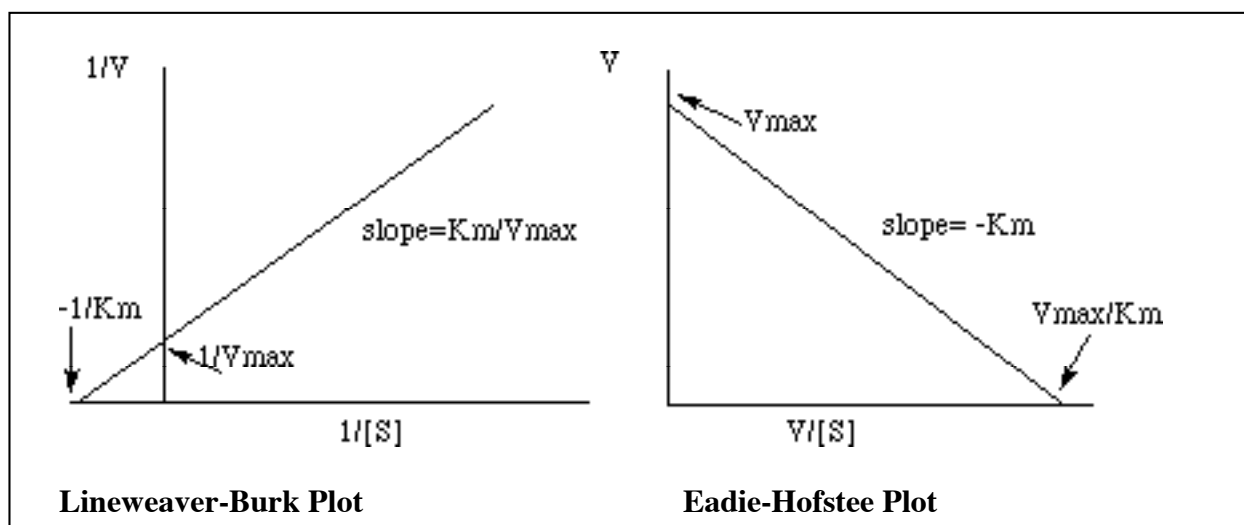
If  $k_{\text{cat}} \ll k_{-1}$ ,  $K_m$  approaches  $K_D$ , the dissociation constant for the enzyme-substrate complex. In these cases,  $K_m$  becomes a measure of the affinity of the enzyme for its substrate. The lower the value of the  $K_m$ , the greater is the affinity.

The Michaelis-Menten equation holds only when  $[S] \gg [E]$ , i.e., when a steady-state concentration of ES may be achieved, and only for initial velocities. Under these conditions, the substrate concentration may be expressed as  $[S]_0$ , the initial substrate concentration. Furthermore, at  $t = 0$ , and shortly thereafter, the concentration of P will be small, and thus the possibility that the reverse reaction will interfere is eliminated.

The original Michaelis-Menten equation may be transformed in several manners to yield the equation of a straight line. Two common transformations are:

$$\text{Lineweaver-Burk: } \frac{1}{V} = \frac{K_m}{V_{\text{max}}} \frac{1}{[S]} + \frac{1}{V_{\text{max}}} \quad (\text{E-3})$$

$$\text{Eadie-Hofstee: } V = -K_m \frac{V}{[S]} + V_{\text{max}} \quad (\text{E-4})$$



These two linear transformations may be used in the determination of  $K_m$  and  $V_{\text{max}}$  for an enzyme, which, in turn, reveal the affinity of the enzyme for its substrate and the catalytic effectiveness of the enzyme. Note that modern tools such as EXCEL Solver can also perform a non-linear least-square fit to the Michaelis-Menten equation. If you take an advanced biochemistry course, you may use non-linear methods to solve for key kinetic parameters.

The study of possible inhibitors of enzyme-catalyzed reactions can provide information concerning the mechanism of the enzyme-catalyzed reaction and the manner in which the enzyme's activity is controlled in the cell. The major types of inhibitors are competitive, uncompetitive, and mixed. Competitive inhibitors usually resemble the substrate and compete with it for the active site. The extent of the inhibition is a function of the relative concentrations

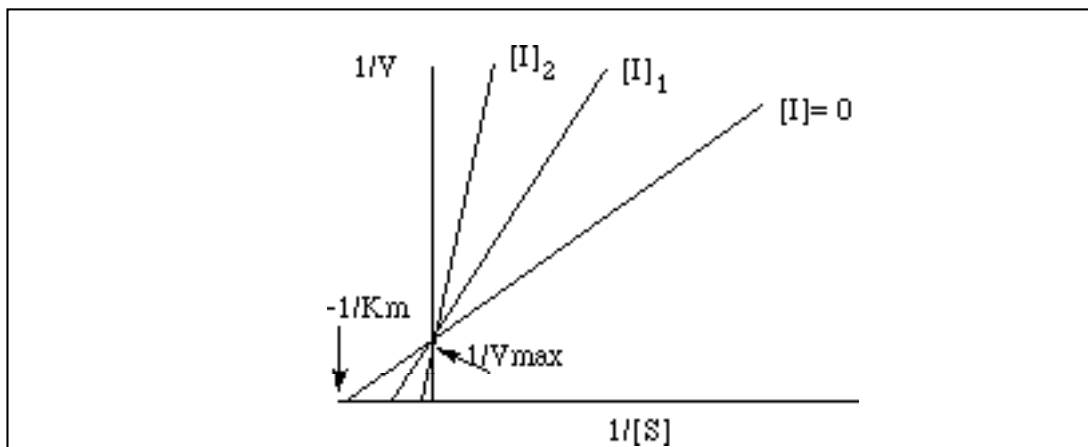
of the substrate and the inhibitor. As the substrate concentration is increased, the extent of inhibition decreases, until at infinitely high substrate concentrations the inhibitor has no effect at all. Therefore, in the presence of a competitive inhibitor,  $V_{\max}$  remains the same, but  $K_m$  is increased.

Competitive inhibition may be treated using the Michaelis-Menten approach, however, additional parameters appear in the Lineweaver-Burk transformation of the equation:

$$\frac{1}{V} = \frac{K_m}{V_{\max}} \left( 1 + \frac{[I]}{K_I} \right) \frac{1}{[S]} + \frac{1}{V_{\max}} \quad (\text{E-5})$$

where  $I$  = concentration of inhibitor;  $K_I$  = dissociation constant of EI complex.

Graphically, competitive inhibition may be readily recognized from Lineweaver-Burk plots of kinetic data at several inhibitor concentrations:



As mentioned above and illustrated in the equation and graph,  $V_{\max}$  is unaltered by competitive inhibition, but  $K_m$  is increased by a factor of

$$1 + \frac{[I]}{K_I}$$

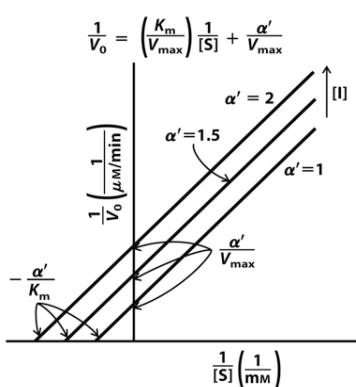
Since the  $K_m$  in the presence and absence of inhibitor may be determined graphically, the  $K_I$  may be calculated from the following relationship:

$$K_p = K_m \left( 1 + \frac{[I]}{K_I} \right)$$

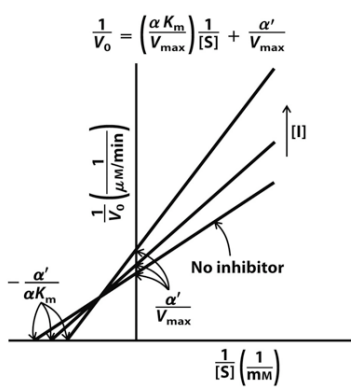
where  $K_p$  is the apparent  $K_m$  in the presence of a competitive inhibitor.

Uncompetitive, mixed, and noncompetitive inhibitors, in contrast to competitive inhibitors, generally bind at a site other than the binding site for the substrate, and thus the inhibitor and substrate are not in direct competition. In this case, the inhibition is independent of the substrate concentration and depends only on the inhibitor concentration. The effect of inhibitor is simply to remove some of the enzyme molecules from participation in the catalytic process. The enzyme molecules bound by inhibitor, however, still generally possess the capability of binding substrate but not of converting it to product. An uncompetitive inhibitor binds only to the ES complex, whereas mixed and noncompetitive inhibitors bind to either the free enzyme E or the ES complex. Check your textbook for the factors by which  $K_m$  and  $V_{max}$  change for these types of inhibitors.

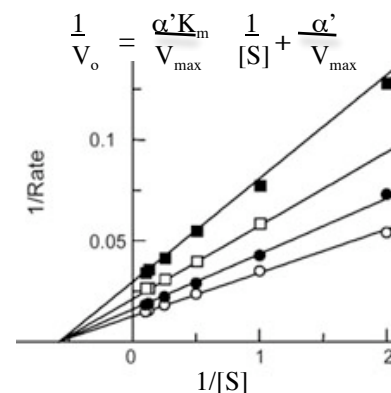
Graphically, uncompetitive, mixed, and noncompetitive inhibition may be recognized from Lineweaver-Burk plots of kinetic data at several different inhibitor concentrations as shown below:



**Uncompetitive inhibition**



**Mixed inhibition**



**Noncompetitive inhibition**

### **Experimental Procedure**

Steady-state kinetic expressions such as the Michaelis-Menten equation hold only for initial velocities. It is therefore necessary when performing kinetic experiments to follow the reaction as a function of time and to extrapolate the initial velocity from the results. A number of artificial substrates for hydrolytic enzymes have been developed which liberate a chromophore (a group that absorbs light) upon hydrolysis. For example, p-Nitrophenylphosphate (PNPP) itself has no absorbance in the visible spectrum, whereas p-nitrophenolate ion, one of the products of enzymic hydrolysis of p-nitrophenylphosphate, has intense absorbance in the blue region. Thus, the activity of a phosphatase can be monitored by following the increase in absorbance at 410 nm due to release of the p-nitrophenolate ion.

Light absorbance by p-nitrophenol is pH-dependent. Fully associated p-nitrophenol does not absorb blue light, but the p-nitrophenolate ion absorbs strongly. At pH 7.2, the  $pK_a$  for p-nitrophenol, the extinction coefficient is only half that which would be observed at pH 9.0, since only half of the molecules are dissociated. However, pH 7.2, rather than a higher pH, was selected for use in this experiment as a compromise between the effect of pH on the extinction coefficient of the chromophore and its effect on the activity of the acid phosphatase. Enzymatic

activity decreases sharply above pH 7.2, thereby more than offsetting the effect of increasing pH on the absorbance of p-nitrophenol. In this experiment, first the  $K_m$  and  $V_{max}$  of acid phosphatase for p-nitrophenylphosphate will be determined. Second, the nature of the inhibition of the acid phosphatase by inorganic phosphate (Pi) will be investigated. Finally, you will choose some aspect of the reaction (e.g., another inhibitor) to study independently.

### Part I. $K_m$ and $V_{max}$ Determinations- Week 1

Reaction mixtures for the enzyme (phosphatase) assays will contain 10 mM Tris-HCl (pH 7.2), 5 mM  $MgCl_2$ , and a PNPP (substrate) concentration of 0.25, 0.5, 0.75, 1.0, 2.5 or 5.0 mM. The PNPP stock solution (50 mM) should be kept on ice to slow non-enzymatic hydrolysis.

1. Each reaction mixture should be prepared immediately prior to use in a cuvette. **All assays should be run in duplicate.** If duplicate values are not relatively consistent, run a third trial.
2. Add the appropriate volume of each reagent, minus enzyme, as specified in Table I and in the order indicated. You may prepare solutions in bulk, but do not add the PNPP until just before running a trial. Once the appropriate amount of the substrate (PNPP) has been added, insert the cuvette into an Ocean Optics spectrophotometer and “calibrate” to zero the instrument. Remove the cuvette from the spectrophotometer, add the enzyme solution (phosphatase) and mix thoroughly but gently (do not vortex the solution; instead you may wish to cover the tube and quickly invert). Make sure the stir bar is stirring uniformly. Collect data for 1 minute at 410 nm as outlined in the Ocean Optics kinetics directions, which you should bring to lab with you.

**Table I**

Reagents*	Reaction Mixtures:					
	1	2	3	4	5	6
0.1 M Tris-HCl, pH 7.2; mL	0.30	0.30	0.30	0.30	0.30	0.30
0.1 M $MgCl_2$ ; mL	0.15	0.15	0.15	0.15	0.15	0.15
H <sub>2</sub> O; mL	2.19	2.34	2.43	2.445	2.46	2.475
50 mM PNPP; mL	0.30	0.15	0.06	0.045	0.030	0.015
<b>zero (“calibrate”) spectrophotometer for each trial before adding enzyme!</b>						
5 mg/mL phosphatase; mL	0.06	0.06	0.06	0.06	0.06	0.06

**\*Pipet reagents as carefully as possible**

3. For each trial, determine the initial velocity (the slope of the absorbance vs. time-in-minutes plot). At the lower substrate concentrations, the data points may begin to deviate from a straight line at the longer time intervals. If so, use only the data points in the initial time interval (where they form a straight line) to obtain the initial velocity.

## Part II. Inhibition of Acid Phosphatase by Inorganic Phosphate- Week 1

Product inhibition is encountered in acid and alkaline phosphatases from many different sources, including the enzyme from wheat germ. The inhibition of wheat germ acid phosphatase by inorganic phosphate ( $P_i$ ) is certainly the major reason for the deviation from linearity of the time course of the enzyme activity. In this experiment, the nature of the inhibition of wheat germ acid phosphatase will be examined. From the data obtained, you should be able to distinguish the type of inhibition and, furthermore, to calculate  $K_i$  for  $P_i$  by two different graphical methods.

Essentially, this experiment is a repetition of the  $K_m$  and  $V_{max}$  determinations (part I), except that a final concentration of 1 mM phosphate buffer (pH 7.2) is present at each substrate concentration (0.25, 0.5, 0.75, 1.0, 2.5 and 5.0 mM). When preparing each inhibited reaction mixtures, reduce the  $H_2O$  added by 0.06 mL and substitute 0.06 mL of 0.05 M potassium phosphate buffer, pH 7.2. Calculate the new volumes of  $H_2O$  to be added before coming to laboratory and include a modified form of Table I in your notebook for this part of the experiment. Again, all assays should be run in duplicate. If the duplicate assays do not agree, then a third trial should be run. Determinations of the initial velocities should be performed as described in the procedure for the uninhibited reaction.

### Analysis- to be completed prior to Week 2 for your pre-lab assignment

- From the data acquired in Part I, determine the  $K_m$  and  $V_{max}$  of the acid phosphatase for p-nitrophenylphosphate using both the graphical methods of Lineweaver-Burk and Eadie-Hofstee. One method may be superior, as judged by the  $R^2$  value, for your data.
- From the data acquired in Part II, determine what type of inhibition  $P_i$  displays and calculate  $K_i$  for  $P_i$ . These parameters can be best determined by plotting the data from this part of the experiment and the data from part I on the same Lineweaver-Burk and Eadie-Hofstee graphs.

## Part III. Effect of Reaction Conditions or Other Inhibitors on Acid Phosphatase Kinetics- Weeks 2 and 3

Search the primary literature for information regarding acid phosphates, particularly from wheat germ. Based on the information you have collected, choose a reaction condition (e.g.,  $Mg^{2+}$  concentration, other divalent metal ions, or addition of reducing agents) or a potential inhibitor other than inorganic phosphate to investigate. **You will discuss this choice with your instructor (bringing literature references) for approval during Week 2.** Prior to this meeting, plan out the procedural details for how you will investigate the effect of this reaction condition/inhibitor on the kinetic activity of wheat germ acid phosphatase. You may wish to model your experimental design on that used during Week 1, although ideas for improvement are encouraged.

After your plan meets the approval of your instructor, carry it out during Week 3 of laboratory. Don't forget to bring the Ocean Optics instructions to lab with you. Perform a similar analysis for your own experiment as for the Week 1 data. Do your findings make sense in the context of the literature? Explain.

**Part IV. Computer Modeling of Acid Phosphatase- Week 2**

Working around your meeting time with your instructor to discuss your experiment for next week, you will perform computer modeling of acid phosphatase in the biochemistry lab. Please refer to the separate handout for instructions to guide you through this exercise.

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