Kinetic studies with alkaline phosphatase in the presence and absence of inhibitors and divalent ations

ARTICLE *in* BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION · NOVEMBER 2002

Impact Factor: 0.65 · DOI: 10.1002/bmb.2002.494030060138

1 AUTHOR:

Rob L. Dean
The University of Western Ontario
28 PUBLICATIONS **391** CITATIONS

SEE PROFILE

BIOCHEMISTRY AND MOLECULAR BIOLOGY EDUCATION
Vol. 30, No. 6, pp. 401–407, 2002

Laboratory Exercises

Kinetic Studies with Alkaline Phosphatase in the Presence and Absence of Inhibitors and Divalent Cations

Received for publication, June 7, 2002, and in revised form, July 15, 2002

Rob L. Dean‡

From the Biology Department, University of Western Ontario, London, Ontario N6A 5B7, Canada

A very robust and inexpensive kinetic assay for determining rates of hydrolysis of *p*-nitrophenyl phosphate by the enzyme alkaline phosphatase is presented. The reaction increases in rate with increase in pH. The enzyme is competitively inhibited by the reaction products, uncompetitively inhibited by L-phenylalanine, and responds to the presence of two cofactors, magnesium and zinc ions. The reaction rate increases as Mg^{2+} concentration is increased from 1–5 mM. With increasing Zn^{2+} concentration, the reaction rate is stimulated and then depressed. Experimental work on the interaction between Mg^{2+} and Zn^{2+} in the reaction is suggested for more capable students.

Keywords: Alkaline phosphatase, enzyme kinetics, enzyme inhibition, divalent cation cofactors.

Experience has shown that enzyme assays used to teach introductory enzyme kinetics should ideally meet a number of criteria. (i) The assay should be reliable and show a prolonged linear rate of reaction to maximize the possibility that inexperienced students will obtain satisfactory data; (ii) given the funding situation at most institutions, the reagents should be inexpensive; and (iii) if preparation time is limited because multiple laboratory classes are offered throughout the week, reagents should be stable. For 2 years we have successfully used variants of one such kinetic assay as the basis for teaching enzyme kinetics in a 2nd-year undergraduate laboratory class. The assay presented in this article involves the hydrolysis of *p*-nitrophenyl phosphate by a commercially available preparation of the enzyme alkaline phosphatase.

A number of publications describe class experiments with acid phosphatase [1–4], which is easily prepared by aqueous extraction of wheat germ and which can be colorimetrically assayed. However, these enzymatic studies all involve a fixed-time assay where a single measurement of product concentration is made after stopping the reaction following a given period of time. Fixed-time assays can be time-consuming because a basic assumption in the Michaelis-Menten approach to enzyme kinetics is that the enzyme is substrate-saturated. Consequently, initial velocities must be determined from reaction data that are linear with respect to the decline in substrate concentration (or increase in product concentration) over time. In fixed-time assays considerable effort is required to establish the duration in which the reaction between enzyme and substrate is linear. Moreover, the validity of the linear relationship must be confirmed each time that the reaction mixture is changed to accommodate the study of, for example, enzyme inhibitors or cofactors. Conversely, if one uses a kinetic assay, in which the reaction is continuously monitored, each data set reveals how long the reaction is linear, and any deviations from linearity are readily apparent. It is not possible to run a kinetic assay with acid phosphatase using *p*-nitrophenyl phosphate as substrate because the product, *p*-nitrophenol, is colorless at acidic pH. I consequently suggest that if the actual enzyme to be used is not critical, alkaline phosphatase be used instead of acid phosphatase. Alkaline phosphatase is inexpensive, is very stable, and shows interesting responses to millimolar concentrations of magnesium and zinc ions both separately and together. For enzyme inhibition studies, alkaline phosphatase is competitively inhibited by both of its reaction products and is uncompetitively inhibited by the L-isomer of the amino acid phenylalanine.

The experimental work described here was developed for, and tested in, a 2nd-year undergraduate laboratory course designed primarily to provide students with laboratory experience. However, enzyme assays are so widely used that the work with alkaline phosphatase would be an appropriate component of a laboratory class associated with an introductory course in biochemistry or cell biology.

A BRIEF INTRODUCTION TO THE PHOSPHATASES

Phosphatases are enzymes that catalyze the hydrolysis of esters of phosphoric acid. They occur in the cells and extracellular fluids of a wide range of organisms. This large and complex group of enzymes falls into four general types based on the chemical nature of the substrate or the type of hydrolytic reaction that is catalyzed. One group, the phosphomonoesterases, hydrolyzes monoesters of phosphoric acid such as α-glycerophosphate or glucose 6-phosphate. Some phosphomonoesterases are highly substrate-specific. For example, in gluconeogenesis, fruc-

‡ To whom correspondence should be addressed: Biology Dept., Biology & Geology Bldg., University of Western Ontario, London, Ontario N6A 5B7, Canada. E-mail: rdean1@uwo.ca.

TABLE I
Typical reaction mixtures and the resulting substrate concentrations

Tube no.	1.35 mM *p*-nitrophenyl phosphate in reaction buffer	Reaction buffer	Resulting substrate concentration
	ml	*ml*	*mM*
1	2.0	3.0	0.54
2	1.0	4.0	0.27
3	0.6	4.4	0.162
4	0.4	4.6	0.108
5	0.3	4.7	0.081
6	0.2	4.8	0.054

tose-1,6-bisphosphatase specifically converts fructose 1,6-bisphosphate to fructose 6-phosphate and inorganic phosphate. Other phosphomonoesterases react with a broad range of substrates, which share common structural motifs. The phosphomonoesterases that lack substrate specificity are classified as acid or alkaline phosphatases based on their pH optima. Acid phosphatases function best at around pH 5.0 and are inhibited by fluoride ion but not by divalent cation-chelating agents. The alkaline phosphatases have pH optima of about 9.0 and are not generally sensitive to fluoride ion but are inhibited by divalent cation-chelating agents like EDTA (ethylene diamine tetraacetic acid, disodium salt).

EXPERIMENTAL PROCEDURES

Materials

Students prepare reaction mixtures and solutions for standard curves from the following previously prepared stock solutions.

Enzyme: bovine and calf intestinal alkaline phosphatase (phosphoric monoester phosphohydrolase (alkaline optimum), EC 3.1.3.1) (Sigma) diluted 1:10,000 with reaction buffer containing 1 mg/ml bovine serum albumin (Sigma).

Substrate: Sigma 104 phosphatase substrate (*p*-nitrophenyl phosphate, disodium, hexahydrate), 1.35 mM (0.5 mg/ml) in reaction buffer.

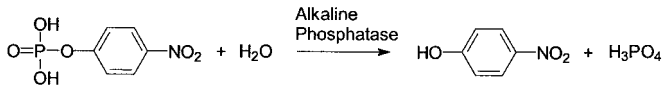
Product: *p*-nitrophenol (Sigma), 1 mM in reaction buffer.

Reaction buffer: Tris-HCl buffer (see figure legends for concentrations and pH) with or without 5 mM $MgCl_2{\cdot}6H_20$.

Enzyme inhibitors: 25 mM monobasic sodium phosphate ($NaH_2P0_4{\cdot}H_20$), 0.5 mM *p*-nitrophenol, 50 mM L-phenylalanine (Sigma). All were dissolved in reaction buffer at the concentrations and pH values given in the figure legends.

The Basic Assay

Alkaline phosphatase hydrolyzes the colorless, synthetic substrate *p*-nitrophenyl phosphate to produce a yellow-colored product, *p*-nitrophenol and inorganic phosphate:



REACTION 1

The initial velocity (v_o) of the reaction, in micromoles of product produced/minute, can be calculated from the increase in absorbance at 400 nm (ΔA_{400}) measured at timed intervals during the reaction.

Typical reaction mixtures to which the enzyme is added are shown in Table I. These mixtures provide a satisfactory concentration range for making a Lineweaver-Burk plot. In reactions involving the addition of inhibitors or cofactors dissolved in reaction buffer the volume of reaction buffer is reduced to give a total volume of 5 ml. The volume of enzyme preparation added to start the reaction is one that will generate a ΔA_{400} of ~0.3–0.4 in the tube with the highest substrate concentration during the time that the reaction is monitored.

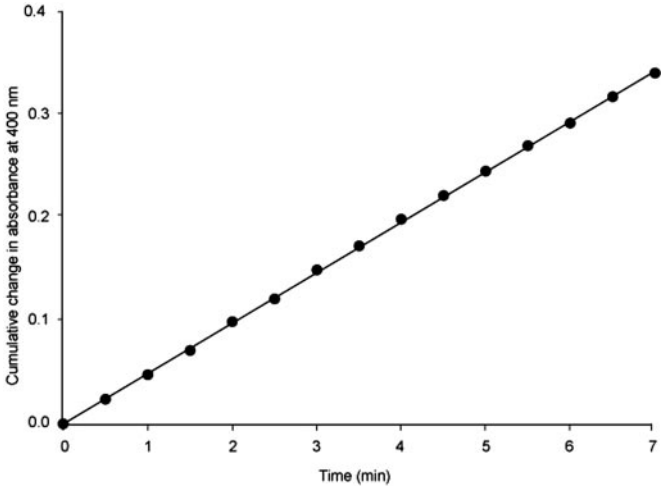


FIG. 1. **The relationship between the increase in absorbance at 400 nm (ΔA_{400}) and time in the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase.** Twenty microliters of the enzyme preparation were added to 7.0 ml of 1.08 mM substrate in 1.0 M Tris-HCl buffer (pH 8.0) containing 5 mM $MgCl_2{\cdot}6H_20$. The prolonged linear relationship in this reaction is very useful for teaching inexperienced students, who tend to take a long time before taking the initial A_{400} reading.

To analyze the rate data, a standard curve of A_{400} against *p*-nitrophenol concentration is needed. The linear relationship between absorbance and *p*-nitrophenol concentration holds up to a concentration of about 40 μM. If reaction buffers of different pH values are used, a separate standard curve must be made using reaction buffer at each pH. As with many colored compounds, the absorbance of *p*-nitrophenol is pH-sensitive.

Assay Protocol

Use the reaction mixture without enzyme to adjust the needle on the spectrophotometer meter to zero absorbance. Add a volume of enzyme preparation (typically 20 μl). Cover the tube with Parafilm, invert three times (do not shake) to mix the contents, and return the tube to the spectrophotometer. The absorbance value will be increasing. When the absorbance reaches a convenient value (*e.g.* 0.010 or 0.020), start timing. Record absorbance values at 30-s intervals for 3 min. (Assays can be run for longer periods if high concentrations of enzyme inhibitors are present).

Data Analysis

a. Graph the change in absorbance (ΔA_{400}) against time to ensure that the reaction is linear over the time that the reaction is monitored. An important assumption in the derivation of the Michaelis-Menten equation is that the concentration of the enzyme-substrate complex ([ES]) instantly reaches a steady state and remains unchanged during initial velocity measurements. In this assay, this presents no problem as the linear relationship between the increase in absorbance and time holds for at least 7 min (Fig. 1), twice the duration of the assay. This prolonged linear phase is particularly useful when measuring reaction rates in the presence of high enzyme inhibitor concentrations.
b. Determine the ΔA_{400}/minute for the initial linear part of the reaction.
c. The ΔA_{400}/minute corresponds to an increase in the con-

centration of the product, *p*-nitrophenol, per minute (Δc/minute) and an equal decrease in the concentration of the substrate. The corresponding Δc/minute can be determined by: (i) interpolation of the standard curve or (ii) using the absorption coefficient (ϵ) for *p*-nitrophenol derived from the slope of the standard curve. If the *x* axis of the standard curve is expressed in moles/liter then:

$$\epsilon = \frac{A}{c} \qquad \text{(Eq. 1)}$$

where A = absorbance, c = concentration in moles/liter, and ϵ = liters/mole/centimeter. Therefore

$$c = \frac{A}{\epsilon} \qquad \text{(Eq. 2)}$$

and

$$\Delta c/\text{minute} = \frac{\Delta A_{400}/\text{minute}}{\epsilon} \text{ moles/liter minute} \qquad \text{(Eq. 3)}$$

d. To find the number of moles of *p*-nitrophenol produced per minute, correct for the total volume of the reaction mixture; *e.g.* if 20 μl of enzyme preparation are added to 5 ml of buffered substrate (total volume = 5.02 ml) then

$$\text{moles/minute} = \Delta c/\text{minute} \times \frac{5.02 \text{ ml}}{1000 \text{ ml/liter}} \qquad \text{(Eq. 4)}$$

Convert this value to micromoles/minute = initial velocity (v_o).

e. V_{max}, the maximum initial velocity (or limiting velocity), and K_m, the Michaelis constant, which is defined as the substrate concentration that produces an initial velocity equal to one-half of V_{max}, can be determined from a double reciprocal (Lineweaver-Burk) plot. The reciprocal of the substrate concentration (1/[S]) on the *x* axis is plotted against the reciprocal of v_o on the *y* axis. A straight line is fitted through the points and extrapolated through the *y* axis and the negative side of the *x* axis. The intercept on the *y* axis = $1/V_{max}$, and the intercept of the *x* axis = $-1/K_m$. (The Lineweaver-Burk plot has been justifiably criticized as a means for determining K_m and V_{max} [5, 6]. However, it is a useful starting point for students being introduced to enzyme kinetics.)

RESULTS AND DISCUSSION

Stability of the Enzyme and Buffered Substrate Preparation

To demonstrate that enzymes only catalyze reactions that proceed slowly in their absence it is instructive to leave a tube of buffered substrate in the lab between classes. This shows that the substrate is slowly converted to product at room temperature in the absence of enzyme. A 5.0-ml solution of 0.54 mM *p*-nitrophenyl phosphate in buffer at pH 9.0 increased in A_{400} by 0.013 each day over a 3-day period. This is ~15,000 times slower than the rate of conversion of substrate to product in the presence of 20 μl of our enzyme preparation.

At lower temperatures, both the buffered substrate solution and the diluted enzyme preparation are remarkably stable. To check for long term stability, substrate solution and enzyme preparation were stored at −8 °C for 3 months. At the end of this time, the rate of hydrolysis of substrate by the enzyme was still 70% of that using freshly prepared solutions.

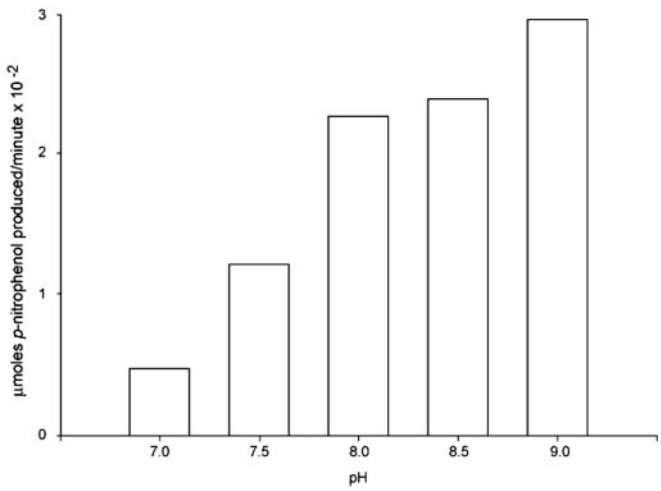


FIG. 2. **The initial velocity of the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase increases with increasing pH in the range from pH 7.0–9.0.** Twenty microliters of enzyme preparation were added to 0.77 mM substrate in 7.0 ml of 1.0 M Tris-HCl buffer containing 5 mM $MgCl_2 \cdot 6H_20$ at pH 7.0, 7.5, 8.0, 8.5, or 9.0.

Effect of pH on the Reaction

As its name indicates, alkaline phosphatase is highly pH-sensitive. In the effective buffering range for Tris-HCl, the initial velocity of the hydrolysis of substrate by alkaline phosphatase increases more than 6-fold from pH 7.0 to pH 9.0 (Fig. 2). Furthermore, the values K_m and V_{max} are clearly affected by pH (Fig. 3). For further information on the effect of pH on enzyme-catalyzed reactions see Refs. 7 and 8 for reviews.

Competitive Inhibition

Inorganic phosphate (P_i) and *p*-nitrophenol, the products of the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase, both competitively inhibit the enzyme. A Lineweaver-Burk plot for the reactions, in 1 M Tris buffer (pH 8.0) containing 5 mM Mg^{2+} in the presence and absence of 30 μM *p*-nitrophenol, shows that the V_{max} remains the same but that the K_m is increased from 0.1 to 0.125 mM in the presence of the inhibitor (Fig. 4). Similarly, a Lineweaver-Burk plot for the reactions, in 0.5 M Tris buffer (pH 9.0) containing 5 mM Mg^{2+} in the presence and absence of 0.1 mM monobasic sodium phosphate (P_i), shows that the V_{max} is the same and that the K_m is increased from 0.2 to 0.5 mM (Fig. 5).

Lineweaver-Burk plots that show the same V_{max} but increased K_m in the presence of the inhibitor are characteristic of competitive inhibition. In competitive inhibition, the inhibitory molecule is thought to bind reversibly at the substrate-binding site on the enzyme consequently preventing the substrate from binding. Since both P_i and *p*-nitrophenol appear to bind at the active site on the enzyme we might reasonably deduce that the substrate-binding site on the enzyme recognizes both the phosphate group and some other part of the *p*-nitrophenyl phosphate molecule when forming the enzyme-substrate complex.

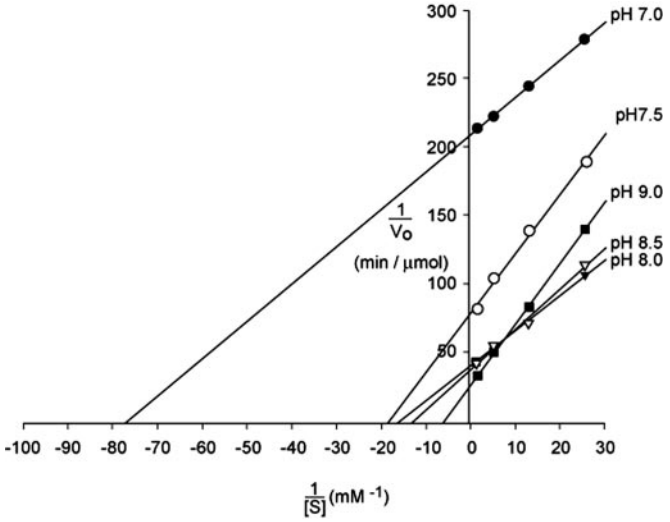


FIG. 3. **Lineweaver-Burk plots for the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase at pH 7.0, 7.5, 8.0, 8.5, and 9.0.** Twenty microliters of enzyme preparation were added to 0.77, 0.193, 0.077, and 0.039 mM substrate in 5.0 ml of 1.0 M Tris-HCl buffer (at pH 7.0, 7.5, 8.0, 8.5, or 9.0) containing 5.0 mM $MgCl_2 \cdot 6H_20$.

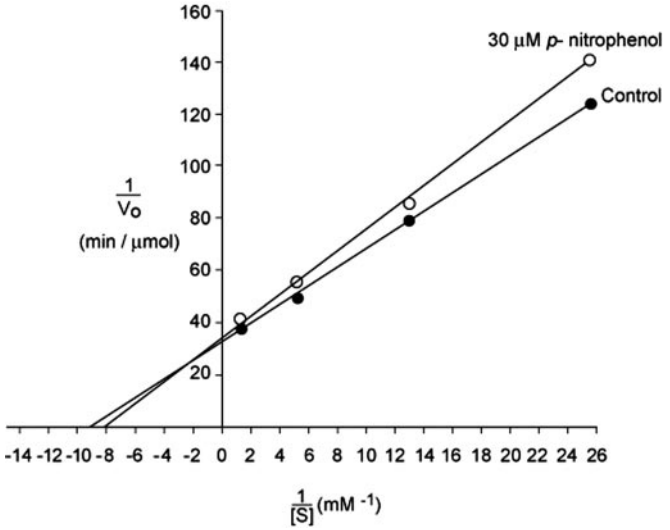


FIG. 4. ***p*-Nitrophenol competitively inhibits the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase.** Twenty microliters of enzyme preparation were added to 0.77, 0.193, 0.077, and 0.039 mM substrate in 7.0 ml of 1.0 M Tris-HCl buffer at pH 8.0 in the presence or absence of 30 µM *p*-nitrophenol.

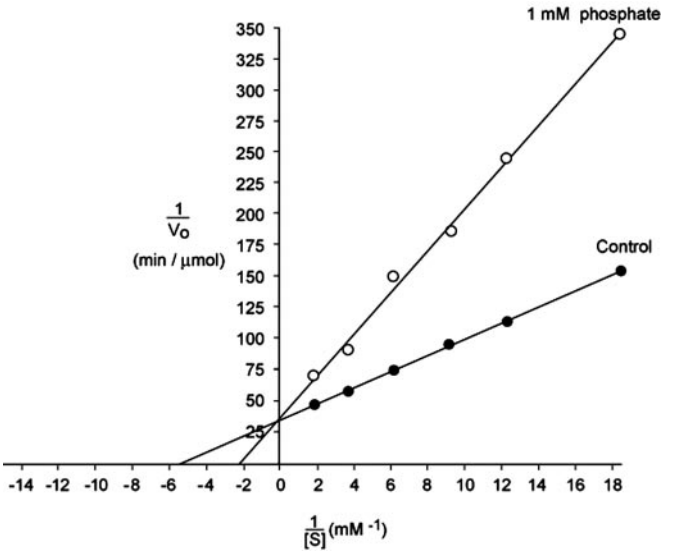


FIG. 5. **Inorganic phosphate competitively inhibits the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase.** Twenty microliters of enzyme preparation were added to 0.54, 0.27, 0.162, 0.108, 0.081, and 0.054 mM substrate in 5.0 ml of 0.5 M Tris-HCl buffer (pH 9.0) containing 5 mM $MgCl_2 \cdot 6H_20$ in the presence or absence of 1 mM monobasic sodium phosphate.

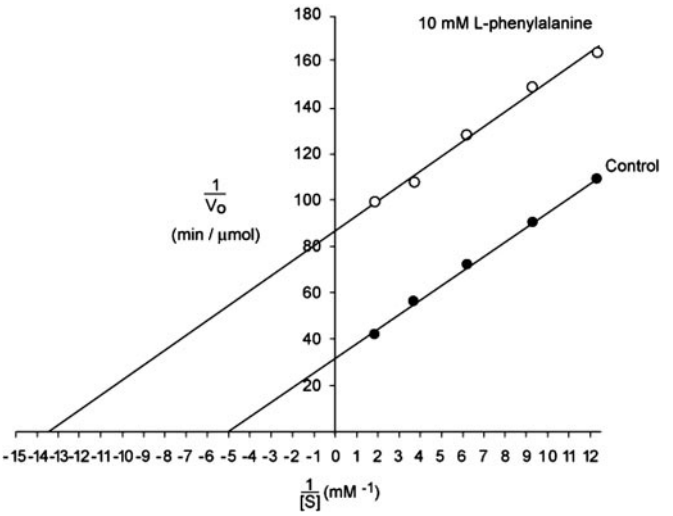


FIG. 6. **L-Phenylalanine uncompetitively inhibits the hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase.** Twenty microliters of enzyme preparation were added to 0.54, 0.27, 0.162, 0.108, and 0.081 mM substrate in 5.0 ml of 0.5 M Tris-HCl buffer (pH 9.0) containing 5 mM $MgCl_2 \cdot 6H_20$ in the presence or absence of 10 mM L-phenylalanine.

Uncompetitive Inhibition

Examples of uncompetitive inhibition in enzyme reactions that involve a single substrate are rare. In uncompetitive inhibition, the inhibitory molecule is thought to bind to the enzyme-substrate complex but not to the free enzyme. The inhibited ES complex is non-functional so the V_{max} decreases when the inhibitor is present. When the inhibitor is present, the K_m value also declines because the reduced number of uninhibited ES complexes require a lower substrate concentration to half-saturate the enzyme. Consequently, in a Lineweaver-Burk plot, the inhibited slope is parallel to, and shifted to the left of, the uninhibited slope. The L-isomer of the amino acid phenylalanine at 5 mM is an uncompetitive inhibitor of alkaline phosphatase (Fig. 6). Interestingly, the D-isomer of phenylalanine is not an inhibitor [9]. This fact should permit an investigation of steric specificity in the uncompetitive inhibition of alkaline phosphatase by phenylalanine.

Magnesium and Zinc Ions Are Cofactors of Alkaline Phosphatase

Some enzymes are catalytically active without a requirement for additional ions or molecules. In others, activity depends on the presence of a non-protein cofactor. Cofactors fall into two major groups; they are either metal ions or organic compounds. A familiar example of the latter

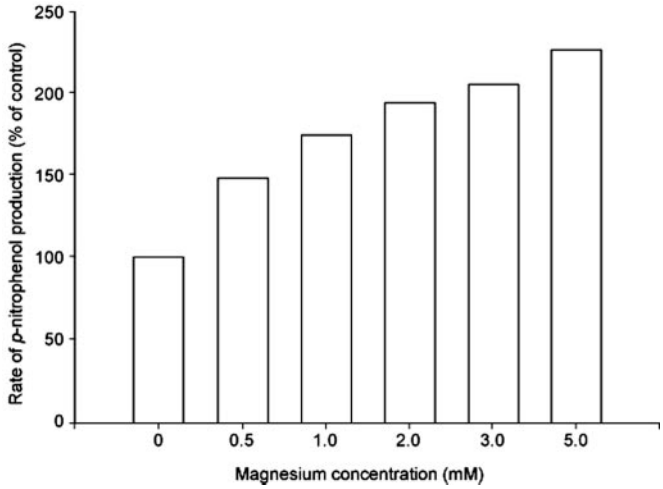


FIG. 7. **Magnesium ion enhances the rate of hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase in a concentration-dependent manner.** Twenty microliters of enzyme preparation were added to 0.54 mM substrate in 5.0 ml of 0.5 M Tris-HCl buffer (pH 8.0) containing 0, 0.5, 1.0 2.0, 3.0, and 5.0 mM $MgCl_2 \cdot 6H_20$.

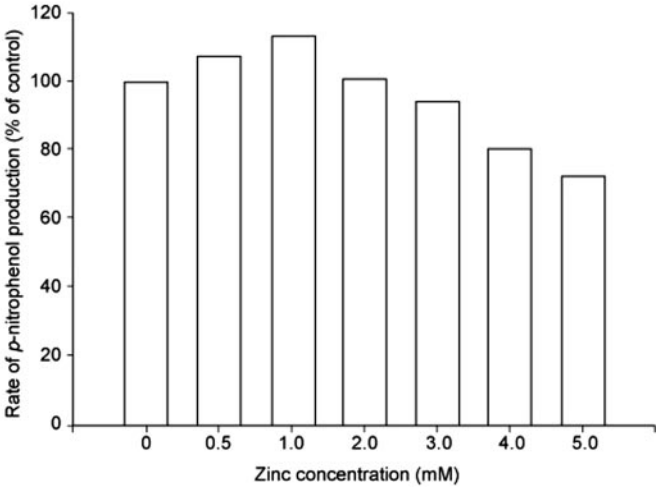


FIG. 8. **At low concentrations, zinc ion enhances the rate of hydrolysis of *p*-nitrophenyl phosphate by alkaline phosphatase; at higher concentrations, zinc ion inhibits the reaction.** Twenty microliters of enzyme preparation were added to 0.54 mM substrate in 5.0 ml of 0.5 M Tris-HCl buffer (pH 9.0) containing 0, 0.5, 1.0, 2.0, 3.0, 4.0, and 5.0 mM zinc chloride.

group is flavin-adenine dinucleotide (FAD). Intestinal alkaline phosphatase uses Mg^{2+} and Zn^{2+} ions as cofactors. Alkaline phosphatase is a glycoprotein with a molecular weight of ~140,000. It is a dimer of two very similar or identical subunits. Each subunit (~69,000 molecular weight) contains a tightly bound zinc atom that contributes to the structural integrity of the polypeptide and a less tightly bound zinc atom that is required for catalysis. Each subunit also contains a different binding site for Mg^{2+} ions that stimulate enzymatic activity [10, 11]. If magnesium is omitted from the reaction mixture, both K_m and V_{max} are reduced. Typical values for K_m and V_{max} are ~0.25 and ~0.1 mM and ~0.03 and ~0.01 μmol of product/min, respectively, in the presence and absence of 5 mM magnesium chloride. The reaction rate is clearly enhanced in the presence of additional Mg^{2+} ion. However, it should not be assumed that no Mg^{2+} or Zn^{2+} ions are present when the reaction mixture is not supplemented with these ions. The Sigma alkaline phosphatase preparation contains 1 mM Mg^{2+} and 0.1 mM Zn^{2+} so that diluted enzyme preparations would be expected to carry some bound Mg^{2+} and Zn^{2+}.

In the descriptions of the effects of adding Mg^{2+} and Zn^{2+} ions to the reaction mixtures that follow it should be emphasized that the concentrations of these ions are nominal. It is difficult to make small volumes of dilute solutions accurately, and both magnesium chloride and zinc chloride are extremely hygroscopic. Even when stored in a vacuum desiccator, the stock chemicals absorb water. As a consequence, we are unable to make solutions of known concentration by dissolving a given weight of reagent in a known volume of solvent. As a result, there can be considerable variation in the magnitude of the response at the reported ion concentrations. Fortunately, the trend of the response to increasing ion concentration is consistent.

A dramatic example of the effect of magnesium concentration is shown in Fig. 7. The initial velocity of the reaction more than doubles as the Mg^{2+} concentration is increased up to the nominal value of 5 mM. In some other cases, the effect of Mg^{2+} concentration has been less pronounced, probably because of the difficulty of producing stock solutions of precisely known concentration.

The effects of different concentrations of zinc are more complex. Low concentrations (~0.5–1.0 mM) produce a slight stimulation in the initial velocity, whereas concentrations above 3.0 mM are inhibitory (Fig. 8). The explanation for this response involves a competitive interaction between magnesium and zinc ions. There is a binding site for magnesium on each enzyme subunit, and when Mg^{2+} is bound the activity of the enzyme increases. Each subunit also contains a separate zinc-binding site that is saturated at low concentrations. Zinc binding also enhances the catalytic activity of the enzyme. However, at higher zinc concentrations, zinc ions bind to the magnesium-binding site with a higher affinity. This displacement of Mg^{2+} by Zn^{2+} results in the loss of the magnesium-enhanced activity [10]. A sample experiment using different Mg^{2+} and Zn^{2+} concentrations, both separately and together, illustrates this phenomenon. Fig. 9 shows that 1 mM Mg^{2+} approximately doubles the initial velocity of the reaction, which is also somewhat enhanced by 1 and 2 mM Zn^{2+}. At 3 mM, zinc ions become inhibitory, reducing activity to 93.9% of the control value. The inhibitory effect of 3 mM Zn^{2+} is reduced to 97.4% of the control value in the presence of 1 mM Mg^{2+}.

Do in Vitro Studies on Enzyme Kinetics Inform Us about How Enzymes Function in Vivo?

Like most instructors, I try to find relevance in the lab work that I offer to students. However, we should be skeptical about any conclusions drawn about the behavior of alkaline phosphatase, in the intracellular or extracellular environment, from the type of studies described in this article. Many of the reaction components and conditions are not found in nature. As examples, (*a*) the substrate, *p*-nitrophenyl phosphate, is synthetic, (*b*) the buffer contains Tris, which is also synthetic, (*c*) Tris stimulates the

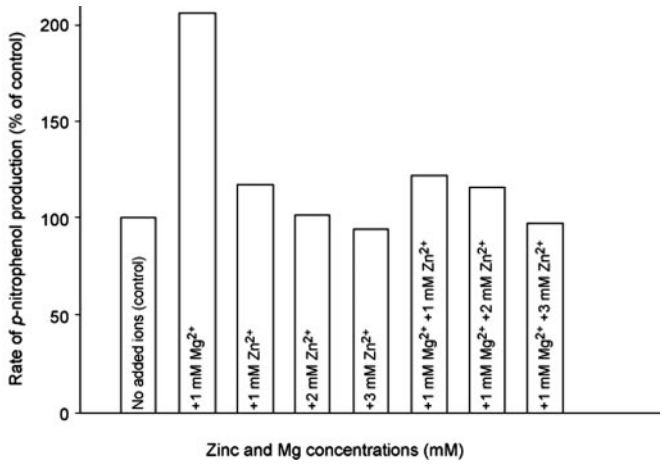


FIG. 9. **The rate of hydrolysis of *p*-nitrophenyl phosphate is increased by Mg^{2+}, increased by low Zn^{2+} concentration, and inhibited by higher Zn^{2+} concentration.** The inhibitory effect of Zn^{2+} can be reversed in the presence of Mg^{2+}. Twenty microliters of enzyme preparation were added to 0.54 mM substrate in 5.0 ml of 0.5 M Tris-HCl buffer (pH 9.0) containing zinc chloride and/or magnesium chloride at the stated concentrations.

activity of alkaline phosphatase in a concentration-dependent manner [12], (*d*) the molar and ionic concentrations of the reaction mixtures are unphysiological, (*e*) the pH used may or may not reflect that occurring *in vivo*, and (*f*) the temperature is ~12 °C too low for a mammalian enzyme.

Moreover, the uncritically repeated explanations for kinetic phenomena "written by people more interested in molecular biology than in kinetics who think that nothing has been learned. . . . since 1934" [8] are frequently oversimplified or unsupported. Examples include: (*a*) the suggestion that the K_m value gives an indication of the degree of affinity of the enzyme for its substrate (it may or may not), (*b*) the perfectly logical suggestion that the K_m value gives an indication of the substrate concentration that the enzyme would encounter *in vivo* (largely unsupported), and (*c*) the suggestion that changes to K_m and V_{max} in the presence of inhibitors can tell us about the *mechanism* of inhibition. It has been argued [8] that terms like competitive and uncompetitive inhibition should be "operational" in that they only describe the observations. (These authors avoid any discussion of noncompetitive inhibition!)

I do not intend to suggest that enzyme kinetic studies have no value in undergraduate education. As long as we do not overinterpret the results and emphasize that kinetic studies "*in conjunction with other techniques*" [7] can provide useful insights into the mechanisms of enzyme action, studies of this type are a valuable introduction to the design and execution of laboratory experiments.

CONCLUSION

The kinetic assays using alkaline phosphatase described in this article have been performed successfully by a large number of 2nd-year undergraduates over a 2-year period. The assay is completely reliable and shows a prolonged linear reaction rate with no lag phase. The latter property is particularly useful for inexperienced students, who tend to procrastinate before taking the first absorbance reading and also permits reactions to be run for longer periods as necessary in the presence of high concentration of enzyme inhibitors. The reagents are inexpensive; a 30-μl sample of the enzyme as purchased from Sigma is more than enough for over 600 students working in groups of three to four students to run assays for 13 weeks in a year. Similarly, 5 g of substrate exceeds the requirements for the same numbers. The great stability of the reagents allows us to make up batches large enough for 2 or more weeks of classes and store them at −8 °C in appropriately sized aliquots. Moreover, for the reasons outlined in the Introduction, kinetic assays are superior to fixed-time assays because the rate of reaction can be continuously monitored. These assays can also be performed very rapidly. As an example, Fig. 6 contains data points from only 10 reactions. Ten such reactions and the data analysis fit comfortably into the usual duration of a laboratory class.

Before working on kinetic assays our students spend three laboratory classes gaining experience in spectrophotometry and, equally importantly, pipetting technique. After this, the majority of students can manage most of the experimental work described.

An exception is the work involving different concentrations of zinc ions where differences between controls and treated values are often too subtle to be detected by less experienced hands. The work with zinc is consequently better suited to upper level undergraduates. However, if more able students can be identified in advance, they can be offered the opportunity to investigate zinc and magnesium ions as cofactors, while the remainder of the class works with only magnesium.

Finally, student comprehension of the preceding work is greatly enhanced if they are assigned readings on enzyme kinetics, enzyme inhibition, and cofactors from one of the many excellent biochemistry laboratory books [13–15] or standard course texts [16–20] before attending class.

Acknowledgments—I am grateful to Fawzi Razem, who prepared the figures, and to Professor Mark Bernards for helpful comments on a draft of the manuscript.

REFERENCES

[1] P. Abramoff, R. G. Thomson (1996) An experimental Approach to Biology, 2nd ed., W. H. Freeman, San Francisco.
[2] G. Litvak (1960) Experimental Biochemistry. A Laboratory Manual, Wiley, New York.
[3] R. P. Kuniewicz, in K. P. Heston, B. Y. Stewart, Eds. (1991) PEW Undergraduate Laboratories in Biology. A Collection of Introductory Experiments in Cellular and Molecular Biology, Biology Curriculum Development Group, PEW Science Program in Undergraduate Education, Mid-Atlantic Cluster, pp. 1–11.
[4] J. M. Clark, R. L. Switzer (1977) Experimental Biochemistry, 2nd ed., W. H. Freeman, San Francisco.
[5] F. Ranaldi, P. Vanni, E. Giachetti (1999) What students must know about the determination of enzyme kinetic parameters, *Biochem. Educ.* **27,** 87–91.
[6] C. G. Whiteley (2000) Enzyme kinetics: partial and complete uncompetitive inhibition, *Biochem. Educ.* **28,** 144–147.
[7] N. C. Price, L. Stevens (1989) Fundamentals of Enzymology, 2nd ed., Oxford University Press, Oxford.
[8] A. Cornish-Bowden, C. W. Wharton (1988) Enzyme Kinetics, IRL Press, Oxford.
[9] N. K. Ghosh, W. H. Fishman (1966) On the mechanism of inhibition of intestinal alkaline phosphatase by L-phenylalanine, *J. Biol. Chem.* **241,** 2516–2522.
[10] J.-P. Bretaudiere, T. Spillman, in H. V. Bergmeyer, Ed. (1984) Methods of Enzymatic Analysis, Vol. 4, Verlag Chemie, Weinheim, Germany, pp. 75–92.
[11] M. Fosset, D. Chappelet-Tordo, M. Lazdunski (1974) Intestinal alka-

line phosphatase. Physical properties and quaternary structure, *Biochemistry* **13,** 1783–1788.
[12] R. L. Dean (2002) It's laboratory class time, do you known what your buffer is doing?, *Am. Biol. Teach.* **64,** 540–547.
[13] R. F. Boyer (2000) Modern Experimental Biochemistry, 3rd ed., Benjamin/Cummings, San Francisco.
[14] A. J. Ninfa, B. P. Ballou (1998) Fundamental Laboratory Approaches for Biochemistry and Biotechnology, Fitzgerald, Bethesda, MD.
[15] R. L. L. Switzer, L. F. Garrity (1999) Experimental Biochemistry, 3rd ed., Freeman, New York.
[16] J. M. Berg, J. L. Tymoczko, L. Stryer (2002) Biochemistry, 5th ed., Freeman, New York.
[17] R. F. Boyer (2002) Concepts in Biochemistry with the interactive Concepts in Biochemistry CD-ROM, 2nd ed., John Wiley, New York.
[18] D. Voet, J. G. Voet, Pratt, C. W. (2001) Fundamentals of Biochemistry, Upgrade Edition, Wiley, New York.
[19] C. K. Mathews, K. E. Van Holde, K. G. Ahern (2000) Biochemistry, 3rd ed., Benjamin Cummings, Redwood City, CA.
[20] A. L. Lehninger, D. L. Nelson, M. M. Cox (2000) Principles of Biochemistry, 3rd ed., Freeman, San Francisco.