ENZYME KINETICS: THEORY

A. Introduction

Enzymes are protein molecules composed of amino acids and are manufactured by the living cell. These molecules provide energy for the organism by catalyzing various biochemical reactions. If enzymes were not present in cells, most of the chemical reactions would not proceed at measurable rates at the temperatures of living systems.

Each enzyme has at least a single active site which is the location where the enzyme binds to the substrate. In this way the substrate is held rigidly in the most favorable orientation. Within the active site there are various chemical groups that are involved in the reaction. It is important to remember that enzymatic reactions usually result in the addition or removal of some molecule or radical such as H_2O, -OH, -H, $-NH_2$, etc.

Each enzyme possesses a pH and a temperature optimum for its activity. This optimum pH and temperature can be easily determined in the laboratory by carrying out the reaction in buffers over a wide range of pH or conducting tests at different temperatures.

Enzymes demonstrate a rather high degree of specificity with respect to their substrates. The degree of specificity varies from enzyme to enzyme: some enzymes carry out a reaction in only one direction (e.g., dehydrogenation) but some will catalyze a reaction in both forward and reverse directions although usually at greatly different rates (e.g., hydrogenation in addition to dehydrogenation). Some enzymes will accept only one or two specific substrate molecules; others accept whole classes or subclasses of molecules as substrates. This specificity is the basis for enzyme nomenclature: according to the kind of reaction performed (e.g., hydrogenase) or, even more specifically, according to the substrate acted upon (e.g., succinic dehydrogenase).

The simplest possible case of an enzyme (E)-catalyzed reaction involves a single substrate (S) molecule giving rise to one product (P):

$$E + S \text{ ---> } P + E \qquad (1)$$

and we find that the amount of P formed increases with time until a plateau is reached as shown in Figure 4.

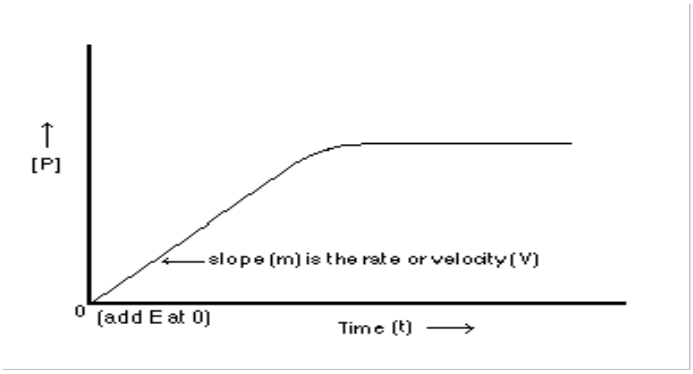

Figure 4. Product concentration as a function of time for an enzyme catalyzed reaction. (Square brackets denote molar concentration.)

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We are usually concerned with the initial rate (or initial velocity) value (V_0) which is the slope measured very near t=0; it is an important value for characterizing an enzymatic reaction. It is observed that the velocity depends on the concentration of E as depicted in Figure 5.

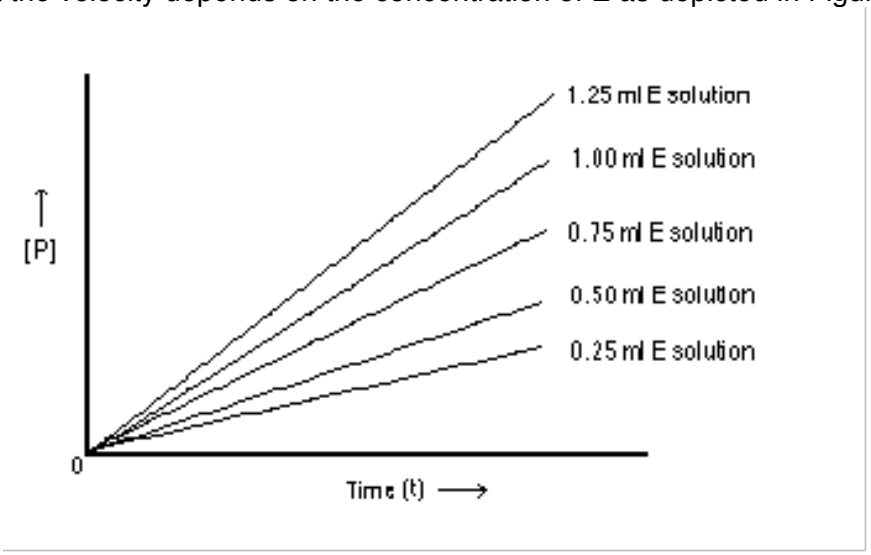

Figure 5. Product concentration as a function of time and enzyme concentration.

A more critical demonstration of the effect of enzyme concentration upon the reaction in question is given by varying [E] and holding time constant. This produces a curve similar to that of Figure 4 but with [E] replacing t at the abscissa.

Now we may ask what happens when [E] is held constant and [S] is varied. Here we will obtain a curve as in Figure 6. In this graph, the rate of product formation is called the velocity of the reaction. Notice how the velocity (O.D. measured by a spectrophotometer) reaches a maximum as substrate concentration reaches a saturation level.

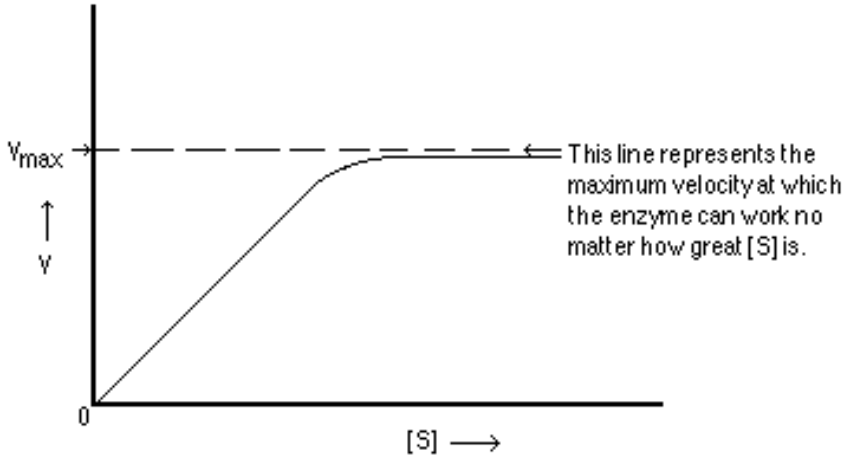

Figure 6. Velocity of an enzymatic reaction as a function of substrate concentration.

Doubling [E], under certain conditions, doubles V_{max}, but the reaction rate always reaches a plateau at high [S]. Actually, E and S interact to form a complex, from which P emerges:

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$$E + S \underset{k_2}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_3} E + P \qquad (2)$$

This formulation is for a simple enzymatic reaction where k_1, k_2, and k_3 denote the kinetic constants for individual reaction components indicated by the arrows. The possible back reaction from E + P ---> ES is assumed to be negligible and so no k_4 is required for accurate description of the equilibrium. Enzyme reactions often require the participation of some small molecules such as ATP, NAD, Mg^{++} or other cofactors.

Concerning equation (2), we can say that:

1. The rate of forming ES is k_1 $[E_f]$ [S], where
   E_f (free enzyme) = ([E] - [ES]), that is total E minus bound E.
   Thus, the rate of forming ES = k_1 ([E] - [ES]) [S].

2. The rate of losing ES is the sum of 2 pathways:
   - k_2 [ES] (the rate of going back to E + S)
   - k_3 [ES] (the rate of going forward to E + P).

   Thus, the rate of losing ES = (k_2+k_3) [ES].

3. Looking at the two above parts, we see that the reaction in equation (2) when the forward and backward rates around ES are in equilibrium, we have:

$$k_1 ([E] - [ES]) [S] = (k_2+k_3) [ES]$$

which can be written as:

$$\frac{k_2 + k_3}{k_1} = \frac{([E]-[ES])[S]}{[ES]} = \left\{\frac{[E]}{[ES]} - 1\right\}[S] \qquad (3)$$

The German biochemist, Leonor Michaelis stated that the ratio of (k_2 + k_3)/k_1, denoted by K_m, is a characteristic quantity for a given enzyme on a given substrate under fixed conditions of pH, temperature, etc. Thus:

$$K_m = \frac{k_2 + k_3}{k_1} \qquad (4)$$

K_m is called the Michaelis constant and is the measure of the efficiency of the enzyme. When $k_2 > k_1$, P formation slows down and K_m is large. In contrast, when $k_1 > k_2$, P formation is accelerated and K_m is a smaller value. This means that *the more efficient an enzyme is, the lower its K_m value.*

Michaelis also found that for the mechanism indicated in equation (2), the rate of production of P, or velocity (V) of the reaction can be written as:

$$V = k_3 [ES] \qquad (5)$$

and

$$V_{max} = k_3 [ES]_{max}$$

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But for a given [E], the fastest rate of the reaction would be when all the E is complexed. This occurs when [S] is much much greater than [E]; i.e., every enzyme molecule easily finds a substrate molecule, so that:

$$[ES]_{max} = [E]$$

and $$V_{max} = k_3 [E] \quad (6)$$

Then, by dividing (5) by (6) we get:

$$\frac{V}{V_{max}} = \frac{[ES]}{[E]} \quad (7)$$

B. Lineweaver-Burk equation

We note from equation (3) that:

$$K_m = \left\{ \frac{[E]}{[ES]} - 1 \right\} [S]$$

Therefore,

$$\frac{K_m}{[S]} = \frac{[E]}{[ES]} - 1$$

Or

$$\frac{[E]}{[ES]} = \frac{K_m}{[S]} + 1 \quad (8)$$

If we take the reciprocal of equation (7) we obtain:

$$\frac{[E]}{[ES]} = \frac{V_{max}}{V} \quad (9)$$

Note that the left sides of equations (8) and (9) are equal. Thus:

$$\frac{V_{max}}{V} = \frac{K_m}{[S]} + 1$$

If we divide the above equation by V_{max}, we get:

$$\frac{1}{V} = \frac{K_m}{V_{max}} \frac{1}{[S]} + \frac{1}{V_{max}} \quad (10)$$

which is called the Lineweaver-Burk equation and is represented graphically in Figure 7. In this graph, when x = 0, y = $1/V_{max}$ and when y = 0, x = $-1/K_m$. This plot is created using the same data from which Figure 6 was constructed, by simply taking the reciprocals of all V (O.D. values) and [S] values. The solid line is drawn through the experimental points and the dashed line

represents extrapolation to determine the x and y intercepts. K_m and V_{max} are readily calculated from the values of these intercepts.

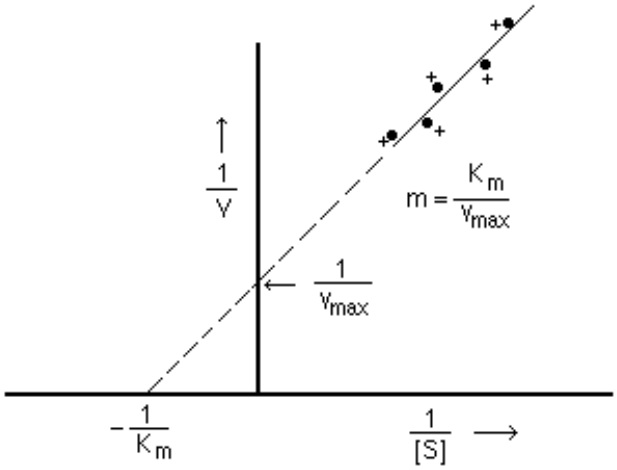
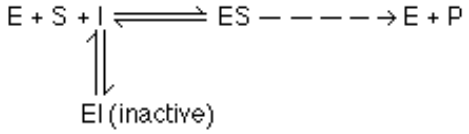


Figure 7. Experimental data graphed on a Lineweaver-Burk plot.

C. Enzyme inhibitors

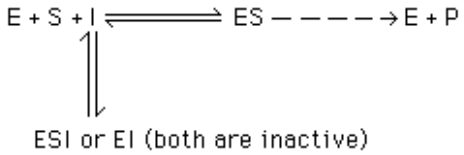
One of the important facets of enzyme kinetics is the phenomenon of enzyme inhibition. Substances can be found which when added to a reaction mixture result in lower velocities; i.e., the reaction is inhibited. Two common mechanisms are competitive inhibition and noncompetitive inhibition.

If the inhibitor (I) is a substance whose shape permits it to bind at the substrate binding site of E, then competition occurs between S and I leading to mutually exclusive states.



The inhibitor combines with free enzyme. It cannot combine with the enzyme when the substrate is already attached to E, hence the term competitive inhibition. Since the interaction between E and S is being interfered with, one might expect the K_m for the inhibited reaction to be different (larger). You remember that the more efficient the enzyme, the lower would be the K_m. In the presence of an inhibitor, the enzyme works slower, so K_m should be higher (larger). But since EI may dissociate, excess S can overcome the inhibition by effectively excluding I and so one may also expect that the V_{max} of the uninhibited reaction may ultimately be attained.

However, if the inhibitor attaches to E at some site other than the active site, and by this binding causes a shape change in E that renders E catalytically inactive or less active, then we have noncompetitive inhibition:



The interaction of E and I does not interfere with the S binding to E, so that ESI may be formed in addition to EI. But ES is sensitive to the presence of I, rendering ESI catalytically inactive or less active. Since the interaction between E and S is not being interfered with, any E not bound by I carries out its normal reaction at its normal rate so that one does not expect K_m to be altered. But, since there is no way to exclude the binding of I, the inhibitor actually lowers the effective enzyme concentration and this will, of course, alter (lower) V_{max}.

D. Enzyme assays

Implicit in all of the preceding discussions has been the idea that we can somehow isolate enzymes at will for study. In practice this is not always so easy. Biochemists obtain enzymes and measure their activities by various methods. The enzyme to be investigated is first extracted from some living material. It may then be assayed in the crude extract or after various degrees of purification, using standard methods for fractionating proteins. The processes of extraction and purification may vary from fairly simple to quite difficult, depending upon the particular enzyme and its purity.

An enzyme assay consists of mixing the enzyme with a substrate in a solution of controlled pH with any additional substance whose effect is to be tested, incubating the reaction mixture at a suitable temperature for a suitable time, stopping the reaction precisely, and then somehow measuring the amount of reaction that has occurred. In general, the amount of reaction that has taken place may be quantified in one of two ways: in terms of the disappearance of substrate or the appearance of product, depending upon which is chemically the more advantageous.

The actual method of measurement depends on some biochemical or biophysical property of the molecule being assayed. For example, titration of acid or base or changes in absorption or rotation of light in the reaction mixture as some molecule decreases or increases in concentration, measurement of evolved gas (a product), or transfer of a radioactive isotope from substrate to product can all be used. Frequently, some reagent is used which combines with a product of the enzyme reaction to produce a color. The intensity of the color can then be measured spectrophotometrically and compared with color produced by known amounts of product to provide a basis for expressing units of enzyme activity.

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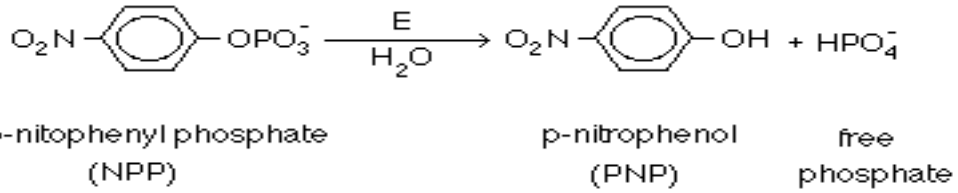
The preceding discussion is presented as a theoretical introduction underlying the study of any enzyme. In the exercises that follow, we will take one enzyme in particular (namely acid phosphatase) and apply the principles that we have discussed so far on it.

There are two general classes of phosphatases found variously distributed in plant and animal tissues. These enzymes hydrolyze a wide variety of phosphoric monoesters. One class, termed acid phosphatase (the one we will be using in our exercise), has a pH optimum in the neighborhood of pH 5 and is inhibited by fluoride but not by metal chelating agents. The other, termed alkaline phosphatase, has a pH optimum around pH 9 and is generally not inhibited by fluoride but is inhibited by metal chelating agents.

Wheat germ (the wheat embryo that is removed from the wheat grain before milling so that the flour will keep better) contains a phosphatase that can be extracted readily in water. The enzyme is not as specific as some other enzymes and will act on a wide range of possible substrates. We will use p-nitrophenyl phosphate (NPP) as substrate because it allows us a simple assay procedure: wheat germ phosphatase acting on NPP produces p-nitrophenol (PNP) as the product and PNP is yellow at alkaline pH. By adding Na_2CO_3 to our reaction mixtures, the pH can be raised enough to stop the reaction (an important requirement in enzyme studies) and to make the product colored for spectrophotometric quantification. The reaction can be written as:



E. Construction and use of a standard graph

You remember that if we measure the optical density (O.D.) of a solution at all the possible wavelengths and plot the data of wavelengths (λ) vs. O.D., we will obtain a bell-shaped curve with a single peak. The wavelength at which the peak occurs, called lambda-max (λ_{max}) is the wavelength at which the molecules have the highest absorption of light. Every solution has its own characteristic λ_{max}. For example, the λ_{max} of PNP is 420 nm.

According to Beer's Law, if we prepare a solution of a substance at a known concentration, dilute it to several additional known concentrations, measure the absorbance of each of these samples at the λ_{max} of the substance and plot optical density as a function of concentration, we should obtain a straight line that passes through the origin. This line is called the standard graph of the substance. Consequently, if someone handed us a solution of the substance of unknown concentration, we could quickly determine its concentration by measuring its absorbance. This could be done for as many unknowns as we wish.

Reading such data from a standard graph may be done by simple inspection aided by a ruler and pencil. But a more precise method would be to establish an equation of two ratios with one unknown using an unambiguous point from the standard graph.

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