Unit - 5

Enzyme kinetics

Importance of enzyme kinetics

- Kinetics is the study of reaction rates.
- Study of enzyme kinetics is useful for measuring *concentration* of an enzyme in a mixture (by its catalytic activity), its *purity* (specific activity), measurement of the *catalytic efficiency* and/or the *specificity* of an enzyme, *comparison* of different forms of the same enzyme in different tissues or organisms, effects of *inhibitors* (which can give information about catalytic mechanism, structure of active site, potential therapeutic agents...)
- Dependence of velocity on [substrate] is described for many enzymes by the Michaelis-Menten equation.
- kinetic parameters:
    - K_m (the Michaelis constant)
    - k_{cat} (the turnover number, which relates V_{max}, the maximum velocity, to $[E_t]$, the total active site concentration)
    - k_{cat}/K_m (the catalytic efficiency of the enzyme)
- Kinetic parameters can be determined graphically by measuring velocity of enzyme-catalyzed reaction at different concentrations of substrate.

Enzyme Kinetics

- **REVIEW: How do enzymes reduce the activation energy ($DG^{‡}$)?**
  1) by **lowering the free energy of the transition state (‡), e.g., by *binding* the transition state tightly**
  2) by **changing the reaction pathway by which reactants react to form products**
    - e.g., taking a 1-step uncatalyzed reaction and accomplishing the same result by a different route, with several intermediate reactions en route.
    - ***Each reaction step has its own transition state with its own activation energy ($DG^{‡}$).***
    - If all of the individual steps' $DG^{‡}$s are lower than the activation energy of the uncatalyzed reaction, the overall rate of product formation will be greater in the presence of the catalyst.
    - The overall rate of the catalyzed reaction is dictated by the ***slowest step*** in a multistep reaction.
        - Given a free energy diagram like the one in *Lehninger Principles* Fig. 8-3 (previous lecture), **how do you identify the rate-limiting (slowest) step on the reaction coordinate?** (Remember to note the ***corrected*** versions of Figures 8-3 and 8-6 in the previous set of lecture notes; the textbook should have the activation energy $\mathbf{DG^{‡}}$ for each step ***starting from the "trough" at the beginning of that step on the free energy diagram***, not starting from the beginning of the reaction coordinate.)

- **The essence of enzyme action is BINDING**, **binding of the SUBSTRATE to form an ES COMPLEX**, and **binding of the TRANSITION STATE even more tightly than the substrate.**

- The binding occurs at the **ACTIVE SITE** of the enzyme, where the subsequent chemical events can then occur.
- **Active site:**
  - a relatively small part of the whole enzyme structure
  - 3-dimensional cleft with participating components from different parts of the primary structure
  - water often excluded so substrates and intermediates are in a non-aqueous environment (unless H_2O is a reactant)
  - Binding is through multiple weak interactions -- hydrogen bonds, salt links, hydrophobic "interactions", van der Waals interactions.

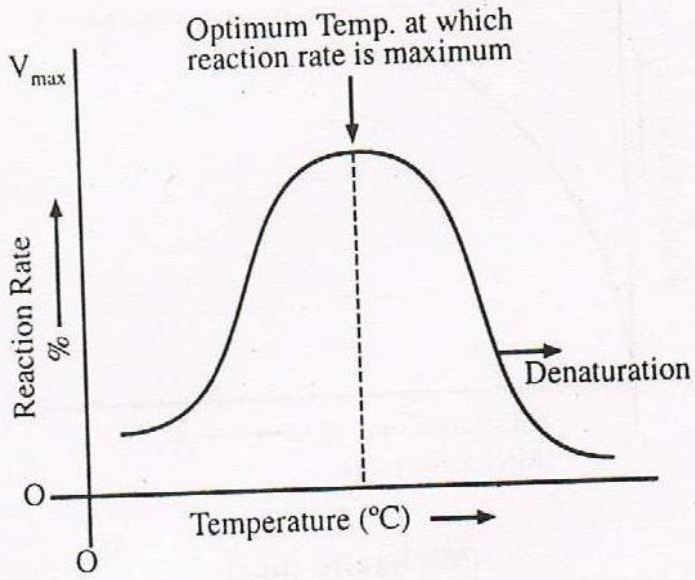
(We'll come back to binding and reduction of $DG^{\ddagger}$ when we discuss enzyme ***mechanisms***, how (chemically) enzymes catalyze reactions.)

- **Why study enzyme kinetics (reaction rates)?**
  - **Compare** enzymes under different conditions, or from different tissues or organisms (understand how differences relate to physiology/function of organism -- e.g., differences in the kinetic properties of the enzyme *hexokinase* that catalyzes phosphorylation of glucose to form glucose-6-phosphate to prepare the sugar for degradation through the glycolytic pathway in all tissues, vs. the enzyme *glucokinase*, that catalyzes the same reaction but is found primarily in liver and involved in maintenance of blood glucose levels)
  - Compare activity of same enzyme with different substrates (understand **specificity**)
  - **Measure** amount or concentration of **one enzyme in a mixture** by its **activity** (measurement of **VELOCITY, i.e., reaction rate**)
  - Measure enzyme **purity** (**specific activity**, amount of activity/amount of protein)
  - Study/distinguish different types of **INHIBITORS**
    - info about **enzyme active sites** & **reaction mechanism**
    - development of **specific DRUGS (enzyme inhibitors)**

- **Factors Affecting Enzyme Activity**

Knowledge of basic enzyme kinetic theory is important in enzyme analysis in order both to understand the basic enzymatic mechanism and to select a method for enzyme analysis. The conditions selected to measure the activity of an enzyme would not be the same as those selected to measure the concentration of its substrate. Several factors affect the rate at which enzymatic reactions proceed - temperature, pH, enzyme concentration, substrate concentration, and the presence of any inhibitors or activators.

(1) Effect of Temperature :



Graph

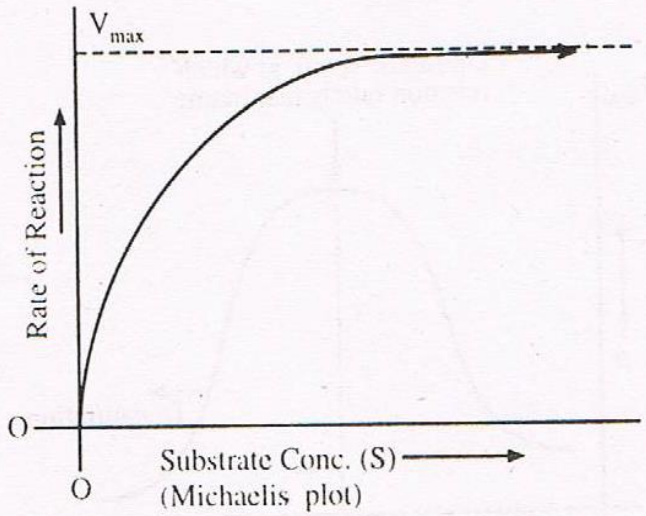
The reaction velocity is increased as the temperature is increased. The rise in temperature accelerates an enzyme catalysed reactions, but at the same time causes inactivation of the enzymes due to denaturation, since the enzymes are proteinous in nature. The activity is maximum at certain temperature known as '**optimum temperature**

(37° - 45° C). At higher temperature, product formation declines due to conformational changes in the enzyme by thermal denaturation which reduces the effective enzyme. At high temperature, the enzymes inactivate and the reaction which it catalyzes slows down and ultimately stops. If the temperature is lowered the rate of enzyme reaction is diminished but does not destroy the enzyme. It's activity is reversible if heated to optimum temperature. Thus 'optimum temperature may be defined as, 'the temperature at which enzymatic activity is maximum.

(2) Effect of Substrate Concentration :

The rate of enzyme activity is influenced by the concentration of substrate. With the fixed amount of enzyme, the reaction rate is proportional to the substrate concentration, but upto a certain concentration, after which the increase in concentration of substrate does not further increase the velocity of the reaction. As the substrate concentration increases, more and more active sites of enzyme molecule will be used for formation of enzyme-substrate (ES) complex. The rate of reaction will be more.

If the concentration of substrate is increased further there will be more active sites for binding and the reaction is lowered.



3. Effects of pH

Enzymes are affected by changes in pH. The most favorable pH value - the point where the enzyme is most active - is known as the optimum pH. This is graphically illustrated in Figure.

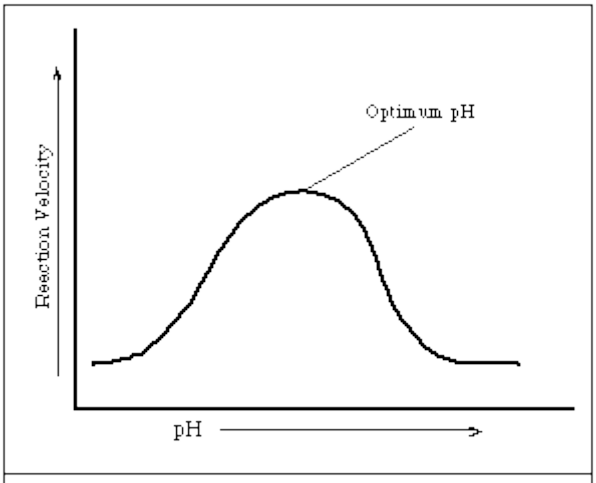


FIGURE 14
Effect of pH on reaction rate

Extremely high or low pH values generally result in complete loss of activity for most enzymes. pH is also a factor in the stability of enzymes. As with activity, for each enzyme there is also a region of pH optimal stability.

4. Effect of Enzyme Concentration

In order to study the effect of increasing the enzyme concentration upon the reaction rate, the substrate must be present in an excess amount; i.e., the reaction must be independent of the substrate concentration. Any change in the amount of product formed over a specified period of time will be dependent upon the level of enzyme present. Graphically this can be represented as:

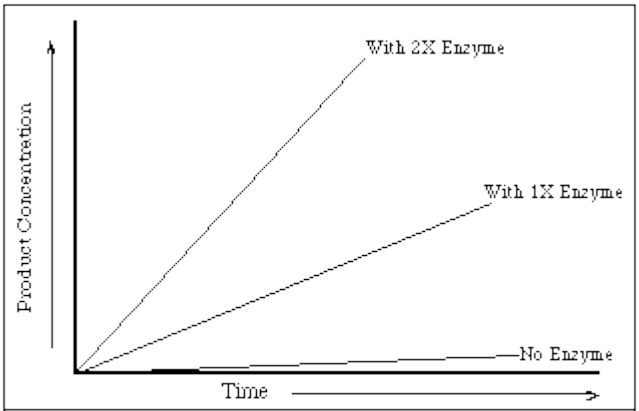


FIGURE 4
"Zero order" reaction rate is independent of substrate concentration.

These reactions are said to be "zero order" because the rates are independent of substrate concentration, and are equal to some constant k. The formation of product proceeds at a rate which is linear with time. The addition of more substrate does not serve to increase the rate. In zero order kinetics, allowing the assay to run for double time results in double the amount of product.

5. Effect of metal ions

Most of the enzymes only become active in the presence of certain ions e.g. cations such as Mg, Ca, Zn, Na,or K. In some cases cations may get loosly bound to enzymes, while in other they get bound to substrate. Anions have also been found to increase thye enzyme activity. E.g. Cl enhances the activity of salivary amylase.

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- # Enzyme kinetics

In 1913, a general theory of enzyme action and kinetics was developed by L. Michaelis and M.L. Menten, which was later extended by J.E. Briggs and J.B.S. Haldane. This theory is developed for the reaction in which there is only one substrate.

Michaelis-Menten equation

This theory assumes that the enzyme **'E'** first combines with substarte **'S'** to form the enzyme-substrate complex **'ES'.** The ES then breaks down in a second step to form free enzyme and the product **'P'.**

$$E + S \underset{K_{-1}}{\overset{K_{+1}}{\rightleftharpoons}} ES \quad \text{---------} \quad (1)$$

$$ES \underset{K_{-2}}{\overset{K_{+2}}{\rightleftharpoons}} E + P \quad \text{--------} \quad (2)$$

These reactions are assumed to be reversible. The rate constants for the forward and backward directions respectively have the positive and negative subscripts.

Michaelis-Menten equation expresses the mathematical relationship between the initial rate of enzyme-catalysed reactions and the concentration of the substrate and certain characteristics of the enzyme. This equation is the rate equation for reactions catalysed by enzymes having single substrate. In this derivation—

[E] ⟶ Concentration of free enzyme

[ES] ⟶ Concentration of enzyme bound to substrate

[E_T] ⟶ Total enzyme concentration (E + ES)

[S] ⟶ Substrate concentration, which is assumed to be far greater than [E].

The amount of S bound to E at any given time is negligible compared to with total concentration of S.

Now initial velocity V_0 is equal to the rate of breakdown of the enzyme substrate complex ES. According to equation (2) we can write the first order rate equation—

$$V_0 = K_{+2} [ES] \quad \text{----------} \quad (3)$$

However, we cannot determine K_{+2} **&** [ES] directly. Therefore we have to find out alternate expression for V_0 in terms of other variable terms, which can be measured more easily.

For that, we first write a second order rate equation for the formation of ES from E & S.

$$\frac{d[ES]}{dt} = K_{+1} [E] [S]$$

$$\frac{d[ES]}{dt} = K_{+1} ([E_T] - [ES]) [S] \quad \text{----------} \quad (4)$$

K_{+1} is the second order rate constant.

Although ES can also be formed from E and P, the rate of this reaction is neglected as we are considering the beginning of the reaction, when P is zero or close to zero.

Now, we may write rate equation for the breakdown of ES by the sum of two reactions; first the reaction yielding the product (forward direction) and second the reaction yielding E + S.

We then have ---

$$-\frac{d[ES]}{dt} = K_{-1} [ES] + K_{+2} [ES] \quad \text{----------} \quad (5)$$

At steady state (concentration of ES remains constant) the rate formation of ES is equal to rate of breakdown.

$$K_{+1}\,([E_T] - [ES])\,[S] = K_{-1}\,[ES] + K_{+2}\,[ES] \quad \text{--------- (6)}$$

Rearranging equation (6), we get

$$\frac{([E_T] - [ES])\,[S]}{[ES]} = \frac{K_{-1} + K_{+2}}{K_{+1}} = K_M \quad \text{---------- (7)}$$

K_M is called Michaelis Menten constant. From equation (7) the steady state concentration of the ES complex can be obtained by solving for [ES].

$$\frac{[E_T]\,[S]}{[ES]} - \frac{[ES]\,[S]}{[ES]} = K_M$$

$$\frac{[E_T]\,[S]}{[ES]} - [S] = K_M$$

$$\frac{[E_T]\,[S]}{[ES]} = K_M + [S]$$

$$[ES] = \frac{[E_T]\,[S]}{K_M + [S]} \quad \text{----------} \quad (8)$$

We can substitute the value of ES in equation (3)

$$V_0 = K_{+2} \frac{[E_T]\,[S]}{K_M + [S]} \quad \text{---------- (9)}$$

When the substrate concentration is so high that the entire enzyme is present as ES complex i.e. when enzyme is saturated, we reach the maximum initial velocity V_{max}.

$$V_{max} = K_{+2}\,[E_T] \quad \text{---------- (10)}$$

Now substituting for $K_{+2}\,[E_T]$ its value from equation (10), we obtain

$$V_0 = \frac{V_{max}\,[S]}{K_M + [S]} \quad \text{---------- (11)}$$

This is the Michaelis Menten equation, the rate equation for one substrate enzyme catalysed reaction.

When initial velocity $V_0 = \frac{1}{2} V_{max}$

$$\frac{V_{max}}{2} = \frac{V_{max}\,[S]}{K_M + [S]}$$

By dividing V_{max} we get

$$\frac{1}{2} = \frac{[S]}{K_M + [S]}$$

On rearranging it becomes

$$K_M + [S] = 2[S]$$

$$K_M = [S]$$

Thus Michaelis Menten constant K_M is equal to the substrate concentration at which the initial velocity is half maximum.

Significance of K_m

i) The K_m value is very useful in evaluating affinity of enzyme for substrate.
ii) The K_m value also gives an idea regarding the type of inhibition of an enzyme caused by inhibitor.
iii) It is used in medical research.
iv) This equation is used for experimental determination of rate of substrate concentration.
v) It is used to understand enzyme regulatory mechanism.
vi) It is useful in quantitating enzyme activity and purification.

- # Enzyme inhibition

From the study of enzyme inhibitors, valuable information can be obtained on the mechanism and pathway of enzyme catalysis, the substrate specificity of the enzymes, and the nature of functional groups at the active site and participation of certain functional groups in maintaining active confirmation of the enzyme molecule. Moreover, inhibition of certain enzymes by specific metabolites is an important element in the regulation of intermediatory metabolism. The enzyme inhibition can be reversible or irreversible.

A) Reversible enzyme inhibition

There are 3 major types of reversible enzyme inhibitions—

I) Competitive
II) Uncompetitive
III) Non-competitive

These 3 types can be experimentally distinguished by the effects of inhibitor on the reaction kinetics of the enzyme, which may be analysed in terms of basic Michaelis-Menten equation. For valid kinetic analysis, the inhibitor must combine rapidly and reversibly with the enzyme.

I) Competitive inhibition

The competitive inhibition is that the inhibitor can combine with the free enzyme in such a way that it competes with the normal substrate for the binding at the active site. A competitive inhibitor reacts reversibly with the enzyme to form an enzyme-inhibitor complex, analogous to the enzyme-substrate complex.

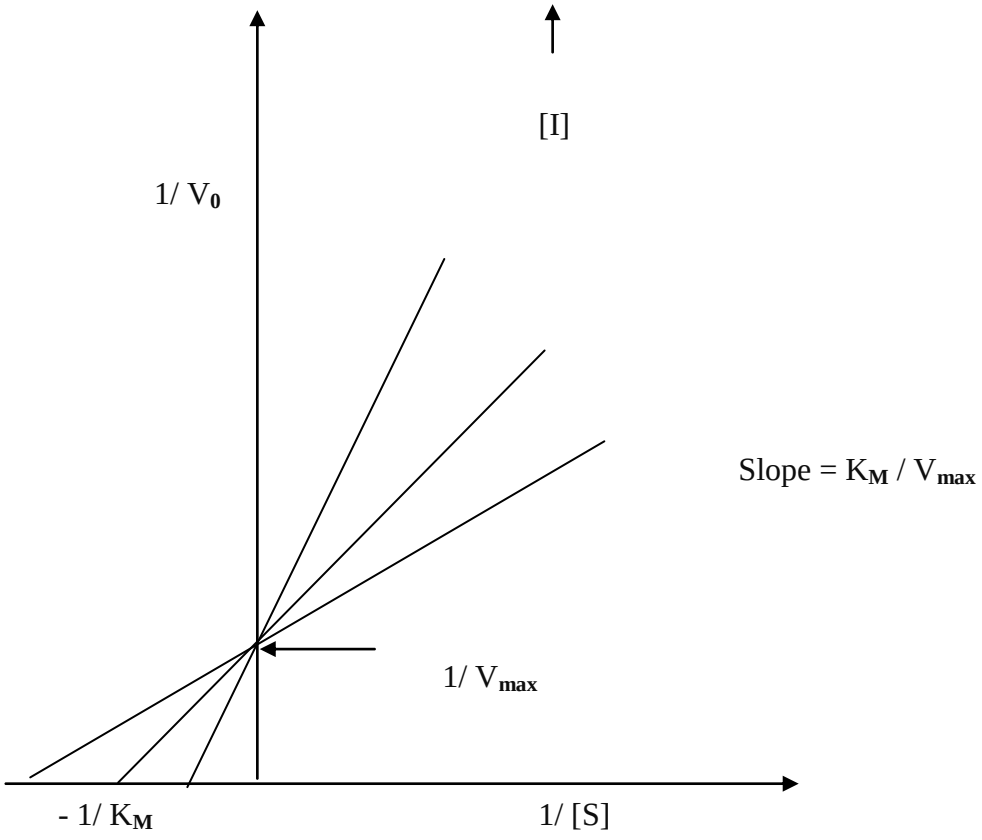
$$E + I \rightleftharpoons EI$$

The inhibitor molecule is not chemically changed by the enzyme. Following the Michaelis-Menten equation, we can define the inhibitor constant KI as the dissociation constant of enzyme-inhibitor complex.

$$KI = \frac{[E]\,[I]}{[EI]}$$

Competitive inhibition is easily recognized experimentally because the percent inhibition at the fixed inhibitor concentration is decreased by increasing the substrate concentration. For quantitative kinetic analysis, the effect of varying substrate concentration [S] on the initial velocity [V_0] is determined at a fixed concentration of inhibitor. This experiment is then repeated with a different concentration of inhibitor; often several series of such experiments are carried out, each at a different concentration of inhibitor. Plots of 1/ V_o versus 1/ [S] are then prepared, one for each concentration of inhibitor. These plots characteristically give a family of straight lines intersecting at a common intercept on 1/ V_0 axis. The presence of competitive inhibitor thus increases the apparent K_M of the enzyme for substrate that is causes it to require a higher substrate concentration to achieve its maximum velocity.

The competitive inhibitor does not affect V_{max} indicating that it does not interfere with the rate of the breakdown of [ES] complex.



The classic example of competitive inhibition is the inhibition of **succinate dehydrogenase** by **malonate and other dicarboxylate anions.**

Some competitive inhibitors of succinate dehydrogenase are malonate, oxaloactate, and pyrophosphates. Note that all contain 2 anionic groups whose spacing resembles that of succinate.

II) Uncompetitive inhibition

In uncompetitive inhibition, which is not very aptly named, the inhibitor does not combine with the free enzyme or affects its reaction with its normal substrate. However, it

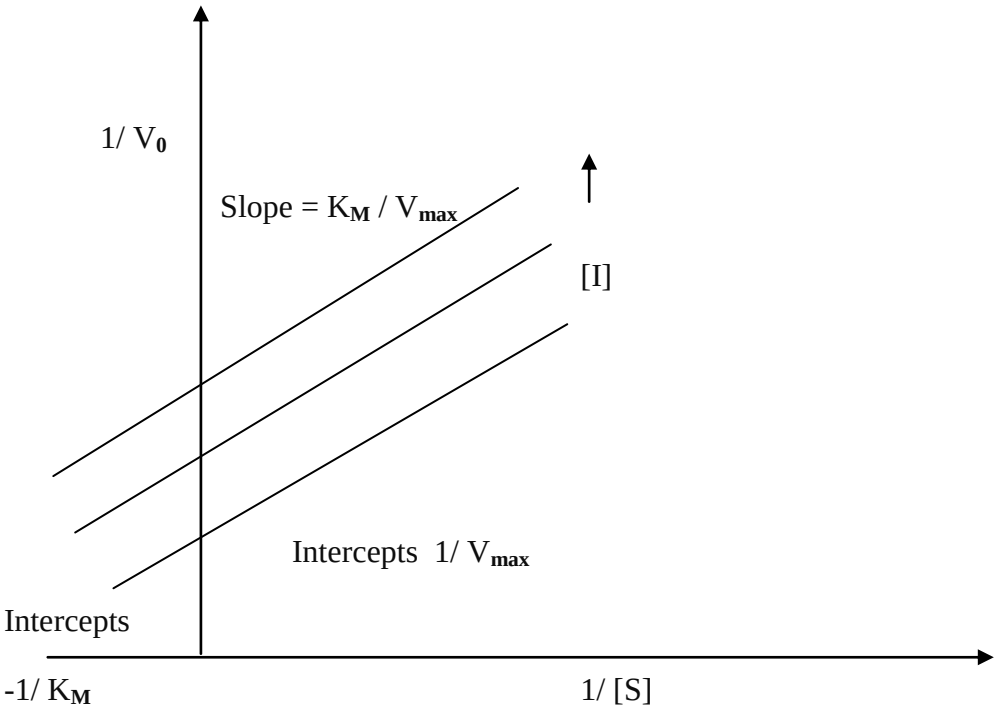
combines with enzyme-substrate complex to give an inactive enzyme-substrate-inhibitor complex, which cannot undergo further reaction to yield the normal product.

$$ES + I \rightleftharpoons ESI$$

The inhibitor constant is thus

$$KI = \frac{[ES]\,[I]}{[ESI]}$$

These relationships show that the degree of inhibition may increase when the substrate concentration is increased. Uncompetitive inhibition is most easily recognised from plots of $1/V_o$ versus $1/[S]$ at fixed inhibitor concentrations.

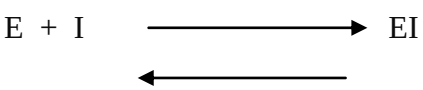


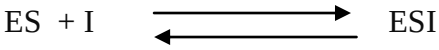
As the plot shows, it is typical uncompetitive inhibition that the slope of the plots remains constant at increasing concentrations of inhibitor, but V_{max} decreases. Uncompetitive inhibition is rare in substrate reactions but common in two substrate reactions.

III) Non-competitive inhibition

A non-competitive inhibitor can combine with either free enzyme or enzyme-substrate complex, interfering with action of both. Non-competitive inhibitors bind to a site of the enzyme other than the active site, often to deform the enzyme, so that it does not form the ES complex at its normal rate and once formed, the ES complex does not decompose at the normal rate to yield the products. These effects are not reversed by increasing the substrate

concentration. In non-competitive inhibition, the reaction with inhibitor yields two inactive forms EI and ESI.





The inhibitor constants are

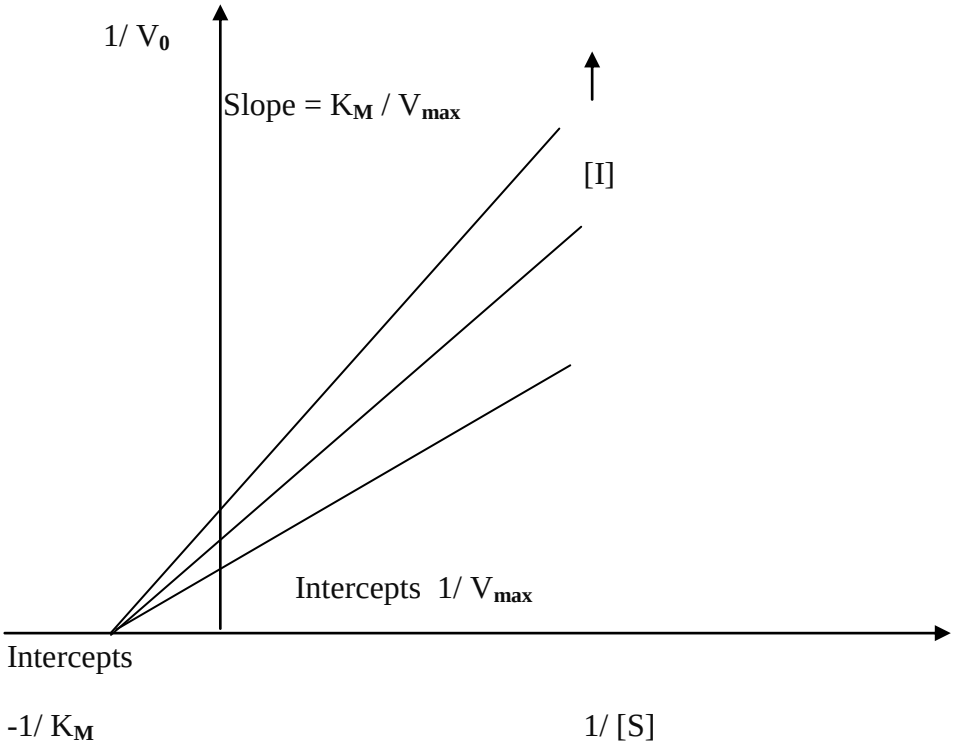
$$K_I^{EI} = \frac{[E]\,[I]}{[EI]}$$

$$K_I^{ESI} = \frac{[ES]\,[I]}{[ESI]}$$

Which may or may not be equal. Non-competitive inhibition is also most easily recognized from $1/V_o$ versus $1/[S]$ in the presence of different fixed concentrations of inhibitor. The plots differ in the slope but do not share a common intercept on $1/V_o$ axis. The intercept on $1/V_o$ axis is greater for the inhibited than the uninhibited enzyme, indicating that V_{max} is decreased by the inhibitor and cannot be restored regardless of how high the substrate concentration may be.

The most common type of non-competitive inhibition is given by reagents that can combine with reversibly with some functional groups of the enzymes (outside the active site) that are essential for maintaining the catalytically active 3 dimensional conformation of the enzyme molecule. Some enzymes possessing an essential –SH groups are non-competitively inhibited by heavy metal ions suggesting that such –SH groups must be essential for enzyme to retain its normal active conformation.

Some enzymes that require metal ions for activity are inhibited non-competitively by agents capable of binding the essential metal. E.g. The chelating agents Ethylenediamine tetraacetate (EDTA) reversibly binds Mg^{++} and other divalent cations and thus non-competitively inhibits some enzymes requiring such ions for activity.



B) Irreversible enzyme inhibition (Enzyme modification)

Some enzymes undergo irreversible inactivation when they are treated with agents capable of covalently and permanently modifying a functional group required for catalysis, making the enzyme molecule inactive. This type of inhibition cannot be treated by Michelis-Menten principles, which assumes reversible formation of EI or ESI complexes. Such an irreversible inhibition sets in slowly compared with normal reaction kinetics of the enzyme, so that the inhibition is incomplete at first but continuously increases with time because chemical modification of an increasing fraction of the enzyme molecule takes place.

C) FEED - BACK INHIBITION: -

This refers to the inhibition of the activity of an enzyme in a biosynthetic pathway. It is a control mechanism operating in living cells.

The product (P) acts to inhibit some early step in the pathway, but not necessarily the first. Substance 'B' can be frequently converted to more than one product; the intermediate products are C and D. By feedback control it is possible not only to inhibit the production of 'P', but also to divert the flow of B from one pathway to another.

- **Lineweaver- Burke's plot and equation**

Michaelis-Menten equation can be transformed into other forms that are more useful in plotting experimental data.

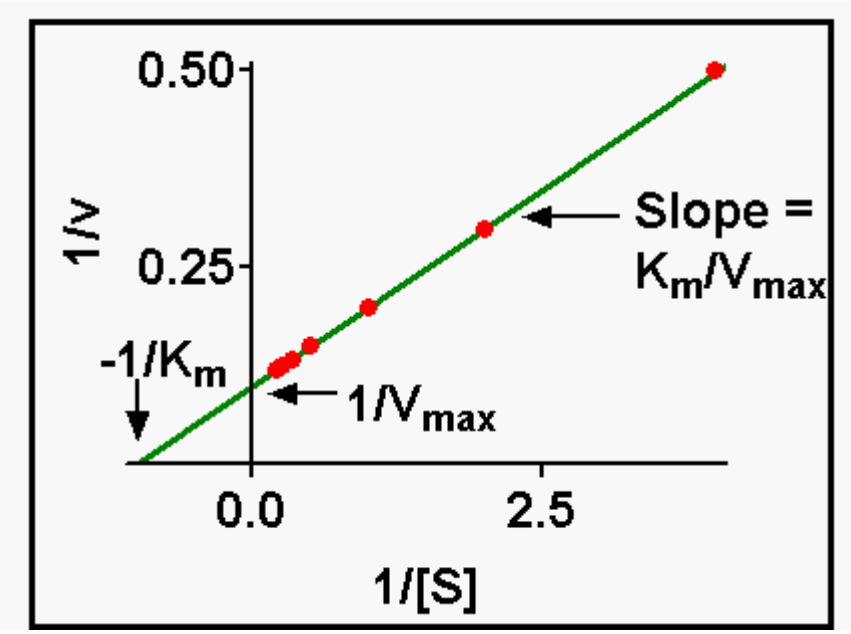
One transformation is derived by taking reciprocal of both sides of Michaelis-Menten equation.

$$V_0 = \frac{V_{max}\,[S]}{K_M + [S]} \qquad \text{----------} \quad (11)$$

$$\frac{1}{V_0} = \frac{K_M + [S]}{V_{max}\,[S]}$$

$$\frac{1}{V_0} = \frac{K_M}{V_{max}\,[S]} + \frac{1}{V_{max}} \qquad \text{----------} \quad (12)$$

Equation (12) is the Lineweaver- Burke's equation. When $1/ V_0$ is plotted against $1/ [S]$, a straight line is obtained. This line will have slope of K_m / V_{max} and an intercept of $1/ V_{max}$ on the $1/ V_0$ axis and an intercept of $– 1/ K_M$ on $1/ [S]$ axis. Such a double reciprocal plt has the advantage of allowing a much more accurate determination of V_{max}. We get only approximate value of V_{max} by plotting V_0 versus [S].



- **Eadie- Hofstee plot**

Another useful transformation of Michaelis-Menten equation is obtained by multiplying both sides of equation (12) by V_{max} and rearranging

$$\frac{1}{V_0} = \frac{K_M}{V_{max}[S]} + \frac{1}{V_{max}} \quad \text{---------- (12)}$$

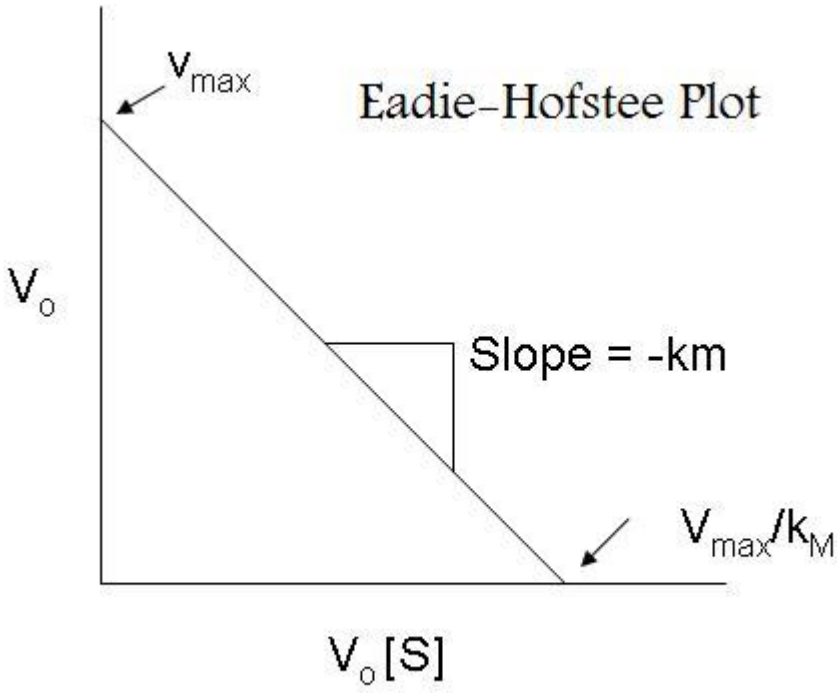
Multiply by V_{max}

$$\frac{V_{max}}{V_0} = \frac{K_M}{[S]} + 1$$

$$V_{max} = \frac{K_M V_0}{[S]} + V_0$$

$$V_0 = -K_M \frac{V_0}{[S]} + V_{max} \quad \text{---------- (13)}$$

Equation (13) is Eadie- Hofstee equation. A plot of V_0 against V_0 / [S] called Eadie- Hofstee plot, gives V_{max} and K_M in a very simple way



- **Michaelis-Menten Kinetics and Briggs-Haldane Kinetics**

The Michaelis-Menten model (*1*) is the one of the simplest and best-known approaches to enzyme kinetics. It takes the form of an equation relating reaction velocity to substrate concentration for a system where a substrate *S* binds reversibly to an enzyme *E* to form an enzyme-substrate complex *ES*, which then reacts irreversibly to generate a product *P* and to regenerate the free enzyme *E*. This system can be represented schematically as follows:

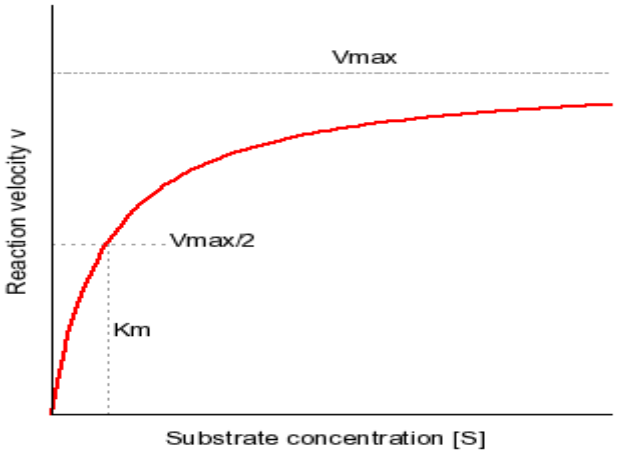
$$E + S \rightleftharpoons ES \rightarrow E + P$$

The Michaelis-Menten equation for this system is:

$$v = \frac{V_{\max}[S]}{K_M + [S]}$$

Here, $V_{\max}$ represents the maximum velocity achieved by the system, at maximum (saturating) substrate concentrations. K_M (the Michaelis constant; sometimes represented as K_S instead) is the substrate concentration at which the reaction velocity is 50% of the $V_{\max}$. [*S*] is the concentration of the substrate *S*.

This is a plot of the Michaelis-Menten equation's predicted reaction velocity as a function of substrate concentration, with the significance of the kinetic parameters $V_{\max}$ and K_M graphically depicted.



The best derivation of the Michaelis-Menten equation was provided by George Briggs and J.B.S. Haldane in 1925 (*2*), and a version of it follows:

$$E + S \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

For the scheme previously described, k_{on} is the bimolecular association rate constant of enzyme-substrate binding; k_{off} is the unimolecular rate constant of the *ES* complex dissociating to regenerate free enzyme and substrate; and k_{cat} is the unimolecular rate constant of the *ES* complex dissociating to give free enzyme and product *P*.

Note that k_{on} has units of concentration^{-1}time^{-1}, and k_{off} and k_{cat} have units of time^{-1}. Also, by definition the dissociation binding constant of the *ES* complex, K_D is given by k_{off}/k_{on} (and so has units of concentration).

Once these rate constants have been defined, we can write equations for the rates of change of all the chemical species in the system:

$$\frac{d[S]}{dt} = -k_{on}[E][S] + k_{off}[ES]$$

$$\frac{d[E]}{dt} = -k_{on}[E][S] + (k_{off} + k_{cat})[ES]$$

$$\frac{d[P]}{dt} = k_{cat}[ES]$$

$$\frac{d[ES]}{dt} = k_{on}[E][S] - (k_{off} + k_{cat})[ES]$$

The last of these equations – describing the rate of change of the *ES* complex – is the most important for our purposes. In most systems, the *ES* concentration will rapidly approach a steady-state – that is, after an initial burst phase, its concentration will not change

appreciably until a significant amount of substrate has been consumed. This **steady-state approximation** is the first important assumption involved in Briggs and Haldane's derivation. This is also the reason that well-designed experiments measure reaction velocity only in regimes where product formation is linear with time. As long as we limit ourselves to studying *initial* reaction velocities, we can assume that [*ES*] is constant:

$$\frac{d[ES]}{dt} = 0$$

$$\Rightarrow k_{on}[E][S] = \left(k_{off} + k_{cat}\right)[ES]$$

In order to determine the rate of product formation ($d[P]/dt = k_{cat}[ES]$), we need to rearrange the equation above to calculate [*ES*]. We know that the free enzyme concentration [*E*] is equal to the total enzyme concentration [E_T] minus [*ES*]. Making these substitutions gives us:

$$k_{on}\left([E_T] - [ES]\right)[S] = \left(k_{off} + k_{cat}\right)[ES]$$

$$k_{on}[E_T][S] - k_{on}[ES][S] = \left(k_{off} + k_{cat}\right)[ES]$$

$$k_{on}[E_T][S] = \left(k_{off} + k_{cat}\right)[ES] + k_{on}[ES][S]$$

$$[ES] = \frac{k_{on}[E_T][S]}{\left(k_{off} + k_{cat}\right) + k_{on}[S]} = \frac{[E_T][S]}{\left(\frac{k_{off} + k_{cat}}{k_{on}}\right) + [S]}$$

$$\Rightarrow v = k_{cat}[ES] = \frac{k_{cat}[E_T][S]}{\left(\frac{k_{off} + k_{cat}}{k_{on}}\right) + [S]}$$

We now make a couple of substitutions to arrive at the familiar form of the Michaelis-Menten equation. Since V_{max} is the reaction velocity at saturating substrate concentration, it is equal to k_{cat} [*ES*] when [*ES*] = [E_T]. We also define K_M in terms of the rate constants as follows:

$$V_{\max} = k_{cat}[E_T]; K_M = \frac{k_{off} + k_{cat}}{k_{on}}$$

$$v = \frac{V_{\max}[S]}{K_M + [S]}$$

Note that [*S*] here represents the free substrate concentration, but typically is assumed to be close to the total substrate concentration present in the system. This second assumption is the **free ligand approximation**, and is valid as long the total enzyme concentration is well below the K_M of the system. If this condition is not met (for instance, with a very high-affinity substrate), then the **quadratic** (or 'Morrison') equation must be used instead.

Comparing K_M [= (k_{off} + k_{cat})/k_{on}] and K_D [= k_{off}/k_{on}], it is obvious that K_M must always be greater than K_D. Michaelis and Menten assumed that substrate binding and dissociation occurred much more rapidly than product formation (k_{cat} << k_{off}, the **rapid equilibrium approximation**), and that therefore the K_M would be very close to the K_D. The larger the k_{cat} is relative to k_{off}, the greater the difference between K_D and K_M. Briggs and Haldane made no assumptions about the relative values of k_{off} and k_{cat}, and so Michaelis-Menten kinetics are a special case of Briggs-Haldane kinetics. The opposite extreme, where k_{cat} >> k_{off}, is called Van Slyke-Cullen behavior

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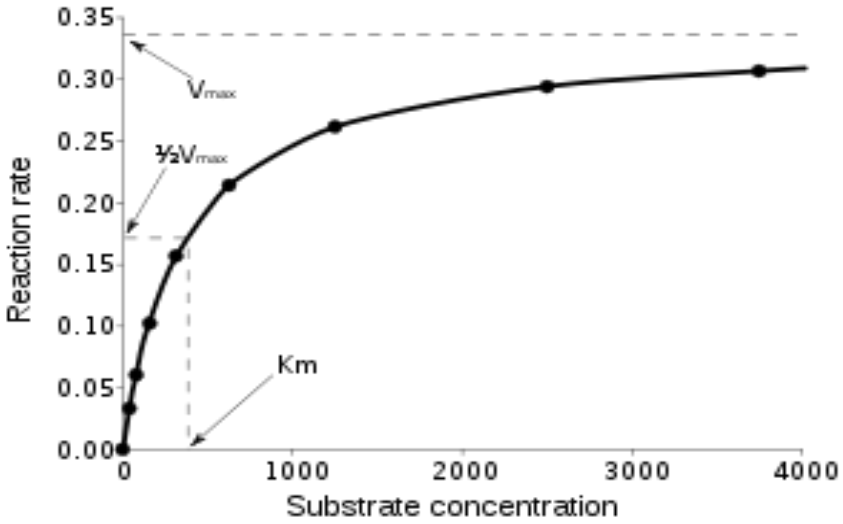
Non-Michaelis–Menten kinetics (sigmoidal kinetics steady state kinetics and transient phases of enzyme reaction)

Some enzymes produce a **sigmoid** *v* by [S] plot, which often indicates **cooperative binding** of substrate to the active site. This means that the binding of one substrate molecule affects the binding of subsequent substrate molecules. This behavior is most common in **multimeric** enzymes with several interacting active sites.[27] Here, the mechanism of cooperation is similar to that of **haemoglobin**, with binding of substrate to one active site altering the affinity of the other active sites for substrate molecules. Positive cooperativity occurs when binding of the first substrate molecule *increases* the affinity of the other active sites for substrate. Negative cooperativity occurs when binding of the first substrate *decreases* the affinity of the enzyme for other substrate molecules.

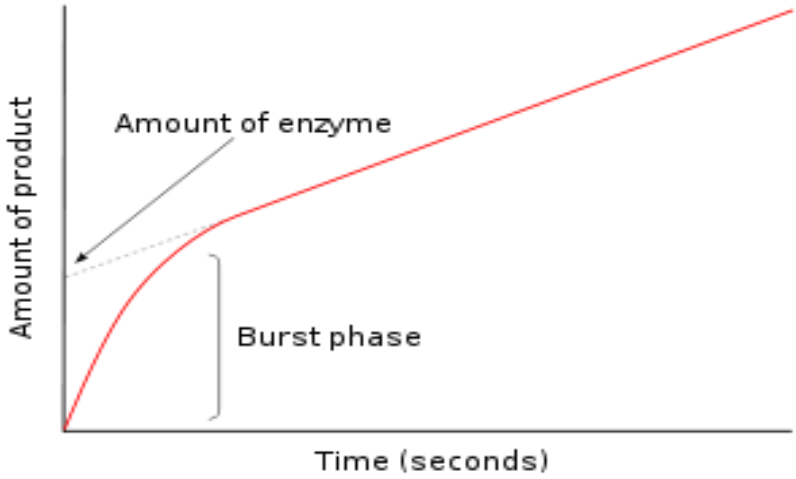
Allosteric enzymes include mammalian tyrosyl tRNA-synthetase, which shows negative cooperativity, and bacterial **aspartate transcarbamoylase** and **phosphofructokinase**, which show positive cooperativity.

Cooperativity is surprisingly common and can help regulate the responses of enzymes to changes in the concentrations of their substrates. Positive cooperativity makes enzymes much more sensitive to [S] and their activities can show large changes over a narrow range of substrate concentration. Conversely, negative cooperativity makes enzymes insensitive to small changes in [S].

The **Hill equation (biochemistry)**[31] is often used to describe the degree of cooperativity quantitatively in non-Michaelis–Menten kinetics. The derived Hill coefficient *n* measures how much the binding of substrate to one active site affects the binding of substrate to the other active sites. A Hill coefficient of <1 indicates negative cooperativity and a coefficient of >1 indicates positive **cooperativity**.



Pre-steady-state kinetics



Pre-steady state progress curve, showing the burst phase of an enzyme reaction.

In the first moment after an enzyme is mixed with substrate, no product has been formed and no **intermediates** exist. The study of the next few milliseconds of the reaction is called Pre-steady-state kinetics also referred to as **Burst kinetics**. Pre-steady-state kinetics is therefore concerned with the formation and consumption of enzyme–substrate intermediates (such as ES or E*) until their **steady-state concentrations** are reached.

This approach was first applied to the hydrolysis reaction catalysed by **chymotrypsin**.[321] Often, the detection of an intermediate is a vital piece of evidence in investigations of what mechanism an enzyme follows. For example, in the ping–pong mechanisms that are shown above, rapid kinetic measurements can follow the release of product P and measure the formation of the modified enzyme intermediate E*. In the case of

chymotrypsin, this intermediate is formed by an attack on the substrate by the **nucleophilic** serine in the active site and the formation of the acyl-enzyme intermediate.

In the figure to the right, the enzyme produces E* rapidly in the first few seconds of the reaction. The rate then slows as steady state is reached. This rapid burst phase of the reaction measures a single turnover of the enzyme. Consequently, the amount of product released in this burst, shown as the intercept on the *y*-axis of the graph, also gives the amount of functional enzyme which is present in the assay.