Al-Mustaqbal University

College of Medical and Health Techniques

Medical Laboratories Techniques Departments

Biochemistry Lectures for 2nd Year Students

(2 Credit Hrs. Theory + 2 Credit Hrs. Practice / Week = 3 Credit Unit

Academic Year: 2024 - 2025

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Lecture No. 3

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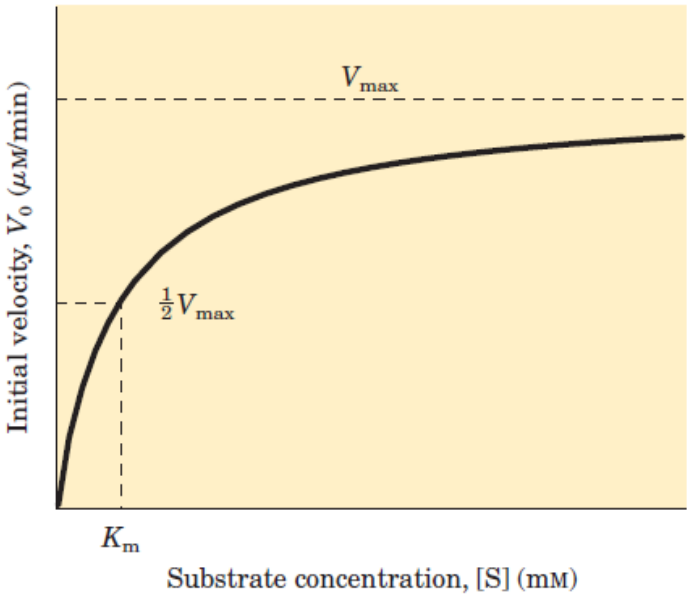
Interpreting V_{max} and k_m:-

Figure below shows a simple hyperbolic curve as a graphical method for obtaining an approximate value for each V_{max} and k_m.



The k_m can vary greatly from enzyme to enzyme, and even for different substrates of the same enzyme. The term is sometimes used as an indicator of the **affinity of an enzyme for its substrate**. The actual meaning of k_m depends on specific aspects of the reaction mechanism such as the number and relative rates of the individual steps. For reactions with two steps,

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \quad \text{.....(1)}$$

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \quad \text{.....(2)}$$

and because the rate constant k_{-2} is very small, therefore the k_m value is:

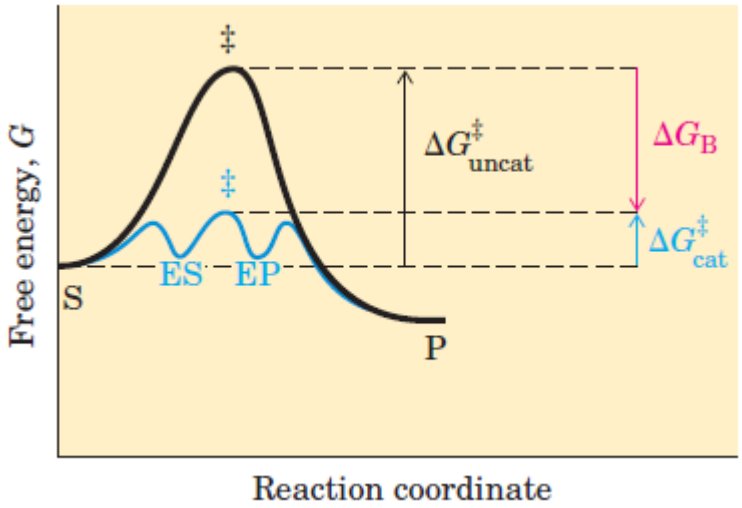
$$k_m = \frac{k_2 + k_{-1}}{k_1}$$

The quantity V_{max} also varies greatly from one enzyme to the next. If an enzyme reacts by the two-step Michaelis-Menten mechanism, $V_{max} = k_2[E_t]$, where k_2 is rate-limiting. However, the number of reaction steps and the identity of the rate-limiting step(s) can vary from enzyme to enzyme.

For example, consider the quite common situation where product release, EP -------► E + P, is rate-limiting. Early in the reaction (when [P] is

very low), the overall reaction can be described by the scheme, see Fig below:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} EP \overset{k_3}{\rightleftharpoons} E + P \quad \text{..... (3)}$$



In this case, most of the enzyme is in the EP form at saturation, and $V_{max} = k_3[E_t]$. It is useful to define a more general rate constant, $\boldsymbol{k_{cat}}$, to describe the limiting rate of any enzyme-catalyzed reaction at saturation. If the reaction has several steps and one is clearly rate-limiting, k_{cat} is equivalent to the rate constant for that limiting step.

For the simple reaction of Equation 2, $k_{cat} = k_2$. For the reaction of Equation 3, $k_{cat} = k_3$. When several steps are partially rate-limiting, k_{cat} can become a complex function of several of the rate constants that define each individual reaction step. In the Michaelis-Menten equation, $k_{cat} = V_{max}/ [E_t]$, and Michaelis-Menten equation becomes:

$$\boldsymbol{U_o = \frac{k_{cat}\,[E_t]\,[S]}{k_m + [S]}} \quad \text{.....(4)}$$

The constant k_{cat} is a first-order rate constant and hence has units of reciprocal time. It is also called the **turnover number.** It is equivalent to the number of substrate molecules converted to product in a given unit of time on a single enzyme molecule when the enzyme is saturated with substrate.

Understanding k_m

1. k_m is a constant derived from rate constants with units M.
2. k_m is, under true Michaelis-Menten conditions, an estimate of the dissociation constant of E from S
3. Small k_m means tight binding; high k_m means weak binding

Understanding V_{max}, *theoretical maximal velocity*

1. V_{max} is the theoretical maximal rate of the reaction constant with units s^{-1}
2. To reach V_{max} would require that all enzyme molecules are tightly bound with substrate, and is asymptotically approached as [S] is increased.

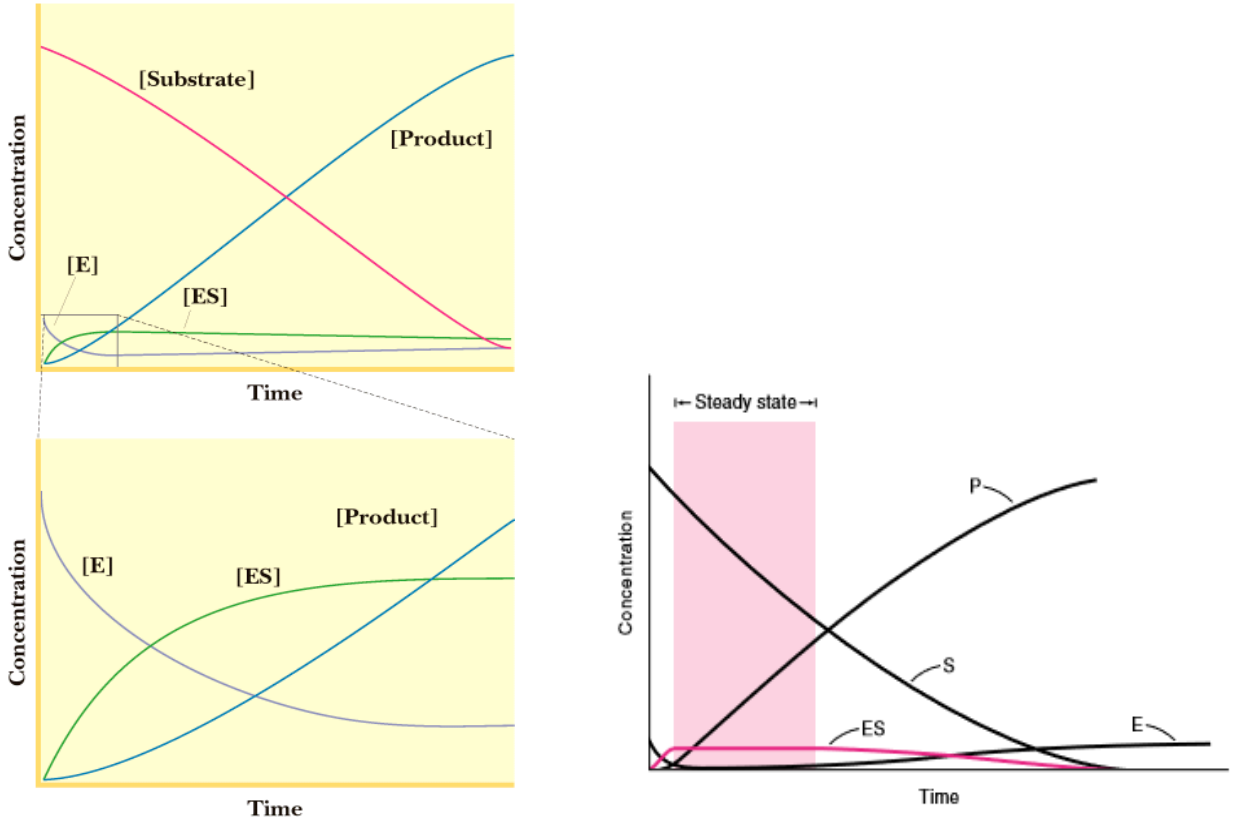


Fig. 1: Plot of the concentrations of the various species in an enzyme-catalyzed reaction E + S ◄=====► ES --------► P + E versus time. We assume that the initial substrate concentration is much larger than the enzyme concentration and that the rate constants k_1; k_{-1}, and k_2 are of comparable magnitudes.

Michaelis-Menten Constant:

According to Michaelis theory, the formation of enzyme substrate complex is a reversible reaction, while the breakdown of the complex to enzyme + product is irreversible.

Salient features of k_m value are shown in Box 3. The lesser the numerical value of k_m, the affinity of the enzyme for the substrate is more. To cite an example, k_m of glucokinase is 10 mmol/L and that of hexokinase is 0.05 mmol/L.

Hexokinase or Glucokinase

Glucose + ATP ----------► Glucose-6-phospate + ADP

ATP: Adenosine-5- triphosphate ; ADP: Adenosine -5-diphosphate

Therefore, 50% molecules of hexokinase are saturated even at a lower concentration of glucose. In other words, hexokinase has more affinity for glucose than glucokinase.

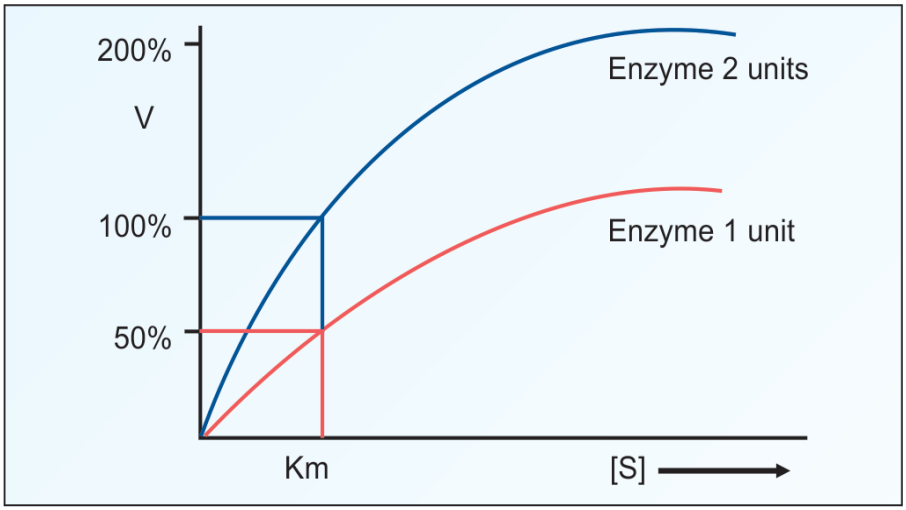


Fig. 7: Effect of enzyme concentrate on k_m

Box 3

1. ***k_m* value is substrate concentration** (expressed in moles/L) **at half-maximal velocity**
2. **It denotes that 50% of enzyme molecules are bound with substrate molecules at that particular substrate concentration.**
3. ***k_m* is independent of enzyme concentration.** If enzyme concentration is doubled, the V_{max} will be double. But the ½ V_{max} (k_m) will remain exactly same. In other words, irrespective of enzyme concentration, 50% molecules are bound to substrate at that particular substrate concentration.
4. ***k_m* is the signature of the enzyme.** k_m value is thus a constant for an enzyme. It is the **characteristic feature of a particular enzyme** for a specific substrate.
5. The **affinity of an enzyme** towards its substrate **is inversely related** to the dissociation constant, K_d for the enzyme substrate complex.

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$

$$K_d = k_1 / k_2 \quad ; \quad k_m = k_2 + k_3 / k_p$$

Therefore, the smaller the tendency for the dissociation of the complex, the greater is the affinity of the enzyme for the substrate.

6. ***k_m* denotes the affinity of enzyme for substrate.** The *lesser the numerical value of k_m, the affinity of the enzyme for the substrate is more.*

The Turnover Number is *a measure of catalytic activity.*

1. k_{cat}, the turnover number, is the number of substrate molecules converted to product per enzyme molecule per unit of time, when E is saturated with substrate.
2. Values of k_{cat} range from less than 1/sec to many millions per sec

Enzyme Inhibition:

Enzymes are protein and they can be inactivated by the agents that denature them. The chemical substances which inactivate the enzymes are called as ***inhibitors*** and the process is called as ***enzyme inhibition.*** Inhibitors are sometimes referred to as ***negative modifier***. They may be small inorganic ions, or organic substances, poisons, toxic compounds, pollutants, xenobiotic ….. etc. Enzyme inhibition is classified under **three major groups:**

1. **Competitive inhibition (Reversible).**
2. **Non-competitive inhibition (Irreversible or reversible).**
3. **Allosteric inhibition.**

Generally, there are two broad classes of enzyme inhibitors: reversible and irreversible.

Reversible versus irreversible

a. **Irreversible inhibitors** interact with an enzyme via covalent, associations for eg, Nerve agents like sarin, cyanide are irreversible inhibitors of acetylcholine esterase and cytochrome a3 respectively.

b. **Reversible inhibitors** interact with an enzyme via non-covalent associations. For therapeutic drug design we're almost always interested in reversible inhibitors.

Enzyme inhibitors are molecular agents that interfere with catalysis, slowing or halting enzymatic reactions. Enzymes catalyze virtually all cellular processes, so it should not be surprising that enzyme inhibitors are among the most important pharmaceutical agents known. For example, aspirin (acetylsalicylate) inhibits the enzyme that catalyzes the first step in the synthesis of prostaglandins, compounds involved in many processes, including some that produce pain.

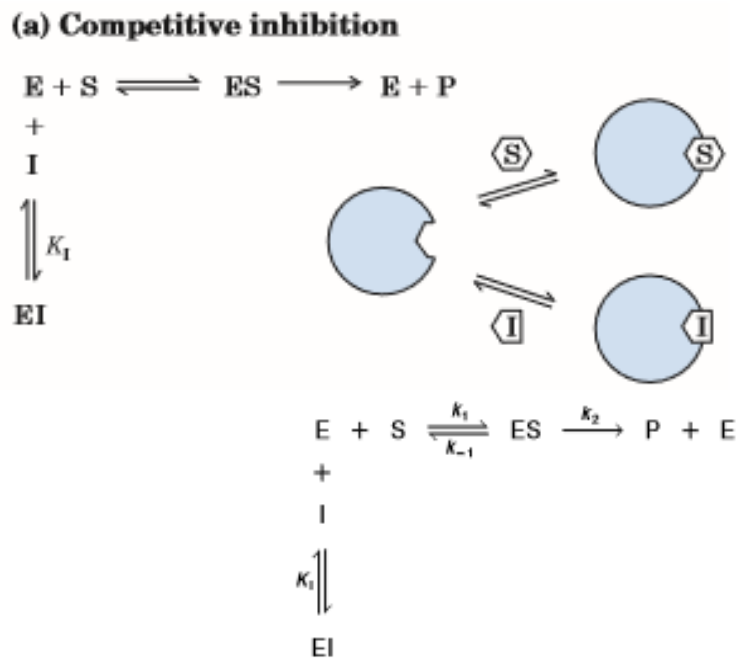
Reversible Inhibition:

There are three important types of reversible inhibition:

1. Competitive inhibition,
2. Non-competitive inhibition
3. Un-competitive inhibition.

Competitive Inhibition:

A competitive inhibitor competes with the substrate for the active site of an enzyme. While the inhibitor (I) occupies the active site it prevents binding of the substrate to the enzyme. Many competitive inhibitors are compounds that resemble the substrate and combine with the enzyme to form an EI complex, but without leading to catalysis. Even fleeting combinations of this type will reduce the efficiency of the enzyme. By taking into account the molecular geometry of inhibitors that resemble the substrate.



In this case, both the substrate S and the inhibitor I compete for the same active site :

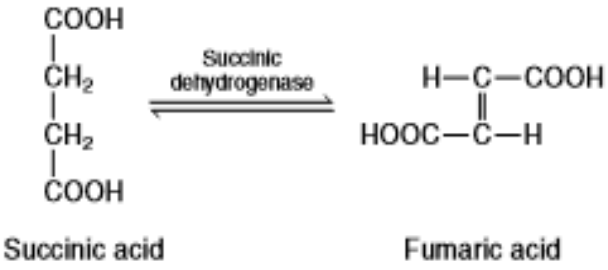
$$\upsilon_0 = \frac{V_{max}[S]}{\alpha k_m + [S]} \quad \text{where} \quad \alpha = 1 + \frac{[I]}{k_i} \quad ; \quad k_i = \frac{[E][I]}{[EI]}$$

The experimentally determined variable αk_m, the k_m observed in the presence of the inhibitor, is often called the "apparent" k_m. Note that the complex EI does not react with S to form products. Applying the steady-state approximation for ES, we obtain:

$$\upsilon_o = \frac{V_{max}\,[S]}{k_m\,(1 + [I] / k_i) + [S]}$$

The measured values of k_m in the presence of the inhibitor are altered, and are called the apparent k_m. The apparent k_m varies depending on the inhibitor concentration involved.

A well-known example of a competitive inhibitor is malonic acid, $CH_2(COOH)_2$, which competes with succinic acid in the dehydrogenation reaction catalyzed by succinate dehydrogenase.



Succinic acid Fumaric acid

Because malonic acid resembles succinic acid in structure, it can combine with the enzyme, although no product is formed in this reaction.

A medical therapy based on competition at the active site is used to treat patients who have ingested methanol, a solvent found in gas-line antifreeze. The liver enzyme alcohol dehydrogenase converts methanol to formaldehyde, which is damaging too many tissues, blindness. Ethanol competes effectively with methanol as an alternative substrate for alcohol dehydrogenase. The effect of ethanol is much like that of a competitive inhibitor, with the distinction that ethanol is also a substrate for alcohol dehydrogenase and its concentration will decrease over time as the enzyme converts it to acetaldehyde. The therapy for methanol poisoning is slow intravenous infusion of ethanol, at a rate that maintains a controlled concentration in the bloodstream for several hours. This slows the formation of formaldehyde, lessening the danger while the kidneys filter out the methanol to be excreted harmlessly in the urine.

The inhibition of the hexokinase-catalyzed reaction between glucose and ATP by fructose or mannose is an example of competitive inhibition by alternate substrate. Glucose, fructose and mannose are all substrate of

hexokinase and can be converted to product (hexose-6-phosphate). All three hexoses combine with the enzyme at the same active site. Consequently, the utilization of any one of the hexoses is inhibited in the presence of either of the other two.

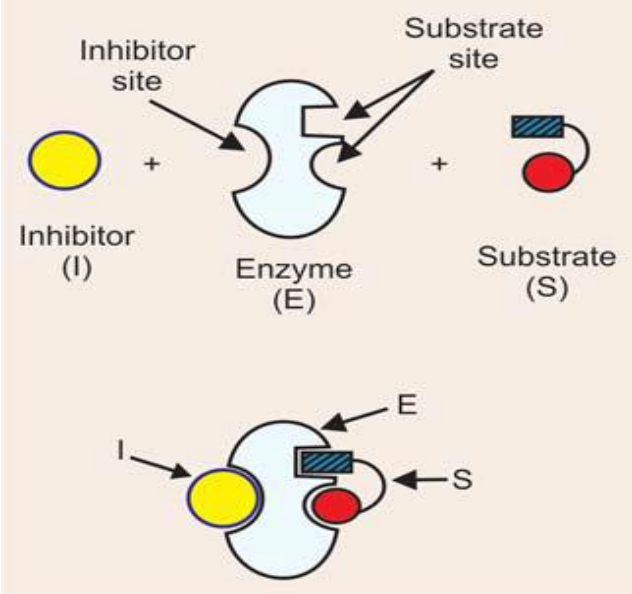
Examples of Competitive Inhibitors in Biological System:

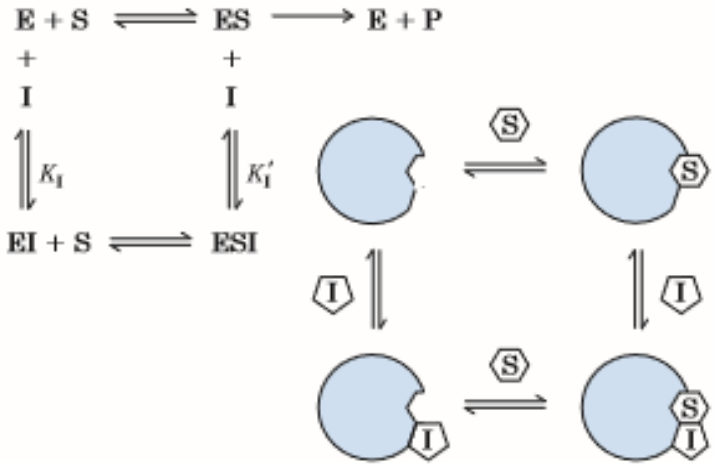
1. **Allopurinol:** A drug used for treatment of Gout. Uric acid is formed in the body by oxidation of hypoxanthine by the enzyme **Xanthine oxidase. Allopurinol structurally resembles hypoxanthine** and thus by competitive inhibition, the drug inhibits the enzyme **xanthine oxidase** thus reducing uric acid formation.
2. **Sulphonamides:** A very commonly used antibacterial agent. Para-aminobenzoic acid (PABA) is essential for synthesis of folic acid by the enzyme action. Folic acid is needed for bacterial growth and survival. Bacterial wall is impermeable to folic acid. Sulphonamide drugs are structurally similar to PABA and competitively inhibit enzyme action. Thus, folic acid is not synthesized and growth of bacteria suffers and they die.
3. **Methotrexate:** A drug used for cancer therapy. Chemically it is 4-amino-N10-methyl folic acid. The drug structurally resembles folic acid. Hence it competitively inhibits **"folate reductase"** enzyme and prevents formation of FH4. Hence, DNA synthesis suffers.
4. **Dicoumarol:** Used as an anticoagulant. It is structurally similar to vitamin K and can act as an anticoagulant by competitively inhibiting vitamin K.
5. **Succinylcholine:** It is used as a muscle relaxant. Succinylcholine is structurally similar to acetylcholine. It competitively fixes on post-synaptic receptors. As it is not hydrolyzed easily by the enzyme **acetyl cholinesterase,** produces continued depolarization with consequent muscle relaxation.

Non-Competitive Inhibition:

This is of two different types namely (i) ***reversible*** and (ii) ***irreversible***. This occurs when the substances not resembling the geometry of the substrate do not exhibit mutual competition. Most probably the ***sites of attachment of the substrate and inhibitor are different.*** The inhibitor binds reversibly with a site on combine with both free enzyme and ES complex. This probably brings about the changes in three-dimensional structure of the enzyme inactivating it catalytically. In noncompetitive inhibition V_{max} ***is lowered***, but k_m ***is kept constant***.

If the inhibitor can be removed from its site of binding without affecting the activity of the enzyme, it is called as ***Reversible-Non-competitive Inhibition***. However, if the inhibitor can be removed only at the loss of enzymatic activity, it is known as ***Irreversible Non-competitive Inhibition***. However, the kinetic properties in case of both are the same.





$$v_0 = \frac{V_{max}}{\dfrac{1 + [I]}{k_i}[S]}{k_m + [S]}$$

Neither EI nor ESI forms products. Because I do not interfere with the formation of ES, noncompetitive inhibition cannot be reversed by increasing the substrate concentration. The initial rate is given by noncompetitive inhibition is independent of [S] and depends only on [I] and k_i.

Examples of Non-competitive Irreversible Inhibitors

1. **Iodoacetate:** An irreversible inhibitor of enzymes like glyceraldehyde-3-P dehydrogenase and papain. It **combines with–SH group** at the active site of the enzyme inactivating the enzyme.
2. **Heavy metal ions** like Ag, Hg also act as irreversible noncompetitive inhibitor.
3. **Fluoride:** Inhibits the enzyme emolase by removing Mg^{++} and Mn^{++} and stops glycolysis.

4. **Di-isopropyl fluorophosphate (DFP):** Inhibits enzymes with serine in their active site e.g. acetylcholine esterase.

Table gives the differences that are observed between competitive and non-competitive inhibition.

Competitive inhibition	*Non-competitive inhibition*
1. Reversible	1. Reversible or Irreversible
2. Inhibitor and substrate resemble each other in structure	2. Does not resemble
3. Inhibitor binds the active site	3. Inhibitor does not bind the active site
4. V_{max} is same	4. V_{max} lowered
5. K_m increased	5. K_m unaltered
6. Inhibitor cannot bind with ES complex	6. Inhibitor can bind with ES complex
7. Lowers the substrate affinity to enzyme	7. Does not change substrate affinity for the enzyme
8. Complex is E-I	8. Complex is E-S-I or E-I
9. Michaelis-Menten equation changed to $V = \frac{V_{max}[S]}{K_m \frac{1+(I)}{K_I} + S}$	9. Michaelis-Menten equation changed to: $V = \frac{V_{max}[S]}{K_m\left[\frac{1+(I)}{K_I}\right][K_m] + [S]}$

Suicide Inhibition

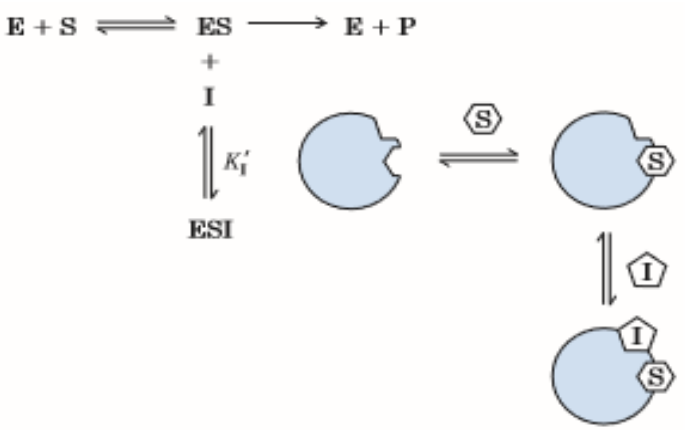
It is a ***special type of irreversible noncompetitive inhibition.*** In this type of inhibition, ***substrate analogue is converted to a more effective inhibitor*** with the help of the enzyme to be inhibited. The so formed new inhibitor binds irreversibly with the enzyme.

Examples

1. **Allopurinol.** The best example of suicide inhibition. The drug is used in treatment of gout, as it inhibits the enzyme xanthine oxidase thus decreasing the uric acid formation. But ***allopurinol gets oxidized by the enzyme xanthine oxidase itself to form "alloxanthine" a more potent effective and stronger inhibitor*** of xanthine oxidase thus potentiating the action of allopurinol.
2. **Aspirin.** Most commonly used drug for relieving pain. Anti-inflammatory action of aspirin is also based on the suicide inhibition. Aspirin acetylates a serine residue in the active centre of cyclooxygenase thus inhibiting the PG synthesis and the inflammation.
3. **5-Fluorouracil.** Used in cancer therapy, 5-fluorouracil (5-FU) is converted to fluorodeoxyuridylate (FdUMP) by the enzymes of the salvage pathway. FdUNP so formed **inhibits the enzyme *thymidylate synthase*** thus inhibiting nucleotide synthesis.

Uncompetitive Inhibition:

An uncompetitive inhibitor does not bind to the free enzyme; instead, it binds reversibly to the enzyme–substrate complex to yield an inactive ESI complex. The reactions are:



$$\text{Where} \qquad k_i = \frac{[ES][I]}{[ESI]}$$

The ESI complex does not form a product. Again, because I do not interfere with the formation of ES, uncompetitive inhibition cannot be reversed by increasing the substrate concentration.

$$\upsilon_o = \frac{\dfrac{V_{max}}{\dfrac{}{}}[S]}{1 + [I]/k_i} \qquad \upsilon_o = \frac{V_{max}[S]}{\dfrac{k_m}{(1 + [I]/k_i)} + [S]}$$

Allosteric Inhibition and Allosteric Enzymes

There is a mixed kind of inhibition when the inhibitor binds to the enzyme at a site other than the active site of the enzyme molecule called ***allosteric site***. ***Allosteric inhibition does not follow the Michaelis-Menten hyperbolic kinetics. Instead it gives a sigmoid kinetics*** **(Figure 2 below)**. Allosteric inhibitors shift the substrate saturation curve to the right.

However as opposite to inhibitors, the presence of activators shifts the curve to the left.

Types: Allosteric enzymes are of *K* and *M* series according to their kinetics.

1. In ***K-enzymes***, e.g. *aspartate carbamoylase* and *phosphofructokinase*, the allosteric inhibitor lowers the substrate affinity to raise the k_m of the enzyme; but the V_{max} is unchanged.
2. ***In M-enzymes***, e.g. ***acetyl-CoA carboxylase***, the allosteric inhibitor reduces the maximum velocity but no change in k_m or substrate affinity. Allosteric activators produce a fall in K enzymes and a rise in V_{max} in *M* enzymes.
3. When the final product allosterically inhibits the enzyme, it is called as feed-back allosteric inhibition.

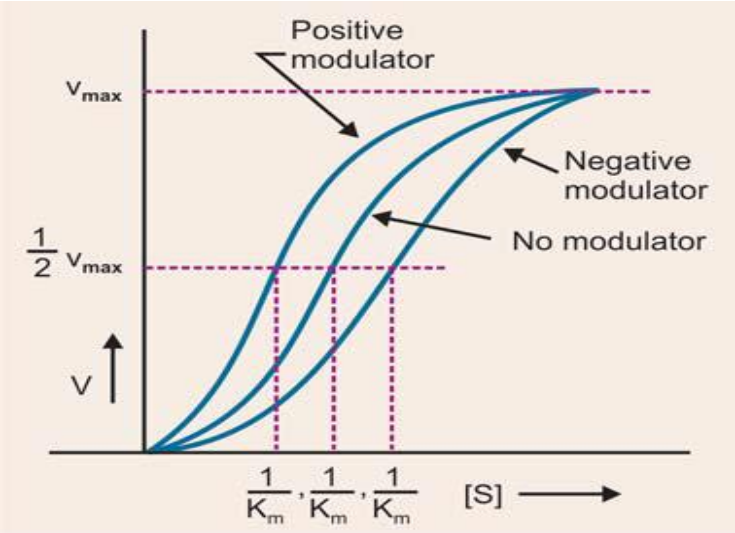


Fig. 2: Sigmoid kinetics, allosteric inhibition

Aspartate transcarbamoylase is a model allosteric enzyme

Aspartate transcarbamoylase (ATCase) catalyzes the first reaction unique to pyrimidine biosynthesis. ATCase is feedback inhibited by cytidine triphosphate (CTP). Following treatment with mercurials, ATCase loses its sensitivity to inhibition by CTP but retains its full activity for carbamoyl aspartate synthesis. This suggests that CTP is bound at a different (allosteric) site from either substrate. ATCase consists of multiple catalytic and regulatory protomers. Each catalytic protomer contains four aspartate (substrate) sites and each regulatory protomer atleast two CTP (regulatory sites).

Another example of allosteric enzyme and inhibition:

Synthesis of isoleucine from threonine involves at least 5 steps of enzymatic reactions. Isoleucine, the end product, inhibits the first enzyme ***threonine deaminase*** and stops its own synthesis.

A metabolite may also cause feed-forward allosteric activation of an enzyme for a subsequent step of its metabolism, e.g. Fructose-1,6-biphosphate allosterically activates *pyruvate kinase* catalyzing subsequent step.

In oligomeric enzymes, the allosteric site and active site are located on different subunits. ***Changes in the enzyme-substrate interaction due to the allosteric effects of regulatory molecules other than the substrate are called heterotropic allosteric modulations***. Allosteric activators and inhibitors exhibit respectively positive and negative cooperativities with the substrates.

Binding of substrate to one protomer enhances the binding of the same to another protomer or another substrate binding site on the same enzyme molecule. ***When the binding of a substrate enhances the interaction between the allosteric enzyme and more molecules of the same substrate it is homotropic allosteric effect.***

Feedback Regulation Vs Feedback inhibition:

Feedback regulation and feedback inhibition are not synonymous and they are different.

In both mammalian and bacterial cells, end-products "feedback" and control their own synthesis. In many instances, this involves feedback inhibition of an early biosynthetic enzyme. It is necessary to distinguish between "feedback regulation" and feedback inhibition, a mechanism for regulation of many bacterial and mammalian enzymes.

Example

Dietary cholesterol restricts the synthesis of cholesterol from acetate in mammalian tissues. This is feedback regulation. This feedback regulation, however, does not appear to involve "feedback inhibition" of an early enzyme of cholesterol biosynthesis. An early enzyme *'HMG-CoA reductase'* is affected, but the mechanism involves curtailment by cholesterol or a cholesterol metabolite of the expression of the gene that encodes *'HMG-CoA reductase'*, i.e. enzyme repression. Cholesterol added directly to *'HMG-CoA reductase'* has no effect on its catalytic activity.

Enzyme Regulation:

The activity of certain enzymes is regulated by a feedback mechanism such that an end product inhibits the enzyme's function in an initial stage of a sequence of reactions. In (Figure below) control of regulatory enzymes frequently involves feedback mechanisms. In this sequence of reactions catalyzed by enzymes, the first enzyme in the series is inhibited by product

F. At the early stages of the reaction, the concentration of F is low and its inhibitory effect is minimal. As the concentration of F reaches a certain level, it can lead to total inhibition of the first enzyme and hence turns its own source of production.

The glycolytic pathway is an example of this feedback mechanism. In effect, enzyme inhibition controls the amount of products formed. The action of an inhibitor on an enzyme can be described as either reversible or irreversible. In reversible inhibition, equilibrium exists between the enzyme and the inhibitor. In irreversible inhibitions, inhibition progressively increases with time. Complete inhibition results if the concentration of the irreversible inhibitor exceeds that of the enzyme.