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Enzyme Inhibition: Mechanisms and Scope

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1. Introduction

Enzyme is a protein molecule acting as catalyst in enzyme reaction. Enzyme inhibition is a science of enzyme-substrate reaction influenced by the presence of any organic chemical or inorganic metal or biosynthetic compound due to their covalent or non-covalent interactions with enzyme active site. It is well known that all these inhibitors follow same rule to interplay in enzyme reaction. Present chapter introduces beginners with basic tenets of classic presumptions of enzyme inhibition, types of enzyme inhibitors, different models of enzyme inhibition with established examples cited in literature, and scientific basis of emerging immobilized enzyme technology in different applications. In the end, limitations of using classic presumptions and variants of enzyme inhibition are highlighted with new challenges to achieve best results. Present time, best approach is 'customize new technology with detailed analysis to make it highly efficient' in both drug discovery and enzyme biosensor industry. However, other applications are described in following chapters on pesticides, herbicides.

2. What are enzyme inhibitors?

The enzyme inhibitors are low molecular weight chemical compounds. They can reduce or completely inhibit the enzyme catalytic activity either reversibly or permanently (irreversibly). Inhibitor can modify one amino acid, or several side chain(s) required in enzyme catalytic activity. To protect enzyme catalytic site from any change, ligand binds with critical side chain in enzyme. Safely, chemical modification can be done to test inhibitor for any drug value.

In drug discovery, several drug analogues are chosen and/or designed to inhibit specific enzymes. However, detoxification or reduced toxic effect of many antitoxins is also accomplished mainly due to their enzyme inhibitory action. Therefore, studying the aforementioned enzyme kinetics and structure-function relationship is vital to understand the kinetics of enzyme inhibition that in turn is fundamental to the modern design of pharmaceuticals in industries [Sami et al. 2011]. Enzyme inhibition kinetics behavior and inhibitor structure-function relationship with enzyme active site clarify the mechanisms of

enzyme inhibition action and physiological regulation of metabolic enzymes as evidenced in following chapters in this book. Some notable classic examples are: drug and toxin action and/or drug design for therapeutic uses e.g., iodoacetamide deactivates cysteine amino acid in enzyme side chain; methotrexate in cancer chemotherapy through semi-selectively inhibit DNA synthesis of malignant cells; aspirin inhibits the synthesis of the proinflammatory prostaglandins; sulfa drugs inhibit the folic acid synthesis essential for growth of pathogenic bacteria and so many other drugs. Many life-threatening poisons, e.g., cyanide, carbon monoxide and polychlorinated biphenols are all enzyme inhibitors.

Conceptually, enzyme inhibitors are classified into two types: non-specific inhibitors and specific inhibitors.

The enzyme inhibition reactions follow a set of rules as mentioned in following rules. Presently, computer based enzyme kinetics data analysis softwares are developed using following basic presumptions.

1. Enzyme interacts with substrate in 1:1 ratio at active site to catalyze the reaction.
2. Enzyme binds with substrate at active site in the form of a lock-key 3D arrangement for induced fit.
3. Inhibitor active groups compete with substrate active groups and/or active groups at enzyme allosteric catalytic site in a synergistic manner or first cum first preference (competition) to make enzyme-inhibitor-substrate/enzyme-substrate/enzyme-inhibitor complexes.
4. Enzyme-inhibitor-substrate complex formation depends on active free energy loss and thermodynamic principles.
5. Enzyme and substrate or inhibitors react with each other as active masses and reaction progresses in kinetic manner of forward or backward reaction.
6. Kinetic nature of inhibitor or substrate binding with enzyme is expressed as kinetic constants of a catalytic reaction.
7. Enzyme reaction(s) are highly depend on physiological conditions such as pH, temperature, concentration of reactants, reaction period to determine the rate of reaction.
8. Substrate and inhibitor molecules arrange over enzyme active site on specific subunit(s) in 3D manner. As a result enzyme-substrate-inhibitor exhibit binding rates depend on allosteric sites or subunit-subunit homotropic or heterotropic interactions.
9. Intermolecular forces between enzyme subunits, substrate or inhibitor active group interactions, physical properties of binding nature: electrophilic, hydrophilic, nucleophilic and metalloprotein nature; hydrogen bonding affect the overall enzyme reaction rates and mode of inhibition (3D orientation of inhibitor molecule on enzyme active site).

Other factors are also significant in determining enzyme inhibition reaction as described in each individual inhibitor in following sections. For basic principles of enzyme units (apoenzyme, holoenzyme, co-factor, co-enzyme) in enzyme catalysis, active energy loss, Michaelis-Menton Equations, LeChatelier's principle, Lineweaver-Burk and semi-log plots, apparent and actual plots, readers are requested to read text books [Schnell et al. 2003, Nelson, et al. 2008, Jakobowski 2010a, Strayer et al. 2011]. Our focus is enzyme inhibition mechanisms with examples in following description. For multisubstrate enzymes, ping-pong mechanism, allosteric mechanisms, and diffusion kinetics, readers are requested to read original papers [Pryciak 2008, Bashor 2008, Jakobowski 2010b]

These inhibitors may act in reversible or irreversible manner. Non-specific irreversible non-competitive inhibitors include all protein denaturing factors (physical and chemical denaturation factors). The specific inhibitors attack a specific component of the holoenzyme system. The action depends on increased amount of substrate or by other means of physiological conditions, toxins. Specific inhibitors can be described in several forms including; 1) *coenzyme inhibitors*: e.g., cyanide, hydrazine and hydroxylamine that inhibit pyridoxal phosphate, and, dicumarol that is a competitive antagonist for vitamin K; 2) *inhibitors of specific ion cofactor*: e.g., fluoride that chelates Mg^{2+} of enolase enzyme; 3) *prosthetic group inhibitors*: e.g., cyanide that inhibits the heme prosthetic group of cytochrome oxidase; and, 4) *apoenzyme inhibitors* that attack the apoenzyme component of the holoenzyme; 5) *physiological modulators* of reaction pH and temperature that denature the enzyme catalytic site.

The apoenzyme inhibitors are of two types; i) *Reversible inhibitors*; their inhibitory action is reversible because they make reversible association with the enzyme, and, ii) *Irreversible inhibitors*; because they make inactivating irreversible covalent modification of an essential residue of the enzyme. Apoenzyme inhibitors show effect on K_m and V_{max} . The reversible apoenzyme inhibitors are also called metabolic antagonists. They are of three subtypes; a) *competitive*, b) *uncompetitive* and c) *non-competitive* or mixed type. For example: enzyme inhibitors are used in drug design.

Discovery of useful new enzyme inhibitors used to be done by trial and error through screening a huge library of compounds against a target enzyme at allosteric catalytic site. This approach is still in use for compounds with combinatorial chemistry and high-throughput screening technology as described in following description based on recent concepts [El-Metwally et al. 2010]. However, rational drug design as an alternative approach uses the three-dimensional structure of an enzyme's active site or transition-state conformation to predict which molecules might be ideal inhibitors as given an example of urease in chapter 11 in this book. 3D-structure shortens the long screening list towards a right set of novel inhibitor which kinetically characterizes and allows specific structural changes in amino acids of catalytic site chain to optimize inhibitor-enzyme binding. Alternatively, molecular docking and molecular mechanics are computer-based methods that predict the affinity of an inhibitor for an enzyme. In following description, a glimpse of these mechanisms is given on different types of inhibitors based on recent classic book [El-Metwally et al. 2010]. Readers are requested to read other classic details from advanced text books [Dixon and Webb, 1979].

3. Irreversible inhibition

The irreversible apoenzyme inhibitors have no structural relationship to the substrate and bind covalently. They also bind stable non-covalently with the active site of the enzyme or destroy an essential functional group of active site. So, irreversible inhibitors are used to identify functional groups of the enzyme active sites at which location they bind. Although inhibitors have limited therapeutic applications because they are usually act as poisons. A subset of irreversible inhibitors called *suicide irreversible inhibitors*, are relatively inactive compounds. They get activated upon binding with the active site of a specific enzyme. After such binding, the suicide irreversible inhibitor is activated by the first few intermediary

steps of the biochemical reaction - like the normal substrate. However, it does not release any product because of its irreversible binding at the enzyme active site. Inhibitors make use of the normal enzyme reaction mechanism to get activated and subsequently inactivate the enzyme. Due to this very nature, suicide irreversible inhibitors are also called *mechanism-based inactivators* or *transition state analog inhibitors*. Thus, inhibitor exploits the transition state stabilizing effect of the enzyme, resulting in a better binding affinity (lower K_i) than substrate-based designs. An example of such a transition state inhibitor is active form of the antiviral drug oseltamivir (Tamiflu; see Figure 1); this drug mimics the planar nature of the ring oxonium ion in the reaction of the viral enzyme neuraminidase [El-Metwally et al. 2010]. After drug activation in the liver, the drug replaces sialic acid as the normal substrate found on the surface proteins of normal host cells. It prevents the release of new viral particles from infected cells. It has been used to treat and prevent Influenza virus A and Influenza virus B infections. Most of such inhibitors are classified as tight-binding competitive inhibitors in other references of enzymes. However, their reaction kinetics is essentially irreversible.

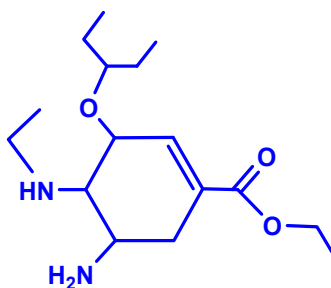


Fig. 1. The transition state analog oseltamivir - the viral neuraminidase inhibitor.

The present art of drug discovery and design of new drugs is based on suicidal irreversible inhibitors. Chemicals are synthesized based on knowledge of 3D conformation of substrate-active site binding at specific binding rates in presence of co-factors, co-enzyme (enzyme reaction mechanisms) to inhibit at specific enzyme active site with minimal side-effects due to its non-specific binding nature. Transition state analogs are extremely potent and specific inhibitors of enzymes because they have higher affinity and stronger binding to the active site of the target enzyme than the natural substrates or products. However, exact design of drugs that precisely mimic the transition state is a challenge because of unstable structure of transition state in the free-state. Prodrugs undergo initial reaction(s) to form an overall electrostatic and three-dimensional intermediate transition state complex form with close similarity to that of the substrate. These prodrugs serve as guideline for drug development to form transition state suitable for stable modification; or, using the transition state analog to design a complementary catalytic antibody; called *Abzyme*. Example: Abzymes are used in catalytic antibodies and ribozymes in catalytic ribosomes [El-Metwally et al. 2010].

- Abzymes are antibodies generated against analogs of the transition state complex of a specific chemical. The arrangement of amino acid side chains at the abzyme variable regions is similar to the active site of the enzyme in the transition state and work as artificial enzymes. For example, an abzyme was developed against analogs of the transition state complex of cocaine esterase, the enzyme that degrades cocaine in the body [El-Metwally et al. 2010]. Thus, this abzyme has similar esterase activity that is

- used as injection drug to rapidly destroy cocaine in the blood of addicted individuals to decreasing their dependence on it.
- Thrombin inhibition is common in saliva of leeches and other blood-sucking organisms. They contain the anticoagulant hirudin that irreversibly inhibits thrombin, and, to regain thrombin action synthesis of new thrombin molecules is required. This made it unsafe as an anticoagulation drug. However, based on hirudin structure, rational drug design synthesized 20-amino acids peptide known as bivalirudin that is safe for long-term use because of its reversible effects on thrombin; despite its high binding affinity and specificity for thrombin.
 - Ornithine decarboxylase by difluoromethylornithine is used to treat African trypanosomiasis (sleeping sickness). The enzyme initially decarboxylates difluoromethylornithine instead of ornithine and releases a fluorine atom, leaving the rest of the molecule as a highly electrophilic conjugated imine. The later reacts with either a cysteine or lysine residue in the active site to irreversibly inactivate the enzyme.
 - Inhibition of thymidylate synthase by fluoro-dUMP. Imidazole antimycotic drugs are examples of such group that inhibit several subtypes of cytochrome P450 [Sharma, 1990]. The mechanisms of toxicities and antidotes of irreversible inhibitors are of medical pathological importance. Because of the irreversible inactivation of the enzyme, irreversible inhibition is of long duration in the biological system because reversal of their action requires synthesis of new enzyme molecules at the enzyme gene-transcription-translation level.
 - Inhibition of acetylcholine esterase (ACE) by diisopropylfluorophosphate (DPFP), the ancestor of current organophosphorus nerve gases (e.g., Sarin and Tabun) and other organophosphorus toxins (e.g., the insecticides Malathion and Parathion and chlorpyrifos). ACE hydrolyzes the acetylcholine into acetate and choline to terminate the transmission of the neural signal from the neuromuscular excitatory acetylcholine presynaptic cell to somatic neuromuscular junction (see Figure 2). DPFP as a potent neurotoxin inhibits ACE and acetylcholine hydrolysis. Failure of hydrolysis leads to persistent acetylcholine excitatory state and improper vital function particularly respiratory muscles that may lead to suffocation; with a lethal dose of less than 100 mg. DPFP inhibits other enzymes with the reactive serine residue at the active site, e.g., serine proteases such as trypsin and chymotrypsin, but the inhibition is not as lethal as that of acetylcholine esterase. Similar to DPFP, malaaxon the toxic reactive derivative from Malathion (after its metabolism by the liver) binds initially reversibly and then irreversibly (after dealkylation of the inhibitor) to the active site serine and inactivates ACE and other enzymes. Lethal doses of oral Malathion are estimated at 1 g/kg of body weight for humans.
 - Inhibition of ACE by these poisons leads to accumulation of acetylcholine that overstimulates the autonomic nervous system (including heart, blood vessels, and glands), thereby accounting for the poisoning symptoms of vomiting, abdominal cramps, nausea, salivation, and sweating. Acetylcholine is also a neurotransmitter for the somatic motor nervous system, where its accumulation resulted in poisoning symptom of involuntary muscle twitching (muscle fasciculation), convulsions, respiratory failure and coma. Intoxication of Malathion is treated by the antidote drug Oxime that reactivates the acetylcholine esterase and by intravenous injection of the anticholinergic (antimuscarinic) drug atropine to antagonize the action of the excessive amounts of acetylcholine [El-Metwally et al. 2010].

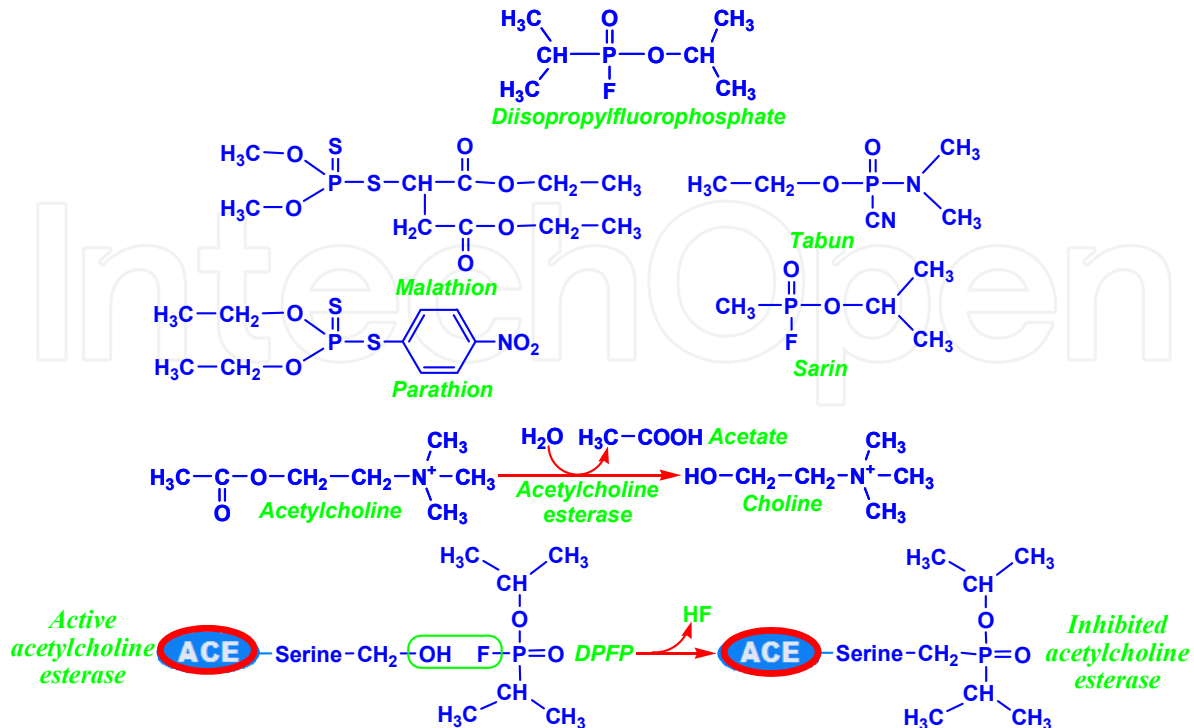


Fig. 2. Organophosphorus compounds and the suicidal irreversible mechanism-based inhibition of the enzyme acetylcholinesterase by diisopropylfluorophosphate. Malathion and parathion are organophosphorus insecticides. The nerve gases Tabun and Sarin are other organophosphorus compounds.

Another example of irreversible inhibition is iodoacetate inhibition of the glycolytic glyceraldehyde-3-phosphate dehydrogenase (GPD). Iodoacetate is a sulfhydryl compound that covalently alkylates and blocks the sulfhydryl group at the active site of the enzyme. Iodoacetate also inhibits other enzymes with -SH at the active site (Figure 3).

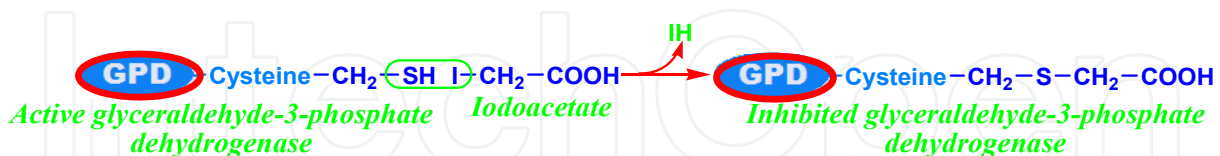


Fig. 3. The suicidal irreversible mechanism-based inhibition of the enzyme glyceraldehyde-3-phosphate dehydrogenase by iodoacetate.

- Allopurinol - the anti-gout drug - is a suicidal irreversible mechanism-based inhibitor of the enzyme xanthine oxidase that works as oxidase or dehydrogenase. The enzyme commits suicide by initial activating allopurinol into a transition state analog - oxypurinol - that bind very tightly to molybdenum-sulfide (Mo-S) complex at the active site (Figure 4). This enzyme accounts for the human dietary requirement for the trace mineral molybdenum. The molybdenum-sulfide (Mo-S) complex binds the substrates and transfers the electrons required for the oxidation reactions.

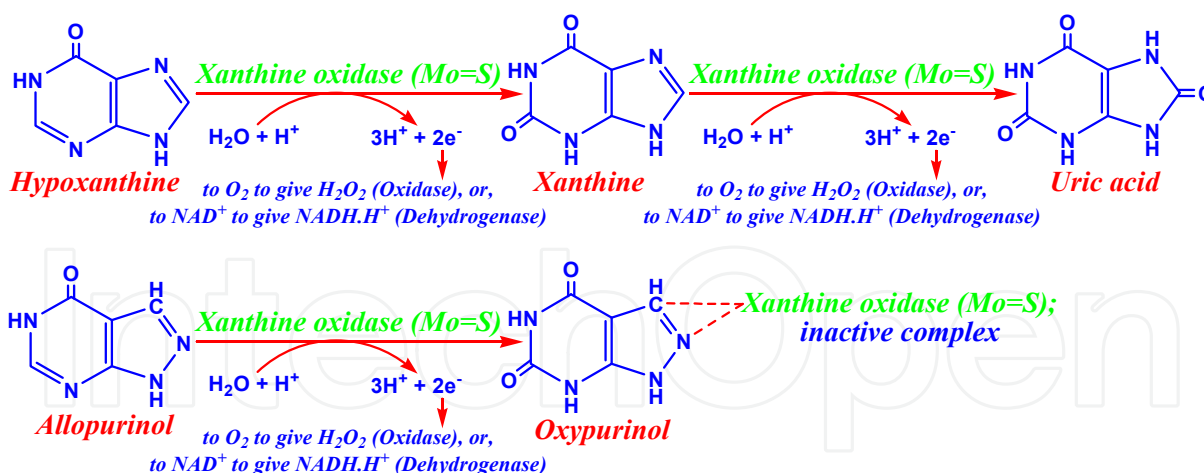


Fig. 4. The suicidal irreversible mechanism-based inhibition of the enzyme xanthine oxidase by allopurinol.

- Guanosine analogue antiviral drug aciclovir - acycloguanosine (2-amino-9-((2-hydroxyethoxy)methyl)-1H-purin-6(9H)-one), as one of the most commonly-used antiviral drugs, it is primarily used for the treatment of herpes simplex and herpes zoster (shingles) viral infections. Aciclovir (see Figure 5) started a new era in antiviral therapy, as it is extremely selective and low in cytotoxicity. Aciclovir as a prodrug differs from previous nucleoside analogues in that it contains only a partial nucleoside structure: the sugar ring is replaced by an open-chain structure. It is selectively converted into acyclo-guanosine monophosphate (acyclo-GMP) by viral thymidine kinase, which is far more effective (3000 times) in phosphorylation than cellular thymidine kinase. Subsequently, the monophosphate form is further phosphorylated into the active triphosphate form, acyclo-guanosine triphosphate (acyclo-GTP), by cellular kinases. Acyclo-GTP is a very potent inhibitor of viral DNA polymerase; it has approximately 100 times greater affinity for viral than cellular polymerase. As a substrate, acyclo-GTP is incorporated into viral DNA, resulting in chain termination. Acyclo-GTP is fairly rapidly metabolized within the cell, possibly by cellular phosphatases.

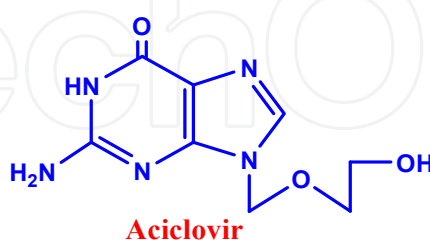


Fig. 5. Aciclovir; the prodrug for the suicidal irreversible inhibition of the viral DNA polymerase.

- The antibiotic penicillin is another transition state analog suicidal inhibitor that binds irreversibly covalently to serine at the active site of the bacterial enzyme glycopeptide transpeptidase. The enzyme is a serine protease required for synthesis of the bacterial cell wall and is essential for bacterial growth and survival. It normally cleaves the

peptide bond between two D-alanine residues in a polypeptide. Penicillin structure contains a strained peptide bond within the β -lactam ring that resembles the transition state of the normal cleavage reaction, and thus penicillin binds very readily to the enzyme active site. The partial reaction to cleave the imitating penicillin peptide bond activates penicillin to bind irreversibly covalently to the active site serine (Figure 6).

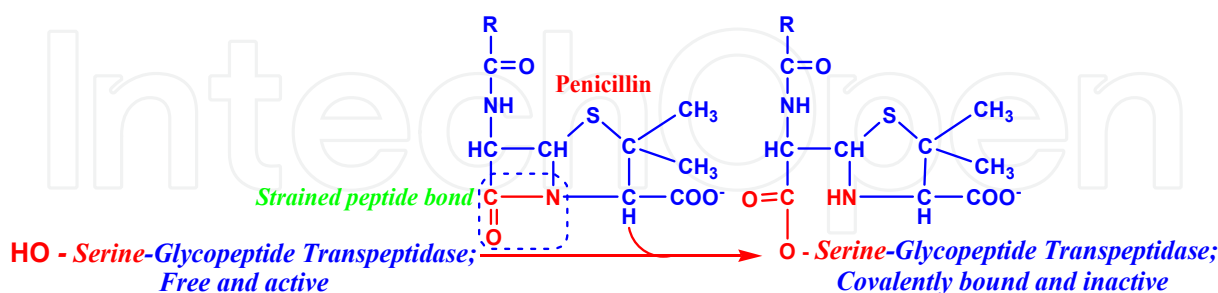


Fig. 6. The suicidal irreversible mechanism-based inhibition of the bacterial enzyme glycopeptide transpeptidase by the antibiotic penicillin.

- *Aspirin (acetylsalicylic acid)* provides an example of a pharmacologic drug that exerts its effect through the covalent acetylation of an active site serine in the enzyme cyclooxygenase (prostaglandin endoperoxide synthase). Aspirin resembles a portion of the prostaglandin precursor that is a physiologic substrate for the enzyme.
- *Heavy metal toxicity* is caused by tight binding of a metal such as mercury, lead, aluminum, or iron, to a functional group at the active site of an enzyme. At high concentration of the toxin, heavy metals are relatively nonspecific for the enzymes they inhibit and inhibit a large number of enzymes. For example, it is impossible to specify which particular enzyme is implicated in mercury toxicity that binds reactive -SH groups at the active sites. Lead developmental and neurologic toxicity is caused by its ability to replace the normal functional metal in target enzymes; particularly Ca^{2+} in important enzymes, e.g., Ca^{2+} -calmodulin and protein kinase C. Because of their irreversible effect, heavy metals are routinely use as fixatives in histological preparations.

Kinetically, the irreversible inhibitors decrease the concentration of active enzyme and in turn decrease the maximum possible concentration of ES complex with ultimate reduction in the reaction rate of the inactivated individual enzyme molecules. The remaining unmodified enzyme molecules are normally functional considering their turnover number and K_m . For example: Natural poisons act as Enzyme inhibitors and Inhibitory enzymes

In nature, animals and plants are rich in poisons as secondary metabolites, peptides and proteins that can act as enzyme inhibitors. Natural toxins are small organic molecules and act as natural inhibitors for enzymes in metabolic pathways and non-catalytic proteins.

- Neurotoxins are natural inhibitors, toxic but valuable for therapeutic uses at lower doses. For example, glycoalkaloids from Solanaceae family plants (potato, tomato and eggplant) act as acetylcholinesterase inhibitors to increase the acetylcholine neurotransmitter, muscular paralysis and then death. Many natural toxins are secondary metabolites. These neurotoxins also include peptides and proteins. An example of a toxic peptide is alpha-amanitin, found in death cap mushroom and acts

potent enzyme inhibitor, in this case preventing the RNA polymerase II enzyme from transcribing DNA. The algal toxin microcystin is also a peptide and is an inhibitor of protein phosphatases. This toxin can contaminate water supplies after algal blooms and is a known carcinogen that can also cause acute liver hemorrhage and death at higher doses. Proteins can also be natural poisons or antinutrients, such as the trypsin inhibitors that are found in some legumes, potato, and tomato. Several invertebrate and vertebrate venoms contain protein and peptide enzyme inhibitors for, e.g., plasmin, renin and angiotensin converting enzymes. Inhibitory enzymes are enzymes that irreversibly inhibit other enzymes by chemically modifying them. In the broad sense, they include all proteases and lysosomal enzymes. Some of them are toxic plant products, e.g., ricin, a glycosidase that is an extremely potent protein toxin found in castor oil beans. It inactivates ribosomes by cleavage the eukaryotic 28S rRNA and reduces protein synthesis and a single molecule of ricin is enough to kill a cell.

4. Reversible inhibition

Reversible inhibitors may be competitive, noncompetitive, or uncompetitive inhibitors relative to a particular substrate. Products of enzymatic reactions are reversible inhibitors of the enzymes. A decrease in the rate of an enzyme caused by the accumulation of its own product plays an important role in the balance and most economic usage of metabolic pathways. It prevents one enzyme in a sequence of reactions from generating a new product more than the capacity of the next enzyme in that sequence, e.g., inhibition of hexokinase by accumulating glucose 6-phosphate.

With the reduction in the inhibitor concentration, the enzyme activity is regenerated due to the non-covalent association and the reversible equilibrium with the enzyme. The equilibrium constant for the dissociation of enzyme inhibitor complexes is known as K_i that equals $[E][I]/[EI]$ [Cheng et al. 1973]. The inhibition effect of K_i on the reaction kinetics is reflected on the normal K_m and or V_{max} observed in Lineweaver-Burk plots; in a pattern dependent on the type of the inhibitor [Nelson et al. 2008]. The inhibitor is removable by several ways. *The three common types of reversible inhibitions are:*

- Competitive reversible inhibition.
- Uncompetitive reversible inhibition.
- Mixed reversible inhibition (or non-competitive inhibition).

4.1 Competitive reversible inhibition

The competitive inhibitor is structurally related to the substrate and binds reversibly at the active site of enzyme and occupies it in a mutually exclusive manner with the substrate. Therefore, the competitive inhibitor competes with the substrate for the active site. The binding is mutually exclusive because of their free competition. According to the law of mass action, relatively higher inhibitor concentration prevents the substrate binding. Since the reaction rate is directly proportional to $[ES]$, reduction in ES formation for EI formation lowers the rate. Increasing substrate towards a saturating concentration alleviates competitive inhibition. In the time enzyme-substrate complex releases the free enzyme and a product, the enzyme-inhibitor complex does release neither free enzyme nor a product.

Reversible inhibition is of short duration in the biological system because it depends on substrate availability and/or rate of the catabolic clearance of the inhibitor (Figure 7).

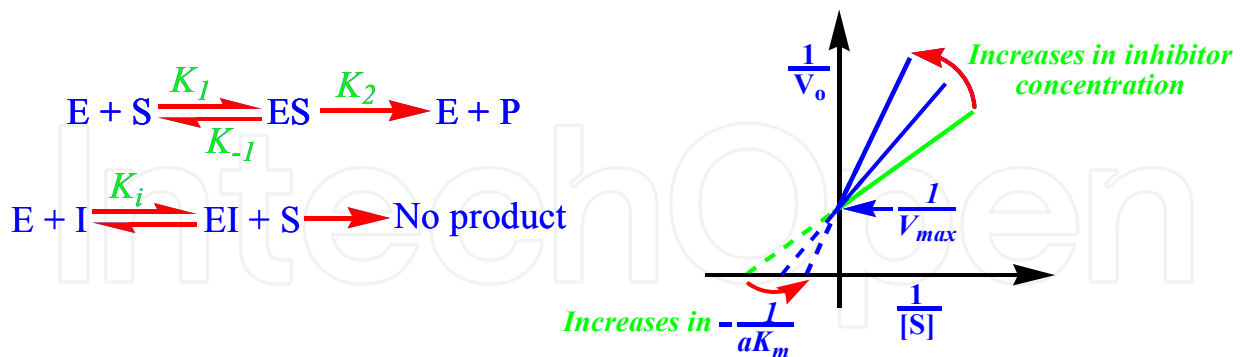


Fig. 7. The equation and the effect of the competitive inhibitor on the double reciprocal plot of the substrate-reaction rate relationship.

Kinetically, the inhibitor (I) binds the free enzyme reversibly to form enzyme inhibitor complex (EI) that is catalytically inactive and cannot bind the substrate. The competitive inhibitor reduces the availability of free enzyme for the substrate binding. Thus, the K_m of the normal reaction is increased to a new K_m (aK_m) as a function of the inhibitor concentration (expressed in the "a" factor - apparent K_m in presence of the inhibitors), where the substrate concentration at $V_o = \frac{1}{2} V_{max}$ is equal to aK_m . The "a" can be calculated from the change in the slope of the line at a given inhibitor concentration;

$$a = 1 + \frac{[I]}{K_i}, \text{ where, } K_i = \frac{[E][I]}{[EI]} \quad (1)$$

Therefore, competitive inhibitors do not affect the turnover number (active site catalysis per unit time) or the efficiency of the enzyme because once enzyme is free, enzyme behaves normally. The Michaelis-Menten equation for competitive inhibitors becomes

$$V_o = \frac{V_{max}[S]}{aK_m + [S]} \quad (2)$$

Consequently, the double reciprocal form of the equation is also modified so as the line slope becomes $\frac{aK_m}{V_{max}}$ and the intercept with y-Axis stays at $\frac{1}{V_{max}}$ but the intercept with the x-axis at $-\frac{1}{aK_m}$ will differ according to the concentration of the competitive inhibitor.

The later property is characteristic for competitive inhibitors.

Examples include the classical competitive inhibitory effect of *malonic acid* on succinate dehydrogenase (SD) of the Krebs' cycle that reversibly dehydrogenates succinate into fumarate. Other less potent competitive inhibitors of succinate dehydrogenase include; oxalate, glutamate and oxaloacetate. The common molecular geometric feature of these compounds is the presence of two negatively charged $-COOH$ groups suggesting that the active site of the flavoprotein SD has specifically positioned two positively charged binding groups (Figure 8).

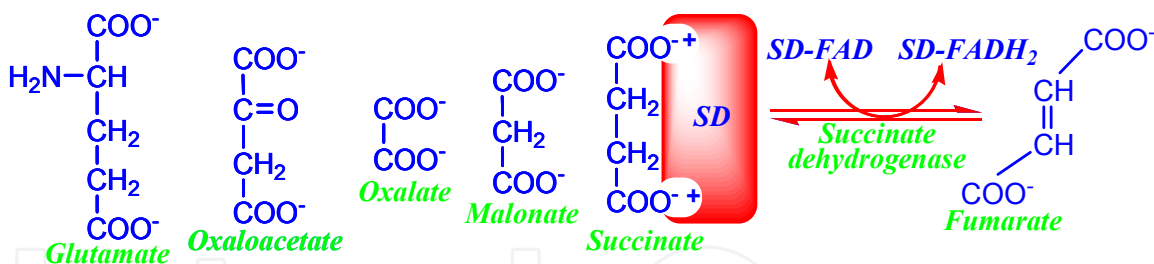


Fig. 8. The substrate and different competitive inhibitors of succinate dehydrogenase (SD).

Methotrexate - competitive inhibitor of dihydrofolate reductase (DHFR) is another example. The drug is used as anticancer antimetabolite chemotherapy particularly for pediatric leukemia. It hinders the availability of tetrahydrofolate as a carrier for one-carbon moieties important for anabolic pathways -particularly synthesis of purine nucleotides for DNA replication (Figure 9).

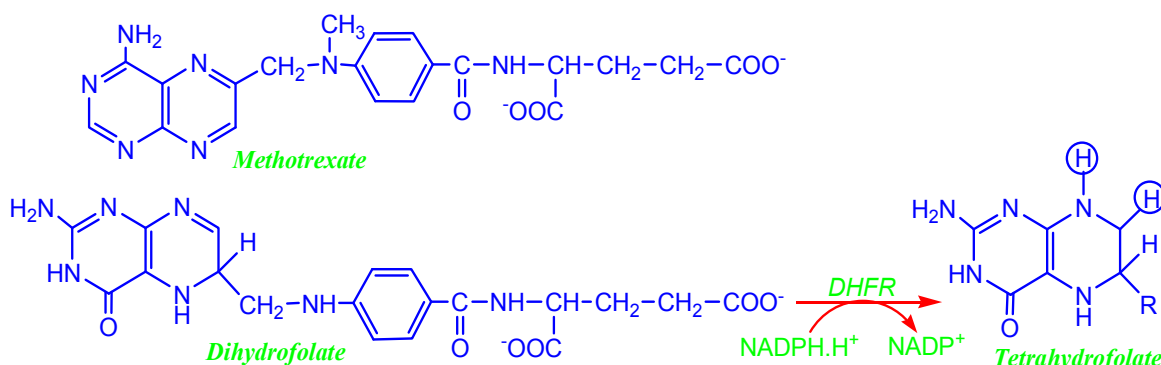


Fig. 9. The substrate and methotrexate as a competitive inhibitor for dihydrofolate reductase.

Sulfanilamides - the simplest form of Sulfa drugs - were among earliest antibacterial chemotherapeutic drugs classified as enzyme inhibitors. They are competitive inhibitors of the bacterial folic acid synthesizing enzyme system from *p*-aminobenzoic acid. Bacteria cannot absorb pre-made folate that is necessary to be synthesized *de novo*. Structural similarity of sulfanilamide (and other sulfas derived from it) to *p*-aminobenzoic acid made them competitive inhibitors to the enzyme (Figure 10).

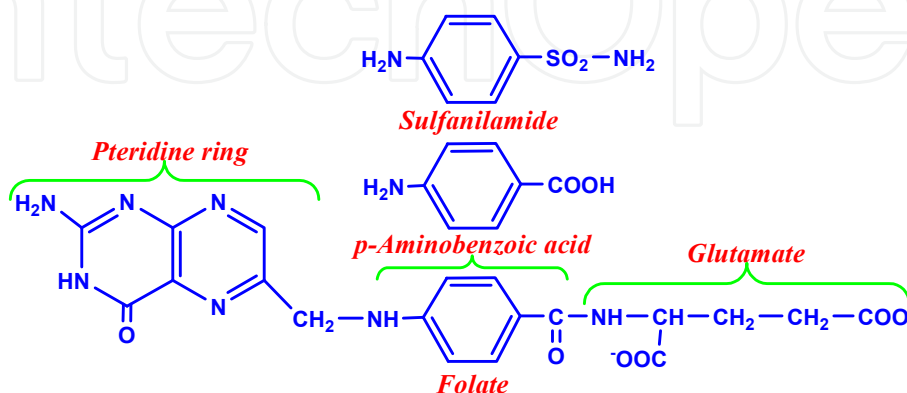


Fig. 10. The *p*-aminobenzoic acid substrate and sulfanilamide as a competitive inhibitor during the bacterial folate synthesis.

Male erectile impotence was a major medical problem. Now a group of chemicals with molecular structural similarity to cGMP is promising that competitively inhibit the cGMP-phosphodiesterase-5. They include sildenafil citrate (Viagra; Figure 11), vardenafil (Levitra) and tadalafil (Cialis). The inhibition of this enzyme that has a limited tissue distribution including the penile cavernous tissue spares cGMP. Accumulation of cGMP leads to smooth muscle relaxation (vasodilation) of the intimal cushions of the helicine arteries, resulting in increased inflow of blood and an erection.

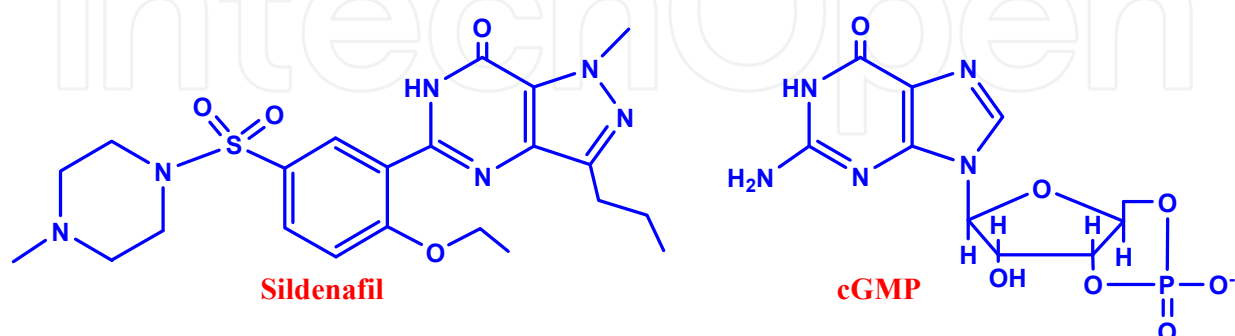


Fig. 11. The cGMP substrate and sildenafil a competitive inhibitor of the cGMP-phosphodiesterase-5.

Another example of these substrate mimics competitive inhibitors are the peptide-based protease inhibitors, a very successful class of antiretroviral drugs used to treat HIV, e.g., ritonavir that contains three peptide bonds (see Figure 12).

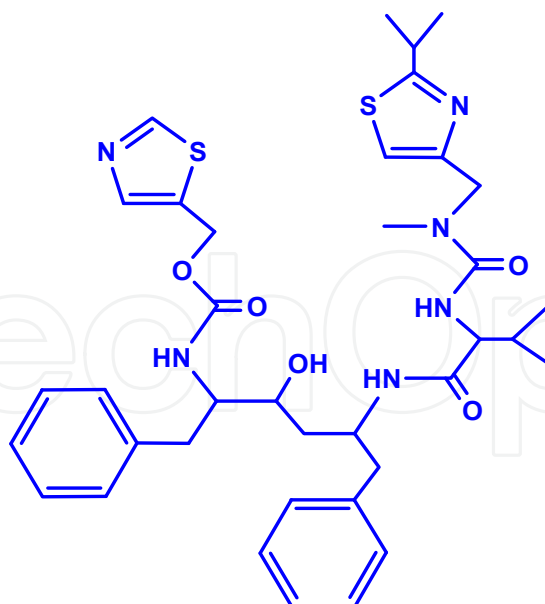


Fig. 12. The peptide-based competitive protease inhibitor ritonavir.

Reversible competitive inhibitors of acetylcholinesterase, such as edrophonium, physostigmine, and neostigmine, are used in the treatment of myasthenia gravis and in anesthesia. The carbamate pesticides are also examples of reversible acetylcholinesterase inhibitors.

4.2 Uncompetitive reversible inhibition

Uncompetitive inhibitor has no structural similarity to the substrate. It may bind the free enzyme or enzyme substrate complex that exposes the inhibitor binding site (ESI). Its binding, although away from the active site, causes structural distortion of the active and allosteric sites of the complexed enzyme that inactivates the catalysis. This leads to a decrease in both K_m and V_{max} . Increasing substrate towards a saturating concentration does not reverse this type of inhibition and reversal requires special treatment, e.g., dialysis. This type of inhibition is also encountered in multi-substrate enzymes, where the inhibitor competes with one substrate (S2) to which it has some structural similarity and is uncompetitive for the other (S1). The reaction without the inhibitor would be; $E + S_1 \leftrightarrow ES_1 + S_2 \leftrightarrow ES_1S_2 \Rightarrow E + Ps$ and with uncompetitive inhibitor becomes; $E + S_1 \leftrightarrow ES_1 + I \Rightarrow ES_1I$ (prevents S2 binding) \Rightarrow **no product**. It is a rare type and the inhibitor may be the reaction product or a product analog.

Kinetically, uncompetitive inhibition modifies the Michaelis-Menten equation by (a') factor that proportionates with the inhibitor concentration to be:

$$V_o = \frac{V_{max}[S]}{K_m + a'[S]} \quad (3)$$

and in the double-reciprocal equation to be:

$$\frac{1}{V_o} = \frac{a'}{V_{max}} + \frac{K_m}{V_{max}} \times \frac{1}{[S]} \quad (4)$$

while y-intercept is at $\frac{a'}{V_{max}}$ and x-intercept is at $-\frac{a'}{K_m}$, whereas, the line slope stays $\frac{K_m}{V_{max}}$.

This gives a number of lines in the Lineweaver-Burk plot that are parallel to the normal line with decreased $1/V_{max}$ and $-a'/K_m$ proportional to concentrations of the uncompetitive inhibitor. The later is characteristic to uncompetitive inhibition (Figure 13).

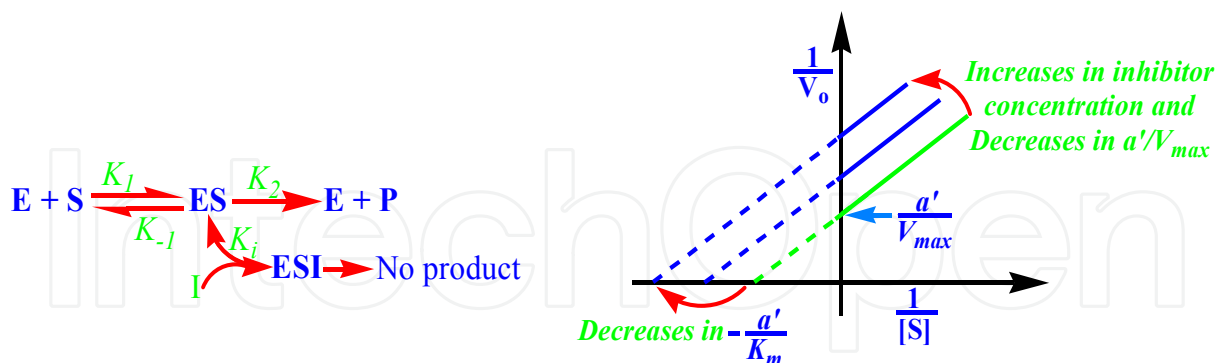


Fig. 13. The equation and the effect of the uncompetitive inhibitor on the double reciprocal plot of the substrate-reaction rate relationship.

Uncompetitive reversible inhibition is rare, but may occur in multimeric enzymes. Examples of uncompetitive reversible inhibitors include; inhibition of lactate dehydrogenase by oxalate; inhibition of alkaline phosphatase (EC 3.1.3.1) by L-phenylalanine, and, inhibition of the key regulatory heme synthetic enzyme; δ -aminolevulinate synthase and dehydratase and heme synthetase by heavy metal ion, e.g., lead. Heavy metals, e.g., lead, form mercaptides with -SH at the active site of the enzyme ($2 R-SH + Pb \Rightarrow R-S-Pb-S-R + 2H$).

Oxidizing agents, e.g., ferricyanide also oxidizes -SH into a disulfide linkage ($2 \text{R-SH} \Rightarrow \text{R-S-S-R}$). Reversion here requires treatment with reducing agents and/or dialysis.

4.3 Mixed (noncompetitive) inhibition

The mixed type inhibitor does not have structural similarity to the substrate but it binds both of the free enzyme and the enzyme-substrate complex. Thus, its binding manner is not mutually exclusive with the substrate and the presence of a substrate has no influence on the ability of a non-competitive inhibitor to bind an enzyme and *vice versa*. However, its binding - although away from the active site - alters the conformation of the enzyme and reduces its catalytic activity due to changes in the nature of the catalytic groups at the active site. EI and ESI complexes are nonproductive and increasing substrate to a saturating concentration does not reverse the inhibition leading to unaltered K_m but reduced V_{max} . Reversal of the inhibition requires a special treatment, e.g., dialysis or pH adjustment. Some classifications differentiate between non-competitive inhibition as defined above and *mixed inhibition* in that the EIS-complex has residual enzymatic activity in the mixed inhibition.

Kinetically, mixed type inhibition causes changes in the Michaelis-Menten equation so as

$$V_o = \frac{V_{max} [S]}{aK_m + a'[S]} \quad (5)$$

Mixed type inhibition - as the name imply - has a change in the denominator with K_m modified by factor (a) as in competitive inhibition, and $[S]$ modified by factor (a') as in uncompetitive inhibition. In the double reciprocal equation 6,

$$\frac{1}{V_o} = \frac{a'}{V_{max}} + \frac{aK_m}{V_{max}} \times \frac{1}{[S]} \quad (6)$$

A line slope is $\frac{aK_m}{V_{max}}$, and the intercept with y-axis is at $\frac{a'}{V_{max}}$ and with x-axis is at $\frac{a'}{aK_m}$. This results in progressive decreases in V_{max} and progressive increases in K_m proportional to the increase in the mixed inhibitor concentration. The double reciprocal plot shows a number of lines reflecting decreases in V_{max} /increases in K_m but their intercept is to the left of the y-axis. Mixed type inhibitor would be called non-competitive only if $[a = a']$, where, it will only lower V_{max} without affecting the K_m (Figure 14).

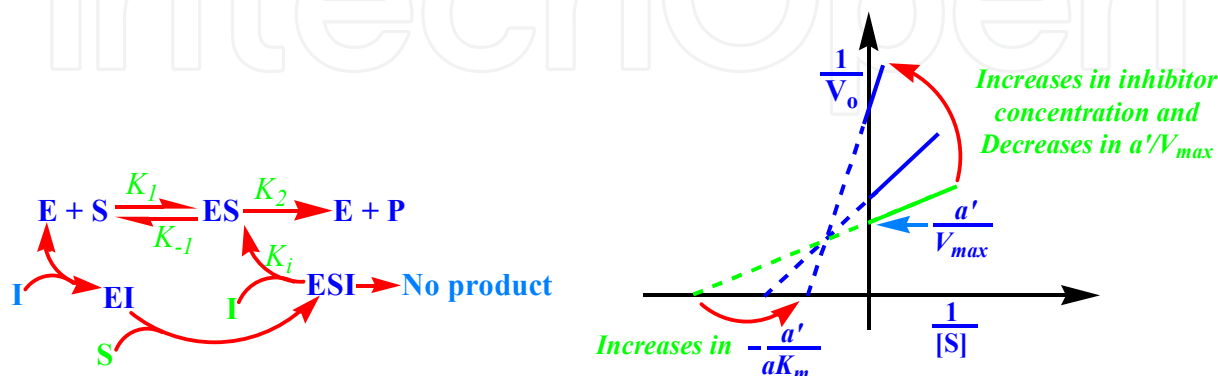


Fig. 14. The equation and the effect of the mixed type (noncompetitive) inhibitor on the double reciprocal plot of substrate-reaction rate relationship.

Examples of noncompetitive inhibitors are mostly poisons because of the crucial role of the targeted enzymes. Cyanide and azide inhibits enzymes with iron or copper as a component of the active site or the prosthetic group, e.g., cytochrome c oxidase (EC 1.9.3.1). They include the inhibition of an enzyme by hydrogen ion at the acidic side and by the hydroxyl ion at the alkaline side of its optimum pH. They also include inhibition of; carbonic anhydrase by acetazolamide; cyclooxygenase by aspirin; and, fructose-1,6-diphosphatase by AMP. Cyanide binds to the Fe^{3+} in the heme of the cytochrome aa_3 component of cytochrome c oxidase and prevents electron transport to O_2 . Mitochondrial respiration and energy production cease, and cell death rapidly occurs. The central nervous system is the primary target for cyanide toxicity. Acute inhalation of high concentrations of cyanide (e.g., smoke inhalation during a fire and automobile exhaust) provokes a brief central nervous system stimulation rapidly followed by convulsion, coma, and death. Acute exposure to lower amounts can cause lightheadedness, breathlessness, dizziness, numbness, and headaches. Cyanide is present in the air as hydrogen cyanide (HCN), in soil and water as cyanide salts (e.g., NaCN), and in foods as cyanoglycosides. Comparison of the three types of the reversible enzyme inhibitors is presented in Table 1.

In a special case, the mechanism of *partially competitive inhibition* is similar to that of non-competitive, except that the EIS complex has catalytic activity, which may be lower or even higher (partially competitive activation) than that of the enzyme-substrate (ES) complex. This inhibition typically displays a lower V_{\max} , but an unaffected K_m value. We compare three main types of inhibitors in terms of reaction properties as shown in Table 1 and Figure 15.

Competitive inhibitor	Uncompetitive inhibitor	Mixed (noncompetitive inhibitor)
<ul style="list-style-type: none"> • The inhibitor binds the catalytic/substrate binding site. • It competes with substrate for binding. • Inhibition is reversible by increasing substrate concentration. • V_{\max} constant, the substrate concentration has to be increased as reflected on increased K_m. 	<ul style="list-style-type: none"> • Substrate binding exposes the inhibitor binding site away from the catalytic/substrate binding site. • Increasing substrate concentration does not reverse the inhibition. • The inhibited reaction rate parallel the normal one as reflected on decreased both V_{\max} and K_m. 	<ul style="list-style-type: none"> • The inhibitor binds each of the free enzyme and the substrate-enzyme complex away from the catalytic/substrate binding site. • Increasing substrate concentration does not reverse the inhibition. • Only V_{\max} is decreased proportionately to inhibitor concentration, • K_m is unchanged since increasing substrate concentration is ineffective.

Table 1. Comparison of the different types of reversible inhibition is shown in Table with a quick view of mechanism in sketches as below.

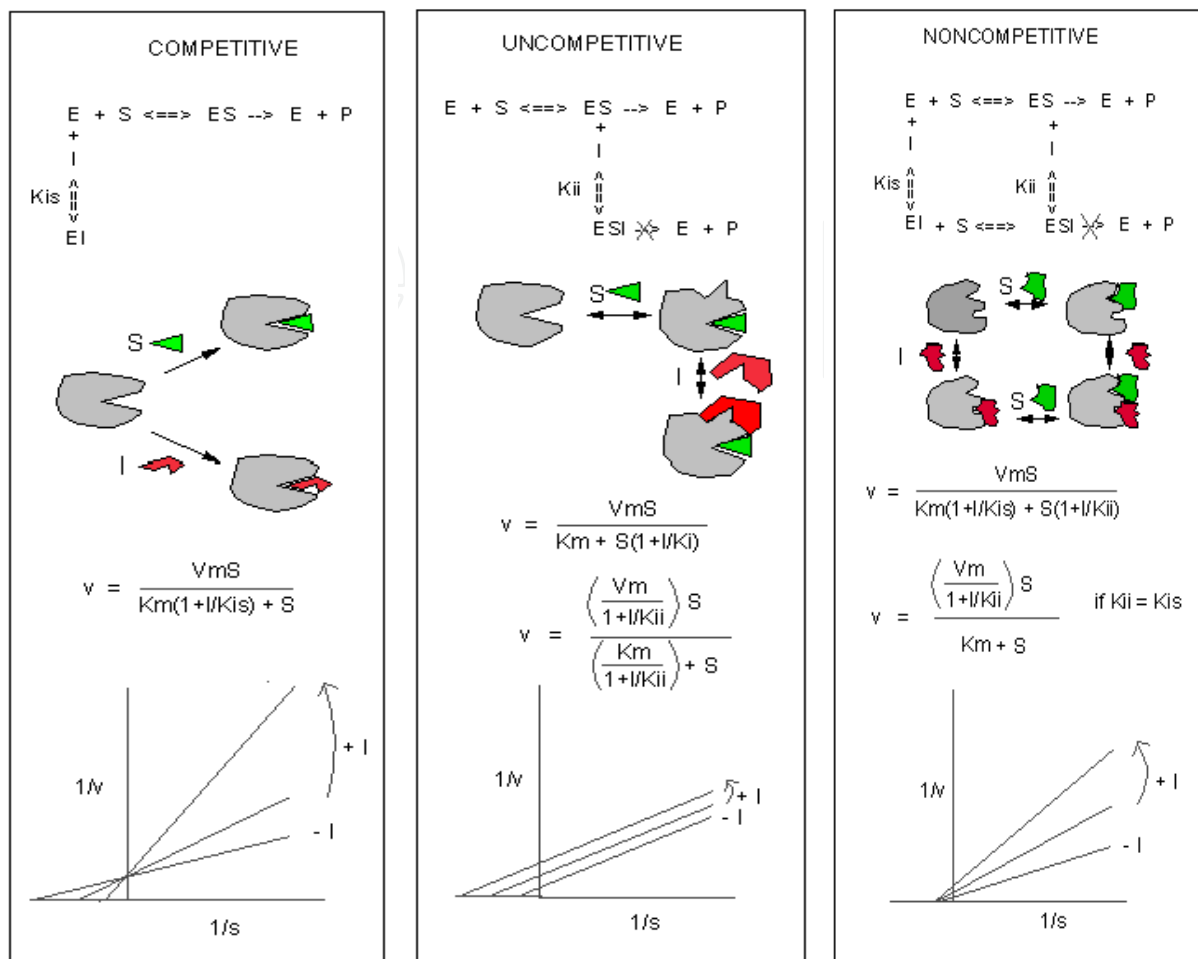


Fig. 15. Sketch of three different enzyme inhibition by competitive, uncompetitive and noncompetitive types are shown with illustration of enzyme-substrate or inhibitor binding, kinetics and graphs.

In last decade, role of membrane receptors was explored in relation with enzyme inhibition. Membrane receptors or transmembrane proteins bind with natural ligands such as hormones, neurotransmitters in tissue membranes. Receptor-ligand binding modulates the binding of drugs with enzyme. Such ligand binding behavior also influences the analysis of competitive, uncompetitive and noncompetitive inhibition by biological effect of prodrugs on enzymes. It usually involves a shape change in the receptor, a transmembrane protein, which activates intracellular activities. The bound receptor usually does not directly express biological activity, but initiates a cascade of events which leads to expression of intracellular activity. However, occupied receptor actually expresses biological activity itself. For example, the bound receptor can acquire enzymatic activity, or become an active ion channel with similar competitive, noncompetitive behavior. Drugs targeted to membrane receptors can have biological effects similar to the natural ligands, they are called **agonists**, or conversely they may inhibit the biological activity of the receptor, they are called **antagonists** [Jakobowski 2010a].

4.4 Agonist

An **agonist** or test drug or substrate is similar to natural ligand and binds with receptor to produce a similar biological effect as the natural ligand. Agonist binds at the same binding site in competition with natural ligand to show full or partial response. So, it is called **partial agonist**. If receptor has a basal (or constitutive) activity in the absence of a bound ligand, it is called **inverse agonist**. If either the natural ligand or an agonist binds to the receptor site, the basal activity is increased. If an inverse agonist binds, the activity is decreased. Ro15-4513 and benzodiazepines (Valium) bind with the GABA receptor. As a result, GABA receptor is "activated" to become a ion channel allowing the inward flow of Cl^- into a neural cell, inhibiting neuron activation. Ro15-4513 binds to the benzodiazepine site, which leads to the opposite effect of valium, the inhibition of the receptor bound activity - a chloride channel as shown in Figure 16.

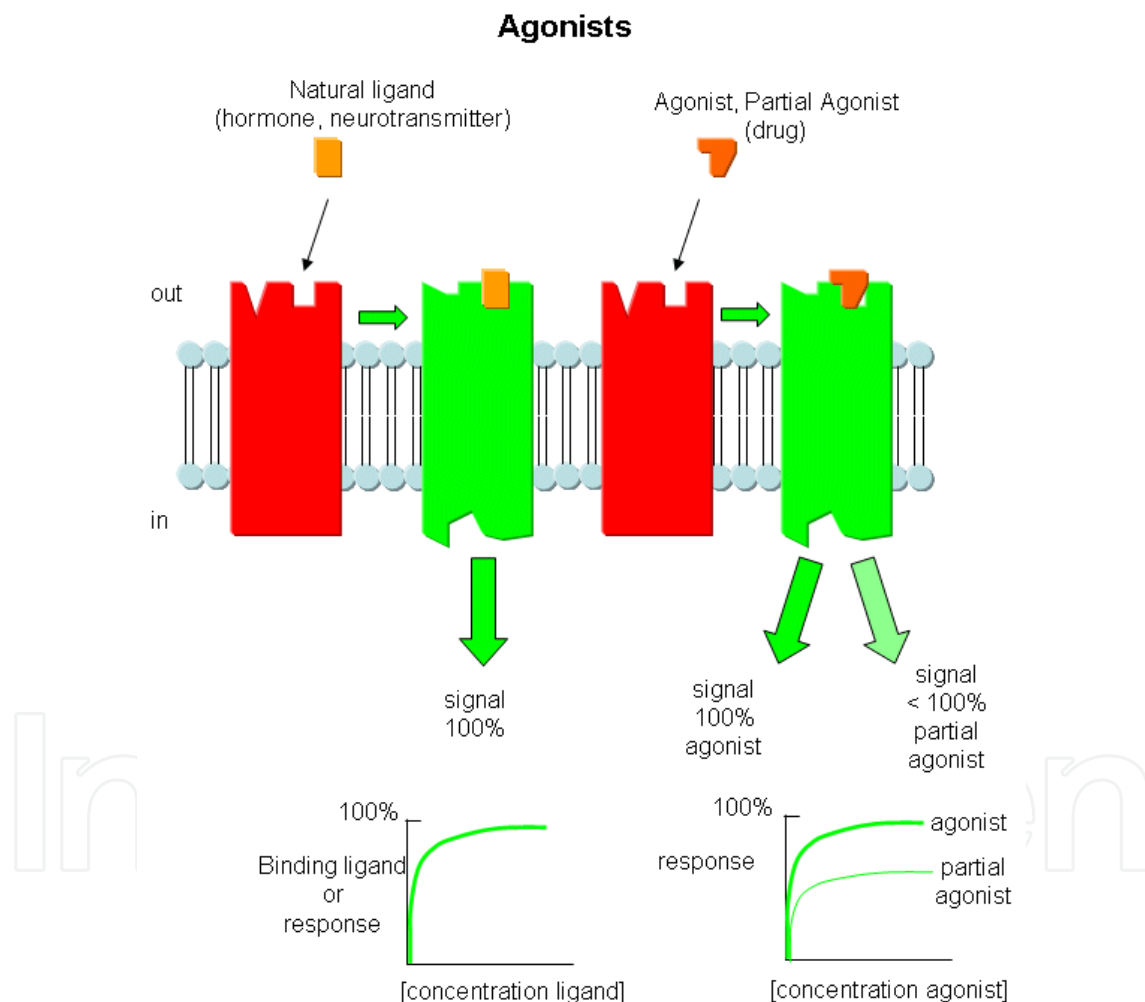


Fig. 16. A sketch is shown for membrane receptor binding with ligand (agonist) acting like as enzyme. Reproduced with permission [Jakobowski 2010a].

4.5 Antagonist

Antagonist or test inhibitor can inhibit the effects of the natural ligand (hormone, neurotransmitter), agonist, partial agonist, and inverse agonists. We can think of them as

inhibitors of receptor activity behaving as competitive, noncompetitive and irreversible antagonists as shown in Figure 17. For further details, readers are requested to read advanced text book [Nelson et al. 2008, Dixon and Webb 1979]

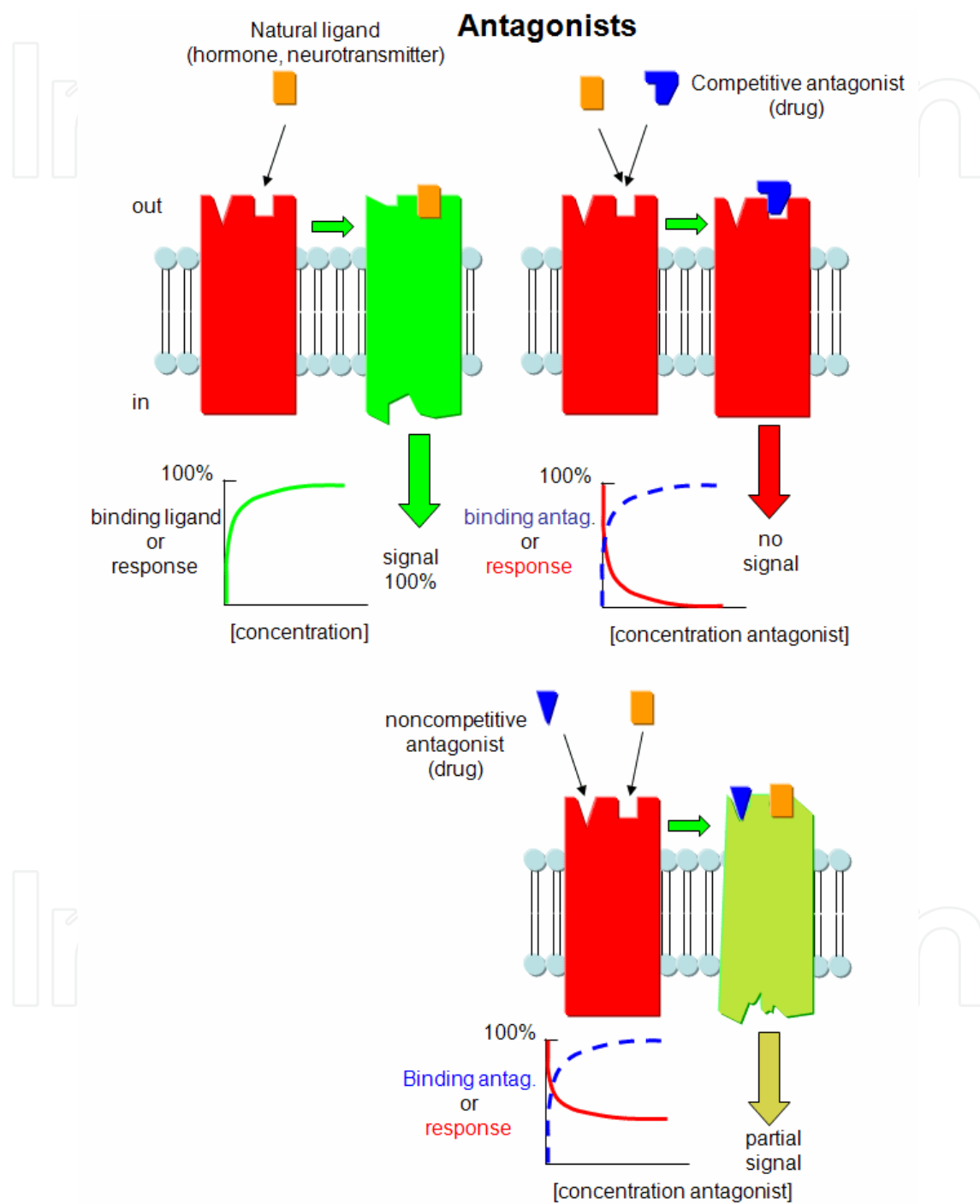


Fig. 17. Sketch is shown for membrane receptor binding with ligand (acting as agonist) and antagonist (acting as inhibitor) in competition with agonist to bind with enzyme. Reproduced with permission [Jakobowski 2010a]

5. Inhibition by physiological modulators

5.1 Temperature of reaction

Some endothermic or exothermic chemical compounds change the temperature of reaction. Enzyme reaction experiences inhibition at higher or lower than optimal physiological temperature. For example, human body optimal temperature of human body is 37 °C. For most of the enzyme reactions, enzyme activity usually increases at 0 to about 40-50 °C in the absence of catalysts. As a general rule of thumb, reaction velocities double for each increment of 10°C rise. At higher temperatures, the activity decreases dramatically as the enzyme denatures as shown in Figure 18.

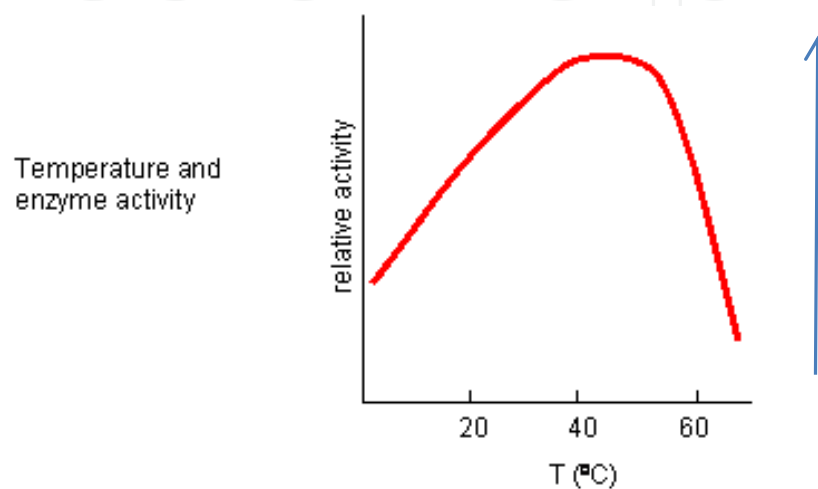


Fig. 18. Figure shows the effect of temperature change on the rate of enzyme reaction. Notice the initial rise of rate of reaction and sudden fall near to optimal temperature 37-42 °C.

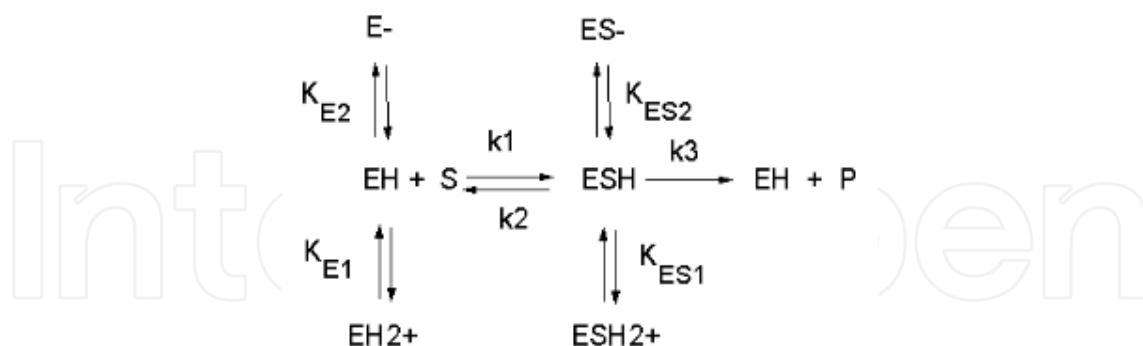
5.2 Hydrogen ion concentration or pH of reaction

Think of all the things that pH changes might affect. Many chemicals such as acids or alkaline chemical compounds if mixed in enzyme reaction medium can change the pH. As a result, reaction rate changes. It might

- affect E in ways to alter the binding of S to E, which would affect K_m
- affect E in ways to alter the actual catalysis of bound S, which would affect k_{cat}
- affect E by globally changing the conformation of the protein
- affect S by altering the protonation state of the substrate

The easiest assumption is that certain side chains necessary for catalysis must be in the correct protonation state. Thus, some side chain, with an apparent pK_a of around 6, must be deprotonated for optimal activity of trypsin which shows an increase in enzyme activity with the increase in range centered at pH 6. Which amino acid side chain would be a likely candidate to participate in enzyme inhibition? It all depends on net charge on active group of each amino acid in the active site chain. The pH of reaction thus depends on net pK_a value of amino acids and presence of acid or alkaline nature of substrate effects on enzyme kinetics by formation of EH, ESH as shown in Figure 19. It can be modeled at the chemical and mathematical level to calculate velocity(v), V_m (apparent) and K_m (apparent) as shown in Equations 7-9. Different enzymes show different behavior of enzyme catalyzed reactions such

as chymotrypsin, cholinesterase, papain, and pepsin show distinct graphs (see Figure 20). For further details, readers are requested to read text books [Nelson et al. 2008, Berg et al. 2011]



$$V = \frac{V_m \text{ app } S}{K_m \text{ app } + S} \quad (7)$$

$$V_m \text{ app} = \frac{V_m}{1 + H^+ / K_{E1} + K_{E2} / H^+} \quad (8)$$

$$K_m \text{ app} = \frac{K_m(1 + H^+ / K_{E1} + K_{E2} / H^+)}{1 + H^+ / K_{E1} + K_{E2} / H^+} \quad (9)$$

Fig. 19. Chemical equations showing the mechanism of pH effects on enzyme catalyzed reactions. Different mathematical equations 7-9 illustrate the modeling pH effects on enzyme catalyzed reactions.

5.2.1 Three dimensional nature of enzyme-inhibitor complex at enzyme active site

The role of non-covalent interactions such as hydrogen bonding, hydrophobic interaction and orientation of inhibitor and enzyme in an organized fashion was well described in classic paper [Amtul et al., 2002]. 3D nature of enzyme reaction can be understood as following. There are two sites on enzyme molecule: 1. at allosteric site, inhibitor binds with enzyme, and 2. at active site, substrate binds with enzyme. However, substrate and inhibitor interact with each other by non-covalent interactions of their chemical groups. Inhibitors interact at allosteric site and known as 'pharmacohores'. Presently, structure-based design and testing, mechanistic biological approach is a state-of-art to develop new pharmacohores. The non-covalent interactions determine the chemoselectivity of the substrate and enzymes during formation of the ESI complex. In other words, ESI complex provides enzyme as a platform to perform catalysis. 3D geometrical shape and topology of active site match with orientation of chemical groups in substrate molecule that fit together in a 'lock and key' arrangement. Several possibilities happen to make enzyme-inhibitor complexes such as bidentate, tri-, tetra- and polydentate, trigonal, pyramidal, tetrahedral, polyhedral charge transfer complexes due to co-ordinate interactions between metallic co-factor with hydrophilic groups on inhibitor(s). In this process, geometry of amino acid side chains at allosteric site changes due to hydrogen bonding between amino acid residues. Suboptimal

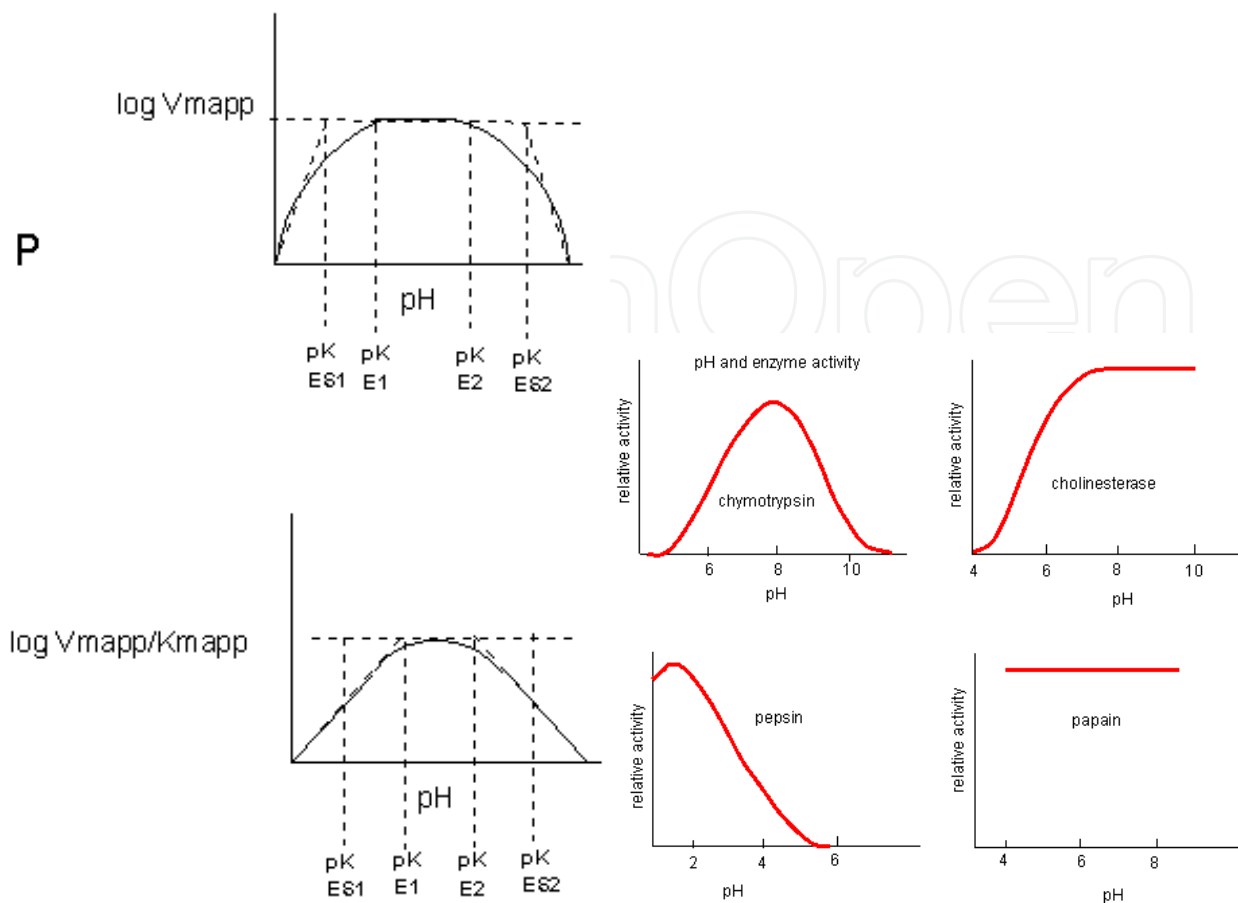


Fig. 20. Graphs of different pH effects on enzyme catalyzed reactions as $\log V_m(app)$ and $V_m/K_m(app)$ are shown on left. Different enzymes such as chymotrypsin, cholinesterase, pepsin and papain are illustrated with different rates of enzyme reaction. Reproduced with permission [Jakobowski 2010a]

interactions of metal-solvent, oxygen-water molecular bridge, free energy content loss, subunit-subunit biophysical interactions as a result play a significant role in inhibitor-enzyme complex formation and completion of enzyme catalysis.

For more details, readers are requested to read recent reference papers on 3D mechanistic studies on enzymes. Specific example on urease is cited in chapter 11 in this book. Now science is shifting to develop crystallized enzyme molecules, better structural-functional relationship in enzyme catalysis and immobilized enzyme chips.

In following description, factors are discussed on different practical considerations that influence the enzyme reaction rates, enzyme inhibition kinetics, % binding efficiency on enzyme solid support with a glimpse of known theories and concepts on real-time, cheaper, economic, user-friendly immobilized enzyme technology.

When actual and practical considerations are analyzed to work in enzyme reactor, the scenario becomes complicated. Several factors such as inhibitor chemical state, substrate structure, enzyme 3D conformation or peptide subunit interactions, physiological reaction

conditions in reactor and enzyme carrier supports also contribute in inhibition kinetics and rates of reaction to form ES,ESI and P. Every year list of new factors grows in new enzyme systems.

Author believes that more and more contributory factors introduced, will influence enzyme reaction rate kinetics and more and more additive kinetic constants are introduced with new variants to define the action of inhibitors on enzyme catalysis.

Other factors to keep in mind for new possibilities are:

1. enzyme autoinhibition and enzyme molecular structural-functional factors affecting 3D conformation of active site compatible with active groups of substrate or inhibitor
2. porosity and diffusion across the enzyme support material and availability of exposed active sites to react
3. real-time recording the instant formation of ESI or ES or EP or EI on solid phase enzyme support organic chip
4. substrate-inhibitor interactions, % binding of active site with each additive
5. computer based semi-corrected or averaged calculations of kinetic constants of inhibition kinetics
6. thermodynamic states of the enzyme reaction in reactor and fluctuating physiological and physical states of substrate, inhibitor, enzyme complexes in reactor.
7. synergy of inhibitors, substrate, subunits in enzyme on active site

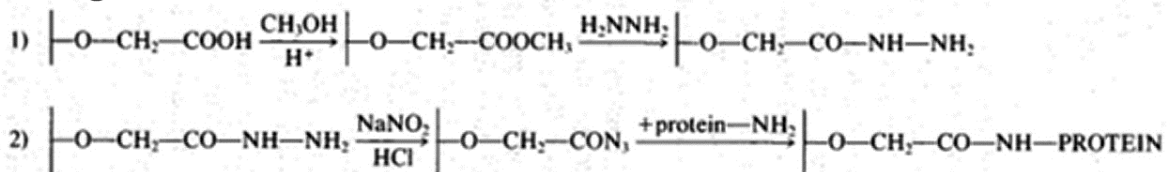
For all these factors and details, readers are expected to read advanced text books on enzyme inhibition and enzyme engineering. Readers will experience a wide variation in the scientific analysis of enzyme inhibition data in different enzyme reactors used in different studies. High efficiency with desired results of enzyme inhibitors is the new challenges to optimize reaction, scale-up, and phase out unwanted physiological factors from reaction. In following section, these issues are addressed. Author believes that above mentioned description is just iceberg from a large hidden treasure or unknown factors contributing enzyme inhibition to give desired outcome.

6. Immobilized enzyme systems

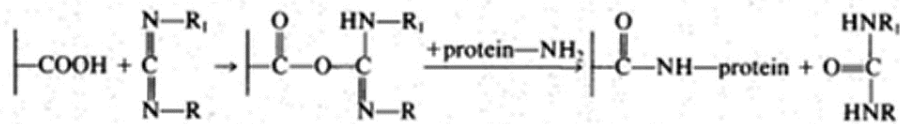
In search of economic, efficient and practical enzyme platforms to test enzyme inhibitors, new user-friendly immobilized enzyme technology is available now. It is based on principle that an enzyme molecule is contained within confined space for the purpose of retaining and re-using enzyme on solid medium in processing system or equipment. There are many advantages of immobilized enzymes and methods of immobilization such as low cost, suitability of reusable model system in membrane-bound enzymes in cell. However, some disadvantages are expansive methods of adsorption or covalent bound or matrix trapping or membrane trapping immobilization methods, low measurement of enzyme activity with mass transfer limitations. For knowledge sake, the entrapment of enzyme molecules on matrix, diffusion phenomenon and kinetics are important to understand. A brief description is given for interested readers on classic concepts and scientific basis of porous or non-porous enzyme supports, theory of enzyme immobilization and efficiency of reaction outcome. For more details of each aspect, readers are requested to read individual research papers.

Matrix entrapment is done by mixing enzyme solution with polymer fluid in matrices such as Ca-alginate, agar, polyacrylamide, collagen. **Membrane entrapment** is done by confining enzyme solutions between semi-permeable membrane hollow fibers made of nylon, cellulose, polysulfone, polyacrylate etc. Surface immobilization by **adsorption** is done by attaching enzymes on stationary solids such as alumina, porous glass, cellulose, ion-exchange resin, silica, ceramic, clay, starch etc. by physical forces keeping active sites intact. **Covalent bonding** is done by enzyme retention on support surfaces by covalent binding between functional groups such as amino, carboxylic, sulfhydryl, hydroxyl groups on the enzyme and those on the support surface keeping enzyme active site(s) free (see Figure 21) [Laidler et al. 1980].

a. Using via azide derivative



b. Using a carbodiimide



supports containing anhydrides

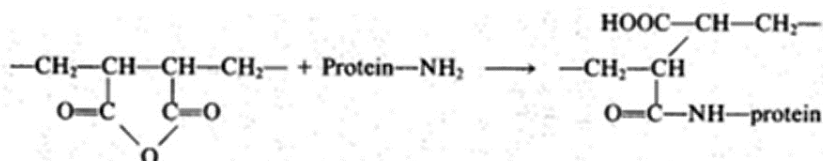


Fig. 21. Scheme of immobilization of enzyme is shown with chemical groups involved in binding of enzyme on solid surface. Reproduced with permission from reference Lieder et al.1980.

Diffusional limitations are observed to various degrees in all immobilized enzyme systems. This occurs because substrate must diffuse from the bulk solution up to the surface of the immobilized enzyme prior to reaction. The rate of diffusion relative to enzyme reaction rate determines whether limitations on intrinsic enzyme kinetics is observed or not as shown in Figures 22 [Laidler et al.1980]. However, rate of diffusion across and within matrix is determinant of immobilized enzyme reaction as shown in Figure 22 and 23.

In immobilized enzyme reaction, two major effects due to diffusion and product inhibition are first observed by Lineweaver-Burk plots in classic study [Rees, 1984]. The diffusional effects and product inhibition both influenced the shape of Lineweaver-Burk plot (see Figure 22). In case of substrate inhibition effects binding of more than one substrate molecule(s) lead to inhibition showing same type of curved Lineweaver-Burk plot as those observed for diffusional limitation and product inhibition in immobilized enzymes. Combination of these two effects lead to intermediate behavior, such as normal Michaelis-Menten kinetics as shown

in Figure 24, 25 by curves [Rees, 1984]. However, immobilized enzyme system also suffers from both diffusion and product inhibition effects. As a consequence, it is important to consider diffusion effects and product inhibition effects while extracting catalytic parameters from kinetic data for immobilized enzyme systems. Use of non-porous support in enzyme immobilization minimizes the diffusion effects to some extent.

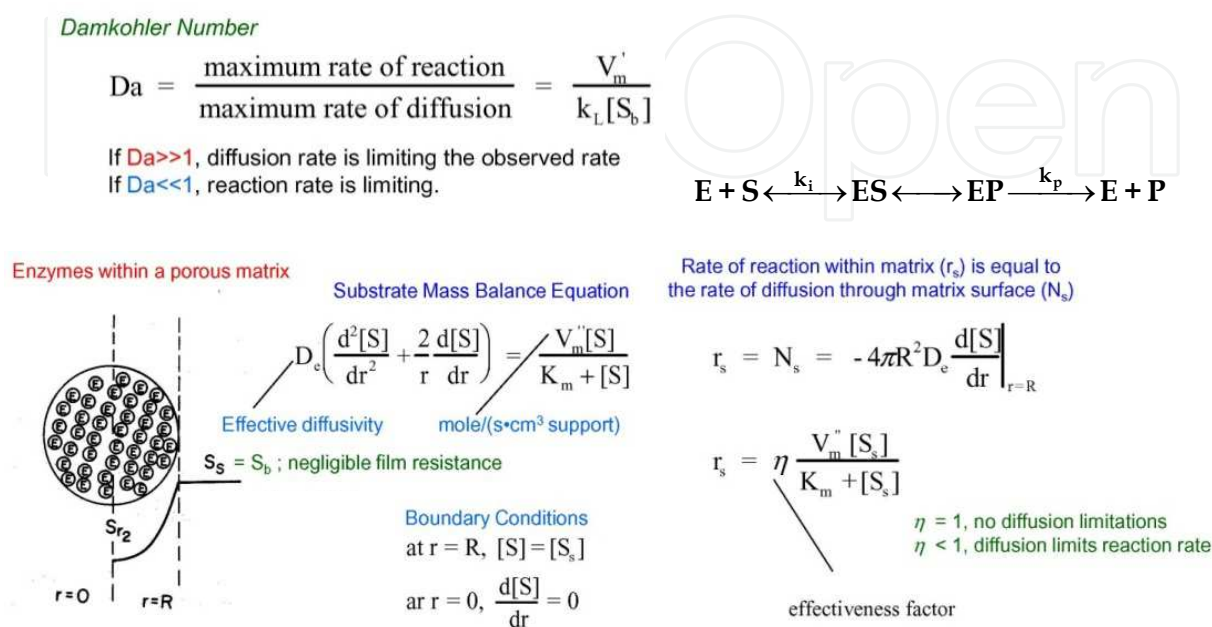


Fig. 22. A sketch of porous matrix is shown (on left) and a scheme of substrate mass balance Equation to calculate rate of immobilized enzyme reaction r_s is shown (on right)

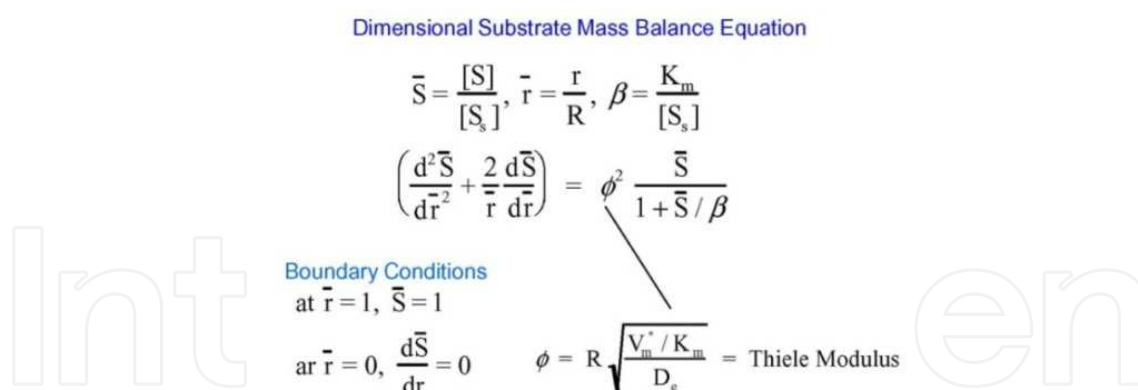


Fig. 23. A scheme of substrate mass balance is shown to calculate S with boundary conditions.

Enzyme kinetics predicts the efficiency of reaction. Kinetics of immobilized enzymes depends on conformational alterations within the enzyme due to the immobilization procedure, or the presence and nature of the immobilization support. Immobilization can greatly affect the stability of an enzyme such as any strain into the enzyme will inactivate the enzymes under denaturing conditions (e.g. higher temperatures or extremes of pH). An example of unstrained multipoint binding between the enzyme and the support to cause substantial stabilization is illustrated in Figure 20. From mechanistic standpoint, a lesser

conformational change within the protein structure will initiate enzyme inactivation. As a result, covalent immobilization processes involve an initial freely-reversible stage. Covalent links may form, break and re-form till an unstrained covalently-linked structure is created. However, additional stabilization is derived from maximum enzyme-support compatibility, least enzyme molecule interactions, least proteolytic and microbiological attacks.

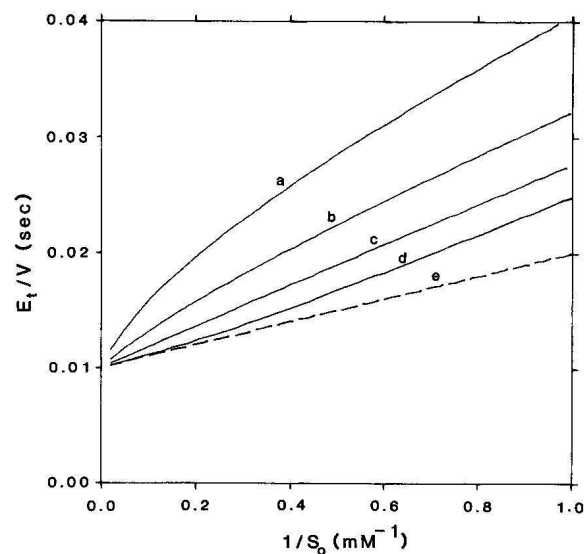


Fig. 24. Effect of one or more inhibitor molecules on enzyme kinetics and their inhibition effect dependent on $1/S_0$. Reproduced with permission from Rees et al. 1984.

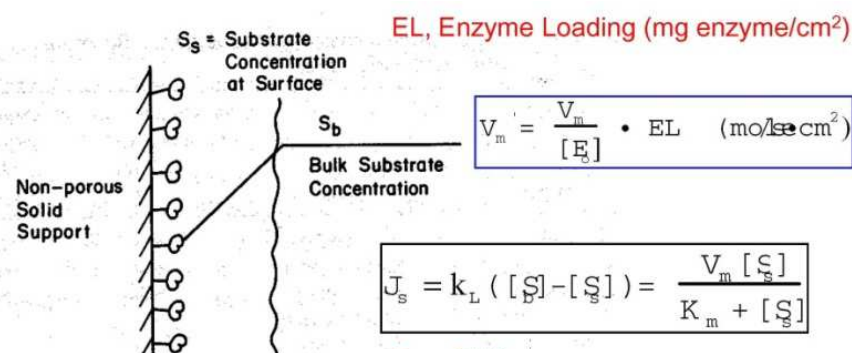


Fig. 25. A scheme of immobilized enzyme action is shown on non-porous solid support. Notice the dependence of V_m on available immobilized enzyme active sites (E_L).

The kinetic constants (e.g. K_m , V_{max}) of immobilized enzymes may be altered by the process of immobilization due to internal structural changes and restricted access to the active site. Thus, the intrinsic specificity (k/K_m) of such enzymes may well be changed relative to the soluble enzyme. An example of trypsin is illustrated in Figure 21, where the freely soluble enzyme hydrolyses fifteen peptide bonds in the protein pepsinogen but the immobilized enzyme hydrolyses only ten. The apparent value of these kinetic parameters, when determined experimentally, may differ from the intrinsic values. This fact may be due to

changes in the properties of the solution in the immediate vicinity of the immobilized enzyme, or the effects of molecular diffusion within the local environment. The relationship between these intrinsic and apparent parameters is shown below in Figure 26. Typically, nonporous microenvironment consists of the internal solution plus part of the surrounding solution which is influenced by the surface characteristics of the immobilized enzyme. Partitioning of substances occurs between these two environments. Substrate molecule (S)

Intrinsic parameters of the soluble enzyme
 ↓
 Intrinsic parameters of the immobilized enzyme
 ↓
 Apparent parameters due to partition and diffusion

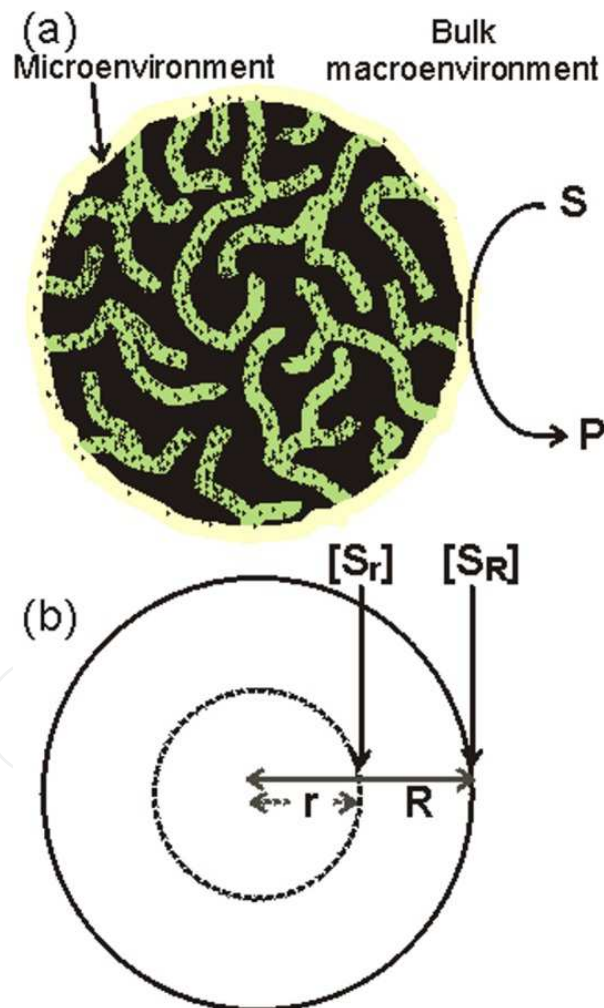


Fig. 26. A schematic cross-section of an immobilized enzyme particle (a) shows the macroenvironment and microenvironment. Triangular dots represent the enzyme molecules. Courtesy: Pangandai V. Pennirselvam, Ph.D UFRN, Lagoa Nova-Natal/RN Campus Universitário. North East, Brazil.

diffuses through the surrounding layer (external transport) in order to reach the catalytic surface and gets converted to product (P). In order for all immobilized enzyme to be utilized, substrate must diffuse within the pores in the surface of the immobilized enzyme particle (internal transport) [Pryciak 2008]. The degree of stabilization is determined by strength of the gel, and hence the number of non-covalent interactions. As a result, intrinsic parameters of enzyme result with specific apparent parameters dependent on partition and diffusion as shown in Figure 27.

- The porosity (ϵ) of the particle can be expressed as ratio of the volume of solution contained within the particle to the total volume of the particle. The tortuosity (t) is the average ratio of the path length, via the pores, between any points within the particle to their absolute distance apart.
- The tortuosity, which is always greater than or equal to unity, depends on the pore geometry. The diagram exaggerates dimensions for the purpose of clarity.
- The concentration of the substrate at the surface of the particles $[S_r]$ depends on radius R or internal concentration $[S_i]$ at any smaller radius (r) is the lower value.

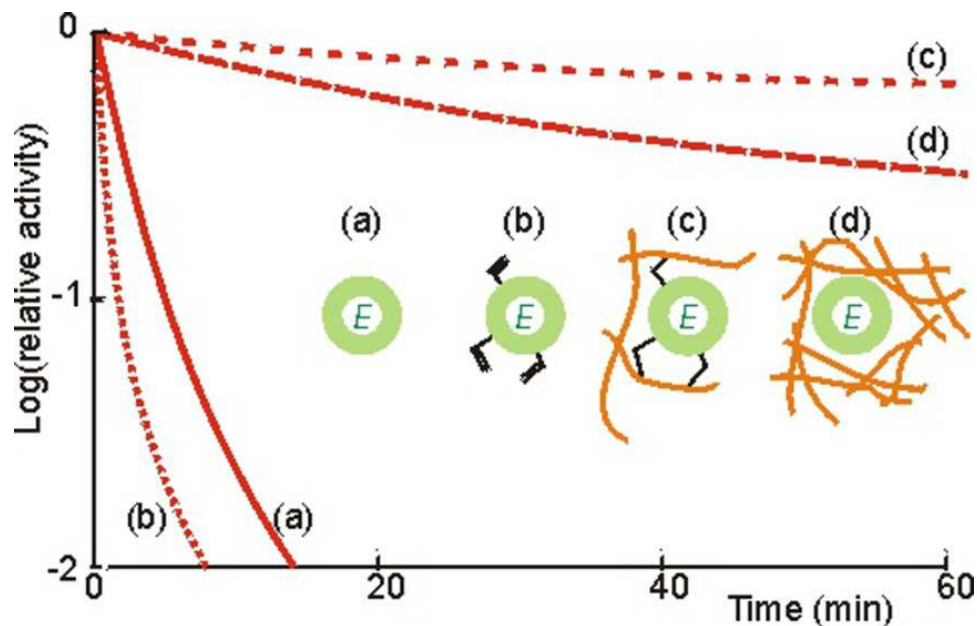


Fig. 27. Illustration of the use of multipoint interactions for the stabilization of enzymes. (a) — activity of free un-derivatized chymotrypsin. (b) activity of chymotrypsin derivatized with acryloyl chloride. (c) - - - activity of acryloyl chymotrypsin copolymerized within a polymethacrylate gel. Up to 12 residues are covalently bound per enzyme molecule. Lower derivatization leads to lower stabilization. (d) - · - activity of chymotrypsin non-covalently entrapped within a polymethacrylate gel. All reactions were performed at 60°C using low molecular weight artificial substrates. The immobilized chymotrypsin preparations showed stabilization of up to 100,000 fold, most of which is due to their multipoint nature although the consequent prevention of autolytic loss of enzyme activity must be a significant contributory factor. Reproduced with permission from Martinek et al, 1977a,b.

In general, the use of immobilized enzyme can be divided into two major categories of applications: in biosensors and bioreactors. However, list is growing in the other fields of ecological, environmental, agriculture, health, oceanic, space and earth sciences.

7. New developments in art of enzyme inhibition

Now a day, immobilized enzymes are used in industries and have value as medicinal and industrial enzyme products. Good examples of industrial enzymes are amylase, glucoamylase, trypsin, pepsin, rennet, glucose isomerase, penicillinase, glucose oxidase, lipase, invertase, pectinase, cellulase in medicinal use. With emergence of new inhibitors in the quest of drug discovery, several new inhibition mechanisms are expected in case of new substrate analogues. New substrate-enzyme active site interactions are envisaged due to different binding intricacies. Some examples of emerging concepts are outlined in following description and readers are expected to read advanced literature on these applications.

- **Slow-tight inhibition:** Slow-tight inhibition occurs when the initial enzyme-inhibitor complex EI undergoes isomerizing conformational change to a more tightly binding complex. However, the overall inhibition process is reversible. This manifests itself as slowly increasing enzyme inhibition. Under these conditions, traditional Michaelis-Menten kinetics gives a false value of a time-dependent K_i . The true value of K_i can be obtained through more complex analysis of the on (k_{on}) and off (k_{off}) rate constants for inhibitor association.
- **Substrate and product inhibition:** Substrate and product inhibition is where either the substrate or product of an enzyme reaction inhibits the enzyme's activity. This inhibition may follow the competitive, uncompetitive or mixed patterns. In substrate inhibition there is a progressive decrease in activity at high substrate concentrations. This may indicate the existence of two substrate-binding sites in the enzyme. At low substrate, the high-affinity site is occupied and normal kinetics is followed. However, at higher concentrations, the second inhibitory site becomes occupied, inhibiting the enzyme. Product inhibition is often a regulatory feature in metabolism and can also be a form of negative feedback; see allosteric regulation [Pryciak 2008, Bashor 2008].
- **Antimetabolites:** They are chemicals that interfere with the normal metabolism of normal biochemical metabolite(s). This in most of case is due to their structural similarity to such physiological substrates and therefore works as competitive enzyme inhibitors. They include antifolates such as methotrexate, hydroxyurea and purine and pyrimidine analogues. They are mainly used as cytotoxic anticancer drugs through inhibiting DNA and RNA synthesis and cell division. An example of nitroimidazole is described in detail on its metabolic effects at cellular level in this book [Sharma 2012a].
- **Antienzyme:** Intestinal parasites, e.g., Ascaris, protect themselves from digestion by expressing on their surface substances that are protein in nature which inhibit the action of digestive enzymes, e.g., pepsin and trypsin. The blood plasma and extracellular fluids are containing several types of protease inhibitors particularly important in controlling the blood clot formation and dissolution and matrix and cytokine homeostasis. Most of these inhibitors are peptides and several of them are also isolated from raw egg white, potatoes, tomatoes and Soya bean and other plant sources. Most of the natural peptide protease inhibitors are similar in structure to the amino acid sequence of the peptide substrates of the enzyme. Designed peptide protease inhibitors are important drugs, e.g., captopril that is a metalloprotease angiotensin-converting enzyme peptide inhibitor. Inhibiting this enzyme prevent activation of angiotensin and therefore prevent vasoconstriction to lower blood pressure. Crixivan is an anti-retroviral aspartyl protease peptide inhibitor used in the treatment of Human

Immunodeficiency Virus (HIV)-induce acquired immunodeficiency syndrome (AIDS). It inhibits the HIV protease that cleaves the large multidomain viral protein into active enzyme subunits. Because these peptide inhibitors may not be specific, they have several side-effects as drugs.

- **Antibodies** against several nonfunctional plasma enzymes have clinical diagnostic importance since they are longer living than the enzyme itself and hence reflect the disease history better. In this respect, autoimmune antibodies are clinically important in diagnosis of autoimmune diseases, e.g., anti-glutamic acid decarboxylase antibodies in type 1 diabetes mellitus.
- **Biosensors:** Light inhibits most enzyme activity although some enzymes, e.g., amylase are activated by red or green light and also specific DNA repairing enzymes (e.g., UV-specific endonuclease) are activated by the blue and UV light. Ultraviolet rays and ionizing radiations cause denaturation of most enzymes. Most enzymes contain sulfhydryl (-SH) groups at their active sites which upon oxidation by oxidants and free radicals by oxidants and free radicals inactivate the enzyme. Examples: Effect of radiations, light and oxidants on the rate of the enzyme catalyzed reaction.
- Other application of membrane bound redox enzymes constitutes them as a scaffolding enzyme arrangement into systems for multi-step catalytic processes. The reconstruction of portions of this redox catalytic machinery, interfaced to an electrical circuit leads to novel biosensing devices or biosensors. An example of nitric oxide synthase enzyme is cited in this book [Sharma, 2012b].
- In EzNET® water purifying system, nitrate pollution is eliminated. Enzyme is immobilized on “beads” with an electron-carrying dye as shown in Figure 28. Reduction of nitrate to environmentally safe nitrogen gas is driven by a low voltage direct current.

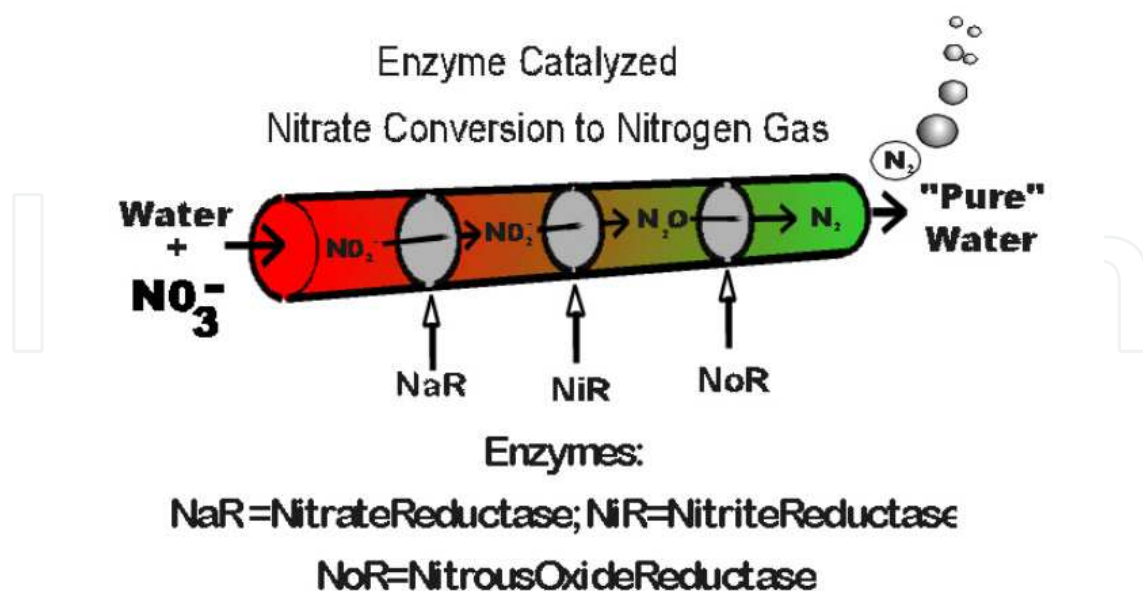
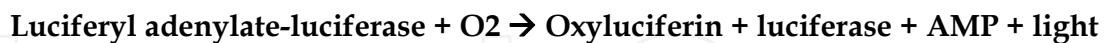
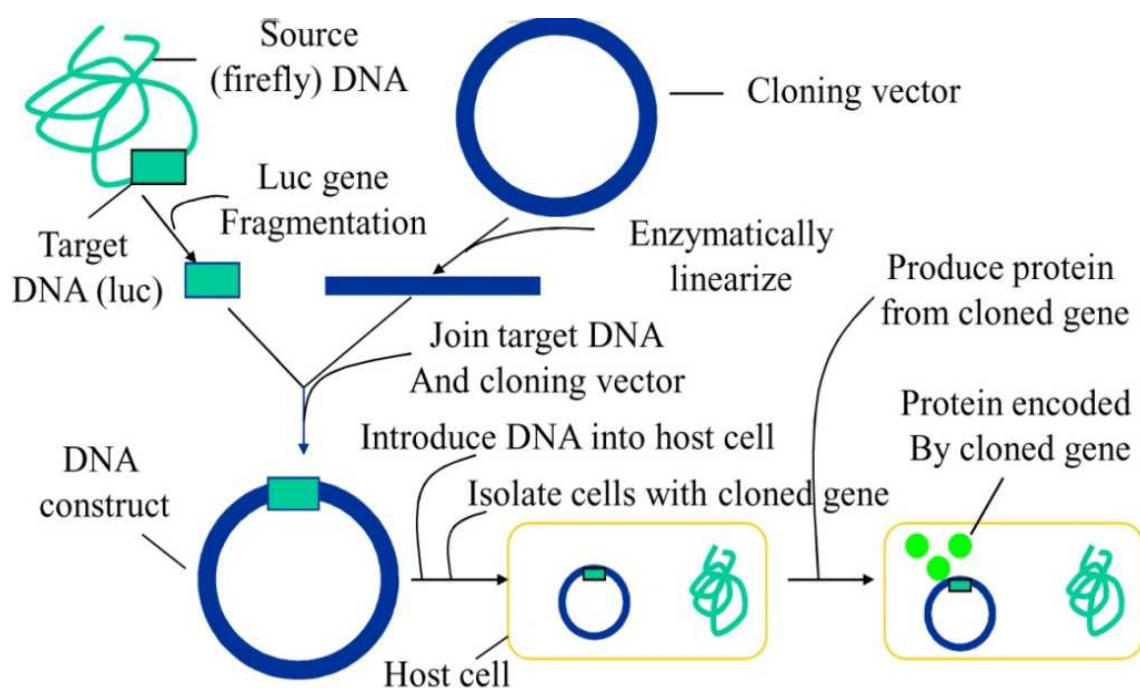


Fig. 28. EzNET® system shows immobilized enzyme on “beads” with an electron-carrying dye. In this system, reduction of nitrate generates environmentally safe nitrogen gas driven by a low voltage direct current. Source: The Nitrate Elimination Co., Inc. 2000.

- In bioluminescence detection for toxicity of HPV chemicals or drug development, 62 kDa MW oxygenase (yellow green light emitted at 560 nm) enzyme gives 88 photon/cycle light output proportional to [ATP] according to:



Strong inhibition of luciferase by chloroform or HPV chemicals indicates the efficiency of immobilized recombinant luciferase enzyme system as shown in Figure 20. Inhibition by chloroform is much reduced in the mutant Luciferase compared to the wild type Luciferase as shown in Figures 29, 30.

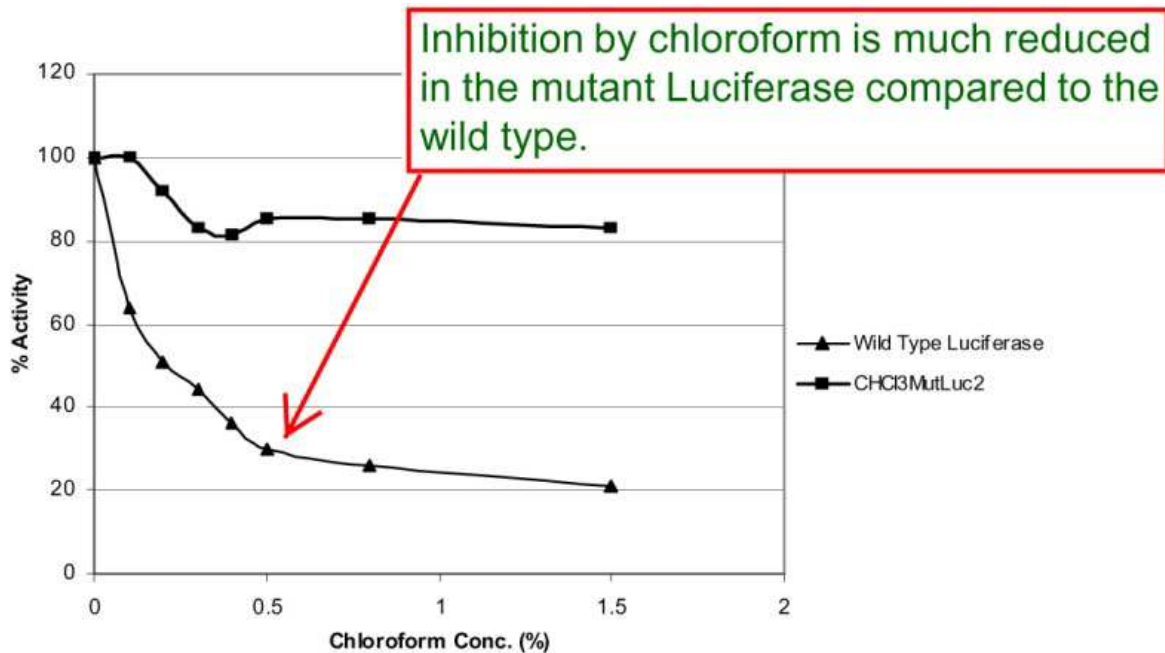


Source: Kim et al. AIChEngg Annual Meeting 2003, San Francisco, CA.

Fig. 29. A sketch of recombinant luciferase is shown illustrating the gene clone.

- In the search for new therapeutics, the high throughput screening (HTS) of ligands for key target proteins, enzymes represent the principal hit identification tool for early drug discovery [Bartolini et al. 2009]. However, output depends on cost-based or amount-based limitation of target availability, need of speed, automation and easy coupling of the enzyme assay with separation systems (affinity chromatography of immobilized proteins) and appropriate detectors. Good example is targeting in drug discovery represented by enzyme inhibition mechanism in monolithic immobilized enzyme reactors (IMERs) to represent different phases of the drug discovery pathway-starting with active compounds (hit) identification, through drug development and lead optimization, early ADMET (absorption, distribution, metabolism, excretion, toxicity) studies and quality control of protein drugs. Some details are described in chapters in this book [Bartolini et al. 2005, 2007]. Interested readers are requested to read advanced text books on these aspects. Different IMER have own requirements for optimal performances to show an

increased data output, reliability and stability to translate into cost reduction for potential applications in pharmacy industry [Bartolini et al. 2005, 2007].



Source: Kim et al. AIChE Annual Meeting 2003, San Francisco, CA.

Fig. 30. Inhibition of luciferase activity by increasing the concentration of chloroform.

8. Softwares and computerization in enzyme inhibition kinetics

Recently softwares have popped up to visualize custom visual interface to see curve fits in real-time, graph transforms, equations using kinetic data entry in terms of substrate, inhibitor, activator, velocity, and standard deviation of the velocity. Data tables are directly generated linked to the Fitting Panel of software. The data and results analysis is transferred in user-friendly lay-out, ANOVA window, % inhibition using Monte-Carlos fits, and receptor or ligand binding calculator. For interested readers, VISUALENZYMICS 2010® is available for statistical analysis for enzyme kinetics. [<http://www.softzymics.com/visualenzymics.htm>].

9. Limitations and challenges

Above mentioned description on mechanism and applications shows a clear issue on need of careful analysis for enzyme inhibition factors, presumptions of enzyme reaction, use of new immobilized enzyme support and enzyme recording/monitoring methods. Challenge is that most of times, basic presumptions do not hold true in enzyme reactors and addition of new factors further complicate the calculation of reactor outcome. Most of the times, computer based kinetic calculations average out outcome as less realistic with more chances of variants. Other major challenge is that each time enzyme reactor outcome depends on individual inhibitor and individual enzyme reactor at different times. It is less reproducible

and unpredictable because of synergy, interplay of known and unknown physical, physiological, biological, molecular factors affecting reaction kinetics.

10. Impact of enzyme inhibition science in business

The major current and emerging therapeutic markets for enzyme inhibitors used in human therapeutics are very high. New information is available on biochemistry for enzyme inhibitors and classes of enzyme inhibiting products with broad current or potential therapeutic applications in large markets. However, more than 100 enzyme inhibitors are currently marketed and double than those are under development. A better understanding of the emerging enzyme inhibitors on enzyme mechanism is main key. These include selected indications for asthma and chronic obstructive pulmonary disease (COPD), cardiovascular diseases, erectile dysfunction, gastrointestinal disorders, hepatitis B virus infection, hepatitis C virus infection, herpesvirus infections, human immunodeficiency virus (HIV)/acquired immune deficiency syndrome (AIDS) and rheumatoid arthritis and related inflammatory diseases. Key information from the business literature and thorough enzyme inhibition research is the basis of expert opinion on commercial potential and market sizes from enzyme industry professionals. Since initial reports on chemical immobilization of proteins and enzymes first appeared ~30 years ago, immobilized proteins are now widely used for the processing of products in industries from food business to environmental control. In recent years, use of chemical immobilization was extended to immobilized antibodies or antigens in bioaffinity chromatography. In coming years, it is speculated that immobilization techniques of proteins and enzymes will have greater impact on point-of-care medical and health business.

11. Conclusion

Enzyme inhibition is significant biological process to characterize the enzyme reaction, extraction of catalysis parameters in bio-industry and bioengineering. Conceptual models of inhibition define the interactions of substrate-enzyme or inhibitor-enzyme or both substrate-enzyme-inhibitor in the moiety of active site. In recent years, application of enzymes and enzyme inhibition science have gone in healthcare, pharmaceutical, bio-industries, environment, and biochemical enzyme chip industries with great impact on healthcare and medical business. Last decade has shown the measurement and accuracy of enzyme detection up to the scale of picometer and enzyme industry is entering in the area of picotechnology. Immobilized enzyme technology has given a new way of economic tools in drug discovery and biosensor industry. Every year new enzyme inhibitors are discovered useful as drugs but success still needs to minimize challenges.

12. Acknowledgements

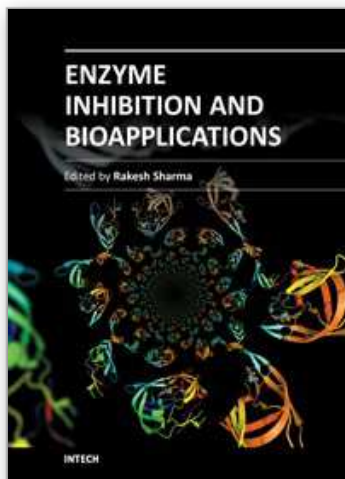
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Enzyme Inhibition and Bioapplications is a concise book on applied methods of enzymes used in drug testing. The present volume will serve the purpose of applied drug evaluation methods in research projects, as well as relatively experienced enzyme scientists who might wish to develop their experiments further. Chapters are arranged in the order of basic concepts of enzyme inhibition and physiological basis of cytochromes followed by new concepts of applied drug therapy; reliability analysis; and new enzyme applications from mechanistic point of view.

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