Chapter 4

Kinetic Modelling of Enzyme Catalyzed Biotransformation Involving Activations and Inhibitions

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Additional information is available at the end of the chapter

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Abstract

To achieve transition from lab scale enzyme studies to industrial applications, understanding of enzyme kinetics plays a critical role. The widely applied Michaelis Menten equation of the single substrate kinetics, sequential and double replacement mechanism of bisubstrate reaction and the relevant kinetics, inhibition and activation of enzyme are all integral parts of this discussion. In this chapter, we have discussed different types of inhibition and kinetic modelling. Systematic approach to generate data and its interpretation as well as designing of inhibitors is also explained.

Keywords: enzyme catalysis, mechanism, activation, deactivation, assay methods, inhibition, kinetic modelling, microwave irradiation

1. Introduction

The chapter gives brief introduction of enzyme, its classification and kinetics. It also discusses about the different types of inhibitions and activations involved in the enzymatic reactions. The classification of different types of enzyme inhibitors and activators and their mechanism of action will also be explained with appropriate examples. It will also give gist of different methods to identify inhibition or activation of specified reaction by given molecule. Different types of kinetic models used for the enzyme inhibition and activation will be systematically discussed with case studies. The applications of bioinformatics and computational modelling for *in-vitro* designing of the modulators and the study of transition states of enzyme-inhibitor complex will also be brought into light. The new developments and future challenges are summarized in this chapter. Enzyme catalysis, classification and effect of physiological conditions are also discussed.

Continuous rigorous efforts have been put into the understanding of enzymatic kinetics in order to use them for reactor design and industrial applications. The systematic study of the

reactivity of enzymes under different controlled external conditions reveals the reaction mechanism, activation and deactivation of enzymes and their reactivity towards a given set of substrates [1–4]. The crystallographic data supporting three-dimensional structural properties and mechanism in combination with the kinetic data serve as a powerful tool to understand working principles of enzyme in cellular fluids and thereby provide an opportunity to design the therapeutic molecules [1, 2]. International Commission on Enzymes was instituted by the joint efforts of International Union of Pure and Applied Chemistry (IUPAC) and International Union of Biochemistry (IUB) which have classified and assigned an EC number to each enzyme. The respective four-digit enzyme commission (EC) number associated with the enzyme signifies the details about the reactions catalysed by the enzyme. The readers can refer the biochemistry literature present in the books written by Stryer, Voet and Voet [3], Nelson and Cox [4] and the official website of the enzyme classification [5, 6]. Enzymes as the biological catalyst are mainly classified in six classes, namely, oxidoreductase, ligase, lyase, transferases, isomerase and hydrolases [3–5, 7, 8]. More importantly, enzymes do not alter the equilibrium constants of chemical reaction as they also have the ability to catalyse backward and forward reactions. Hence, they only affect the rate at which the equilibrium is established. However, as almost all the biological systems in human body work at the steady state, the net effect of enzyme to convert the substrate into the product occurs rapidly as the products are removed instantaneously [4, 8, 9]. Various factors and physiological conditions affect the activity and efficiency of enzymes to the various extents. Therefore, it is essential to understand their effects on the enzymes [10, 11].

1.1. Effect of temperature

The fluctuations in temperature can affect the activity of enzymes in either way, i.e. they may cause activation or deactivation, depending on the nature of enzymes and properties of the solution. In most of the catalytic reactions, the average kinetic energy and velocity of molecules rise with increase in temperature, which results in higher probability of effective collisions and the cumulative changes lead to increase the rate of reaction with temperature [12–14]. Below the denaturation temperature, on an average, rate increases with per 10°C increase in temperature. Ratio by which rate changes for 10°C rise is known Q_{10} (approximately from 1.7 to 2.5). Generally, as the enzymes are evolved to work with optimum efficiency at physiological conditions of the source microorganism, the same conditions are observed to be the optimal conditions for most of the enzymes at which the rate of reaction is maximum. [18] concluded that in the case of macro-heterogeneous systems such as lipase, the rate of reaction of enzymes is partly regulated by diffusion and partly by temperature. Inactivation by heat expends on stability of the enzyme up to certain temperature range at different experimental conditions. However, purified enzymes have higher probability of deactivation than crude enzymes. This is because the liability is often subsidized by the protective protein and their colloids associated with the enzymes of interest. Therefore, the critical temperature and temperature coefficient depend upon properties and composition of the enzyme preparation along with the properties of enzyme itself. The critical temperature is at which the activity of its enzyme is destroyed to half of the reactions. If the enzymes are used for the reaction at higher

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temperature than optimum, it catalyses the reaction and will get deactivated over the period of time. If the temperature is lowered to optimum, reaction will be completed earlier. When the optimum temperature is maintained slightly above the critical temperature of inactivation, reaction will reach to the completion at a faster rate. Practically, optimum temperature gives rough idea of heat stability of enzymes and there are no other significant data available at this temperature. Hence, it does not serve as a definite character even for the given enzyme system. In some cases, increase in catalase activity in a yeast cell suspension occurs by 49 times after heating for an hour. This is attributed to the destruction of an inhibitory substance at higher temperatures. Similar properties are observed for the blood catalase due to hemolysis as corpuscular enzyme differs from the intra-corpuscular enzyme in its properties [15].

1.2. Action of radiations on enzyme

The exposure of enzyme solution to the various radiations generally leads to an irreversible destruction, occasionally an activation of the enzymes [16]. The rate of destruction is practically independent of temperature during the treatment of enzyme preparation with radiations. Perhaps, it is affected by pH and chemical conditions like presence of substrate, optical sensitizers and oxygen [17, 18]. While enzymes are very stable to heat inactivation near their optimal pH, the reverse is often the case with regard to ultraviolet (UV) radiations. Generally, diluted solutions are more affected by the UV radiations than the concentrated ones due to absence of protective colloids and ions. The deactivating effect of X- and γ-rays may be due to the electrons ejected by them which lead to the inactivation of enzymes [19]. In case of radioactive sources, the amount of deactivation can be calculated. If Q is the amount of enzyme present, then

$$\frac{I}{Q}\frac{dQ}{dW} = c \tag{1}$$

where W is an average activity of radioactive source and c is a constant multiplied by time over which it acts.

1.3. Effect of microwave radiations

Since last five decades, microwave irradiations have been established as an efficient source of heat energy for chemical reactions, wherein rate of reactions has been enhanced by a multiple fold than conventional heating and has led to the reduction in overall reaction time drastically with high selectivity. The electromagnetic components of microwave irradiation lead to the vibration of bonds to chemical reactants and catalysts resulting into the lowering of activation energy and increase in collision frequency [20, 21]. Microwave irradiation triggers the effect that cannot be created with the help of thermal heating. Especially, in case of polar solvent molecules, it acts as a bridge between two molecules through absorption energy [22–24]. The enzyme hydration layer can be used for the improvement of the process or modification of the selectivity as it interacts with the microwave radiations [25–27]. Hence, the microwave irradiation affects synergistically to the enzymes.

1.4. Effect of pH

The optimal pH of the enzyme is generally the same as that of fluid at which enzyme functions *in vivo*. Enzyme activity profile is bell shaped curve signifying that before and after the optimal pH, the activity of the enzyme decreases drastically. The presence of charged amino acids in enzyme structure, chemical nature of substrate and enzyme-substrate complexes is the reason for the pH dependence of enzyme [28]. Hence, pH causes dissociation or undissociation of the charged amino acids that affect catalysis. Ionization of these groups depends upon *pKa* values. The pH and nature determine the *pKa* value of the medium. The change in pH alters interaction of the substrate with an active site and rate of breakdown of enzyme-substrate complex into product [29–31]. The effect of pH on the biocatalysed reaction can be represented with the change in $V_{\max}$ and K_{m} which is summarized in Scheme 2.1 and Expression 2.1.

$$\begin{array}{ccccc} E- & & ES- & & \\ \uparrow\downarrow K_{\mathrm{E2}} & & \uparrow\downarrow K_{\mathrm{ES2}} & & \\ EH+S & \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} & ESH & \xrightarrow{k_2} & EH+P \\ \uparrow\downarrow K_{\mathrm{E1}} & & \uparrow\downarrow K_{\mathrm{ES1}} & & \\ EH2+ & & ESH2+ & & \end{array} \tag{2}$$

Scheme 2.1: Mathematical equations modelling pH effects on enzyme catalysed reactions.

$$v = \frac{V_{\mathrm{mapp}}[S]}{K_{\mathrm{mapp}} + [S]} \tag{3}$$

$$V_{\mathrm{mapp}} = \frac{V_{\mathrm{m}}}{1 + \frac{[H+]}{K_{\mathrm{es1}}} + \frac{K_{\mathrm{es2}}}{[H+]}} \tag{4}$$

$$K_{\mathrm{mapp}} = \frac{K_{\mathrm{m}}(1 + \frac{[H+]}{K_{\mathrm{e1}}} + \frac{K_{\mathrm{e2}}}{[H+]})}{1 + \frac{[H+]}{K_{\mathrm{es1}}} + \frac{K_{\mathrm{es2}}}{[H+]}} \tag{5}$$

Expression 2.1: Kinetic expression for the effect of pH on enzyme catalysed reactions.

1.5. Effect of immobilization

With the purpose of reusability, enzymes are immobilized on porous and non-porous solid medium using various methods. This immobilization along with the stabilization of the enzyme brings the extrinsic and intrinsic diffusional limitations and increased probability of substrate or product inhibition. The kinetic constants (e.g. K_{m}, $V_{\max}$) of immobilized enzymes may be altered by the process of immobilization, intrinsic specificity of enzymes, properties of the solution and molecular diffusion within the local environment [32–34]. The relationship between these intrinsic and apparent parameters is shown below.

$$\text{Damkohler number } (D_a) = \text{Maximum rate of reaction/maximum rate of diffusion} = \frac{V'_{\max}}{K_{\mathrm{L}}[S_{\mathrm{b}}]} \tag{6}$$

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If $D_a >> 1$, diffusion rate is limiting the observed rate.

If $D_a << 1$, reaction rate is limiting the observed rate.

The Michealis Menten equation for the immobilized enzyme is represented in equation (7).

$$v_s = \eta \frac{V'_{max}[S_b]}{K_m + [S_b]} \tag{7}$$

Expression 2.2: Modified Michealis Menten equation for immobilized enzymes.

The substances are partitioned in the two environments, internal solution and surrounding of the immobilized enzyme. Substrate molecule (S_b) diffuses to the micro-environment of catalytic centre from the macro-environment to convert into product (P) [35].

2. Study of enzyme mechanism and kinetics

2.1. Study of enzyme mechanism

To study the mechanism of enzyme catalysis, one should be able to estimate the inferences of the experimentally observed effects with respect to the change in molecular events. The basic mechanism can be predicted and validated for the enzymatic reaction under investigation by analysing the data available from a number of techniques. Combination of the basic physicochemical properties with steric structure and quantum mechanical calculations gives solid foundation for the prediction of mechanism [36]. The kinetic data and graphical representation validate the data and play a crucial role in establishing the mechanism of the enzyme. Additionally, the study of enzyme inhibition not only unveils the mode of action of enzyme but also opens up other crucial information which is boon to the other fields of science. Some of the methods that are used for the prediction of the mechanism of the enzymatic catalysis are isotope exchange, irreversible inhibition, pH dependence, fluorescence labelled substrates, etc. [37].

2.2. Study of enzyme kinetics

To upscale the laboratory level research to an industrial scale with improved productivity and reduction in cost of the process, it is very necessary to study the kinetic models of the desired biocatalytic reactions, which facilitate process designing and intensification [38, 39]. The understanding of enzyme kinetics is also essential to design modulator that can help in designing various drugs. Transient-state kinetics, steady-state kinetics and rapid-equilibrium kinetics are the three major classes of kinetic studies [40–42]. Transient-state kinetics is applied to the very fast reactions of which mechanisms are dependent on the enzyme structure [43, 44]. The steady state of enzyme kinetics is derived with the hypothesis that each enzymatic step is in steady state and remains in steady state, even though the outer environment continuously changes in a given catalytic system [36, 45]. For rapid equilibrium kinetics, the reaction components of the step, preceding to the rate determining step, are in equilibrium with various

enzyme forms like the enzyme, enzyme-substrate complex and substrate [46–48]. The initial velocity of the enzyme catalysed reaction gets affected by the modifiers [49]. The addition of single type of modifier changes the kinetics and yields two rate constants, whereas addition of two types of modifiers in two different reactions; five independent equilibrium conditions and three routes for synthesizing products are observed [50]. The chemical equilibrium is significantly affected by the external conditions such as drugs, activators, metals, toxins and pH, some of which were discussed earlier in Section 2. However, the enzyme kinetics can differ marginally in a cellular environment because of the dynamic nature of system [41].

2.2.1. Single substrate kinetics

In 1902, Brown found that at high concentration of sucrose, reaction follows zero-order reaction. Therefore, he proposed that the reaction is composed of two elementary steps. (1) Pre-steady-state: the formation of ES complex (2) At steady state: formation of product. This initiated the study of enzyme kinetics and derivation of the relevant rate expression (6 and 7). The next significant efforts were put forth by Michaelis and Menten in 1913 which were further improved by Briggs and Haldane [3, 7]. The reaction rate is directly proportional to [*E*] if excess of free substrate concentration is present. At low substrate concentration, reaction follows first-order kinetics. Enzyme substrate interaction obeys the mass action law. For given [*E*], reaction velocity increases initially with increasing substrate concentration up to certain maxima and further addition does not change the velocity anymore. Plotting and shaping of rectangular hyperbola characterize the shape of non-allosteric enzymes [8, 33]. The progress curve for the simple enzyme for catalysed reaction is represented in **Figure 1**.

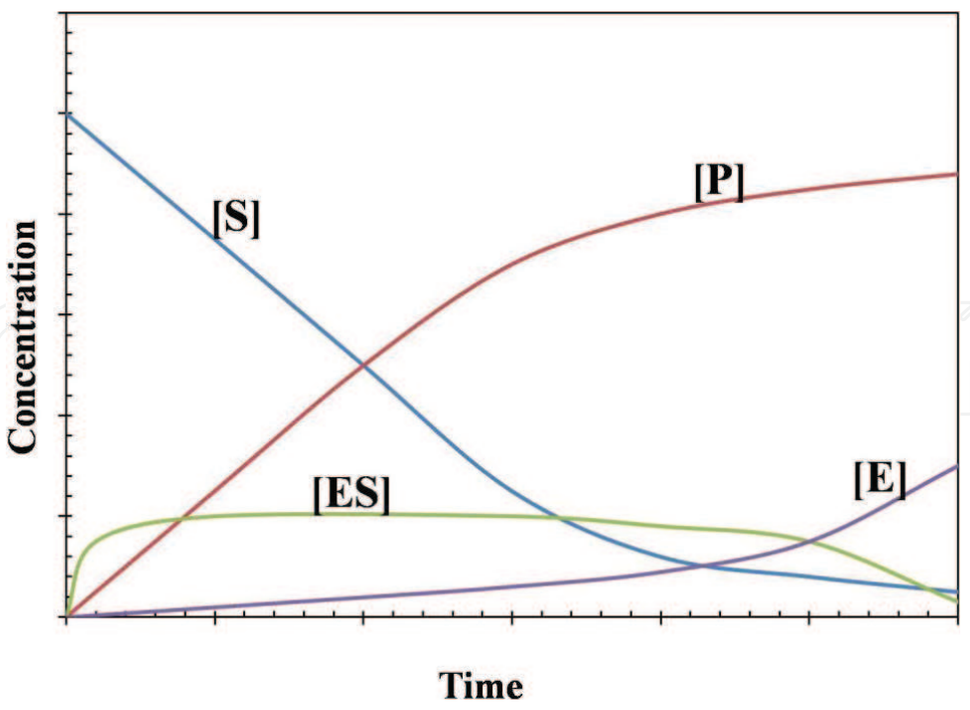

Figure 1. Progress curve for simple enzyme for catalysed reaction [7].

Unfortunately, steady-state kinetics measures are incapable of revealing the number of intermediates. It is referred as 'black box'. Perhaps, it provides phenomenal description of enzymatic behaviours. The nature of intermediate remains indeterminate. Hence, even though steady-state kinetics is not helpful for predicting the mechanism, it is useful to eliminate the proposed mechanisms [8, 51]. This developed expression relates initial rate of reaction with the substrate concentration and rate constants. The representation of reaction is as follows:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} ES \xrightarrow{k_2} E + P \tag{8}$$

In the given rate expression, the first step is reversible, whereas second step became irreversible. The second step is not reversible as the sufficient amount of energy is not available to cross the barrier as the energy liberated during the reaction. To derive the rate expression for the desired single substrate enzymatic reaction, following assumptions are made [3, 4].

1. Concentration of substrate ([S]) is greater than the concentration of enzyme ([E]): concentration of substrate is not so large that all enzyme become enzyme-substrate (ES) complex form. But, it must be sufficiently large so that the concentration of substrate does not rapidly become so small that concentration of substrate becomes larger than the concentration of enzyme. Under these conditions, concentration of ES complex rapidly becomes constant, so that the rate of formation of ES complex is equal to the rate of breakdown of ES complex wherein steady-state kinetics can be applied.

2. Only initial velocities are measured so that the concentration of substrate is not depleted during the measurement (velocity measured before more than ~10% of conversion). Initial velocity measured below ~10% minimizes interference by parameters like reversible nature of reactions, inhibition of the enzymes by its product and progressive inactivation of the enzymes.

3. Concentration of the ES complex is static throughout the process which infer that:

a. Sufficient time has passed, which is needed to build the concentration of the ES complex after mixing of E and S.

b. Adequate time has been given to the reaction for the rate of formation of ES complex resulting into the depletion of substrate.

c. If it is $k_2 >> k_{-1}$, ES complex breakdown occurs as rapidly as it is produced to form product and thus steady state can never be achieved. When $k_2 << k_{-1}$, the concentration of ES can build up inside the system and then,

$$\text{Rate of formation of ES complex} = \frac{d[ES]}{dt} = k_1[E][S] \tag{9}$$

$$= k_1([E_T - [ES])[S] \tag{10}$$

$$\text{Rate of breakdown of ES complex} = \frac{d[ES]}{dt} = k_{-1}[ES] + k_2[ES] \tag{11}$$

Under steady state conditions,

Rate of formation of ES complex = rate of breakdown of ES complex

$$k_1([E_T] - [ES])[S] = (k_{-1} + k_2)[ES] \tag{12}$$

Rearranging the Eq. (12)

$$\frac{([ET] - [ES])[S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m \tag{13}$$

Wherein, K_{m} = Michealis Menten constant which is a ratio of rate constant and not an equilibrium constant.

$$[S][E_T] - [S][ES] = K_m[ES] \tag{14}$$

$$[S][ET] = (K_{\mathrm{m}} + [S])[ES] \tag{15}$$

$$[S] = \frac{[ES]}{[E_{\mathrm{T}}]}(K_{\mathrm{m}} + [S]) \tag{16}$$

At any point of time, it is very difficult to measure the concentration of enzyme-substrate complex in the system. Hence, Eq. (16) is not useful experimentally to deduce any quantitative results. On the contrary, various experimental methods can be used for quantify the velocity (v) and maximum velocity (V_{max}). Therefore, V_{max} is the limiting value, if $[S] \rightarrow \infty$. In this case, all enzymes are bound to the substrate and $[E] = 0$, $[E_{\mathrm{T}}] = [ES]$. Thus,

$$v = k_2[ES] \tag{17}$$

$$V_{\mathrm{max}} = k_2[E_{\mathrm{T}}] \tag{18}$$

$$k_2 = \frac{v}{[ES]} \tag{19}$$

$$\mathrm{k}_2 = \frac{V_{\mathrm{max}}}{[E_{\mathrm{T}}]} \tag{20}$$

$$\frac{v}{V_{\mathrm{max}}} = \frac{[ES]}{[E_{\mathrm{T}}]} \tag{21}$$

So, after putting these substitutes, Eq. (16) becomes,

$$[S] = \frac{v}{V_{\mathrm{max}}}(K_{\mathrm{m}} + [S]) \tag{22}$$

This expression is known as Michealis Menten equation for the prediction of enzyme kinetics. This is the most widely accepted and applied enzyme kinetics to the various systems *in vitro* and *in vivo*.

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Deductions derived from the Michealis Menten expression:

i. Michealis Menten constant (K_m) does not change for desired enzyme and is independent of the concentration of substrate and enzyme.

ii. Maximum velocity (V_{max}) depends on concentration of enzyme and at saturating concentration it is independent of substrate concentration.

iii. Both K_m and V_{max} may be affected by temperature, pH, immobilization and other physical constraints.

iv. A graph of velocity (v) versus concentration of substrate [S] fits hyperbolic rectangular function.

v. If enzyme can catalyse more than one reaction, then K_m values can be used to measure the relative affinity towards particular substrate. If K_m increases, affinity of substrate towards enzyme decreases. In metabolic pathway studies, rate limiting step from the sequential pathway is determined with the values of K_m for that enzyme for different substrate.

vi. When $k_{-1} >> k_2$, concentration of ES complex is implied that it reaches to equilibrium with concentration of enzyme and substrate and ES is dissociated to yield the product. Under these conditions, the dissociation constant of enzyme substrate complex (K_s) is,

$$K_S = \frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} \tag{23}$$

$$K_m = \frac{k_{-1} + k_2}{k_1} \tag{24}$$

$$K_m = \frac{k_{-1}}{k_1} = K_S \quad k_{-1} \gg k_2 \tag{25}$$

vii. When $k_2 >> k_{-1}$, the rate of dissociation is low so that the rate of product formation is high, reaction sequence becomes irreversible at both steps.

$$E + S \xrightarrow{k_1} ES \xrightarrow{k_2} E + P \tag{26}$$

As overall rate of reaction is determined by the concentration of ES complex,

- At low substrate concentration, formation of ES complex follows second-order reaction. The rate of reaction is proportional to the concentration of enzyme and substrate.
- At high substrate concentration, [ES] complex remains constant and reaction rate is proportional to the [ES] complex. The reaction follows the first-order reaction rate.

viii. The turnover number is defined as the rate at which an enzyme can accomplish its catalytic activity per unit time or it can also be defined as a frequency at which the ES

complex leads to the formation of product. When $[S] >> K_m$, the turnover number for the enzyme is calculated. It can be determined under saturation conditions as,

$$\frac{dP}{dt} = V_{max} = k_2[ES] = k_2[E_T] \tag{27}$$

If $[E_T]$ increases x-times, while concentration of substrate is established at the saturating levels as compared to the total concentration of enzyme, then V_{max} increases x-times. The rate of reaction is proportional to the concentration of total enzyme concentration. The reaction tends to become first order.

In saturation condition, the rate of reaction is maximum. The rate constant k_2 is denoted as k_{cat} at saturation levels and gives the value of turnover number. It ranges from 1 to 10^4 per second. Sometimes, it reaches to 10^5. Upper limit to the value of k_{cat}/K_m cannot be greater than k_2 i.e. decomposition of ES complex to E and P. The most efficient catalyst has values of k_{cat}/k_m near the controlled diffusion limits of 10^{-8}–10^{-9} m^{-1} s^{-1}. These enzymes catalyse reaction almost every time they colloid with substrate and achieve virtual catalytic perfection.

$$k_{cat} = \frac{V_{max}}{[E_T]} \tag{28}$$

The ability of enzyme to produce a given amount of product to use in given time is changes proportionally with turnover number and total amount of enzyme present in cell. However, turn over number is measured for purified enzymes. Hence, the enzyme activity is measured as a specific activity (μmol of substrate converted per minute per mg of enzyme to form product). That is also denoted as Katal (kat). It is defined as concentration of enzyme that transforms 1 mol of substrate into product per second. As for most of the clinical disorders, activity is measured in biological fluids. It is redefined as 1 μmol of substrate to product per minute at optimal conditions (IU).

ix. When concentration of substrate is far less that the value of K_m, the product formation rate rises linearly with rise in substrate concentration and reaction becomes first order with reference to the concentration of substrate.

At low substrate concentrations, reaction rate is proportional to the total enzyme concentration. Reaction velocity at low concentration of substrate can be represented as,

$$v = k'[E][S] \tag{29}$$

$$k' = \frac{k_{cat}}{K_m} \tag{30}$$

Therefore, the rate equation must specify a second-order dependence on the concentration of substrate and total enzyme. When concentration of substrate is small there is first order dependence in concentration of total enzyme alone. When concentration of substrate is large, rate constant is k_{cat} for first order and k_{cat}/K_m for second order in which

K_m is the value of the concentration of substrate. When $v = \frac{1}{2}V_{max}$, k_{cat}/K_m measures total activity of the enzyme, which includes the ability of enzyme to bind with a particular substrate.

x. When $K_m = [S]$, results in the velocity that is $V_{max}/2$,

$$\frac{V_{max}}{2} = \frac{V_{max}[S]}{K_m + [S]} \tag{31}$$

$$K_m + [S] = 2[S] \tag{32}$$

Initial velocity increases with increase in V_{max} at constant concentration of the substrate and K_m and Initial velocity decreases with increase in K_m at constant concentration of the substrate and V_{max}.

- Limitation of Michealis Menten relation:

It is very tedious to quantify the velocity of the enzyme which can catalyse the multiple reactions. In other cases, the enzyme may be inactivated by its substrate or impurities in it. The enzyme that consists of several components and velocity-substrate relationship depend upon their ratios (e.g. urease). In these cases, the use of Michealis Menten equation cannot be accurately predicted.

- Representation of Michealis Menten Data:

For enzyme catalysis the data mostly fit into a rectangular hyperbola wherein the initial velocity of reaction is plotted against the concentration of substrate in the system [52, 53]. It can be represented as follows (**Figure 2**):

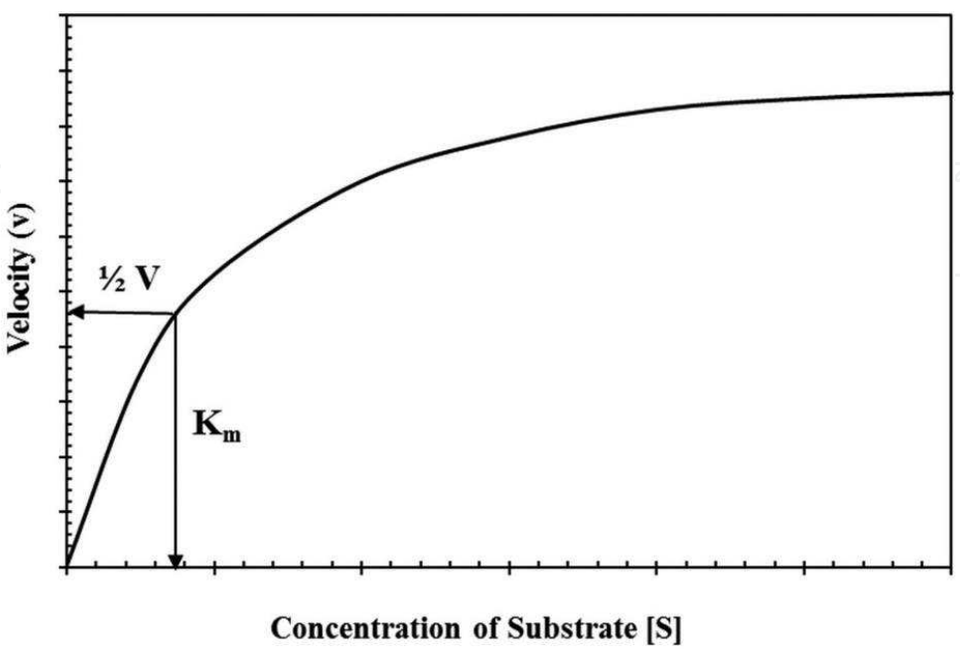

Figure 2. Initial velocity of reaction versus the concentration of substrate [4].

In the direct diagram, V_{max} and K_m are calculated by extrapolating the graph. As from the given data points, there are numerous ways to represent the data. Secondly, as the experiment is conducted at the saturation level, it is often that velocity is overlooked and therefore K_m. Also, the inhibitory effect of the excessive substrate or the limited solubility of the substrate causes the misinterpretation of the values of kinetic parameters. Practical prediction of this error prone data becomes more tedious as it becomes very difficult to predict and evaluate the experimental error from data. Hence, the need of accurate prediction of the data resulted into development of various types of reciprocal and logarithmic plots derived on the basis of Michealis Menten equation. Because straight lines are easier to evaluate, the efforts have been taken to interpret that hyperbolic data in the form of a straight line by various ways like Lineweaver Burk plot, Eadie Hofstee plot, Hanes plot, Dixon plot, etc. [47, 53].

• Lineweaver Burk plot (Double reciprocal plot)

It is based on the reciprocal of the Michealis Menten Equation and represented as,

$$\frac{1}{v} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}} \frac{1}{[S]} \tag{33}$$

The plot is developed by plotting the inverse of velocity of reaction against the inverse of concentration of substrate with abscissa at $1/K_m$. This method is the most frequently used method for the prediction of kinetic parameters. The major disadvantage is the irregular distribution of data. The reciprocal action causes distortion of equally distributed substrate concentrations either in the compressed form towards the co-ordinates or over-extended in the other direction. To overcome the limitation, if the analysis is carried out at small range for uniform dispersal of data, the results do not cover the range of study at satisfactory levels. The tangible advantage of the linear graph is that the variables (v and $[S]$) are displayed on the separate co-ordinators from each other. The external disturbances and change in mechanism pattern cause the deviations from straight lines nature of the plot. This plot serves as primary step to distinguish the inhibition in the system as well as it is useful to understand the mechanism followed by the reactions [7, 8, 54].

• Eddie-Hofstee plot

Eddie-Hofstee plot is more equally placed in comparison with Lineweaver Burk plot. The equation can be obtained as,

$$v\left(\frac{k_m + [S]}{[S]}\right) = V_{max} \tag{34}$$

$$v\left(\frac{k_m}{[S]}\right) + v = V_{max} \tag{35}$$

$$v = V_{max} - K_m\left(\frac{v}{[S]}\right) \tag{36}$$

Plotting v against $v/[S]$ gives V_{max} from the ordinate intercept and $-K_m$ from the slope. This method of linearization is not only associated with distortion of error limits at higher concentrations of substrates but also there is no separation of variables from each other [3, 55].

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• Hanes plot

The expression is derived by simple multiplication of the concentration of the substrate with the reciprocal Michaelis-Menten equation. It is represented as,

$$\frac{[S]}{v} = \frac{K_m}{V_{max}} + \frac{[S]}{V_{max}} \tag{37}$$

Plotting of $[S]/v$ against $[S]$ gives K_m/V_{max} (ordinate intercept) and $1/V_{max}$ (slope). The error limits are only slightly deflected at low substrate concentrations when simple linear regressions can be applied. However, substrate concentrations variable is represented on both the coordinates [7, 33].

• Eisenthal and Cornish-Bowden plot

Eisenthal and Cornish-Bowden have derived over a period of time in various forms. The final equation of this type of direct linearization is represented as,

$$\frac{1}{K_m} = \frac{V_{max}}{K_m v} - \frac{1}{[S]} \tag{38}$$

By entering $1/v$ against $1/[S]$, $1/K_m$ is quantified from the intercept and V_{max}/K_m from the slope of the graph. The reciprocal transformation of the equation leads to the distortion of scale. The deviation of the graph from the ideal behaviour is difficult to trace as it is overlapped by the distortion error and gives the false values of kinetic constants [52, 56, 57].

• Dixon plot

In a Dixon analysis, two types of graphs are plotted to evaluate the type of inhibition that is caused by the addition of the inhibitor in the reaction system. In 1953, the first Dixon plot of $1/v$ versus concentration of inhibitor at static substrate level was studied. Based on the nature of lines, this plot can be used to differentiate between the partial and complete inhibition (**Table 1**).

In some cases, the plots create confusion in the prediction of non-competitive and competitive inhibition. This led to the development of second Dixon plot ($[S]v-1$ is plotted versus [I]) (1972), which requires change in the turnover rate with respect to the inhibitor concentration at a static saturating concentration of the substrate. In this case, the enzyme activity follows hyperbolic decrease reaching to zero at complete inhibition. In case of the competitive inhibition, the lines plotted remain parallel to each other while in case of uncompetitive inhibition, the Dixon plot showed the presence of intersecting lines [33, 55].

Type of inhibition	Nature of Dixon plot I
Competitive inhibition	Intersect with each other at I = −Ki
Non-competitive inhibition	Intersect on abscissa at I =−Ki
Uncompetitive inhibition	Remain parallel with each other

Table 1. Analysis of Dixon plots.

There are some generalized methods that are used for determining various rate and inhibition constants along with the prediction of mechanism that is followed by the reaction under particular set of conditions.

2.2.2. Kinetics of two substrate reaction

The study of enzyme kinetics was initiated with the single substrate molecule which can be further applied to study the bisubstrate reactions which constitute 60% of biochemical bisubstrate reactions in nature [8].Two substrate reactions are more complex than single substrate reactions. For example, most of dehydrogenase and aminotransferase follow bisubstrate enzyme kinetics. The Cleland notation is used for the representation of higher order enzymatic reactions. According to which, the substrates are assigned with A, B, C and D letters based on the sequence of binding to the enzyme, products are assigned by letters P, Q, R and S on the basis of release sequence. Enzyme is designated with letter E, and sometimes with F, which is a slightly modified form of a stable enzyme observed in double-displacement reaction [58–60]. Hence forward, this notation is used for the representation of bisubstrate reaction. To study reaction kinetics by applying Michaelis Menten kinetic to bisubstrate enzyme reactions, concentration of one of the substrates (*B*) is kept constant, whereas another one (*A*) is varied and *vice versa*. As concentration of substrate *B* is kept arbitrarily constant, the obtained values of the kinetic parameters for concentration of substrate *A* can be erroneous. On the other hand, varying the concentration of both the parameters at a single point of time intricates large complexity in data acquisition and analysis. Bisubstrate reactions can be broadly classified into two groups, namely, sequential and double-displacement reactions. In sequential reaction mechanism, both substrates bind to the enzyme which leads to the development of transition state complex followed by the product. The binding of substrate decides further classification of sequential reaction mechanism. When binding of one substrate *A* becomes obligatory prior to other substrate *B*, then the reaction follows ordered sequential mechanism (**Figure 3**). On the other hand, the sequence of binding of the substrates to the enzyme has very less importance, the reaction undergoes random sequential reaction mechanism (**Figure 4**) [8, 33, 61].

These types of sequential mechanisms are further bifurcated into different sub-groups based on the location rate limiting step. In case of a rapid equilibrium mechanism, the rate-limiting step is chemistry of the reaction; on the other hand, step except chemistry of reaction controls the rate of reaction for a steady-state ordered mechanism. In Theorell-Chance mechanism, every step is fast except the release of the second product, *Q*, in a reaction pathway. For steady-state random mechanism, the substrate binds with the enzyme in any order [55, 62].

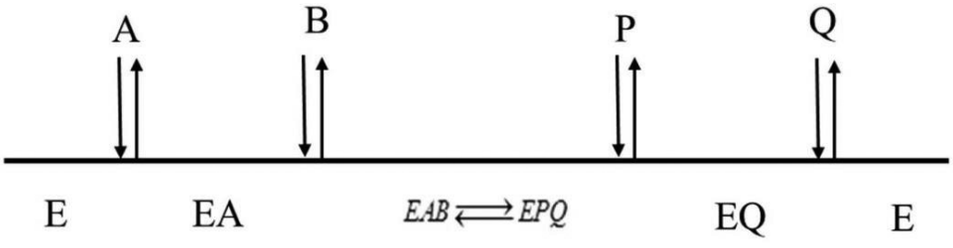

Figure 3. Ordered sequential mechanism.

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As the name suggests, a double-displacement mechanism occurs when a catalytic process can proceed with binding of one of the two substrates to the enzyme (**Figure 5**). On completion of first catalytic event, first product (*P*) leaves from the active site leaving some of its portion inside the active site. After the release of first product, some chemical group of substrate *A* left behind in the catalytic site of the enzyme creating new form of stable enzyme (*F*). After binding of second substrate, the catalysis proceeds further to produce product *Q* with the regeneration of an original enzyme *E*. The plot of initial velocity and substrate concentration does not signify any noticeable change in different types of mechanisms by visual inspection and hence, not helpful in assigning any proper rate equation. However, the double-reciprocal plot, i.e. $1/v_0$ versus $1/[A]$ at the different concentrations of *B*, significantly distinct between a sequential or ping-pong mechanism [13, 63, 64].

In case of sequential mechanism (ternary complex mechanism) of reaction, the lines on the double reciprocal plot intersect with each other on the left of the *x*-axis (**Figure 6**), while that of the double-displacement mechanism (ping-pong mechanism), the double reciprocal plot is represented by the parallel lines (**Figure 7**) [58, 65, 66].

Thus, the double reciprocal plot gives clear indication of mechanism of reaction with some exception [67]. The different rate equations and plot information are presented in **Table 2**.

The rapid-equilibrium random, steady-state ordered and Theorell-Chance mechanisms are represented with the same rate expression and the primary plot or the double reciprocal plot cannot differentiate between these mechanisms from each other. They only differ at the location of rate limiting step and can be differentiated on the basis of kinetics studies such as dead-end inhibition studies, isotope effects and pre-steady-state kinetics [55, 59, 68]. The bisubstrate

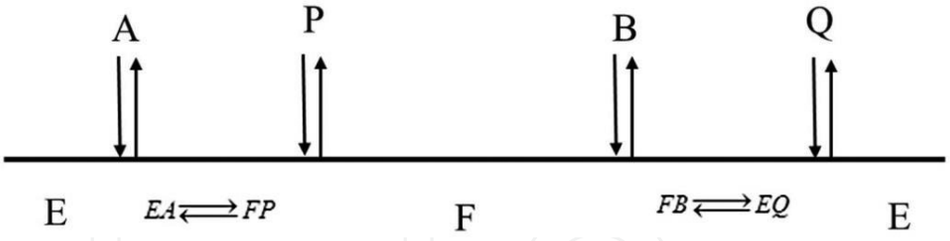

Figure 4. Random sequential mechanism.

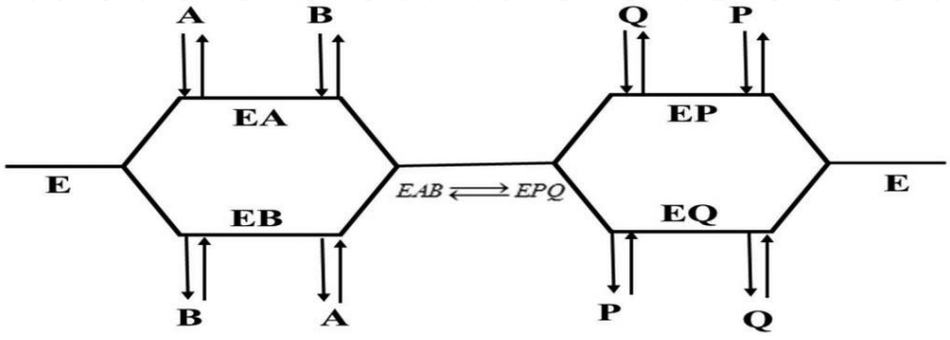

Figure 5. Double-displacement reaction mechanism.

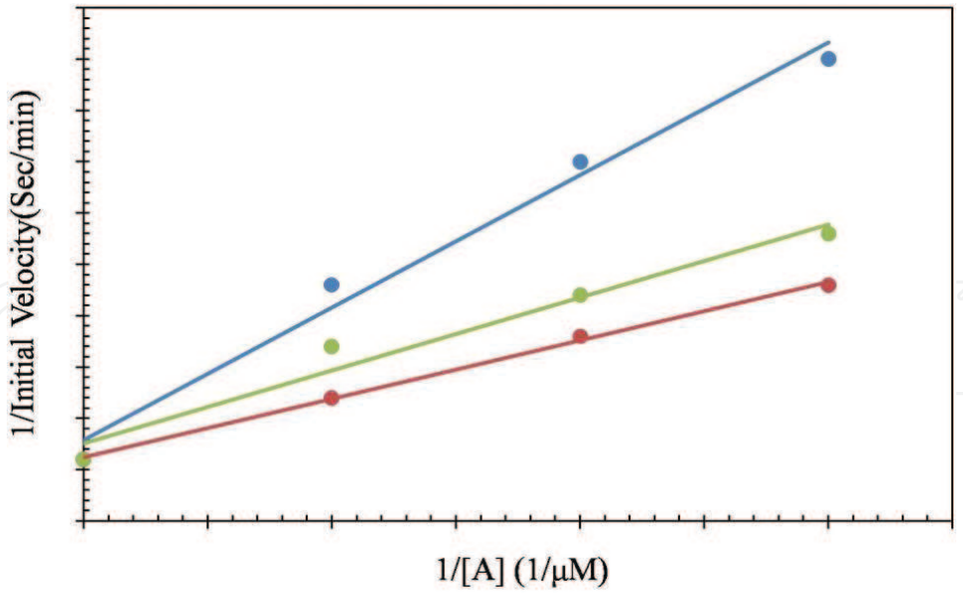

Figure 6. Double reciprocal plot for double-displacement mechanism (at different levels of *B*).

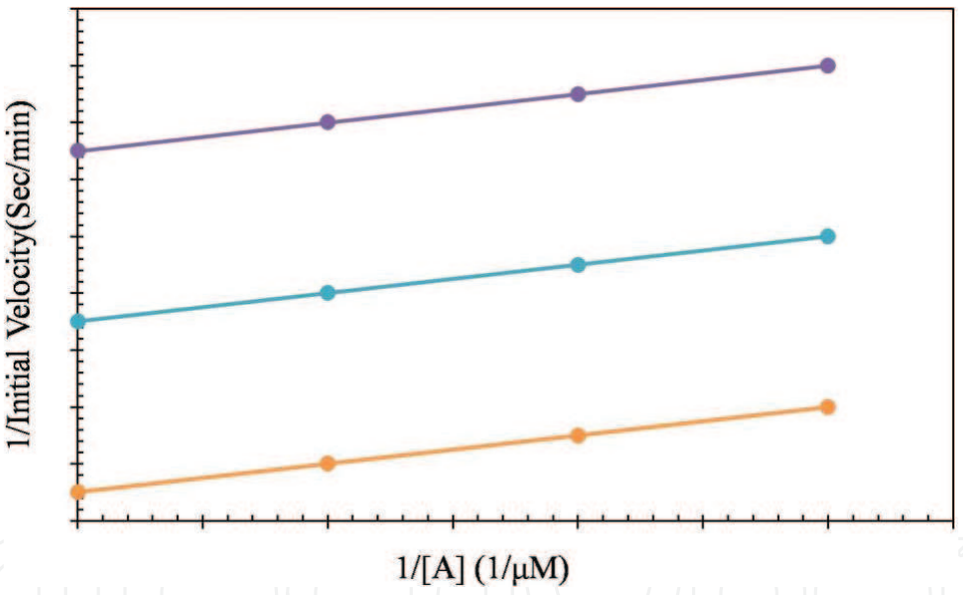

Figure 7. Double reciprocal plot for double-displacement mechanism (at different levels of *B*).

Mechanism	Rate equation	Double reciprocal plots
Rapid equilibrium random sequential mechanism	$v = \frac{VAB}{(K_{ia}+K_bA+K_aB+AB)}$	Intersecting lines
Steady state ordered sequential mechanism	$v = \frac{VAB}{(K_{ia}K_b+K_bA+K_aB+AB)}$	Intersecting lines
Theorell-Chance mechanism	$v = \frac{VAB}{(K_{ia}K_b+K_bA+K_aB+AB)}$	Intersecting lines
Rapid equilibrium ordered sequential mechanism	$v = \frac{VAB}{(K_{ia}K_b+K_bA+AB)}$	Intersecting lines
Double-displacement mechanism	$v = \frac{VAB}{(K_aB+K_bA+AB)}$	Parallel lines

Table 2. Rate expressions for different type of bisubstrate mechanisms.

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kinetic study also reveals which form of enzyme can exist and which cannot, which has significant implications in the study of dead-end inhibition and therapeutic application [68, 69]. The rapid equilibrium ordered mechanism is a unique sequential mechanism as the chemistry step is the slowest step along the reaction pathway. This results in the absence of kinetic term $K_a[B]$ from the rate of expression.

$$v_0 = \frac{V_{max}[A][B]}{K_{ia}K_b + K_b[A] + [A][B]} \tag{39}$$

This mechanism cannot be differentiated from other on the basis of double-reciprocal patterns.

$$\frac{1}{v_0} = \frac{1}{[A]}\frac{K_{ia}K_b}{V_{max}[B]} + \frac{K_b}{V_{max}[A]} + \frac{1}{V_{max}} \tag{40}$$

As discussed previously, both the slope ($K_{ia}K_b/V_{max}[B]$) and intercept ($K_b/V_{max}[B] + 1/V_{max}$) terms reveal a dependency on the concentration of substrate B. In this instance, the graph of intercept will not differ much. By contrast, the slope value ($y = 1/[B](K_{ia}K_b/V_{max})$) when plotted as the slope versus $1/[B]$, a line that passes directly through the origin as K_a/V_{max} does not exist. However, the slope-of-the-slope value reflects free enzyme and is equivalent to $K_{ia}K_b/V_{max}$. Similarly, the implications are valid when bisubstrate reaction is studied by varying concentration B at various levels of the concentration of A, are kept constant.

This distinctive character works as a diagnostic tool to distinguish rapid-equilibrium ordered mechanism from all the other double-reciprocal plots. For various rate expressions for other mechanism, please refer the above section. The double-displacement or ping-pong mechanism, which represents the other type of bireactant mechanism, has symmetrical equation as it can be broken into two separate equations representing each half of the complete reaction [70]. For example, at saturating concentrations of substrate, the equation can be simplified as,

$$v_0 = \frac{V_{max}[A]}{K_a + [A]} \quad \text{at saturating concentration of } B \tag{41}$$

$$v_0 = \frac{V_{max}[B]}{K_a + [B]} \text{at saturating concentration of } A \tag{42}$$

The diagnostic double-reciprocal pattern for a double-displacement mechanism is a series of parallel lines. When varying the concentration of A, the double-reciprocal equation becomes,

$$\frac{1}{v_0} = \frac{1}{[A]}\frac{K_a}{V_{max}} + \left(\frac{K_b}{[B]} + 1\right)\frac{1}{V_{max}} \tag{43}$$

When the intercept of intercept is plotted, it provides the values of K_b/V_{max} and $1/V_{max}$. By contrast, the slope value from the primary plot is defined as $y = (K_a/V_{max})$.

When reaction mechanism is predicted based on the initial velocity data, one has to be sceptical as value of K_{ia} is much greater than that of K_a, rapid-equilibrium random mechanism gets degenerated into a rapid-equilibrium ordered mechanism. If the value of K_{ia} is very less, then the sequential mechanism degenerates into a ping-pong mechanism [11, 45, 55, 61].

Examples

— Ternary complex mechanism

Cinnamyl acetate is a major ingredient of food and cosmetic products as a flavouring or fragrance agent [71]. Yadav and Devendran [71] discuss transesterification of cinnamyl alcohol with vinyl acetate to produce cinnamyl acetate. The parameters were optimized to minimize the errors in the prediction of kinetic constants due to change in temperature and solvent system. The mass transfer resistance was removed to accurately estimate the kinetic constant. Hence, the reaction was conducted in toluene with 10 mg novozym 435 as a catalyst. The mole ratio was maintained at 1:2 of cinnamyl alcohol to vinyl acetate. Under these optimized conditions, the reaction kinetics was studied. The detailed experimental process is explained in the section below. The kinetics was predicted by systematically changing the concentrations of reactants on a wide range of the concentrations keeping others constant. The Lineweaver Burk plot was plotted with the initial velocities of reaction (**Figure 8**). It was observed that for given system under given set of conditions, reaction followed ternary complex mechanism as the plot showed the lines intersecting with each other [71].

The formation of dead-end complex with alcohol at saturating level of cinnamyl alcohols is observed in double reciprocal graph with linear increase in slope and intercept with rise of concentration of cinnamyl alcohol (**Figure 8**). The equation obtained with this mechanism is represented as,

$$v = \frac{V_{\max}[A][B]}{(K_{\text{ia}}K_{\text{mb}}(1 + ([B]/K_{\text{ib}})) + K_{\text{mb}}[A] + K_{ma}[B](1 + ([B]/K_{ib})) + [A][B])} \tag{44}$$

Where v is the velocity of reaction, $V_{\max}$ is the maximum velocity of reaction and $[A]$ and $[B]$ represent the initial concentration of vinyl acetate and cinnamyl alcohol, respectively. K_{ma} and

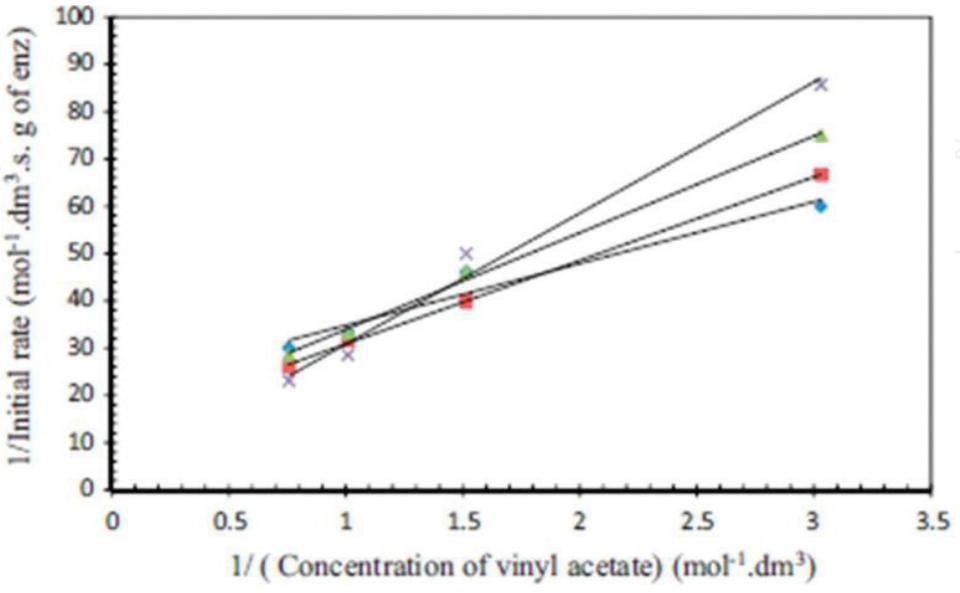

Figure 8. Lineweaver Burk plot of initial velocity versus concentration of vinyl acetate.

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K_{mb} are the Michaelis constants for vinyl acetate and cinnamyl alcohol, respectively; K_{ia} and K_{ib} are the inhibition constants for vinyl acetate and cinnamyl alcohol, respectively. The kinetic constants were predicted using Polymath 5.1 software. The model was validated by plotting the simulate versus experimental rate of reactions for proposed reaction mechanism.

Ping-pong Bi-Bi mechanism

The reaction between ethyl-3-phenylpropanoate and n-butanol was studied using novozyme 435. The reaction conditions of transesterification are optimized for maximum conversion and initial rates to understand the kinetics and mechanism. There is synergism between enzyme catalysis and microwave irradiation. The reaction kinetics was studied after eliminating the external mass transfer limitation. The Lineweaver Burk plot showed that the lines are parallel to each other. The reaction follows the ping-pong bi-bi mechanism with inhibition by n-butanol as the double reciprocal graph represents the data in the form of parallel lines (**Figure 9**) [72].

With experimental data analysis and Lineweaver Burk plot, the reaction rate equation was predicted as,

$$v_0 = \frac{V_{max}[A][B]}{K_{mb}[A] + K_{ma}[B](1 + \frac{[B]}{K_i}) + [A][B]} \tag{45}$$

where K_{ma} and K_{mb} are the respective Michaelis constants for ethyl-3-phenylpropanoate and n-butanol. v_0 and V_{max} represent the respective initial and maximum velocity of the reaction. The data are validated with a parity plot.

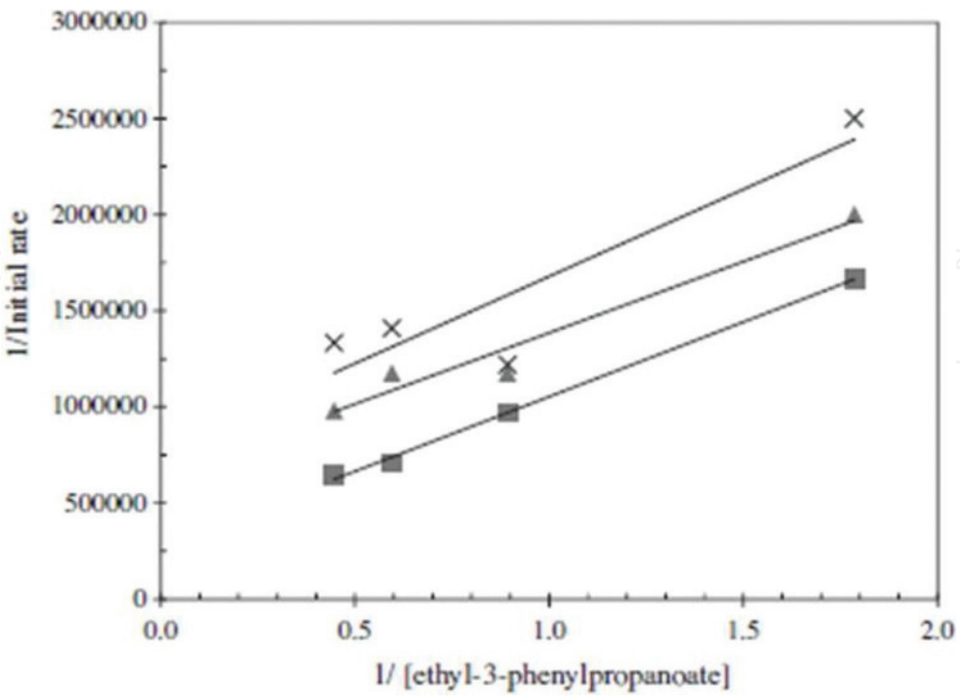

Figure 9. Lineweaver Burk plot of initial velocity versus concentration of ethyl-3-phenylpropionate.

Ordered Bi-Bi mechanism

For the production of perlauric acid, Novozyme 435 was used as catalyst in toluene solvent. The conversion and initial velocity are optimized with reference to the different parameter of reaction. Lineweaver-Burk plots indicated the formation of a ternary complex. The reaction undergoes ordered bi-bi mechanism based on which the kinetic parameters were calculated (**Figure 10**) [73].

Reusability studies indicated that there was enzyme deactivation and from the preliminary study of the deactivation, it was observed that the deactivation obeys a pseudo first-order model. The deactivation was observed at higher levels of hydrogen peroxide wherein the methionine and cysteine amino acid got oxidized due to the hydrogen peroxide. The ordered Bi-Bi mechanism showed the best fit for the given data and it is represented as,

$$v = \frac{V_{\max}[A][B]}{(K_{\text{ia}}K_{\text{mb}}(1 + ([B]/K_{\text{ib}})) + K_{\text{mb}}[A] + K_{ma}[B](1 + ([B]/K_{ib})) + [A][B])} \tag{46}$$

Some of the examples for the bisubstrate reaction are presented in **Table 3**.

2.2.3. Termolecular reaction

Termolecular reactions are the reactions wherein three reactants react simultaneously to form a desired product. They are unusual because the simultaneous collision of three molecules is a rare event. Fourth and higher-order reactions are unknown. These types of reaction are given in literature [74].

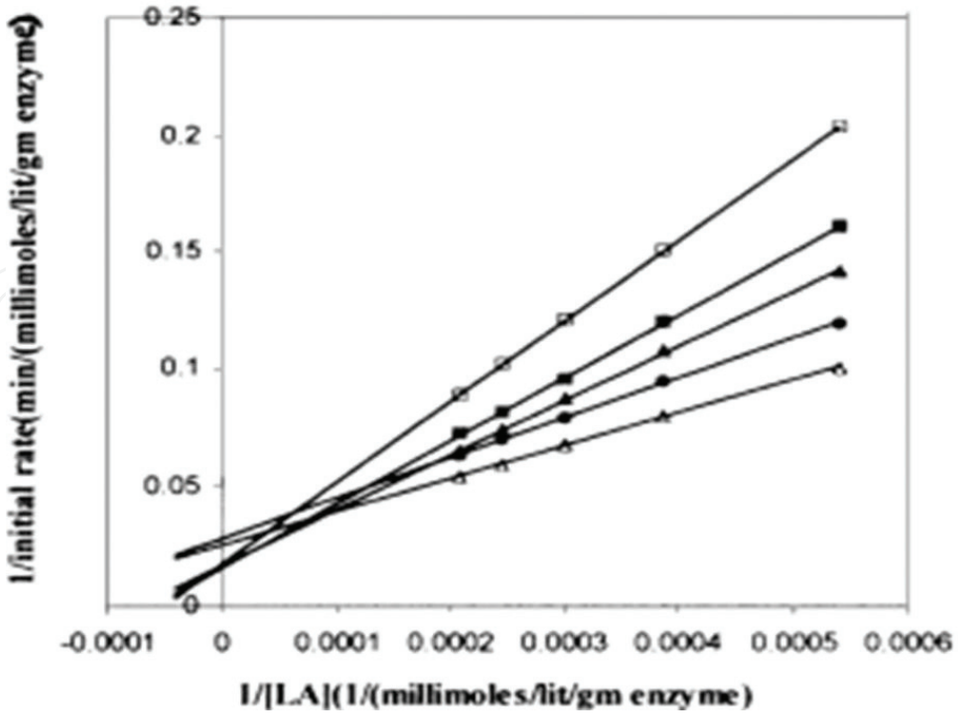

Figure 10. Lineweaver Burk plot of initial velocity versus concentration of lauric acid.

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Sr. no.	Enzyme	Reaction	Kinetic model	Inhibiting molecule	Reference
1.	Mucor Miehei lipase	Esterification of pentanol with caprylic acid	Ping-pong Bi-Bi mechanism	n-Pentanol	[66]
2.	Candida antarctica lipase B	Reaction of citronelly alcohol and vinyl acetate	Ping-pong Bi-Bi mechanism	Both citronelly alcohol and vinyl acetate	[28]
3.	Candida antarctica lipase B	Reaction of n-butyl amine with benzyl acetate	Ternary complex mechanism	n-butyl amine	[77]
4.	Candida antarctica lipase B	Esterification of cinnamyl alcohol with lauric acid	Ternary complex mechanism	Cinnamyl alcohol	[78]
5.	Candida antarctica lipase B	Resolution of (RS)-methyl mandelate	Ordered Bi-Bi mechanism	S-methyl mandelate	[79]
6.	Candida antarctica lipase B	Esterification of butyl alcohol and levulinic acid	Ternary complex mechanism	Levulinic acid	[80]
7.	Candida antarctica lipase B	Reaction of Styrene with perlauric acid	Ternary complex mechanism	Lauric acid	[81]
8.	Candida antarctica lipase B	Transesterification of n-octanol with vinyl acetate	Ordered bi-bi mechanism	n-octanol	[17]
9.	Candida antarctica lipase B	Reaction of (DL)-(+/−)-3-phenyl lactic acid with vinyl acetate	Ordered bi-bi mechanism	(DL)-(+/−)-3-phenyl lactic acid	[27]
10.	Candida antarctica lipase B	Reaction of (+/−)-1-(1-naphthyl) ethanol with vinyl acetate	Ping-pong Bi-Bi mechanism	Inhibition by both substrate	[82]
11.	Candida antarctica lipase B	Hydrolysis of methyl mandelate	Ordered bi-bi mechanism	(RS)-methyl mandelate	[83]

Table 3. List of bisubstrate reaction with their mechanism and inhibition.

3. Enzyme inhibition

The inhibition of enzymes is a naturally occurring phenomenon that control and regulate body's defence and repair system along with various other essential functionalities. It also helps in regulating the optimal use of limited resources available within the cell. Naturally occurring inhibition processes are blood coagulation, blood clot dissolution, complement activation, connective tissue turnover, inflammatory reaction, etc. [57]. Hence, it is very essential to study the nature of inhibitor, mode of action, quantitative estimation of the inhibitory effect of the inhibitor on the enzyme. Such mechanistic and kinetic observations provide the information for the designing of various inhibitors. The inhibitors are usually classified into two groups namely, reversible and irreversible. In case of reversible, inhibitors bind with the

enzyme with non-covalent interaction which can be reversed at any point of time by dilution or dialysis. In second class of inhibitors, i.e. irreversible inhibitors, the inhibitors bind covalently or tightly with the enzyme which cannot revert back causing permanent damage to the catalytic site [68, 75, 76]. The detailed classification of the inhibition is shown in **Figure 11**.

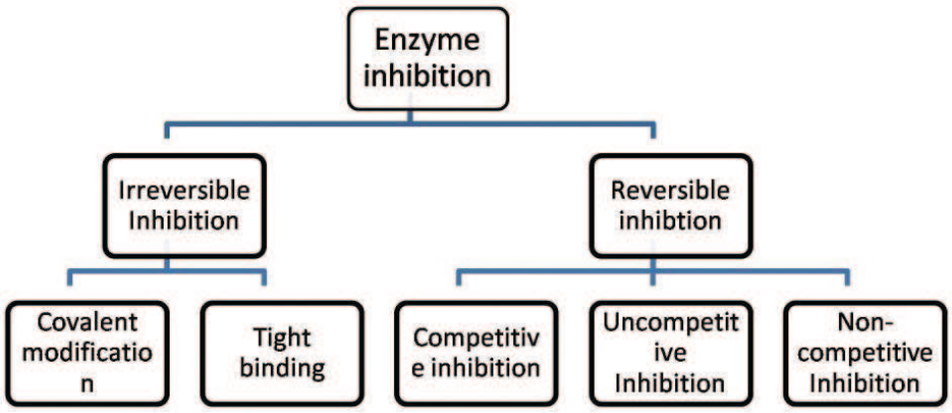

Figure 11. The classification of enzyme inhibition.

3.1. Reversible inhibition

The reversible inhibition is the class of inhibitors which bind with the enzymes and this binding can be reversed with the complete regain of enzyme activity. The reversible inhibition further is mainly classified in three categories competitive, uncompetitive and non-competitive inhibition based on its binding with direct enzyme, enzyme-substrate complex or both [77].

3.1.1. Competitive inhibition

The competitive inhibition occurs due to either binding of substrate or inhibitor to the active site. Both substrate and inhibiting molecule compete for the same active site on the enzyme leading to the reduction in the velocity of the reaction. The diverse set of substrate analogues studied for their activity in the presence of different kinds of inhibitors reveals immense information useful to understand the interaction of the active site and catalytic mechanism of the enzyme. The basic assumption in the study of this type of inhibition is that the inhibitor can only bind to the active site of the free enzyme [3, 74, 78].The Cleland notation for the competitive inhibition can be written as,

$$\begin{array}{c} E + A \underset{k2}{\overset{k1}{\rightleftarrows}} EA \xrightarrow{k2} E + P \\ + \\ I \\ \uparrow\downarrow \\ EI \end{array} \tag{47}$$

which can also be represented as,

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$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EA \xrightarrow{k_2} E + P \tag{48}$$

$$E + I \overset{k_i}{\rightleftarrows} EI \tag{49}$$

The Michealis Menten equation for the competitive inhibition can be written as,

$$v = \frac{V_{\max}[S]}{[S] + K_{\mathrm{m}}(1 + \frac{[I]}{K_i})} \tag{50}$$

Hence, the Lineweaver Burk equation can be derived as,

$$\frac{1}{v} = \frac{(1 + \frac{[I]}{K_i})K_{\mathrm{m}}}{V_{\max}[S]} + \frac{1}{V_{\max}} \tag{51}$$

After plotting $1/v$ versus $1/[S]$, the slope and intercept will give the values of apparent K_{m} and $V_{\max}$ (**Figure 12**).

In this case, K_{m} changes with $V_{\max}$ unchanged. Increase in K_M depends upon the concentration of I. The competitive inhibitors can be applied as targeted blockers of enzyme in the pharmaceutical industry. Product can also act as a competitive inhibitor in various regulation pathways in the cells which increases the efficiency of cellular processes by eliminating the accumulation product and diverting the substrate to another pathway [79]. Both structural analogue and in some cases, unrelated compounds act as competitor inhibitors. For example, alkaline phosphatases are inhibited by the inorganic inhibitors where both substrate and inhibitor have similar affinities. Because of high selectivity, it provides many *in vivo* application opportunities, e.g. penicillinase, prostaglandin cyclooxygenase.

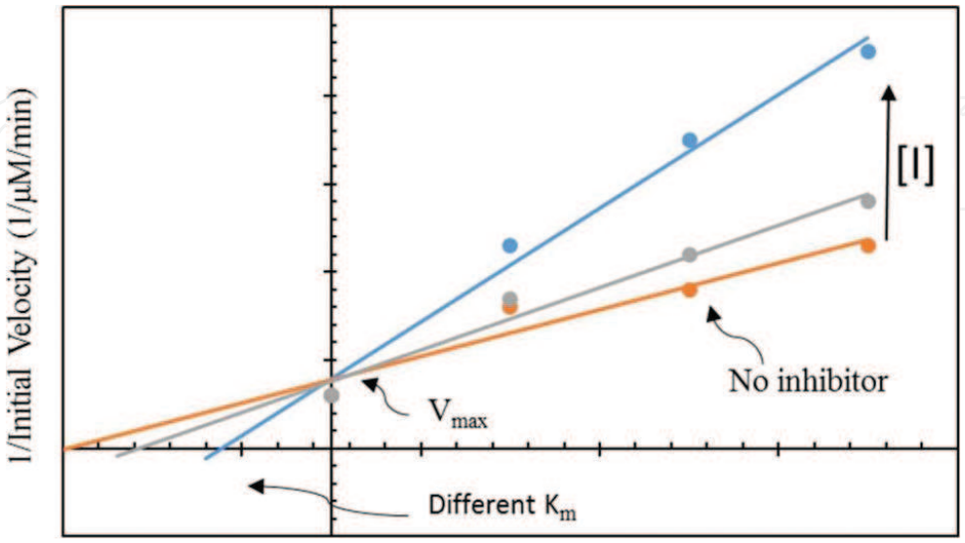

Figure 12. Graphical representation of competitive inhibition.

The reactions requiring the presence of metal ion as co-factors compete with similar ones for the catalytic site on the enzyme, e.g. Ca ions compete with Mg requiring enzyme. Similarly, Na requiring enzymes are inhibited by the Li and K ions. In double-displacement reaction mechanism, high concentration of the second substrate acts as a competitive inhibitor with reference to binding of first substrate, e.g. aminotransferase.

Adulteration of ethanol with methanol makes it unsuitable for human consumption, commonly known as denatured alcohol. Methanol is oxidized in liver and kidney to form formaldehyde and formic acid. This causes damage to retinal cells that may cause blindness which is followed by severe acidosis which lead to death. This may also lead to depression of CNS. Retardation of first step in oxidation of methanol can be achieved by administration of ethanol. The removal of methanol is done by gastric lavage, haemodialysis and administration of exogenous bicarbonate. Ethylene glycol is an anti-freezing agent used in automobiles. Ingestion in the body leads to depression of CNS and causes metabolic acidosis with severe renal damage after oxidation by alcohol dehydrogenase which is inhibited by ethanol or 4-methyl pyrazole. Kidney damage is resulted due to the deposition of oxalate crystal into convulsed tubules. Elevated anion-gap metabolic acidosis is caused by glycolic acid and lactic acid. The shift in redox potential causes the production of lactic acid instead of pyruvate. The treatment is same as that of methanol adult injection. Fomepizole drug (4-methylpyrazole) can be used in the treatment without any side effect that is caused by the ethanol. Isopropanol is major constituent in rubbing alcohols such as hand lotion and anti-freezing preparations. If ingested accidentally is oxidized and converted into acetone, a toxic non-metabolized product by alcohol dehydrogenase. It also causes depression in CNS, coma, gastritis, vomiting and haemorrhage. This can be treated by haemodialysis.

Toxicity by these substrates is done by evaluation of following serum components: Na, K, Cl, HCO_3, glucose, urea nitrogen, blood osmolality, blood gap, anion gap and metabolic acidosis along with pertinent medical history. Serum osmolal gap is difference between measured osmolality and calculated osmolality.

Serum osmolal gap = Measured osmolality-calculated osmolality

Calculated osmolality=2 $\times$ Na^+ (mM/L) + glucose (mM/L) + Urea nitrogen (mM/L)

Small rise in toxic substances increase osmolality significantly. Hence, it becomes very easy to detect the toxicity in the body based on this test.

3.1.2. Uncompetitive inhibition

Uncompetitive inhibition occurs when instead of enzyme, inhibitor binds with enzyme substrate complex to inhibit the reaction. The binding of inhibitor is possible only after the binding of substrate to the enzyme. The binding site of the inhibitor forms when the substrate binds with enzyme. This type of inhibition is rare where inhibitor binds with the enzyme substrate complex [7, 52]. This can be represented in the form of Cleland notation as,

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$$\begin{array}{c} E + A \underset{k2}{\overset{k1}{\rightleftarrows}} EA \xrightarrow{k2} E + P \\ + \\ I \\ \uparrow\downarrow \\ EAI \end{array} \tag{52}$$

can also be represented as,

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EA \xrightarrow{k_2} E + P \tag{53}$$

$$EA + I \overset{k_i}{\rightleftarrows} EAI \tag{54}$$

The Michealis Menten equation for the uncompetitive inhibition becomes,

$$v = \frac{V_{\max}[S]}{K_{\mathrm{m}} + [S](1 + \frac{[S]}{K_{\mathrm{i}}})} \tag{55}$$

The Lineweaver Burk equation can be written as,

$$\frac{1}{v} = \frac{K_{\mathrm{m}}}{V_{\max}[S]} + \frac{(1 + \frac{[I]}{K_{\mathrm{i}}})}{V_{\max}} \tag{56}$$

The plot is shown in **Figure 13**.

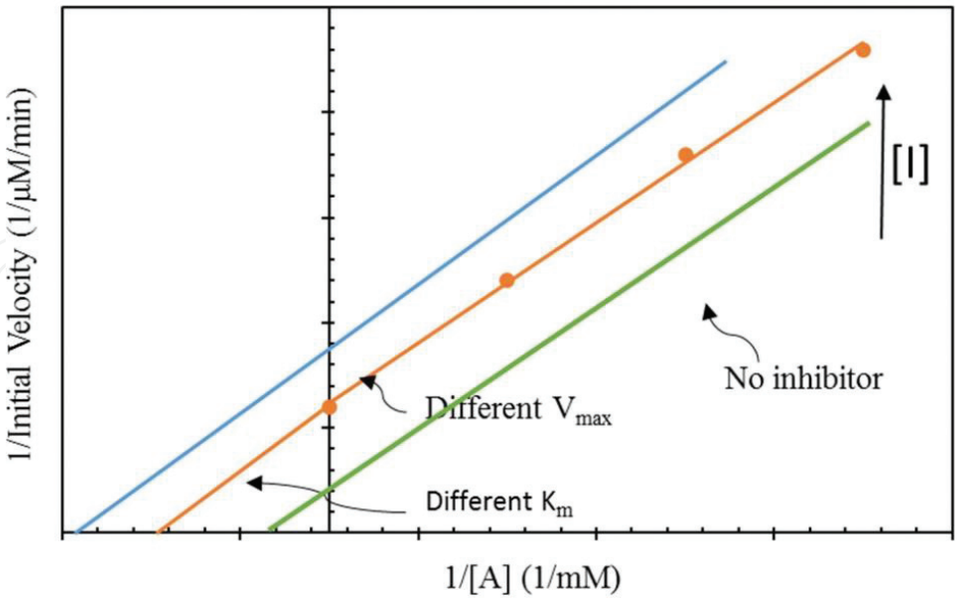

Figure 13. Graphical representation of uncompetitive inhibition.

The binding with ES complex yields parallel lines wherein both slope and intercept change. The apparent K_m and V_{max} include division by (1+ $[I]/K_i$). This type of inhibition is rarely observed in single substrate reactions. The classical example of this type of inhibition is an intestinal alkaline phosphatase which is inhibited by l-phenylalanine.

3.1.3. Non-competitive inhibition

Non-competitive inhibition is observed when inhibitor can bind with both enzymes and enzyme-substrate complex. The inhibitor bears no structural resemblance to substrate and bind to distinct site than the substrate. There is no competition between substrate and inhibitor for the active site of the enzymes. This type of inhibition cannot be overcome by increasing the substrate concentration. This may bind to enzyme or enzyme-substrate complex making both of them catalytically inactive [8, 80]. This can be denoted as,

$$A + E \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EA \overset{k_2}{\rightarrow} E + P \tag{57}$$

$$E + I \overset{k_i}{\rightleftarrows} EI \tag{58}$$

$$EA + I \overset{k_i}{\rightleftarrows} EAI \tag{59}$$

The Cleland notation is denoted for the non-competitive inhibition.

$$E + A \underset{k2}{\overset{k1}{\rightleftarrows}} EA \overset{k2}{\rightarrow} E + P + I \rightleftarrows EI + I \rightleftarrows EAI \tag{60}$$

The double reciprocal plot for the non-competitive inhibition is represented in **Figure 14**.

Examples:

• Enzymes with sulfhydryl group that participate in maintenance of three-dimensional confirmation of the molecules are non-competitively inhibited by heavy metal ions such as silver (Ag), lead (Pb) and mercury (Hg).

$$ESH + Hg^{+2} \rightleftarrows ESHg^{+} + H^{+} \tag{61}$$

Heavy metal ions react with S-, O- and N-containing ligand. Hence, they can inhibit enzymes in the metabolic pathway (see **Table 4**).

3.2. Irreversible inhibition

Irreversible inhibition occurs, when inhibitor molecule bind with enzyme so strongly that it does not dissociate from the enzyme. This kind of inhibitor binds rapidly with the enzyme and deactivated the enzyme completely. The activity decreases exponentially with binding of the inhibitor, at saturating levels of inhibitor concentration. At lower concentration, the rate of reaction decreases linearly. The covalent modification and tight binding ($K_d < 10^{-8}$ M) are two

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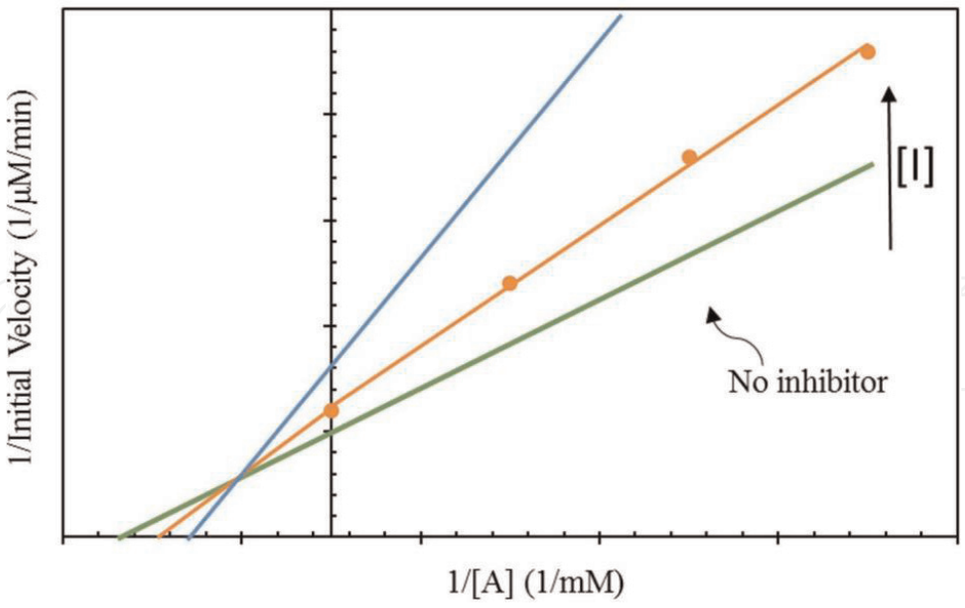

Figure 14. Graphical representation of noncompetitive inhibition.

types of irreversible inhibitions; for practical purposes, there is no dissociation of E and I. Thus, physical separation processes are ineffective in removing the irreversible inhibitor from the enzyme [33, 75, 81]. Reaction is written as,

$$E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \xrightarrow{k_2} EI^* \tag{62}$$

3.2.1. Transition state analogues

Transition state is a state in which substrate is strongly bound and interacting with enzyme for short period (in picosecond). Therefore, the transition state analogues bind tightly with transition state enzyme and cannot be easily dissociated from the enzyme [82–84, 94, 102, 103, 104, 105]. The rate expression for such type of inhibition is represented as,

$$E_t = Ee^{-\frac{k_2[I]}{[I]+k_i}} \tag{63}$$

3.2.2. Suicide inhibition

The suicide substrate binds covalently with an active site of the enzyme and blocks the enzyme completely. The mechanism of binding of suicide substrate with the active site of the enzyme gives the understanding of enzyme mechanism [84–86].

$$\begin{array}{c} E + I \underset{k_{-1}}{\overset{k_1}{\rightleftarrows}} EI \xrightarrow{k_2} EI^* \xrightarrow{k_3} E + I^* \\ \downarrow k_{+4} \\ E - I \end{array} \tag{64}$$

Sr. no.	Enzyme	Substrate	Inhibitor	Mechanism of action	Reference
Competitive inhibition					
1.	Cytochrome c oxidase	Oxygen	Cyanides	Competes with active site	[92]
2.	Succinate Dehydrogenase	Succinate	Malonate, oxaloacetate, oxaloacetate	Competes with active site	[93, 94]
3.	HMG-CoA reductase	HMG-CoA	Lovastatin	Competes with active site	[54]
4.	Sucrase	Sucrose	Acarbose, nojirimycin, and deoxynojirimycin	Competes with active site	[95]
5.	Influenza neuraminidase	Neuraminic acid	Oseltamivir carboxylate	Competes with active site and prevent escape of viral flu particles (Influenza)	[96, 97]
6.	Dihydropteroate synthetase	p-Aminobenzic acid	Sulfonamides	Competes with active site (bacterial infection)	[98]
7.	Dihydrofolate reductase	Folate	Methotrexate	Competes with active site and prevent rapid DNA synthesis (HIV)	[98]
8.	Xanthine oxidase	Hypoxanthine	Allopurinol	Competes with active site to block formation of uric acid (Gout, hemotological disorders, antineoplastic therapy)	[99, 100]
9.	Cytochrome P450 enzyme	Range of substrate	Biapigenin (hypericum perforatum extracts)	Competes with active site	[101]
Non-competitive inhibition					
1.	Porphobilinogen synthase and ferrocene lactase	Heme group synthesis pathway	Lead (Pb)	Bind with sulfuryl group of enzyme	[102, 103]
2.	Enzyme requiring divalent ions	Magnesium and calcium	Ethylenediamine tetracetate (EDTA)	Chelates the metallic ion	[104]
3.	Enolase (magnesium and manganese complexes	2-phosphoglycerate	Fluorides	Competes with magnesium and manganese complexes and prevent glycolysis pathway.	[105, 106]
4.	Acetylcholinesterase	Acetylcholine	cis-2-dimethylaminocyclohexanol, (hydroxyphenyl)-trimethylammonium derivatives	Binds with esterase active site	[107]
Uncompetitive inhibition					
	Inositol monophosphatase	Inositol	Lithium	Interfere with polyphosphoinositide metabolism in brain	[108, 109]

Table 4. List of examples of different types of inhibitions.

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Examples:

- Enzymes containing free sulfhydryl group at active site of the enzymes. These groups react with alkylating agent such as iodoacetic acid. This cause inactivation of enzymes. Imidazole ring also showed the reactivity with the iodoacetic acid, e.g. Ribonuclease, two residue Histidine 12 and Histidine 19 loses activity by reacting with iodoacetic acid at pH 5.5 [8].
- Seryl containing hydroxyl group at active site modified by organophosphorus diisopropyl phosphate fluoridate (DFP) and it inactivates serine hydrolase. Acetylcholine esterase has two types of active sites: (1) esteric activity: site contains Seryl OH sites whose nucleophilicity has been enhanced by properly placed imidazolium group. Generally, function as base catalyst (2) site containing negative charges. The seryl hydroxyl site reacts with DFP and inactivates its activity. The inhibition of acetylcholine esterase can be reversed and enzyme can be reactivated. The reactivation by hydrolysis is very slow. Therefore, nucleophilic reagents such as hydroxyl amine, hydroximic acid and oximes can reactivate enzyme more rapidly [87].
- In activation and deactivation of cytochrome oxidase, cyanides are most rapidly acting toxic substances. It inhibits intracellular respiration and causes tissue hypoxia by binding to terminal component of mitochondrial electron transport chain. This chain uses molecular oxygen to generate energy. Cyanides severely impede mitochondrial respiration. It causes cell death and affects CNS and respiratory system. It is an oxidized form of cytochrome oxidase has high affinity for CN^{-} forms and loses complex with $Fe^{+2}$of porphyria which forms stable complex with negative cyanides ions. It prevents oxygen uptake, e.g. methemoglobin [88].
- Proteinase inhibitor and their applications: Mostly, proteinases are proteinaceous in nature. They are present in intracellular and extracellular fluids in the form of enzymes and peptides. Other protein inhibitors are very rare. Within the cells, it contributes in the control of blood clot, activation of complement cascades, formation and destruction of peptide hormones. Protein inhibitor combines with target proteinase. It converts then into non-dissociable form. It binds to reactive or/and active site. Specific amino acid is recognized as primary binding site. For example, elastase is inhibited by alpha-1 protein inhibitors, which inhibit serine-containing proteinase. Alpha-2 macroglobins are present in plasma of mammals. It can combine with the variety of proteinase. It only inhibits proteolytic enzymes acting towards large protein. It is very useful in metastasis of cancer. It requires remodelling of extracellular matrix (ECM). Viral proteinase offers unique target HIV protease inhibitors. Reverse transcription of RNA to dsDNA is inhibited by nucleoside analogue such as zidovudine, didanosine, zalcitabine, stavudine and lamivudine. After integration, it produces protease, i.e. aspartly protease, which is a homodimer cleaves at phenylalanine-proline bonds and is observed in mammals [88] (see **Table 5**).

3.3. Inhibition in combination with each other

The kinetics expression for mutually exclusive inhibitors is studied for single substrate system. The overall reaction velocity for the system in presence of types of inhibition is given by,

Sr. no.	Enzyme	Substrate	Inhibitor	Mechanism of action	Reference
1.	ATPase	Phosphoenolpyruvate, ATP	Rutamycin, bongkrekic acid	Tight binding inhibitions	[116]
2.	Human mast-cell tryptase	Benzamidine	CRA-001390 inhibitor	Tight binding inhibitions	[113]
3.	Adenosine deaminase	Adenosine (purine metabolism)	Deoxycoformycin, 1,6-dihydro-6-hydroxymethyl purine ribonucleoside	Tight binding inhibitions	[117]
4.	Human caspases	Proteins	Peptide based (4 amino acid sequence)	Tight binding inhibitions	[118]
5.	Serine proteases	Proteins	Phenylmethanesulfonyl fluoride	Suicide inhibition	[36]
6.	Thymidylate synthase and Cofactor methylene tetrahydrofolate	Deoxyuridine monophosphate	Fluorouracil	Suicide inhibition	[36]
7.	Chymotrypsin	Protein	Tosyl phenylalanyl chloromethylketone	Suicide inhibition	[119]
8.	Monoamine oxidase (MAO)	Norepinephrine, serotonin	Clorgyline, deprenyl, pargyline	Suicide inhibition	[120]
9.	Cyclooxygenase 2	Arachidonic acid	Vioxx, celebrex	Suicide inhibition	[121–123]

Table 5. Examples of irreversible inhibitions.

$$\frac{1}{v_{1,2,\ldots n}} = \sum_{i=1}^{n} \frac{1}{v_i} - \frac{n-1}{v_0} \tag{65}$$

Wherein, v_0 is the velocity in the absence of inhibition and 'n' is the number of inhibitors used in combination. Similarly, the kinetic expression is derived for the two substrate systems. For example, two substrate reaction with two inhibitors: ping-pong Bi-Bi mechanism.

Two inhibitors: I_1 is competitive with respect to the substrate A (binds to E) and uncompetitive with respect to the substrate B; I_2 is competitive with respect to the substrate A (binds to E) and uncompetitive with respect to the substrate B.

$$\frac{1}{v_0} = \frac{(AB + K_{\mathrm{b}}A + K_{\mathrm{m}}B)}{VAB} \tag{66}$$

$$\frac{1}{v_1} = \frac{(AB + K_{\mathrm{b}}A + K_{\mathrm{m}}B(1 + \frac{I_1}{K_{I_1}}))}{VAB} \tag{67}$$

$$\frac{1}{v_2} = \frac{(AB + K_bA + K_mB(1 + \frac{I_2}{K_{I_2}}))}{VAB} \tag{68}$$

$$\frac{1}{v_{1,2}} = \frac{(AB + K_{\mathrm{b}}A + K_{\mathrm{m}}B(1 + \frac{I_1}{K_{I_1}} + \frac{I_2}{K_{I_2}}))}{VAB} \tag{69}$$

Overall velocity of the reaction is given as,

$$\frac{1}{v_{1,2}} = \frac{1}{v_1} + \frac{1}{v_2} - \frac{1}{v_0} \tag{70}$$

Synergism:

$$\frac{1}{v_{1,2}} \rangle \frac{1}{v_1} + \frac{1}{v_2} - \frac{1}{v_0} \tag{71}$$

Antagonism:

$$\frac{1}{v_{1,2}} \langle \frac{1}{v_1} + \frac{1}{v_2} - \frac{1}{v_0} \tag{72}$$

The expression is useful to understand the effect of inhibitors on each other's activity when used in combination. The overall velocity of the reaction is independent of mechanism of reaction, type of inhibition and number substrate used in the study [89].

4. Analytical aspects of enzyme inhibition

4.1. Assay conditions for single substrate enzyme kinetics

To estimate the reaction rate, it becomes very essential to quantify accurately the change in the concentration of either substrate or product or both during the progress of reaction. If the substrate and product generate different signals, the spectroscopic techniques like UV-visible spectroscopy and fluorescence spectroscopy can be applied to estimate these parameters [90]. If there is no significant difference between the products and substrate signal, then the better alternative is a discontinuous assay which measures the rates by intermittent sampling. This sampling can be done with removal of aliquots of a reaction mixture or sampling from the batch reactor. These reaction samples represent the time points of a reaction, and their respective concentration of product formed and substrate consumed. In some cases, labelling the molecules with chromophore or the fluorescent dye can serve as a better option. The estimation of amount of an ATP can be quantified by determining the amount of Pi present by the Malachite Green assay with absorbance at 660 nm [91]. Chromatography techniques can separate the substrate from the product which is then estimated with the spectroscopic method in continuous or discontinuous manner. In depth understanding, the enzyme mechanism is studied by radiolabelling the substrate, which is tracked for the transition of substrate through intermediate state to the product during the reaction progress [92]. The crystallographic structures of separated intermediate complexes and the various states of enzymes during the reaction progress reveal most of the mechanistic aspect of the reaction. With the advent of instrumentation technology, the product can be separated or analysed by thin-layer chromatography, separation techniques such as high-performance liquid chromatography, electrophoresis and gel filtration. The high-performance liquid chromatography is the most applied system as it gives the separation and quantification of substrate as well as product in real time.

Recent studies showed that the mass spectrometric (MS) techniques are applied to study the conversion of substrate into product and their behaviour in the presence of modulators. Matrix-assisted laser desorption ionization-time of flight-mass spectroscopy (MALDI-TOF-MS) is proved as more effective technique when compared to other mass spectrometric techniques, such as electronspray ionization MS, as it showed negligible interference due to the presence of buffers and reagents. MALDI-TOF-MS can quantify the ratio of substrate to product form. Greis et al. studied the phosphorylation catalysed by kinases using this technique for accurate prediction of the kinetics of reaction [93]. This technique has been used in combination with various chromatographic techniques such as capillary isoelectric focusing, frontal affinity chromatography and size exclusion chromatography to analyse the inhibition of the enzyme in direct or indirect manner [93–96]. This method is free of laborious work of labelling or derivatization and can be done in short period of time.

To estimate the enzyme inhibition and relative kinetic parameters, the stepwise process has to follow with the aim of minimum human error, which is discussed in the following sections.

4.1.1. Experimental measurement of V_{max}, k_{cat}, *and* K_m

As discussed in the above sections, the quantification of the initial velocity, the region of the curve wherein velocity does not change with time (up to 10% of the conversion), is of prime importance to estimate the reaction kinetics under the influence of other factors such as product inhibition. This region varies with the type of enzyme, nature of substrate and medium conditions and type of modulator. Hence, for any given system, it cannot be predetermined. The instrument capacity needs, therefore, has to be very precise and effective to detect the signal generated from the build-up of product and to plot it. As the measurement needs to be done at a very constrained range, it may severely compromise on measurements because of the poor instrument capacity.

The linear range for the product of an instrument can be estimated by measuring the response of product at various known concentration and preparing the standard curve. Before the start of experimentation for evaluating enzyme kinetics, it is very essential to optimize the conditions of the reactions so that the enzyme remains stable throughout the reaction progress. If the maximum plateau value of product formed does not reach the same for all levels of tested enzyme, it is likely due to the enzyme instability over time [10, 53].

Conditions required for measuring initial velocity of an enzyme reaction:

- Equilibrate and maintain all the reaction reagents to the optimum temperature.
- Keep the reaction medium condition static (previously optimized or derived from literature) for the optimum performance of enzyme.
- Replicate the same reaction for minimum three times to avoid the manual errors.
- Proper analytical technique is developed for the estimation of product formed or substrate consumed below 10%.
- Record the signal of the reaction mass at time zero for the background.

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For kinase assays, generally, the background can be recorded without the enzyme or the substrate. The condition with highest background level should be used for further estimation of the parameters. In case of EDTA, background is taken with control without EDTA during validation of a kinase assay. Once the assay has been validated, if the background measured with EDTA is the same as that of no enzyme and no substrate control, then EDTA could be used.

Measurement of K_m and V_{max}

After primary experiments, wherein the initial velocity conditions have been established and change in velocity with respect to time has been estimated, the substrate concentration should be varied. These data will generate a saturation curve and can be used for the determination of K_m and V_{max} values. The Michaelis Menten kinetic model shows that the $K_m = [S]$ at $V_{max}/2$. In order for competitive inhibitors in which the substrate competes, the substrate concentration should be maintained around or below the K_m. At the substrate concentration higher than the K_m, the identification of competitive inhibitors itself becomes more difficult.

For kinase assays, two set of experiments are carried out. In first experiment, the K_m for ATP is determined at saturating levels of the substrate concentration. Subsequent reactions need to be conducted with optimum ATP concentration (around or below the K_m value). However, simultaneous determination of K_m for ATP and specific substrate gives more accurate estimation of kinetic constants with maximum information and any potential co-operativity between substrate and ATP [91]. To achieve steady state, ratio of substrate to enzyme is maintained in between 100 and one million.

How to measure K_m

- Minimum eight experiments should be conducted with various substrate concentrations from 0.2 to 5.0 K_m (from the literature, if available).
- The initial estimate is established by using the wide range of six substrate concentrations when no data are available in the literature. This is then followed by the first step.
- For bisubstrate reaction, the K_m of the substrate of interest is measured in replicates by keeping the other substrate at saturating concentrations and vice-versa.
- The data are fitted in rectangular hyperbola function using non-linear regression analysis.

The reaction product is measured at various times for eight different levels of substrate. The product generated (Y-axis) is plotted against the reaction time (X-axis) with each curve of different concentration of substrate. The slope of the line is the initial velocity (v) of the reaction curve.

These resulting initial velocities (Y-axis) are plotted against the concentration of substrate (X-axis) and fitted with a non-linear regression analysis in a rectangular hyperbola (**Figure 2**). The K_m is one-half the maximum velocity determined under saturating substrate concentrations.

The linear plots are generated to determine the kinetic constants besides fitting the data by non-linear regression such as Lineweaver–Burk plot wherein the reaction rates ($1/v_o$) are plotted against reciprocal substrate concentrations ($1/[S]$) with y-intercept equivalent to $1/V_{max}$; and the slope of K_m/V_{max}. Various linearization plots such as Eisenthal–Cornish–Bowden

plot, Dixon plot and Hanes plot can also be used to estimate kinetic and inhibition constant (see Section of Enzyme Kinetics).

Optimization experiments

Published literature information should be used in selecting these factors. The following parameters should be optimized prior to kinetic study so that the enzyme remains stable throughout the experiments.

- Divalent cations, for example Ca^{2+}, Mg^{2+}, Mn^{2+}
- Salts, for example NaCl, KCl
- Reducing agents, such as β-ME, DTT and glutathione
- Bovine serum albumin
- Detergents, such as Triton, CHAPS and DMSO
- Buffer source, for example HEPES versus acetate buffer
- pH

The assay conditions should be validated to avoid the loss of enzyme activity due to the wrong selection of buffer, pH or temperature [97].

4.2. Assay conditions for bisubstrate reaction

The plotting of Lineweaver Burk plot is more important to distinguish between a sequential reaction mechanism (plotted lines intersect each other) and a double-displacement or 'ping-pong' kinetic mechanism (plotted lines remain parallel). In some cases, these graphs gave preliminary evidences in different types of sequential kinetic constants. In the process of predicting kinetic and inhibition constant for the bisubstrate reaction, it is compulsory to understand the mechanism of the reaction to predict the appropriate rate equations. The data were generated by plotting the reciprocals of the initial rate of product formation versus [A] at the differing [B] and vice-versa. Most importantly, the values of kinetic parameters such as K_m and V_{max} change for substrate A with the change in substrate B concentration [12, 41, 63]. In case of random sequential reaction, the double reciprocal plot of rate expressions gives the apparent value of slopes and intercept which include the term for concentration B.

$$\frac{1}{v_0} = \frac{1}{[A]}\left(\frac{K_{ia}K_b}{V_{max}[B]} + \frac{K_b}{V_{max}}\right) + \frac{K_a}{V_{max}[B]} + \frac{1}{V_{max}} \quad [B] = \text{constant} \tag{73}$$

The double reciprocal plot gives the confirmation of the mechanism followed by the reaction. But, as the slope $\left(\frac{K_{ia}K_b}{V_{max}[B]} + \frac{K_b}{V_{max}}\right)$ and intercept $\left(\frac{K_a}{V_{max}[B]} + \frac{1}{V_{max}}\right)$ terms involve the concentration term, it signifies the dependency on the concentration B. Also, it is noted that the slope and intercept are the linear equations and can be plotted against 1/[B]. When the intercept is plotted against 1/[B], it gives the values of $1/V_{max}$ and K_a/V_{max} and this plot is called as intercept of intercept (**Figure 15**). At different concentration of substrates various different forms of

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enzyme exist in the system. To obtain the intercept of intercept of the secondary plot, concentration of both *A* and *B* are maintained at higher concentrations which give rise to higher concentration of EAB Form.

To calculate the K_a/V_{max} of desired enzymatic reaction, the EA form of enzyme should be predominant. Hence, the slope of the intercept is calculated by maintaining the concentration of *A* at high levels while concentration of *B* is kept low. The plot of slope versus 1/[*B*] will give the values of K_b and K_{ia}, which are measured graphically (**Figure 16**).

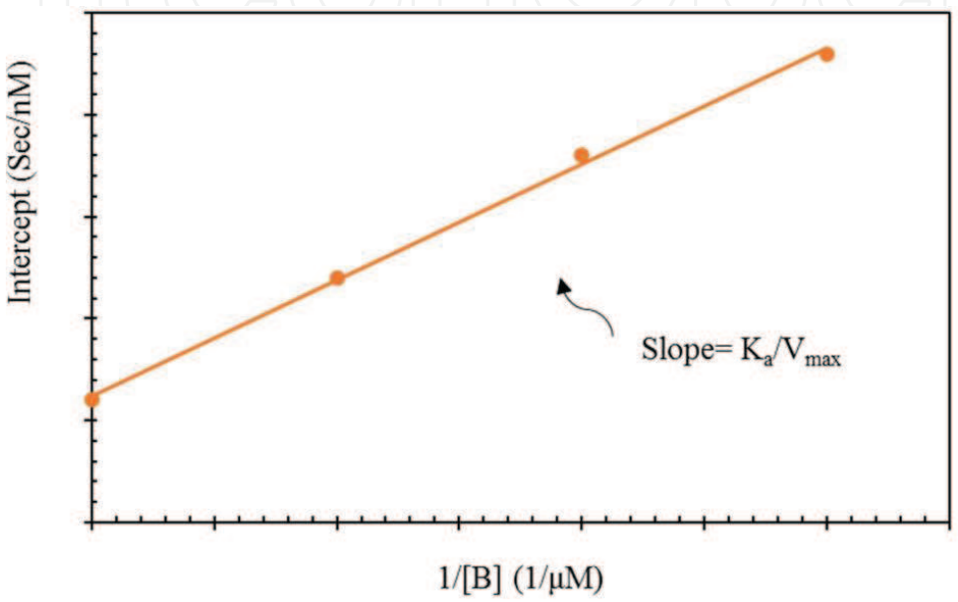

Figure 15. Secondary plot of intercept versus reciprocal of concentration of *B*.

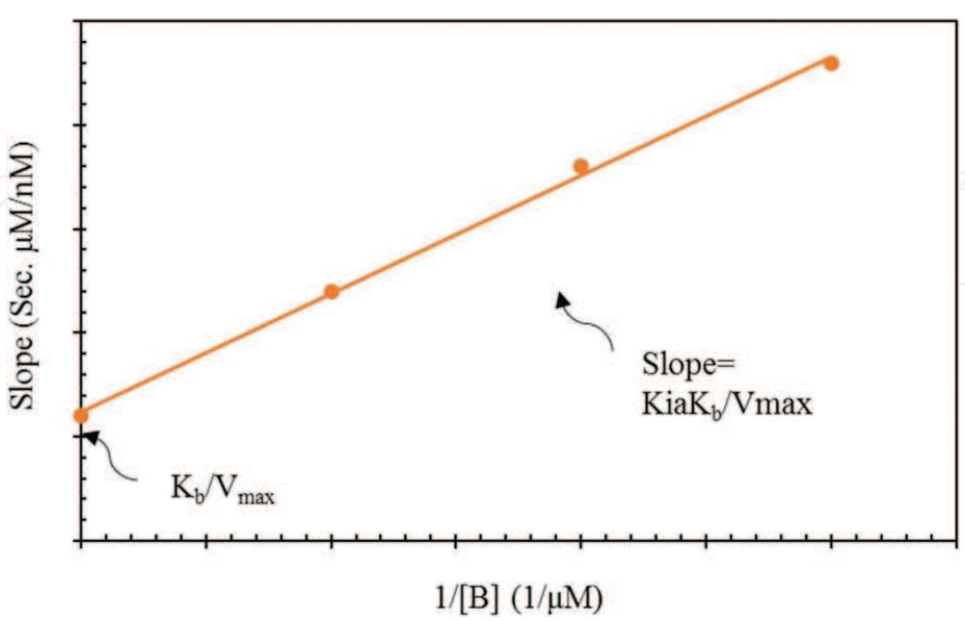

Figure 16. Secondary plot of slope versus reciprocal of concentration of *B*.

Similarly, same kind of analysis can be performed to find out the V_{max} and K_b/V_{max} by keeping high levels of B and A. The plot of intercept of intercept, i.e. intercept versus 1/[A], will give the values of $1/V_{max}$ and K_b/V_{max}. The slope plotted against the 1/[B] was studied for deriving the values of K_a and K_{ib} by analysing at high levels of B and low concentrations of A (building up the concentration of EB). These secondary plots elaborate various aspects of enzyme kinetics some of which are discussed in the kinetics section.

4.3. Software used in kinetic data analysis

The data-fitting process can be accomplished by using a software program that provides non-linear regression-fitting capability. Various programs such as Kaleidagraph are developed wherein the user put the experimental data along with probable fitting function for the prediction of rate constants. Some other software such as dynafit, mathematica, sigmaplot and prism are applied for the estimation of rate expression and effect of inhibition or activator on the enzyme [98]. Alternatively, some of the programs like Enzfitter have a predefined library of equations which can also be used for the prediction kinetics of enzyme. While using the software for data prediction, one should be able to differentiate in errors added due to the lack of fit and pure error as they contribute as a source of error [99]. Although both sources of error normally contribute to the sum of squares of deviations from a model, they can be separated. The inconsistencies between replicate observations are unaffected by the choice of model and thus allow calculations of how much of the total sum of squares is due to the pure error, and from this one can calculate the contribution of lack of fit [57, 80, 100].

The basic assumption during the development of related software is that the rate of reaction is zero in the absence of substrate. The buffer solution used for the reaction should be selected so that the *pKa* is not greater than 1 unit of pH that the desired one. Generally, the operational pH should be less than *pKa*. This will maintain the desired configuration required for the optimal activity of the enzyme. Also, the used buffer system should not react with the enzyme used for the catalysis or the buffer interfering with the analysis method [10, 52, 88]. Primary assumptions that any developed software have done are as follows:

1. Enzyme to substrate interaction ratio is maintained as 1:1.
2. Inhibitor compete with enzyme active site/or active groups at enzyme allosteric site to form enzyme-inhibitor-substrate and/or enzyme-inhibitor complex.
3. The loss of active free energy decides the formation of enzyme-inhibitor-substrate complex.
4. Reaction is reversible at every stage of the interaction between enzyme-substrate, enzyme inhibitor and enzyme-substrate-inhibitor interaction.
5. The binding of inhibitor or substrate with enzyme is expressed in terms of kinetic constants of a catalytic reaction.
6. The physiological conditions such as pH, temperature, concentration of reactants and reaction period are kept constant while determining the kinetic constants.

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7. The overall enzyme reaction rates and mode of inhibition are depended upon the intermolecular forces between enzyme subunits, substrate or inhibitor.

While deriving the kinetics and mechanistic parameters in appropriate conditions at various points in iterative manner, various well established programs make some primary assumption during the development of the rate expression. For example, the popular program sigmaplot can fit Michaelis-Menten data very easily, but if used in its default state it incorporates assumptions that: (1) the errors in the observed rates are subjected to a normal (Gaussian) distribution and substrate concentration are exactly known; and (2) all of the rates have the same standard deviation and are independent of each other as magnitude of error in one rate measurement do not affect the measurement of any other rate.

Some of the assumptions like no deviation in standard deviation cause the problem that need to be eliminated from the model. Very less number of software databases allows doing so. Hence, the selection of the software to process the data has to be done very meticulously. It is always preferable to derive the rate expression and related constant manually with desired set of assumptions for error free fitting of data which may be crossed checked with the computerized data fitting. The computer-based data fitting serves as boon to researchers when more complex and complicated rate expression are observed (e.g. bisubstrate reaction with inhibition and termolecular reactions). In the gist, software-based studies of kinetics become essential part of the system [2].

5. Design of inhibitors and activators

The use of inhibitors usually has two outputs when used against the target enzyme as drug. First, the inhibition of enzyme leads to the accumulation of substrate that it meant to process and second wherein the concentration of metabolite decrease as the enzyme is inactivated by the presence of inhibitors. The inhibition of enzyme results into accumulation of the substrate or the metabolite which then can be detected by the various analytical methods [101]. The reduction of the metabolite due to inhibition of the selective enzyme from the pathway leads to the blocking of further activation sequence and therefore the consequences are resulted due to the same. For example, allopurinol inhibits the action of xanthine oxidase in the treatment of gout disease while inhibition of co-enzymes in the pathway leads to the prevention of undesired cancerous growth. The combination of two inhibitors may work in synergism or may not. It has to be studied thoroughly before using it for the human treatment. Most of the anti-cancerous and antibacterial drugs (e.g. cotrimoxazole) are used in synergism to fight against the targeted disease. Some of the inhibitor (e.g. 5-fluorouracil and doxorubicin) acts on the enzyme-co-enzyme complexes and DNA-enzyme complexes to form dead-end complex instead of binding to the enzyme [85, 102, 103]. Other 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.
4. Apoenzyme inhibitors that the molecules which attack the apoenzyme component of the holoenzyme and
5. Physiological modulators of reaction such as pH and temperature denature the enzyme and disturb the catalytic activity drastically.

5.1. Rational approach to the design of enzyme inhibitors

5.1.1. General discovery method for lead inhibitor

A lead inhibitor with low potency and selectivity acts as a base scaffold molecule for designing the highly potent and selective inhibitors. The pool of initial molecule (5000–10,000 related compounds) to screen as a lead compound can be decided by two ways. Either by studying the existing drug molecules creating side effects by reacting with proposed enzyme or from the compounds having low potency in pharmacological, anti-bacterial, anti-parasitic, or anti-viral effects in the screening experiments against the proposed target enzyme.

The selection of molecules to study for primary screening of lead compound can be decided on the basis of prior knowledge of (1) modification of the structural scaffold of a lead inhibitor, (2) information of substrate enzyme and inhibitor interactions and their catalytic mechanism and (3) use of molecular modelling software. Among this pool, the compounds are then studied for their inhibitory action. The molecules showing dissociation constant less than 1 μM against the desired enzyme are designated as lead compound. These molecules are then studied with various permutation and combination of approaches to enhance their activity against the selective target. Some of the approaches that can be used for the redesigning of the molecules are replacing existing functional group, enhancing the hydrophobic characters, removal of steric hindrances as well as stereogenic centres. While applying the approaches, it needs to understand the solubility of the drug molecule and its adsorption within the body. After modifications, if the selected molecules are designed with the intent of pharmaceutical applications, then they tested and studied on the animal model. After clearing the animal studies, it the molecules fail in the clinical trials, it requires further structural and manipulation of to improve in vivo profiling [55].

5.1.1.1. Process of development of lead inhibitor

The complementary structure of the molecule to the active site of the enzyme forms non-covalent bonds such as hydrogen bonds, ionic bonds and hydrophobic interaction to bind with the enzyme. The structure specific requirements of the molecules are maintained by replacing the isosteric replacement of groups. Isosteric replacement is done by replacing the atoms or the group with similar electronic and steric configurations which can be the elements within the same vertical group of the periodic table. The replacement of groups is done on the basis of the outer electronic configuration as well as by the ring equivalents without disturbing the spatial arrangement of the lead molecule. In the process of improving potency of the lead

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molecule, the atoms or the groups can be replaced by the non-specific substitute which may increase the hydrophobicity or hydrophilicity of the molecule to enhance the membrane penetration or the solubility. It is necessary to understand the dissociation state of the functional group at the physiological pH during the process of modification of the lead compound [68, 102].

The strategy of replacement leads compound group with the analogous group (═F, ═OH, ═NH_2 and ═CH_3) and is successfully applied to design the hypoglycemic agents, inhibitory compounds such as aminopterin, 6-mercaptopurine, 6-thioguanine, etc. The cis- and trans-configurational similarities can also serve as the way to design better lead compound. For example, replacement of ester functionality of the anaesthetics prolongs their action in body which led to the group of drugs such as anticholinergic spasmolytics and anti-depressant drugs. The pyridyl containing compound has more hydrophilicity as compared to the benzene because of the presence of p-electron deficiency which improve the reactivity of most of the drugs. This concept has already applied to the development of tricyclic, anti-histaminic and neuroleptic (major tranquilizing) drugs as well as in sulfide-containing groups [69, 104].

Ring replacement of ═N< by ═HC< and its subsequent modification to >C— have resulted in a variety of useful drugs. This is seen in the development of psychotherapeutics (chlorprothixene), the anti-inflammatory drug (sulindac). Several other modifications such as reversal of group, ring opening and closure and groups with similar polar effect can lead to the development of more potent lead compound [97].

5.2. Design from a knowledge of the catalytic mechanism

In most of the examples, the prior data on the enzyme active site are not available. The new lead compounds are designed based on the knowledge of substrate and catalytic mechanism followed by the reaction. The design of the molecules and their interaction with predicted structure of enzymes can also serve as better option to design the lead compound wherein molecular modelling plays an essential role [105–107]. The enzymes about which no data are available, structure of substance acts as a guiding line. For example, before discovery of the structural data of the angiotensin-converting enzyme (ACE), the inhibitors are decided based on the knowledge of substrate and poison (snake venom) and its similarity with the carboxypeptidase A enzyme. The discovery that D-benzylsuccinic acid was a potent inhibitor of carboxypeptidase A was challenged to the molecule similar to snake venom with the attachment of terminal. Ala-Pro sequence leads to the development of inhibitor methyl glutamyl proline. For the further improvements to enhance similarity between the substrates, increasing the solubility of the compound and reduction of side effect caused the drug molecule, it undergoes a series of changes to develop highly potent and efficient drug [78, 94, 108, 109].

Nowadays, application of computer-based molecular modelling techniques has gained the relative importance over the conventional techniques. The highly resolved crystal structure of the enzyme and enzyme-inhibitor complex reveals the change structural configurations, the spatial arrangement of the inhibitor within the active site of the enzyme along with the binding site of the enzyme and inhibitors [110–112]. On the basis of analysis of these data, the molecular modelling software showed the best match for the given enzyme from the large pool for chemical molecules in its database. These might be the completely new structures which need

to explore for their catalytic activity. It can also suggest the best modification that can serve for the developing potent inhibitor from the existing drugs. By using data from various sources such as structure of inhibitors from the various crystallographic databases and crystal structures of enzymes, enzyme-substrate and enzyme-inhibitor complex from the various PBD databases can be imported and analysed for the prediction of inhibition capacities of the various molecules [113]. Various *ab initio* and drawing softwares are used for the prediction of the structure of the inhibitors which are not present in the database. There spatial arrangement and group and chain positions are studied and model is minimized to confirm with lowest energy status. This is then docked inside the active site of the enzyme and positioned to have a maximum favourable interaction and tried for the energy minimization of the system. Thus, the finalized inhibitor then modified by various methods discussed earlier (Section 7.1) and analysed again in the software [77, 84, 114]. The use of high-resolution NMR studies or by homology modelling (minimum 30%) can give the relative desired protein structure. This can be used for the further docking experiments against the library of the inhibitors using computerized modelling software. The prediction of the active site on the surface of enzyme (pharmacophore) is done by docking the multiple inhibitor molecules. Because of the basic assumption of the rigid structure of the protein, the predicted inhibitor may differ in activity with the dynamic enzymes molecule [115, 116]. Thus, the developed lead compounds then can be applied in pharmaceutical, agrichemical industries and in the study of metabolic flux, genetic engineering and molecular biology for the betterment of life.

6. Enzyme activators

Activators are the molecules that enhance the rate of reaction by multi-fold which may or may not get utilized during the catalytic reaction progress. Depending upon their interaction with the enzymes, they are classified into various groups. The prosthetic groups generally bind covalently with the enzyme and act as a source of hydrogen or phosphorus group. Prosthetic

Sr. no.	Reaction type	Activators used
1	H-transfer reaction (vitamin-based activators)	(Vitamin-based activators) NAD, NADP, FMN, FAD
2	H-transfer (non-vitamin-based activators)	Lipoic acid, biopterin, coenzyme Q
3	Group transfer (vitamin based activators)	CoA, CoASH, TPP, pyridoxal phosphates, tetrahydrofolic acid (FH4), biotin, cobamide coenzyme, vitamin K, vitamins C
4	Group transfer	ATP, CDP, VDP, phosphoadenosine phosphosulfate
5	Transfer (non-vitamin-based activators)	(PAPs), heme-group, s-adenosylmethionine
6	Metals	Mg^{2+}—ATP utilizing enzymes Ca^{2+}—requires in functioning of muscle and blood clotting, essential for formation of cAMP Fe^{2+} and Fe^{3+}—heme-nonheme containing enzymes Cu^{1+} and Cu^{2+}—electron transport systems Zn^{2+}—present in dehydrogenase, polymerase Mo^{6+}—required for functioning of xanthanine oxidase

Table 6. Different types of enzyme activator and their examples.

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group forms part of active group undergoes changes during reaction. The co-enzymes do not bind covalently with enzymes and very essential for the activity of the enzymes. Co-enzymes get chemically altered temporarily during the reactions which get reconverted into its original form by the same or another enzyme. The enzymes associated co-enzyme usually catalyses bisubstrate reactions [3, 4]. The co-factors are complex organic molecules or metal ions. Some of the activators are listed in **Table 6**.

7. New developments in enzyme inhibition

In the 1970s and 1980s, the stopped flow and continuous flow experiments brought about the major experimental advancement in enzyme kinetics field by allowing the process to reach steady state. The complicated enzyme kinetics and intermediates formed during the process are decoded using rapid sample mixing and high time resolution monitoring [50, 117, 118]. The application of single-molecule fluorescence imaging at room temperature for single-molecule manipulation has been applied to study the single molecule enzyme in 1990s [119, 120]. It gave a major breakthrough in the field of enzyme kinetics. Single-molecule enzymology has elaborated on molecular level insights on molecular motors and nucleic acid enzymes. In single molecule experiments, wherein the time pattern of enzymatic turnovers was analysed, it is observed that the catalytic activity is not consistent over time. The reaction follows parallel reaction pathway through different enzymatic conformation with different rate constants and rate constant for the rate-limiting step might become a function of time. This phenomenon is called dynamic disorder [120–123]. Single molecule experiments have shown that the stochastic waiting time of an enzymatic reaction exhibits a distribution of an exponential rise followed by an exponential decay [124, 125]. For a single molecule with slowly interconverting conformational states with different k_{cat} and K_m, it follows that

$$\frac{1}{\langle T \rangle} = \frac{k_{cat}[S]}{[S] + K_m} \tag{74}$$

Where $\langle T \rangle$ is the mean of the stochastic waiting time and the overbars denote the weighted averages of k_{cat} and K_m of different conformational state.

In case of reversible reactions, Hill showed that forward and backward reaction fluxes (J^+ and J^-, respectively) can be related to the chemical potential difference, $\Delta\mu$, between product and the substrate:

$$\Delta\mu = -\mathrm{k}T\ln\left(\frac{J+}{J-}\right) \tag{75}$$

where k is the Boltzmann constant and T is the absolute temperature. It also serves as bridge between the thermodynamic driving force and enzymatic kinetics.

A most commonly used fluorogenic substrate, resorufin â-D-galactopyranoside (RGP), is also hydrolysed by the enzyme, yielding a fluorescent product, resorufin. The fluorescent burst released during the process is used for the prediction of the single enzyme kinetics. The location,

brightness and spectral properties of the fluorophore-labelled molecule affect the measurement of the signal significantly. This resolution directly yields kinetic information, for example, when measuring: (a) diffusion coefficients with respect to changes in the position of molecule (b) time-lapse experiments to analyse the systematic change in the brightness of fluorogenic substrate turnover with time or (c) temporal changes in molecule conformation or configuration when observing changes in FRET between a judiciously placed donor-acceptor pair.

The microscopic validity of chemical master equation (CME) has also been tested and verified by molecular dynamics for dilute chemical systems and using Brownian dynamics simulations for non-dilute crowded systems. The major assumption underlying the CME is that reactions are occurring in well-mixed environments. The characteristics of reactivity fluctuations in individual enzyme molecules relate to the second moment of turnover time statistics which can be defined by the randomness parameter R and time statistics. The randomness parameter R tends to become 1, when the waiting time distribution is a single exponential decay function. Any deviation of R from this predicted value is an indication of dynamic disorder [121]. When this is applied for the inhibition reaction, the expressions for the waiting time distributions for inhibition reactions became cumbersome. If more than one intermediate steps are present in the given set of reactions, then the chemical master equations become more and more complex. To avoid such complications, Chaudhary has used a simple analytical model based on the first passage time distribution between successive catalytic turnover events. This probability distribution function (PDF) is the moment of one monitored transition. If $\varphi(t)\mathrm{d}t$ is the probability of observing the next monitored transition between time t and $(t+\mathrm{d}t)$ after this time moment. The assumption made is that there is formation of product molecules with regeneration of the free enzyme. Such events are actually detectable in single-molecule fluorescence experiments [126]. These experiments have given the final expression for competitive inhibition can be given as

$$\langle t_{\text{competitive}} \rangle = \frac{[S] + [I]\frac{k_3(k_{-1}+k_2)}{k_1 k_{-3}} + \frac{(k_{-1}+k_2)}{k_1}}{k_2[S]} \tag{76}$$

$$\langle t_{\text{uncompetitive}} \rangle = \frac{[S] + [I][S]\frac{k_3}{k_{-3}} + \frac{(k_{-1}+k_2)}{k_1}}{k_2[S]} \tag{77}$$

Various researchers are applying various approaches to derive rate expression for the enzymatic reaction, which are under continuous evaluation to finalize more generalized and simplistic equations.

8. Conclusion

Biocatalysis has evolved as a promising lead to make the chemical process more sustainable, green and environment friendly. The main challenge in biocatalysed reactions is their industrial viability and economical aspects. The advancement in genetic engineering, medium engineering and immobilization technology has made them more robust to sustain the industrial environment. To transform the lab scale process to industrial scale, the understanding of the

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enzyme kinetics, inhibition and activation becomes very essential. The fragile nature of the enzymes makes them more sensitive towards the changes in medium and physical parameters of the surrounding environment. The various physical parameters such as temperature, pH, microwave and other radiations exposure, immobilization on different support affect the activity of the enzyme and in turn the kinetics of the reaction. The kinetics of the enzymes become more complex when the reaction becomes multi-substrate compared to single substrate reactions. The different kinds of methodologies and plots are used to predict the reaction kinetics of the multi-substrate reactions. The pattern observed during the analysis of the obtained experimental data is used to predict the model for the reactions such as ping-pong bi-bi or ternary complex model and mechanism of the reaction. The obtained model is then solved using various non-linear regression softwares available for the prediction of kinetic constants for the reaction. The inhibition or activation of the enzyme with the chemical entities or the reactant itself further modifies the reaction kinetics and in turn model of the reaction. To study these phenomena, a systematic research methodology needs to be followed to predict the exact reaction kinetics. The finding then can be used to design new enzyme inhibitors, activators which can be applied as possible drug molecules or for the prediction of the mechanism of the enzyme from the same family. This opens up the new area of the application such as pharmaceutical, molecular modelling, genetics studies and industrial production of fine and bulk chemicals. The use of the continuous and stopped flow experiments is becoming a new field to explore and understand enzyme kinetics and its inhibition to better extend.

Abbreviations

E	Enzyme
S, A	Substrate
H	Hydrogen
H+	Hydrogen ion
ES	Enzyme-substrate form
P	Product
ESH	Enzyme-substrate-hydrogen form
k_1	Rate constant for forward reaction to form ES
k_2	Rate constant for reaction to form P
k_{-1}	Rate constant for backward reaction to form ES
v	Initial velocity
V_{max}	Maximum velocity
K_m	Michaelis Menten constant
V_{mapp}	Apparent maximum velocity
K_{mapp}	Apparent Michaelis Menten constant
k_{e1}, k_{e2}, k_{es1}, k_{es2}	Equilibrium constant for hydrogen binding with various enzyme form
η	Effectiveness factor
S_b	Amount of substrate at boundary layer
V'_{max}	Maximum velocity within matrix
v_s	Reaction velocity within matrix

S_s	Amount of substrate in the solution
E_T	Total enzyme
K_a	Michaelis Menten constant for substrate *A*
K_{ia}	Intrinsic dissociation constant for *A*
K_{ib}	Intrinsic dissociation constant for *B*
k_i	Inhibition rate constant
B	Second substrate
P	Product 1
Q	Product 2
F	Stable modified form of enzyme
K_b	Michaelis Menten constant for substrate *B*

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