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MBC-004: ENZYMES AND THEIR APPLICATIONS

Block

II

ENZYME KINETICS

BLOCK II: ENZYME KINETICS

As you know enzymes acts as biocatalyst and its kinetics elucidates the rates of the biochemical reactions and the factors influencing them. The enzyme kinetics is important to elucidate the mechanistic action of the enzyme, how enzyme interacts with the substrate and how its activity can be regulated or controlled. Studying enzyme kinetics is important for several reasons to predict the behavior of enzyme action. The core concepts of enzyme kinetics involve understanding the relationship between enzyme concentration, substrate concentration, and the reaction rate. The different kinetic constants are vital for a reasonable understanding of mechanistic enzymatic reaction for regulating the bioconversion process.

The opportunities presented by single-molecule kinetic measurements permits to record the trajectories of individual enzyme molecules as they bind, interconvert, and release substrates/products. The most common model used to explain simple enzyme kinetics is the Michaelis–Menten model. Further questions of interest include the catalytic mechanism in presence of two or more substrates. How the rate of the catalyzed reaction is affected in presence of different types of inhibitor? All related studies provide vital information to determine a mechanistic enzymatic reaction.

Unit-4 of the block gives the basis of the derivation of one of the most widely used models of enzyme kinetics i.e Michaelis-Menten equation for mono substrate enzyme catalyzed reaction. Several other graphical methods of plotting enzyme kinetics have been discussed in the unit. Most of the biochemical reactions involve two or more substrates; Unit-5 dwells on bisubstrate enzyme catalyzed reactions. Enzyme activity needs to be firmly regulated to guarantee that levels of the product do not increase to undesired levels. This is accomplished by enzyme inhibition. Unit-6 of the block describe about different types of enzyme inhibition- reversible and irreversible.

Expected Learning Outcomes:

After studying this block, you should be able to:

- derive Michaelis-Menten equation,
- plot enzyme kinetics data using Lineweaver-Burk, Hanes-Woolf, Woolf-Augustinsson-Hofstee, Eadie-Scatchard; Direct linear plot,
- discuss bisubstrate enzyme catalyzed reactions,
- explain the different mechanisms of enzyme catalysis,
- describe different types of inhibitors regulating enzyme activity.

UNIT 4

KINETICS AND CHARACTERIZATION

Structure

4.1 INTRODUCTION

The oldest and the important approach to understand enzyme mechanisms is the discipline known as enzyme kinetics. Kinetics is the study of reaction rates, their quantitative measurement and a systematic study of the factors influencing the activity of enzymes. In this unit, you will gain an insight of the enzymatic mechanisms as well as role played by enzyme activity in regulating metabolic pathways. Enzymes convert substrates to products through a series of steps known as enzymatic mechanism. Therefore the effect of substrate concentration on enzyme activity is one of key concepts in enzyme kinetics. Several models have been proposed to explain the kinetics of enzyme catalyzed reactions. Classical experimental work for single enzyme catalysed reactions is Henri-Michaelis-Menten plot, Briggs Haldane equation, Lineweaver-Burk plot, etc. The kinetics parameters such as K_m, V_{max}, K_{cat} and K_{cat}/K_m are vital to understand the enzyme kinetcis. These kinetics parameters can be determined by the various methods such as Hanes-Woolf plot, Eadie-Hofstee plot and Eadie-Scatchard plot. Eisenthal-Cornish-Bowden plot or direct linear plot does not need any calculations; it can be used in routine in lab to measure K_m and V_{max} without assumptions or distortions.

Several Flow-Based Systems are being developed for enzyme kinetics studies. Classification of flow strategies is based on the manipulation of the mixing zone after enzyme and substrate solutions merge. In the unit we have tried to discuss several methods of flow based techniques with a comparative view of their advantages and disadvantages.

Expected Learning Outcomes

After studying this unit, you should be able to:

- derive Michaelis-Menten equation;
- explain the mechanisms of enzyme catalysis;
- draw Lineweaver-Burk plot;
- describe K_m and $V_{max;}$
- derive Eadie–Hofstee equation;
- give a graphical representation of Eadie-Scatchard plot;
- discuss the advantages of direct linear plot; and
- compare different flow techniques.

4.2 ENZYME KINETICS

Kinetic analysis helps to disclose the number and order of the individual steps involved in the transformation of substrates to products. In the past, data generated from the experiments of enzyme catalyzed reactions was collected and analyzed to determine the rate of a reaction. It was found out that at low concentrations of substrate, the reaction was of first-order with respect to the substrate. However, at the higher concentrations of substrate, the reaction became zero-order. Please recall from your chemistry books regarding the zero order or first order enzyme catalyzed reactions. Generally all single substrate enzyme catalyzed reactions and even multi-substrate reactions where concentrations of all but one were kept constant follows the same order. At constant enzyme concentration, graph of initial velocity [v_o] (on y-axis) against substrate [S] concentration (on x-axis) was found to exhibit a hyperbolic curve (Fig. 4.1).

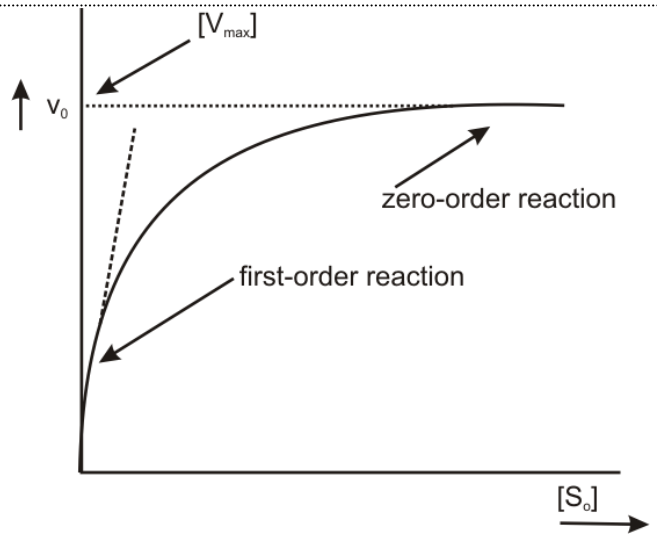


Fig. 4.1: Graph of initial velocity against substrate concentration for a single substrate enzyme catalyzed reaction.

The general equation from the graph is

$$v_o = \frac{V_{max}[S_o]}{[S_o] + b}$$

V_{max} = Maximum velocity = maximum value of v_o

b = constant = value of [S_o] where v_o = ½ V_{max}

In general terms, in a mono substrate enzyme catalyzed reaction and considering just one substrate binding site per enzyme molecule, substrate [*S*] comes in physical contact with enzyme [*E*] to form an enzyme substrate complex [*ES*] complex which eventually undergoes a further reaction and leads to the formation of product [*P*]. It can be represented as:

$$E + S \xrightarrow{\text{rate constant } k_1} ES \xrightarrow{\text{rate constant } k_2} E + P$$

k_1 = rate constant for the association of substrate and enzyme

k_2 = rate constant for the breakdown of enzyme and product

k_{-1} = rate constant for the dissociation of [*ES*] complex to form free enzyme and substrate

The overall rate of reaction is limited by two factors:

1) The amount of concentration of enzyme

2) The breakdown of enzyme-substrate complex

At low substrate concentrations, the overall rate of reaction will be limited by the rate at which enzyme and substrate molecules react to form enzyme-substrate complex. At constant enzyme concentration the rate of reaction will be proportional to the substrate concentration (first-order reaction). However, at high substrate concentration enzyme will be saturated with the substrate and therefore no free enzyme will be available. So the overall rate of reaction

will be independent of the substrate concentration. The maximum initial velocity possible will be

$$V_{max} = k_2 [E_o]$$

SAQ 1

How does substrate concentration affect the rate of enzymatic reactions?

4.2.1 Michaelis-Menten Equation

Kinetic models used to explain above mentioned findings were proposed by Michaelis and Menten (1913). The Michaelis-Menten equation demonstrates the relationship between initial reaction velocity and substrate concentration. Derivation of this equation begins with the generalized scheme of events considering a single substrate enzyme catalyzed reaction as stated earlier; Substrate [*S*] interacts with enzyme [*E*] to form an enzyme substrate complex [*ES*] complex which eventually breaks down to free enzyme [*E*] and the formation of product [*P*]. The Michaelis and Menten set out the following scheme as given below:

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

The term k_1 denotes the rate constant for the formation of *ES* complex. *ES* complex has two fates, it can dissociate back to enzyme and substrate with rate constant k_{-1}, or proceed to form product and release the free enzyme with a rate constant k_2. In any enzyme catalyzed reaction, the concentration of substrate should be five or six orders higher than that of enzyme. The above model also assumes that $k_2 << k_1$. There is every possibility that reaction can go backward but if we consider only the initial rate of a reaction, we can ignore the backward reaction.

The overall rate of reaction is termed as initial velocity (v_0) and it will depend on two factors – the rate of formation of product (k_2) and the concentration of enzyme bound with the substrate i.e [*ES*]

So $v_o = k_2[ES]$ .....equation 1

Michaelis and Menten made two assumptions in their model. First, the availability of excess substrate [*S*] >> [*E*]. Secondly a rapid equilibrium is established between the reactants ([*E*] + [*S*]) and [*ES*] complex. Moreover, the breakdown of enzyme-substrate complex is too slow to cause any change in equilibrium. Hence Michaelis and Menten model is also known as "Rapid equilibrium model". Thus at the equilibrium state:

$$k_1[E][S] = k_{-1}[ES]$$ ..... equation 2

$$\frac{[E][S]}{[ES]} = \frac{k_{-1}}{k_1} = K_s$$ …… equation 3

K_s is the dissociation constant. If E_o is the total enzyme concentration, it is equal to the sum of free enzyme [*E*] and enzyme bound to the substrate [*ES*] as represented in the following equation:

$$E_o = [E] + [ES]$$ ……..equation 4

$$E = [E_o] - [ES]$$

Substituting the value of *E* in equation equation 3

$$\frac{([E_o] - [ES])[S]}{[ES]} = K_s$$

$$([E_o] - [ES])[S] = K_s[ES]$$

$$[E_o][S] - [ES][S] = K_s[ES]$$

$$[E_o][S] = K_s[ES] + [ES][S]$$

$$[E_o][S] = [ES](K_s + [S])$$

$$[ES] = \frac{[E_o][S]}{(K_s + [S])}$$

Substituting the value of [*ES*] in equation 1 we get

So $v_o = k_2 \frac{[E_o][S]}{(K_s + [S])}$ ……..equation 5

Maximum rate of enzyme reaction will be achieved when all the enzyme molecules are bound to the substrate molecules.

So $V_{max} = k_2 [E_o]$ …..equation 6

Substituting this value in equation 5 we get

$$v_o = \frac{V_{max}[S]}{(K_s + [S])}$$

Michaelis and Menten also made the supposition that initial substrate concentration [S_o] is much higher than the initial enzyme concentration [E_o], in such a scenario the formation of enzyme-substrate complex will have no such big change in free substrate concentration. The expression for v_o will be:

$$v_o = \frac{V_{max}[S_o]}{(K_s + [S_o])}$$ …..equation 7

The above equation equation 7 is well known as Michaelis-Menten equation

Briggs Haldane modified Michaelis-Menten Plot:

The Michaelis-Menten model is dependent on the assumption of rapid equilibrium approach in any enzyme catalyzed reaction. It limits the

applicability of the equation to only rapid kinetic reactions which might not be the case with many of the other enzyme reactions. Most of enzyme catalyzed reactions assume a constant concentration of enzyme-substrate complex [*ES*].

Generally if an enzyme is mixed with high concentration of a substrate there is an initial period expressed as pre-steady state (lasts in micro seconds) where concentration of [*ES*] slowly builds up. Eventually the concentration of [*ES*] builds up and attains a steady state, remains constant over time. The **steady-state concept** was introduced by Briggs and Haldane in 1925, a modified Michaelis-Menten method. This concept was considered more valid assumption than the earlier ones. The Michaelis-Menten model gave importance to the formation of [*ES*] while Briggs-Haldane method focuses on the consistency of [*ES*] complex, its maintenance at constant concentration and breakdown to products.

Considering the single substrate enzyme catalyzed reaction one more time. In this reaction at steady state rate of formation of [*ES*] will be equal to the rate of its decomposition to products. Therefore

$$k_1[E][S] = k_{-1}[ES] + k_2[ES]$$ ……..equation 8

Separating the constant from variables:

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2[ES]}{k_1} = K_m$$ ……..equation 9

Where K_m = Michaelis constant

Substituting K_s with K_m in the above equations (4-7)

$$E = [E_o] - [ES]$$

Substituting the value of *E* in equation 9

$$\frac{([E_o] - [ES])[S]}{[ES]} = K_m$$

$$([E_o] - [ES])[S] = K_m[ES]$$

$$[E_o][S] - [ES][S] = K_m[ES]$$

$$[E_o][S] = K_m[ES] + [ES][S]$$

$$[E_o][S] = [ES](K_m + [S])$$

$$[ES] = \frac{[E_o][S]}{(K_m + [S])}$$

Substituting the value of [*ES*] in equation 1 we get

So $v_o = k_2 \frac{[E_o][S]}{(K_m + [S])}$

Since $V_{max} = k_2 [E_o]$

Substituting this value in above equation

$$v_o = \frac{V_{max}[S]}{(K_m + [S])}$$

Since substrate concentration is much higher than the enzyme concentration [*S*]~ [S_o] so

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])} \qquad \text{.....equation 10}$$

The above equation equation 10 is similar to well known Michaelis-Menten equation. The only change is the K_m instead of K_s in the denominator. So the equation retains its previous name of Michaelis-Menten equation and constant K_m is known as Michaelis-Menten constant.

A graph of v_o at y-axis vs substrate concentration [*S*] at x-axis will be in the form of a hyperbolic curve (Fig. 4.2).

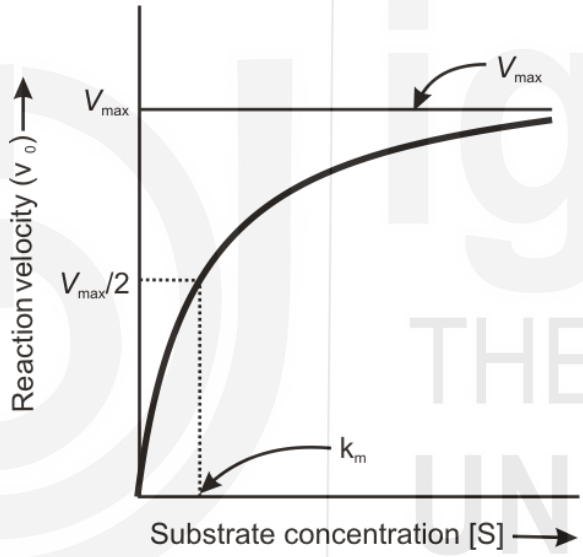


Fig. 4.2: Michaelis-Menten plot of a single substrate catalyzed enzyme reaction.

SAQ 2

Explain how the rate of an enzyme-catalyzed reaction reaches a maximum value at high substrate in a Michaelis-Menten equation?

4.2.2 Lineweaver Burke Plot

If you look at the Michaelis-Menten plot, you will observe that v_o approaches V_{max} in a tangential manner at higher substrate concentrations. So if you want to determine V_{max} and K_m from the plot, it will be difficult and unsatisfactory. A hyperbolic curve nature of the graph makes it difficult to determine the accurate value of V_{max} and K_m. Therefore, to overcome this difficulty, Lineweaver and Burk (1934) suggested a straight line graph for enzyme catalyzed reactions obeying Michaelis-Menten equation. They did not made any new assumptions and derive the Lineweaver-Burk plot, which is also

known as double reciprocal plot. Lineweaver and Burk took the Michaelis-Menten equation and inverted it.

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])}$$

$$\frac{1}{v_o} = \frac{(K_m + [S_o])}{V_{max}[S_o]}$$

$$\frac{1}{v_o} = \frac{[S_o]}{V_{max}[S_o]} + \frac{K_m}{V_{max}[S_o]}$$

$$\frac{1}{v_o} = \frac{1}{V_{max}} + \frac{K_m}{V_{max}[S_o]}$$

This equation is known as Lineweaver-Burk equation. It is in the form of y = mx+c . ..... equation 11

This is known as Lineweaver-Burk plot or double reciprocal plot. This is of the form of $y = mx + c$, which is the equation of a straight line graph. Plot of $1/v$ against $1/S_o$ is linear and obeys Michaelis-Menten equation (Fig. 4.3).

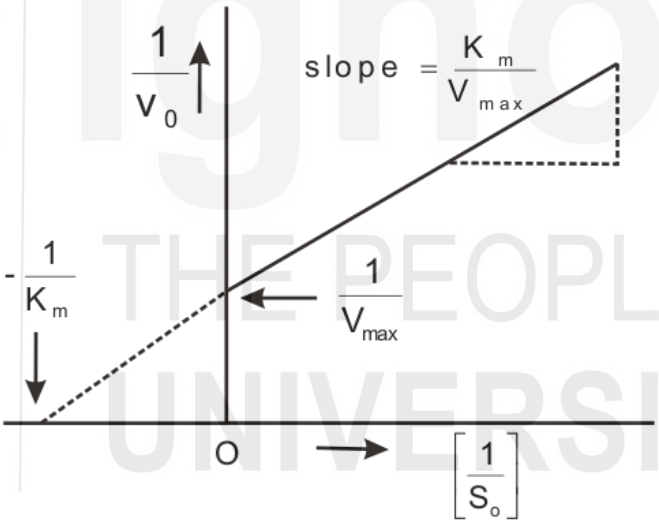


Fig. 4.3: Double reciprocal plot of the Michaelis-Menten equation.

Lineweaver-Burk plot is highly applicable for determining kinetic parameters. Y-axis intercept is represented by $1/V_{max}$ while x-axis intercept is represented by the $-1/K_m$. The reciprocal of the y-axis intercept gives information about V_{max} while reciprocal of the x-axis gives information about K_m. Lineweaver-Burk plot is extensively used to determine different types or reversible inhibition such as competitive, non-competitive and mixed inhibition.

Lineweaver-Burk method has its own limitation. First, as y-axis is the reciprocal of the v_o hence if there is even a small error in the v_o calculation it will have its ramifications as it gets enlarged. The situation is precarious at lower low [S]. Second, the reciprocal of the [S], bunches at the axis of the $1/v_o$ therefore the lower [S] i.e its reciprocal 1/[S] will be higher and leads to a profound effect on the plot. The concentration of the substrate preferred to make reciprocal plot should be near or close to the K_m value. When [S] chosen is much lower than K_m value, the rate of reaction/velocity is roughly

proportional to the substrate concentration and rate of reaction is held to be first order. However, it result into curve intercepting both X and Y axes too close to the origin to permit either V_{max} or K_m to be determined exactly. When [S] is much greater than K_m value, the velocity is constant and equal to V_{max}. The rate of reaction is becomes independent of [S] and is of zero order. The curve becomes horizontal. This will permit calculation of V_{max} but the slope of the line will be near zero therefore it is difficult to determine K_m precisely.

4.2.3 Significance of K_m and V_{max}

K_m is also called as Michaelis constant. It is expressed as the substrate concentration at which velocity is half of V_{max} (maximal velocity) refer Figure 4.2. Here you must note that K_m has the units of concentration but it is independent of the enzyme and substrate concentration. As you know K_m is equal to the substrate concentration at ½ V_{max}. And since at V_{max} all the enzyme molecules are bound with substrate to form *ES* complex, thus the substrate concentration (K_m) required to convert half of the enzyme molecules into *ES* complex signifies the affinity of the enzyme for the substrate. When K_m value is small, it signifies that enzyme has very high affinity for that substrate i.e. low concentration of substrate is needed to saturate the enzyme. Similarly, a large value of K_m indicates a relatively high concentration of substrate required to saturate the enzyme, thus signifying a low affinity of the enzyme for substrate. This is why K_m is also known as **affinity constant**.

V_{max}, the maximal rate *reveals* the "**turnover number"** of an enzyme, i.e. the rate at which number of substrate molecules is being converted into product by an enzyme molecule in a unit time when the enzyme is fully saturated with substrate. The k_{cat}. is referred as the turnover number for an enzyme. For simple reactions $k_2 = k_{cat}$ whereas for complex reactions k_{cat} will be function of individual rate constants. It ranges from 1 to 10^5 per second.

SAQ 3

Tick [√] mark the correct option:

a) Michaelis-Menten equation relates the rate of an enzyme-catalyzed reaction to substrate concentration/product concentration (pick one).

b) A hyperbolic curve gives an accurate value of V_{max} and K_{max} (True/False).

c) Lineweaver-Burk plot is linear/hyperbolic (Choose one).

d) K_m is equal/more to the substrate concentration at ½ V_{max} (Choose one).

4.2.4 k_{cat} and Turnover Number

To determine the enzyme efficiency in enzyme kinetics, we are interested to know how many maximum molecules of substrate can be converted into product per catalytic site of a given concentration of enzyme per unit time.

$k_{cat} = V_{max}/E_t$

where

k_{cat} = Turnover number,

V_{max} = Maximum rate of reaction when enzyme catalytic site is saturated with substrate E_t =Total enzyme concentration or concentration of total enzyme catalytic sites.

The k_{cat} is a direct measure of catalytic production of product under optimal conditions. The units of **Turn over number (k_{cat})** = (moles of product/sec)/ (moles of enzyme) or sec^{-1}.

Metalloenzyme carbonic anhydrase catalyzes interconversion of carbon dioxide and water to form bicarbonate ions and protons. A turnover number of 400,000 to 600,000 s^{-1} of carbonic anhydrase enzyme suggests that each enzyme molecule can produce up to 600,000 molecules of product (bicarbonate ions) per second.

4.2.5 Hanes-Woolf Plot

Hanes–Woolf plot is a graphical representation of enzyme kinetics in which the ratio of the initial substrate concentration [S] to the reaction velocity v is plotted against [S]. It can be derived from the Michaelis–Menten equation by inverting and multiplying with [S] (Fig. 4.4).

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])}$$

On inverting the above equation and multiplying with $[S_o]$

$$\frac{[S_o]}{v_o} = \frac{[S_o](K_m + [S_o])}{V_{max}[S_o]} = \frac{(K_m + [S_o])}{V_{max}}$$

$$\frac{[S_o]}{v_o} = \frac{[S_o]}{V_{max}} + \frac{K_m}{V_{max}}$$

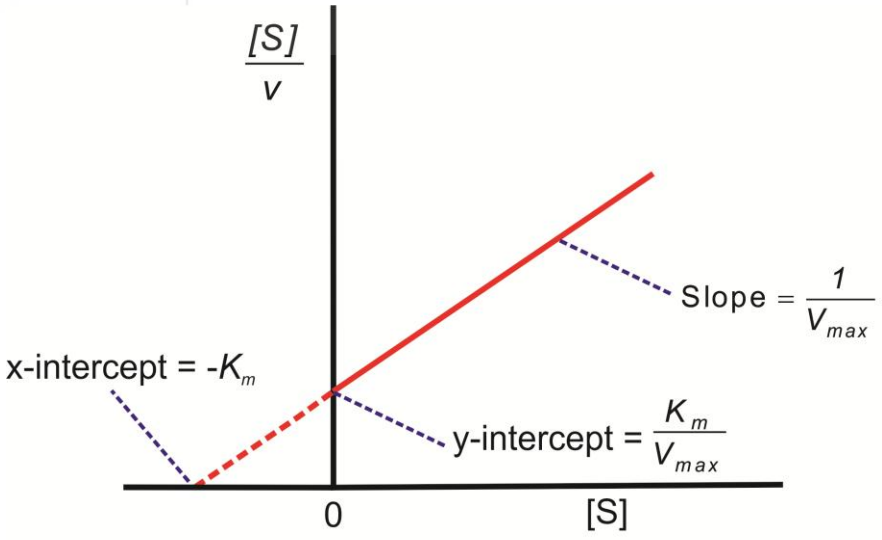


Fig. 4.4: Hanes –Woolf Plot.

Hanes –Woolf equation is in the form of y = mx + c. and graphical representation of its plot shows a straight line of slope $1/V_{max}$, a y intercept of K_m/V_{max} and an x-intercept of $-K_m$. Hanes–Woolf plot is used for determining kinetic parameters K_m, V_{max} and V_{max}/K_m. This plot overcomes the limitation of Lineweaver Burk plot because of use of reverse value of [S]. If the substrate concentration range is very little compared to the K_m, the plot will be almost horizontal. If the substrate concentration range is very high in comparision to the K_m, the plot will intersect the axes near to the origin.

Major limitations of this plot that neither ordinate nor abscissa are independent variables since both are dependent on the substrate concentration. Therefore the typical measure of goodness of fit, the correlation coefficient R remains inapplicable.

4.2.6 Eadie–Hofstee Plot

The Eadie–Hofstee plot is another method of representing enzyme kinetics. In this plot, reaction rate is plotted as a function of the ratio between rate and substrate concentration. It can be derived from the Michaelis–Menten equation by inverting and multiplying with V_{max} (Fig. 4.5).

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])}$$

On inverting the above equation and multiplying it with V_{max}

$$\frac{V_{max}}{v_o} = \frac{V_{max}(K_m + [S_o])}{V_{max}[S_o]} = \frac{(K_m + [S_o])}{[S_o]}$$

$$V_{max} = \frac{v_o(K_m + [S_o])}{[S_o]} = \frac{v_o(K_m)}{[S_o]} + v_o$$

$$v_o = -\frac{v_o(K_m)}{[S_o]} + V_{max}$$

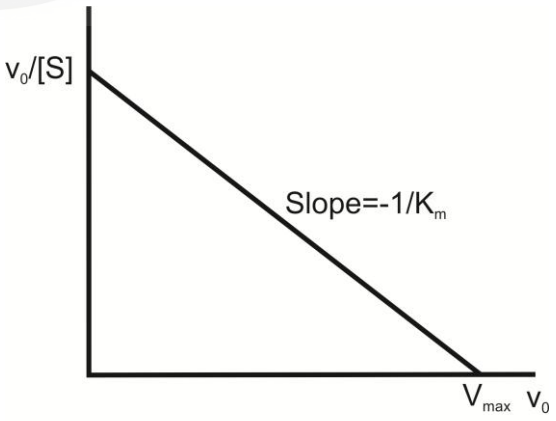


Fig. 4.5: Eadie –Hofstee Plot.

If you see in the above plot when v_o is plotted against $v_o/[S]$ it leads to the determination of V_{max} as the y-intercept. V_{max}/K_m as the x-intercept, and slope is $-K_m$. Similar to lineweaver plot that linearize the Michaelis–Menten equation,

the Eadie-Hofstee plot is also used for the determination of K_m and V_{max}. This plot is considered to be more robust against error-prone data than the Lineweaver–Burk plot, predominantly because it gives equal weight to data points in any range of substrate concentration or reaction rate. Major limitations of this plot that neither ordinate nor abscissa characterizes independent variables since both are dependent on the reaction rate. Any experimental error will affect both the axes and experimental error will unevenly proliferate and become larger over abscissa. So smaller values of $v_o/[S]$ will get more weightage. In such type of scenario, the measure of goodness of fit, correlation coefficient remains inapplicable. Eadie-Hofstee plot has been outdated by nonlinear regression methods that are significantly more exact and no longer computationally inaccessible.

4.2.7 Eadie– Scatchard Plot

Eadie-Scatchard equation can be derived by the Eadie-hofstee equation by dividing both sides of the equation by K_m

$$v_o = -\frac{v_o(K_m)}{[S_o]} + V_{max}$$

$$\frac{v_o}{K_m} = -\frac{v_o(K_m)}{[S_o]K_m} + \frac{V_{max}}{K_m}$$

$$\frac{v_o}{K_m} = -\frac{v_o}{[S_o]} + \frac{V_{max}}{K_m}$$

$$\frac{v_o}{[S_o]} = -\frac{v_o}{K_m} + \frac{V_{max}}{K_m}$$

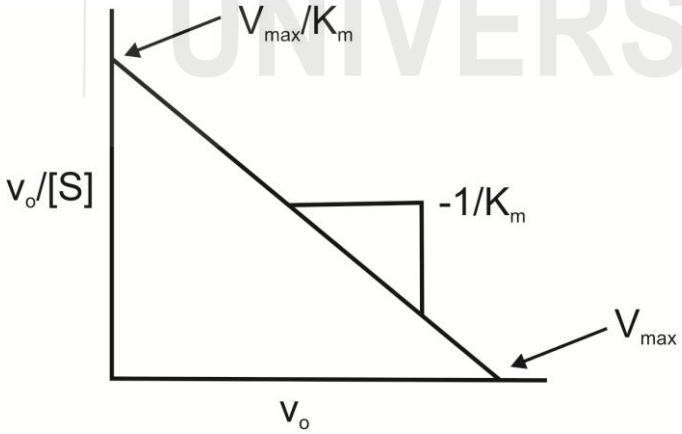


Fig. 4.6: Eadie-Scatchard Plot.

In Eadie-Scatchard plot when v_o is plotted against $v_o/[S]$, its Y-axis intercept represent V_{max}/K_m and x-axis intercept represent V_{max} and slope represent the $-1/K_m$. This plot overcome the limitation of the using reverse value of v_o. Eadie-Scatchard equation aids in determining the interaction affinity of a biomolecule with its ligands.

4.2.8 Eisenthal-Cornish-Bowden Plot or Direct Linear Plot

This is a non-linear method for determining enzyme kinetic parameters such as K_m and V_{max}. In the Lineweaver Burk plot, reciprocal transformations leads to the distortion of data which can be avoided using Eisenthal-Cornish Plot because for accuracy, empirical kinetic data should have low noise for the interpretation of the plot.

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])}$$

On rearranging the Michaelis-menten equation,

$$V_{max} = \frac{v_o K_m}{[S_o]} + v_o$$

In Michelis Menten equation, we considered V_{max} and K_m as constant but if we assume V_{max} and K_m as "variables" (not constants) and v_o / $[S_o]$ and v_o as "constants" (not variables) then Eisenthal-Cornish equation would describe a straight line for every pair of v_o and $[S_o]$ variables.

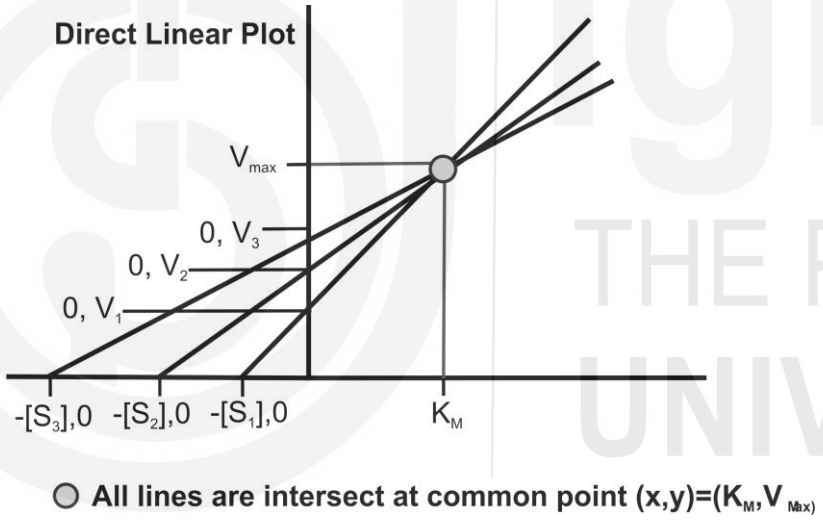


Fig. 4.7: Eisenthal-Cornish Bowden Plot.

Eisenthal–Cornish–Bowden plot (Fig. 4.7) engages directly plotting of the initial velocities of enzyme-catalyzed reactions i.e v_o, v_1, v_2, v_3 at different substrate concentrations i.e S_1, S_2, S_3 without changing the Michaelis-Menten equation. Each data set point is defined by the two sets of coordinates that falls on a line, so for these data points, equations are:

(-[S_1],0) and (0, v_1), line 1 = V_{max} = (v_1/[S_1])K_m + v_1

(-[S_2],0) and (0, v_2), line 2 = V_{max} = (v_2/[S_2])K_m + v_2

(-[S_3],0) and (0, v_3), line 3 = V_{max} = (v_3/[S_3])K_m + v_3

Each of these lines is different, but they all have one general point of intersection characterized by these (x,y) coordinates, (K_m,V_{max}); this point describe the only real value for K_m and V_{max}. So instead of fitting a straight line,

this method is based on the intersection of lines formed by pairs of experimental data points.

The direct linear plot offer several advantages, not only over the double-reciprocal plot, but over the other well-known plots as well. It does not require calculation or mathematical tables, it can be comfortably used in routine in the lab. It allows determination of K_m and V_{max} without assumptions or distortions. The kinetic constants are read off the plot directly, again without calculation. It is extremely useful during the course of an experiment to determine its success or to modify the experimental design. It gives clarity and accurate information about the quality of the observations. The direct linear plot can be applied to binding experiments, where it holds several advantages over conventional graphical methods for estimation of different binding parameters.

SAQ 4

Tick [√] mark the correct option

a) In the graphical representation of Hanes –Woolf plot what does slope and y intercept indicate?

b) Eadie–Hofstee plot is derived from the Michaelis–Menten equation by inverting and multiplying with V_{max}. (True/False)

c) Eisenthal-Cornish equation describes a straight line for every pair of …….and……..variables (Fill in the Blanks).

4.3 FLOW TECHNIQUES

The conventional study of enzyme kinetics involves manual bench top operation by mixing solutions of the assays for enzyme at constant concentration with different concentrations of substrate. Spectrophotometric or fluorometric changes during the course of enzyme reaction mixture need to be recorded immediately to generate data at the initial rate as soon as the reagents are mixed. However, the variation and irregularities of mixing time before detection and the wastage of time during manually placing/moving of cuvette in and out of the spectrometer can lead to errors. Therefore the accuracy of data during kinetic studies will have 5–20% error due to manual operation. How to surpass this inherent wastage of time during the processes? For more precise and to gain an extra improvement of analysis time, semi-to-fully automatic flow-based systems are considered as better substitutes.

Flow techniques employ the continuous flow of reactants and/or solvents through a system to study fast reactions. Flow-based systems bring in the product zone into the detector fast which facilitates measurement close to initial state. It is quite possible that dispersion and dilution of the product zone in the flow line may affect the accuracy of the results. However, several reports have demonstrated successful kinetic studies of enzyme in flow injection systems (Table 4.1). The best way to minimize the dilution and dispersion in flow techniques is to reduce the time taken in moving from the product zone to detector. Let us discuss some of these flow-based systems:

Stopped Flow technique: As you know enzyme kinetics involves many intermediate steps that involves substrate binding several transitional catalytic steps before the release of product. These steps are all fast or quicker than the steady state turnover rate of the enzyme, so to measure these reactions on millisecond to second time scales require usage of techniques such as stopped flow technique. In this technique (Fig 4.8), two or more reactants are mixed swiftly in a chamber, and the flow of solution is stopped after a certain period by stopping the pump. The reaction time is prolonged and the progress of the stopped reaction zone can be continuously monitored over time spectroscopically (absorbance, fluorescence, UV-vis or circular dichroism). For example, an enzyme-substrate mixture can be stopped in a flow-through cell located in the detector in order to follow the enzyme kinetics and track the changes due to inhibitors. In another setting, a number of reactions are performed in batches and stopped at various times by the use of a compound which will halt the reaction.

Stopped flow technique helps to enhance sensitivity in contrast to continuous flow because measurement takes place when a higher amount of product gets accumulated or when the decrease in reactants is more prominent. These techniques has been used extensively to reveal the aspects of enzymatic reactions such as substrate binding, product release or any rate determining steps to understand the enzyme mechanism. Stopped-Flow spectroscopy is one of the fundamental techniques widely used for studying enzyme kinetics which has found wide range applications in drug discovery and development and synthetic biology. The advantages of using this technique lies in its highly sensitive detection and signal processing to measure the spectroscopic signal changes in elucidating enzyme kinetics. The generated data can be analyzed to find out binding affinities, catalytic efficiencies, and infer conformational state changes using kinetic constants. Pre-steady state kinetic analysis can permit us to find out individual chemical intermediates such as the number and state of the cofactors during complex multistep reaction mechanisms associated with redox-active proteins. Other examples include flavoproteins or metal complexes such as heme proteins. Moreover, these techniques have wide range applications in the study the effect of inhibitors, ligands, and other macromolecules on enzymatic activity.

Continuous flow techniques: These techniques involves enzyme assay to determine the progress of a reaction at distinct time points and is generally used for determining initial rates and inhibition values. In this type of flow technique, the reaction mixture is continuously collected and the composition of the reaction is measured continuously normally by absorbance, while the reactants flow and mix continuously. This technique maintains steady-state conditions and is often used to study enzyme kinetics over long periods or to produce compounds at scale.

Continuous flow assays employ apparatus that have reactants initially separated in chambers (Fig 4.8). They are thrusted in a mixing chamber and then move out through a long tube. At discrete points along this tube, measurements can be taken - generally by spectrophotometers. Since the flow rate of the reactants is constant, each point on the tube corresponds to a discrete point in time. During the course of reaction, there will be change in the

absorbance values which are necessary to perform a traditional continuous flow assay. The addition of inhibitors during the course of reaction will change the ratios of reactants and by determining the data at different time points through the output tube, a high level of accuracy can be maintained. This technique has its application in large scale synthesis, monitoring of long term stability of enzymes or in chemical production processes. However, it suffers from disadvantage in a sense that it needs a large amount of reagent for continuous flow which can be very expensive and wastage of reagents.

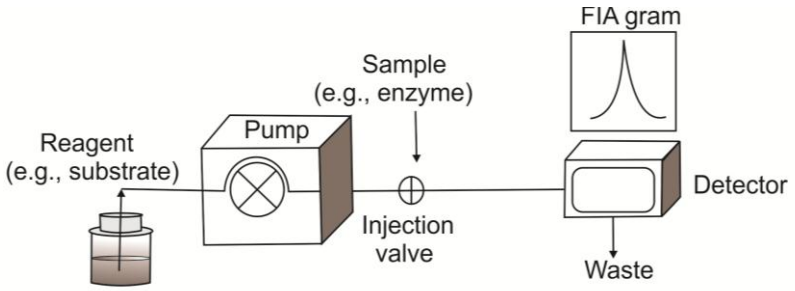


Fig 4.8: Schematic representation of Stopped and Continuous flow techniques.

Quench Flow Technique: Quenching offers a complementary approach for studying the enzyme mechanism as well as supplementing the information obtained from spectroscopic, structural, and computational methods. Usually quenching flow technique is used to study fast reactions. The reactants are mixed to react for a controlled period, and then the enzymatic turnover is swiftly stopped at different stages with a chemical agent (e.g., a strong acid or base), and the individual reaction mixtures at each time point are analyzed for the reactants, products and any intermediates.

Quenching technique (Fig 4.9) is similar to the stopped flow technique where enzyme-substrate mixture is stopped within the flow system for determining the transformation in product or substrate concentration over time. However, the difference lies in the fact that in the stopped flow technique, one reaction mixture resides in the system (e.g., at the detection cell) for continuous monitoring of changes with time (generally spectrophotometrical). In quench flow technique, spectrophotometric changes cannot be recorded during the reaction. Generally, more than one reaction mixture needs to be injected into the part of the system called “delay loop or aging loop’. Several different reaction times are permitted by altering the size of the delay loop for each replicate, ahead of being stopped by the addition of the “quench solution”. Then, the quenched mixtures of different reaction times are collected and analyzed using separate techniques such as gel electrophoresis and chromatography.

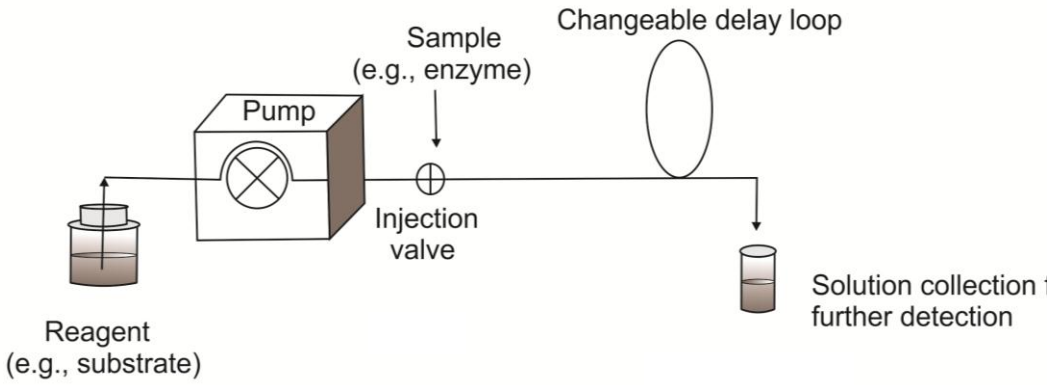


Fig 4.9: Schematic representation of Quench Flow Technique.

The order by which bonds are formed and broken in the reaction is revealed by the identities of the captured intermediates or products, and the rates of individual steps in the enzyme mechanism are determined from the quantities of various chemical species collected at different time points using techniques such as chromatography or mass spectrometry. This technique has extensive applications in the analysis of transitional molecules formed during the course of enzymatic reactions, detailed kinetic studies and determination of rate constants in fast biochemical reactions.

Zone Trapping/Bypass Zone Trapping Flow injection analysis (ByT-FAS): This technique (Fig 4.10) in principle is similar to stopped flow technique. However, it laid more importance to make certain that the part of the sample/reagent plug being determined attain steady-state and is not likely to be affected by laminar flow and dispersion. Generally, it can be done by regulating the sample size that needs to be adequately large and by assembling the flow manifold in such a manner that the plug of mixture holds a portion of physical steady state that can be detected. Remaining parts of the mixture plug that do not attain the steady state are compelled to bypass the detector via a switching valve. Therefore, time control in switching of valve to change the direction of the flow at the beginning and the end of the product zone to bypass the detector is decisive. Usually, detected zone (the middle of the product zone) is passed into and entrapped inside the detector for a preferred period of time, similar to the stopped flow strategy. However, in this type of flow, the trapped zone is justly at steady state. Consequently, it is feasible to determine accurate concentration of analyte in the detection cell at the time of detection.

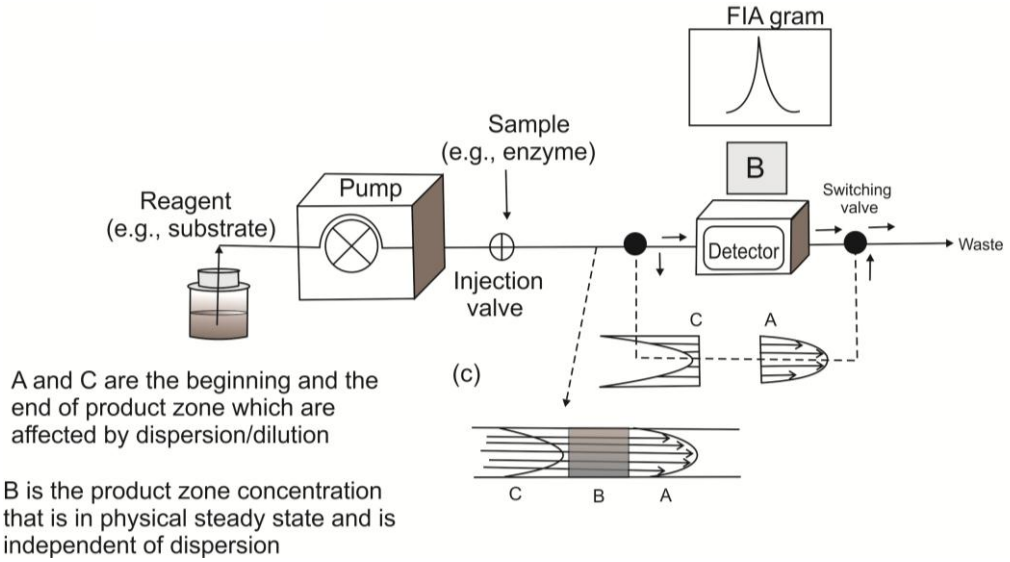


Fig 4.10: Schematic representation of Zone Bypass Flow Technique.

Air-Segmented Flow Technique: With rapid automation flow techniques are undergoing innovative changes. Automated processing of sample, high replicability, adaptability to micro-miniaturization, restraint of chemicals, low wastage, and economical usage of reagents in a system which can function at volumes in microliter are all considered to be worthy assets that put in to the application of flow injection to real-world assays. Air segmented flow technique (Fig. 4.11) is lab automated technique that uses air bubbles to separate samples and reagents for the purpose of eliminating dispersion/diffusion and prolonging the time prior to mixing.

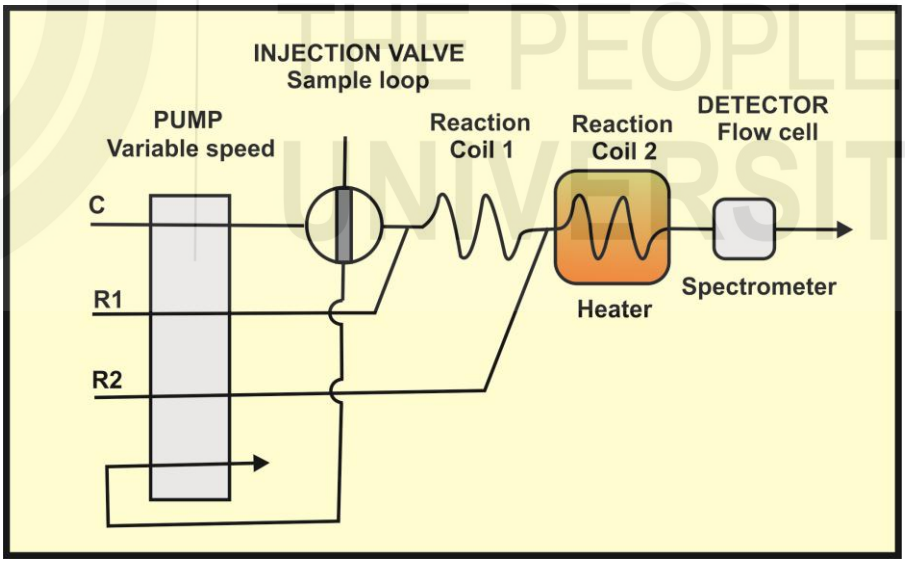


Fig 4.11: Schematic representation of Air Segmented Flow Technique.

A sample or called as analyte is injected into a flowing carrier solution stream using a peristaltic pump. The sample is injected under controlled dispersion in known volumes. To ensure the measurement of enzymatic reaction at initial state, air segments were introduced in between each solution plug to prevent early mixing of the enzyme and substrate zones. An open end reactor was directly coupled to one of the ports of the multiselection valve. The carrier solution and sample then get together at mixing points with reagents for reaction. The reaction time is restricted by a pump and reaction coil. The

product of reaction flows through a detector generally a spectrophotometer. After moving through the detector, the sample then flows to waste.

Table 4.1: Flow-based techniques for study of enzyme kinetics

Flow-based technique	Enzyme	Methodology
Contiunous flow	Tyrosine phosphatase	Use 3 syringe pumps for delivering solutions with constant combined flow rate of 100 µL/min for reaction with kinetic time scale of a minute or more
	Alkaline phosphatase	Randomly and site directed immobilized His-tag alkaline phosphatase on beads were studied using FIA-chemiluminescence system
	Acetylcholinesterase	Online dialysis of product before mixing with chromogenic reagent
	Acetylcholinesterase and angiotensin-converting enzyme	SI-LOV system with microreservoir and fiber optic/stirrer
	Urease	Thermal inactivation, flow colorimetry, and model equation
	α-amylase	Basic FIA system for measuring enzyme activity at different pHs
	Glucanase	Fluorescence probe flow injection
	Alkaline phosphatase	Open-closed flow injection, theophylline as inhibitor to determine Km and inhibitor constant
Stopped flow	Fructose bisphosphatase	Label free, mid IR detection Potentiometric detection for mechanism of different isoenzymes
	Elastase	Conventional stopped flow-spectrometric system for slow binding kinetic approach
	5-enolpyruvoyl shikimate-3-phsophate (EPSP) synthase	Fluorescence measurement at equilibrium to evaluate substrate and inhibitor binding
	Tannase	Immobilized enzyme on glass beads, packed in conductometric

		flow cell to check activity of immobilized tannase.
	Total and prostatic acid phosphatase	Double injection flow analysis
Quench flow	Protein kinase	Phosphorylation of peptide substrate, quench with acetic acid
	3-Deoxy-D-manno-2-octulosonate-8-phosphate synthase	Anion exchange HPLC for detection of radiolabel reaction intermediate
	5-enorpyruvoyl shikimate-3-phophate4 (EPSP) synthase	Radioactively label the enol moiety and then separate and quantitate products with HPLC after acid quench to observe tetrahedral intermediate
Zone trapping/Bypass trapped flow	Hexokinase	Coupled to glucose-6-phosphate dehydrogenase, and monitored production of reduced NADPH fluorometrically
Air segmented flow	Peroxidase	LAV with microreservoir with air segments

A comparison of several advantages and disadvantages of different flow systems regarding their application in enzyme kinetic studies is given in Table 4.2. An over view of flow techniques reveal its several advantages such as

a) Real-time monitoring that allows for continuous or rapid data collection.

b) High precision: Since flow techniques are often automated it minimizes the chances of human error.

c) The flow techniques offer various advantages for determining the rate of fast reactions, kinetic studies, formation of intermediates during the course of reaction

d) Control over reaction conditions: Flow techniques guarantee high regulation by restricting control over factors such as mixing time, temperature, and concentration.

Flow techniques have several disadvantages such as:

a) Complex setup: The setup of flow techniques is usually complex and needs specialized equipment and expertise.

b) Expensive: Usage of flow systems involve high costs

c) Limited Usage: For certain reactions, flow techniques may not be apt for large-scale processes without considerable optimization.

However, we can still hold that flow techniques are essential tools in biochemistry, particularly for enzyme kinetics, protein-ligand interactions, and fast chemical reactions.

Table 4.2: Comparison of the key advantages and disadvantages of different flow formats for use in enzyme kinetics studies.

Flow formats	Advantages	Disadvantages
Continuous flow	i) The most simple system because of no requirement of extra part or flow manipulation ii) High sample throughput with the least time consumption on flow operation	i) Potential for low accuracy due to dispersion, dilution, and laminar flow effect (though, reports showed there are no significant problems) ii) May be lower in sensitivity than other formats, if not enough reaction time due to high flow rate.
Stopped flow	i) Improved sensitivity by increasing reaction time before detection ii) Possible to follow the reaction at various increments of stopped time which may give more information about the reaction	i) Excessive stopped time may cause more dilution/dispersion of the reaction zone which will affect accuracy of the measurement. ii) Lower sample throughput because of longer analysis time due to the stopped time prior to detection
Quench flow	Same as stopped flow format	i) Extra time consumption in collecting aliquots of quenched solutions for further analysis ii) The least automatic due to separated detection step iii) Requires quench solution to stop chemical reaction
Zone merging/bypass flow	i) Possible to calculate for accurate concentration of the detected product because only	i) Requires higher injected volume to gain adequate size of the

	the part of the mixing zone that is not effected by dispersion/dilution is detected	product zone ii) More complicated arrangements, requires extra switching valve to change flow direction of the beginning and the end parts of the mixing zone, and needs precise time controlled operation
Air segmented flow	i) Accurate concentration because there is no dispersion, dilution, and or laminar flow effect on concentration measurement ii) Ensures measurement of initial rate	i) Requires SIA system to precisely control small volume air segment which may not be possible if use FIA system

Reference: S.K. Hartwell & K. Gudpan. Flow-Based systems for rapid and high precision enzyme kinetic studies. Journal of Analytical Methods in Chemistry Volume 2012; 1-10.

4.4 SUMMARY

Let us summarize:

- Enzyme kinetics is the basis for enzyme catalyzed biochemical reactions. Therefore, understanding of enzyme kinetics is important to understand these biological processes as well as several assays being carried out in several research laboratories.
- Kinetics provides a rationale for the complex behavior of enzymes. The rational is based on simple chemical principles. For single substrate reactions, at constant *E*, increasing *S* results in increased product formation to a point where product formation no longer increases. This saturation is presumed to reflect the fact that all *E* is now in the form of *ES*.
- Kinetic model proposed by Michaelis-Menten used equilibrium assumption for deriving Michaelis-Menten equation. The equilibrium assumption was later modified to introduce a more valid steady state assumption. The equation remains the same except the definition of Michaelis constant (K_m).
- The substrate concentration at which velocity is half of V_{max} (maximal velocity) is known as Michaelis constant (K_m). This constant gives an idea about the affinity of the enzyme for its substrate.

- The hyperbolic graph of *v* versus *S* obtained by Michaelis-Menten equation proved inadequate for determining the accurate value of V_{max} and K_m. Lineweaver and Burk gave the solution by inverting the Michaelis-Menten equation to give double reciprocal plot.
- Hanes–Woolf equation is derived from the Michaelis–Menten equation by inverting and multiplying with [S].
- Eadie-Scatchard equation can be derived by the Eadie-hofstee equation by dividing both sides of the equation by K_m.
- Eisenthal–Cornish–Bowden plot or direct linear plot engages directly plotting of the initial velocities of enzyme-catalyzed reactions i.e v_o, v_1, v_2, v_3 at different substrate concentrations i.e S_1, S_2, S_3 without changing the Michaelis-Menten equation.
- Flow techniques employ the continuous flow of reactants and/or solvents through a system to study fast reactions. These are essential tools in biochemistry, particularly for studying enzyme kinetics, protein-ligand interactions, and fast chemical reactions These techniques have been classified in mainly five forms-stopped flow, continuous flow, quench flow, Zone merging/bypass flow and air segmented flow. Each of these techniques has their own advantages and disadvantages.

4.5 TERMINAL QUESTIONS

1. Why is the rate of an enzyme-catalyzed reaction proportional to the amount of *ES* complex?
2. Explain the maximal velocity V_{max} in the v_o vs *S* graph?
3. Derive double-reciprocal equation from Michaelis-Menten equation and give its importance.
4. How a value for K_m can be obtained from the v_o vs *S* graph when v_o = 1/2 V_{max}?
5. Derive Eadie-Scatchard equation and plot.
6. Discuss the advantages and disadvantages of Flow Techniques.

4.6 ANSWERS

Self-Assessment Questions

1. Initially, as substrate concentration increases, the speed of the reaction increases. This happens because free activation centers of the enzyme bind to free substrates. Once all the activation centers of the available enzymes are bound to their substrates, new increases in the substrate concentration will have no effect on the speed of the reaction.
2. At high substrate concentration S_o, $Km <<<< S_o$ (numerically), so the term $K_m + S_o$ in the Michaelis-Menten equation becomes equal to S_o. $v_o = (V_{max}\ S_o)/S_o$, and S_o cancels. Therefore, at high S_o, $v_o = V_{max}$.

3. a- Substrate concentration, b-False, c-Linear, d-Equal

4. Slope is $1/V_{max}$ and Y-intercept is K_m/V_{max}, b-True, c- v_o and $[S_o]$, d-Direct linear plot.

Terminal Questions

1. The product formation takes place after *ES* complex formation in an enzyme catalyzed reaction. Enzyme *E* must bind to the substrate *S* before the product is formed. Therefore, the rate of an enzyme-catalyzed reaction is proportional to the amount of *ES*.

2. At high substrate concentrations, enzyme *E* will be bound to substrate *S*. So the maximum amount of *E.S* is formed under these conditions. Since the rate is proportional to the amount of *ES*, the rate is at a maximum value under these conditions.

3. Refer to section 4.2.1 and 4.2.2

4. When $v_o = V_{max}/2$, then $V_{max}/2 = V_{max}.S/K_m + S$ cancelling V_{max},

   $1/2 = S/(K_m + S)$

   $K_m + S == 2S$ or $K_m = S$ at $v_o = V_{max}/2$
   Refer to section 4.2.7

5. Refer to section 4.3

4.7 FURTHER READINGS

1. David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry6th Ed., W.H. Freeman.

2. Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.

3. Donald J Voetand Judith G. Voet: Principles of Biochemistry 4th ed., John Wiley and Sons, Inc, USA.

4. Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, John Wiley and Sons, Inc, USA.

5. S. Shanmugan and T. Sathishkumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.

6. Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.

UNIT 5

BISUBSTRATE REACTIONS

Structure

5.1 INTRODUCTION

In the previous unit you learnt about the enzyme kinetics of single substrate enzyme catalyzed reactions. The enzyme catalyzed reactions involving two or more substrates and yielding two or more products are more common than the single substrate reaction. Enzyme-catalyzed bi substrate reactions fall under two major mechanistic classifications-sequential and non-sequential. Biochemical reactions with two substrates account for ~60 % of the known biochemical reactions. The study of bisubstrate reactions is necessary for understanding enzyme kinetics, enzyme regulation, as well as vital for designing drugs that can purposely target this mechanism. Therefore it is necessary to consider the kinetics of “bisubstrate reactions”. Bisubstrate enzyme reactions follow complex rate equations as the derivation of equations depend on the order and sequence of binding of substrates.

Expected Learning Outcomes

After studying this unit, you should be able to:

- distinguish two different types of bisubstrate kinetic mechanisms;
- sequential (single displacement) reactions, which can be of either of two subtypes as given below;
- ordered sequential
- random sequential
- ping-pong" (double displacement) reactions
- able to write kinetic mechanisms for different types of bisubstrate reactions using Cleland terminology("shorthand" diagrams for kinetic mechanisms),
- explain *sequential* and *ping-pong* kinetic mechanisms based on the patterns observed on double reciprocal (Lineweaver-Burk) plots.

5.2 BISUBSTRATE REACTIONS

The nomenclature formulated for multisubstrate kinetics is as given in Table 5.1

Table 5.1: Nomenclature of enzyme catalyzed reactions depending on substrates or products involved in the reaction.

S.No.	***Reaction***	***Nomenclature***
1.	$A \longrightarrow P$	Uni uni
2.	$A + B \longrightarrow P$	Bi uni
3.	$A + B \longrightarrow P_1 + P_2$	Bi bi
4.	$A + B + C \longrightarrow P$	Ter uni
5.	$A + B + C \longrightarrow P_1 + P_2$	Ter bi

The derivation of velocity equations for multisubstrate reactions is difficult as compared to single substrate reactions. Kinetic equations for non-rapid multisubstrate systems can be derived algebraically with the aid of steady state assumptions. However, as the number of substrates increases the algebraic manipulations becomes more complicated. In bisubstrate systems isomerization of the central complex and subsequent product release steps may be so rapid that E, EA (enzyme-substrate A), EAB (enzyme-substrate A and substrate B complex), EPQ (enzyme - product P- product Q complex) and EQ (enzyme- product Q) may never attain equilibrium. The kinetic analysis may depend on the distribution of enzyme in different states as per the rate constants of all the steps along with the product release.

Considering the complexity of reactions, we will begin with two substrate two product reactions taking reactions in a series of steps for example serine proteases such as chymotrypsin that catalyses the reaction involving two substrates yielding two products. In the first step, peptide substrate is hydrolyzed to form two peptide fragment molecules and in the second step, the water molecule as substrate indirectly supplies the proton and hydroxyl groups required to complete the hydrolysis. Similarly ATP dependent kinases catalyses the phosphorylation of protein utilizing ATP as energy molecule and yielding ADP and phosphoprotein as two product molecules.

Bisubstrate reactions involving two substrate two product (bi-bi) reactions are generally transfer reactions (including oxidation/reduction reactions) and are represented as:

$$AX + B \rightleftharpoons BX + A$$

Such type of reactions are of two types viz., sequential and non sequential.

5.2.1 Sequential Reactions

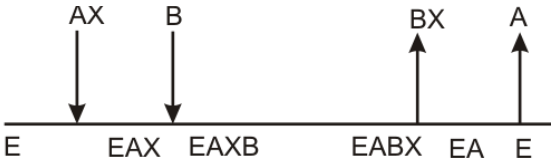
In these types of reactions, both the substrates (reactants) bind to the enzyme before the product is released. If you observe carefully, you will notice that even in sequential reactions, there could be two ways of reaction mechanisms. It may be ordered or random.

Ordered Mechanism: In this type of enzyme mechanism substrate molecules bind to the enzyme thereby forming a ternary complex and then the products are released in a defined ordered sequential manner. In other words the order of binding and leaving the enzyme is compulsory. The order is well précised and specified. So this mechanism is also known as Compulsory order mechanism. Assuming two substrates A and B binds to the enzyme E, the mechanism is represented as:

$$E + AX \rightleftharpoons E.AX \overset{+B}{\rightleftharpoons} E.AX.B \rightleftharpoons E.A.BX \overset{-BX}{\rightleftharpoons} EA \rightleftharpoons E + A$$

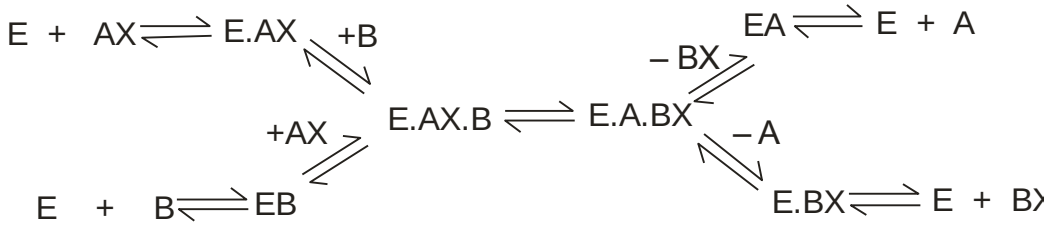
$$E + B \rightleftharpoons EB \overset{+AX}{\rightleftharpoons} E.B.AX \rightleftharpoons E.BX.A \overset{-A}{\rightleftharpoons} E.BX \rightleftharpoons E + BX$$

The diagrammatical summary of the sequences of reactions is also represented in the form of Cleland plot in which the enzyme is represented as a horizontal line and the arrows are used to represent the arrival and departure of substrates and products.

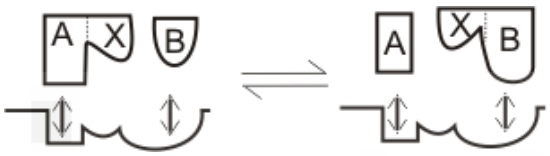


Some of the well known examples include enzyme such as citrate synthase, lactate dehydrogenase and aspartate transcarbamoyalase (ATCase). ATP sensitive K channels and H, K-pumps are also some of the other examples of compulsory order sequential reactions.

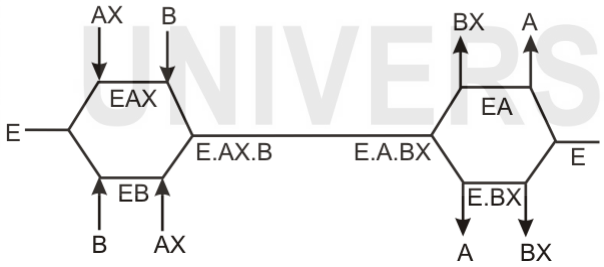
Random Sequential Mechanism: In this type of bisubstrate reactions, there is no specified order. Any substrate can bind first to the enzyme and any product may be released first. The formation of ternary complex (enzyme with two substrates) in this type of mechanism is similar to the ordered mechanism. The reaction mechanism has been shown below:



In this type of reaction mechanism, there are two separates binding sites on the enzyme, one for A/AX and other for B/BX.



The difference between two types of sequential mechanism – Ordered and Random lies in the fact that enzyme may follow ordered sequential pathway if first substrate causes a conformational change in the enzyme. However, in case of random order mechanism there will be separate binding sites on the enzyme, one for one substrate and another site for another substrate. Random sequential mechanism is typical in multienzyme complexes such as hemoglobin, ligand operated channels and tyrosine kinase receptors. The Cleland plot for such type of reaction mechanism is as follows:



SAQ 1

a) Chymotrypsin is an example of an enzyme that catalyzes the…….type of reactions (single substrate/bisubstrate). Pick one

b) Multisubstrate reactions are more common than the single substrate reactions (True/False).

c) Ternary complex is formed in ……. reactions (sequential/non-sequential).

d) Give one example of the enzyme following compulsory order sequential reaction.

5.2.2 Non- Sequential Reactions

Ping-Pong Bi-Bi Reactions: These reactions follow non-sequential mechanism and are also known as double displacement reactions. In such reactions at least one product gets released from the enzyme before both substrates bound to the enzyme. A pictorial representation has been given below:

$$AX + E \rightleftharpoons E.AX \rightleftharpoons EX.A \rightleftharpoons EX + A$$

$$EX + B \rightleftharpoons EX.B \rightleftharpoons E.BX \rightleftharpoons E + BX$$

Firstly, AX binds to the enzyme E forming a binary complex. E.AX. The X represents a small group which on its own cannot participate in the reaction. The active site of the enzyme is full as this substrate (AX) gets converted to products on account of intramolecular reorganization in the active site where the bond E-X is formed and the bonding between X-A is broken. The first product (A) is also formed which is then released from the enzyme E before second substrate (B) binds to the enzyme. The second substrate will not bind free enzyme E but will bind in its modified form E-X. In this type of reaction mechanism enzyme has only one binding site since only one substrate binds at a time. There is no room for the second substrate. The release of first product exposes the binding site on the enzyme to the second substrate. Another phase of intermolecular reorganization takes place in the active site of enzyme where bond B-X is formed and bond E-X breaks down. The release of second product (BX) follows leaving behind the enzyme in its original form. Several enzymes such as co-transporters and pumps work as per double displacement reaction mechanism. Examples include serine proteases such as trypsin, chymotrypsin and amino transferases.

Cleland plot for ping-pong bi-bi enzyme catalyzed reaction is:

SAQ 2

a) The form in which the ping pong mechanism binds substrates is identified as which type of mechanism?

b) What are two characteristics of an enzyme that catalyzes a reaction through the ping-pong mechanism?

5.3 ALBERTY RATE EQUATION

Bisubstrate enzyme catalyzed reactions observe Michaelis-Menten equation when one of the two substrates is at constant concentration with respect to the other substrate. We would like to remind the learner that it will be applicable only when enzyme has one binding site per substrate or multiple binding sites per substrate provided there is no interaction between the binding sites. Alberty derived equation for such reactions is:

$$v_o = \frac{Vmax\,[AXo][Bo]}{K_m^B[AXo] + K_m^{AX}[Bo] + [AXo][Bo] + K_s^{AX}K_m^B}$$

Where V_{max} is the maximum v_o when both the substrates *AX* and *B* are saturating

$K^{AX}{}_m$ is concentration of substrate *AX* which gives ½ V_{max} when substrate *B* is saturating

$K^B{}_m$ is concentration of substrate *B* which gives ½ V_{max} when substrate *AX* is saturating

$K^{AX}{}_s$ is the dissociation constant for *E* + *AX* -→ *EAX*

The total enzyme concentration is much lesser than the concentrations of two substrates.

At saturating concentration of [B_o] the Alberty equation will be

$$v_o = \frac{Vmax\,[AXo]}{K_m^{AX} + [AXo]}$$

Similarly at the saturating concentration of [AX_o] the Alberty equation will be

$$v_o = \frac{Vmax\,[Bo]}{K_m^B + [Bo]}$$

From the above equations, you will observe that two substrate reactions will also obey Michaelis-Menten equation with respect to one substrate when the concentration of other substrate is fixed. So, the corresponding Lineweaver-Burk plot or double reciprocal plot will also be linear.

Several sequential bisubstrate enzyme catalyzed reactions (random order and compulsory order) will observe Alberty equation when the rate limiting step is interconversion of the ternary complex (*E.AX.B* -→ *E.A.BX*). If you remember, the rate limiting step in single substrate reactions is the interconversion of *ES*→ *EP*.

For double displacement reactions or ping-pong bi-bi enzyme catalyzed non-sequential reactions, the release of one product from the enzyme in the initial period of reaction will be irreversible. Hence $K^{AX}{}_s = 0$ and $K^{AX}{}_s$. $K^B{}_m = 0$. The rate of reaction will be

$$v_o = \frac{Vmax\,[AXo][Bo]}{K_m^B[AXo] + K_m^{AX}[Bo] + [AXo][Bo]}$$

5.4 DIFFERENTIATING BI SUBSTRATE MECHANISMS

5.4.1 Primary Plots

The distinguishment of the bisubstrate enzyme catalyzed reactions can be made from the primary plots of reaction mechanisms observing Alberty rate equation. The reaction mechanisms of bisubstrate reaction obeying Alberty rate equation will give linear plots of $1/v_o$ vs $1/[AX_o]$ at constant concentration of other substrate [B_o]. Similarly $1/v_o$ vs $1/[B_o]$ at constant concentration of other substrate [AX_o] will also be a linear plot. The slopes and intercept of

these linear plots includes concentration of the fixed substrate at non-saturating concentrations. Therefore, the slope as well as intercept of these plots will change if the experiments are repeated with fixed substrate concentration at a different value. The linear plots of compulsory or random order sequential enzyme catalyzed reactions are shown in the Figure 5.1 a. The double displacement or Ping-Pong bi bi enzyme catalyzed reaction is also shown in Figure 5.1 b. The learner can see from the graph that the linear plot in figures is independent of the concentration of the fixed substrate. Recall from the last section, you will notice that K^{AX}_s. K^{B}_m =0 in ping-pong bi-bi reaction mechanism. The linear plot of ping-pong bi bi will show several parallel lines if the experiments are repeated at the different concentrations of the fixed substrate.

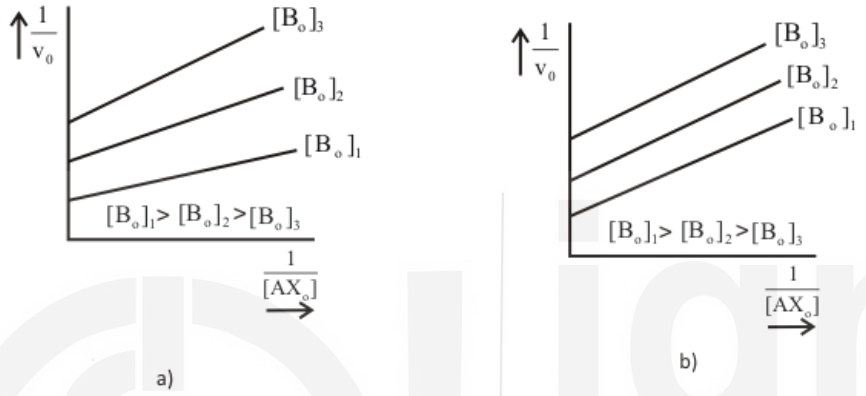


Fig. 5.1: Graph of plot taken at non-saturating concentrations of both substrates of the enzyme.

a) for compulsory-order and random –order ternary complex

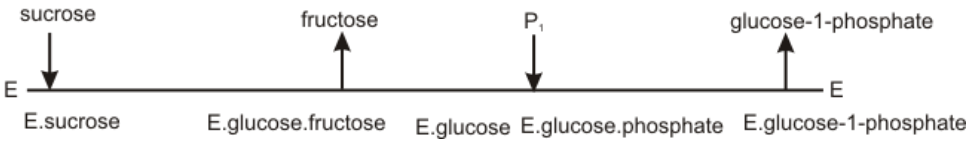
b) for ping-pong bi-bi reaction mechanisms

The limitation of these plots is that compulsory-order and random order can be distinguished from the ping pong bi bi reaction mechanism but not from each other.

5.4.2 Non-Steady State Methods-Isotope Exchange

If you recall from your school books regarding radioisotopes, they are one of the finest techniques which can be effectively used to determine enzyme reaction mechanism. Let us see how? The isotope exchange at chemical equilibrium helps to determine the reaction mechanism. In ping pong non sequential mechanism, one of the products is released before the joining of another substrate to the enzyme. Therefore, exchange of isotope between reactant and product in a reaction in the absence of other reactants and products suggests a ping-pong enzyme catalyzed reaction mechanism. Consider the bisubstrate reaction catalyzed by enzyme sucrose converting orthophosphate to glucose-1-phosphate. The other substrate in this reaction is sucrose and other product is sucrose. The isotope exchange between orthophosphate (substrate) and glucose-1-phosphate (product) in sucrose catalyzed reaction in the absence of sucrose (other substrate) and fructose (other product) suggests a ping pond mechanism. Similarly, isotope exchange between sucrose and fructose in the absence of orthophosphate and glucose-

1-phosphate will also suggests ping pong bi bi enzyme catalyzed reaction mechanism.



Another useful application of isotopes is in distinguishment of compulsory order sequential mechanism from random order sequential mechanism. The perceived change in the rate of equilibrium isotope exchange on account of changes in the concentration of reactants and products without changing the concentration of reactants and products will help to determine whether the bisubstrate enzyme catalyzed reaction mechanism is compulsory or random order.

The general representation of bisubstrate reaction is:

$AX + B \rightleftharpoons BX + A$

When the rate of forward reaction is equal to the rate of backward reaction, the equilibrium will be:

$$\frac{[BX][A]}{[AX][B]} = Keq$$

The addition of a small amount of radiolabel molecule will not affect the equilibrium significantly. Therefore, if we add a small amount of radiolabel molecule *B* in the reaction and then measure the rate of formation of radiolabel *BX*, we can trace the reaction mechanism. In the next step we will increase the concentrations of *A* and *AX*, keeping their ratio [*A*] : [*AX*] constant as well as keeping the equilibrium unchanged. This will lead to change in the rate of isotope exchange between the reactants and products.

Now consider a compulsory order sequential mechanism:

In this bisubstrate reaction, substrate *B* binds first to the enzyme leading to the formation of *EB*. The second substrate *AX* joins the *EB* complex to form a ternary complex *E.B.AX*. An interchange is followed by the release of first product *A* and second product *BX* in compulsory order. A little increase in the concentration of one substrate *AX* and one product *A* will increase the isotope

exchange between *B* and *BX*. The isotope exchange will increase with the increase in the concentration of *A* and *AX* keeping [*A*] : [*AX*] constant. You should see isotope exchange in Figure 5.2. If you can recall, there is formation of ternary complex in compulsory order reactions. Therefore substantial increase in the concentrations of *A* and *AX* will make it difficult for the substrate *B* to dissociate from *E.B* and product *BX* to dissociate from *EBX*. So there will be decrease in the rate of isotope exchange between *B* and *BX*.

Let us look again at the compulsory order mechanism with the first substrate *AX* binding the enzyme *E* to form *E.AX* and then joined by *B* to form *E.AX.B*. An interchange is followed by the release of first product *BX* and second product *A* in a compulsory order. An increase in the concentrations of *AX* and *A* will lead to the free enzyme *E* forming *E.AX* and *EA* complexes. *E.AX* reacts with second substrate *B* and *EA* doesn't affect release of *BX* from *E.A.BX*. Therefore the rate of isotope exchange will increase in a hyperbolic manner on further increase in the concentrations of *A* and *AX*. So you will observe a hyperbolic graph as shown in Figure 5.3

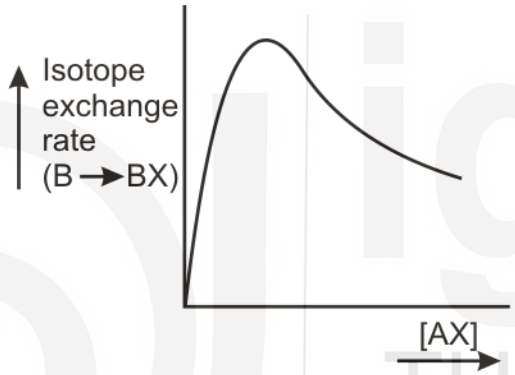


Fig. 5.2: Graph of plot for the isotope exchange from *B*→ *BX* against [*AX*] in a compulsory order bisubstrate enzyme catalyzed reaction when *B* binds first.

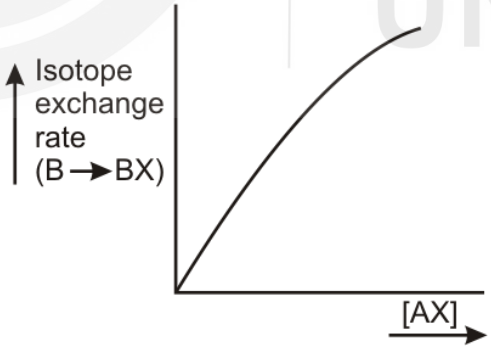


Fig. 5.3: Graph of plot for the isotope exchange from *B*→ *BX* against [*AX*] in a compulsory order bisubstrate enzyme catalysed reaction when *AX* binds first.

Similar results will be obtained in Random Order enzyme mechanism.

Rapid Reaction Studies:

Many biochemical processes are too fast for fitting observation using a standard spectrophotometer. Sometimes these processes can be avoided by alternative of conditions, other than for enzyme reactions that occur in 0.01 - 100 second timescales, stopped flow methods are helpful. Theorell and Chance used stopped flow techniques with a double beam detector for studying enzyme reaction of horse liver alcohol dehydrogenase. The enzyme catalyzes the formation of formaldehyde from ethanol. The entire reaction is represented as:

$$CH_3CH_2OH + NAD^+ \rightleftharpoons CH_3CHO + NADH\ (+H^+)$$

The coenzyme *NADH* shows absorbance at 350 nm and not at 328nm. So measuring the differences in the absorbance at 350nm and 328nm will help to determine the formation of *NADH* from *E.NADH* complex (*E.NADH*--→ *E* + *NADH*). The rate of absorbance at 328 measures the rate of reduction of NAD^+ (NAD^+ -→ *E.NADH*). In this type of compulsory order reaction, Chance found out that interconversion of ternary complex is too fast and the rate limiting step of the reaction is the release of *NADH* from *E.NADH* complex.

SAQ 3

a) The rate equation of Alberty bears resemblance to the Michaelis-Menten equation (True/False).

b) In the Alberty rate equation for bisubstrate reations, V_{max} denotes the maximum v_o when only one substrate is saturating (True/False).

c) NADH shows absorbance at 350 nm. (True/False)

d) Compulsory-order and random order reaction mechanisms can be distinguished from ping pong bi bi mechanisms but not from each other by the primary plots. (True/False)

e) Isotope exchange technique can be used for investigating reaction mechanisms. (True/False)

5.5 SUMMARY

Let us summarize:

- Enzyme kinetics can be complex especially in case of bisubstrate reactions but can be very informative.
- Two substrate enzyme reactions can proceed by several different mechanisms leading to the release of two products. The bisubstrate reaction mechanism can be sequential (single displacement reactions) or non-sequential (double displacement reactions or ping-pong bi-bi).

- Single displacement reactions can also be either of two subtypes: ordered sequential or random sequential.
- The Alberty rate equation for bisubstrate reactions obeys Michaelis-Menten equation with respect to one substrate at fixed concentration of the other.
- The kinetic mechanism of bisubstrate reactions can be determined by steady-state methods and by non-steady state methods such as isotope exchange and rapid-reaction techniques.

5.6 TERMINAL QUESTIONS

1. Why is the difference between random sequential and ordered sequential reaction mechanisms?
2. Derive Alberty rate equation?
3. Draw primary plot for distinguishing sequential and non-sequential rate mechanisms?
4. Discuss rapid- reaction techniques for bisubstrate reactions.

5.7 ANSWERS

Self Assessment Questions

1. a) Bisubstrate, b) True, c) Sequential, d) Lactate Dehydrogenase,
2. a) The ping-pong mechanism is a non-sequential mechanism. A product is released after the first substrate is bound.

   b) The characteristics of enzyme catalyzing ping-pong bi bi reaction mechanism are:

   c) A product is formed before the second substrate binds to the enzyme.

   d) The binding of the first substrate causes the enzyme to change into an intermediate form that will bind the second substrate.

   e) The plot of $1/v$ vs. $1/[A]$ as $[B]$ changes will be parallel lines.
3. a) True, b) False, c) True, d) True, e) True

Terminal Questions

1. Refer to section 5.2.1
2. Refer to section 5.3
3. Refer to section 5.4.1
4. Refer to section 5.4.2 Rapid Reaction Studies

5.8 FURTHER READINGS

1. David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry6th Ed., W.H. Freeman.

2. Robert K. Murray, Daryl K. Granner, Victor W. Rodwell Harper's Illustrated Biochemistry, 27th edition. 2006, McGraw-Hill.

3. Donald J Voet and Judith G. Voet: Principles of Biochemistry 4th ed., John Wiley and Sons, Inc, USA.

4. Eric E Conn, Paul K Stumpf: Outlines of Biochemistry, John Wiley and Sons, Inc, USA.

5. S. Shanmugan and T. Sathish Kumar: Enzyme Technology, I K International Publishing House Pvt Ltd, New Delhi.

6. Nicholas C Price and Lewis Stevens: Fundamentals of Enzymology, Oxford University Press, Oxford, New York, USA.

UNIT 6

ENZYME INHIBITION

Structure

6.1 INTRODUCTION

In the previous unit, you have learnt about the kinetics of enzyme catalyzed reactions. In this unit we will introduce you to important class of molecules called “enzyme inhibitors”. Enzyme inhibitors are the molecules that regulate the activity of enzymes in the cell. Inhibitors alter the catalytic action of the enzyme; as a result slows down the enzyme activity, or in some cases, close the catalysis. The study of these inhibitors provides wealth of information on the working of enzymes and their mechanism. The blockage of enzyme activity can lead to several changes such as correction of metabolic imbalance or killing of a bacteria or pathogen. Therefore, many of the drug molecules are enzyme inhibitors and their discovery as well as improvement has been a major area of research for biochemistry and pharmacology.

This unit will give you an overview about different types of enzyme inhibitors.

Expected Learning Outcomes

After studying this unit, you should be able to:

- determine types of enzyme inhibitor;
- find out how inhibitor interacts with the enzyme;

- ❖ state the role of inhibitor affecting enzyme kinetic parameters;
- ❖ distinguish between reversible and irreversible inhibitors; and
- ❖ explain the role of enzyme inhibitors and their classification according to nature and function.

6.2 ENZYME INHIBITION-REVERSIBLE AND IRREVERSIBLE

Molecules that bind to the enzymes and cause a decrease in their activity are called enzyme inhibitors. These molecules either bind at the active site of enzyme thereby preventing the substrate molecule to bind to the enzyme or they may inhibit the catalytic activity of enzyme. You should know that many of these molecules perform several regulatory roles in the metabolism. Some of them are used as herbicides or pesticides. Most of the drug molecules also act as enzyme inhibitors. Natural enzymes inhibitors e.g. poison are a part of the defense mechanisms in wild life animals.

Enzyme inhibitions are mainly classified into two types:

a) Reversible Inhibition

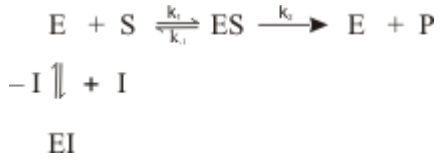
b) Irreversible Inhibition

6.3 REVERSIBLE INHIBITION

In this unit, we will discuss about the inhibition of simple single substrate enzyme catalyzed reactions. In reversible enzyme inhibition, loss of the enzyme activity due to inhibitory molecule is reversible. Enzyme activity gets restored on the removal of inhibitor. These inhibitors bind non-covalently and give rise to different kinds of inhibition. Multiple weak bonds between the inhibitor and the enzyme combine to give strong binding which prevents the formation of product. They can be easily removed by dilution or dialysis to restore full enzyme activity. Reversible inhibitors tend to form equilibrium with an enzyme leading to certain level of inhibition.

6.3.1 Competitive Inhibition

There is a direct competition between the substrate and the inhibitor for the binding site of enzyme in a competitive inhibition. If inhibitory molecule is bound to the enzyme then substrate will not be able to bind it and vice versa. In such kind of scenario, inhibition can be overcome by increasing substrate concentration in comparison to inhibitor. Therefore, the maximum velocity (V_{max}) of the reaction will remain unchanged while K_m will be decreased in a competitive inhibition. Most of the competitive inhibitors are structurally similar to the substrate molecules.



The dissociation constant (K_i) or inhibitor constant for the reaction is

$$\frac{[E][I]}{[EI]} = K_i \text{ or } \frac{[E][I]}{Ki} = [EI] \quad \text{… equation 1}$$

I = Inhibitor

EI = Enzyme bound to Inhibitor

E = Free Enzyme

E_o = Total enzyme

As you know in the previous unit, using steady state assumption, Michaelis- Menten constant for the rate of reaction is given by the following equation.

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m \quad \text{…. equation 2}$$

$$E_o = [E] + [ES] + [EI] \quad \text{…. equation 3}$$

Substituting the value of [*EI*] as given in equation 1

$$E_o = [E] + [ES] + \frac{[E][I]}{K_i}$$

$$E_o = [E](1 + \frac{[I]}{K_i}) + [ES]$$

$$E = \frac{[E_o] - [ES]}{(1 + \frac{[I]}{K_i})}$$

Substituting the value of [*E*] in equation 2:

$$\frac{([E_o] - [ES])[S]}{(1 + [\frac{I}{K_i}])[ES]} = K_m$$

$$\frac{([E_o][S])}{[ES]} - [S] = K_m(1 + [\frac{I}{K_i}])$$

$$\frac{([E_o][S])}{[ES]} = K_m(1 + [\frac{I}{K_i}]) + [S]$$

$$\frac{([E_o][S])}{K_m(1 + [\frac{I}{K_i}]) + [S]} = [ES]$$

$$v_o = k_2[ES] \quad \text{……. equation 4}$$

(Ref: Michaelis Menten equation in the previous Unit-4, Block-2 of BBCT-107)

Putting the value of [*ES*] in equation 4

$$v_o = \frac{k_2[E_o][S]}{K_m(1+[\frac{I}{K_i}])+[S]}$$

$V_{max} = k_2[E_o]$ ……. equation 5

(Ref: Michaelis Menten equation in the previous Unit-4, Block-2 of BBCT-107)

$$v_o = \frac{V_{max}[S]}{K_m(1+[\frac{I}{K_i}])+[S]}$$

Since inhibitor concentration is generally of the same order of magnitude as the substrate concentration and much higher than the enzyme concentration [*I*] ~ [I_o] just as [*S*] ~ [S_o]. The above equation can be expressed as

$$v_o = \frac{V_{max}[S_o]}{K_m(1+[\frac{I_o}{K_i}])+[S_o]}$$ ……. equation 6

This equation is similar to Michaelis- Menten equation and the K_m is increased by a factor

$K_m(1+[\frac{I_o}{K_i}])$ or $K'_m = K_m(1+[\frac{I_o}{K_i}])$

The Lineweaver- Burk equation and plot (Fig. 6.1) of competitive inhibition will be:

$$\frac{1}{v_o} = \frac{K'_m}{V_{max}[S_o]} + \frac{1}{V_{max}}$$

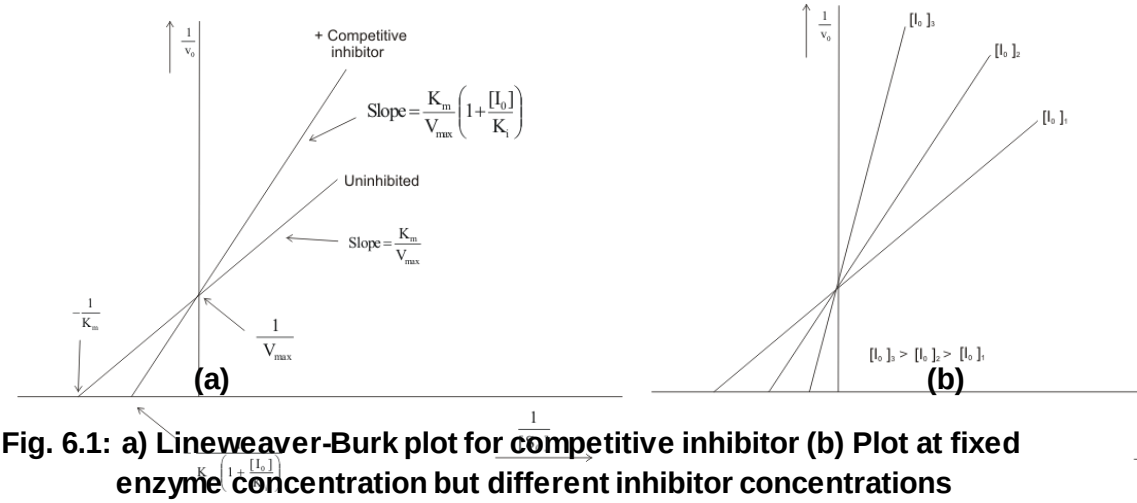


Fig. 6.1: a) Lineweaver-Burk plot for competitive inhibitor (b) Plot at fixed enzyme concentration but different inhibitor concentrations

Examples of Competitive Inhibition: Succinate dehydrogenase (SDH) enzyme converts succinate to fumarate. Succinate is competitively inhibited by

malonate. Malonate ($CO^-_2.CH_2.CO^-_2$) is structurally similar to fumarate having two carboxyl groups. Similarly, inhibition of xanthine oxidase enzyme by allopurinol leads to decreased formation of uric acid and act as a remedy for gout.

$$CO_2.CH_2.CH_2.CO^-_2 \rightleftharpoons CO^-_2.CH{=}CH.CO^-_2$$

Succinate Fumarate

SAQ 1

Draw Lineweaver- Burk plot for different inhibitory concentrations of a competitive inhibitor at the fixed enzyme concentrations.

6.3.2 Un-Competitive Inhibition

Uncompetitive inhibitors are those inhibitors who do not compete with the substrate and do not bind to the free enzyme. They bind to the enzyme substrate complex thereby forming a dead end complex. It is quite likely that binding of substrate to the enzyme may cause some conformational changes and reveals an inhibitor binding site where inhibitor can bind. This type of inhibition cannot be overcome by excess of substrate. Both K_m and V_{max} gets changed in this type of inhibition pattern.

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

$$-I \uparrow\downarrow + I$$

$$ESI$$

The inhibitor constant K_i is as given below:

$$\frac{[ES][I]}{[ESI]} = K_i \text{ or } \frac{[ES][I]}{Ki} = [ESI] \quad \text{..... equation 7}$$

I = Inhibitor

EI = Enzyme bound to Inhibitor

E = Free Enzyme

E_o = Total enzyme

Using steady state assumption, Michaelis- Menten constant for the rate of reaction is given by

$$\frac{[E][S]}{[ES]} = \frac{k_{-1} + k_2}{k_1} = K_m$$

..... equation 2

$$E_o = [E] + [ES] + [ESI] \quad \text{..... equation 8}$$

Substituting the value of [*ESI*] as given in equation 7

$$E_o = [E] + [ES] + \frac{[ES][I]}{Ki}$$

$$E_o = [E] + [ES](1 + \frac{[I]}{Ki})$$

$$E = [E_o] - [ES](1 + \frac{[I]}{Ki})$$

Substituting the value of *E* in equation 2

$$\frac{[E_o] - [ES](1 + \frac{[I]}{Ki})][S]}{[ES]} = K_m$$

$$\frac{[E_o][S]}{[ES]} - (1 + \frac{I}{K_i})[S] = K_m$$

$$\frac{[E_o][S]}{[ES]} = K_m + (1 + \frac{I}{K_i})[S]$$

$$\frac{[E_o][S]}{K_m + (1 + \frac{I}{K_i})[S]} = [ES]$$

$v_o = k_2[ES]$ ……. equation 4

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCT-107)

Putting the value of [*ES*] in equation 4

$$v_o = k_2 \frac{[E_o][S]}{K_m + (1 + \frac{I}{K_i})[S]}$$

$V_{max} = k_2[E_o]$ ……. equation 5

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCT-107)

Since inhibitor concentration is generally of the same order of magnitude as the substrate concentration and much higher than the enzyme concentration [*I*] ~ [I_o] just as [*S*] ~ [S_o]. Thus continuing similarly, above equation can be expressed as

$$v_o = \frac{V_{max}[S_o]}{K_m + (1 + \frac{I_o}{K_i})[S_o]}$$

……. equation 9

Dividing the numerator and denominator by $(1 + \frac{[I_o]}{K_i})$ gives

$$v_o = \frac{\frac{V_{max}[S_o]}{(1+\frac{I_o}{K_i})}}{\frac{Km}{(1+\frac{I_o}{K_i})}+[S_o]}$$

The above equation is similar to Michaelis-Menten equation. However, the constants K_m and V_{max} are changed as following:

$$V'_{max} = \frac{V_{max}[S_o]}{(1+\frac{I_o}{K_i})}$$

and

$$K'_m = \frac{K_m}{(1+\frac{I_o}{K_i})}$$

The Lineweaver-Burk equation in the presence of an uncompetitive inhibitor is:

$$\frac{1}{v_o} = \frac{K'_m}{V'_{max}[S_o]} + \frac{1}{V_{max}}$$

The slope of a Lineweaver-Burk plot will remain unchanged as given by the following equation:

$$\frac{K'_m}{V'_{max}} = \frac{\frac{K_m}{(1+\frac{[I_o]}{K_i})}}{\frac{V_{max}}{(1+\frac{[I_o]}{K_i})}} = \frac{K_m}{V_{max}}$$

The x-intercept as well as y-intercept of the Lineweaver-Burk plot in the presence of an uncompetitive inhibitor (Fig. 6.2) will change but the slope will remain unaltered.

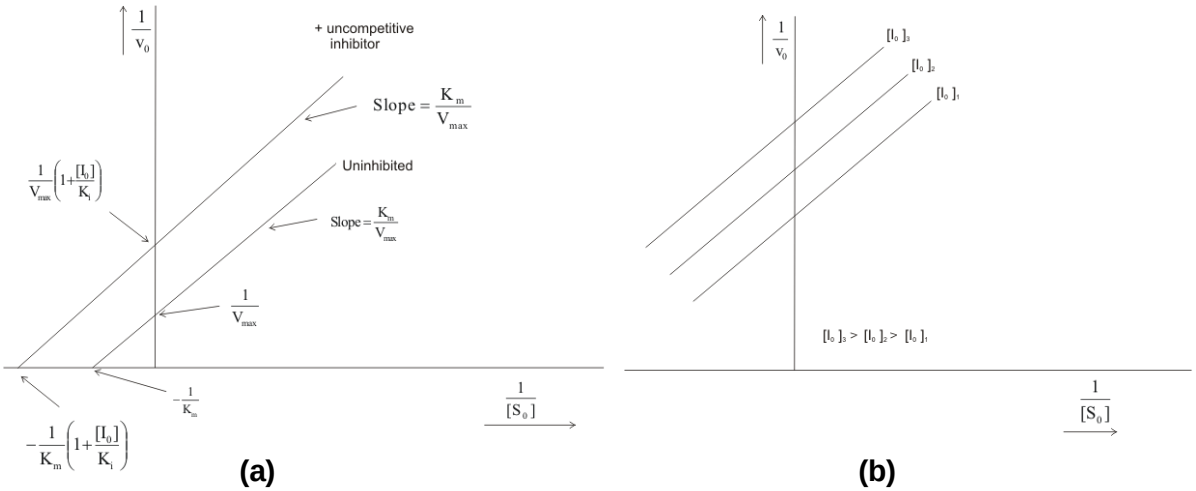


Fig. 6.2: a) Lineweaver-Burk plot of enzyme inhibited by an uncompetitive inhibitor b) Plot at fixed enzyme concentration but different inhibitor concentrations.

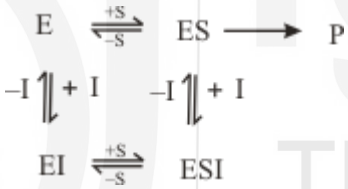
Example of Uncompetitive Inhibitor: Enzyme aryl sulphatase is uncompetitively inhibited by hydrazine. Generally uncompetitive inhibition pattern is not seen in single substrate reactions but several bi-substrate reactions shows this kind of pattern.

SAQ 1

Draw Lineweaver- Burk plot for different inhibitory concentrations of an uncompetitive inhibitor at the fixed enzyme concentrations.

6.3.3 Non-Competitive Inhibition

These inhibitory molecules react with the enzyme at a site other than the active site. The inhibitor can bind to the free enzyme or when it is bounded to the substrate molecule. It destroys the catalytic activity of the enzyme. The impact of inhibitor can not be overcome by increasing the substrate concentration. Apparently, K_m value will not be changed but V_{max} will change. Let us consider a simple situation involving single substrate reaction



Since *ES* can be formed via two alternative routes and considering the fact that inhibitory rates could vary in binding the free enzyme or when it is bound to the substrate the situation will become quite complex. In order to simplify the above model, we assume that substrate binding will have no effect on the binding of inhibitor. The dissociation constant (inhibitor constant) for both the reactions $E + I \rightleftharpoons EI$ and $ES + I \rightleftharpoons ESI$ will then be the same. The total enzyme concentration will be reduced in the presence of inhibitor and it will lead to decrease in V_{max}. The value of K_m will not change because binding of inhibitor does not affect the binding of substrate to the enzyme or vice versa.

In the presence of non-competitive inhibitor

$$\frac{[E][I]}{[EI]} = K_i = \frac{[ES][I]}{[ESI]} \qquad \text{..... equation 10}$$

$$E_o = [E] + [ES] + [EI] + [ESI] \qquad \text{..... equation 11}$$

Substituting the value of [*EI*] and [*ESI*] as given in equation 10

$$E_o = [E] + [ES] + \frac{[E][I]}{K_i} + \frac{[ES][I]}{K_i}$$

$$E_o = [E] + \frac{[E][I]}{K_i} + [ES] + \frac{[ES][I]}{K_i}$$

$$E_o = [E](1 + \frac{[I]}{K_i}) + [ES](1 + \frac{[I]}{K_i})$$

$$E_o = ([E] + [ES])(1 + \frac{[I]}{K_i})$$

$$E = \frac{[E_o]}{(1 + \frac{[I]}{K_i})} - [ES]$$

Substituting the value of *E* in equation 2

$$\frac{(\frac{[E_o]}{(1 + \frac{[I]}{K_i})} - [ES])[S]}{[ES]} = K_m$$

$$\frac{(\frac{[E_o][S]}{(1 + \frac{[I]}{K_i})} - [ES][S])}{[ES]} = K_m$$

$$\frac{\frac{[E_o][S]}{(1 + \frac{[I]}{K_i})} - [ES][S](1 + \frac{[I]}{K_i})}{[ES]} = K_m$$

$$\frac{[E_o][S]}{[ES]} - [S](1 + \frac{[I]}{K_i}) = K_m(1 + \frac{[I]}{K_i})$$

$$\frac{[E_o][S]}{[ES]} = K_m(1 + \frac{[I]}{K_i}) + [S](I + \frac{[I]}{K_i})$$

$$\frac{[E_o][S]}{[ES]} = (K_m + S)(I + \frac{[I]}{K_i})$$

$$\frac{[E_o][S]}{(K_m + [S])(1 + \frac{[I]}{K_i})} = [ES]$$

$$v_o = k_2[ES] \qquad \text{..... equation 4}$$

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCT-107)

Putting the value of [*ES*] in equation 4

$$v_o = k_2 \frac{[E_o][S]}{(K_m + [S])(1 + \frac{[I]}{K_i})}$$

$$V_{max} = k_2[E_o] \quad \text{..... equation 5}$$

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCT-107) Continuing similarly as in previous sections,

$$v_o = \frac{V_{max}[S_o]}{(K_m + [S_o])(1 + \frac{[I_o]}{K_i})} \quad \text{..... equation 12}$$

This is similar to the Michaelis-Menten equation with V_{max} divided by a factor $(1 + \frac{[I_o]}{K_i})$

$$V'_{max} = \frac{V_{max}}{(1 + \frac{I_o}{K_i})}$$

The Lineweaver-Burk equation in the presence of a noncompetitive inhibitor is:

$$\frac{1}{v_o} = \frac{K_m}{V'_{max}[S_o]} + \frac{1}{V'_{max}}$$

The Lineweaver-Burk plot (Figure 6.3) of a non-competitive inhibitor is as given below:

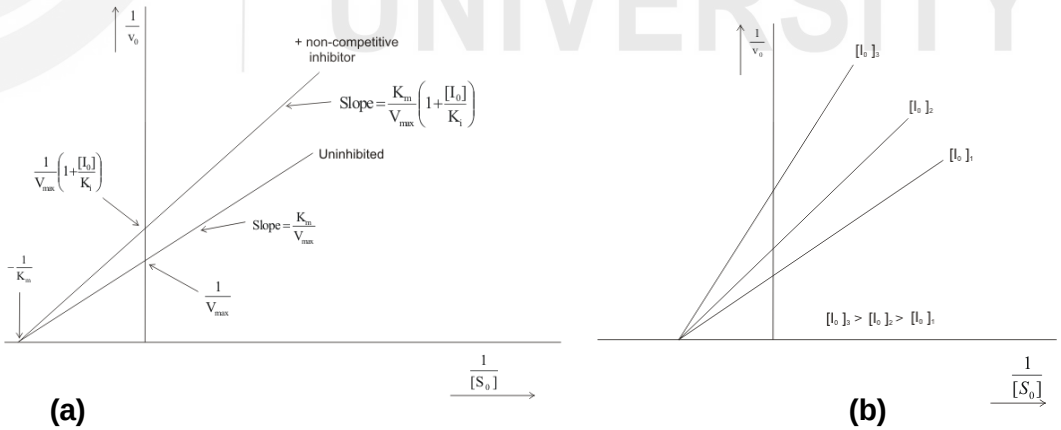


Fig. 6.3 : a) Lineweaver-Burk plot for non-competitive inhibitor (b) Plot at fixed enzyme concentration but different inhibitor concentrations.

Example of Noncompetitive Inhibition: Chymotrypsin is noncompetitively inhibited by H^+. Heavy metal ions and small organic molecules bound to the –SH groups of the cysteine moiety in an enzyme can also lead to the noncompetitive inhibition.

SAQ 3

Draw Lineweaver- Burk plot for different inhibitory concentrations of a noncompetitive inhibitor at the fixed enzyme concentrations.

6.3.4 Mixed Inhibition

Mixed inhibition occurs when an inhibitor may bind to the enzyme as well as enzyme substrate complex. However, the affinity with which inhibitor binds to two different states may varies. It may bind with greater affinity when the enzyme is free than the enzyme substrate complex or vice versa. The inhibition is a mixture of competitive inhibition and uncompetitive inhibition. If the inhibitor has equal affinity for both the states of enzyme (free as well as bound to the substrate), mixed inhibition will become non-competitive inhibition. In mixed inhibition, inhibitor binds to the enzyme at a site different from the active site, the site where substrate binds. As you know, there are two processes by which inhibitor can bind to the enzyme.

$E + I \rightleftharpoons EI$ (inhibitor constant K_i)

and

$ES + I \rightleftharpoons ESI$ (inhibitor constant K_1)

Therefore

$$\frac{[E][I]}{[EI]} = K_i$$

and

$$K_1 = \frac{[ES][I]}{[ESI]}$$

As you know in a single substrate reaction,

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

$$E_o = [E] + [ES] + [EI] + [ESI]$$

……..equation 13

Since K_i and K_1 are not similar, they are different. So

$$E_o = [E] + [ES] + \frac{[E][I]}{K_i} + \frac{[ES][I]}{K_1}$$

$$E_o = [E] + \frac{[E][I]}{K_i} + [ES] + \frac{[ES][I]}{K_1}$$

$$E_o = [E](1 + \frac{[I]}{K_i}) + [ES](1 + \frac{[I]}{K_1})$$

$$\frac{\left([E_0]-[ES]\left(1+\frac{[I]}{K_I}\right)\right.}{\left(1+\frac{[I]}{K_i}\right)} = [E]$$

Substituting for [*E*] in equation 2, the expression for K_m :

$$\frac{\left([E_0]-[ES]\left(1+\frac{[I]}{K_I}\right)\right.)[S]}{\left(1+\frac{[I]}{K_i}\right)[ES]} = K_m$$

$$\therefore [E_0][S] - [S][ES]\left(1+\frac{[I]}{K_I}\right) = K_m[ES]\left(1+\frac{[I]}{K_i}\right)$$

$$\therefore [ES]\left([S]\left(1+\frac{[I]}{K_I}\right)+K_m\left(1+\frac{[I]}{K_i}\right)\right)=[E_0][S]$$

$$\therefore [ES] = \frac{[E_0][S]}{[S]\left(1+\frac{[I]}{K_I}\right)+K_m\left(1+\frac{[I]}{K_i}\right)}$$

$$v_o = k_2[ES]$$

…… equation 4

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCT-107)

Putting the value of [*ES*] in equation 4

Therefore, $v_0 = \frac{k_2[E_0][S]}{[S]\left(1+\frac{[I]}{K_I}\right)+K_m\left(1+\frac{[I]}{K_i}\right)}$

$$V_{max} = k_2[E_o]$$

…… equation 5

(Ref: Michaelis Menten equation section in the previous Unit-4, Block-2 of BBCT-107) Continuing similarly as in previous sections:

$$v_0 = \frac{V_{max}[S_o]}{[S]\left(1+\frac{[I_0]}{K_I}\right)+K_m\left(1+\frac{[I_0]}{K_i}\right)}$$

……..equation 14

Dividing the numerator and denominator by (1 + (I_0]/K_i)),

$$v_0 = \frac{\dfrac{V_{max}}{\left(1+\dfrac{[I_0]}{K_I}\right)}[S_o]}{[S_o] + \dfrac{K_m\left(1+\dfrac{[I_0]}{K_i}\right)}{\left(1+\dfrac{[I_0]}{K_I}\right)}}$$

The above equation assumes the same form as the Michaelis-Menten equation and can be written as:

$$v_0 = \frac{V'_{max}\ [S_o]}{[S_o] + K'_m}$$

where

$$V'_{max} = \frac{V_{max}}{\left(1+\dfrac{[I_0]}{K_I}\right)} \text{ and } K'_m = K_m \frac{\left(1+\dfrac{[I_0]}{K_i}\right)}{\left(1+\dfrac{[I_0]}{K_I}\right)}$$

The Lineweaver-Burk equation will be:

$$\frac{1}{v_0} = \frac{K'_m}{V'_{max}}\frac{1}{[S_o]} + \frac{1}{V'_{max}}$$

From the equation, it appears that the Lineweaver-Burk plot will be linear. However, K_m, V_{max} and slope will be affected by the inhibitor. The Lineweaver-Burk plot will be represented as shown in the Figure 6.4.

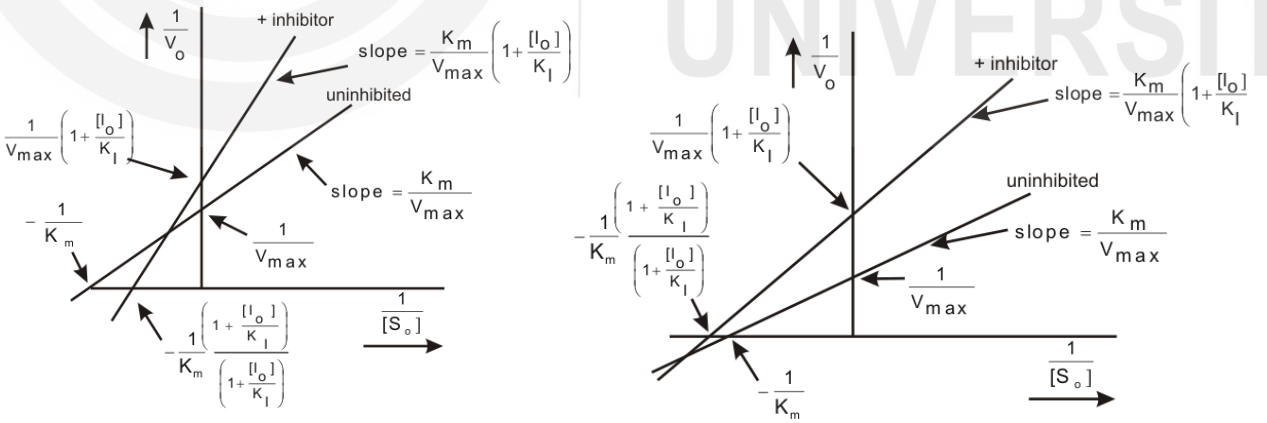
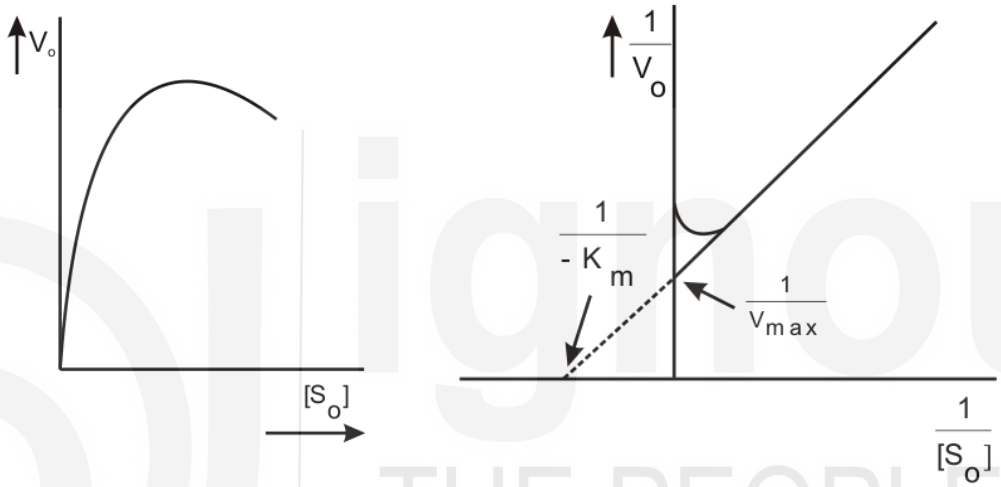


Fig. 6.4 : Lineweaver-Burk plots showing the effect of mixed inhibition: (a) $K_1 > K_i$; (b) $K_1 < K_i$.

6.3.5 Substrate Inhibition

In enzyme catalyzed reactions, when the amount of enzyme is kept constant and substrate concentration is increased gradually the rate of reaction will increase and the velocity curve will reach a maximum. A further increase in

substrate concentration does not increase the rate of reaction and the velocity curve falls. This event occurs on account of substrate inhibition whenever a dead-end-enzyme substrate complex is formed thereby inhibiting its own conversion to the product. Several enzymes are inhibited by their own substrates. For example, enzyme succinate dehydrogenase catalyzes dehydrogenation of substrate succinate. If you will look at the structure of succinate, you will see two carboxyl groups at the two ends of the molecule. The enzyme reaction takes place when both the carboxyl groups of the substrate bind to the enzyme. Excess concentration of succinate will lead to an increased possibility of carboxyl groups from two different molecules of succinate binding to the same enzyme, thereby stopping the enzyme reaction due to the formation of a dead-end complex. The graphs of characteristic substrate inhibition are shown in the Fig. 6.5.



(a) (b)

Fig. 6.5: Graphs on Substrate inhibition.

a) Michaelis-Menten b) Lineweaver-Burk plots; presenting the effects of substrate inhibition

The biological significance of substrate inhibition of phosphofructokinase ensures resources are not dedicated to the formation of ATP when it is in plenty. Another enzyme DNA metyltransferases catalyze transfer of methyl group to DNA. Their substrate inhibition leads to faithfully copy DNA methylation patterns when cells divide while preventing de novo methylation of methyl-free promoter regions.

Regarding the enzyme kinetics, if you recall from the uncompetitive inhibition

$$v_o = \frac{\frac{V_{max}[S_o]}{(1+\frac{I_o}{K_i})}}{\frac{Km}{(1+\frac{I_o}{K_i})}+[S_o]}$$

If the inhibitor is identical to the substrate, the equation will become:

$$v_0 = \frac{\dfrac{V_{max}}{\left(1+\dfrac{[S_o]}{K_i}\right)}\ [S_o]}{[S_o] + \dfrac{K_m}{\left(1+\dfrac{[S_o]}{K_i}\right)}} = \frac{V_{max}[S_o]}{[S_o]\left(1+\dfrac{[S_o]}{K_i}\right)+K_m}$$

When the substrate concentration is low [*S*] the term [*S*]/K_i becomes negligible and the equation will be the normal Michaelis-Menten equation. However, when the substrate concentration is high, then

$$[S_o]\left(1+\frac{[S_o]}{K_i}\right) + K_m \simeq [S_o]\left(1+\frac{[S_o\ ]}{K_i}\right)$$

The rate of equation will be

$$v_0 = \frac{V_{max}}{1+\dfrac{[S_o]}{K_i}} \qquad \text{……..equation 15}$$

As shown in the above equation, v_o will decrease on an increase of substrate concentration due to substrate inhibition.

SAQ 4

a) Mixed inhibition is a mixture of competitive inhibition and uncompetitive inhibition (true/false).

b) If the inhibitor has equal affinity for both the states of enzyme (free as well as bound to the substrate), mixed inhibition will become non-competitive inhibition (true/false).

c) The substrate inhibition of phosphofructokinase ensures resources are not dedicated to the formation of ATP when it is in less/excess (pick one).

d) Succinate has one/two carboxyl groups (pick one).

6.4 IRREVERSIBLE ENZYME INHIBITION

Irreversible inhibition is different from temporary enzyme inactivation by the reversible inhibitor. The enzyme activity is lost on the binding of inhibitor molecule to enzyme and its activity cannot be recovered afterwards. These inhibitory molecules are highly specific and can modify enzyme 3D structure. The enzyme gets inactive or there is time dependent loss of enzyme concentration. Inhibition cannot be removed by dilution or dialysis without losing enzyme activity. They generally form or break covalent bonds with the amino acid residues essential for substrate binding, catalysis or maintenance of enzyme conformation. Irreversible inhibition is progressive and generally

keeps on increasing with time till the all the inhibitor and enzyme present form the enzyme-inhibitor complex.

Examples: Heavy metal ions such as mercury, lead, aldehydes and haloalkanes. Alkylating agents such as iodoacetate and iodoacetamide forms covalent linkages with *–SH* groups of the enzyme.

$$E\text{-}SH + ICH_2.CO^-_2 \text{ ----------} \rightarrow E\text{-}S\text{-}CH_2.CO^-_2 + HI$$

Irreversible inhibitors decrease the available enzyme concentration. Taking the inhibitor concentration as $[I_o]$, the effective enzyme concentration will decrease from $[E_o]$ to $[E_o] - [I_o]$. If the substrate is added after the enzyme inhibitor reaction gets completed then the value of K_m will be same for uninhibited reaction but V_{max} will decrease from V_{max} to V_{max}'.

In the absence of inhibitor, $V_{max} = k_{cat} [E_o]$

In the presence of irreversible inhibitor, $V'_{max} = k_{cat} ([E_o] - [I_o])$

$$\frac{V'_{max}}{V_{max}} = \frac{[Eo] - [Io]}{[Io]}$$

$$V'_{max} = V_{max}[Eo](1 - \frac{[Io]}{[Eo]})$$

The **mechanism-based inhibitors** are modified substrates that are also known as suicide inhibitors or suicide inactivators. They modify the active site of enzyme irreversibly. These suicide inactivators or inhibitors are generally inactive unless they bound to the enzyme at its active site. The inhibitor binds to the enzyme as substrate and is catalyzed in a similar manner. The mechanism of catalysis generates a chemically reactive intermediate that leads to the inactivation of enzyme by covalent modification. These inactivators are used to unravel the mechanism of enzyme reaction and substrate reactivity. This mechanism has proved to be a boon for *rational drug* designing by pharmaceutical companies to determine the molecules which could be synthesized by the chemists as pharmaceutical agents.

Several important drugs are well known examples of irreversible inhibitors. Widely used drugs such as penicillin act by covalently inhibiting the enzyme transpeptidase, thereby preventing the synthesis of bacterial cell walls and thus killing the bacteria. You must have heard of tablet aspirin. The drug aspirin inhibits the enzyme cyclooxygenase thereby reducing the synthesis of inflammatory signals. Enzyme monoamine oxidase deaminates neurotransmitters such as dopamine and serotonin, and lowers the levels of these hormones in the brain. A neurodegenerative disease such as Parkinson's disease is linked with low levels of dopamine, while depression is related with low levels of serotonin. Suicide inhibitor such as drug (-)deprenyl, is widely used to treat Parkinson disease and depression.

6.5 SUMMARY

Let us summarize:

- Enzyme inhibition can be either reversible or irreversible. The different modes of reversible enzyme inhibition can be distinguished by their effects on the kinetic behavior of enzymes: competitive, uncompetitive or non-competitive.
- Competitive inhibitors compete with the substrates for the same binding site available on the enzyme. Therefore the graphs of these inhibitors showed that K_m is increased but V_{max} remain unchanged in the presence of an inhibitor.
- The uncompetitive inhibitor does not compete with the substrate. However, it binds at a site other than the substrate binding site on the enzyme-substrate complex. The graphs of these inhibitors showed that K_m and V_{max} is altered in the presence of an inhibitor but the slope remains unchanged.
- Non-competitive inhibitors also bind at a different site other than the substrate binding site on the enzyme and enzyme substrate complex. In the presence of such inhibitors, K_m remains unchanged, however V_{max} is decreased.
- Mixed inhibition is a mixture of competitive inhibition and uncompetitive inhibition.
- Substrate inhibition seems to be a type of uncompetitive inhibition, the extra substrate molecule acts as an inhibitor.
- Irreversible inhibitors can be used to map the active site of enzyme. They bind to the active site of enzyme and modify it covalently and hence cannot dissociate from the enzyme. They reduce the concentration of enzyme present.
- Mechanism based inhibitors or suicide inhibitors are processed by the enzyme in a catalytic mechanism resulting in the formation of a reactive compound that inactivates or inhibits the enzyme. These inhibitors are widely used for rational drug designing.

6.6 TERMINAL QUESTIONS

1. Discuss uncompetitive inhibition and derive the equation for a competitive inhibitor.
2. Distinguish between reversible and irreversible inhibition?
3. Explain the substrate inhibition.
4. What are the mechanism-based inhibitors? How they help in drug designing?

6.7 ANSWERS

Self-Assessment Questions

1. Refer to figure 6.1 b
2. Refer to figure 6.2 b
3. Refer to figure 6.3 b
4. a) True, b) True, c) Excess, d) Two

Terminal Questions

1. Refer to section 6.3.2
2. Refer to section 6.2
3. Refer to section 6.3.5
4. Refer to section 6.4

6.8 FURTHER READINGS

1. David L. Nelson and Michael M. Cox: Lehninger Principles of Biochemistry 6th Ed., W.H. Freeman.
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