Enzyme kinetics with two substrates

Introduction :

In the previous chapter, the analysis of enzyme-catalyzed reactions only considered reactions with one substrate and one product. However, most biochemical reactions involve two or more substrates and two or more products.

In fact, the kinetic study of two-substrate enzyme reactions aims to determine the order of substrate binding, the kinetic constants characterizing the binding of each substrate in the presence and absence of the other, and the maximum reaction rate.

1. Classification of reaction mechanisms :

There are two classifications : kinetic and chemical.

1.1. Kinetic classification :

Depending on the type of complex formed, two mechanisms can be distinguished :

1. 1. 1. Sequential mechanism :

When the enzymatic reaction only occurs after the formation of a ternary complex between the enzyme and the two substrates. The binding of the substrates can itself be :

- **Ordered :** one of the substrates must bind first. It is always the same substrate that binds first.
- **Random (Hazardous) :** either substrate binds first. Its presence may either not modify, facilitate, or disfavor the binding of the other.

1. 1. 2. Ping-pong mechanism (alternative) :

When the enzymatic reaction takes place after the formation of an active binary complex. This is a specific mechanism in which the binding of the first substrate is followed by its conversion into a reaction product, which also modifies the enzyme.

When the first product leaves the enzyme, it becomes ready to receive the second substrate, which it could not bind before. It binds it, transforms it, and the second reaction product leaves. Thus, it returns to its initial state.

1.2. Chemical classification :

This classification helps explain how the chemical reaction works. So, depending on how the enzyme combines with the substrates, we find :

1.2.1. Single displacement reactions :

They involve the formation of a ternary complex. The experimental conditions are :

- ✓ The reverse reaction is neglected,
- ✓ Both substrates A and B must be present simultaneously,
- ✓ The substrates can bind to the enzyme in different ways.

1.2.2. Double displacement reactions :

Some metabolic reactions involving two substrates occur without the reaction requiring the formation of a ternary complex. This is the case for many group transfer reactions that involve only the formation of binary complexes.

Thus, the enzyme is modified; the group is transferred twice, first from substrate A to the free enzyme E and then from the substituted enzyme to the second substrate B.

2. Experimental approach :

In most cases, an enzymatic reaction belongs to the type :

$$A + B \longrightarrow P + Q$$

To study the kinetics, we use artificial conditions. We measure the variations in reaction speed in the presence of very high concentrations of compound B when we vary the concentration of A, then we do the same with a high, non-limiting concentration of A. This brings us back to single-substrate kinetics. We define a Michaelis constant K_A and K_B for each substrate. In other words, the kinetic study is carried out in two stages :

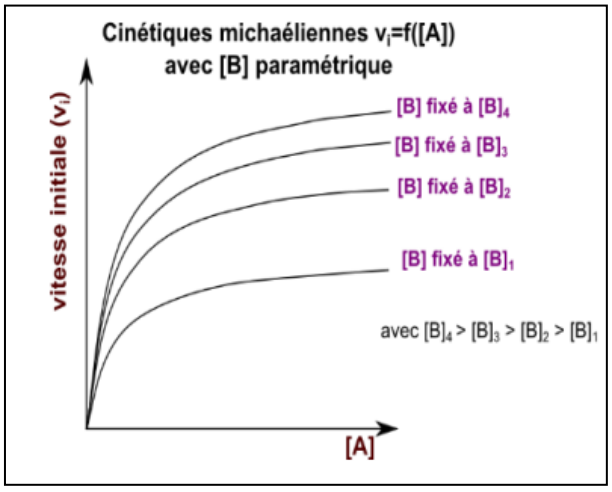
2.1. First step :

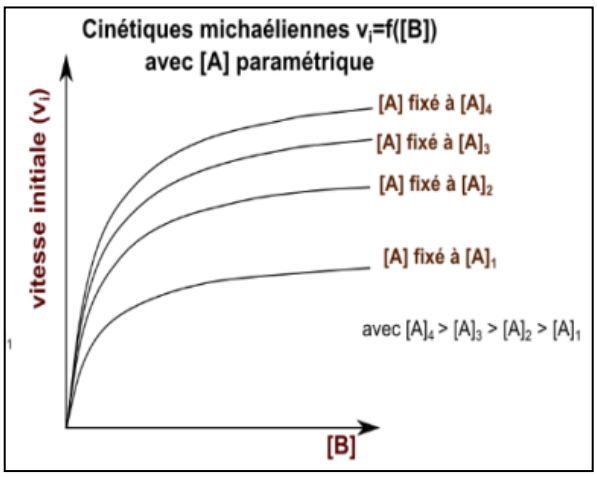
The experiment is carried out with the first substrate (A) in excess, called the fixed substrate, and the speed is measured by varying B (variable substrate) in a stationary phase.

2.2. Second step :

We take B in excess and vary A to measure the speeds in the stationary phase. The two (kinetic) steps are Michaelis-type, but the mathematical treatment is complex, which is why we use Cleland's notation (1963).

The result : two Michaelis-type reactions, but the mathematical treatment is more complicated.



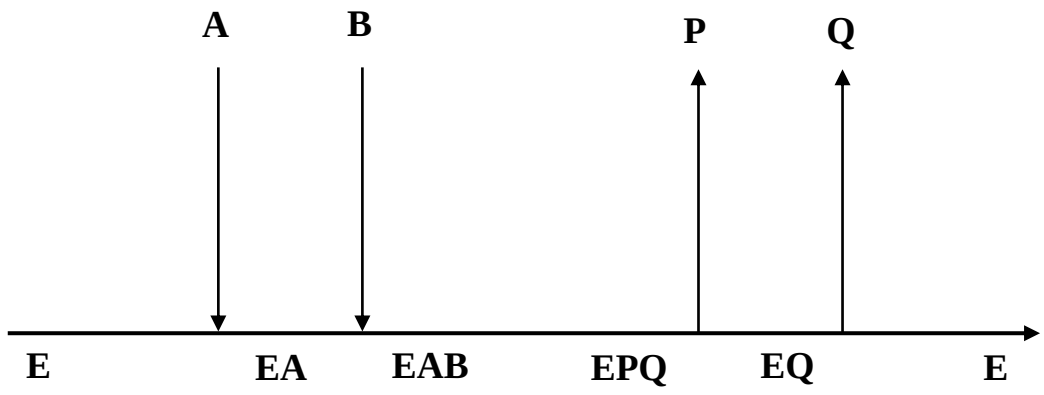


3. CLELAND diagram (1963) :

To better understand reaction mechanisms, **Cleland** diagrams are used to clarify the events that occur when there are multiple substrates.

This diagram consists of a horizontal line representing the enzyme surface and its evolution during the reaction cycle, arrows pointing to this line indicating the substrates in their order of attachment, and arrows pointing away from the line indicating the order in which the reaction products are released.

Example :



This example represents an ordered sequential mechanism.

N.B.

If we consider that enzymes behave in a Michaelis-Menten manner and that the catalytic reaction occurs in a single kinetic step after the formation of the ternary complex EAB to give E+P+Q :

K_A is the equilibrium dissociation constant : EAB $\longleftrightarrow$ EB + A

K_B is the equilibrium dissociation constant : EAB $\longleftrightarrow$ EA + B

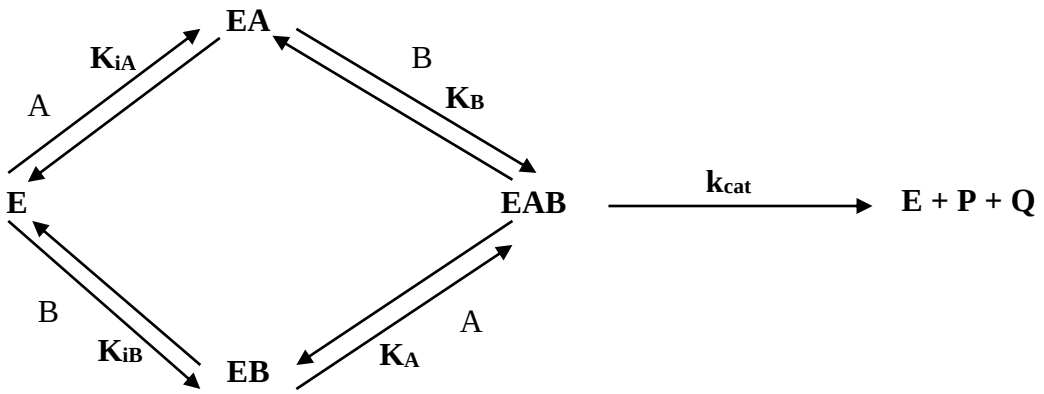
Studying the variation in initial speed as a function of the concentration of each substrate allows us to determine the kinetic constants of the reaction and the order of substrate binding.

The representations of (1/v) as a function of the inverse of the concentration of one of the substrates (the concentration of the other substrate being kept constant) are linear when the enzyme is Michaelis-Menten; these are called ***primary representations***.

STUDY OF REACTION MECHANISMS

1. Sequential mechanisms :

The general diagram of a sequential mechanism and the equilibrium constants that account for kinetics are written as follows :



With : $K_{iA} = [E]\,[A] / [EA]$ and $K_{iB} = [E]\,[B] / [EB]$

$K_A = [EB]\,[A] / [EAB]$ and $K_B = [EA]\,[B] / [EAB]$

These equilibrium constants are called K_i, because each substrate behaves as a competitive inhibitor in the absence of the other.

1.1. Mechanism of random binding (aleatory) :

In this case, one or the other substrate binds first. The enzyme has two binding sites, one for each substrate, and the reaction can only take place when both substrates are present.

Two cases must be considered :

- The associations of A and B with the enzyme are dependent ; that is, the binding of A modifies the affinity of the enzyme for B and vice versa.
- The associations are independent : the association of one of the substrates occurs in the same way in the absence of the second.

1.1.1. Dependent association :

This is the most common case. The binding of A modifies the affinity of the enzyme for B and vice versa. Four equilibrium constants are defined K_{iA}, K_A, K_{iB} et K_B.

❖ ***Kinetics :***

At equilibrium : [A] et [B] >> [E_t] and $K_{iA}.K_B = K_{iB}.K_A$

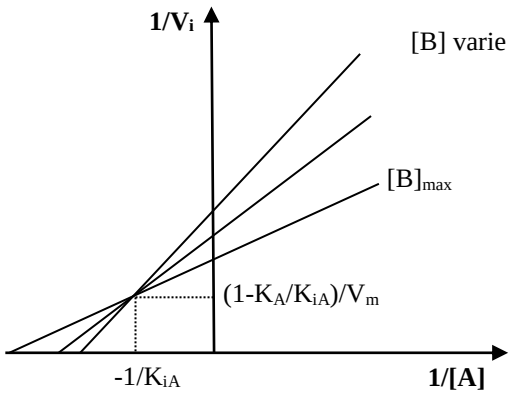
The speed equation is of the form : $\mathbf{v = v_m / 1 + K_A/[A] + K_B/[B] + K_{iA}.K_B/[A]\,[B]}$

❖ ***Graphical representation :***

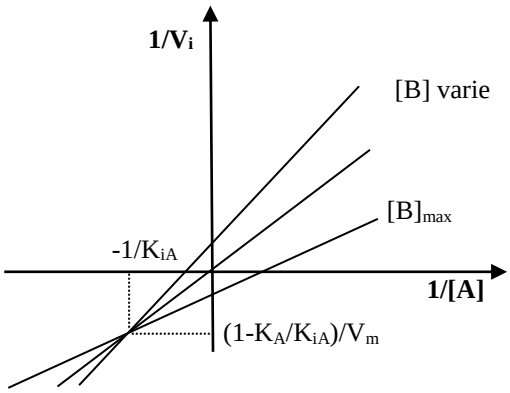
1st case: [B] = constant and [A] variable :

Primary representation :

- **Positive dependence** ($K_{iA} > K_A$):
  The binding of B is facilitated by the presence of A.



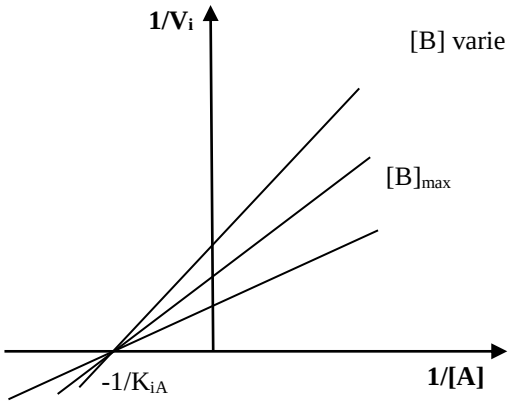
- **Negative dependence** ($K_{iA} < K_A$) :
  The binding of B is hindered by the presence of A.



1.1.2. Independent association :

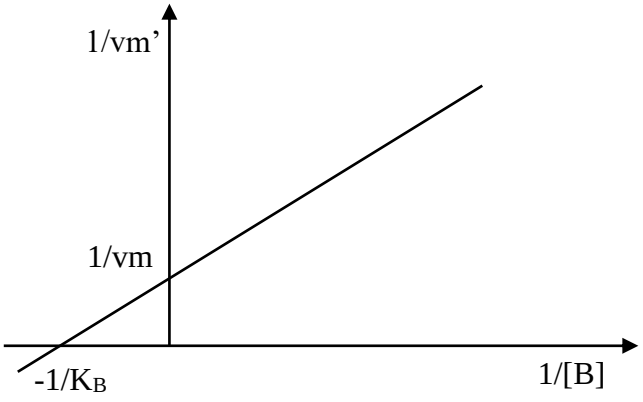
If the binding sites of the two substrates are sufficiently distinct and there are no interactions between them, the binding of one will not affect the association of the other. Therefore, there are two equilibrium constants because : ($K_{iA}=K_A$ and $K_{iB}=K_B$) and the rate equation becomes :

$$v = v_m / 1 + K_A/[A] + K_B/[B] + K_A.K_B/[A]\,[B]$$



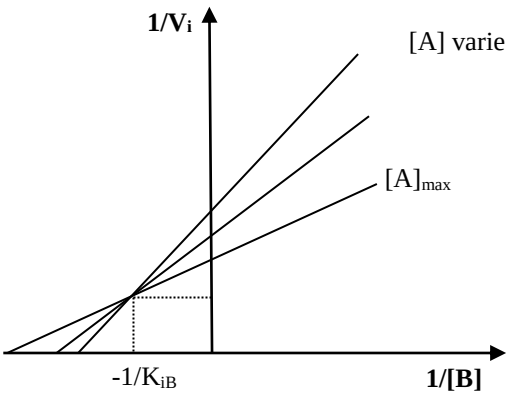
Secondary representation :

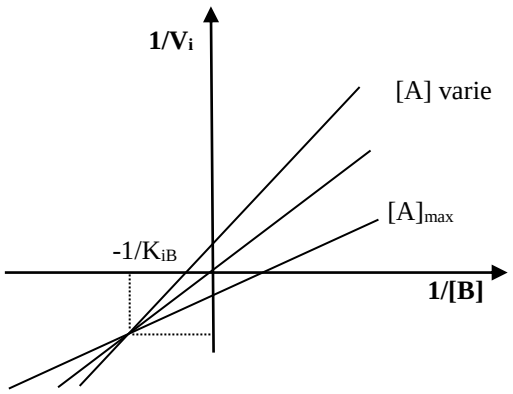
It consists of graphically plotting 1/vm'=f(1/[B]), where vi' is the maximum speed determined from the primary representation 1/vi = f(1/[A]).

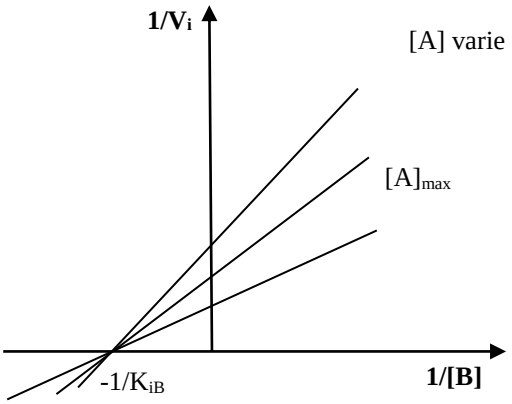


2nd case : [A] = constant and [B] variable :

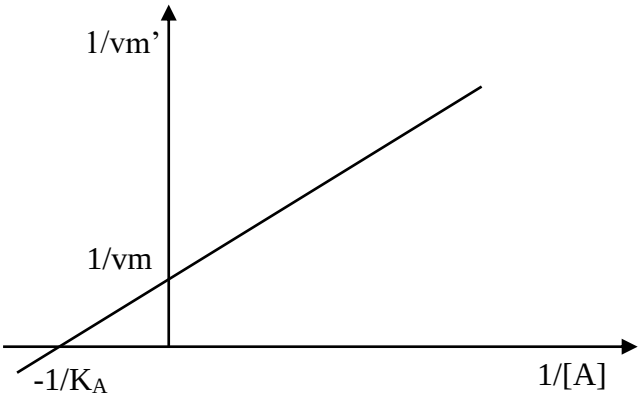
Primary representation :







Secondary representation :



Example :

Hexokinase (EC 2.7.4.3) catalyzes the following reaction :

D-glucose + ATP-Mg^{++} ⟶ D-glucose-6 phosphate + ADP-Mg^{++}

1. 2. Mechanism for an ordered bi-bi binding (obligatory) :

❖ ***Kinetics :***

The general reaction diagram is as follows :

$$E + A \xleftrightarrow{K_{iA}} EA + B \xleftrightarrow{K_B} EAB \xrightarrow{k_{cat}} E + P + Q$$

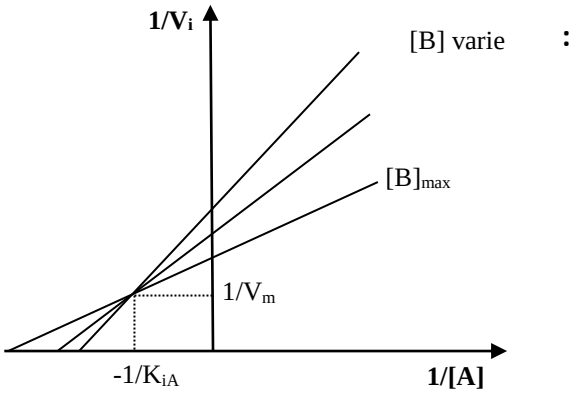
- ✓ If A is the first substrate bound, the EB complex does not exist ;
- ✓ B has no affinity for the free enzyme (B can only bind to the EA complex) ;
- ✓ A cannot dissociate from the EAB complex (K_A=0).
- ✓ The release of the products themselves is ordered ; the second product corresponds to the first substrate ;
- ✓ The rate equation is simplified :

$$v = v_m / 1 + K_B/[B] + K_{iA}.K_B/[A][B]$$

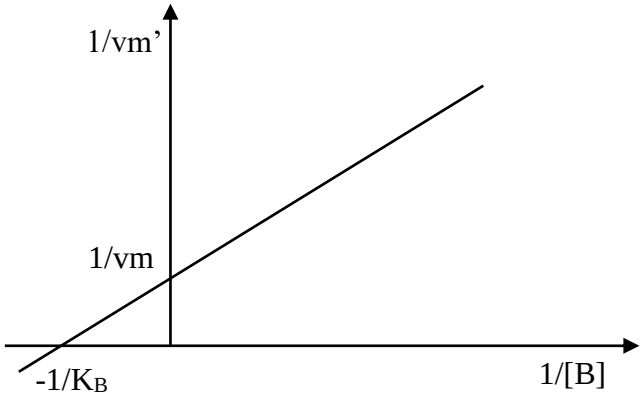
❖ ***Graphical representation :***

<u>1st case</u> : [B] = constant and [A] variable

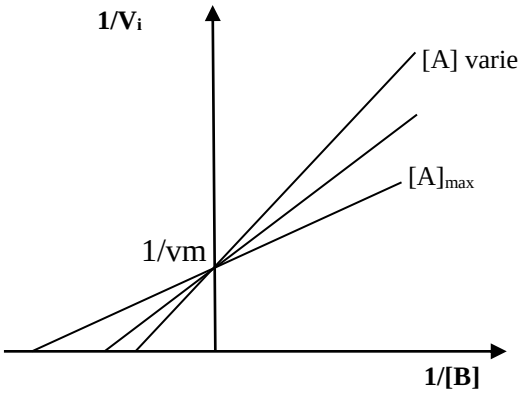
<u>Primary representation :</u>



<u>Secondary representation :</u>



2nd case : [A] = constant and [B] variable



Primary representation :

This representation allows the ordered sequential mechanism to be identified.

In this case, there is no secondary representation.

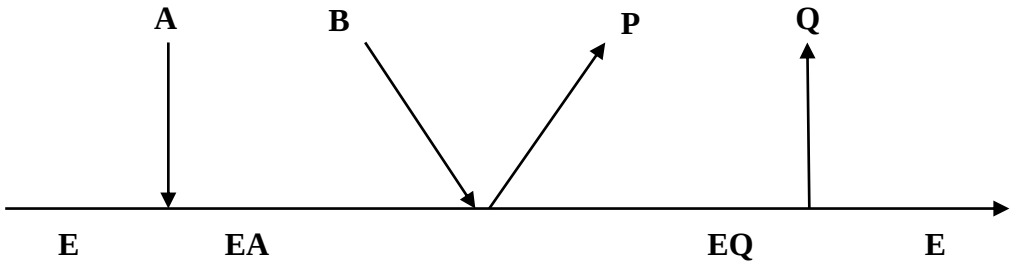
Example :

Lactate deshydrogenase LDH (EC 1.1.1.27) catalyzes the following reaction :

NAD^+ (A) + lactate (B) ⟶ pyruvate (P) + $NADH,H^+$ (Q)

Theorell-Chance mechanism :

- ✓ This is a special case of the ordered mechanism.
- ✓ It is described for some dehydrogenases (alcohol dehydrogenase, lactate dehydrogenase).

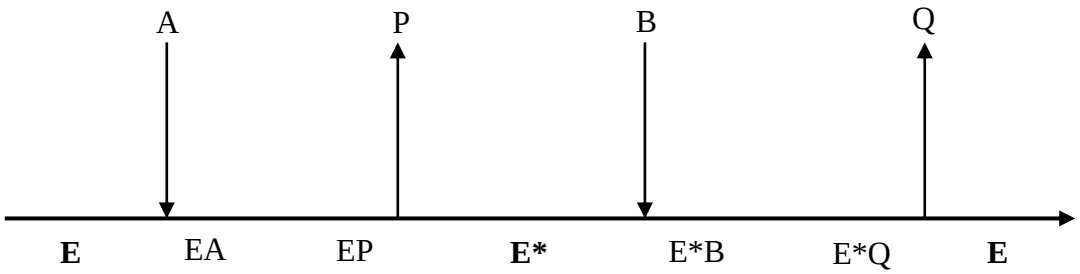


❖ ***Characteristics :***

- ✓ The second substrate establishes very brief contact with the first EA complex and quickly produces the first product.
- ✓ The dissociation constants of the second substrate and the first product are very high compared to those of the first substrate and the second product.
- ✓ The lifetime of the EAB complex is very short compared to that of EA and EQ (ephemeral complex).
- ✓ The relationship between the speeds with A and B and that of the ordered bi-bi mechanism :

$$v = v_m / 1 + K_B/[B] + K_{iA}.K_B/[A][B]$$

2. 2. Non-sequential mechanism (alternative) : ping-pong :



❖ ***Kinetics :***

- ✓ The reaction does not require the formation of a ternary complex.
- ✓ After binding to the first substrate, the enzyme transforms it and releases the corresponding product.
- ✓ The enzyme then binds to the other substrate, transforms it, and releases the second product.
- ✓ In this mechanism, between the binding of the two substrates, there is an irreversible step in the absence of reaction products :

$$E + A \xleftrightarrow{K_A} EA \xrightarrow{k_{cat}} E' + C + B \xleftrightarrow{K_B} E'B \xrightarrow{k_{cat}} E + D$$

The first reaction modifies the enzyme so that the second reaction with the second substrate can take place.

The speed is given by the following equation :

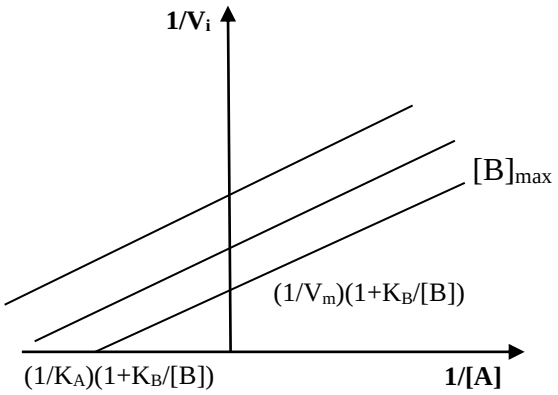
$$\mathbf{v = v_m / 1 + K_A/[A] + K_B/[B]}$$

❖ ***Graphical representation :***

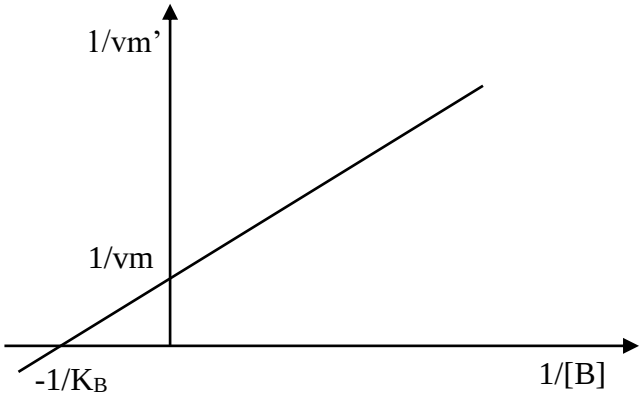
<u>1st case</u> : [B] = constant and [A] variable

<u>Primary representation :</u>

This representation allows the ping-pong mechanism to be identified.

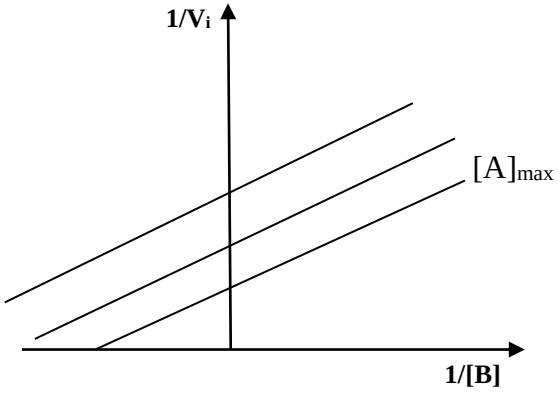


Secondary representation **:**

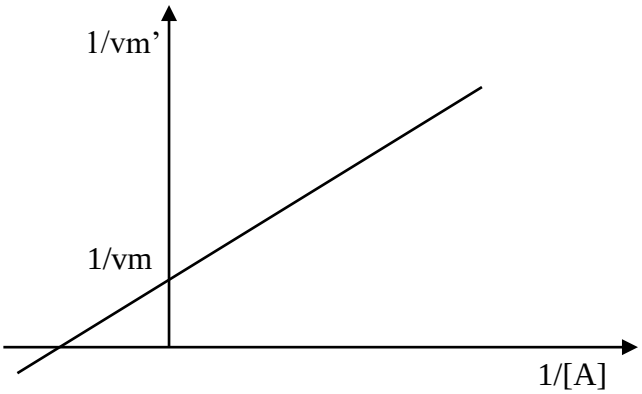


2nd case : [A] = constant and [B] variable

Primary representation :



Secondary representation :



Example :

Glucose oxydase (EC 1.1.3.4) catalyzes the following reaction :

D-glucose (A) + O_2 (B) ⟶ D-glucocno-1,5-lactone (P) + H_2O_2 (Q)