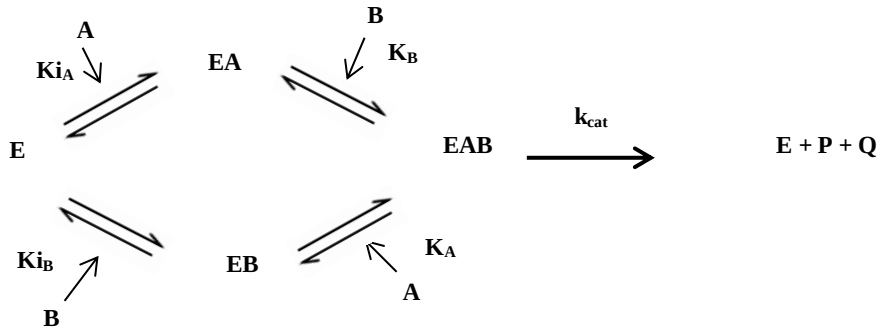
Chapter 2: Two-Substrate Enzyme Kinetics

Introduction

Most enzymatic reactions involve **two substrates**, and sometimes more, leading to the formation of several products. The kinetic study of two-substrate enzymatic reactions aims to determine the **binding order of substrates**, the **equilibrium constants characterizing the binding of each substrate** in the presence and absence of the other, as well as the **maximum reaction velocity** when the concentrations of both substrates are saturating.

In this chapter, only Michaelian-type two-substrate enzymes will be studied.



EA et **EB** are **binary enzyme–substrate complexes**, while **EAB** is the **ternary complex**.

The equilibrium constants are defined as follows:

$\mathbf{KiA} = \frac{[E][A]}{[EA]}$ Ki_A : The dissociation constant of **A** with respect to **the free enzyme**

$\mathbf{KiB} = \frac{[E][B]}{[EB]}$ Ki_B : The dissociation constant of **B** with respect to **the free enzyme**

$\mathbf{KA} = \frac{[EB][A]}{[EAB]}$ K_A : The dissociation constant of **A** with respect to **the enzyme complexed with B**.

$\mathbf{KB} = \frac{[EA][B]}{[EAB]}$ K_B : The dissociation constant of **B** with respect to **the enzyme complexed with A**.

These constants describe the **binding affinities** of substrates A and B, either to the free enzyme or to the enzyme already bound to the other substrate.

1. Non-ordered or Random binding

Both substrates, A and B, bind **randomly** to the active site of the enzyme to form a **ternary complex (EAB)**. The reaction involves four equilibrium constants: Ki_A, Ki_B, K_A and K_B.

For any given concentration of A or B, the reaction rate (v) as a function of [A] or [B] follows **Michaelis-Menten kinetics law**. The true maximum velocity ($\mathbf{V_{max}}$) is only reached when the enzyme is **saturated with both A and B**.

1.1. Dependent non-ordered binding

In a **dependent** random mechanism, although the binding is random, the **binding of one substrate affects the affinity of the enzyme for the other substrate**.

This means:

- The fixation of A modifies the binding of B
- Or the fixation of B modifies the binding of A

As a result, the constants K_A and K_B are **not independent** of Ki_A and Ki_B. This reflects **interactions between substrates** during binding to the enzyme.

$$\frac{1}{v} = \frac{1}{Vmax}\left(1 + \frac{KA}{[A]} + \frac{KB}{[B]} + \frac{KiA.KiB}{[A][B]}\right)$$

The graphical representation of **1/v as a function of 1/[A],** while keeping **[B] constant**, results in the following curve:

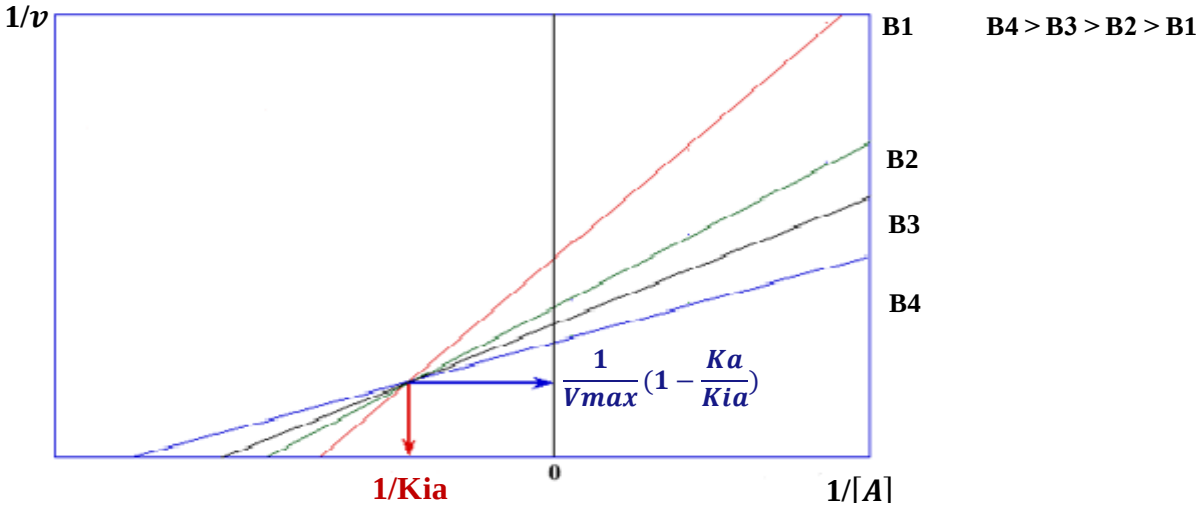


Figure 1. Primary plot of 1/v = f(1/[A]) for different concentrations of B.

Similarly, the **primary representation** of 1/v = f(1/[B]) can be drawn while keeping [A] **constant**.

The **primary plots** allow us to determine the values of the apparent $1/V_{max}$ for one substrate at a specific concentration of the other. These values are then plotted in a **secondary graph** for substrate B (or substrate A).

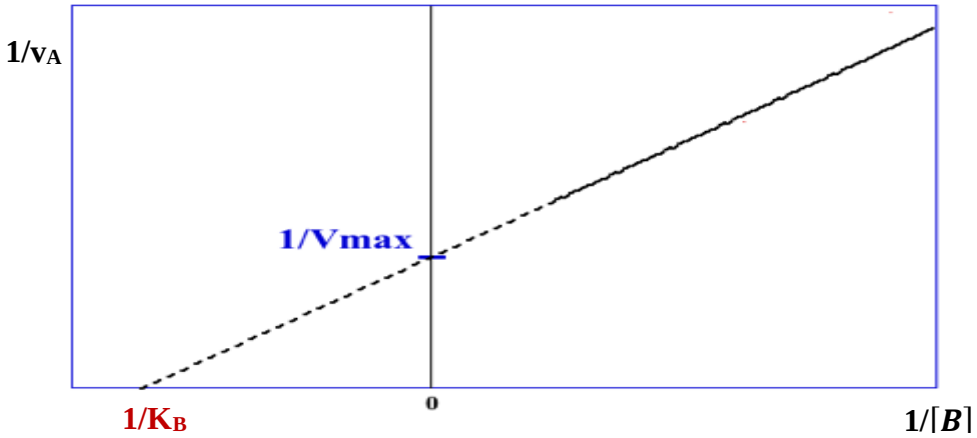


Figure 2. Secondary plot $1/v_A = f\ 1/[B]$

In this mechanism, the binding of the two substrates is **dependent**, meaning that the association of substrate **A** with the enzyme depends on the presence of **B**, and vice versa.

When analyzing this using a **primary plot** (where the intercepts or slopes of a primary plot are plotted against the reciprocal of the second substrate concentration), two cases arise based on the **y- intercept of the intersection point**:

a) Positive y- coordinate of the intersection point

This indicates **positive dependent binding** (Positive cooperativity). In this case, the binding of the first substrate **facilitates** or favors the binding of the second substrate. This indicates a **favorable interaction** between substrates.

b) Negative y-coordinate of the intersection point

This indicates **negative dependent binding** (Negative cooperativity). Here, the binding of the first substrate **reduces** or disfavors the binding of the second substrate. This reflects an **unfavorable interaction** between substrates.

1.2. Random independent binding

In the case of **independent binding**, the two substrates bind **independently**, meaning that each substrate can bind to the enzyme **with the same affinity**, whether the enzyme is **free** or already **complexed** with the other substrate.

In other words:

- Substrate A binds equally well to **E** or **EB**
- Substrate B binds equally well to **E** or **EA**
- There is **no interaction (no influence)** between the two substrates during binding

Therefore:

- **$Ki_A = K_A$**: The inhibition constant (dissociation constant) for substrate A is equal to its Michaelis constant.
- **$Ki_B = K_B$**: Similarly, the dissociation constant for substrate B is equal to its Michaelis constant.

$$\frac{1}{v} = \frac{1}{Vmax}\left(1 + \frac{KA}{[A]} + \frac{KB}{[B]} + \frac{KA.KB}{[A][B]}\right)$$

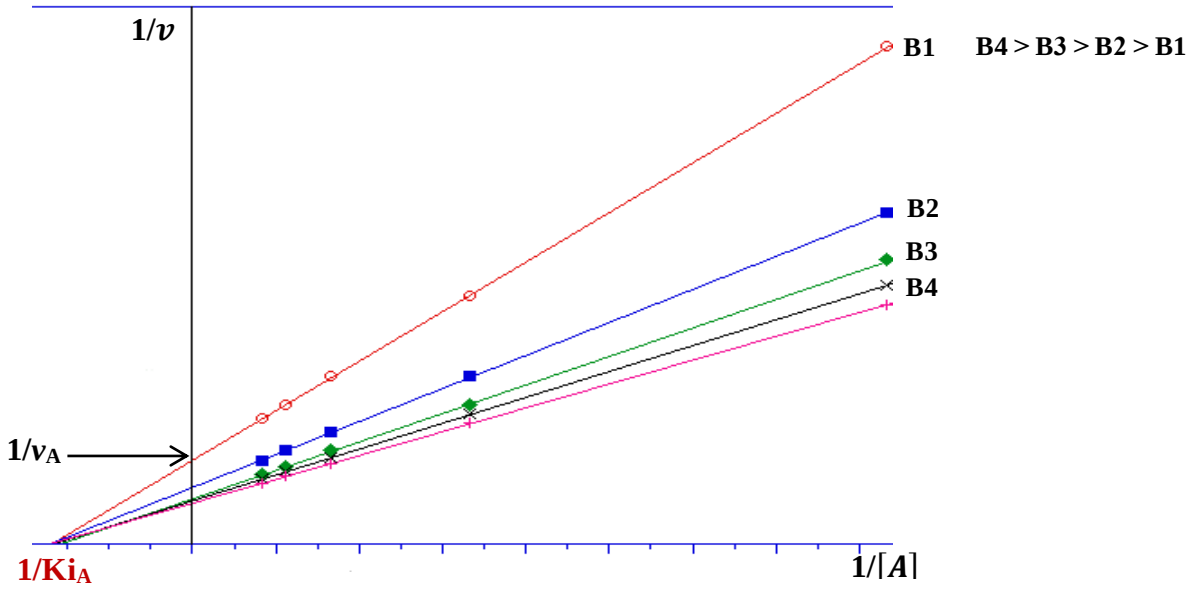


Figure 3. Primary plot $1/v = f\ 1/[A]$ for different concentrations of B

The **y-intercepts** ($1/v_A$) from the primary plot (where 1/v is plotted against 1/[A] at various fixed concentrations of B are used to construct the **secondary plot**.

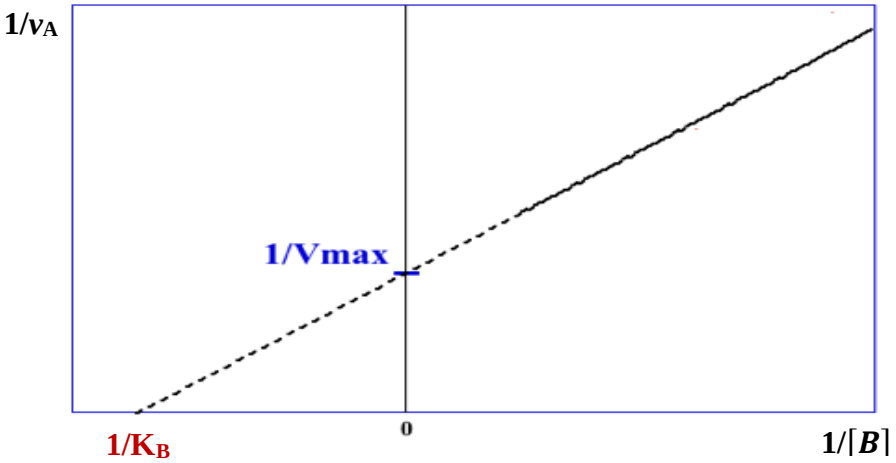


Figure 4. Secondary plot $1/v_A = f\ 1/[B]$

2. Ordered binding (Sequential mechanism)

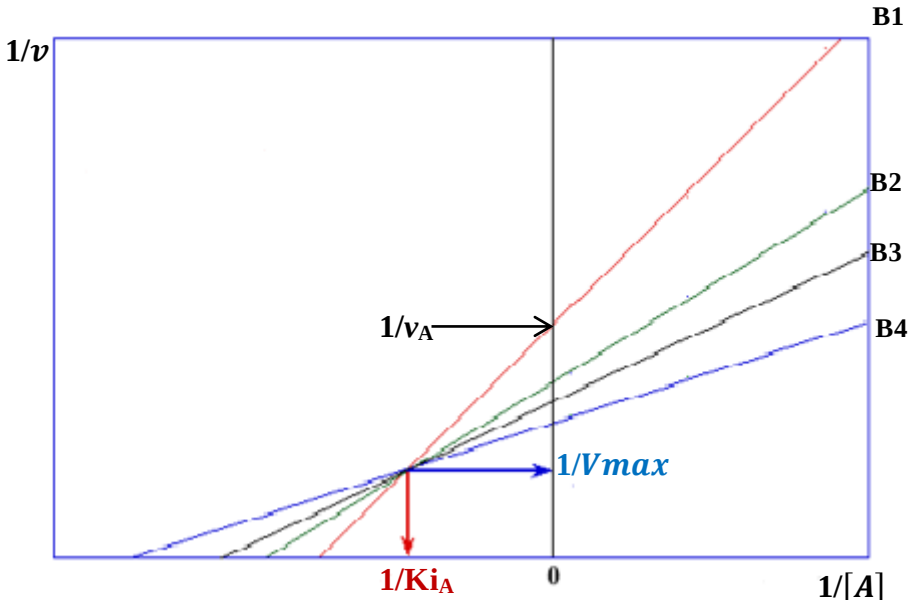
In this type of binding, **one of the two substrates must bind first** to the enzyme **before the other can** join. This follows a specific, mandatory sequence as shown in the following kinetic scheme:

It is assumed that **substrate A binds first**:

And the following equation is obtained:

$$\frac{1}{v} = \frac{Vmax}{1 + \frac{KB}{[B]} + \frac{KiA.KB}{[A][B]}}$$

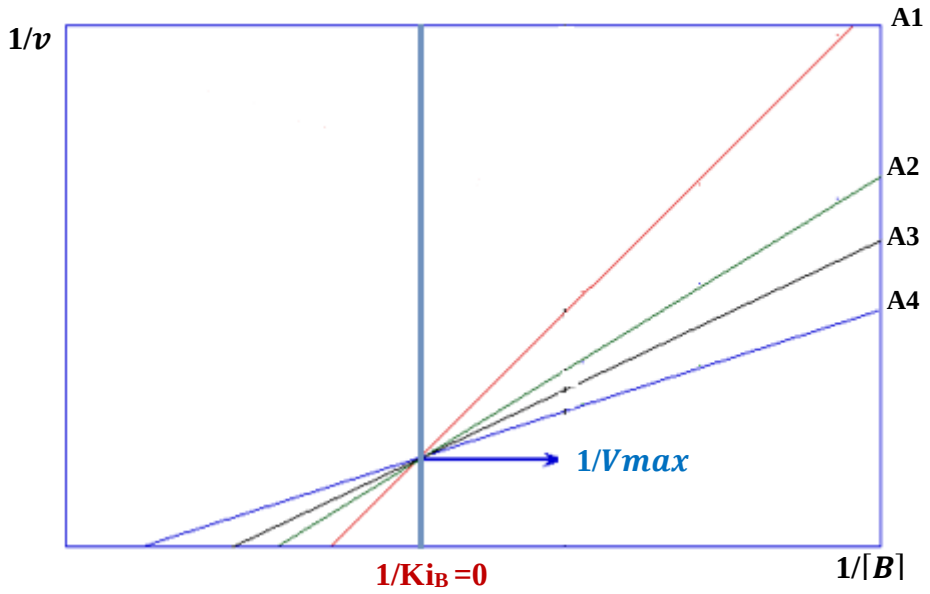
The graphical representation of **1/v as a function of 1/[A]**, while keeping the concentration of the second substrate **[B] constant**, gives the following curve:



B4 > B3 > B2 > B1

Figure 5. Primary plot $1/v = f\ 1/[A]$ for different concentrations of B

The graphical representation of **1/v as a function of 1/[B]**, while keeping the concentration of the second substrate **[A] constant**, gives the following curve:



A4 > A3 > A2 > A1

Figure 6. Primary plot $1/v = f\ 1/[B]$ for different concentrations of $[A]$

By using the data from the curve shown in **Figure 5**, the **secondary plot** is constructed to determine the remaining (missing) kinetic constants.

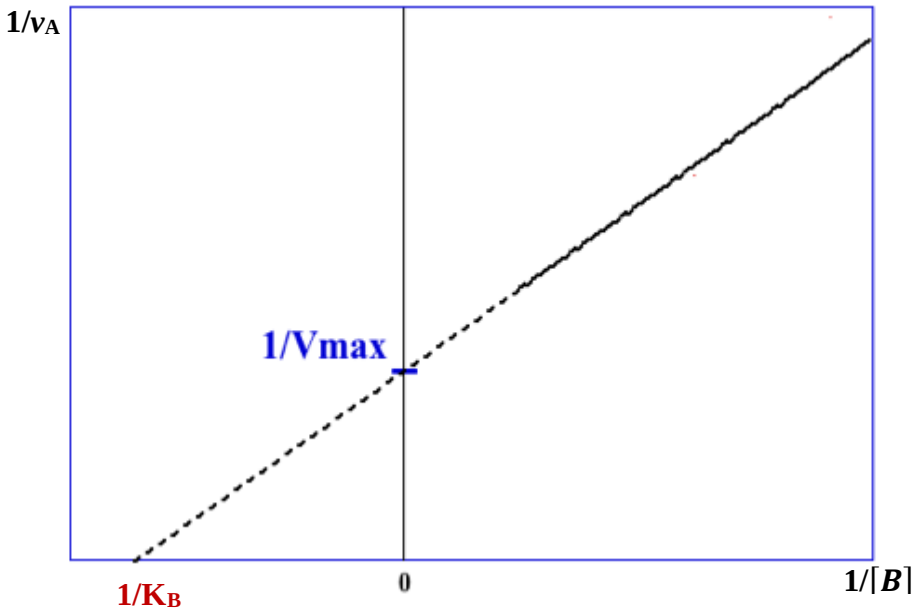


Figure 7: Secondary Plot of $1/v_A = f\ 1/[B]$

3. Ping-Pong binding

In this type of reaction, one substrate must bind first, then it is converted into a product and released **before** the second substrate can bind. After the second reaction step, the second product is released and the enzyme is regenerated (with the original form).

It is considered an **ordered mechanism**, but **without formation of a ternary complex (EAB)**. Instead, the enzyme exists in a modified intermediate state (**E***) between the two substrates.

Assuming that substrate **A binds first**, the mechanism can be written as:

$$E + A \underset{}{\overset{K_{MA}}{\rightleftharpoons}} EA \longrightarrow E^* + P$$

$$E^* + B \underset{}{\overset{K_{MB}}{\rightleftharpoons}} E^*B \longrightarrow E + Q$$

N.B.

(**E***) represents the **modified enzyme intermediate**. This is the state of the enzyme after it has undergone a structural or chemical change (such as phosphorylation or acetylation) following the binding of the first substrate and the release of the first product.

If we assume that all reversible reactions are negligible, the equation of this reaction mechanism is as follows:

$$\frac{1}{v} = \frac{Vmax}{1 + \frac{KA}{[A]} + \frac{KB}{[B]}}$$

The graphical representation of $1/v$ as a function of 1/[A], while keeping [B] constant, gives the following curve.

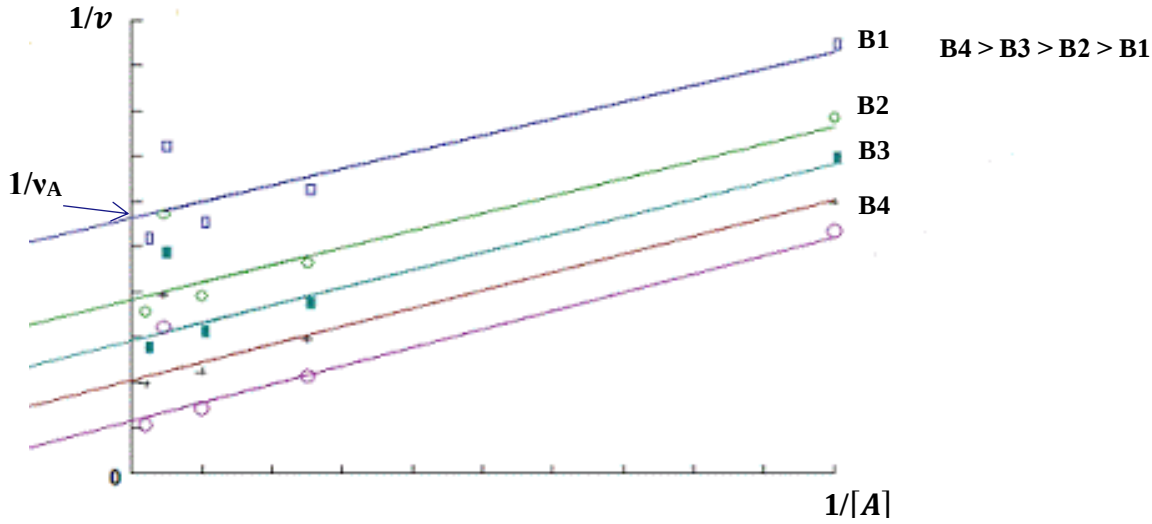


Figure 8. Primary plot $1/v = f\ 1/[A]$ for different concentrations of B

The most defining feature of a Ping-Pong mechanism on a primary plot ($1/v = f\ 1/[A]$) is that the lines at different fixed concentrations of B are **parallel lines**, since no ternary complex is formed, the lines never intersect. The **Intercepts points are Changing,** because the concentration of substrate B increases, the y-intercept decreases (meaning **apparent V_{max}** increases), but the slope (**K_M/V_{max}**) remains constant.

The secondary representation $1/v_A = f\ 1/[B]$ allows the determination of **V_{max}** and **K_B**.

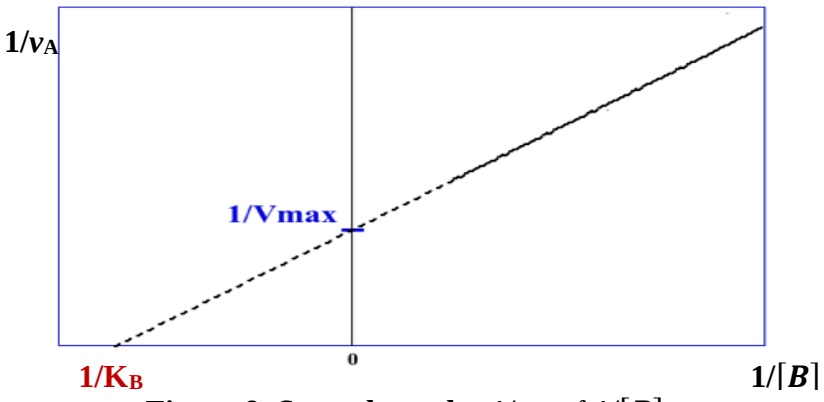
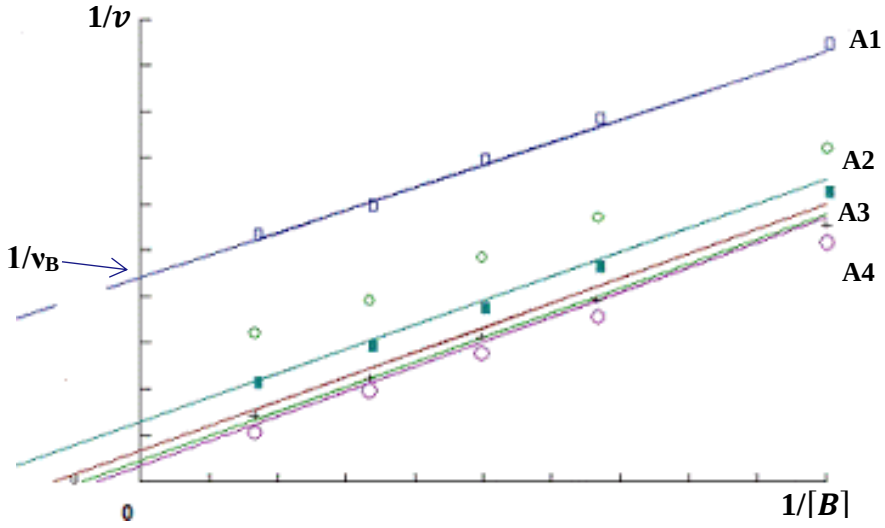


Figure 9. Secondary plot $1/v_A = f\ 1/[B]$

The graphical representation of $1/v$ as a function of 1/[B], while keeping [A] constant, gives the following curve.



A4 > A3 > A2 > A1

Figure 10. Primary plot $1/v = f\ 1/[A]$ for different concentrations of A

When plotting $1/v_B = f\ (1/A)$, this corresponds to the **secondary representation**, from which $\mathbf{V_{max}}$ and $\mathbf{K_A}$ can be determined.

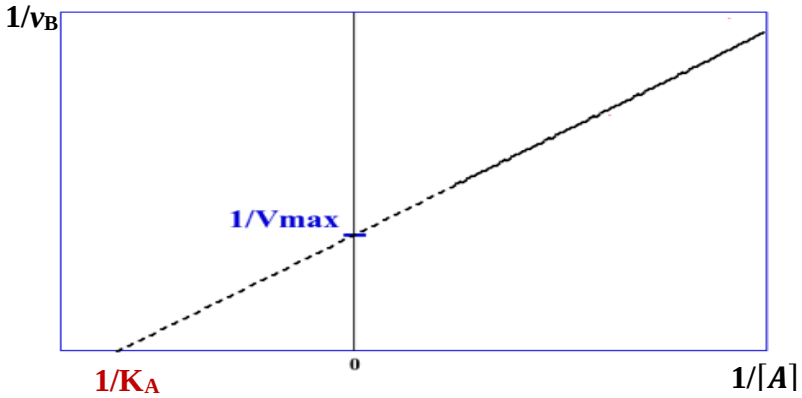


Figure 11. Secondary plot $1/v_B = f\ 1/[A]$.