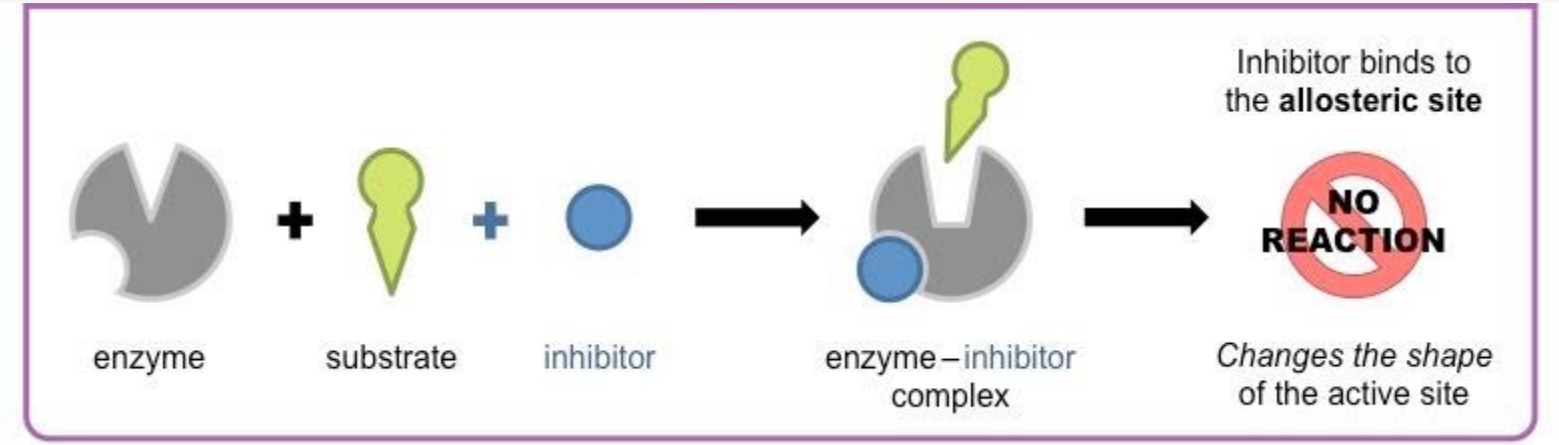
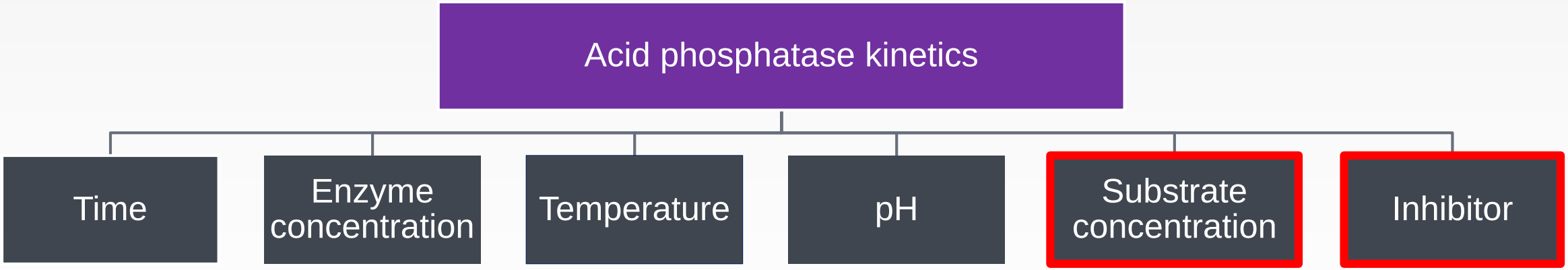
The effect of *substrate concentration* and *inhibitors* on the rate of an enzyme catalyzed reaction



- **In this experiment, we will continue to study acid phosphatase kinetics.**

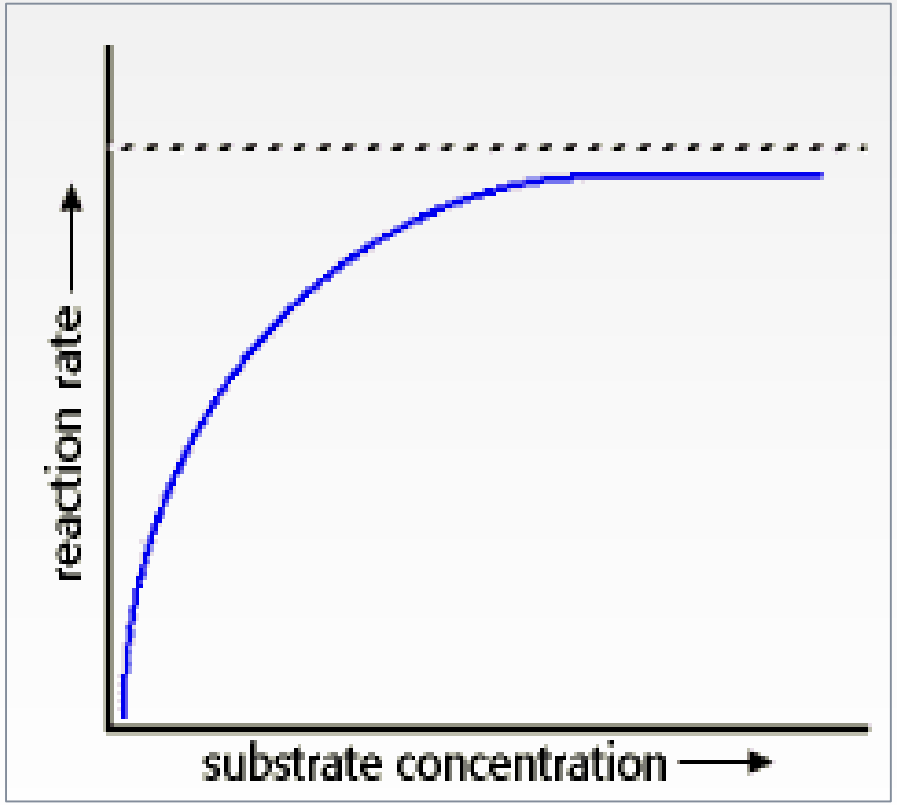


Objectives

- To establish the relationship between substrate concentration and the rate of an enzyme catalyzed reaction.
- To determine the Km and Vmax of the enzyme for a particular substrate.
- To study the effect of inhibitors on the rate of an enzymatic reaction.
- To determine the type of inhibition of acid phosphatase by inorganic phosphate and sodium fluoride.

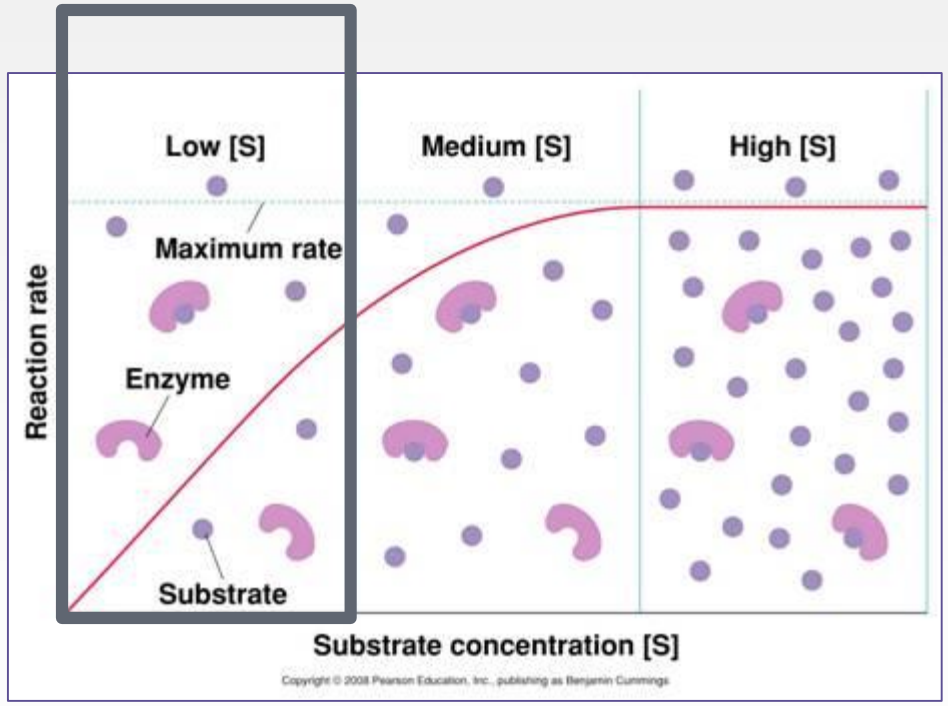
The effect of substrate concentration

- During enzyme substrate reaction, the initial velocity V_0 gradually increases with increasing concentration of the substrate. Finally a point is reached, beyond which the increase in V_0 will not depend on the [S].

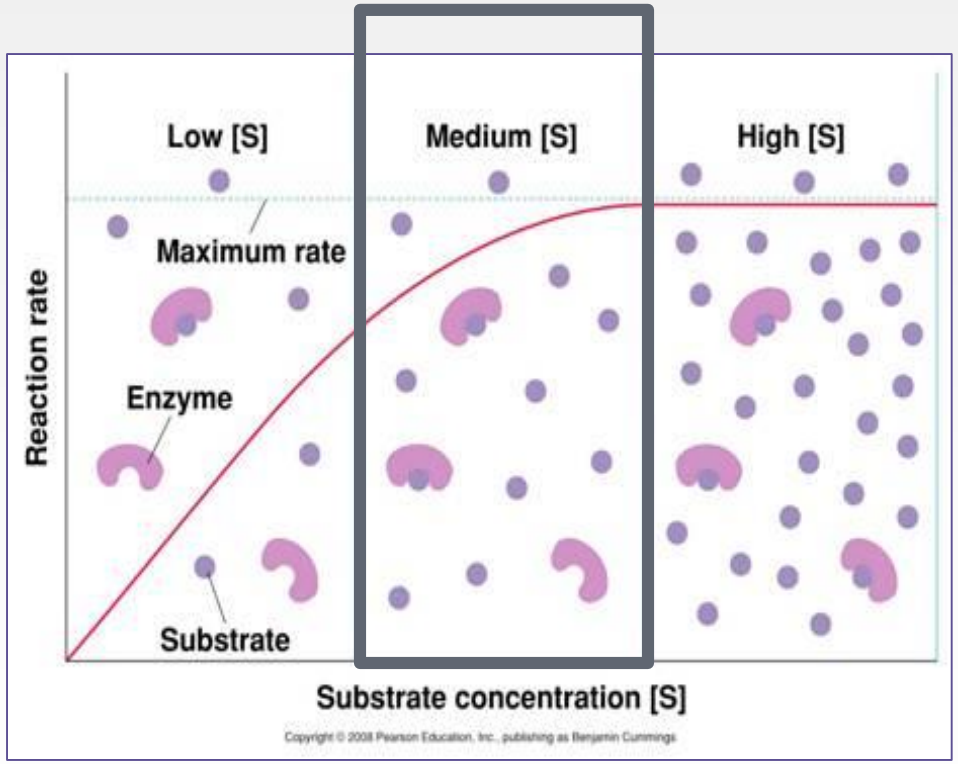


The effect of substrate concentration

- **At relatively low concentration of substrate**, the rate of reaction increase **linearly** with an increase in substrate concentration.
- The catalytic site of the enzyme is empty, waiting for substrate to bind, for much of the time, and the rate at which product can be formed is limited by the concentration of substrate which is available.



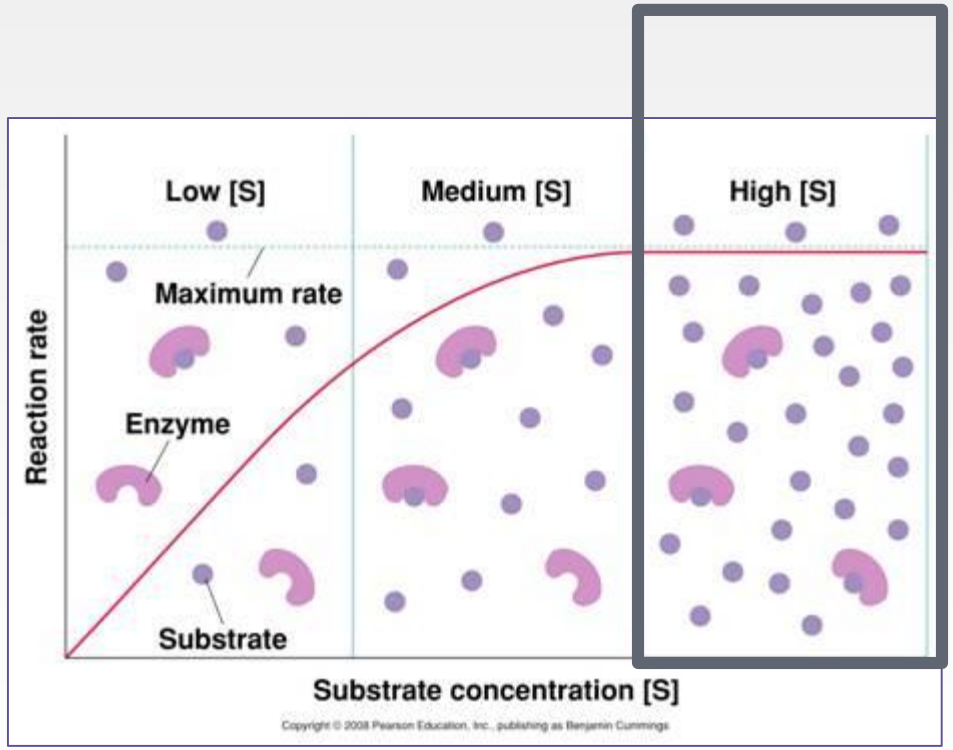
- **At higher substrate concentration**, the rate of reaction increases by smaller and smaller amounts in response to increase in substrate concentration.



- However **beyond a particular substrate concentration**, the velocity remains <u>constant</u> without any further increase.

→This is because as the concentration of substrate increases, **the enzyme becomes saturated with substrate**.

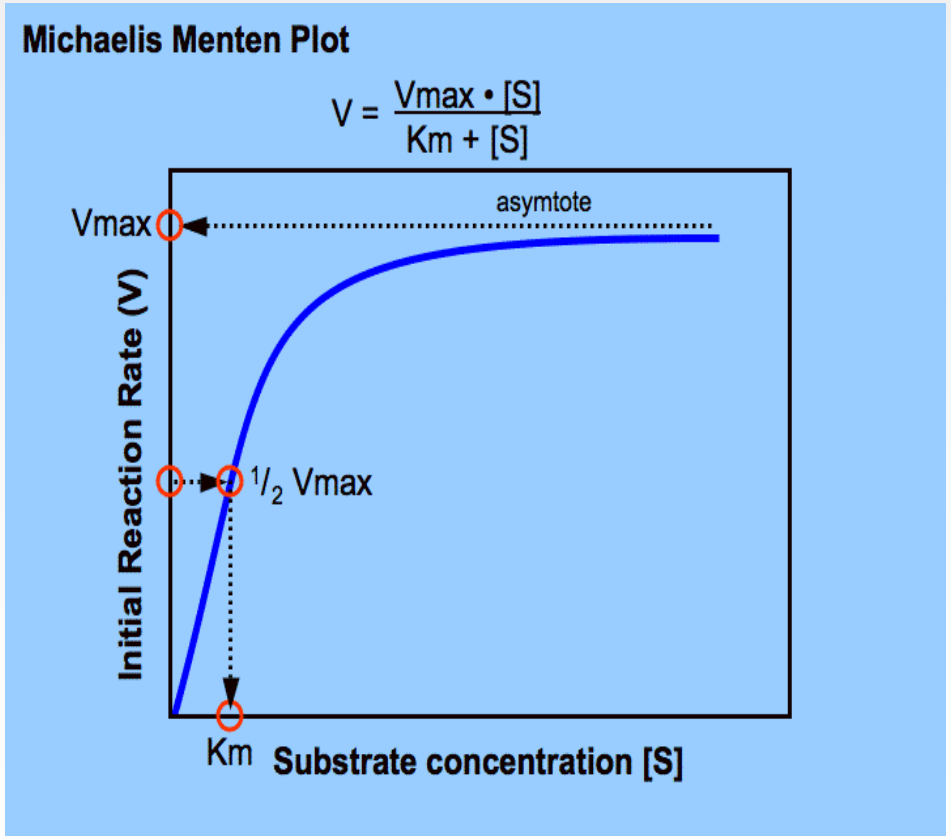
So there is usually a hyperbolic relationship between the rate of reaction and the concentration of substrate



Michaelis–Menten equation

- The rate of reaction when the enzyme is saturated with substrate is the **maximum rate** of reaction,(**maximum velocity**) **Vmax**.
- Michaelis-Menten equation give the relationship between [S] and velocity of enzymatic reaction.
- The hyperbolic shape of this curve can be expressed algebraically by the Michaelis – Menten equation:

$$V = \frac{V_{max}[S]}{K_m + [S]}$$

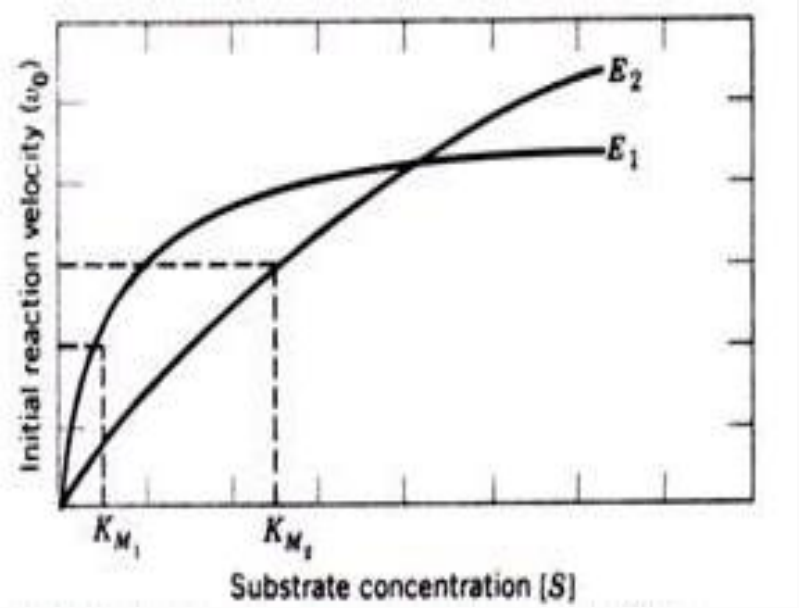


Vi= initial velocity, **V max=** maximum velocity,

[S] = substrate concentration, **Km=** Michaelis constant.

Michaelis constant (Km)

- **Km** is the substrate concentration at half V max.
- The relationship between rate of reaction and concentration of substrate depends on the **affinity of the enzyme** for its substrate. This is usually expressed as the **Km** of the enzyme, an inverse measure of affinity
- The larger the km, the weaker the binding and the larger the [S] needed to reach the half the maximum rate.
- The Km can **vary** greatly from enzyme to enzyme, and even for different substrates of the same enzyme

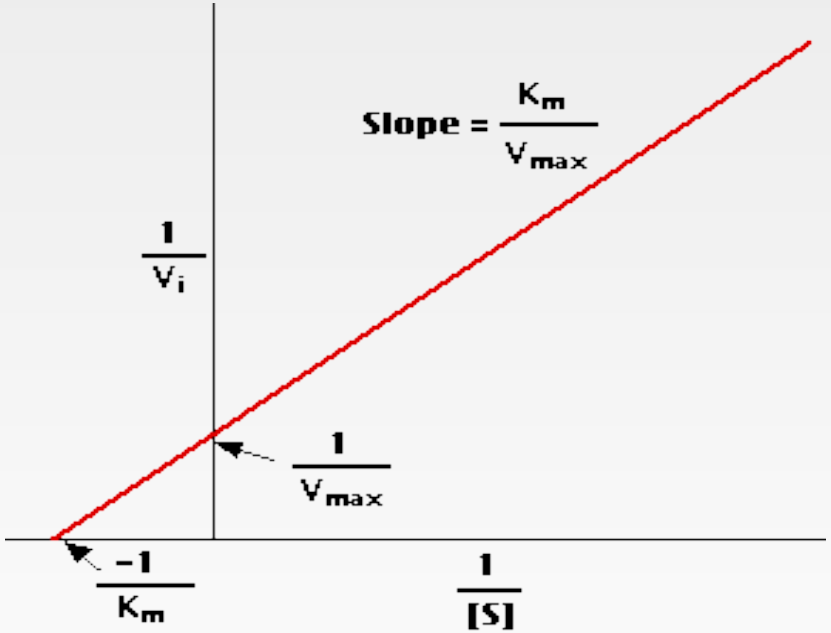


Lineweaver – Burk equation

- The Michaelis -Menten equation can be algebraically transformed into forms that are useful in the practical determination of Km an V max.

- One common transformation is derived simply by taking the reciprocal of both sides of the Michaelis -Menten equation to give Lineweaver – Burk equation:

$$\frac{1}{Vi} = \frac{1}{Vmax} + \frac{Km}{Vmax} \cdot \frac{1}{[S]}$$

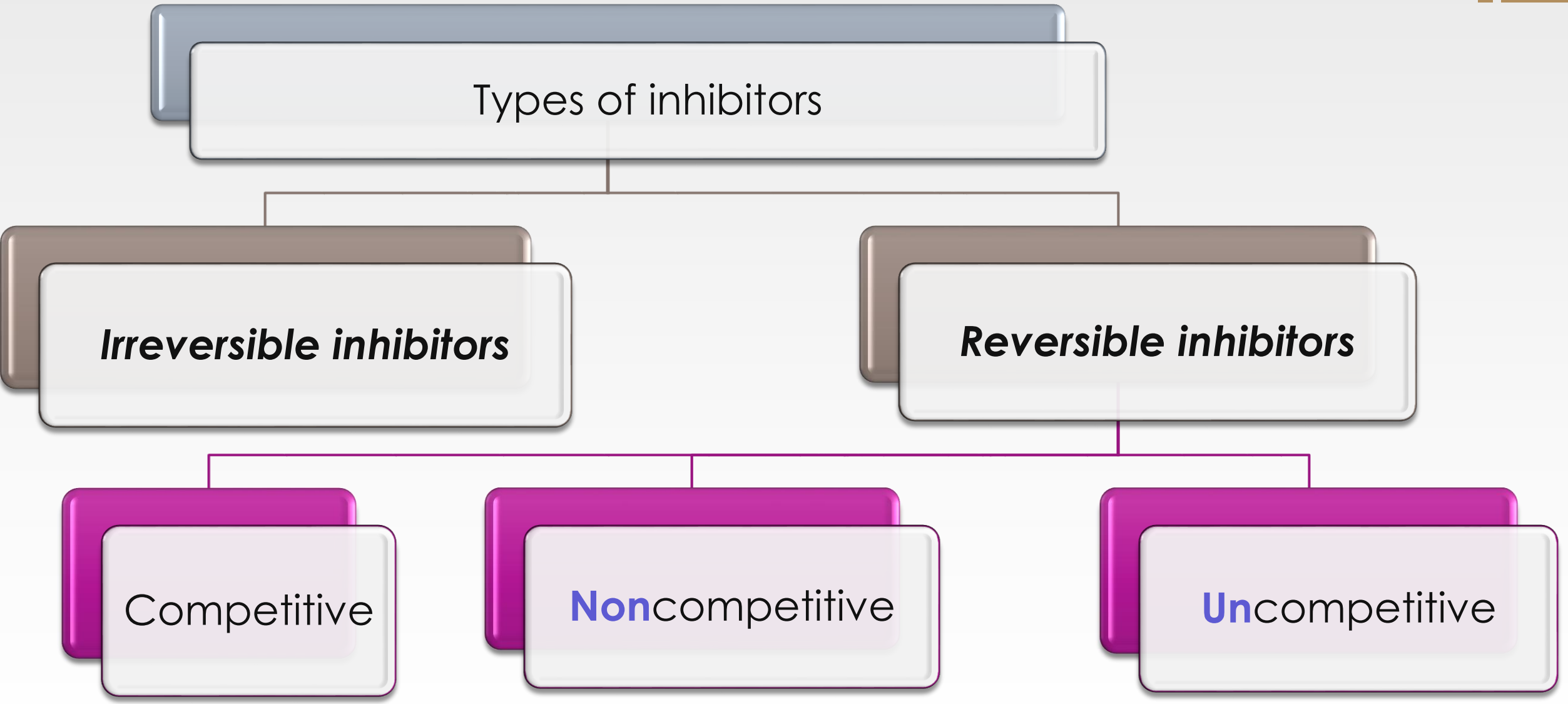


- By plotting 1/v against 1/ [S] a straight line plot, **Lineweaver – Burk plot** is obtained.

- Both V max and Km can be obtained **accurately** from intercepts of the straight line with the y – axis and x-axis

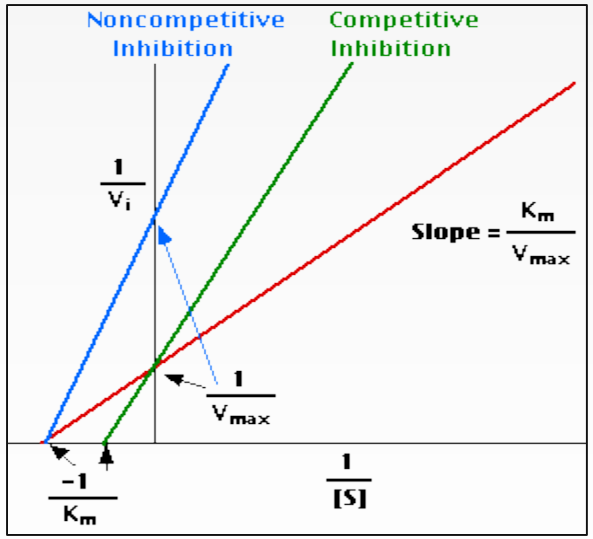
Inhibitors

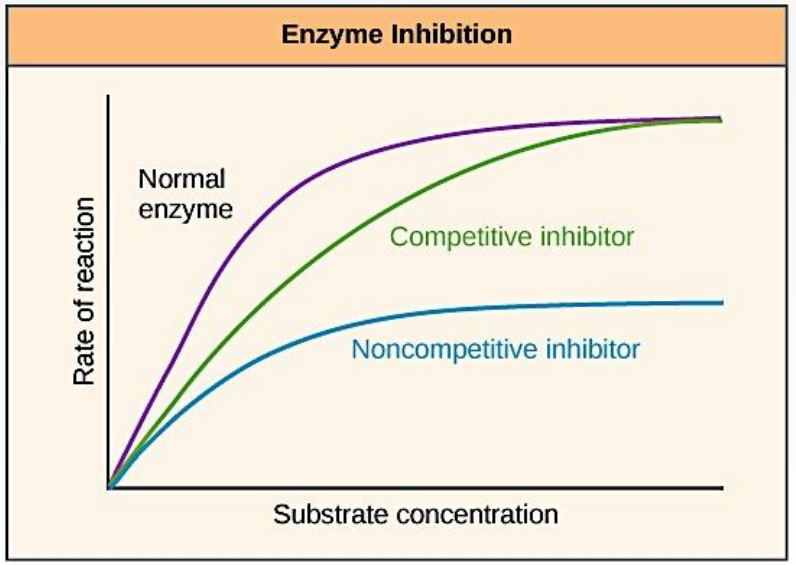
- They are chemicals that reduce the rate of enzymatic reactions.
- They are usually specific and they work at low concentrations.
- They block the enzyme but they do not usually destroy it.
- Since blocking an enzyme's activity can kill a pathogen or correct a metabolic imbalance, *many drugs are enzyme inhibitors*.


Types of inhibitors
Irreversible inhibitors
Reversible inhibitors
Competitive
Noncompetitive
Uncompetitive

	Irreversible inhibitors	Reversible inhibitors
Type of bonds with E	Inhibitors bind **covalently** with enzyme	Inhibitors bind **non-covalently** with enzyme
Removal	Cannot be removed by dialysis or other way	Can be removed by dialysis
Activity Restoration	Permanently modify the active site residues (functional group) which the enzyme become inactive.	Removal of the inhibitor restores enzyme activity.

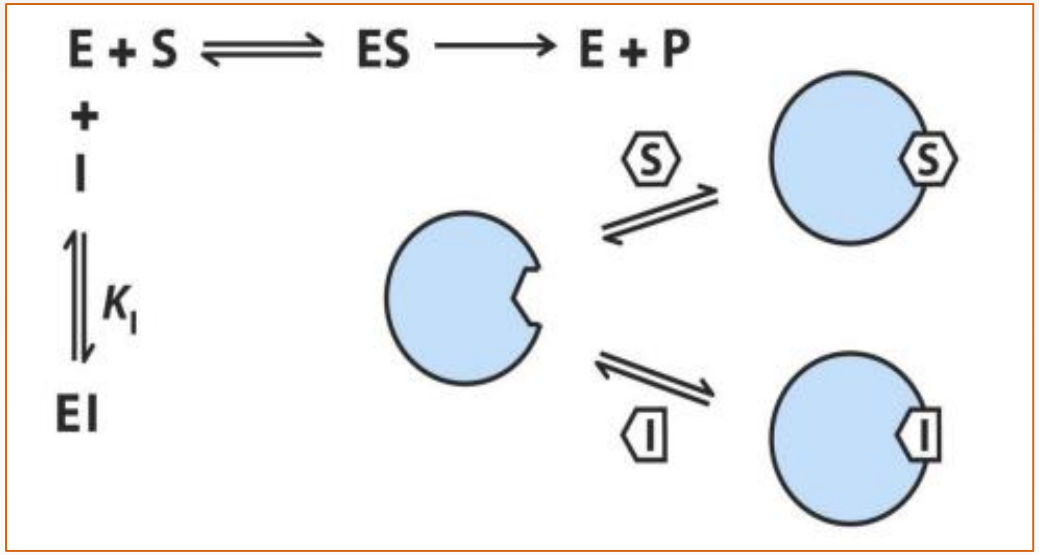
It is relatively simple to **distinguish the three types of reversible inhibition** by comparing the Michaelis-Menten and Lineweaver-Burke kinetics (Vmax and Km) in the presence and absence of the inhibitor.





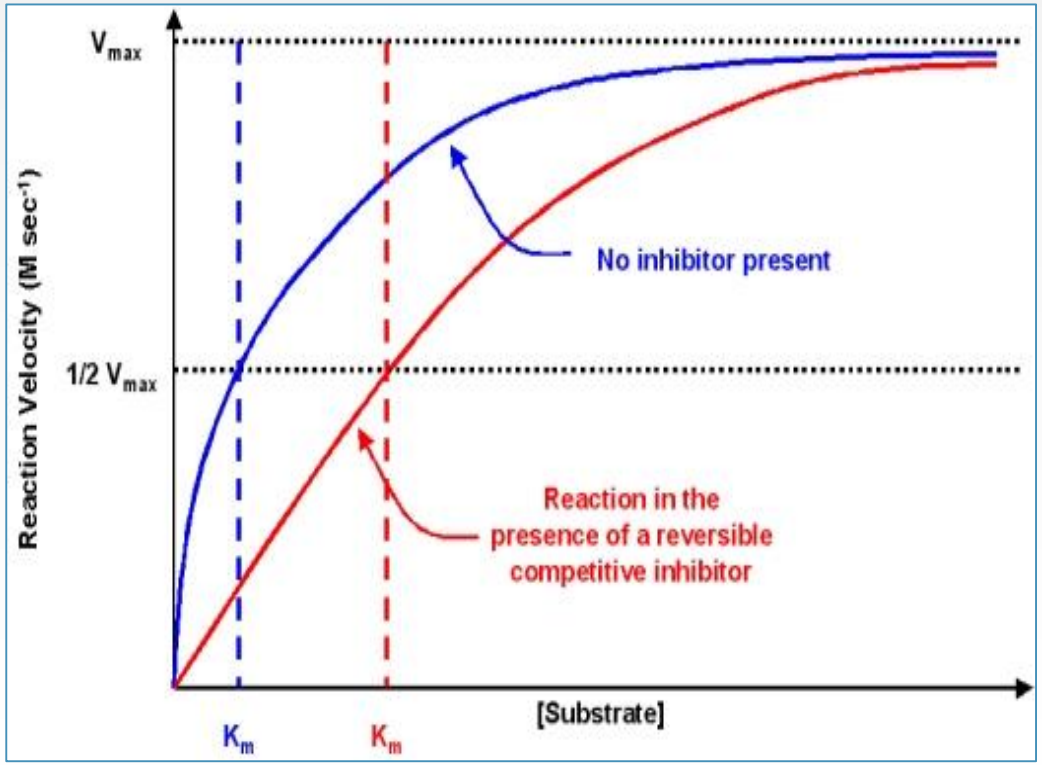
Competitive inhibitors

- As the name implies, the inhibitor compete with the substrate for active site of the enzyme.
- **The structure of substrate** and inhibitors are similar.
- Competitive inhibitor will **not affect** the Vmax
- **Increase the Km** →decrease the affinity
- This type of inhibition can be overcome by sufficiently high concentrations of substrate.

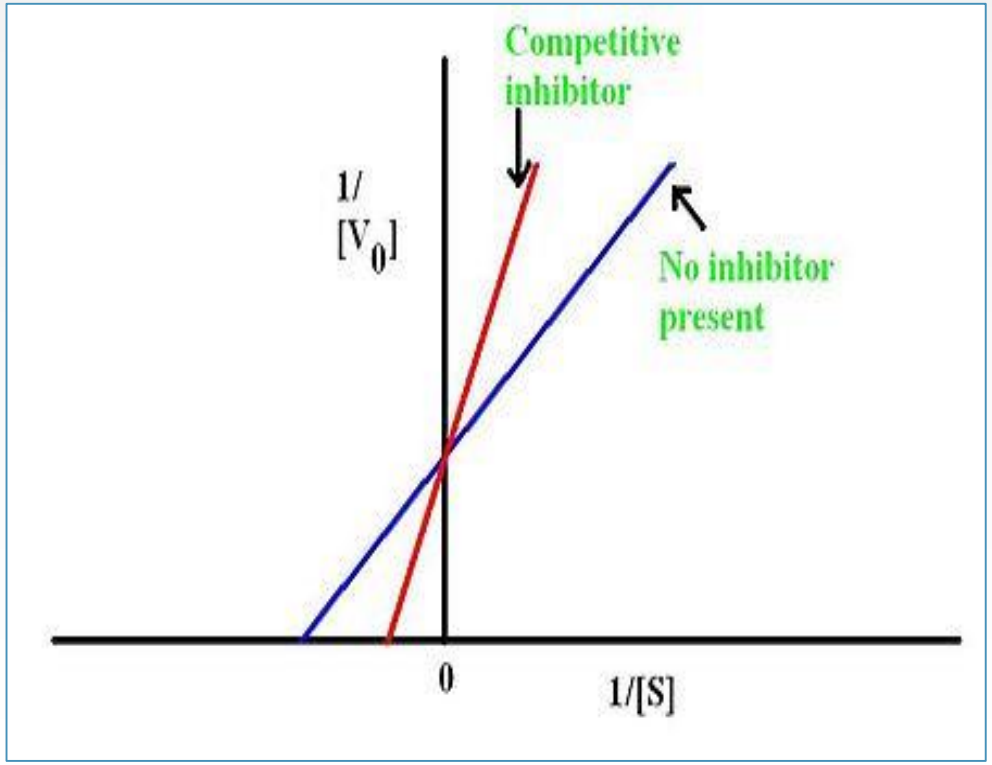


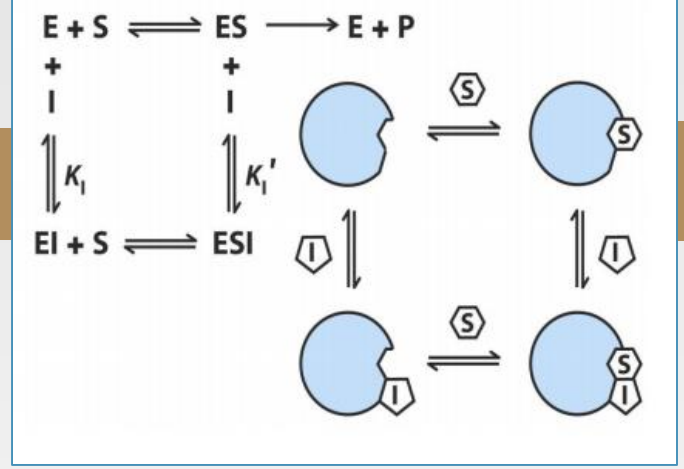
Competitive inhibitors

- Michaelis-Menten



- Lineweaver-Burke



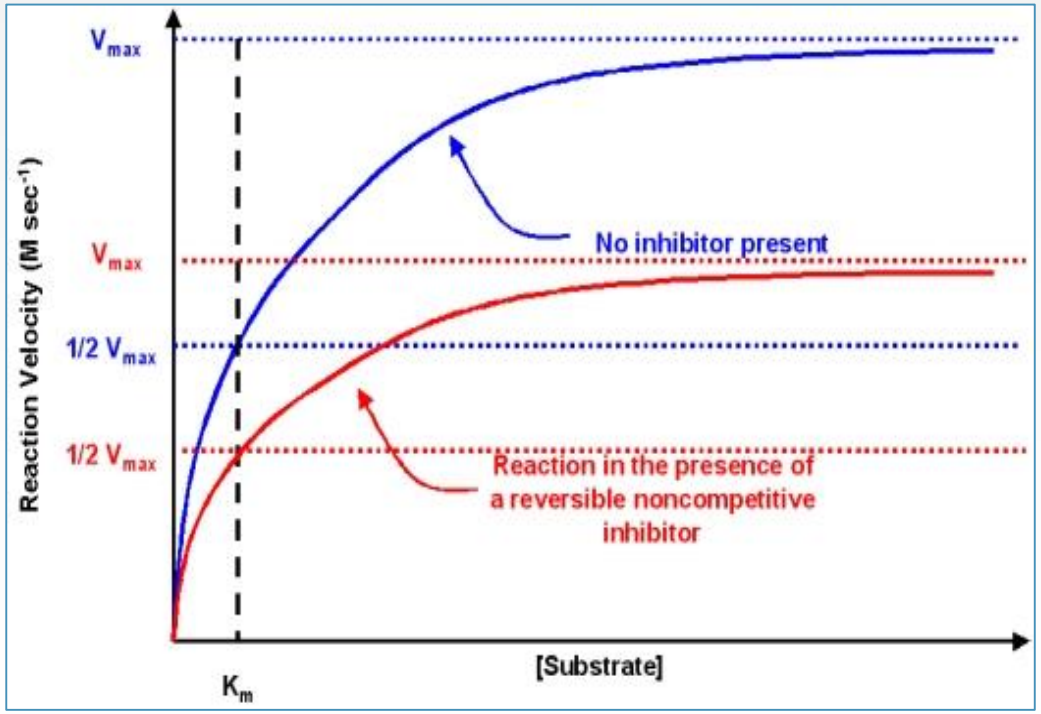


Noncompetitive inhibitors

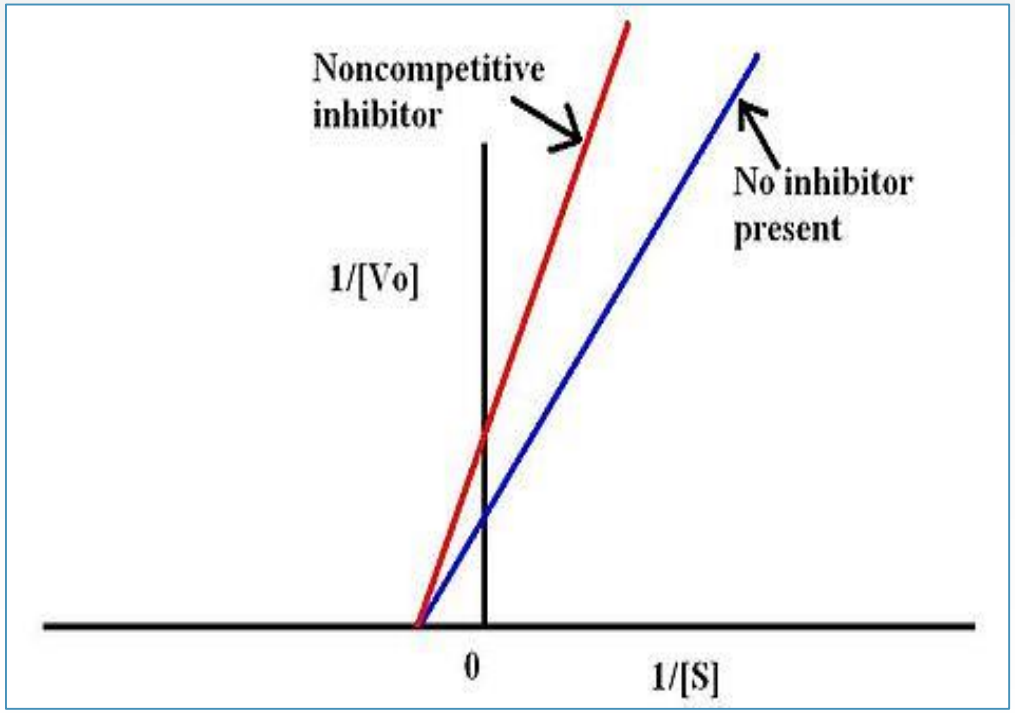
- A noncompetitive inhibitor binds to the enzyme, but not at the active site itself, so that the substrate can still bind at the active site, but there's no catalyzed transformation.
- It causes changes in the overall 3-D shape of the enzyme that leads to a decrease in activity.
- They can bind with E or ES complex.
- Have the **same Km** (with I OR without I)→ substrate can bind
- **low Vmax** (with I)
- This type of inhibition cannot be overcome by a large amount of substrate, thus noncompetitive inhibition

Noncompetitive inhibitors

- Michaelis-Menten

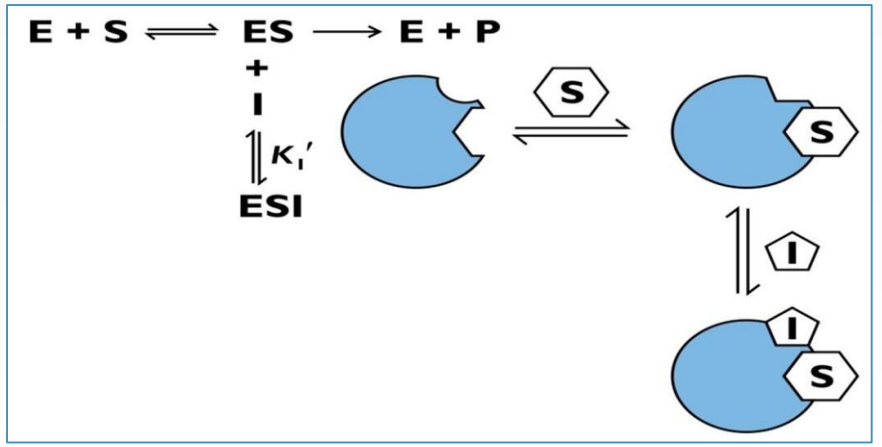


- Lineweaver-Burke



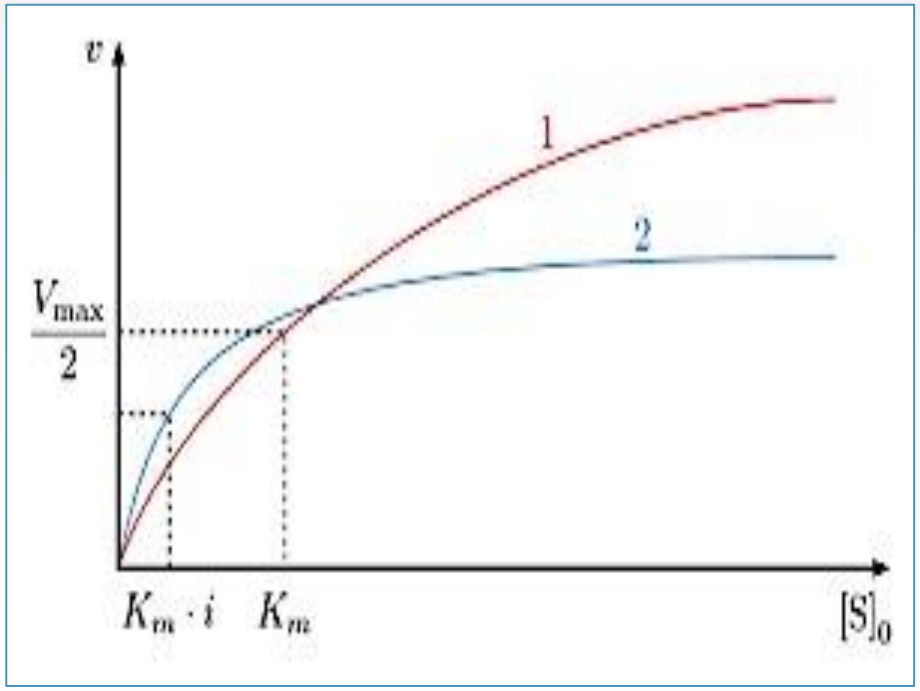
Uncompetitive inhibitors

- The inhibitor binds only to the substrate-enzyme complex
- Both Vmax and Km are low (with I)
- This type of inhibition cannot be overcome by a large amount of substrate, thus uncompetitive inhibition

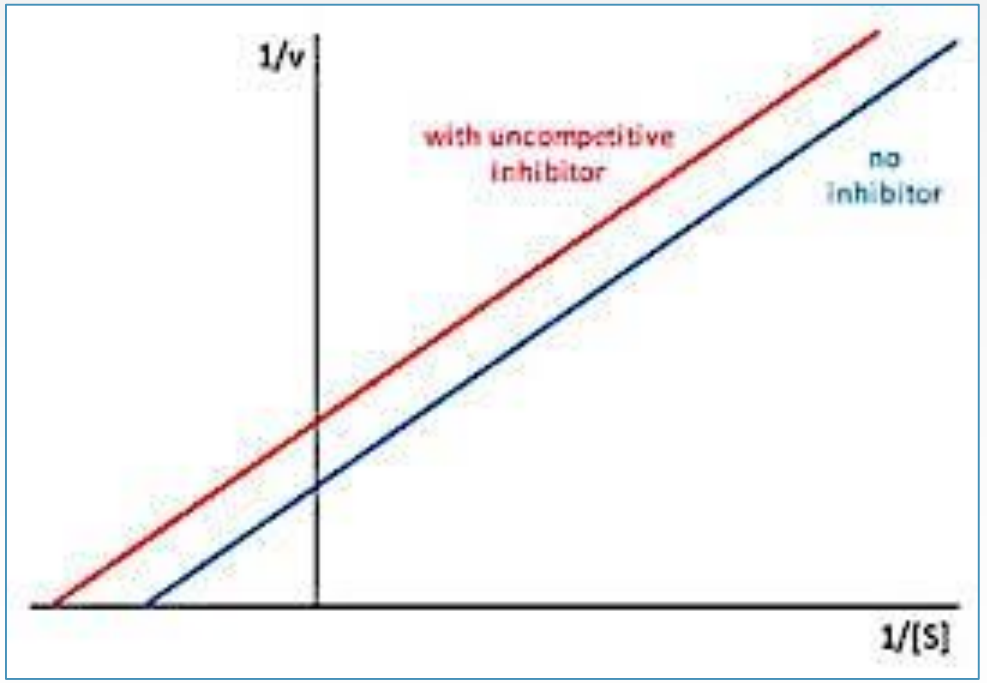


Uncompetitive inhibitors

- Michaelis-Menten



- Lineweaver-Burke



Method:

- **Inorganic phosphate (Pi) and sodium fluoride** are inhibitors of acid phosphatase and it is your task to determine whether they are competitive, noncompetitive, or uncompetitive inhibitor.
- The setup is basically the same as in the experiment for the effect of substrate concentration on reaction velocity, except that a constant amount of inhibitor is added.
- The kinetics for the uninhibited reactions must be **compared** with those of reactions run in the presence of the inhibitor.
- Determinations of V_{max} and K_m will help you to determine the specific mode of inhibition

Method

In order to detect the effect of substrate concentration you must fix all the component except the [S]	
Time (5 minutes)	constant
Enzyme concentration	constant
Substrate concentration	Variable
Temperature (37˚C)	constant
pH (5.5)	constant

Method:

Without I

- Prepare 8 tubes labeled as follows

Tube	A	B	C	D	E	F	G	H
[S] mM	0	0.5	1	2.5	5	10	25	50

- To each of these tubes add

Chemical	Volume (ml)
pH sodium acetate buffer	0.5
0.1M $MgCl_2$	0.5
Corresponding p-nitrophenyl phosphate (pNPP)	0.5
Water	5

With I

- Prepare 8 tubes labeled as follows

Tube	A	B	C	D	E	F	G	H
[S] mM	0	0.5	1	2.5	5	10	25	50

- To each of these tubes add

Chemical	Volume (ml)
pH sodium acetate buffer	0.5
0.1M $MgCl_2$	0.5
Corresponding p-nitrophenyl phosphate (pNPP)	0.5
Water	4
K_2HPO_4 or Sodium fluoride (NaF)	1

- Place the tubes in a test tube rack situated in 37°C water bath and **let stand for 5 min**.

- Start the reaction by adding 0.5 ml enzyme and stop it by adding 0.5 ml KOH as in the following table:

Tube	Start the reaction	Stop the reaction
A	0 min	0 min
B	0 min	5 min
C	2 min	7 min
D	4 min	9min
E	6 min	11 min
F	8 min	13 min
G	10 min	15 min
H	12 min	17 min

- Determine the absorbance at 405 nm for each sample, **using the first tube (0 mM of S) as the blank.**

Results

Tube	[S] (mM)	1/[S] (1/mM)	Abs at 405 nm		V=(A x 10^6) /(18.8 x 10^3x time) (µmole of PNP/min)	1/V (1/ µmole of PNP/min)
			Without I	With I		
A	0					
B	0.5					
C	1					
D	2.5					
E	5					
F	10					
G	25					
H	50					

Results

- Draw the curve using Michaelis-Menten, determine Vmax and Km for acid phosphatase of both inhibited and not inhibited reaction
- Prepare the double –reciprocal plot of Lineweaver-Burk and determine the Km and V max from the x and y intercepts of both inhibited and not inhibited reaction

Discussion

- Describe the shape of the curve and discuss the relationship between substrate concentration and the rate of the reaction

- Comment on the value of **Vmax** and **km** and define each of them, and what the km reflect.

- Compare between the two values (Km and Vmax) in Michaelis-Menten and Lineweaver-Burk for uninhibited reaction. Are they similar or different and which is more accurate.

- **Compare** the Vmax and Km obtained Michaelis-Menten and Lineweaver-Burk graphs of both inhibited and uninhibited reactions with each other to **determine the type of inhibition**

- Determine if inorganic phosphate **and** sodium fluoride are a competitive, noncompetitive, or uncompetitive inhibitor **?** Justify your answer and discuss the difference you find