Kinetics analysis of **β-fructofuranosidase** enzyme

5-EFFECT OF COPPER (II) SULFATE AS INHIBITOR ON RATE OF REACTION CATALYZED BY B-FRUCTOFURANOSIDASE

TYPE OF INHIBITORS

- There exist a number of molecular species which, in the presence of an enzyme and its substrate, have the effect of binding to the enzyme (or to the enzyme-substrate complex) and totally or partially inhibiting the reaction.
- In those cases where the binding is **irreversibly,** the reaction is inalterably inhibited and **not subject to kinetic analysis.**
- If the binding is reversible, however, the specific type of inhibition can be determined by kinetic analysis.

*TYPES OF **REVERSIBLE** ENZYME INHIBITORS*

The three types of revisable inhibitors that can be clearly distinguished in this manner are :

competitive, noncompetitive, and uncompetitive.

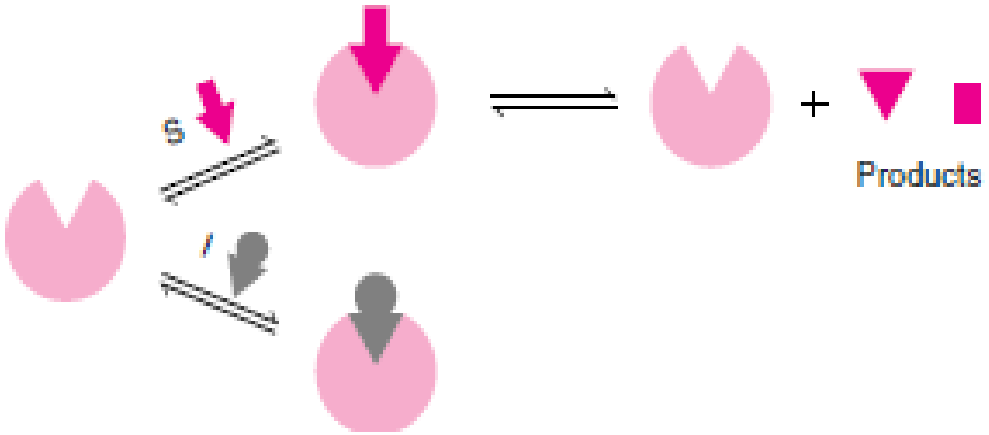
Experimentally, these are distinguished by amount of the inhibitor at ever-increasing concentrations of the substrate.

When the inhibited reaction is compared with the normal reaction using the graphic analyses of Michaelis - Menten or Lineweaver - Burke, the type of inhibition is clearly indicated.

I-COMPETITIVE INHIBITOR

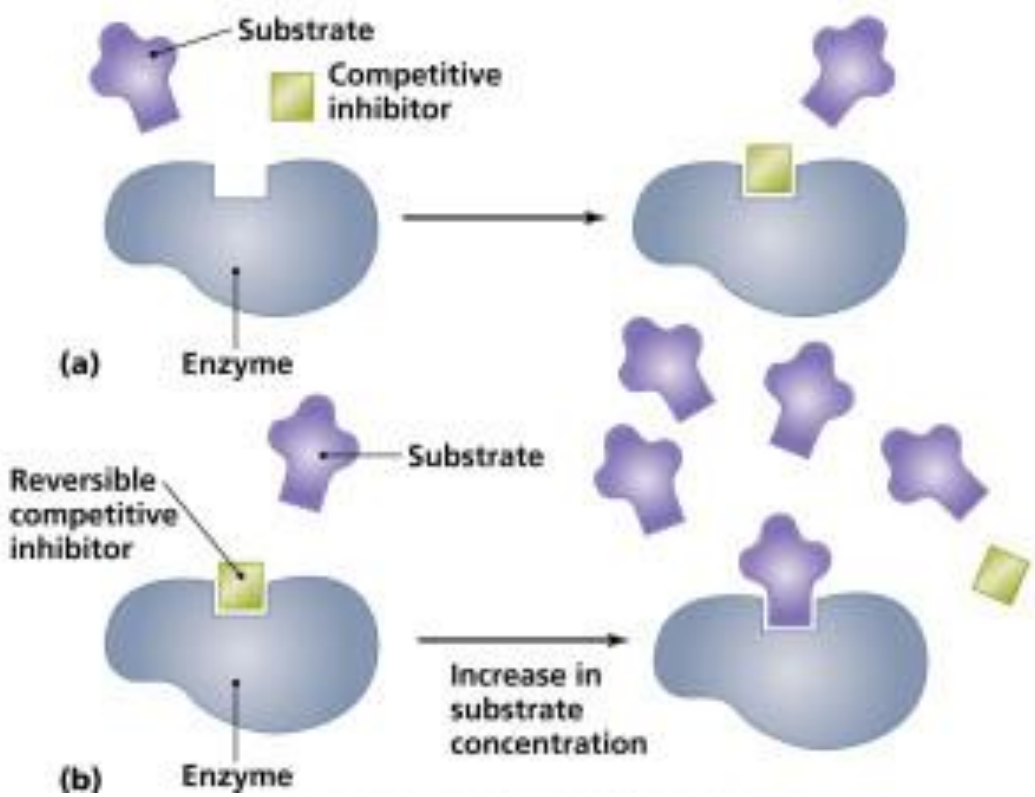
- In the case of competitive inhibition, high substrate concentrations wipe out the inhibitory effect and the V_{MAX} for the inhibited reaction is identical to that for the uninhibited reaction
- The K_M of the inhibited reaction (K_M) is significantly higher than that of the reaction run in the absence of inhibitor, which indicates an apparent decrease I the affinity of the enzyme for its substrate

Competitive inhibitor and substrate compete for the same active site. Only ES complex leads to production on formation

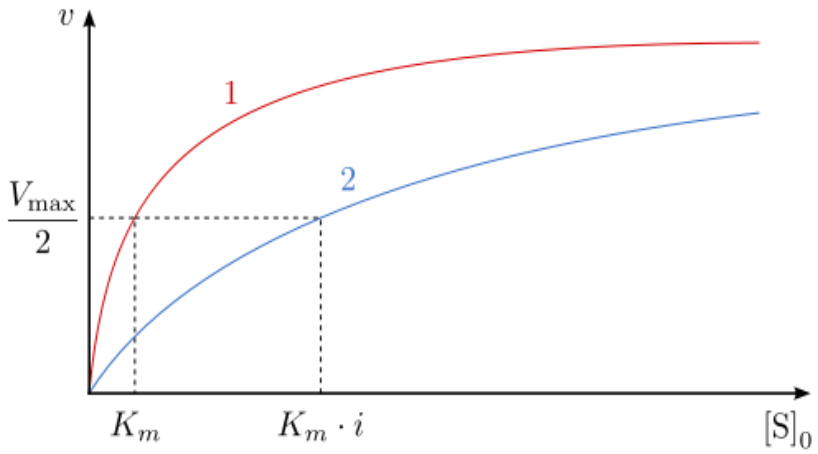


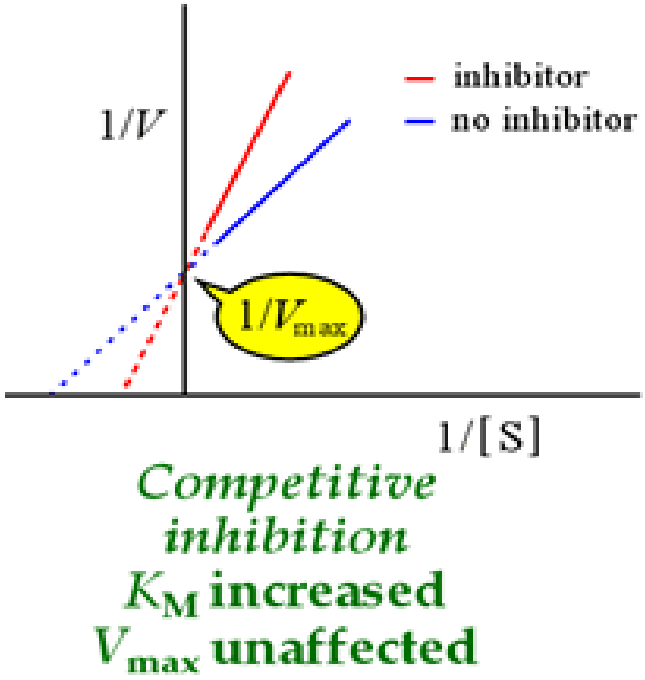
In **competitive inhibition** , the substrate and inhibitor cannot bind to the enzyme at the same time, This usually results from the inhibitor having an affinity for the active site of an enzyme where the substrate also binds; the substrate and inhibitor *compete* for access to the enzyme's active site.

- This type of inhibition can be overcome by sufficiently high concentrations of substrate (Vmax remains constant), i.e., by out-competing the inhibitor. However, the apparent Km will increase as it takes a higher concentration of the substrate to reach the Km point, or half the V max. Competitive inhibitors are often similar in structure to the real substrate



I-COMPETITIVE INHIBITION





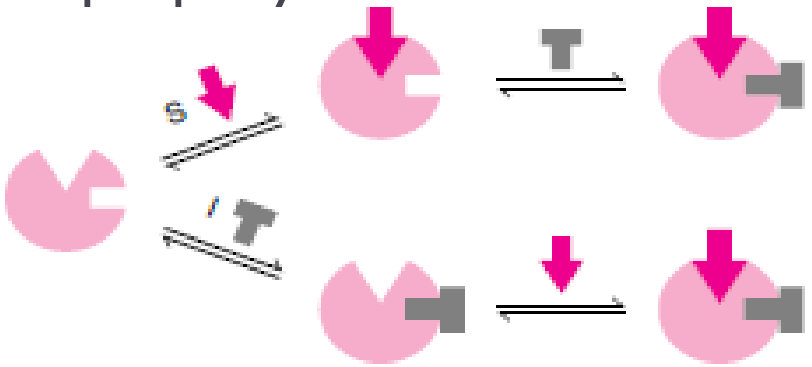
Competitive inhibition in M-M plot

Competitive inhibition in L-B plot

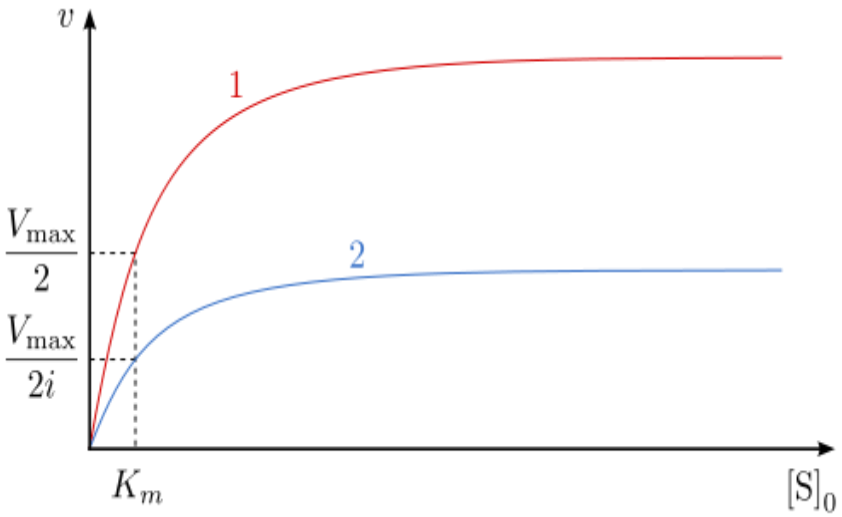
2-NON-COMPETITIVE INHIBITION

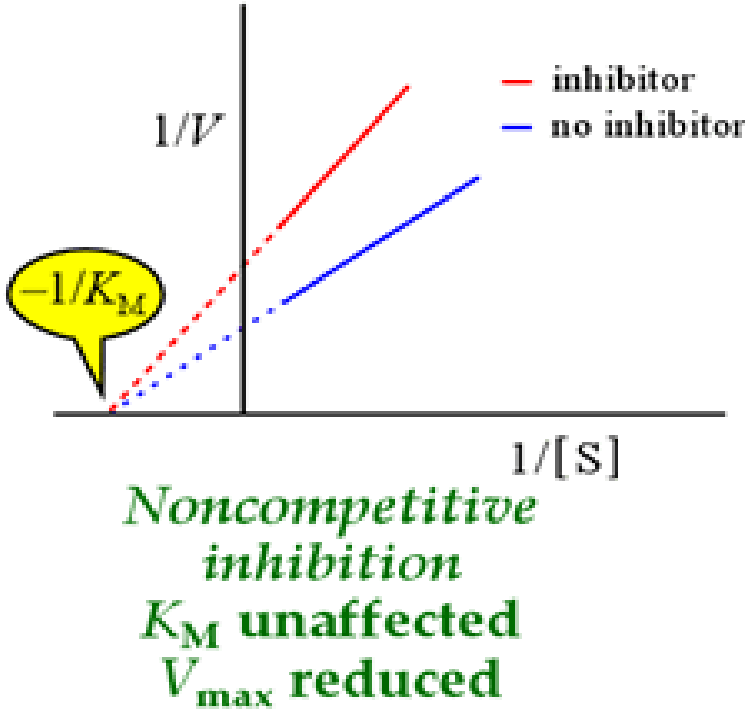
- Noncompetitive inhibition yields the curve indicated, with a lower V_{MAX} and a **K_M identical** to the reaction in the absence of inhibitor.
- The inhibitor bind to the free enzyme or enzyme-substrate complex and reduces Enzyme activity ,but does not affect the binding of substrate
- V max will decrease due to the inability for the reaction to proceed as efficiently, but Km will remain the same as the actual binding of the substrate, by definition, will still function properly

Noncompetitive inhibitor binds to a site other than the active site or the ES complex. The (ESI) complex does not lead to product.



2-NON-COMPETITIVE INHIBITION





Noncompetitive inhibition in M-M plot

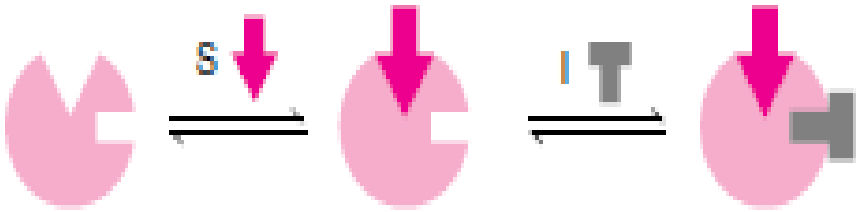
Noncompetitive inhibition in L-B plot

3-UNCOMPETITIVE INHIBITION

- Uncompetitive inhibition is characterized by a low V_{MAX}, a lower K_M and a Michaelis-Menten curve similar to that of noncompetitive inhibition. The best way to distinguish the three types of inhibition graphically is to use the Lineweaver-Burk plot
- Notice that in the case of uncompetitive inhibition the slope of the inhibited curve (K_M/V_{MAX}) is the same as that of the non inhibited curve, whereas in the other two types of inhibition, the slope of the inhibited plot is greater

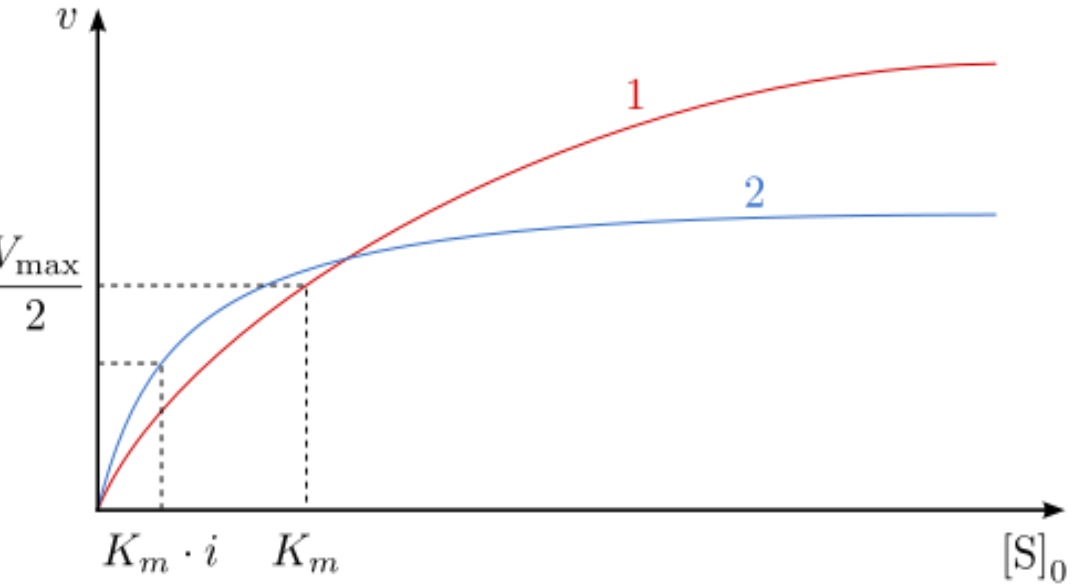
3-UNCOMPETITIVE INHIBITION

- In **uncompetitive inhibition**, the inhibitor binds only to the substrate-enzyme complex; it should not be confused with non-competitive inhibitors. This type of inhibition **causes V max to decrease** (maximum velocity decreases as a result of removing activated complex) **and Km to decrease** (due to better binding efficiency thus decreasing the Km which indicates a higher binding affinity).

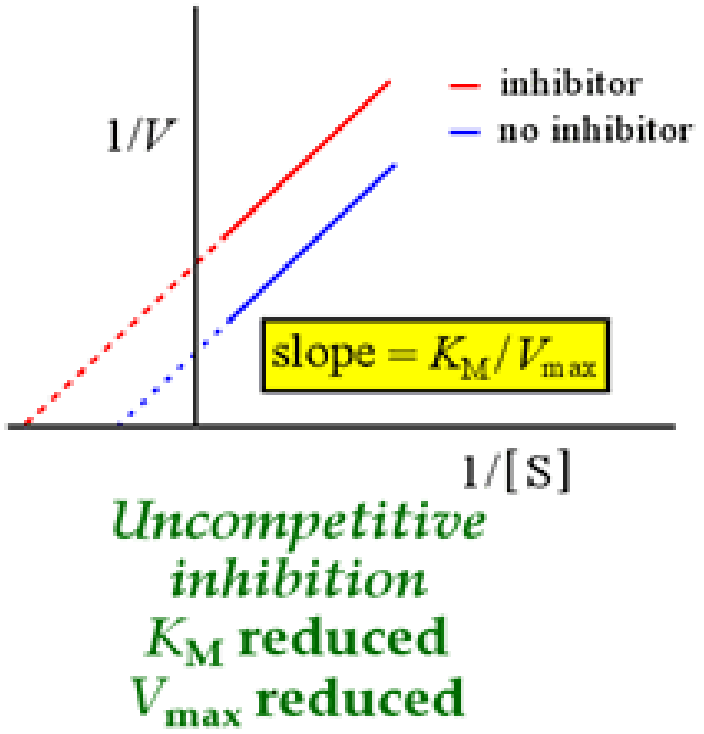


Uncompetitive inhibitor binds only to the ES complex. The (ESI) complex does not lead to produc

3-UNCOMPETITIVE INHIBITION



Uncompetitive inhibition in M-M plot



Uncompetitive inhibition in L-B plot

PRINCIPLE

- Sucrose is a disaccharide composed of -glucose and -fructose joined by an -1,4-glycosidic linkage.
- This bond can be hydrolyzed by fructofuranosidase, an enzyme commonly known as invertase, to yield glucose and fructose in equal proportions .
- In this experiment, the kinetic parameters of -fructofuranosidase, a yeast enzyme that catalyzes the break-down of sucrose, were quantized through dinitrosalicylic acid assay .
- presence of heavy metal ions such as Cu2+ may inhibit the activity of invertase. This is due to the high affinity of the sulfhydryl group on cysteine and methionine residues in a protein for the Cu2+ ion.

PRINCIPLE

- When copper ions form complexes with sulfhydryl groups in amino acid residues, disulde bridges are disrupted, modifying the enzyme's tertiary structure. Since the functions of an enzyme are highly correlated with its form, a change in the tertiary structure of invertase would likely inhibit its function

PRINCIPLE

- Competitive inhibition refers to the type of inhibition in which an inhibitor binds to an enzyme's active site and lowers the affinity of the enzyme for its substrate.
- Competitive inhibition does not change the maximal rate of reaction, measured as Vmax, due to the fact that the presence of a large amount of substrate can saturate the active sites, preventing the inhibitor from binding.

MATERIALS

Solutions :

- 0.05M Sodium Acetate buffer , pH 4.7 .
- 0.25 M Sucrose
- Reducing sugar (0.005M glucose + 0.005M fructose)
- ß- Fructofuranosidase enzyme extract from yeast.
- DNS (dinitrosalicylicacid)Reagent .
- Sodium Bicarbonate .
- Anhydrous copper sulfate

METHOD

- Prepare two sets of test tubes SET I WITHOUT INHIBITOR :
- Prepare 7 tubes of different substrate(sucrose) concentrations by following the table provided.

Tube	Distilled water (ml)	0.25M Sucrose (ml)	Concentration of sucrose M
Blank	2.0	0.0	
A	1.8	0.2	
B	1.6	0.4	
C	1.2	0.8	
D	1.0	1.0	
E	0.5	1.5	
F	0.0	2.0	

• Mix each tube properly then add 1.0ml of the Acetate buffer to each tube mix well

METHOD

Prepare SET2 WITH INHIBITOR :

Tube	Distilled water (ml)	0.25M Sucrose (ml)
Blank Inhibitor	2.0	0.0
G	1.8	0.2
H	1.6	0.4
I	1.2	0.8
J	1.0	1.0
K	0.5	1.5
L	0.0	2.0

• Mix each tube properly then add 1.0ml of the Inhibitor (copper sulphate) to each tube mix well .

METHOD

- Incubate two sets of test tubes at 40°C for 5min.
- Start the reaction by adding 0.05ml of diluted enzyme to all tubes , mix and start the stop clock immediately , incubate each tube for 10min , then stop the reaction by adding 2.0ml of the DNS reagent to each tube and mix well (follow table 2 for adding enzyme and DNS to tubes)
- Note : Mix each tube frequently during the incubation time .

Tube	Start Time (min)	Stop by adding 2.0ml DNS . (min)
Blank	0	10
A	1.0	11
B	2.0	12
C	3.0	13
D	4.0	14
E	5.0	15
F	6.0	16
Blank Inhibitor	7.0	17
G	8.0	18
H	9.0	19
I	10	20
J	11	21
K	12	22
L	13	23

METHOD

- Mix properly , cover each tube by aluminium foil and place in a boiling water bath for 5min to allow the color to develop .

- Remove from water bath cool under tap water , add 20ml of distilled water to each tube , mix properly then measure the absorbance at 540nm .

RESULTS

- Record the absorbance of each test tube.
- Convert the Absorbance reading obtained to micromoles of sucrose hydrolyzed making use of the standard reducing sugars calibration curve.
- Determine the velocity for each tube and record all in table 3
- drawing a graph between the initial velocity v_i and substrate concentration .
- Determine the Km value of the enzyme/ substrate pair .

RESULTS

- Calculate the reciprocals of velocity (1/v) and substrate concentration (1/[S]).

- Prepare the double-reciprocal plot of Lineweaver and Burk and determine the Vmax and Km from the x and y intercepts.

RESULTS

Tube	Absorbance 540nm	Concentration of sucrose *	µmoles of sucrose hydrolyzed	µmoles of sucrose hydrolyzed/min
A				
B				
C				
D				
E				
F				

RESULT : SET I WITHOUT INHIBITOR

Tube	Concentration of sucrose *	µmoles of sucrose hydrolyzed/ min	1/Concentration of sucrose *	1/µmoles of sucrose hydrolyzed/min
A				
B				
C				
D				
E				
F				

RESULT : SET II WITH INHIBITOR

Tube	Concentration of sucrose *	µmoles of sucrose hydrolyzed/ min	1/Concentration of sucrose *	1/µmoles of sucrose hydrolyzed/min
A				
B				
C				
D				
E				
F				

Discussion

- Prepare Michaelis-Menten and Lineweaver-Burke plots that **compare** the inhibited reaction with the uninhibited reaction.

- Determine the K_M and V_{MAX} in the presence of anhydrous copper sulfate

QUESTION

- Did the presence of copper sulfate affect the enzyme activities? If so, is it a competitive, a noncompetitive, or an uncompetitive inhibitor? (Base your argument on appropriate plots, e.g., Lineweaver-Burk plots.)

Thank You

&

Good Luck