Enzyme kinetics

The aim:

To determine the effect of concentration of enzyme and time on the velocity of hydrolysis of sucrose by invertase enzyme

Enzyme inhibition

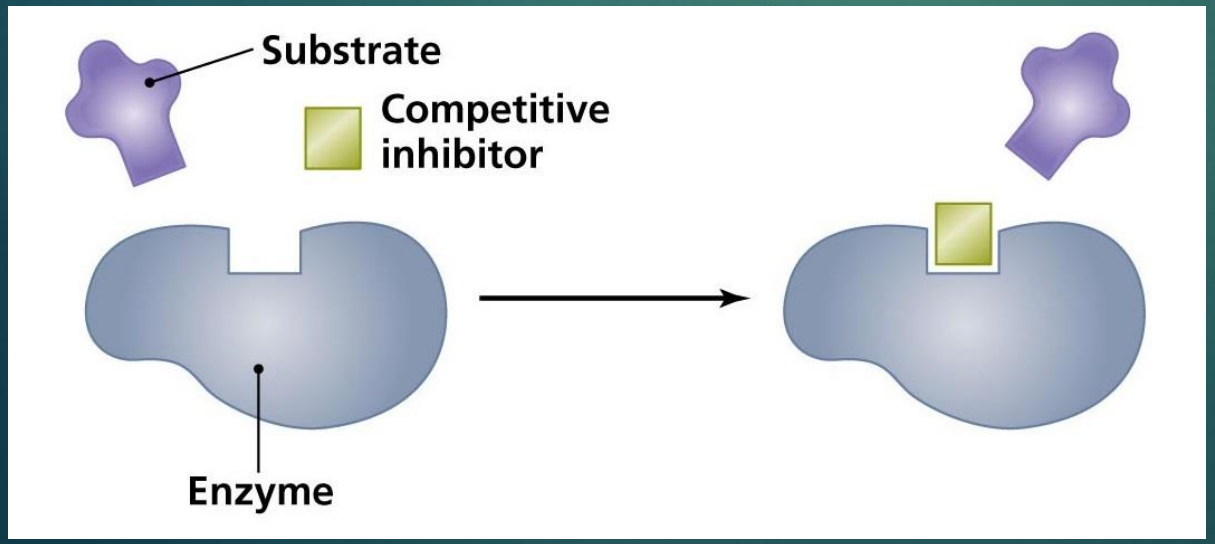
Inhibitor is a molecule that binds to an enzyme and decreases its activity. The binding of inhibitor is either reversible or irreversible.

Enzyme inhibitors are divided into 3 groups:

1. Competitive inhibitors
2. Non-competitive inhibitors
3. Acompetitive inhibitors

Competitive inhibition

- Competitive inhibitors are similar in structure to the real substrate.
- The substrate and inhibitor cannot bind to the enzyme at the same time, therefore they compete for access to the enzyme's active site.
- The extent of inhibition depends on the concentration of the inhibitor.

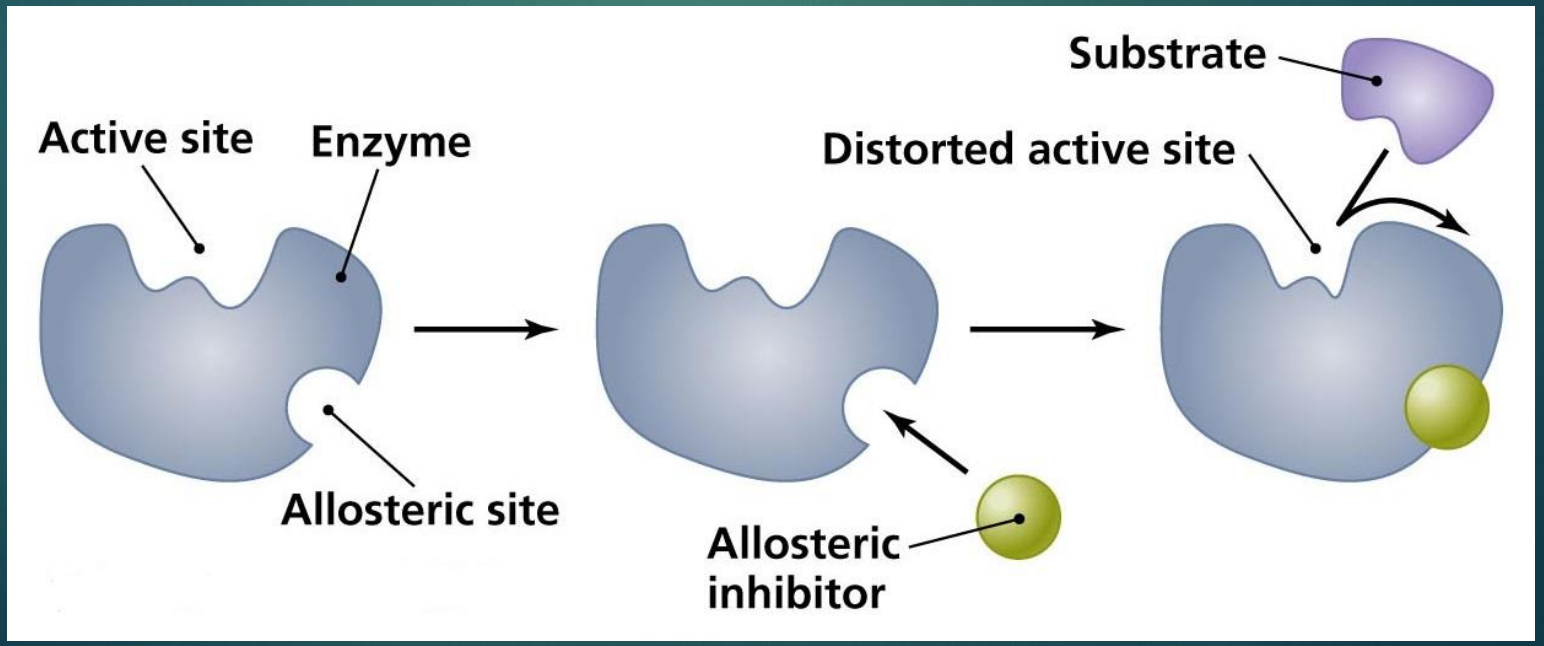


- This type of inhibition can be overcomed by sufficiently high concentrations of substrate

(reversible inhibition)

Non-competitive inhibition

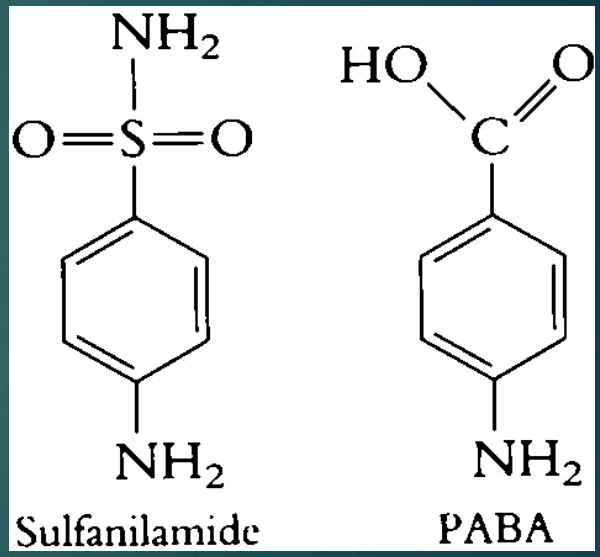
- Non-competitive inhibitors hinder enzymatic reactions by binding to another part of the enzyme, changing its shape and the shape of the active site resulting that it is not receptive to substrate molecules.
- **Non-competitive irreversible** binding occurs, when inhibitor tightly, permanently is bound to the catalytic site



Competitive inhibition – an example

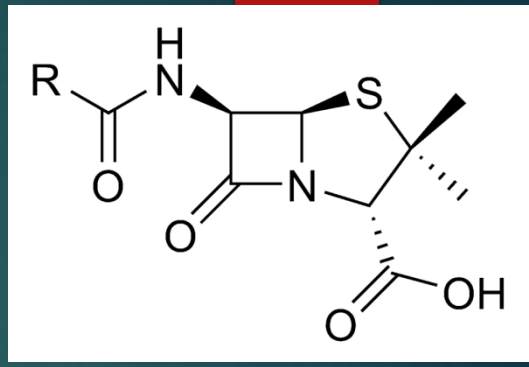
1. Bacterial folic acid synthesis inhibited by sulphonamides

- In bacteria, antibacterial sulfonamides act as competitive inhibitors of the enzyme dihydropteroate synthase (DHPS), an enzyme involved in folate synthesis.
- Sulphonamides resemble PABA (p-aminobenzoic acid) in structure and compete for admission into the active site.
- They inhibit enzymatic activity of the bacteria competitively, thus being unable to produce the necessary amino acids and nucleotides.



- The human body is not capable to synthesize folic acid and therefore the sulfonamides interact selectively on the bacteria.

Competitive inhibition – an example

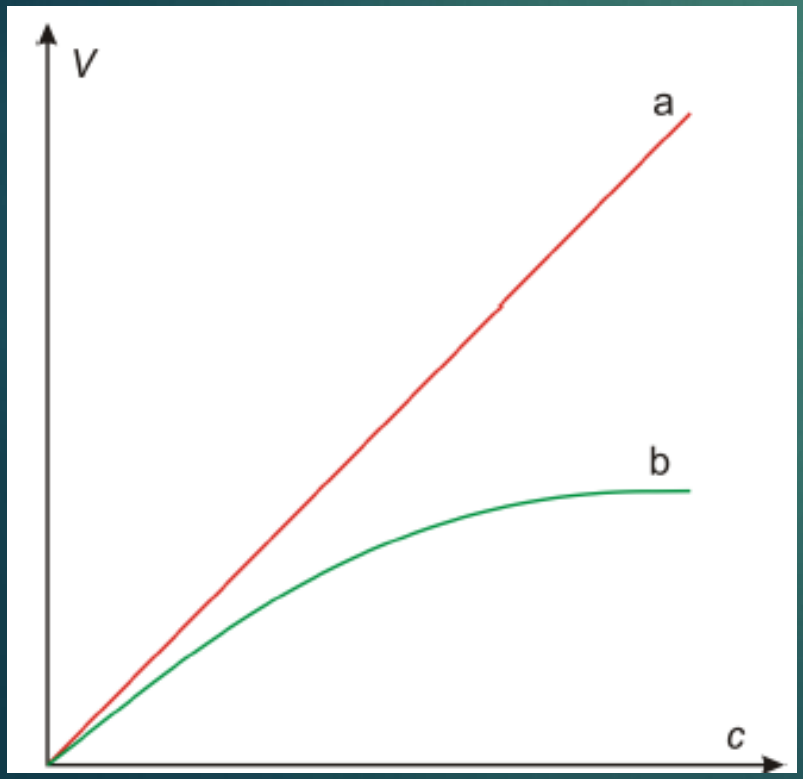


2. **Inhibition of transpeptidase by beta-lactam antibiotics (e.g. Penicillin)**

- Bacterial enzyme called transpeptidase determines the correct formation of some chemical bonds in the bacterial cell wall.
- In the presence of penicillin, bacteria can not effectively divide because they are unable to produce a normal cell wall.
- Meanwhile, human cells do not have both cell wall and transpeptidase enzyme and therefore (except for individual allergic reactions) penicillin is a harmless medicine.

The effects of enzyme concentration on the velocity (V) of enzymatic catalysis

- It is most convenient to observe the effect of enzyme concentration (C_e) on V at high concentrations of substrate (C_s)



when:

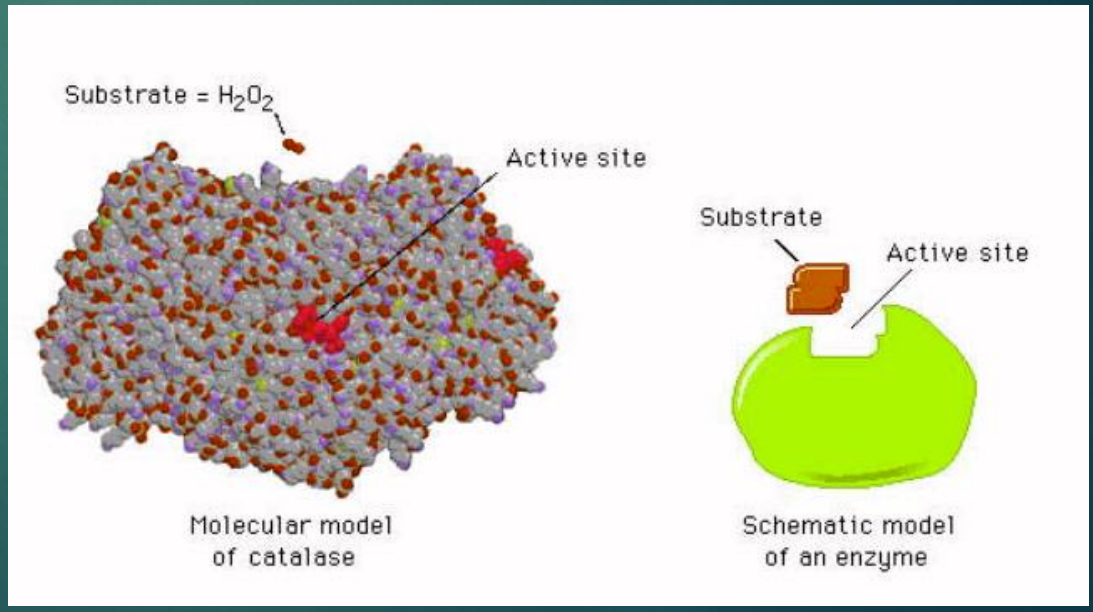
- V is independent of C_s (zero-order reaction)
- V is proportional to C_e (at not very high $C_{e)}$

The dependence of the velocity of enzymatic reaction (V) on enzyme concentration (c) at 2 different substrate concentrations:
a – enzyme is saturated with substrate
b – enzyme is not saturated with substrate

The mechanism of enzymatic catalysis

- In the solution there is a mixture of enzyme and substrate molecules. Substrate molecules as smaller and more mobile than enzyme circulate in the space surrounding the enzyme molecule until they are near the active site.

- Then, thanks to the ionic interactions, polarity and hydrogen bonds, they combine with the active site of the enzyme and they are converted into a product.

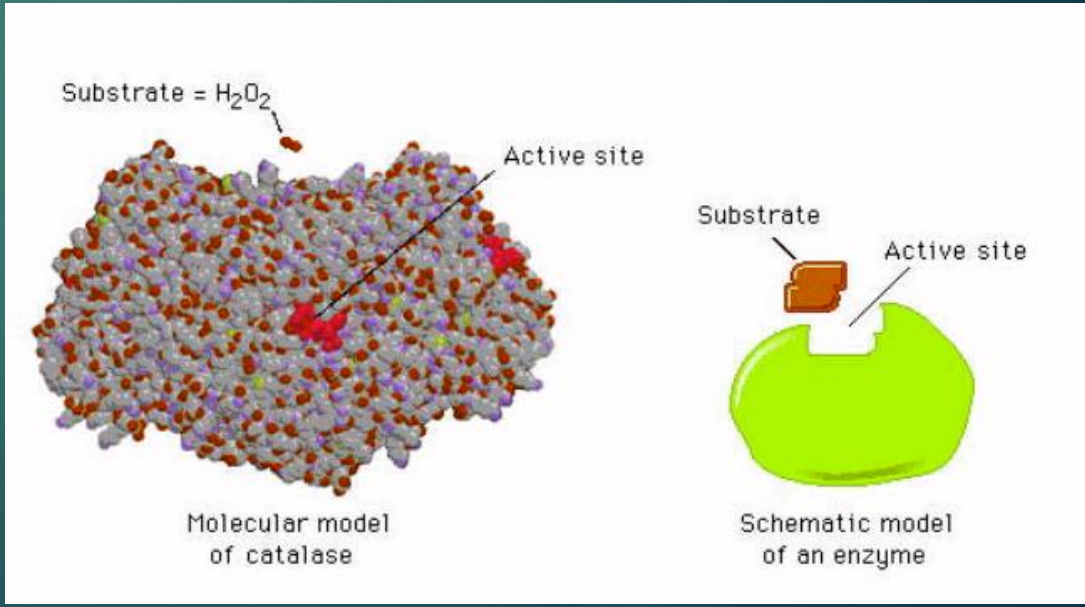


The mechanism of enzymatic catalysis

- The product dissociates and the space in the active site is free for the next substrate molecule.

- The **higher the concentration of substrate** in the solution, the greater the probability of "finding" the active site.

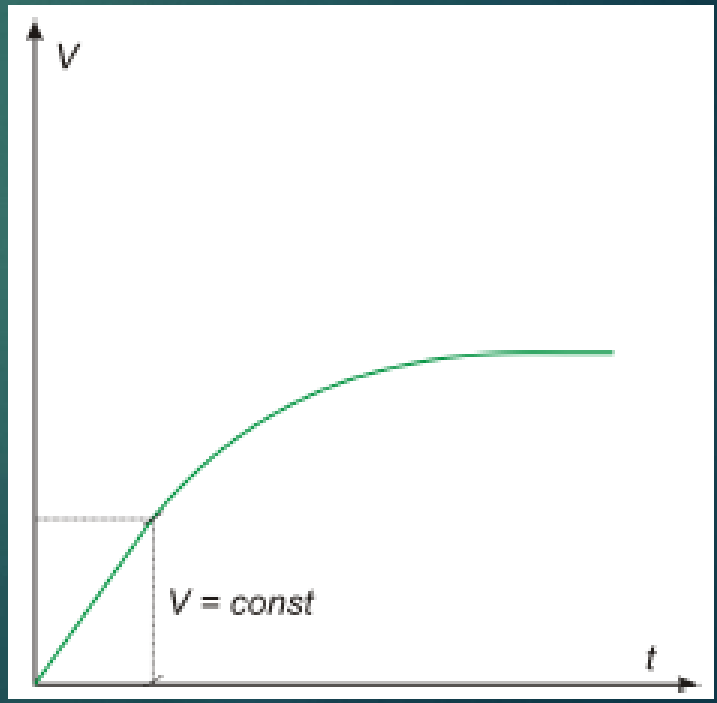
- A similar relationship concerns the amount of enzyme protein in the reaction environment.



The dependence of the velocity of enzymatic reaction (V) on the time (t)

- Enzyme activity only for a certain period of time has a **constant value** (relationship is linear), then it decreases.

- Then the concentrations of the processed substrate (formed product) are no longer proportional to the concentration of the enzyme.



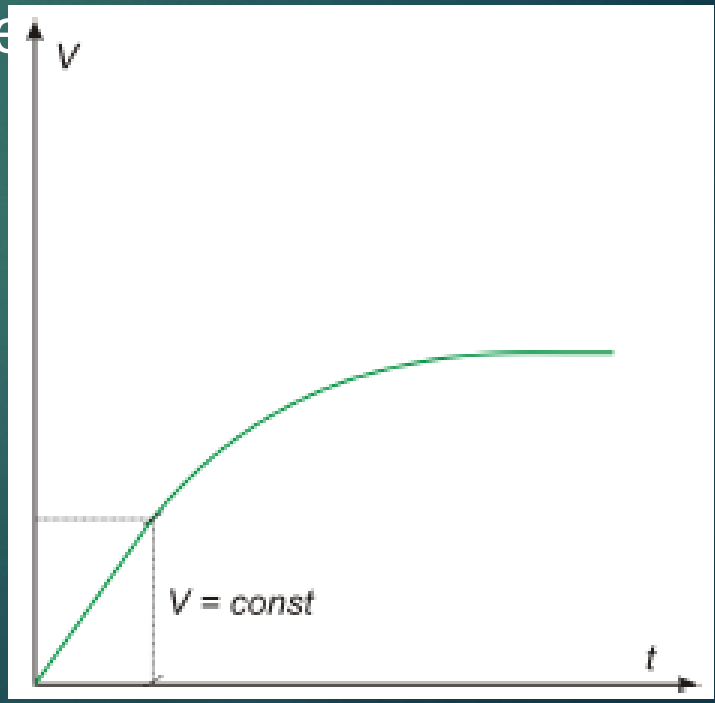
The dependence of the velocity of enzymatic reaction (V) on the time (t)

The dependence of the velocity of enzymatic reaction (V) on the time (t)

- Therefore, the measurements should be carried out, when the reaction rate has a constant value:

- the reaction has zero-order kinetics,
- in a short time from the moment the reaction begins (measurements of the initial velocity).

- **The initial V is independent of the order of reaction.**



The dependence of the velocity of enzymatic reaction (V) on the time (t)

The effects of enzyme concentration on the velocity of enzymatic catalysis

- We know, that the relationship between enzyme and substrate concentrations have an influence on the velocity of enzymatic reaction

- However, the question is whether the "enzyme concentration„ significantly influences the velocity of the reaction ?

- We will get the answer to this question from the experiment in which two solutions containing different concentrations of the same enzyme are used

Units of enzyme activity

The term *"enzyme concentration*" is not used in Biochemistry, because we are not interested in the determination of the concentration of protein in a solution.

Since the velocity of enzymatic reaction is determined by the amount of processed substrate per unit of time, and the process depends on the presence of the active enzyme, the term enzyme activity has been established.

The activity of an enzyme - is the activity which allows to convert a given amount of substrate
per given unit of time.

Units of enzyme activity

Units of enzyme activity:

- **Katal** [mol/sec] activity of enzyme which catalyses the conversion of one mol substrate in one sec

- **IU** [µmol/min], 1IU =16,67x 10^{-9} katal

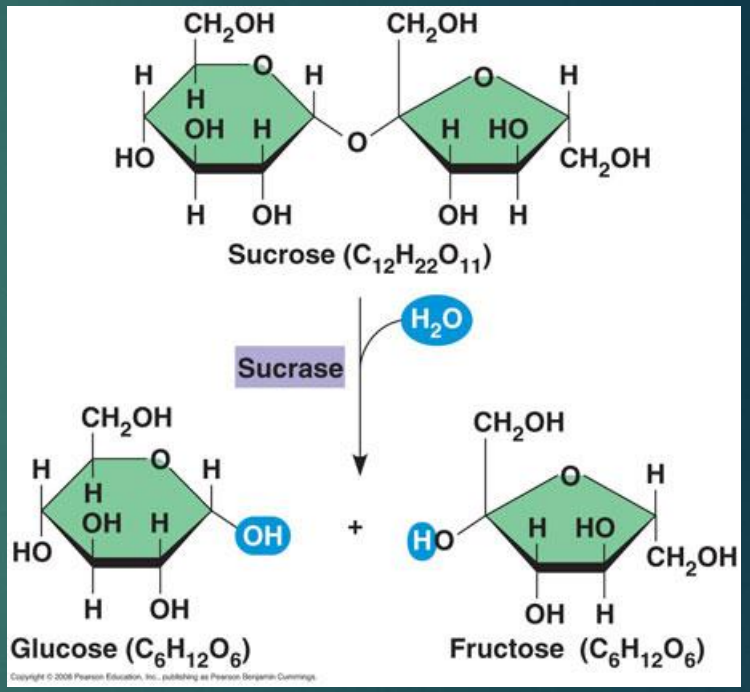
- **Turnover number** [number of substrate/sec]

Invertase (saccharase, sucrase)

- Invertase is an enzyme classified into the group of hydrolases.

- It hydrolyses sucrose only (it is strictly substrate specific) to monosaccharides: glucose and fructose

- Optimum pH: 4.0 and 7.0



After the enzymatic splitting of non-reducing sucrose – as the reducing glycosidic OH-groups of monosaccharides became free – it gives a positive result with the Fehling's test.

Invertase (saccharase, sucrase)

Measurement of enzyme activity:

- The activity of the enzyme can be performed by measuring the increase of new product.

- Determination of the reducing sugar (glucose) is based on the reduction of Cu^{2+} to Cu^{+}, which then reacts with the phosphomolybdenum reagent reducing it to molybdenum blue. The intensity of the blue color is proportional to the amount of reducing sugar.

The principle of the determination of invertase activity

1. The ring of carbohydrates with free glycosidic OH-group (glucose) can be opened.

2. The aldehyde group of the glucose molecule reduces Cu^{2+} ions to Cu^{+} ions.

$$2\ Cu^{2+} + 4\ OH^{-} + R—CHO \rightarrow 2\ CuOH + R—COOH + H_2O$$

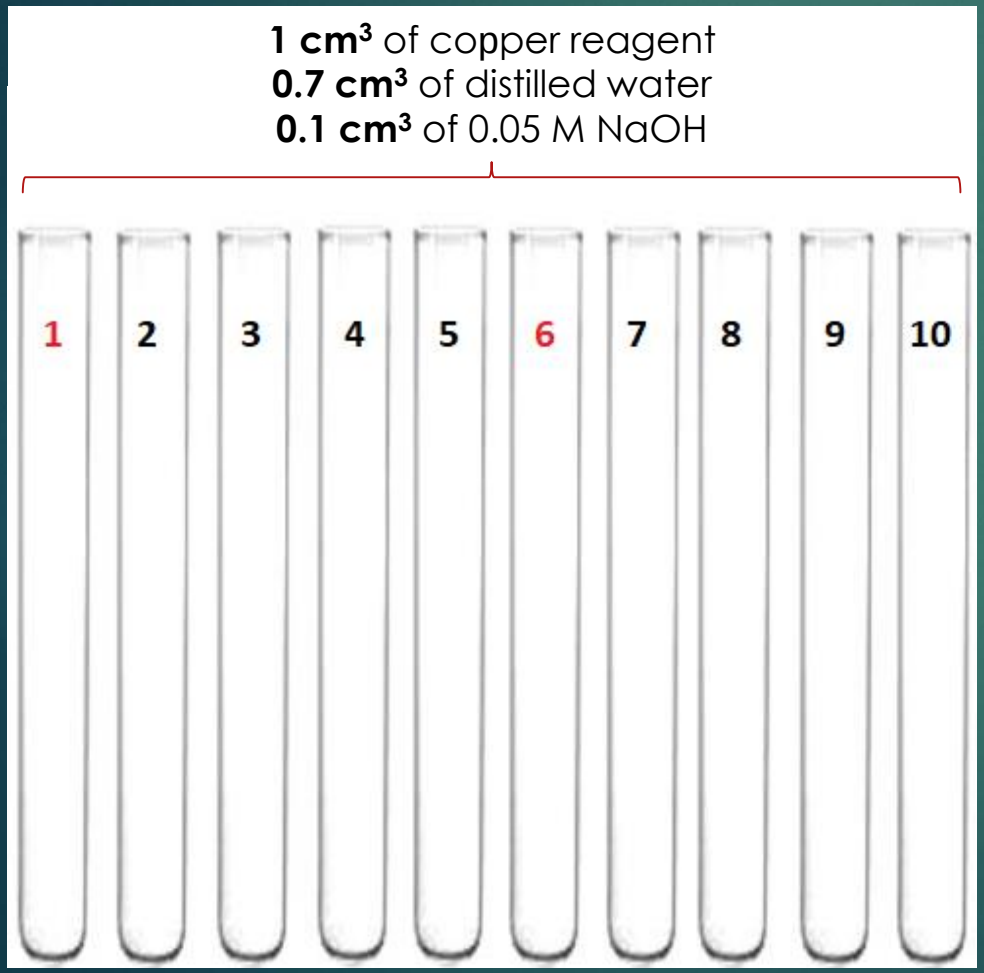
$$2\ CuOH \rightarrow \mathbf{Cu_2O\downarrow} + H_2O \text{ (after heating, brick-red precipitate)}$$

3. Copper (I) oxide reacts with the phosphoromolybdenum reagent and reduces it to molybdenum blue.

4. The intensity of the blue color is proportional to the amount of reducing sugars.

Procedure

1. Prepare the set of 10 test tubes with the following solutions.



Test tube 1 -> add:

- 0.1 cm^3 of undiluted enzyme
- 0.1 cm^3 of distilled water

Test tube 6 -> add:

- 0.1 cm^3 of 2x diluted enzyme
- 0.1 cm^3 of distilled water

Test tube 1 is a control for the tubes: 2, 3, 4, 5.

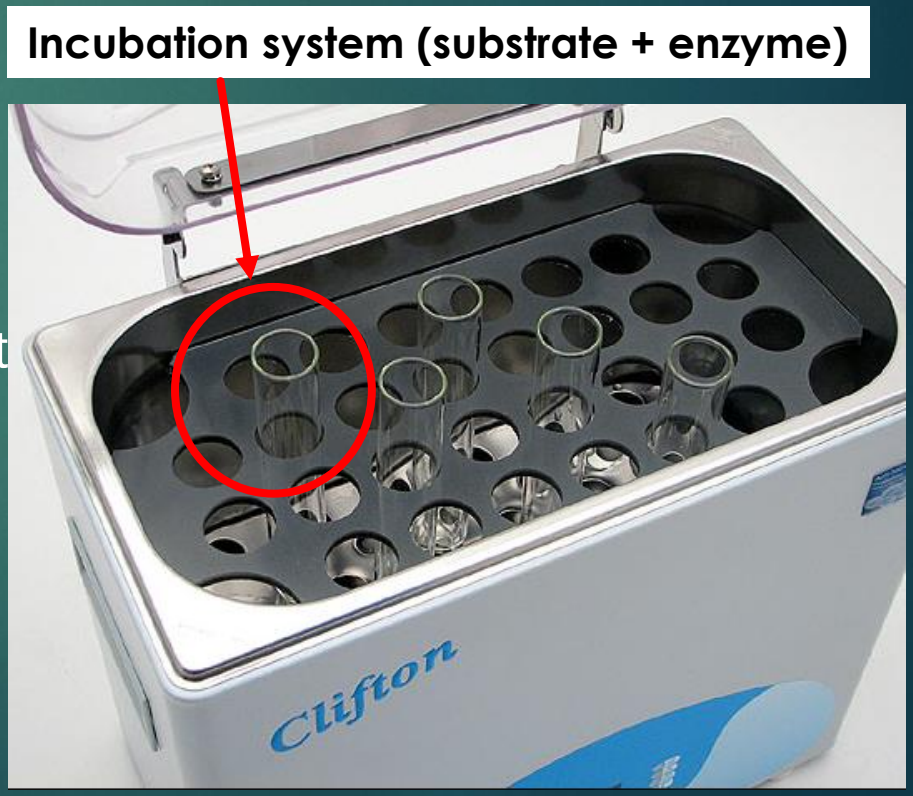
Test tube 6 is a control for the tubes: 7, 8, 9, 10.

Procedure

2. Prepare 2 incubation systems (A & B) in parallel:

Test tube A:
1 cm^3 of 0.8 mol/dm^3 sucrose (preheated to 37°C), and 1 cm^3 of the enzyme solution

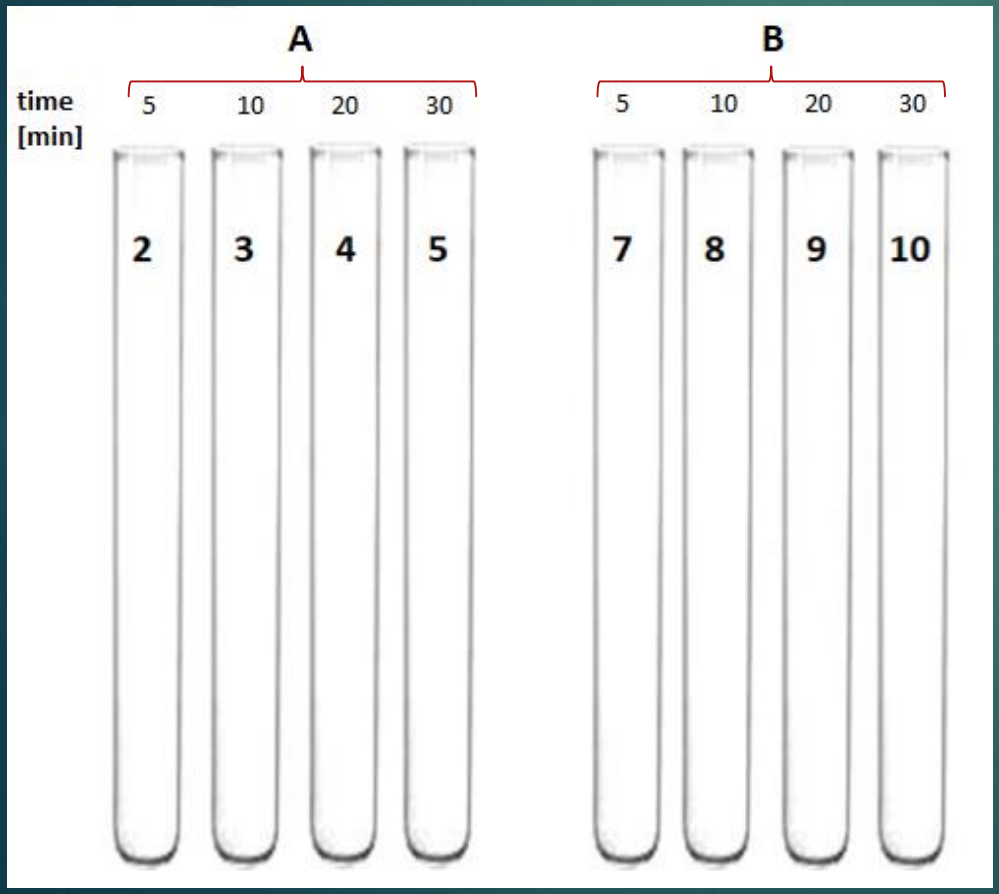
Test tube B
1 cm^3 of 0.8 mol/dm^3 sucrose (preheated to 37°C),
0.5 cm^3 of the enzyme solution,
and 0.5 cm^3 of distilled water (in result enzyme will be 2x diluted)



3. Immediately put the tubes into a water bath (37°C) and **note** time "0" - the moment of mixing the substrate with the enzyme.

Procedure

4. After 5, 10, 20 and 30 mins of incubation, take 0.2 cm^3 of the incubate from the tubes **A** and **B** into previously prepared tubes with the copper reagent, as follows:



after 5 mins
- to the test tube 2 and 7,

after 10 mins
- to the test tube 3 and 8,

after 20 mins
- to the test tube 4 and 9,

after 30 mins
- to the test tube 5 and 10.

Procedure

5. After 30 minutes:

- heat the tubes (10) for 8 minutes in a **boiling water bath**
- cool the tubes (using tap water)
- add 1 ml of phosphomolybdenum reagent to the tubes.
- mix the tubes **thoroughly**!

- dilute the tubes depending on the intensity of the color,
- measure the absorbance of the samples in the spectrophotometer
- at 610 nm:
  - test tubes 2, 3, 4, 5 against sample 1 (control)
  - test tubes 7, 8, 9, 10 against sample 6 (control)

Procedure

- Draw the standard curve, plotting the absorbance on the Y axis, and the time of enzyme action and on the X axis (2 curves for two different concentration of the enzyme in 1 chart).