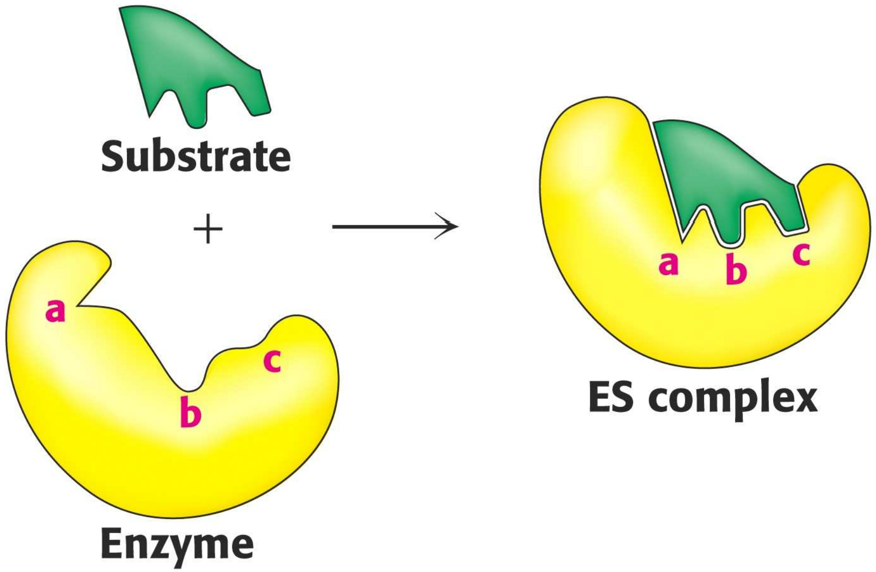
Enzymes – Part II

Biochemistry I
Lecture 2

2009 (J.S.)

The formation of an enzyme-substrate complex is the first step in enzymatic catalysis:



Induced-fit model of enzyme-substrate binding: The enzyme changes shape on substrate binding. The active site forms a shape complementary to the substrate only after the substrate has been bound.

Catalytic mechanisms

depend on the number of substrates.

Monosubstrate (monomolecular) reactions are not very frequent:

$$S \rightleftharpoons P \qquad S + E \rightleftharpoons \left[ES\cdots\text{transition state}\cdots EP \right] \rightleftharpoons P + E$$

Bisubstrate (bimolecular) reactions:

$$S_A + S_B + E \rightleftharpoons \left[ES_{AB}\cdots\text{transition state}\cdots EP_{AB} \right] \rightleftharpoons P_A + P_B + E$$

Multiple substrate reaction can be divided into two classes:

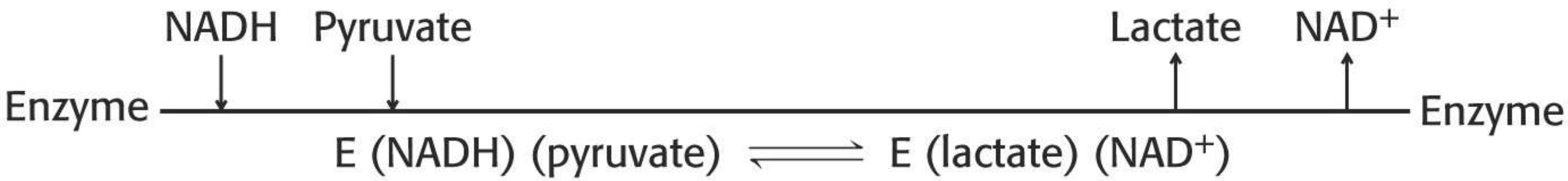
Sequential displacement – in the mechanism, all substrates must bind to the enzyme before any product is released (ternary complex of the enzyme and both substrates forms).

Double-displacement (ping-pong) reactions – one or more products are released before all substrates bind the enzyme (existence of a substituted enzyme intermediate, in which the enzyme is temporarily modified).

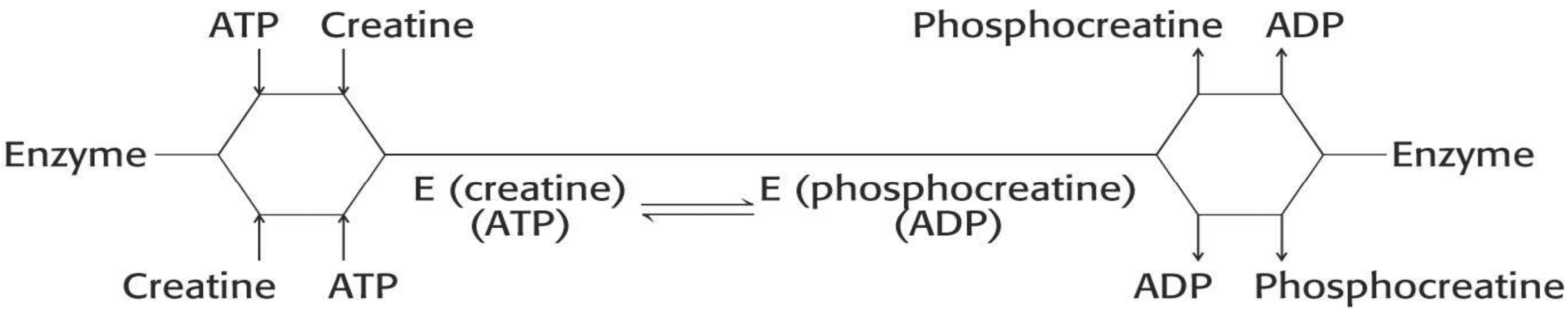
In the Cleland notation:

Sequential reaction

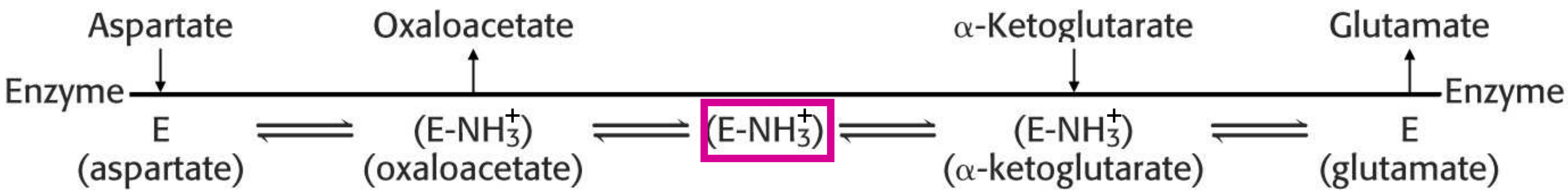
- type **ordered** - the substrates bind the enzyme in a defined sequence



- type **random** – the order of addition of substrates and release of products is random:



Ping-pong reaction:



The details of the catalytic mechanisms of enzymes will not be discussed in the lectures.

A brief general comment:

Decrease of the reaction free energy of activation $\Delta G^{\ddagger}$ is caused by **facilitating the formation of the transition state** of the reactive intermediates in the active site of enzymes and specific preferential binding (stabilization) of it.

Examples of **different types of catalytic mechanisms**:

- Catalysis through proximity and orientation effects (strained reactants)
- Covalent catalysis – formation of transitory covalent bonds between E and S
- Acid-base catalysis – protonization of substrates or catalytic groups of E
- Metal ion catalysis mediating redox reactions or shielding negative el. charges
- Electrostatic catalysis (after excluding water from the active site by binding of substrate)

The great catalytic efficiency arises from the simultaneous use of several of these catalytic mechanisms.

The fundamental terms in general reaction kinetics

Kinetics studies the rates of chemical reactions.

The term **velocity** (symbol v) is the reaction rate expressed in terms of change in the concentrations of reactants:

For the simple reaction S → P, the velocity is defined as

$$v = -\frac{1}{\nu}\frac{\Delta[\mathrm{S}]}{\Delta t} = \frac{1}{\nu}\frac{\Delta[\mathrm{P}]}{\Delta t}$$

S – substrate, P – product, ν – reaction stoichiometric coefficients (if there are any)

Because $v = \dfrac{c}{t}$, velocity is expressed in **mol × l^{-1} × s^{-1}**

Factors affecting velocities of reactions: temperature,
concentrations of reactants,
catalysts or inhibitors.

Velocity depends on the concentrations of reactants

This dependence is described in the **velocity equation**:

For the reaction m**A** + n**B** $\rightarrow$ xC,

$$v = k\,[\mathbf{A}]^{m}\,[\mathbf{B}]^{n}$$

where ***k*** is the **kinetic constant** that includes the specific reaction features as well as the temperature term ($\boldsymbol{k} = A \times e^{-Ea\,/\,\boldsymbol{R}T}$).

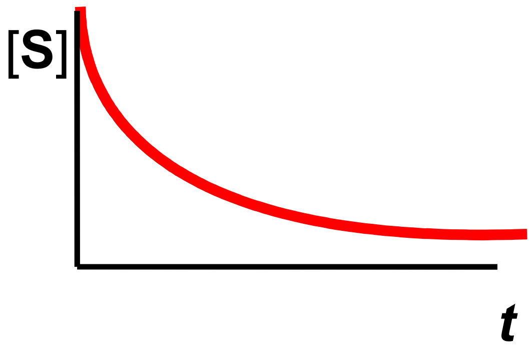
The sum of all exponents in velocity equations (m + n + ….) indicates the **reaction order**.
The equation mentioned above is a $(m+n)^{th}$-order reaction.

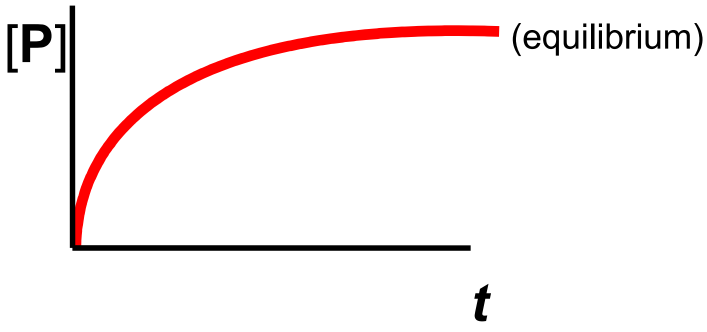
Due to decreasing concentrations of reactants in closed systems, there must be always a **gradual decrease of reaction velocity till the reaction reaches the equilibrium**.

Progress curves (kinetic curves)

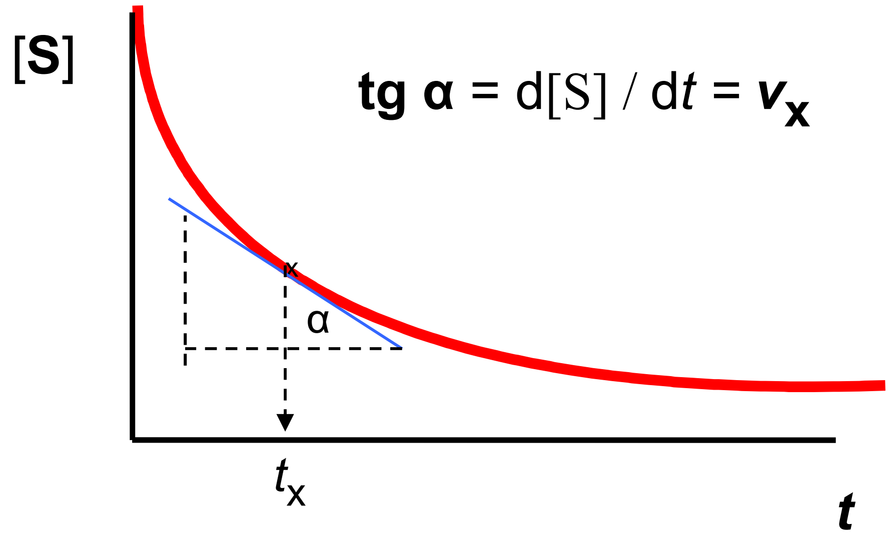
The progress - the time course of a reaction is shown by a plot of the concentration of any of the substrates or products against time.

Example: Both curves hold for the reaction S $\rightarrow$ P. It is a **first-order reaction** according to the velocity equation $v = k\,[\mathrm{S}]$.





The instantaneous velocity $\boldsymbol{v_x}$ at any particular time $\boldsymbol{t_x}$ is then given by the **slope of the tangent** to the curve at that time.



At equilibrium the net reaction velocity is zero.

For the **first-order reactions**

$$[\mathrm{S}]_t = [\mathrm{S}]_0\, e^{-kt} \quad \text{or} \quad v_t = v_0\, e^{-kt}$$

$[\mathrm{S}]_0$ – initial concentration of S,
v_0 – initial velocity, in the first moments of the reaction

Kinetics of enzyme-catalysed reactions

Let us consider an enzyme-catalysed transformation of substrate S to the product P:

$$\mathbf{E + S \rightleftharpoons ES \rightleftharpoons E + P}$$

The overall velocity of the reaction depends on the substrate concentration [S] as well as on the enzyme concentration [E]

Initial reaction velocities

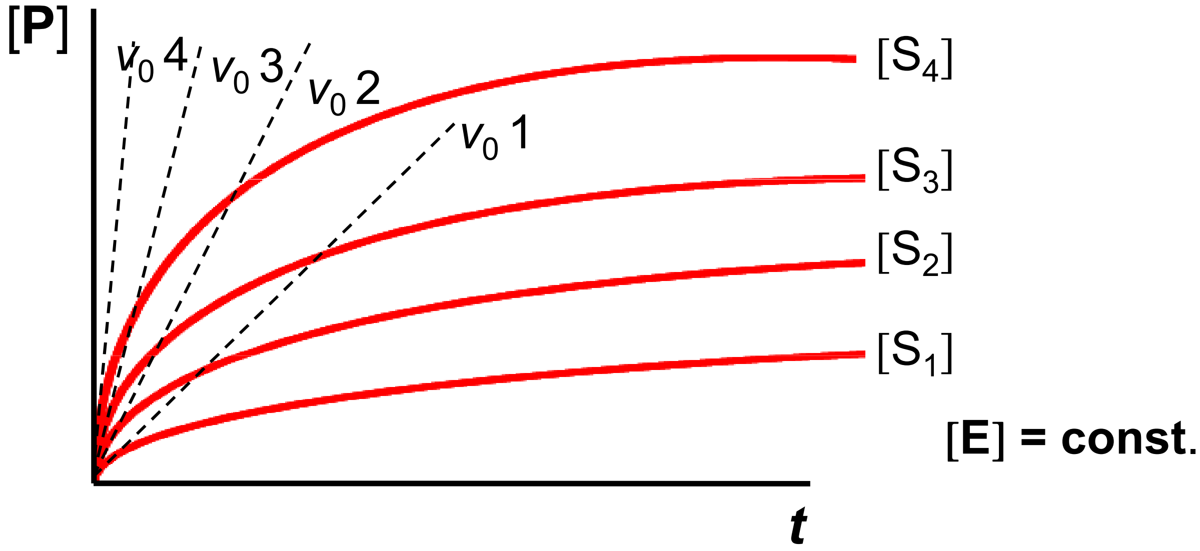
Initial velocities v_0 measured in the short time period after the reaction has started are used preferentially in kinetics studies considering that

- they are the highest under the given conditions (then they decrease),
- they are not influenced by the small decrease of the substrate concentration,
- the product concentration can be neglected (it is very low), and that is why
- there is no need to think of the reverse reaction (it is insignificant).

Dependence of initial velocity on substrate concentration

At a constant enzyme concentration, the velocity v_0 rises linearly as substrate concentration increases, and then begins to level till it reaches a limit value at high substrate concentrations.

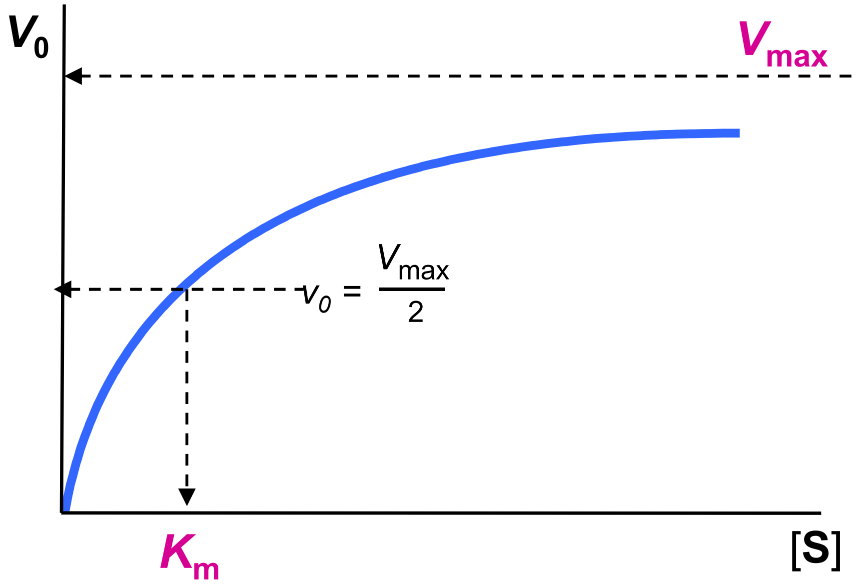
A series of measurements of initial reaction velocity must be arranged at a constant enzyme concentration [E] and different substrate concentrations [S] (in the range of 2 - 3 orders of magnitude).



From the obtained progress curves, the values of v_0 should be estimated and plotted against the corresponding [S] to gain a part of an rectangular hyperbolic curve:

A plot of $\boldsymbol{v_0}$ **against** [**S**] is called

a **saturation curve** or a **Michaelis plot** .



The hyperbole is asymptotic to certain limit value on the v_0 axis called maximal velocity V_{max} for the given concentration [E].

The concentration of substrate which gives half the maximum velocity is the Michaelis constant K_m (of the enzyme half-saturation).

Realize the distinction between progress curves and saturation curves:
A progress (kinetic) curve shows the time-progress of only one experiment, [S] = *f*(t).
A saturation curve (Michaelis plot) is derived from the multiple experiments, v_0 = *f*([S]).

The Michaelis- Menten equation

describes the dependence of v_0 on [S] and [E] in monosubstrate reactions.

$$\mathbf{E + S} \underset{-1}{\overset{1}{\rightleftharpoons}} \mathbf{ES} \underset{(-2)}{\overset{2}{\longrightarrow}} \mathbf{E + P}$$

At initial velocity v_0 in the reaction initial period, the net reaction does not depend on product concentration [P] and the reaction (–2) can be neglected.

If the kinetic constant $k_1 > k_2$, the reaction 2 is decisive for the net reaction and the overall velocity of P appearance is $\boldsymbol{v_0 = k_2\,[ES]}$. When the enzyme is fully saturated by the substrate, then $v_0 = V_{max} = k_2\,[E]_{tot}$.

$$\boxed{v_0 = V_{max}\frac{[S]}{[S] + K_m}}$$

Leonar Michaelis and Maud Menten, 1913

Sometimes the reaction is cited in the form $v_0 = \dfrac{V_{max}}{1 + \dfrac{K_m}{[S]}}$

By separating of K_m from the equation we obtain the definition $\boldsymbol{K_m} = [\mathbf{S}]\left(\dfrac{V_{max}}{v_0} - 1\right)$

Deduction of the Michaelis-Menten equation:

$$\mathbf{E + S} \underset{-1}{\overset{1}{\rightleftharpoons}} \mathbf{ES} \underset{(-2)}{\overset{2}{\longrightarrow}} \mathbf{E + P}$$

At initial velocity v_0 in the reaction initial period, the net reaction does not depend on product concentration [P] and the reaction (–2) can be neglected.

The Michaelis-Menten equation (simply Michaelis kinetics) is based on **assumptions** that

- [S] » $[E]_{tot}$ and so the difference between [S] and ($[S]_{tot}$ - $[S]_{ES}$) can be neglected,
- the kinetic constant $k_1 > k_2$ (reaction 2 is decisive for the net reaction S $\rightarrow$ P i.e. the overall velocity of P formation is $\boldsymbol{v_0 = k_2\,[ES]}$),
- the reaction passes through a state with a steady concentration [ES].

Then velocity of ES formation $v_1 = k_1\,[S]\,([E]_{tot} - [ES])$,
velocity of ES breakdown $(v_2 + v_{-1}) = (k_2 + k_{-1})\,[ES]$.

These two velocities are equal in the supposed steady state, from that

$$([S]\,([E]_{tot} - [ES]) / [ES] = \boldsymbol{(k_2 + k_{-1}) / k_1 = K_m}.$$

After separation of [ES], multiplication of the obtained equation by k_2 and by substitution v_0 for $k_2[ES]_{tot}$ and V_{max} for $k_2[E]_{tot}$ (because v_0 shall reach up to V_{max} if enzyme is completely saturated by the substrate) we get the Michaelis-Menten equation.

If **[S] « K_m**, then $v_0 = V_{max} \frac{[S]}{[S] + K_m} \approx \boxed{\frac{V_{max}}{K_m}} [S] = k\,[S]^1$

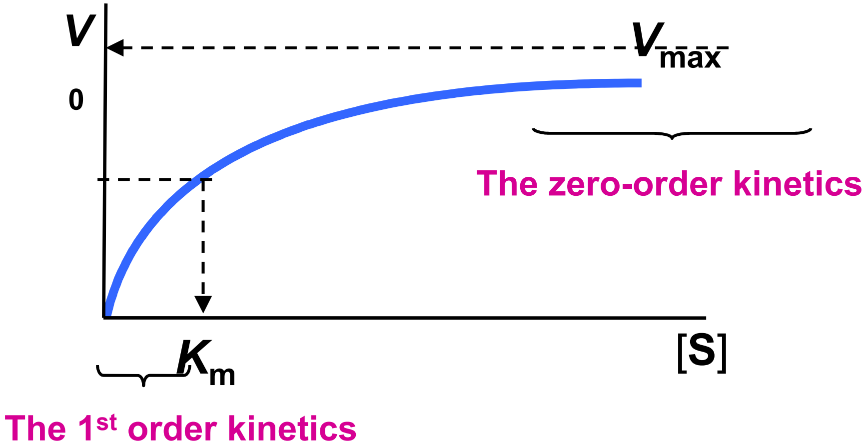
At very low concentrations of the substrate there is the **1st order kinetics**.

If **[S] = K_m**, then $v_0 = V_{max} \frac{[S]}{[S] + [S]} = V_{max} \frac{[S]}{2\,[S]} = \frac{1}{2} V_{max}$

that defines the Michaelis constant K_m

If **[S] » K_m**, then $v_0 = V_{max} \frac{[S]}{[S] + K_m} \approx \boxed{V_{max}} \frac{[S]}{[S]} = k\,[S]^0$

At [S] much higher than the value of K_m it is the **0^{th} order kinetics**
(e.g. at [S] = 10 K_m velocity v_0 equals 0.91 × V_{max}).

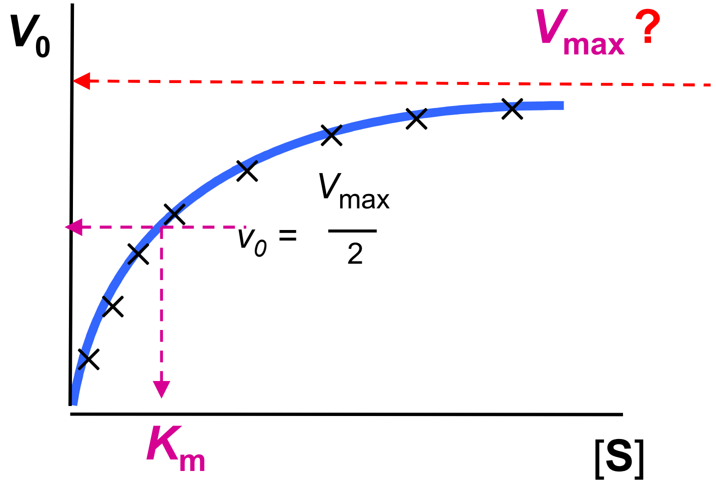


In **zero-order kinetics** the velocity does not depend on substrate concentration [S], $v = k\,[S]^0 = k$.

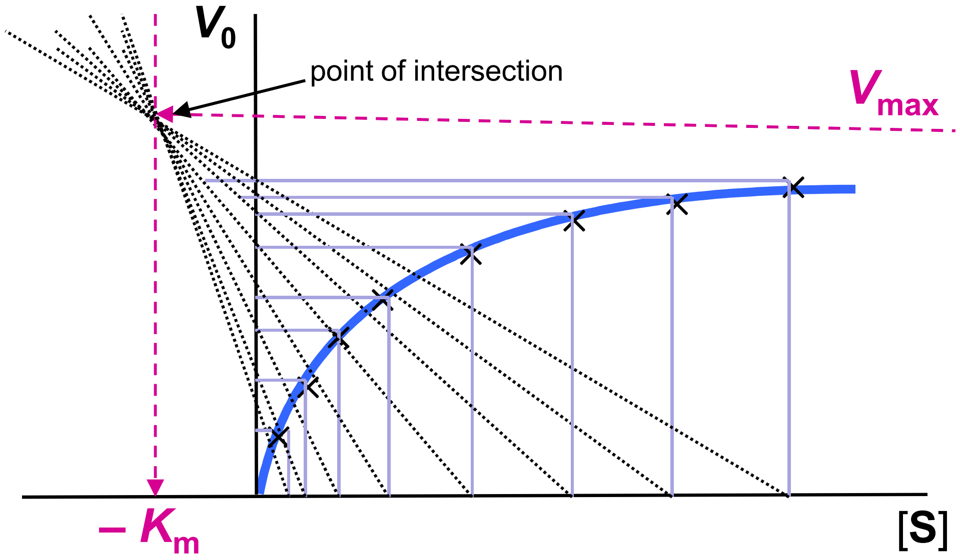
At very high substrate concentrations, the enzyme is fully saturated by an substrate and in addition, there is a surplus of substrate. Then the reaction is of 0^{th} order kinetics until the decrease of [S] is not sufficient to saturate all enzyme molecules fully. After that. the 0^{th} kinetics transforms in the 1^{st} order (or a higher order) kinetics.

Determination of K_m and V_{max}

Rough visual estimate of V_{max}



The plot of Eisenthal and Cornish-Bowden



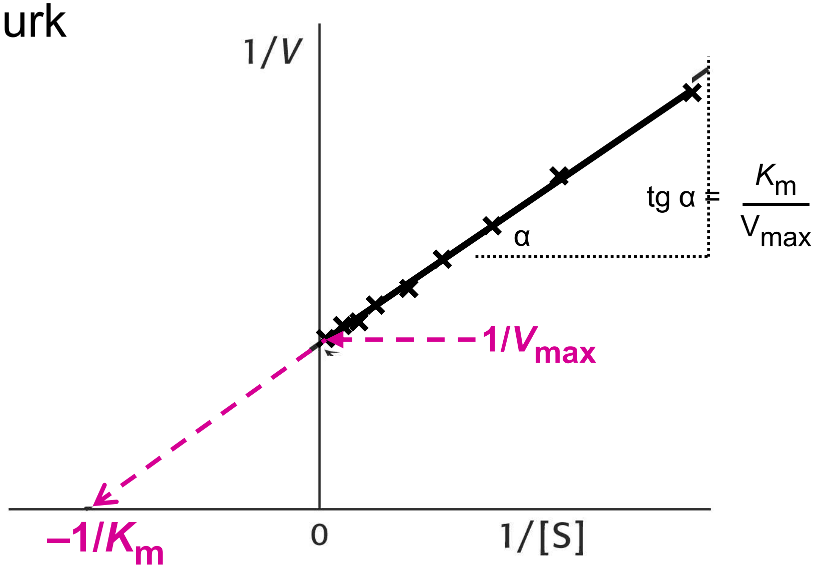
The linear double reciprocal plot of Lineweaver-Burk

Michaelis equation $\quad v_0 = \frac{V_{max}\,[S]}{[S] + K_m}$

The reciprocal form of Michaelis equation

$$\frac{1}{v_0} = \boxed{\frac{K_m}{V_{max}}}\,\frac{1}{[S]} + \boxed{\frac{1}{V_{max}}}$$

(The equation of a line $\quad y = a\,x + b$)



Significance of K_m and V_{max}

The Michaelis constant K_m ("the constant of half-saturation") is the concentration of substrate [S] which gives *half* the maximum velocity V_{max}.

The value of K_m is independent of enzyme concentration and defines the substrate concentration range that an enzyme requires in order to work efficiently.

K_m is inversely related to the affinity of the enzyme for its substrate. If more substrates with similar structure exist, then the best natural substrate is one with the least value of K_m.

If there is a need to measure the catalytic activity of an enzyme in zero-order kinetics reaction, the substrate concentration has to be at least several times higher than the K_m value.

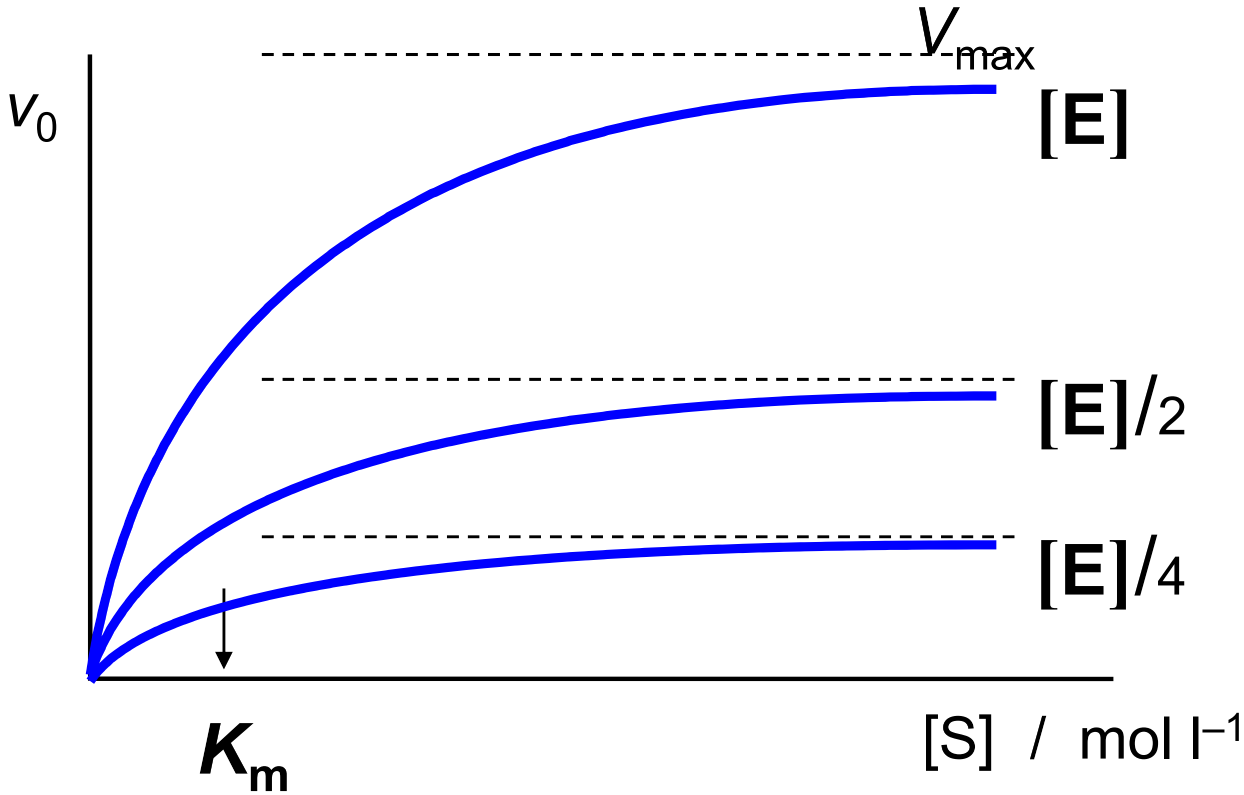
K_M values of some enzymes

Enzyme	Substrate	K_m / µmol l^{-1}
Chymotrypsin	Acetyl-L-tryptophanamide	5000
Lysozyme	Hexa-*N*-acetylglucosamine	6
β-Galactosidase	Lactose	4000
Threonine deaminase	Threonine	5000
Carbonic anhydrase	CO_2	8000
Penicillinase	Benzylpenicillin	50
Pyruvate carboxylase *)	Pyruvate	400
	HCO_3^-	1000
	ATP	60
Arginine-tRNA synthetase *)	Arginine	3
	tRNA	0.4
	ATP	300

*) Take notice that there are different values of the K_m for particular substrates!

Dependence of initial velocity on enzyme concentration

The Michaelis plots at different enzyme concentrations:



V_{max} is directly proportional to the enzyme concentration [E].

K_m does not change at various concentrations [E].

Enzymes differ in efficiency to catalyze.
Two quantities exist for comparing of the ability:

Catalytic constant k_{cat}

The overall velocity of substrate conversion into products in a given reaction when the enzyme is completely saturated by the substrate is $V_{max} = k_{cat}\,[E]$; then

$$k_{cat} = \frac{V_{max}}{[E]}$$

It denotes either the number of substrate molecules transformed in the reaction by one enzyme molecule per second – the **turnover number**, or the catalytic activity (moles of substrate transformed per second) of one mole of the enzyme – the **molar activity** (kat / mol).

The numerical values of both quantities are the same.

Catalytic efficiency k_{cat} / K_m

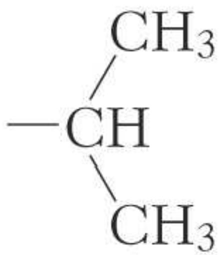
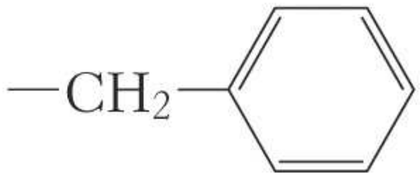
takes into consideration K_m that is inversely related to the enzyme affinity for its substrate.

Examples of the turnover numbers of some enzymes

Enzyme	Turnover number (s^{-1})
Carbonic anhydrase	600,000
3-Ketosteroid isomerase	280,000
Acetylcholinesterase	25,000
Penicillinase	2,000
Lactate dehydrogenase	1,000
Chymotrypsin	100
DNA polymerase I	15
Tryptophan synthetase	2
Lysozyme	0.5

Substrate preferences of chymotrypsin

Efficiency:

Amino acid in ester	Amino acid side chain	k_{cat} / K_m
Glycine	$—H$	1.3×10^{-1}
Valine		2.0
Norvaline	$—CH_2CH_2CH_3$	3.6×10^{2}
Norleucine	$—CH_2CH_2CH_2CH_3$	3.0×10^{3}
Phenylalanine		1.0×10^{5}

Source: After A. Fersht, *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding*

Enzymes for which k_{cat}/K_M is close to the diffusion-controlled rate of encounter

Enzyme	k_{cat}/K_m
Acetylcholinesterase	1.6×10^8
Carbonic anhydrase	8.3×10^7
Catalase	4×10^7
Fumarase	1.6×10^8
Triose phosphate isomerase	2.4×10^8
Superoxide dismutase	7×10^9

Source: After A. Fersht, *Structure and Mechanism in Protein Science: A Guide to Enzyme Catalysis and Protein Folding* (W. H. Freeman and Company, 1999),

Assays of enzymes

Assays of enzymes in a tissue or a body fluid by **measuring the mass** (mass concentrations in µg/l, µg/g tissue) or amount of substance (nmol/l, nmol/g) are rather exceptional.
For that purpose, immunochemical methods are the most convenient..

Assays of enzyme catalytic activities

The amount of an enzyme in a complex mixture is usually determined by **measuring the velocity of the reaction catalysed by a given amount of the sample**, making the assumption that this velocity is proportional to the amount of enzyme present.

Catalytic activity of an enzyme - simply "enzyme activity"

means the velocity of the reaction which can be ascribed to the catalytic action of the enzyme.

The SI unit of catalytic activity is **katal** – the activity that catalyses transformation of one mole of the substrate per one second

1 **kat** = 1 **mol / s**

The older unit is still in use in certain countries,
so-called **international unit** – the activity catalysing transformation of one micromole of the substrate per one minute.

1 **IU** = 1 **μmol / min**

1 μkat = 60 IU 1 IU = 16.6 nkat

Catalytic concentration is the catalytic activity estimated in certain **volume of a liquid sample** (usual units μkat / l, nkat / l).

Specific activity informs of the activity of usually **1 mg of proteins** present in solid samples.

Methods for estimation of enzyme catalytic activities

The common prerequisites: nearly optimal temperature and pH value,
presence of necessary cofactors,
absence of inhibitory factors.

The **zero-order kinetics** is preferred (high substrate concentrations)..

1 The **constant time method**
Reactions proceed for a fixed time, then are stopped by inactivating the enzyme, and the concentration of a substrate (or product) are measured. The average velocity is calculated.

2 The **kinetic method**
Changes in substrate (or product) concentrations are measured continually in the course of the reaction, e.g. by spectrophotometers.

If only the **1st order reaction** can be arranged, kinetic methods are preferred. It is necessary to calculate the value of the kinetic constant k, from which the initial velocity v_0 (that is directly proportional to [E]) can be derived: $k = \ln([S]_{t1} / [S]_{t2}) / (t_2 - t_1)$

Inhibitors of enzyme activity

Inhibitors are substances which reduce enzyme activities.
There are two major classes – irreversible and reversible inhibitors.

Irreversible inhibition

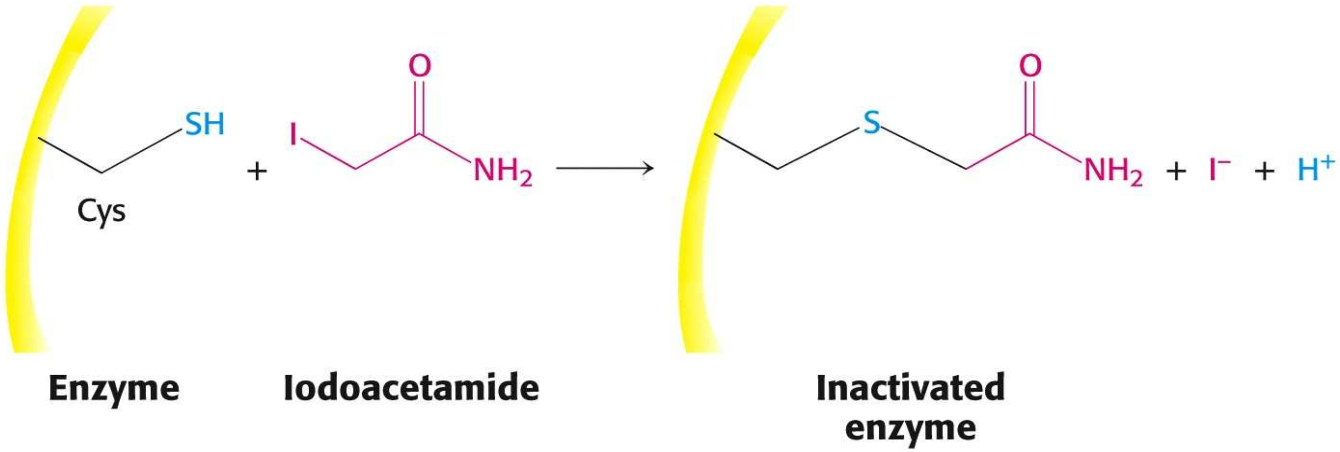
Irreversible inhibitors are usually compounds not of biological origin, which bind onto an enzyme mostly **covalently** and make substrate binding impossible.

Some of them called "active-site directed inhibitors" are used in experimental studies of enzymes because they permit to map the active sites (affinity labels structurally similar to the substrate, other group-specific reagents).

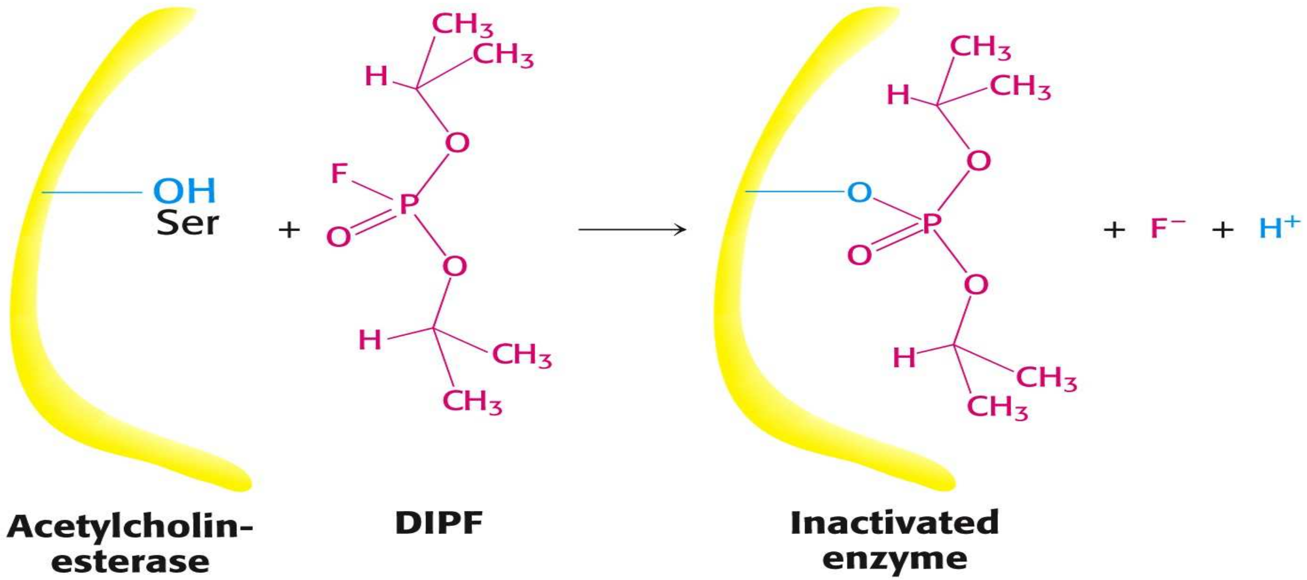
Heavy metal ions bind and inhibit irreversibly enzymes during isolation.

Mechanism-based inhibitors (**suicide inhibitors**) are recognized as substrates, initially processed, but catalysis generates a reactive intermediate that inactivates the enzyme (e.g. α_1-antitrypsin, penicillin, aspirin).

Irreversible inhibition – examples:



Iodoacetamide (specific reaction with –SH groups) can be used to map the active sites.



Diisopropyl fluorophosphate (and similar pesticides and nerve gases) inhibits acetylcholine esterase by phosphorylation of a crucial serine residue.

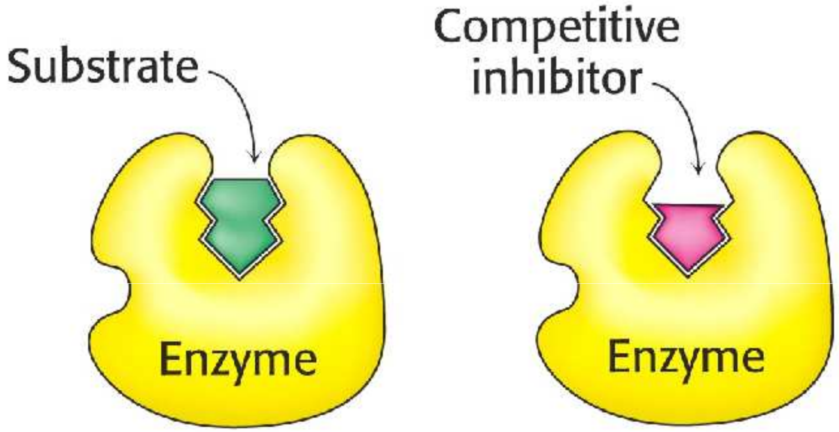
Reversible inhibition

In contrast with irreversible inhibitors, reversible inhibitors bind to the enzyme loosely and can rapidly dissociate from the enzyme-inhibitor complex. These inhibitors are classified as **competitive**, **non-competitive** and uncompetitive.

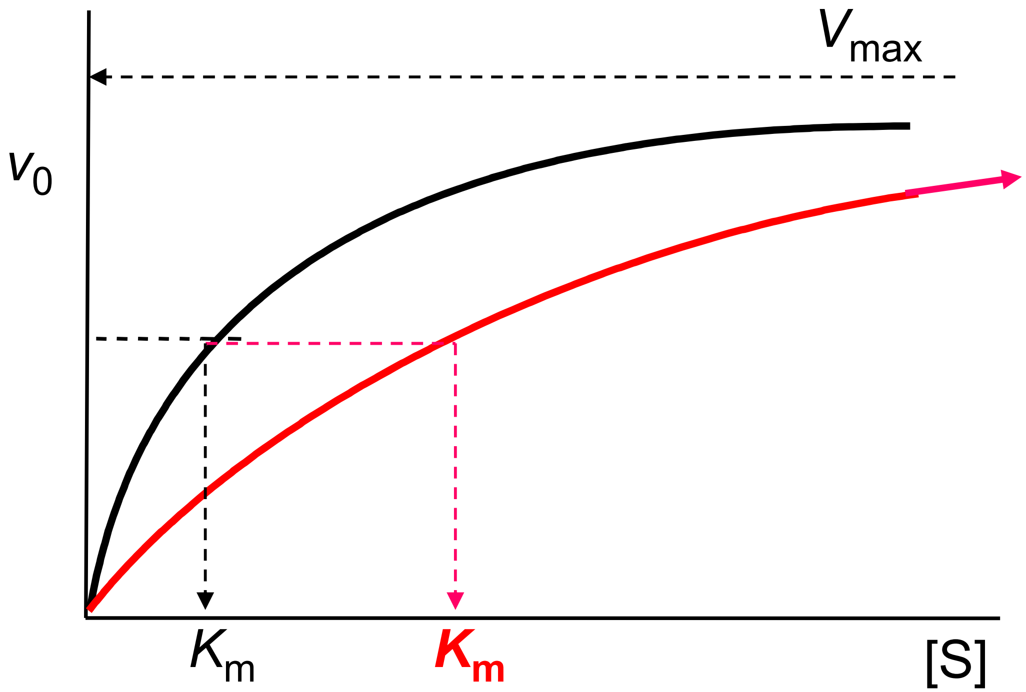
Competitive inhibitors

resemble the substrates and **bind to the active sites**, but the complex is non-reactive.

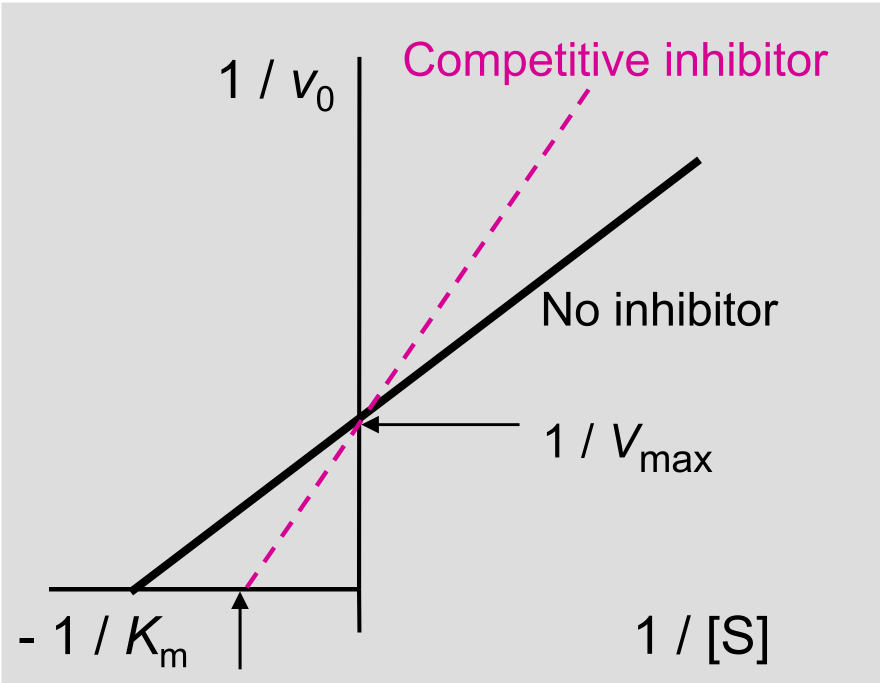
They compete with normal substrates for the active sites.



Competitive inhibitors **increase the value of K_m** without any change in V_{max}



The $\boldsymbol{V_{max}}$ **can be reached** even in the presence of inhibitor, but at much higher concentrations of [S] that have to overcome the competing inhibitor concentration.



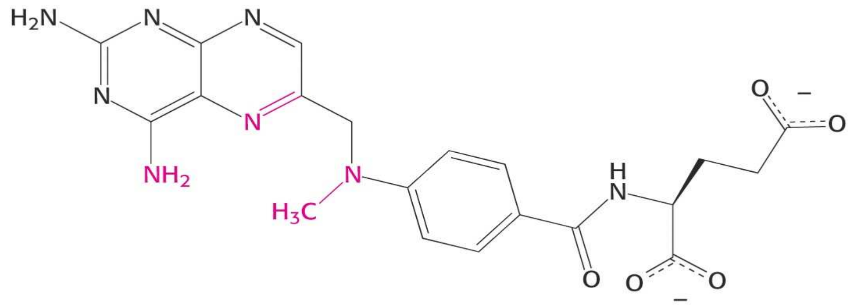
Examples:

Malonate competitively inhibit succinate dehydrogenase

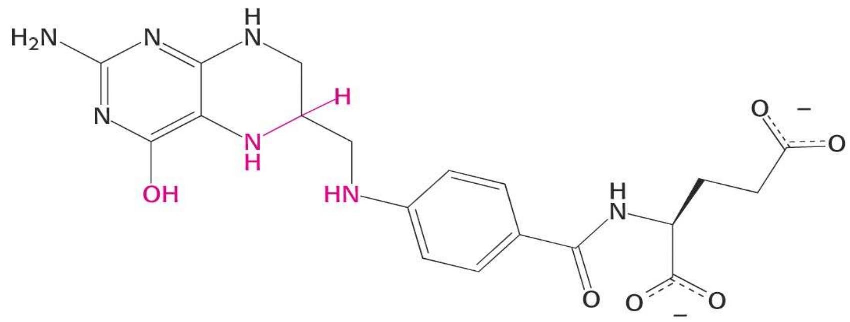
$$\begin{array}{c} COO^- \\ | \\ CH_2 \\ | \\ CH_2 \\ | \\ COO^- \end{array} \qquad \begin{array}{c} COO^- \\ | \\ CH \\ | \\ COO^- \end{array}$$

Succinate **Malonate**

Methotrexate competitively inhibits active sites for tetrahydrofolate of the dihydrofolate reductase in the synthesis of purine and pyrimidine bases of nucleic acids. It is used to treat cancer.



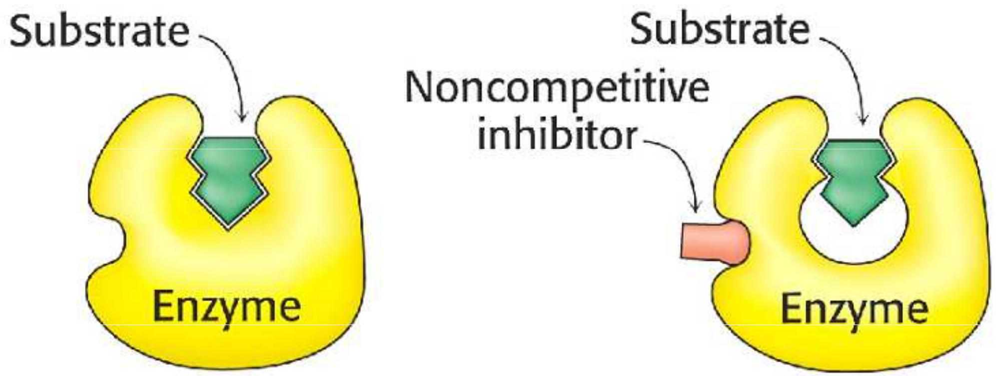
Methotrexate



Tetrahydrofolate

Non-competitive inhibition

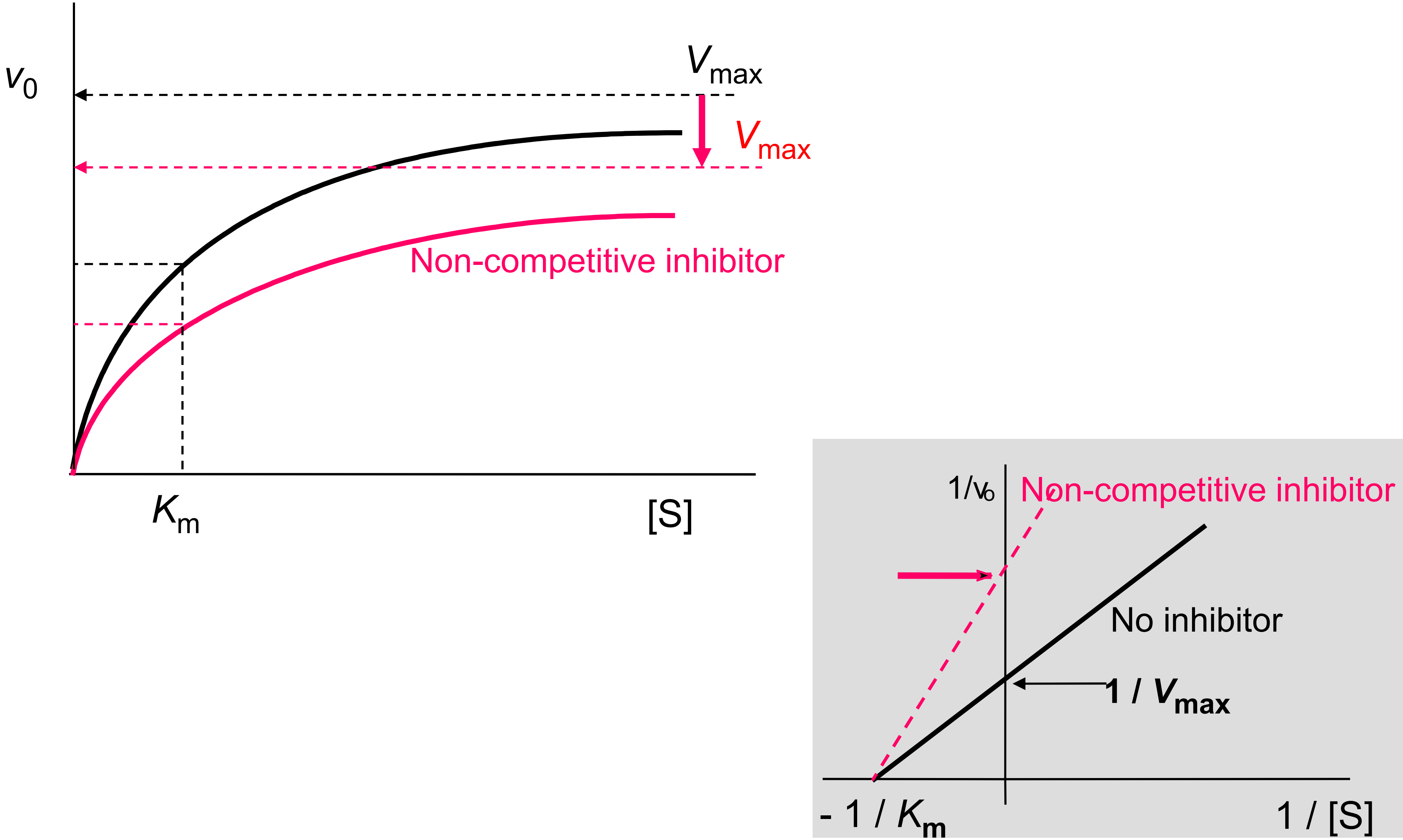
Non-competitive inhibitors **bind to both free enzyme and enzyme-substrate complex**, but in contrast to competitive inhibitors, **not in the active site** (the structures of inhibitors is distinct from the substrates.



Non-competitive inhibition cannot be overcome by increasing the substrate concentration. The non-inhibited remaining molecules of the enzyme behave like a more diluted solution of the enzyme.

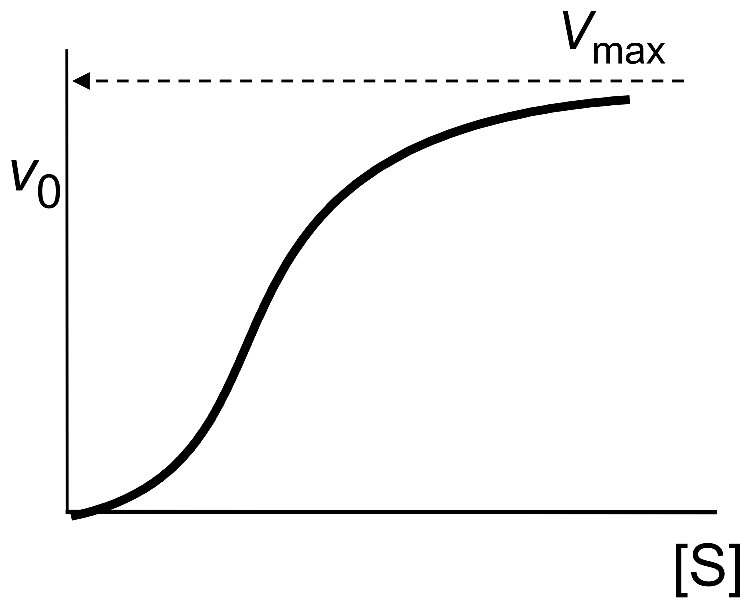
Uncompetitive inhibitors bind only **to the enzyme-substrate complex**
- decrease both K_m and V_{max}.

Non-competitive inhibitors **decrease V_{max}** without any change in K_m



Cooperative effect, allosteric enzymes, allosteric effectors

Not all enzymes obey the Michaelis kinetics (M.-M. equation). Regulatory enzymes are frequently **oligomers** that consist of several subunits (protomers). Those enzymes show saturation curves which deviate from Michaelis (hyperbolic) behaviour – saturation curves exhibit a **sigmoid dependence of v_0 on [S]**.



Cooperative effect

In these **allosteric enzymes** (and also in some not catalysing proteins, e.g. haemoglobin) the **binding of substrate** (oxygen to haemoglobin, resp.) to one active site can affect the properties of other active sites in the same oligomeric molecule.

The binding of substrate becomes **positively cooperative,** when the binding of substrate to one active site facilitates substrate binding to the other sites on other subunits due to induced changes in conformation.

Allosteric effectors

In addition, the activity of such enzymes may be altered by **regulatory molecules that are allosteric to the substrate** (having their structure distinct from the substrate) and bind reversibly to specific sites **other than the active sites** – to the **allosteric sites.**

The binding of an allosteric effector may either stimulate or inhibit the enzyme activity.