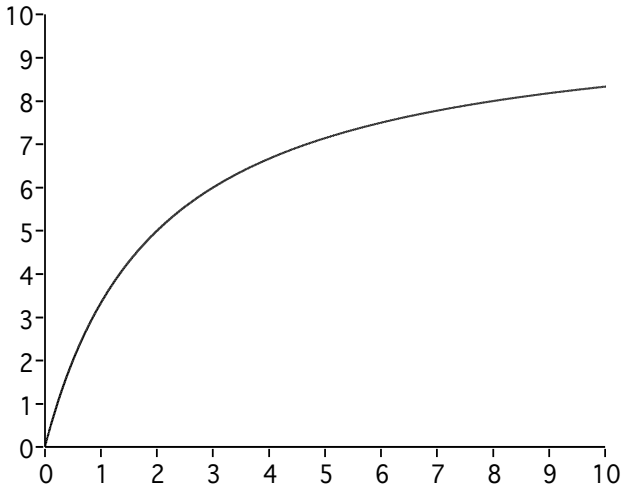
Enzyme kinetics & inhibition

Sherry Mowbray



Today’s motto: Don’t panic!

Some basics of chemical kinetics

$$A + B \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} C$$

Rate constants, *k*, relate the rate of reaction and the concentrations of one or more reactants.

First order rate constants:

$$C \xrightarrow{k_{-1}} A + B \qquad d[C]/dt = -k_{-1}[C]$$

Second order rate constants:

$$A + B \xrightarrow{k_1} C \qquad d[C]/dt = k_1[A][B]$$

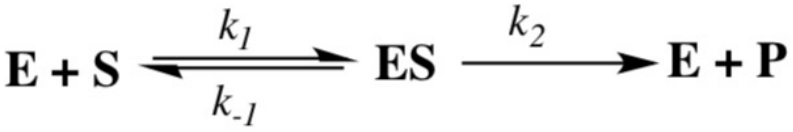
Pseudo-first order rate constants (when B is in large excess):

$$A \xrightarrow{k_1[B]} C \qquad d[C]/dt = k_1[B] \cdot [A]$$

Equilibrium constants, *K*, can be defined by the ratio of rate constants, e.g. for dissociation of C to A + B, $K_d = \frac{k_{-1}}{k_1}$

For enzyme reactions

- We usually simplify (assume k_{-2} is small):



- Second order:
  - E + S -> ES
- First order:
  - ES -> E + S
  - ES -> E + P

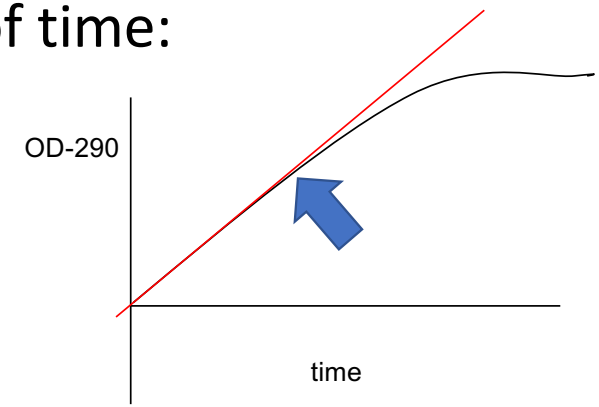
What we will do in the lab…

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

Measure change in [S] or [P] over a period of time:

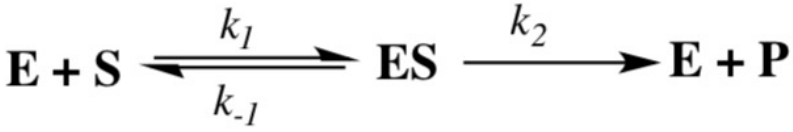
-d[S]/dt = d[P]/dt = v

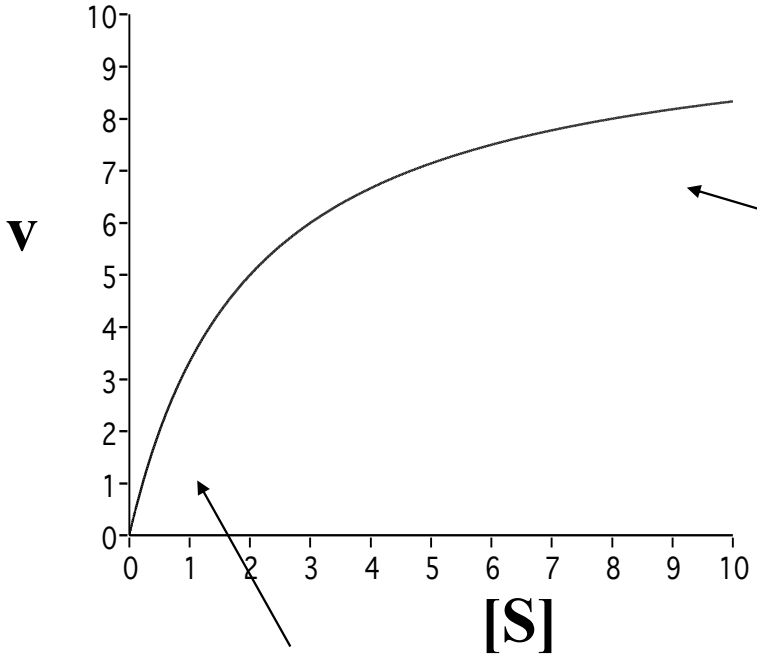
i.e., how fast is something happening?



Measure v at different [S].

What we will see



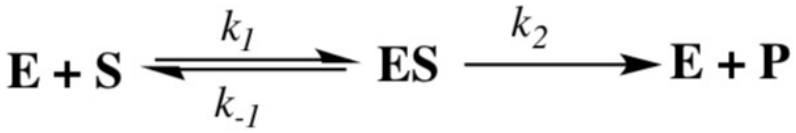


At high [S]
v becomes
nearly constant
= saturation with S, [ES] = [E_{total}].

We call this V_{max}
Equal to k_2 [ES] = k_2 [E_{total}].

At low [S]
v increases linearly

The math that descibes this



- Can usually describe enzyme behavior using the Michaelis-Menten equation, which is based on three assumptions:
  - the steady-state approximation (d[ES]/dt = 0)
  - the free ligand approximation (if [S] >> [E], then $[S]_{free} \sim [S]_{total}$)
  - the rapid equilibrium approximation (k_1 and k_{-1} are both >> k_2)

- The Michaelis-Menten equation

$$v = \frac{V_{max}[S]}{K_m + [S]} \quad \text{or} \quad \frac{k_2[E_{total}][S]}{K_m + [S]}$$

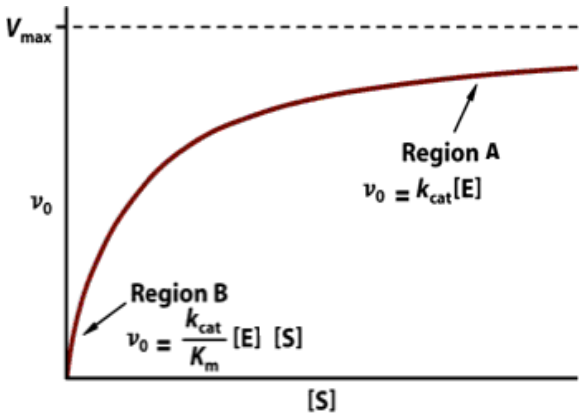
where $V_{max} = k_2[E_{total}]$ (we will usually refer to k_2 as k_{cat})

and. $K_m = \frac{(k_{-1} + k_{cat})}{k_1}$

Michaelis-Menten kinetics, simplified

Region A: [S] high (saturating)
reaction rate depends $[E]_{tot}$, not [S]
$v_o = k_{cat}[E]_{tot} = V_{max}$

Region B: [S] low (most biology)
rate depends on both [S] and $[E]_{tot}$
$v_o = (k_{cat}/K_m)[E]_{tot}[S]$
(2^{nd} order reaction)



You need values in BOTH regions to know both k_{cat} and K_m

You can get the catalytic constants k_{cat} and K_m from nonlinear fitting with the MM equation, or with linear versions like LB and HW

Why do we want catalytic constants?

- k_{cat} (turnover number)
  - the number of molecules of P each E can produce per unit time (usually seconds)

  $= V_{max}/[E]$

  In some cases, there are multiple steps, then it is usually the rate constant for the ”rate-limiting” step of the reaction

- K_m (Michaelis constant)
  - the concentration of S needed to get halfway to V_{max}
  - in many (but not all) cases, an approximation of the K_d for E binding to S (but this fails when E and S associate very quickly, for example)

Big K, little k? It matters! Please use the right one!

K_m is <u>not</u> actually a measure of affinity!

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} E + P$$

$$\frac{k_2+k_{-1}}{k_1} = K_m$$

$K_m \sim K_d$ only when chemistry is slow, $k_2 << k_{-1}$

$$K_m = \frac{k_{-1}}{k_1} = K_d$$

Slide from Prof. Ralf Morgensten, KI

The relation of K_m and K_d

$K_m = K_d$ when chemistry (k_2) is slow (in comparison to k_{-1})

$K_m > K_d$ in very efficient enzymes ($k_2 > k_{-1}$)

$K_m < K_d$ when intermediates occur after ES (Fersht, p.107)

$$\frac{k_2+k_{-1}}{k_1} = K_m \qquad \frac{k_{-1}}{k_1} = K_d$$

Slide from Prof. Ralf Morgensten, KI

- k_{cat}/K_m **(unit is M^{-1} s^{-1})** is a measure of the efficiency of the enzyme (cannot be higher than the diffusion controlled encounter rate 10^9 M^{-1} s^{-1})

- Most enzymes in biology operate in Region B.
Here k_{cat}/K_m gives a measure of how fast S goes to P

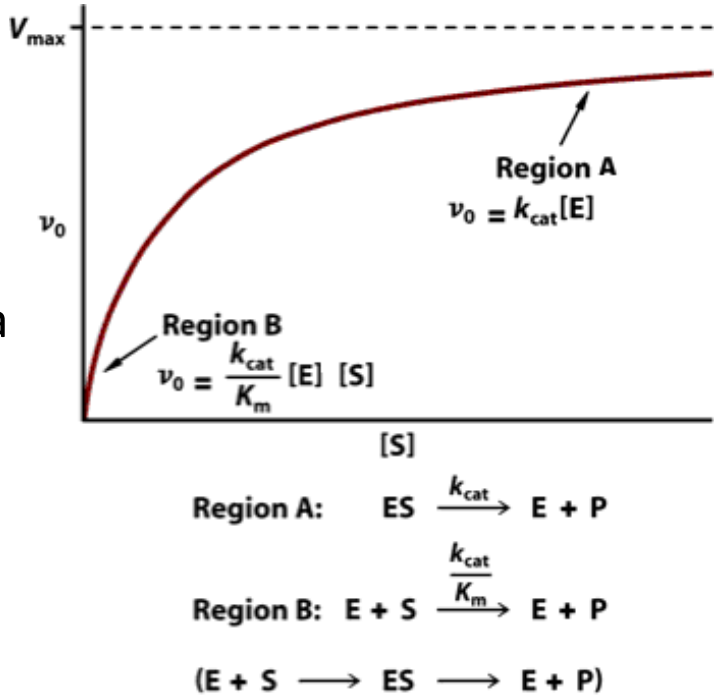


Figure 5-5 Principles of Biochemistry, 4/e
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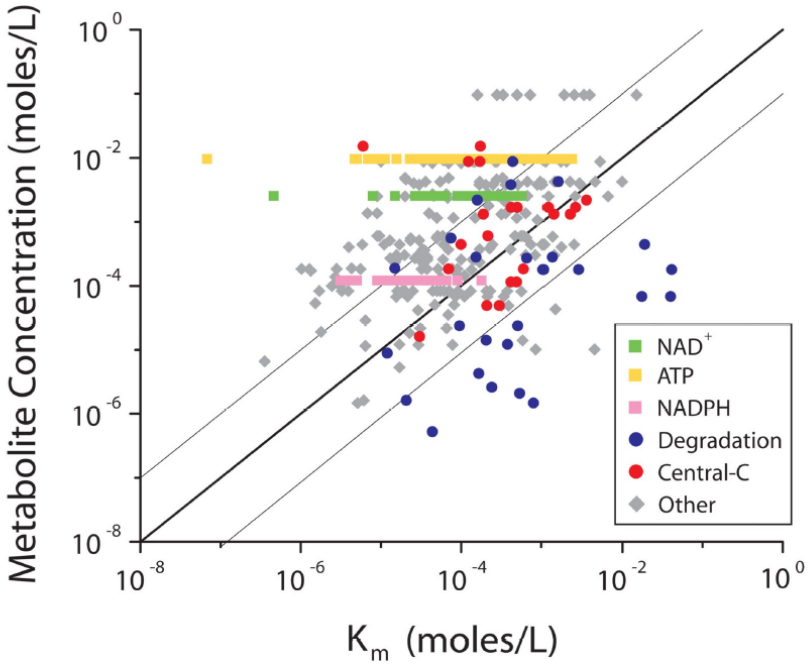


Figure : The relationship of metabolite concentrations and the K_m values of their consuming enzymes in glucose-grown *E. coli*.

Bennett, B. D. et al, vol 5(8) 2009 **Nature CHEMICAL BIOLOGY**

k_{cat}/K_m can be determined directly at low substrate concentrations where:

$$v = k_{cat}/K_m * E_t * [S]$$

In practice: measure v at two low substrate concentrations where v doubles as S is doubled. From 5-6 measurements determine k_{cat}/K_m from

$$\frac{v}{E_t * [S]} = k_{cat}/K_m$$

Slide from Prof. Ralf Morgensten, KI

Determination of k_{cat} and K_m in practice

- Get true initial rates! Don't use more than 10-20% of S
- Choose [S]: 0.2-10 x K_m and at least 5-6 concentrations (0.2, 0.5, 1, 2, 5, 10 x K_m)
- Triplicate determinations, if possible
- Repeat the experiment, consider using two different enzyme batches

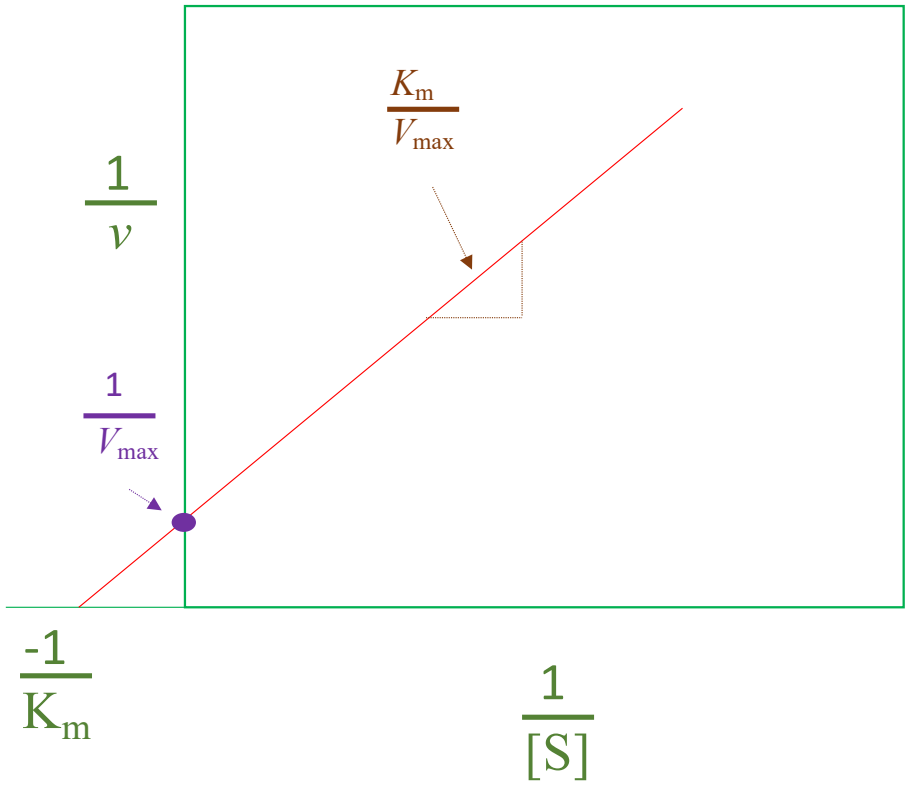
Slide from Prof. Ralf Morgensten, KI

Linear transformations

Lineweaver-Burk plot

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

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$



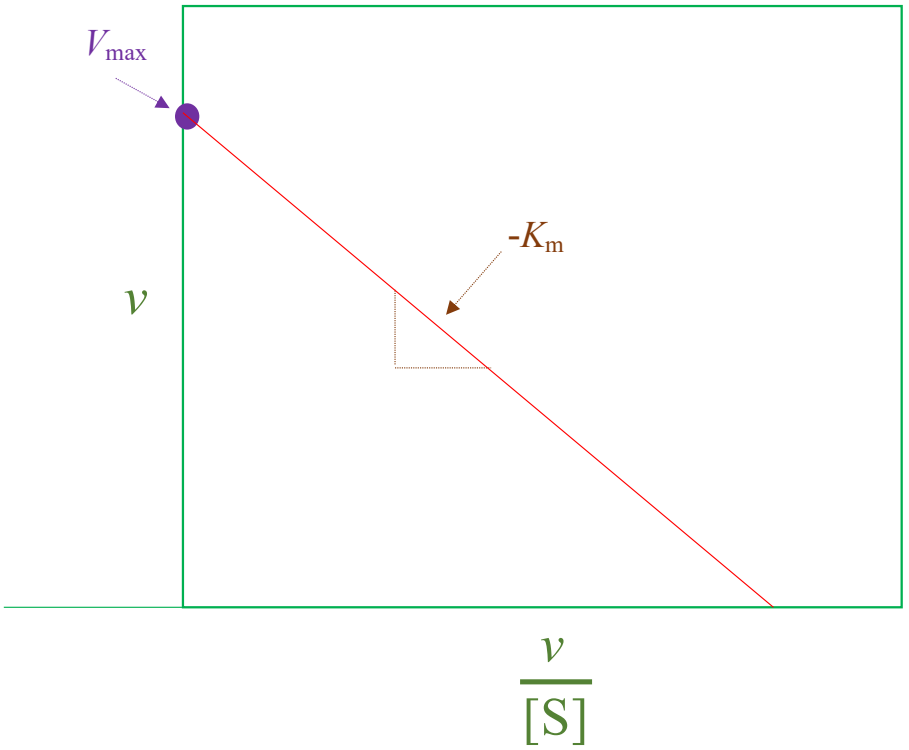
Use the equation in Excel!

Linear transformations

Eadie-Hofstee plot

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

$$v = -K_m \cdot \frac{v}{[S]} + V_{max}$$



Linear transformations

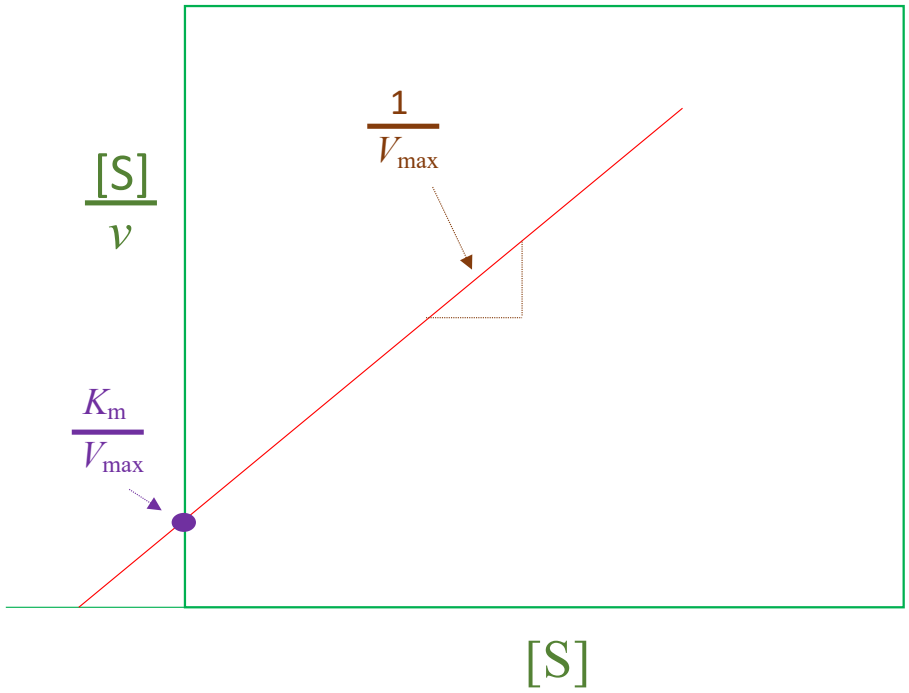
Hanes-Woolf plot

Treats errors better than Lineweaver-Burk plot,
but the two axes are not independent of [S]

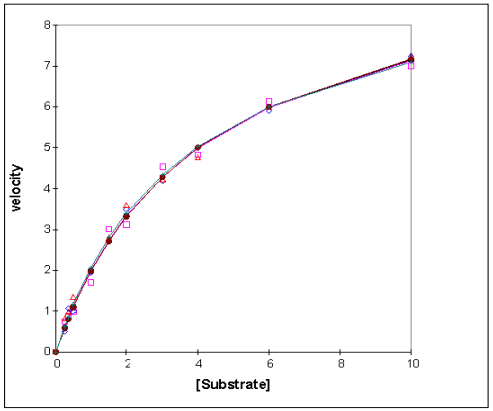
$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$\frac{[S]}{v} = \frac{K_m}{V_{max}} \cdot \frac{[S]}{[S]} + \frac{1}{V_{max}} \cdot [S]$$

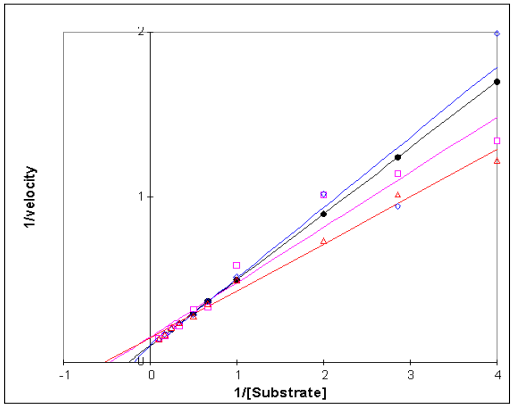
$$\boxed{\frac{[S]}{v} = \frac{1}{V_{max}} \cdot [S] + \frac{K_m}{V_{max}}}$$



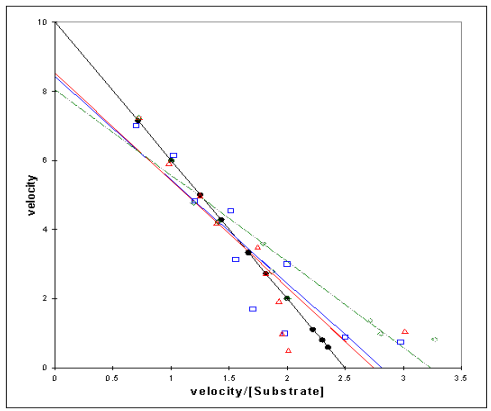
Substrate dependence v vs. [S]



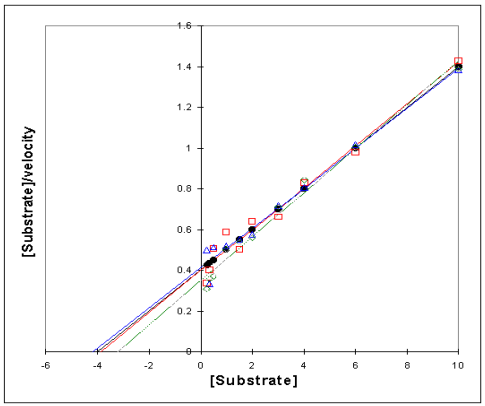
Lineweaver-Burk 1/v vs. 1/[S]



Eadie-Hofstee v vs. v/[S]



Hanes-Wolff [S]/v vs. [S]

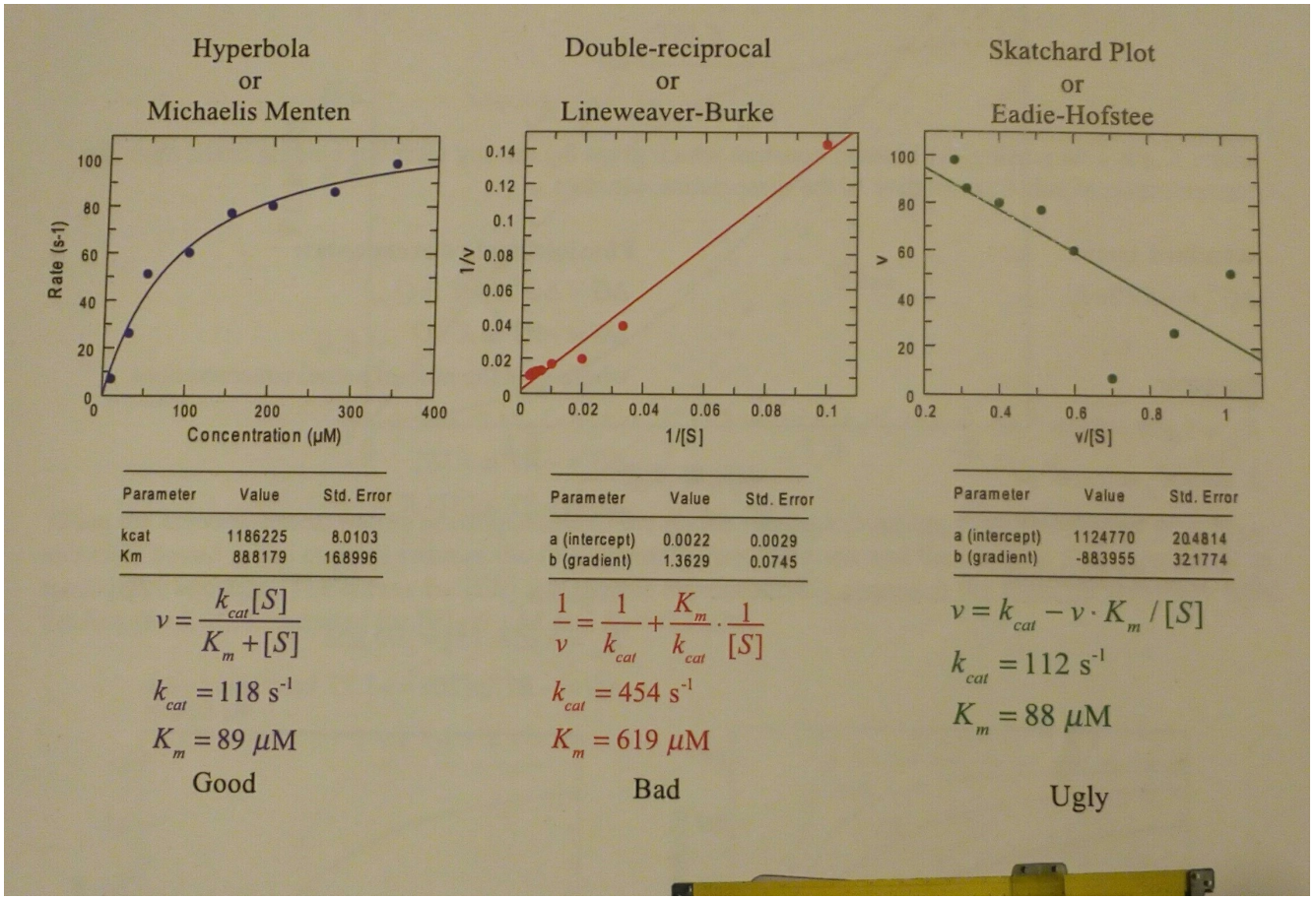


Errors

Three series with errors compared to ideal values in different plots.

Best with non-linear curve fit to substrate dependence plot with computer program (e.g. SIMFIT)

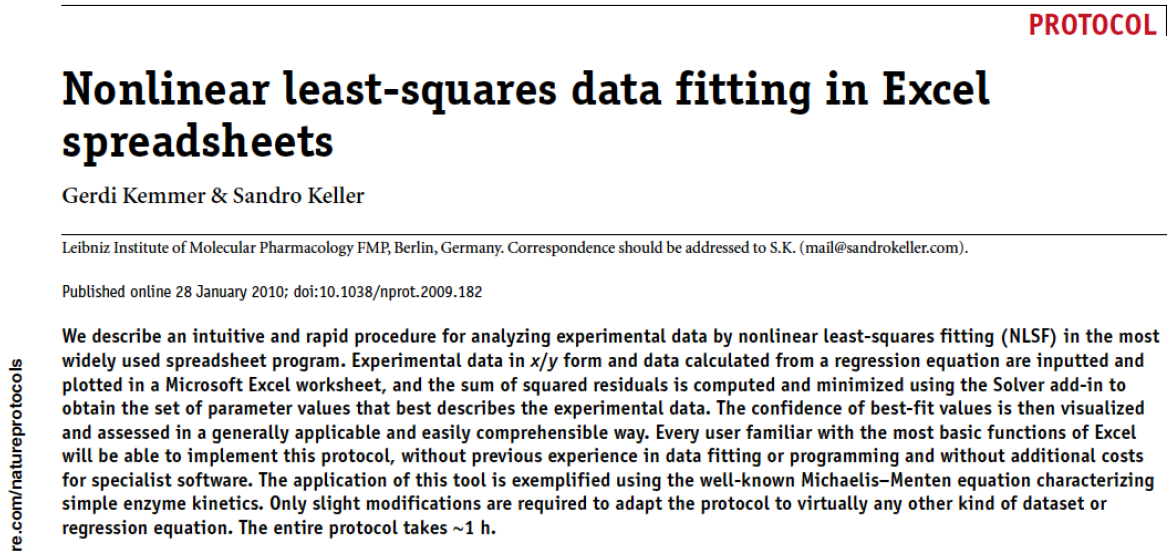
Hanes-Wolff is best among linear plots to cope with errors



(Ref- KAJ-Enzymology workshop handbook)

Non-linear fitting of the MM curve

- e.g. http://www.rose-hulman.edu/~brandt/Chem330/Mathematical_Modeling.pdf
- or:



PROTOCOL

Nonlinear least-squares data fitting in Excel spreadsheets

Gerdi Kemmer & Sandro Keller

Leibniz Institute of Molecular Pharmacology FMP, Berlin, Germany. Correspondence should be addressed to S.K. (mail@sandrokeller.com).

Published online 28 January 2010; doi:10.1038/nprot.2009.182

We describe an intuitive and rapid procedure for analyzing experimental data by nonlinear least-squares fitting (NLSF) in the most widely used spreadsheet program. Experimental data in *x*/*y* form and data calculated from a regression equation are inputted and plotted in a Microsoft Excel worksheet, and the sum of squared residuals is computed and minimized using the Solver add-in to obtain the set of parameter values that best describes the experimental data. The confidence of best-fit values is then visualized and assessed in a generally applicable and easily comprehensible way. Every user familiar with the most basic functions of Excel will be able to implement this protocol, without previous experience in data fitting or programming and without additional costs for specialist software. The application of this tool is exemplified using the well-known Michaelis–Menten equation characterizing simple enzyme kinetics. Only slight modifications are required to adapt the protocol to virtually any other kind of dataset or regression equation. The entire protocol takes ~1 h.

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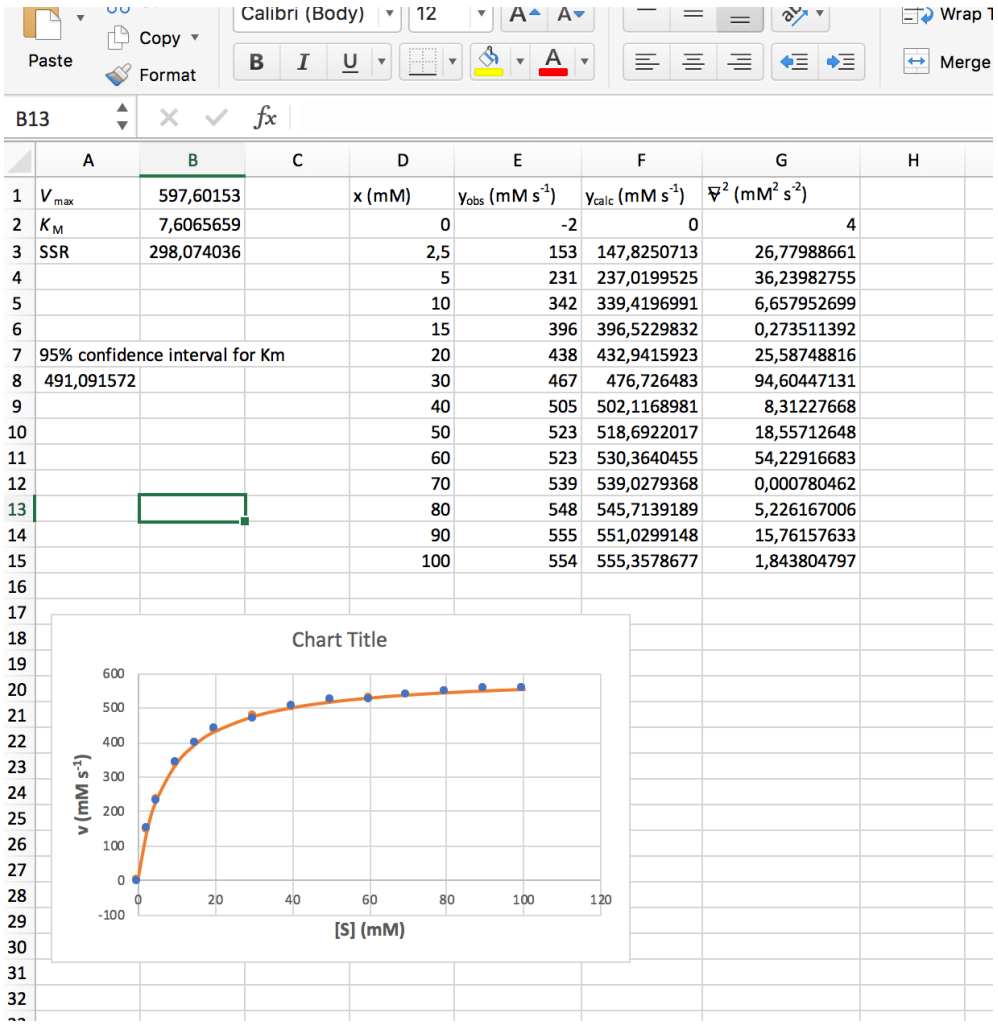
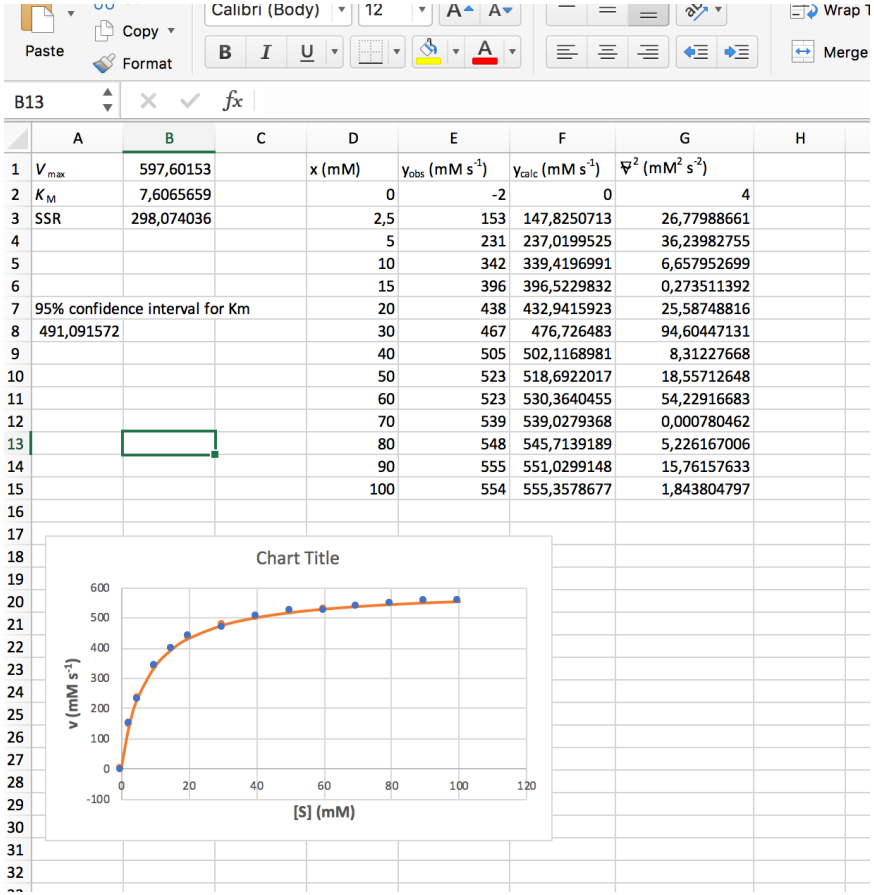
a copy on the course site…



	A	B	C	D	E	F	G	H
1	V_{max}	597,60153		x (mM)	y_{obs} (mM s^{-1})	y_{calc} (mM s^{-1})	∇^2 (mM^2 s^{-2})	
2	K_M	7,6065659		0	-2	0	4	
3	SSR	298,074036		2,5	153	147,8250713	26,77988661	
4				5	231	237,0199525	36,23982755	
5				10	342	339,4196991	6,657952699	
6				15	396	396,5229832	0,273511392	
7	95% confidence interval for Km			20	438	432,9415923	25,58748816	
8	491,091572			30	467	476,726483	94,60447131	
9				40	505	502,1168981	8,31227668	
10				50	523	518,6922017	18,55712648	
11				60	523	530,3640455	54,22916683	
12				70	539	539,0279368	0,000780462	
13				80	548	545,7139189	5,226167006	
14				90	555	551,0299148	15,76157633	
15				100	554	555,3578677	1,843804797	

=v_max*D2/(K_m+D2)



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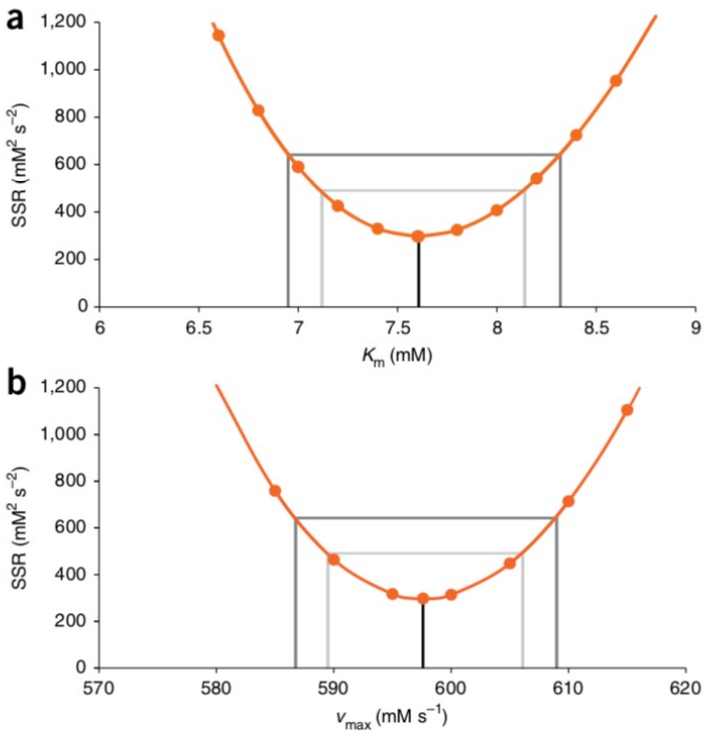
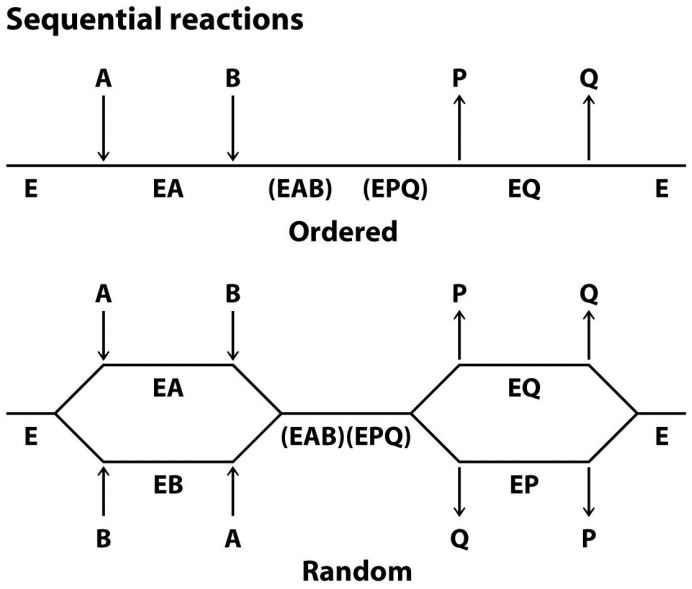


Figure 7 | Confidence assessment of best-fit parameter values. Plots of sum of squared residuals (SSR) against (**a**) K_m and (**b**) v_{max}. Light and dark gray lines mark parameter values at which the SSR amounts to, respectively, 491 and 642 mM^2 s^{-2}. These parameter ranges correspond to the approximate 95% and 99% confidence intervals, respectively. Vertical black lines indicate best-fit values.

Multisubstrate reactions



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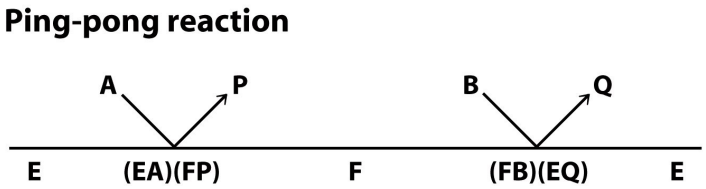

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How do we assay these systems?

Pseudo first order reaction: determine K_m, V_{max} for one S, keeping all other Ss in excess

Binding of effector molecules

In biology, many proteins are regulated by:

- Inhibitors
- Activators

Inhibitors

Why do we use inhibitors?

- As a tool in protein purification (stabilize protein, or prevent activity, e.g. of proteases)
- Determine kinetic mechanism e.g. which substrate binds first…
- Study how the structure acts and reacts
- Drug design
- and much more!!

May be defined as (decided using kinetic assays):

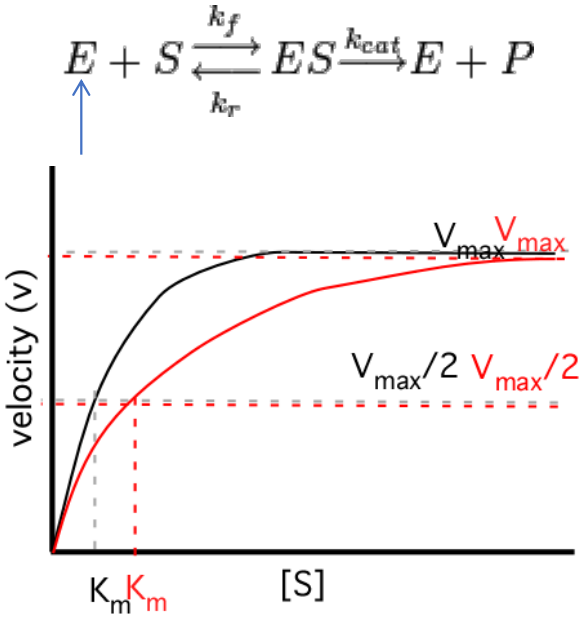
- competitive
- non-competitive
- uncompetitive

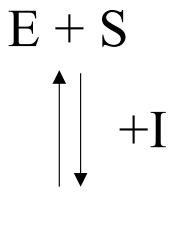
Competitive inhibitors

Often substrate or product-like

-> compete with them in the active site

bind E only, increases K_m, V_{max} is the same





$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$v = \frac{V_{max}[S]}{K_m.(1+\frac{[I]}{K_{ic}}) + [S]}$$

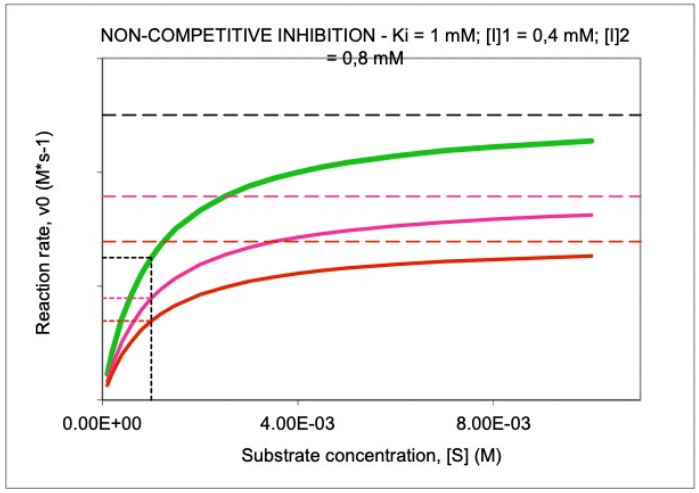
$$\frac{1}{v} = \frac{K_m.(1+\frac{[I]}{K_{ic}})}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

Non-competitive inhibitors

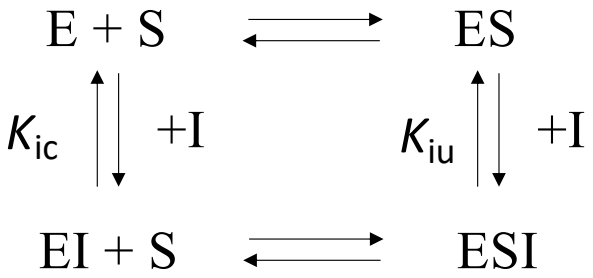
usually bind “outside” the active site

bind both E and ES, V_{max} decreases, K_m is same

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$



If binding to E and ES is not equally strong, the inhibition is “mixed”.



$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$v = \frac{V_{max}[S]}{K_m.(1+\frac{[I]}{K_{ic}}) + [S].(1+\frac{[I]}{K_{iu}})}$$

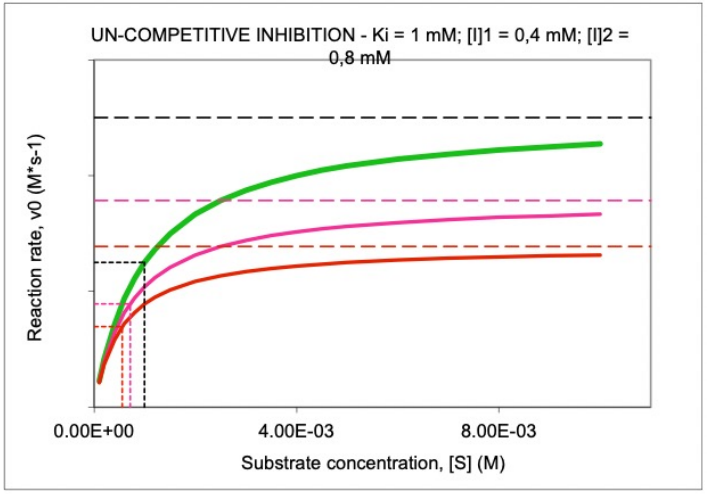
$$\frac{1}{v} = \frac{K_m.(1+\frac{[I]}{K_{ic}})}{V_{max}} \cdot \frac{1}{[S]} + \frac{(1+\frac{[I]}{K_{iu}})}{V_{max}}$$

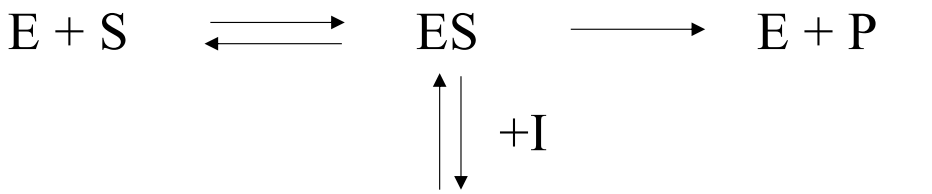
Uncompetitive inhibitors

bind “outside” the active site, quite rare

bind ES only, decrease both V_{max} and K_m

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$





$$v = \frac{V_{max}[S]}{K_m + [S]} \qquad \frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

$$v = \frac{V_{max}[S]}{K_m + [S].(1+\frac{[I]}{K_{iu}})}$$

$$\frac{1}{v} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{(1+\frac{[I]}{K_{iu}})}{V_{max}}$$

SUBSTRATE DEPENDENCE PLOT

$$V_0 = V_{max} \cdot \frac{[S]}{[S] + K_m}$$

DOUBLE RECIPROCAL PLOT (LINEWEAVER-BURKE)

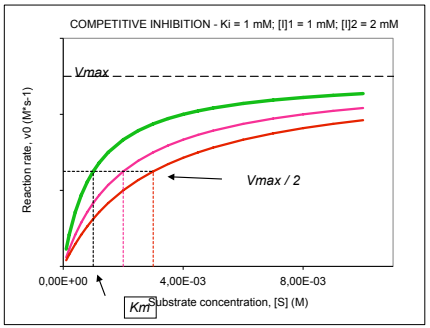
$$\frac{1}{v_0} = \frac{K_m}{V_{max}} \cdot \frac{1}{[S]} + \frac{1}{V_{max}}$$

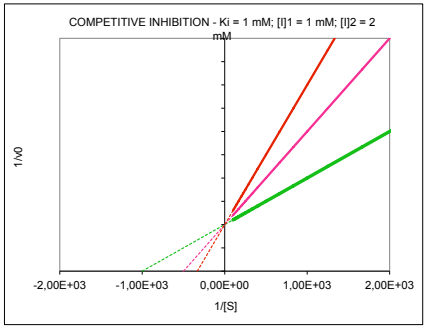
EADIE-HOFSTEE PLOT

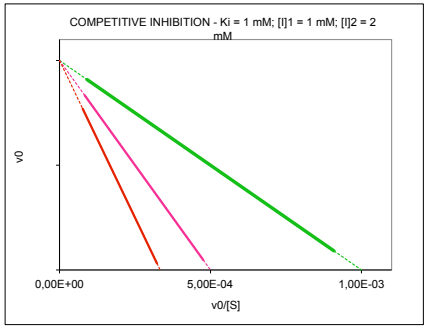
$$v_0 = -K_m \cdot \frac{v_0}{[S]} + V_{max}$$

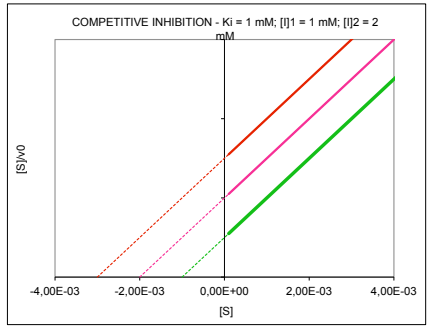
HANES-WOOLF PLOT

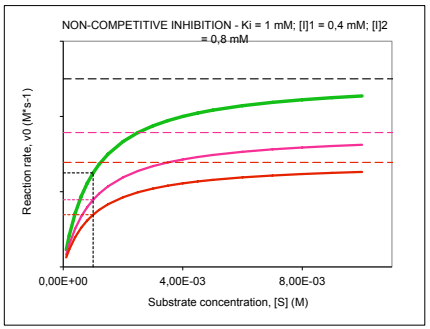
$$\frac{[S]}{v_0} = \frac{1}{V_{max}} \cdot [S] + \frac{K_m}{V_{max}}$$

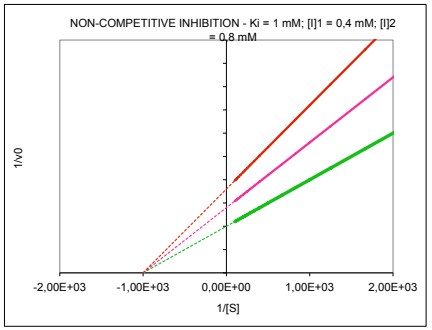


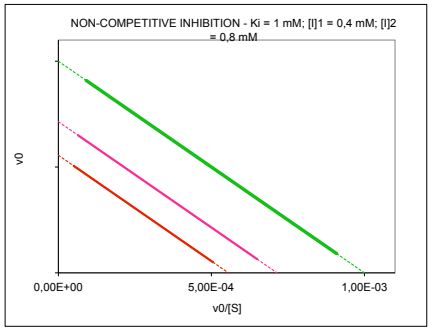


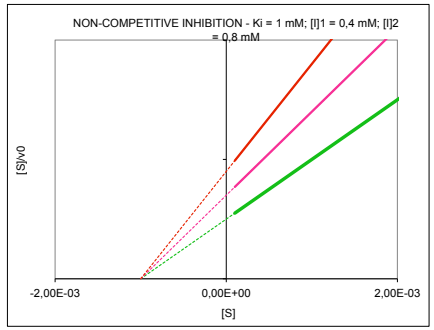


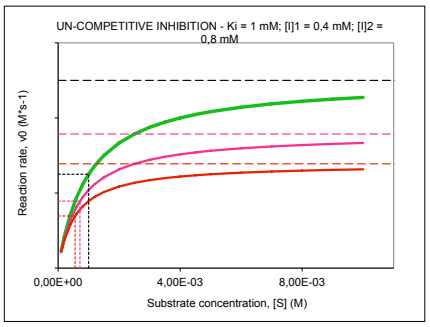


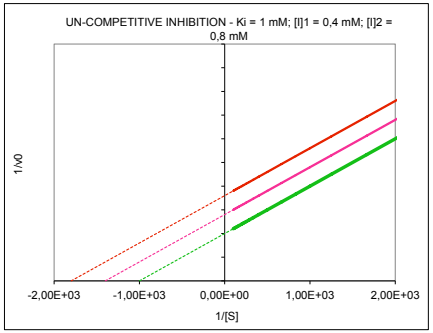


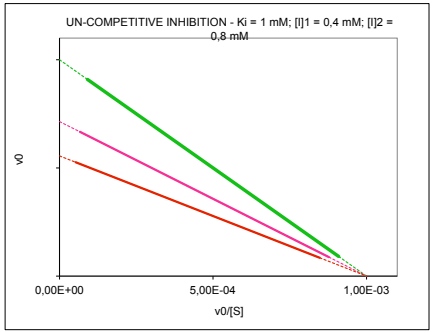


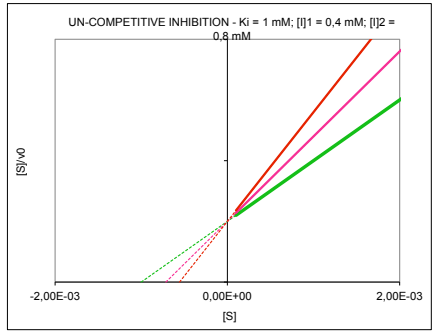










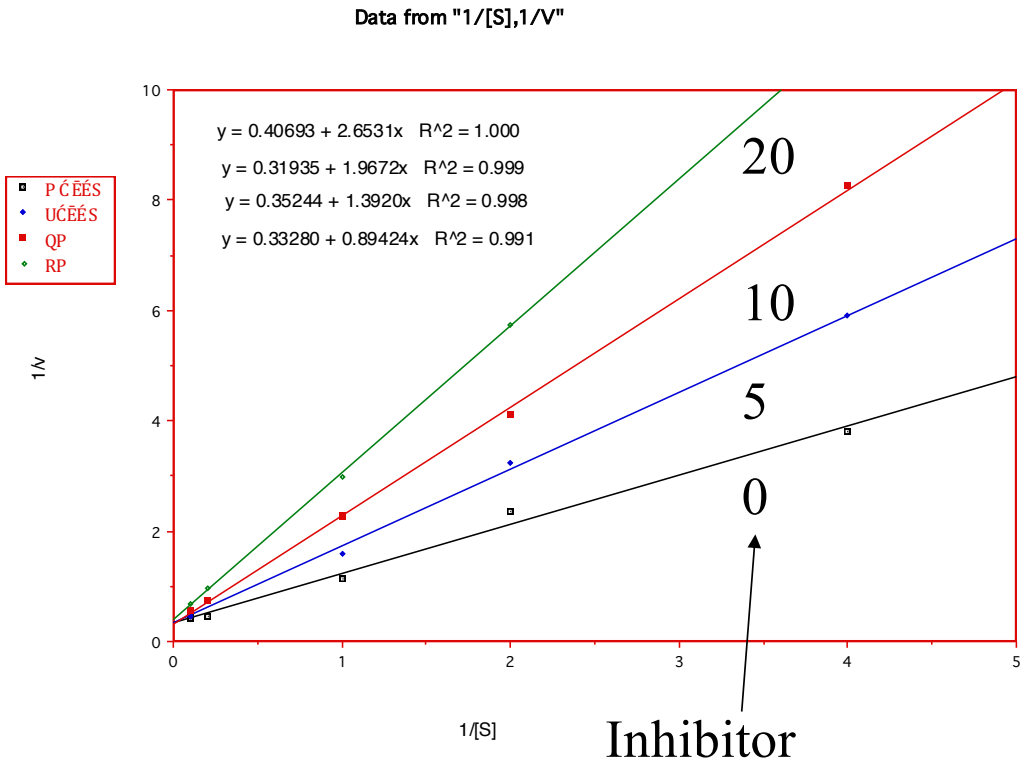


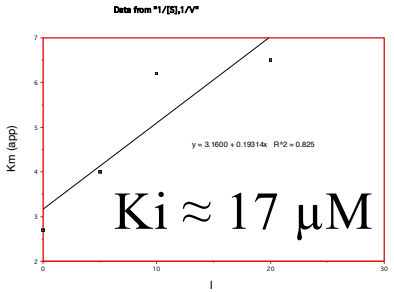
K_i

- The inhibition constant, K_i, indicates how potent an inhibitor is.
- It is the concentration required to produce half maximum inhibition.

$$K_{ic} = \frac{[E]\,[I]}{[EI]} \qquad K_{iu} = \frac{[ES]\,[I]}{[ESI]}$$

An example of competitive inhibition (Lineweaver-Burk plot)





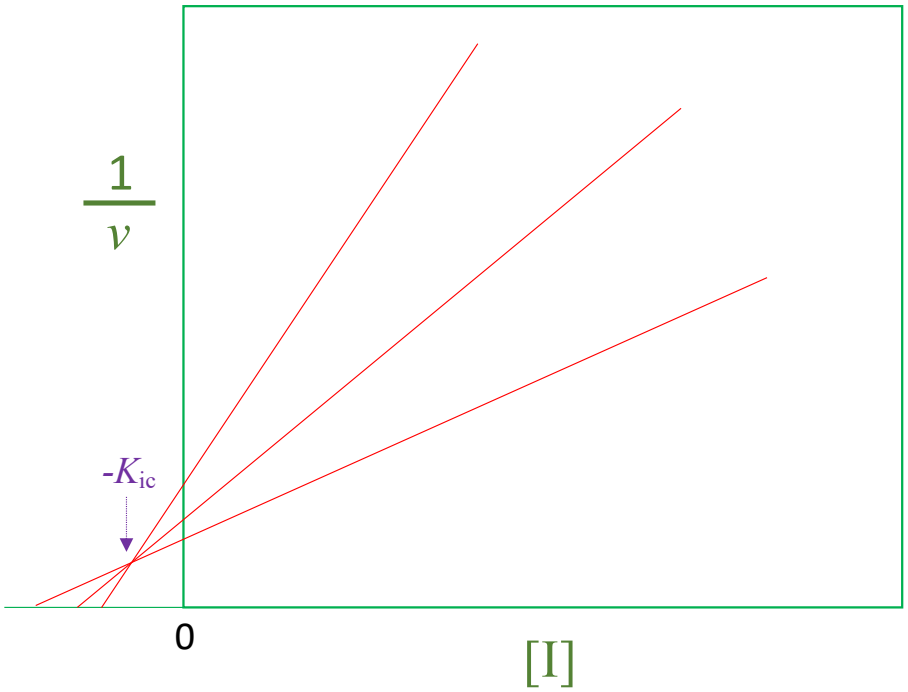
$K_{m(app)}$ is linearly dependent on I where $K_{m(app)} = K_m(1+I/K_i)$
The slope is K_m/K_i

Slide from Prof. Ralf Morgensten, KI

A Dixon plot can be useful

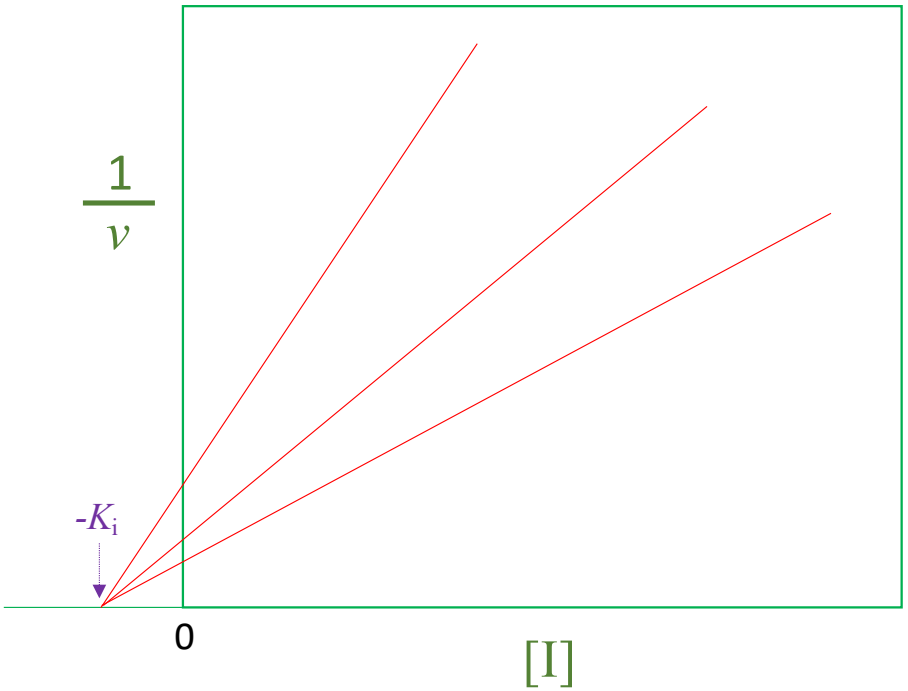
- Plot 1/v against concentration of inhibitor at each [S]
- Gives a family of intersecting lines

Determining K_i for a competitive inhibitor (Dixon plot)

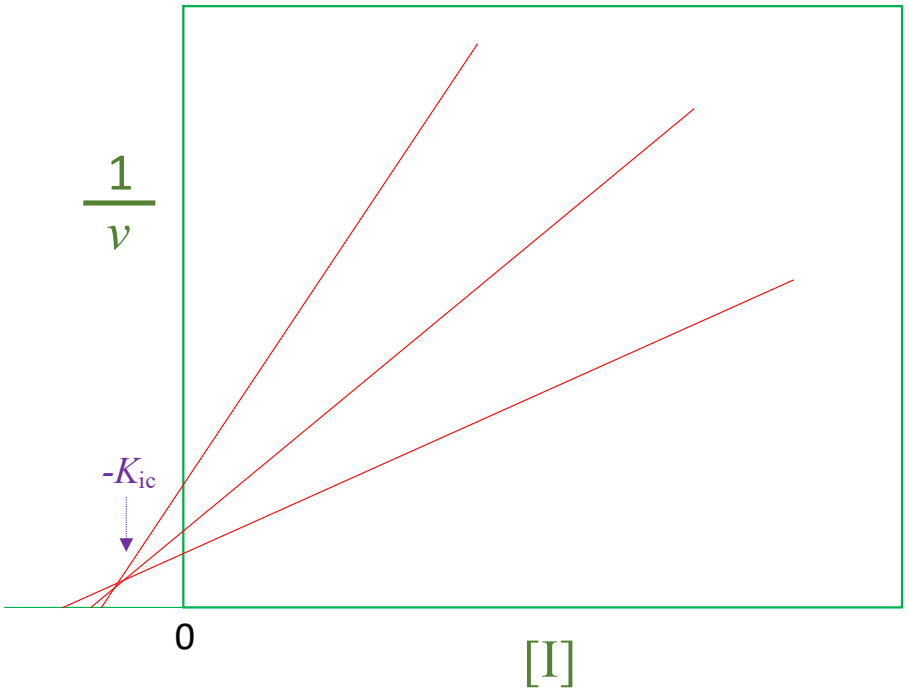


Determining K_i for a noncompetitive inhibitor (Dixon plot)

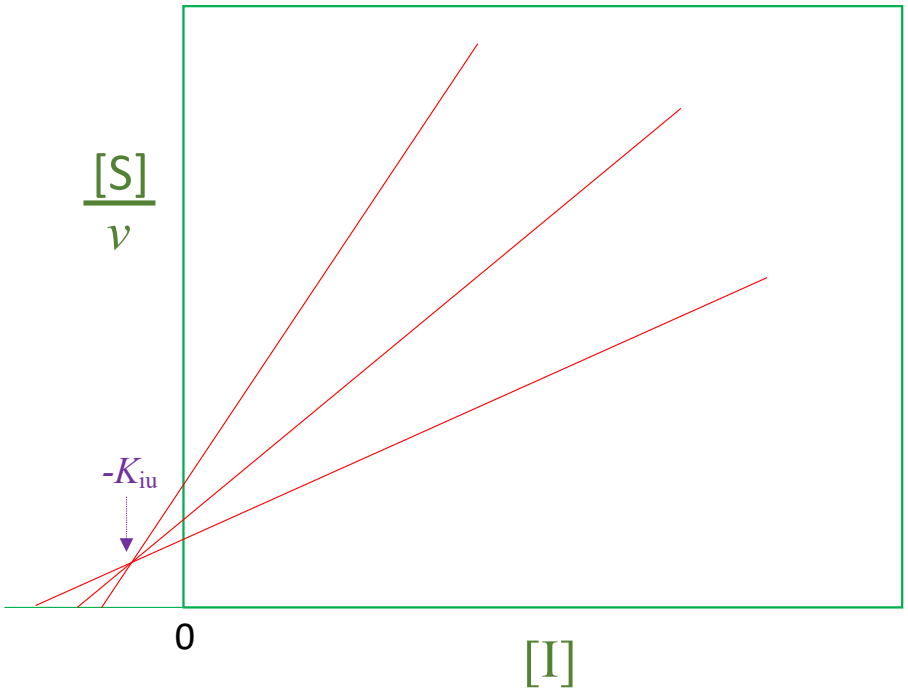
Pure noncompetitive, when $K_{ic} = K_{iu}$



Mixed



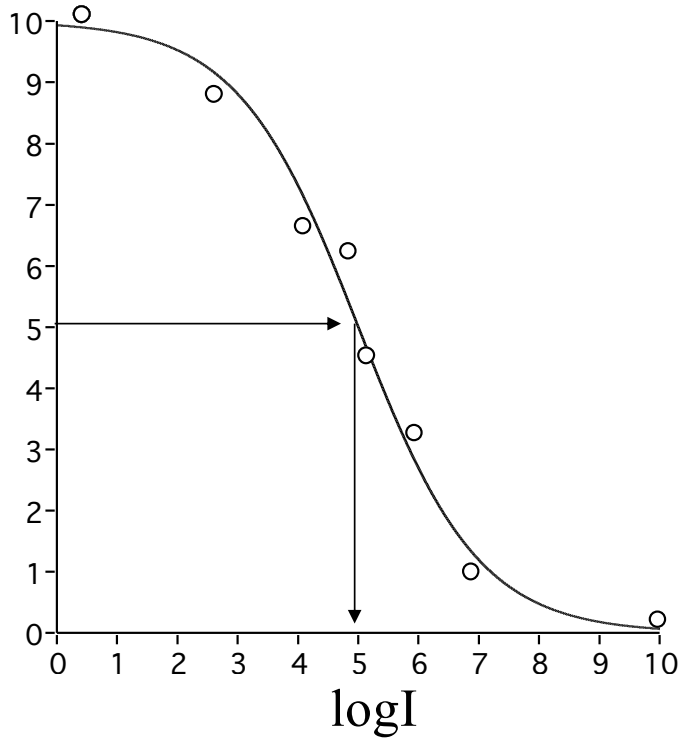
Determining K_i for an uncompetitive inhibitor (Cornish-Bowden, 1974)



IC_{50}

IC_{50} values are useful practical values for screening and comparing inhibitors (chemists and drug design people tend to use IC_{50})

In practice:
3 experiments where 5-6 v values are determined between 30-70% inhibition. IC_{50} is then determined graphically



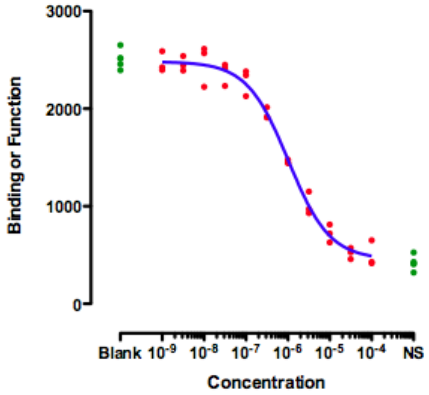
Slide from Prof. Ralf Morgensten, KI

IC_{50} versus K_i

- K_i: the concentration resulting in 50% binding of the enzyme, in the absence of substrate:

  Ki = [I] [E]/ [EI] (i.e. a K_d)

- IC_{50}: the concentration resulting in 50% activity (so it depends on [S], if competitive)



- Can you compare the two?

  yes, easily, in the competitive case $K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_m}}$

  Try to use biologically relevant [S]!

Some enzymes do not follow MM kinetics, because:

- The three assumptions break down
  - e.g. if S and E associate very quickly
- Or the situation is more complicated, like multiple reaction steps, or allostery…

Allosteric enzymes

(literally “other shape”)

Allostery: “Of or involving a change in the shape and activity of an enzyme that results from molecular binding with a regulatory substance at a site other than the enzymatically active one.”

Or: “Allostery involves coupling of conformational changes between two widely separated binding sites. The common view holds that allosteric proteins are symmetric oligomers, with each subunit existing in at least two conformational states with a different affinity for ligands.”

Any protein that *can change conformation in a way that affects its activity* is potentially allosteric!

Allosteric proteins

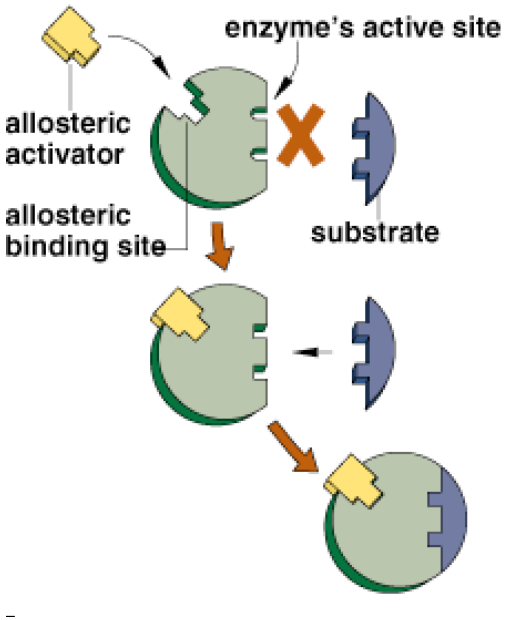
Can be active sites of subunits in oligomers communicating

Can be two kinds of sites (e.g. catalytic and regulatory) on one protein chain

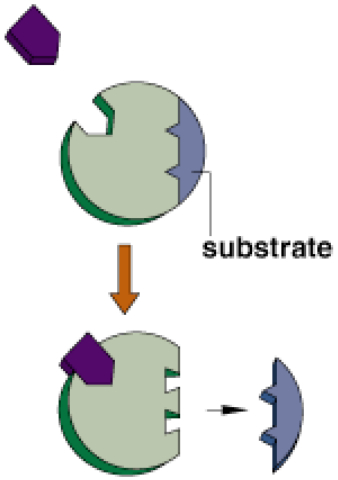
Or both!

The main thing is that the sites talk to each other.

Allosteric effectors (activators *versus* inhibitors)



Activator

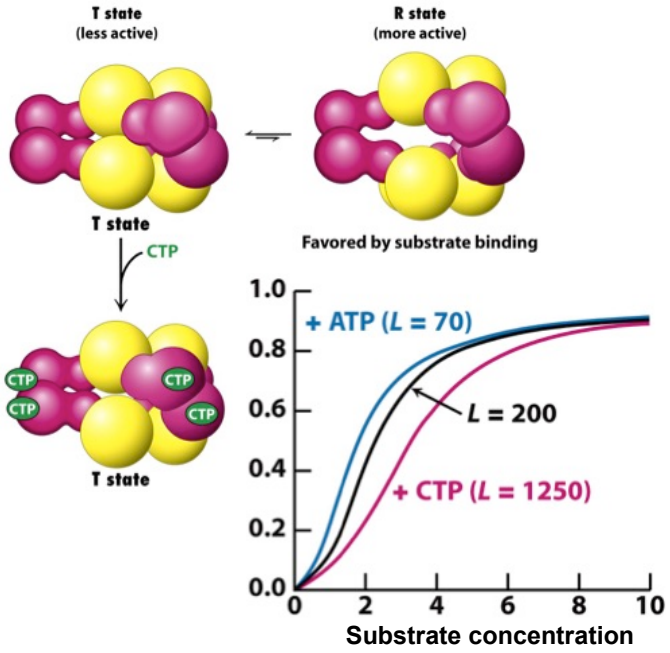


Inhibitor

Allosteric proteins in kinetics

Ones with multiple active sites often do not follow M-M kinetics.
Sigmoidal v_o *vs* [S] plots.
Cooperative substrate binding.

Sigmoidal plot describes transition between two states of the enzyme:
T - low affinity for S
R - high affinity for S



$$L = \frac{[T]}{[R]}$$

Co-operativity

- In a multimeric enzyme, the binding of a substrate or ligand molecule affects the affinity of the others.

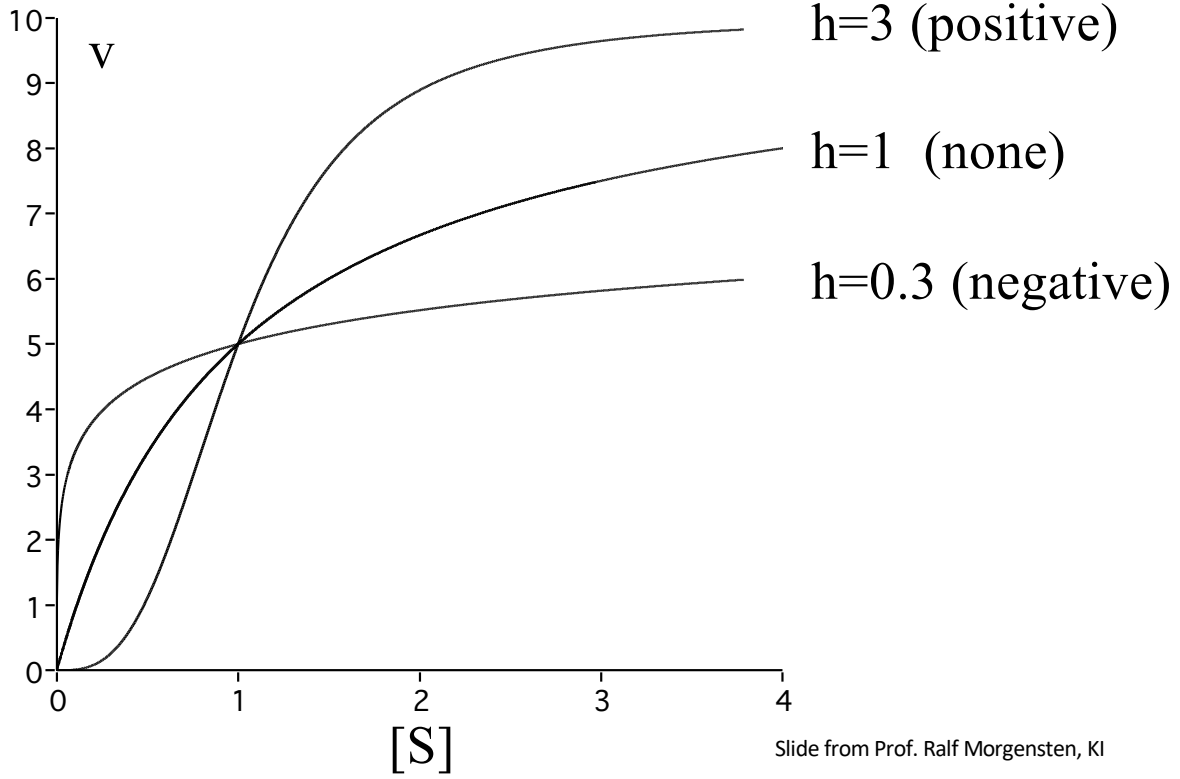
- May be positive (most common) or negative
  - Binding of the first molecule causes a conformational change that changes affinity at others
- Allostery allows a “magnified” response

$$v = \frac{k_{cat} \cdot S^h}{K_{0.5} + S^h}$$

$k_{cat} = 10$

"K_m" = 1

Note!
"$K_{0.5}$" has no real meaning



Slide from Prof. Ralf Morgensten, KI

Ideal assay and conditions, general considerations

- Linear during time period measured (initial rate)
- Linear with [E]
- Saturation with substrate should be possible to obtain (a problem if substrate is not very soluble)

- Choice of temperature, ionic strength, buffer, pH, additives, usually based on previous experience.

- Subtraction of a background rate may be needed.

Slide from Prof. Ralf Morgensten, KI

Enzyme assays

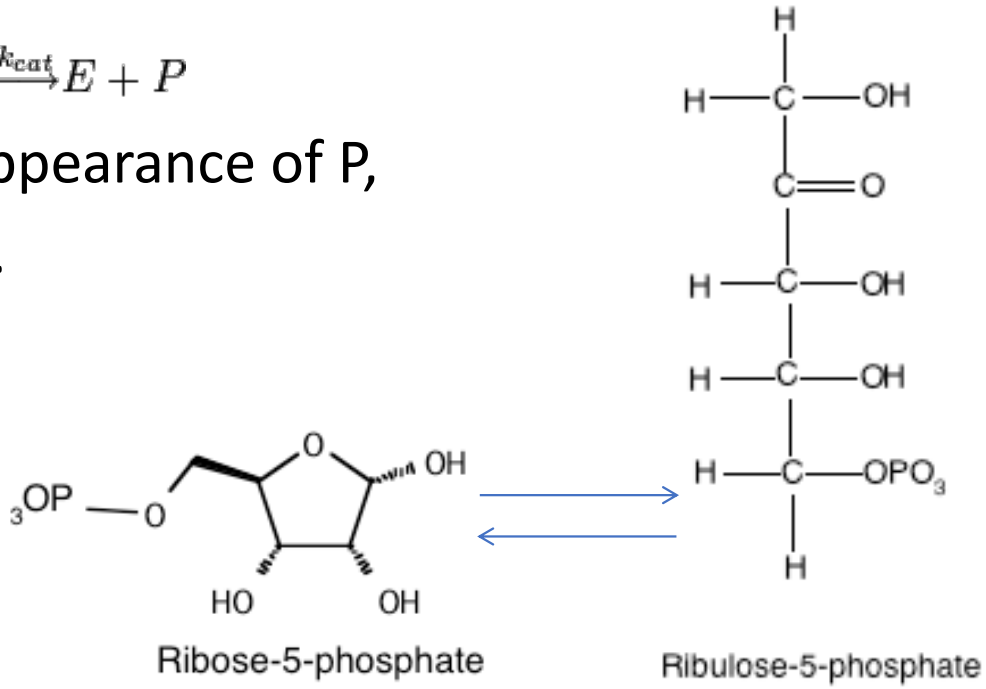
If we want to study enzyme function, we must be able to measure activity.

$$E + S \underset{k_r}{\overset{k_f}{\rightleftharpoons}} ES \xrightarrow{k_{cat}} E + P$$

Can measure either appearance of P,

or disappearance of S.

For the lab protein:



Ribose-5-phosphate

Ribulose-5-phosphate

Usually multiple ways to assay

For the lab protein

Possibility 1

Coupled steps *(ribose 5-phosphate to ribulose 5-phosphate)*:

ribose 5-phosphate <=> ribulose 5-phosphate (Rpi)

ribulose 5-phosphate + ATP ⇔ ribulose-bisphosphate + ADP (phosphoribulose kinase)

phosphoenolpyruvate + ADP <=> pyruvate + ATP (pyruvate kinase)

Pyruvate + NADH + H+ <=> lactate + NAD+ (lactate dehydrogenase)

Possibility 2

Coupled steps *(ribulose 5-phosphate to ribose 5-phosphate)*:

ribulose 5-phosphate <=> ribose 5-phosphate (Rpi)

ribose 5-phosphate + xylulose 5-phosphate ⇔ sedoheptulose-7-phosphate + glyceraldehyde-3-phosphate (transketolase)

glyceraldehyde-3-phosphate <=> dihydroxy-acetone-phosphate (triose-P-isomerase)

NADH + dihydroxy-acetone-phosphate ⇔ NAD+ + glycerol-3-phosphate (glycerol phosphate dehydrogenase)

Possibility 3

(ribose 5-phosphate to ribulose 5-phosphate):

ribose 5-phosphate <=> ribulose 5-phosphate (Rpi)

ribulose 5-phosphate + carbazole (purple color)

Possibility 4

(ribose 5-phosphate to ribulose 5-phosphate):

ribulose 5-phosphate <=> ribose 5-phosphate (Rpi)

ribulose 5-phosphate (direct measurement at OD290)

Today's computer lab, building an Excel file

- It is mandatory that you do it, as we need you to be ready, when you get your own data!
- We don't demand that you do it with us in the computer lab, but it can help a lot to have someone there to answer questions…
- Simplest to keep velocity in terms of change in absorbance per minute while you are working, then convert at the end.
- Please:
  - State all units, so that we know what is what!
  - Label all axes, on all plots!
  - Add words in your report, to say what you are doing!

Determine k_{cat} and K_M for *Mycobacterium tuberculosis* RpiB on Rib5P

- Sample data:

Substrate conc, [r5p]	Reaction rate -/+ inhibitor		
	0 mM 4PEH	0.01 mM 4PEH	0.05 mM 4PEH
mM	ΔA/min	ΔA/min	ΔA/min
0.5	0.004	0.0037	0.0031
1	0.008	0.0061	0.0042
2	0.0099	0.0075	0.0071
5	0.016	0.013	0.011
10	0.0219	0.0182	0.0162
20	0.0223	0.0202	0.0196

- Plot Michaelis-Menten curve, v_0 *vs* [S]
- Plot Lineweaver-Burk
  - Calculate K_M and k_{cat}
- Plot Hanes-Wolff
  - Calculate K_M and k_{cat}

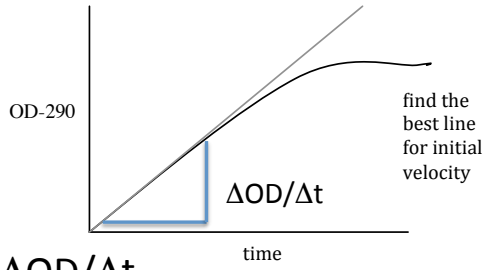
Determine Ki for 4PEH

Kinetics quiz on Studium

- Little extra, mostly entering values from your Excel file.
- A few basic questions
- Do by Wednesday next week.
- Also mail me your Excel file!
- If you have found the errors in your Excel file, you'll get some points back…

YOU will obtain data in the lab

Need to observe initial rates (v) at various [S]



Measure $\Delta OD/\Delta t$.

Since absorbance (OD) is related to concentration, we can convert to [P] using Beer's law: $OD = \varepsilon\ b\ c$

- ε is characteristic of compound and wavelength
  - for ribulose 5-phosphate, it is 72 $cm^{-1}\ M^{-1}$
- b is the distance the light travels through the sample (1 cm)
- c is concentration

-> $\Delta P/\Delta t = v$