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Binding and Kinetics for Experimental Biologists
Lecture 5

Initial rate enzyme kinetics

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Tento projekt je spolufinancován Evropským sociálním fondem a státním rozpočtem České republiky.

EVROPSKÁ UNIE esf MINISTERSTVO ŠKOLSTVÍ, MLÁDEŽE A TĚLOVÝCHOVY OP Vzdělávání pro konkurenceschopnost UNIVERSITAS MASARYKIANA BRUNENSIS

INVESTICE DO ROZVOJE VZDĚLÁVÁNÍ

BioKin L t d.

Lecture outline

- The problem:

  Fit initial rate enzyme-kinetic data to a variety of mechanistic models.
  Avoid algebraic models, which may not even exist for complex cases.
  Select the most plausible model, based on statistical criteria.

- The solution:

  Use generally applicable numerical (iterative) models to represent initial rates
  Use the Akaike Information Criterion for model selection.

- An implementation:

  Software DynaFit (Kuzmic 1996; 2009).

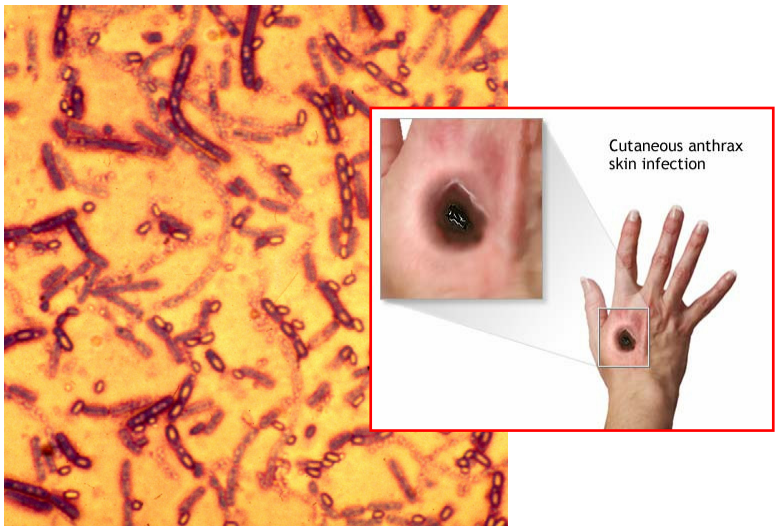
- Two examples:

  1. Inhibition of the Lethal Factor protease from *Bacillus anthracis*.

  2. Inhibition of the $p56^{lck}$ protein tyrosine kinase.

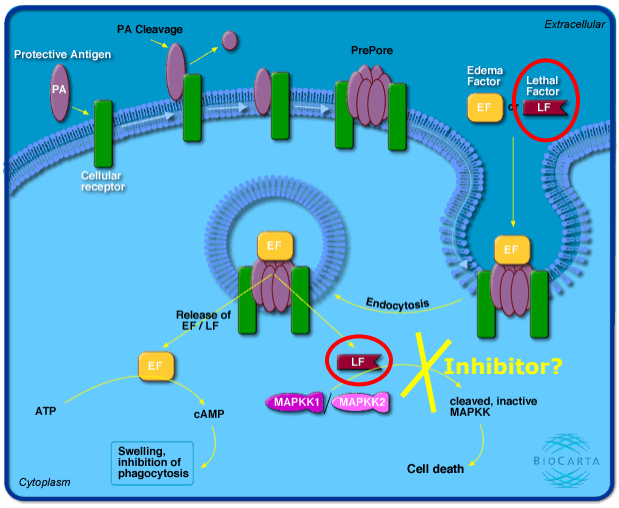
Anthrax bacillus

CUTANEOUS AND INHALATION ANTHRAX DISEASE



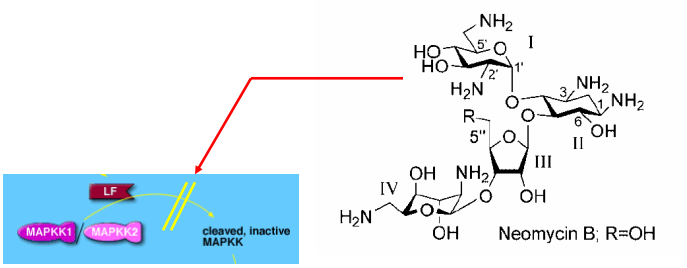
Lethal Factor (LF) protease from *B. anthracis*

CLEAVES MITOGEN ACTIVATED PROTEIN KINASE KINASE (**MAPKK**)



Neomycin B: an aminoglycoside inhibitor

PRESUMABLY A "COMPETITIVE" INHIBITOR OF **LF** PROTEASE

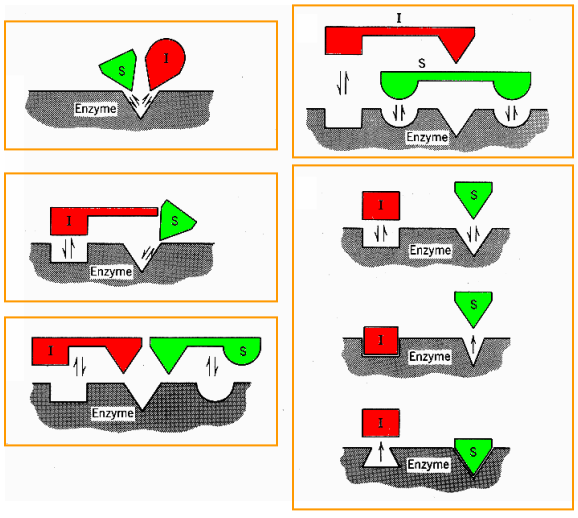


"All the compounds tested were found to be competitive inhibitors."

Fridman *et al.* (2004) *Angew. Chem. Int. Ed. Eng.* **44**, 447-452

Competitive inhibition - Possible mechanisms

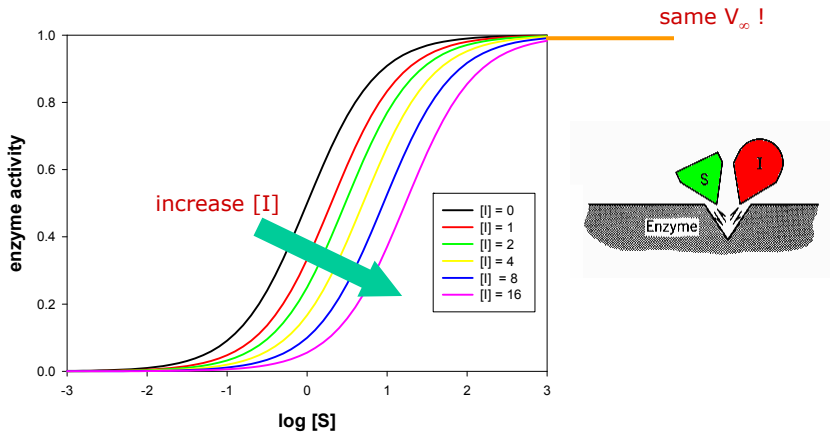
MUTUALLY EXCLUSIVE BINDING TO ENZYME



Segel, I. (1975) *Enzyme Kinetics*, John Wiley, New York, p. 102

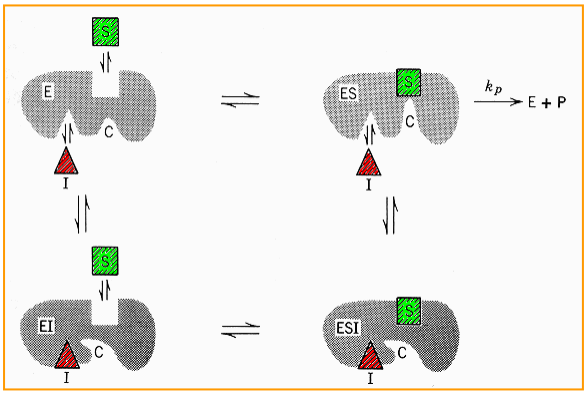
Competitive inhibition - Kinetics

AT VERY HIGH [SUBSTRATE], ANZYME ACTIVITY IS COMPLETELY RESTORED



Non-competitive inhibition - A possible mechanism

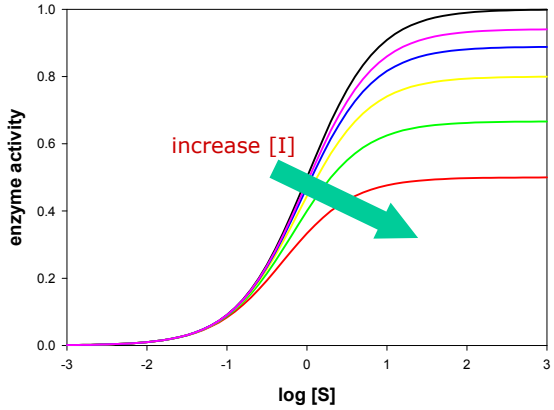
NON-EXCLUSIVE BINDING, BUT TERNARY COMPLEX HAS NO CATALYTIC ACTIVITY

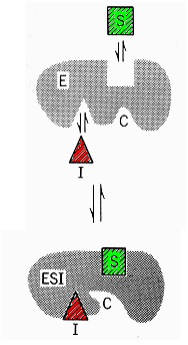


Segel, I. (1975) *Enzyme Kinetics*, John Wiley, New York, p. 126

Non-competitive inhibition - Kinetics

EVEN AT VERY HIGH [SUBSTRATE], ANZYME ACTIVITY IS NEVER FULLY RESTORED

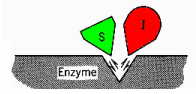


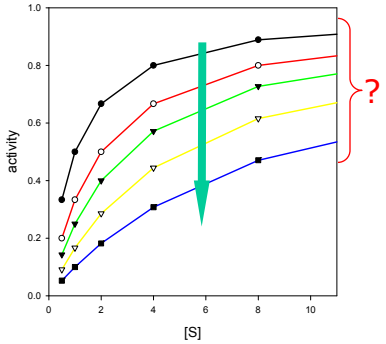


Compare saturation curves

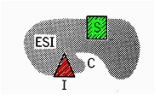
DIAGNOSIS OF MECHANISMS: SAME OR DIFFERENT RATE AT VERY LARGE [S]?

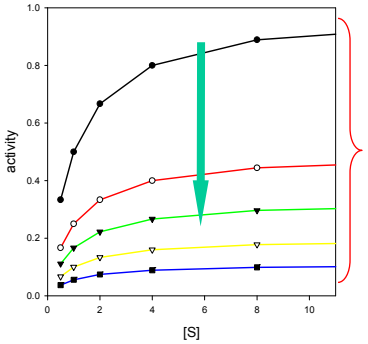
COMPETITIVE





NON-COMPETITIVE

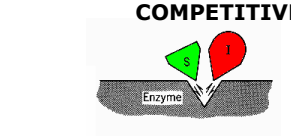


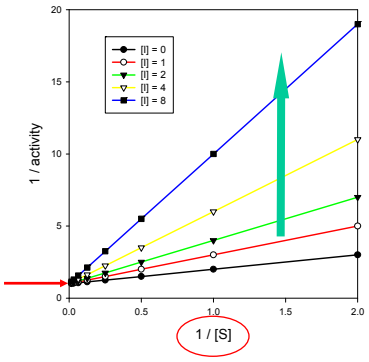


Compare "double-reciprocal" plots

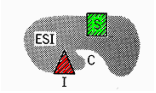
DIAGNOSIS OF MECHANISMS: STRAIGHT LINES INTERCEPT ON VERTICAL AXIS?

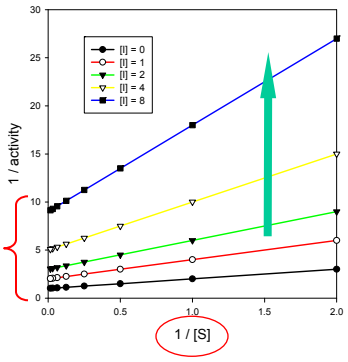
COMPETITIVE





NON-COMPETITIVE





Traditional plan to determine inhibition mechanism

THE TRADITIONAL APPROACH

1. Measure enzyme activity at increasing [S]

   Collect multiple substrate-saturation curves at varied [I]

2. Convert [S] *vs.* activity data to **double-reciprocal** coordinates

3. Perform a **linear fit** of transformed (double-reciprocal) data

4. Check if resulting straight lines **intersect** on the vertical axis

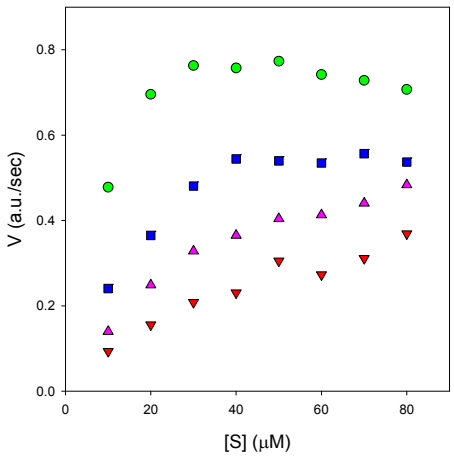
   If yes, declare the inhibition mechanism **competitive**

concentrations of **12** were 0, 16.2, 32.5, and 54.1 nM, the concentrations of all other compounds were 0, 165, 330, and 550 nM). The K_i values were estimated from double-reciprocal plots of initial velocities as a function of substrate concentration. [b] High-salt conditions: potassium HEPES buffer (10 mM), at pH 7.4, KCl (150 mM), LE (≃33 nM), a fluorescent

Fridman *et al.* (2004) *Angew. Chem. Int. Ed. Eng.* **44**, 447-452

Collect experimental data at varied [S] and [I]

THE RAW DATA



[I] = 0

[I] = 0.5 μM

[I] = 1.0 μM

[I] = 2.0 μM

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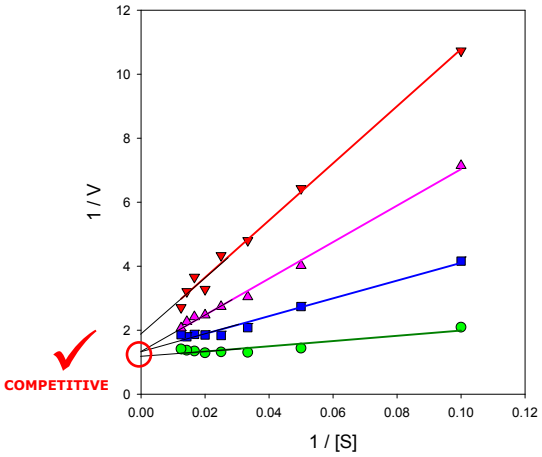
Check for intersection of double-reciprocal plots

DO LINEWEAVER-BURK PLOTS INTERSECT?



[I] = 0

[I] = 0.5 μM

[I] = 1.0 μM

[I] = 2.0 μM

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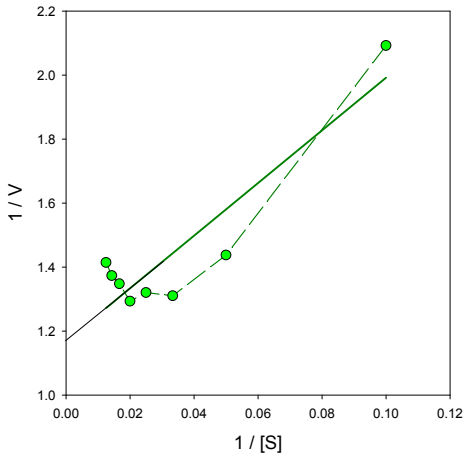
Doubts begin to appear...

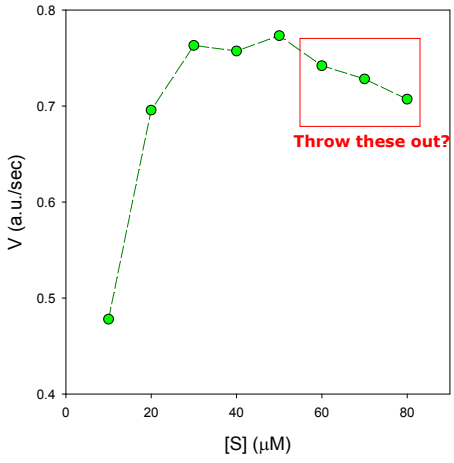
IS THIS A STRAIGHT LINE?



[I] = 0

Mysterious substrate saturation data

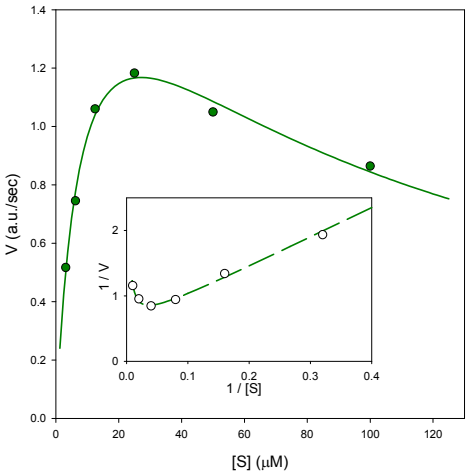
MICHAELIS-MENTEN KINETICS IS NOT SUPPOSED TO SHOW A MAXIMUM !



[I] = 0

Repeat substrate experiment at higher [S]

SEE IF MAXIMUM HOLDS UP AT HIGHER [S]



[I] = 0

Substrate inhibition in LF protease is real

HAS ANYONE ELSE SEEN IT?

THE JOURNAL OF BIOLOGICAL CHEMISTRY
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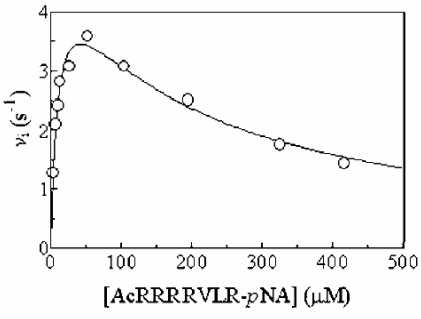
Vol. 278, No. 41, Issue of October 10, pp. 40075–40078, 2003
Printed in U.S.A.

The Metalloproteolytic Activity of the Anthrax Lethal Factor Is Substrate-inhibited*

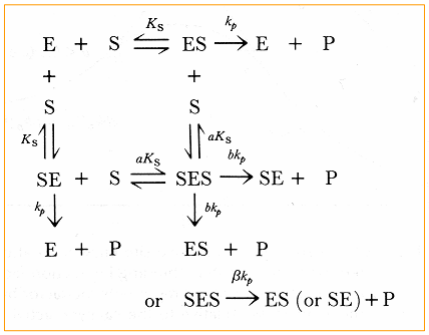
$$v_i = (k_{cat} \times [E] \times [S])/(K_m + [S] + ([S]^2/K_i)) \quad \text{(Eq. 1)}$$



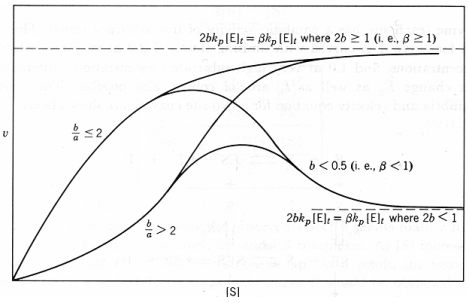
Tonello *et al.* (2003) *J. Biol. Chem.* **278**, 40075-78.

Rate equation for inhibition by substrate

WHAT DOES THE "BIG BLUE BOOK" SAY?



$$\frac{v}{[E]_t} = \frac{k_p \frac{2[S]}{K_S} + 2bk_p \frac{[S]^2}{aK_S^2}}{1 + \frac{2[S]}{K_S} + \frac{[S]^2}{aK_S^2}}$$



Segel, I. (1975) *Enzyme Kinetics*, John Wiley, New York, p. 126

Rate equation for inhibition by substrate + inhibitor

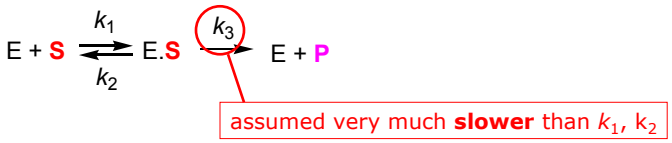
WHAT DOES THE "BIG BLUE BOOK" SAY?

?

Initial rate kinetics

TWO BASIC APPROXIMATIONS

1. **Rapid-Equilibrium** Approximation

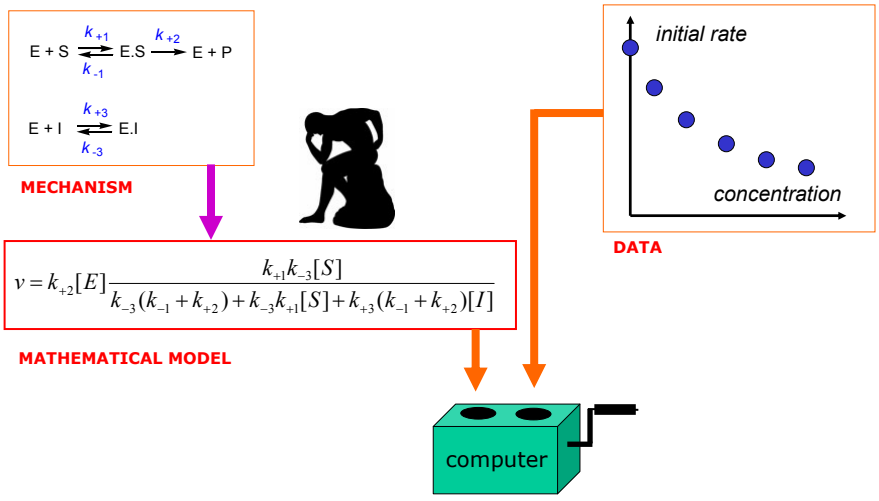


2. **Steady-State** Approximation

- *no assumptions* made about relative magnitude of k_1, k_2, k_3
- concentrations of enzyme forms are *unchanging*

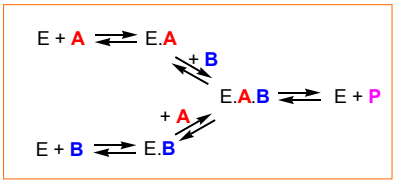
Initial rate kinetics - Traditional approach

DERIVE A MATHEMATICAL MODEL FROM BIOCHEMICAL IDEAS



Problem: Simple mechanisms ...

MERELY FIVE REACTIONS ...



"RANDOM BI-UNI" MECHANISM

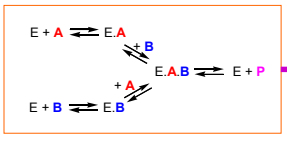
- 2 reactants (A, B)
- 1 product (P)

- 5 reversible reactions
- 10 rate constant

... lead to complex algebraic models

MERELY FIVE REACTIONS ...

Segel, I. (1975) *Enzyme Kinetics*. John Wiley, New York, p. 646.



"RANDOM BI-UNI" MECHANISM

$$\frac{v}{[E]_t} = \frac{K_1[A][B] + K_2[A]^2[B] + K_3[A][B]^2 - K_4[P] - K_5[A][P] - K_6[B][P]}{K_7 + K_8[A] + K_9[B] + K_{10}[A][B] + K_{11}[A]^2 + K_{12}[B]^2 + K_{13}[A]^2[B]}$$

$$+ K_{14}[A][B]^2 + K_{15}[P] + K_{16}[A][P] + K_{17}[B][P] + K_{18}[A][B][P] \qquad (IX\text{-}181)$$

$(k_{-5} = 0)$

where K_1 through K_{18} represent combinations of rate constants:

$K_1 = k_1k_{-2}k_3k_5 + k_{-1}k_2k_4k_5$, $K_2 = k_1k_3k_4k_5$, $K_3 = k_2k_3k_4k_5$,

$K_4 = k_{-1}k_{-2}k_{-3}k_{-5} + k_{-1}k_{-2}k_{-4}k_{-5}$, $K_5 = k_{-1}k_{-3}k_4k_{-5}$,

$K_6 = k_{-2}k_3k_{-4}k_{-5}$, $K_7 = k_{-1}k_{-2}k_{-3} + k_{-1}k_{-2}k_{-4} + k_{-1}k_{-2}k_{-5}$,

$K_8 = k_1k_{-2}k_{-3} + k_1k_{-2}k_{-4} + k_1k_{-2}k_5 + k_{-1}k_{-3}k_4 + k_{-1}k_4k_5$,

$K_9 = k_{-1}k_2k_{-3} + k_{-1}k_2k_{-4} + k_{-1}k_2k_5 + k_{-2}k_3k_{-4} + k_{-2}k_3k_5$,

$K_{10} = k_1k_{-2}k_3 + k_{-1}k_2k_4 + k_1k_3k_{-4} + k_2k_{-3}k_4 + k_3k_4k_5$,

$K_{11} = k_1k_{-3}k_4 + k_1k_4k_5$, $K_{12} = k_2k_3k_{-4} + k_2k_3k_5$, $K_{13} = k_1k_3k_4$,

$K_{14} = k_2k_3k_4$, $K_{15} = k_{-1}k_{-2}k_{-5} + k_{-1}k_{-4}k_{-5} + k_{-2}k_{-3}k_{-5}$,

$K_{16} = k_{-1}k_4k_{-5} + k_{-3}k_4k_{-5}$, $K_{17} = k_{-2}k_3k_{-5} + k_3k_{-4}k_{-5}$, $K_{18} = k_3k_4k_{-5}$

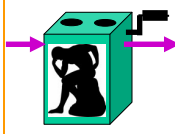
Initial rate kinetics in DynaFit

GOOD NEWS: MODEL DERIVATION CAN BE FULLY AUTOMATED!

DynaFit input file

```
[task]
   task = fit
   data = rates
   approximation = ...

[mechanism]

   E + A   <==> E.A     :  k1   k2
   E.A + B <==> E.A.B   :  k3   k4
   E + B   <==> E.B     :  k5   k6
   E.B + A <==> E.A.B   :  k7   k8
   E.A.B   <==> E + P   :  k9   k10

[constants]
   ...
```

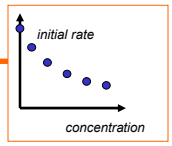
MECHANISM



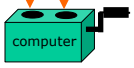
MATHEMATICAL MODEL

$$0 = [E] + [E.A] + [E.B] + [E.A.B] - [E]_{tot}$$
$$0 = [A] + [E.A] + [E.A.B] - [A]_{tot}$$
$$0 = [B] + [E.B] + [E.A.B] - [B]_{tot}$$

$$0 = +k_1[E][A] - k_2[E.A] - k_3[E.A][B] + k_4[E.A.B]$$
$$0 = +k_5[E][B] - k_6[E.B] - k_7[E.B][A] + k_8[E.A.B]$$
$$0 = +k_3[E.A][B] + k_7[E.B][A] + k_{10}[E][P] - (k_4+k_8+k_9)[E.A.B]$$

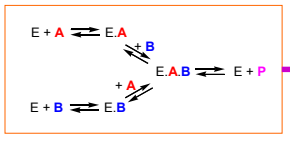


DATA



Initial rate kinetics in DynaFit *vs.* traditional method

WHICH DO YOU LIKE BETTER?




```
[task]
   task = fit
   data = rates
   approximation = king-altman

[mechanism]

   E + A   <==> E.A     :  k1   k2
   E.A + B <==> E.A.B   :  k3   k4
   E + B   <==> E.B     :  k5   k6
   E.B + A <==> E.A.B   :  k7   k8
   E.A.B   <==> E + P   :  k9   k10

[constants]
   ...

[concentrations]
   ...
```

Rapid-equilibrium approximation in enzyme kinetics

I. Segel (1975) "Enzyme Kinetics", J. Wiley, New York, pp. 22-24

The Michaelis-Menten mechanism and **rate** equation:

$$E + S \underset{}{\overset{K_S}{\rightleftharpoons}} ES \xrightarrow{k_p} E + P$$

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

How is this derived?

v is equal to the concentrations of all product-forming species, each multiplied by its catalytic rate constant.

$$v = k_p[ES]$$

Rate is proportional to the **equilibrium concentrations** of reactive complexes!

Enzyme kinetics treated as simple "binding equilibria"

1. Compute the composition at equilibrium.
2. Look up all enzyme-substrate complexes that do form products.
3. Multiply their concentrations by an appropriate proportionality constant:

   constant = molar instrumental response of the product × relevant k_{cat}
4. Compute the sum total of all such terms.

The result is the *initial rate* under the rapid equilibrium approximation.

Rapid-equilibrium enzyme kinetics in DynaFit

TWO EQUIVALENT WAYS TO REPRESENT RAPID-EQUILIBRIUM ENZYME KINETICS **DYNAFIT**

See "DynaFit Scripting Manual" on http://www.biokin.com/

METHOD 1: initial rate formalism

```
[task]
   data = rates
   approximation = rapid-equilibrium

[mechanism]
   E + S <==> E.S  :  Ks    dissoc
   E.S ---> E + P  :  kcat

[constants]
   Ks = ...
   kcat = 3

[responses]
   P = 4

...
```

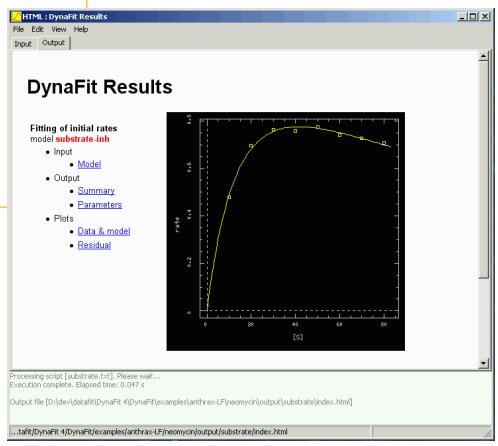
METHOD 2: equilibrium formalism

```
[task]
   data = equilibria


[mechanism]
   E + S <==> E.S  :  Ks    dissoc


[constants]
   Ks = ...


[responses]
   E.S = 12     ;     = 3 × 4

...
```


DynaFit model for inhibition by substrate

```
[task]
   task = fit
   data = equilibria

[mechanism]

   E + S <===> E.S       :  Ks
   E.S + S <===> E.S.S   :  Ks2

[responses]
   ES = 1.234 ?
...
```

the only product-forming molecular species



DynaFit model for inhibition by substrate + inhibitor

ENZYME KINETICS MADE **EASIER**

```
[task]
   task = fit
   data = equilibria

[mechanism]

   E + S <===> E.S       :  Ks    dissoc
   E.S + S <===> E.S.S   :  Ks2   dissoc
   E + I <===> E.I       :  Ki    dissoc
   E.S + I <===> E.S.I   :  Kis   dissoc

[constants]

   Ks = 1 ?, Ks2 = 1 ?
   Ki = 1 ?, Kis = 1 ?

...
...
```

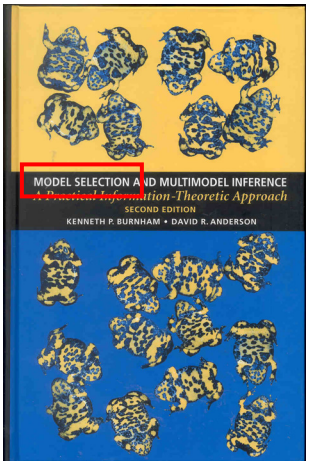
optimization flag

initial estimate

How do we know which mechanism is "best"?

COMPARE ANY NUMBER OF MODELS IN A SINGLE RUN

```
[task]
   task = fit
   model = mixed-type ?

...

[task]

   task = fit
   model = competitive ?

...

[task]

   task = fit
   model = uncompetitive ?

...
```



Akaike Information Criterion

Review: Burnham & Anderson (2004)

The best model: mixed-type noncompetitive

NEOMYCIN B IS **NOT** A COMPETITIVE INHBITOR OF LETHAL FACTOR PROTEASE

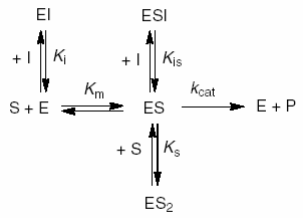
the FEBS Journal

Mixed-type noncompetitive inhibition of anthrax lethal factor protease by aminoglycosides

Petr Kuzmic[1], Lynne Cregar[2], Sherri Z. Millis[2] and Mark Goldman[2,*]

1 BioKin Ltd, Pullman, WA, USA
2 Hawaii Biotech Inc., Aiea, HI, USA

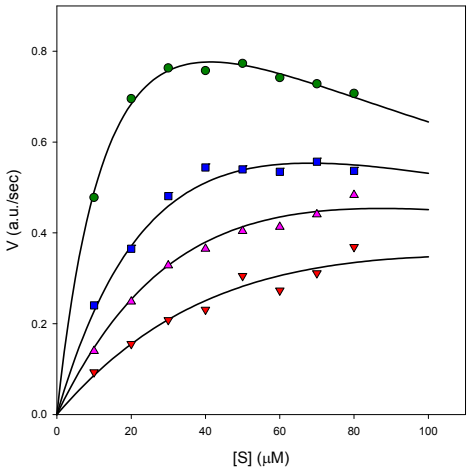
P. Kuzmic *et al.*



Scheme 5. Mixed-type mechanism.

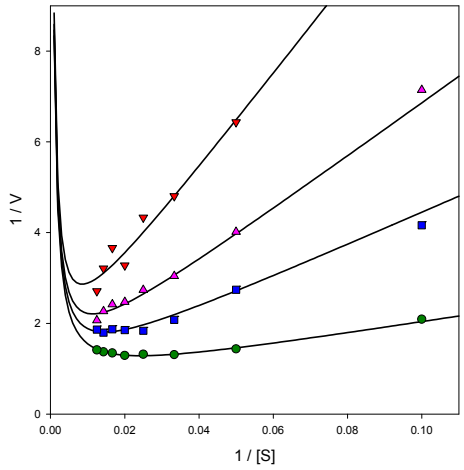
Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Direct plot: maximum on dose-response curves



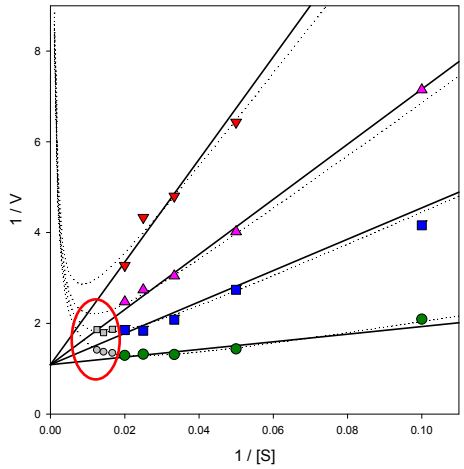
Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Double-reciprocal plot is nonlinear



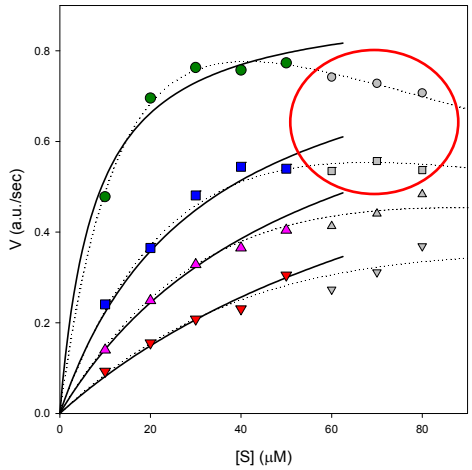
Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

DR plot obscures deviations from the model



Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Direct plot makes model departures more visible



Kuzmic *et al.* (2006) *FEBS J.* **273**, 3054-3062.

Summary and conclusions

1. Initial rate enzyme kinetics *can* be handled by numerical methods.
2. DynaFit software implements both rapid-equilibrium *and* steady-state.
3. Neglecting substrate inhibition distorts overall mechanistic analysis:

   E.g., a "competitive" enzyme inhibitor might actually be mixed-type noncompetitive.