Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2017

Lecture 16:

Data Analysis for Reversible Inhibitor Experiment:

and

Introduction to Irreversible Inhibitors

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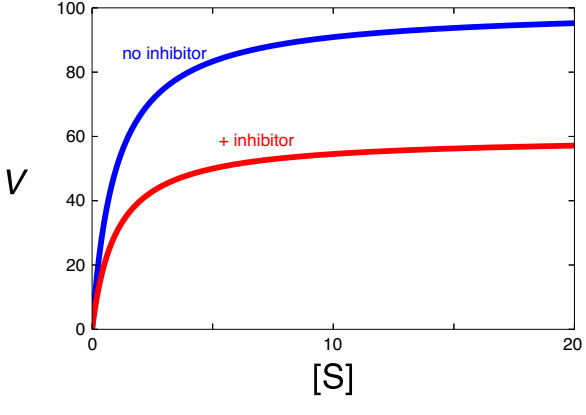
University of Utah
goldenberg@biology.utah.edu

Clicker Question #1

Which species does this inhibitor bind to?

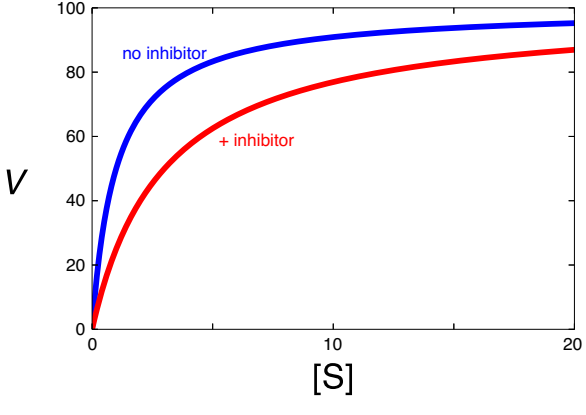


1. The free enzyme.
2. The enzyme-substrate complex.
3. Both the free enzyme and the enzyme-substrate complex.

Velocity is reduced by the same proportion at all substrate concentrations.

Clicker Question #2

Which species does this inhibitor bind to?

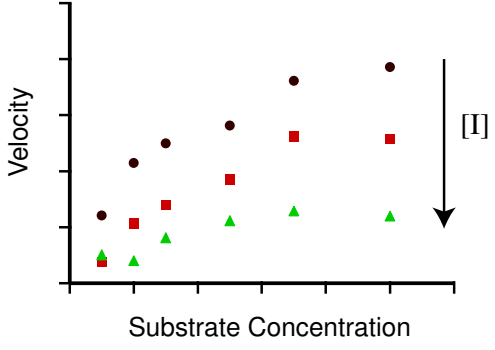


1. The free enzyme.
2. The enzyme-substrate complex.
3. Both the free enzyme and the enzyme-substrate complex.

Velocity is reduced most at low substrate concentrations.

Strategy for Analyzing Reversible Inhibition

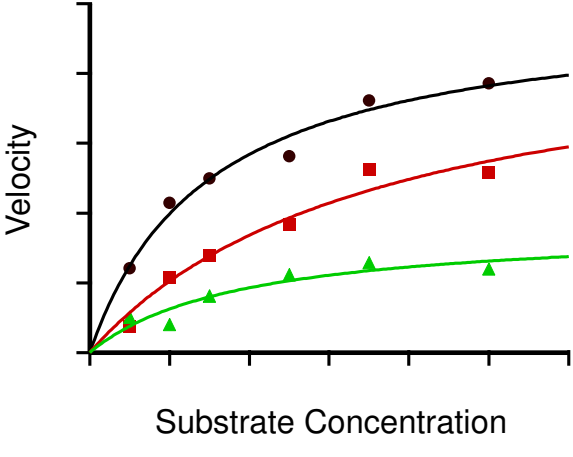
- Measure velocity versus [S] in the presence of different inhibitor concentrations



- Does the inhibitor change the apparent values of K_m, V_{max} or both?
- Experimental scatter can make it hard to tell!

Step 1

- Determine K'_m and V'_{max} from non-linear least squares fit to V vs [S] data



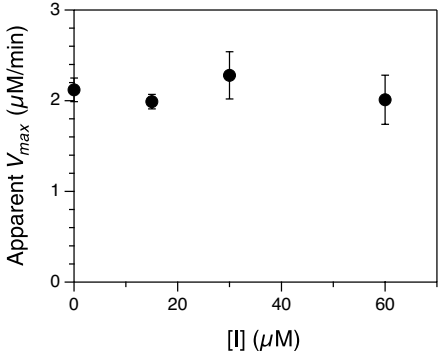
Step 2

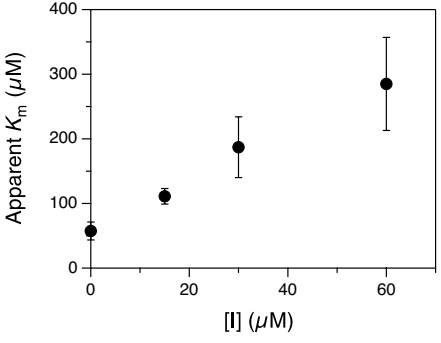
- Tabulate K'_m and V'_{max} data:

[I] (μM)	K'_m (μM)	V'_{max} (μM/min)
0		
15		
30		
60		

- Include small amount of benzamidine from the trypsin stock.
- Because of scatter in experimental data, patterns may not be obvious from inspection.

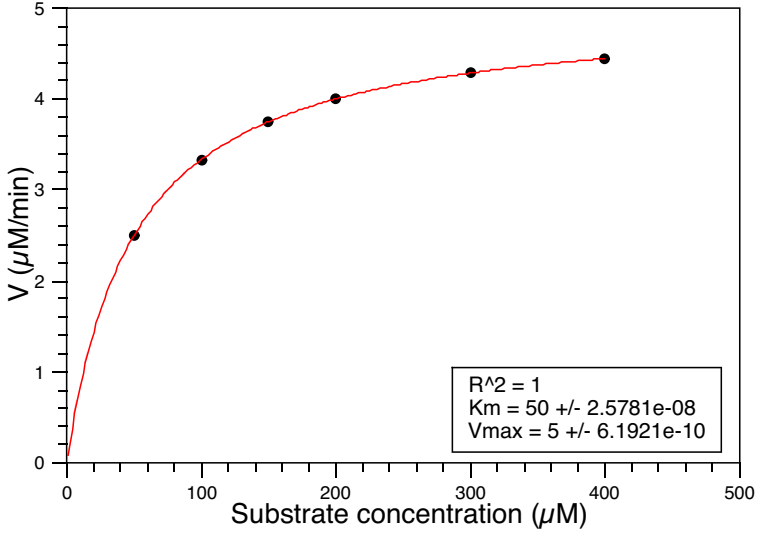
Step 3: Replot V'_{max} and K'_m versus Inhibitor Concentration



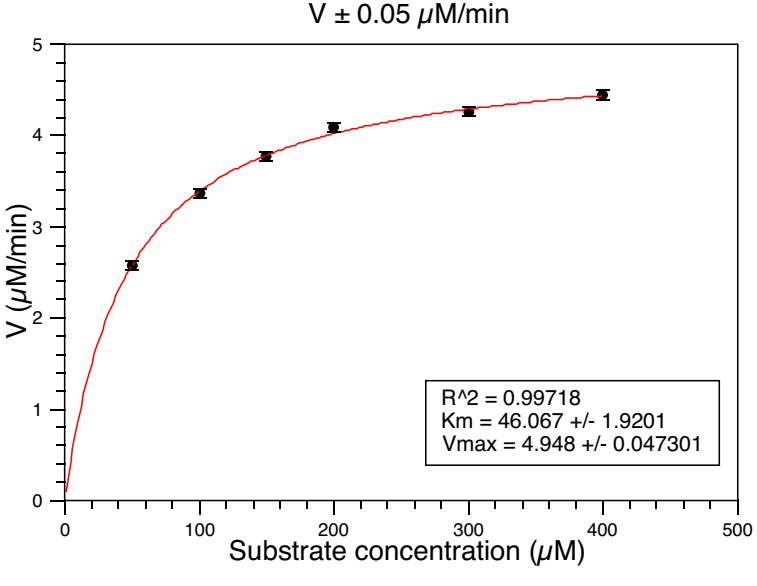


- Which parameter changes systematically?
- Error bars indicate uncertainty in the estimates of V'_{max} and K'_m.
- Error bars come from non-linear least-squares fit of V versus [S] data.

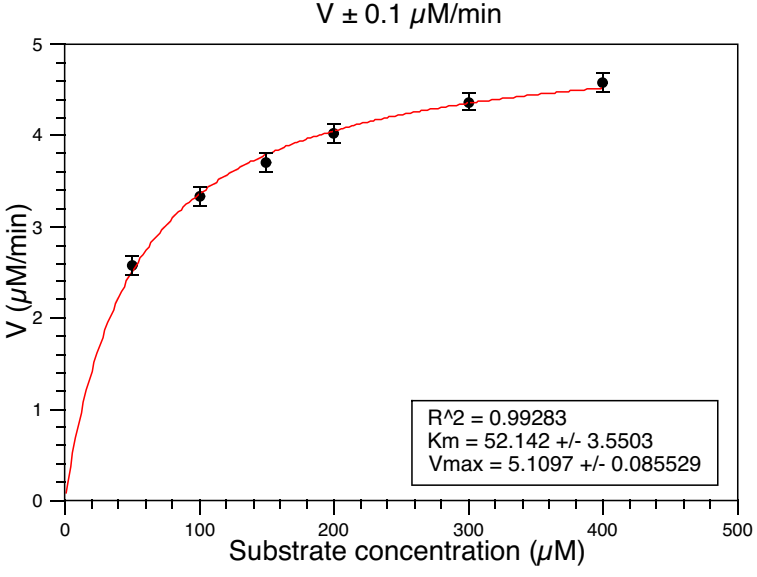
A Perfect Fit to Perfect Data



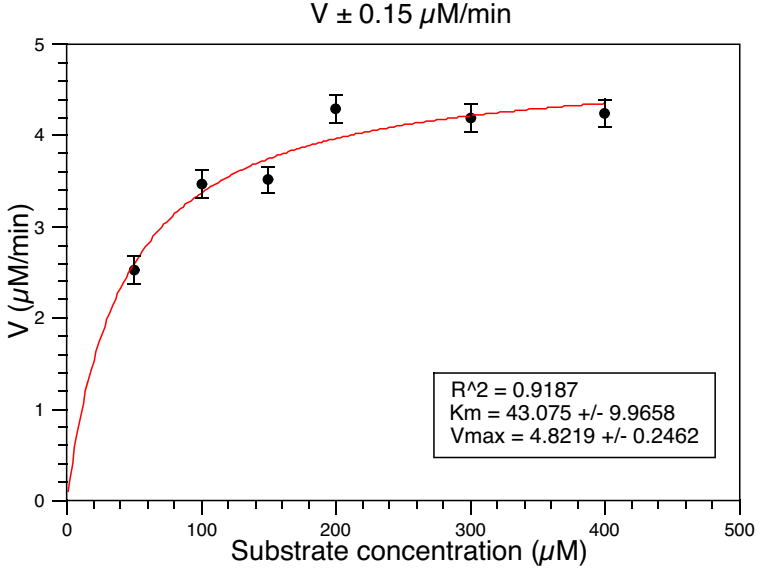
A Fit to Data with Simulated Errors



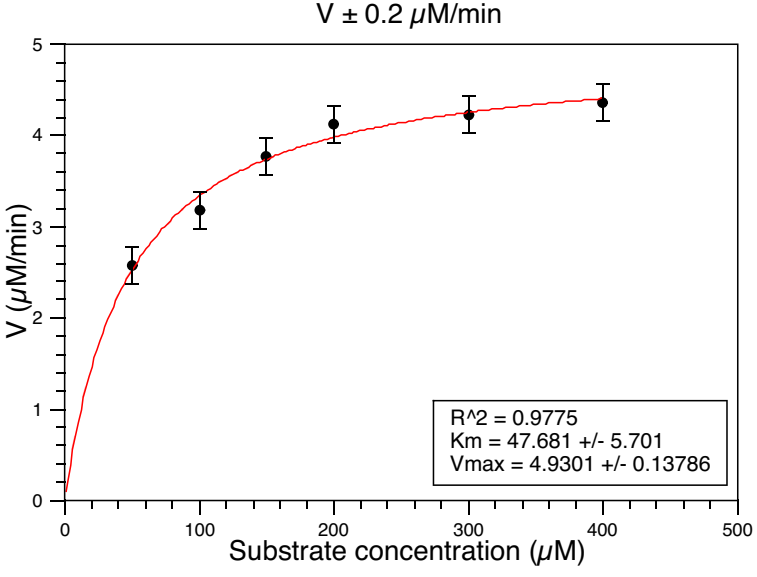
A Fit to Data with Simulated Errors



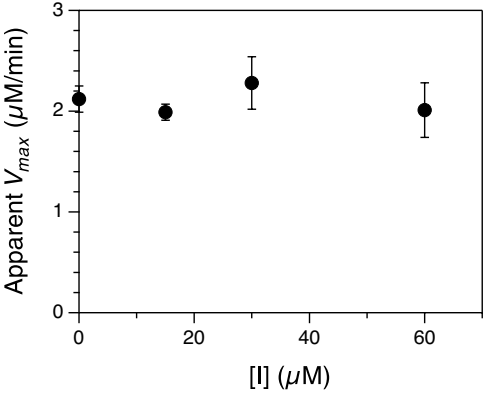
A Fit to Data with Simulated Errors



A Fit to Data with Simulated Errors

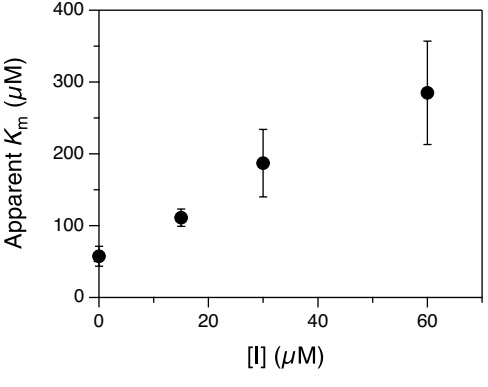


Replot #1: Apparent V_{max} versus Inhibitor Concentration



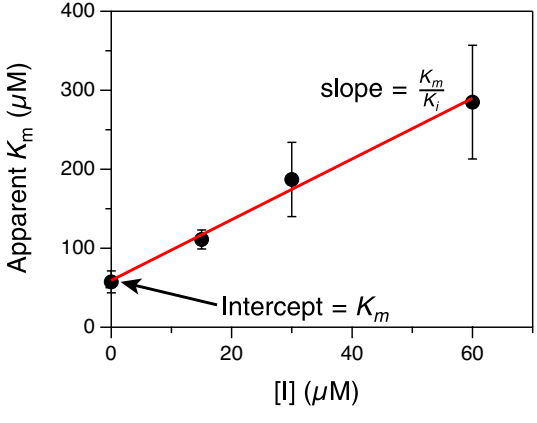
- Error bars from least-squares fits of V *vs* [S] (analogous to standard error of the mean.)
- Does V_{max} change consistently with the inhibitor concentration?
- Probably not in this case!

Replot #2: Apparent K_m versus Inhibitor Concentration



- Does K_m change consistently with the inhibitor concentration?
- Probably yes in this case!

For Competitive Inhibitor:



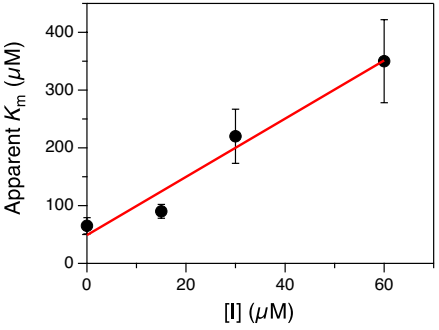
- Estimate K_m and K_i from fit of apparent K_m versus [I]

$$K'_m = K_m\,(1 + [I]/K_i)$$
$$= \frac{K_m}{K_i}[I] + K_m$$

- Use error estimates for K_m to weight data in fit.

Weighted vs. Un-weighted Least-Squares Fits

Data points weighted equally

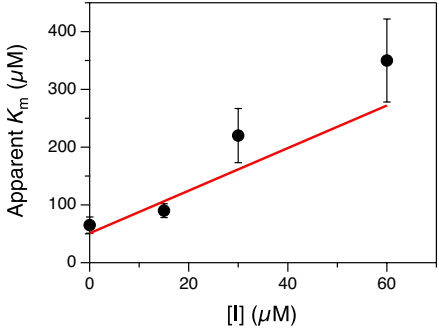


$R^2 = 0.96$

$K_m = 49 \pm 25\,\mu\text{M}$

$K_m/K_i = 5 \pm 0.7, \quad K_i = 10\,\mu\text{M}$

Data points weighted inversely by errors



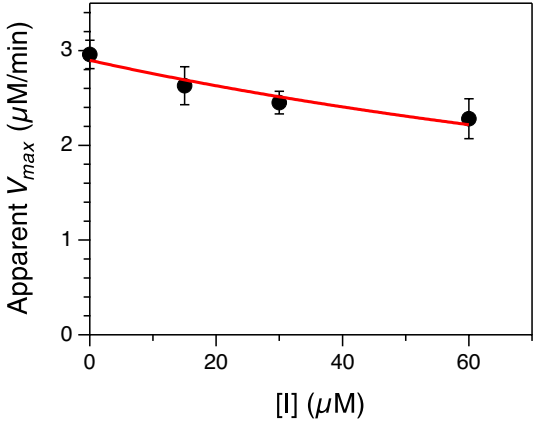
$R^2 = 0.47$

$K_m = 51 \pm 12\,\mu\text{M}$

$K_m/K_i = 3.7 \pm 0.9, \quad K_i = 14\,\mu\text{M}$

- Which fit gives the right estimates for K_m and K_i?
- We don't know! But, the weighted fit is *more likely* to give better estimates.

If Apparent V_{max} Decreases with [I]



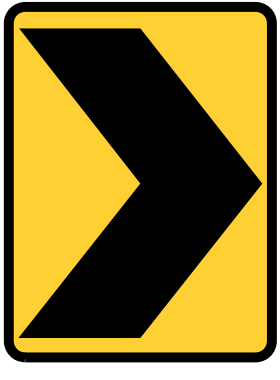
- For both noncompetitive and uncompetitive inhibition

$$V'_{max} = V_{max}/(1 + [I]/K_i)$$

- Is this real? Does K'_m:
  - Stay constant as [I] increases? (noncompetitive inhibitor)
  - Decrease as [I] increases? (uncompetitive inhibitor)
- If it looks real:
  - Estimate V_{max} and K_i from non-linear least-squares fit.

    Use error estimates for V_{max} to weight data in fit.

Warning!

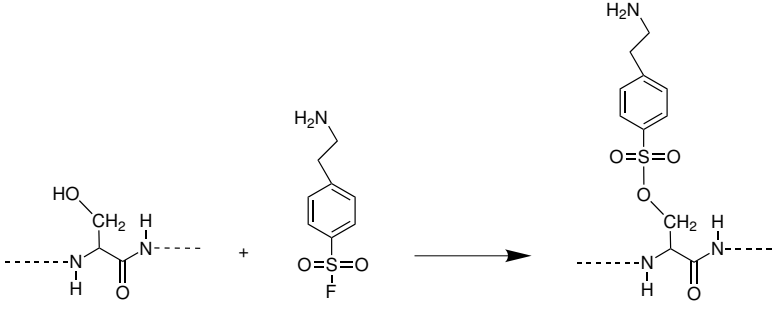


Direction Change

Irreversible Inhibitors

Irreversible Inhibition of Trypsin by AEBSF

4-(2-aminoethyl)-benzenesulfonyl fluoride



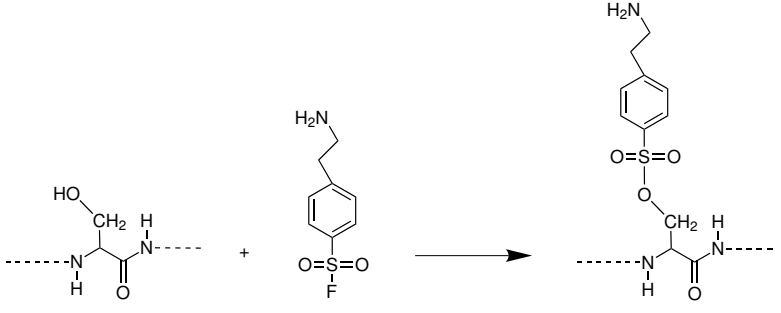
- Ser -

AEBSF

- Reaction is specific for the catalytic Ser residue.
- Reaction is irreversible.

Clicker Question #3

Is this reagent likely to be good for you?



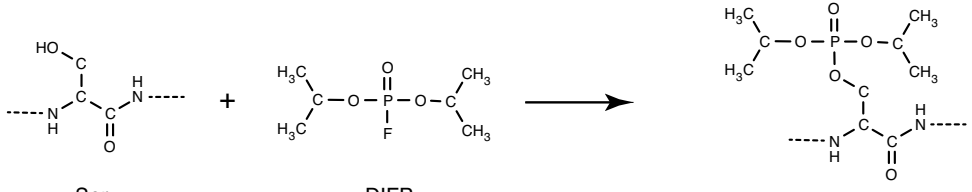
- Ser -

AEBSF

1 Yes

2 No

An Earlier Irreversible Inhibitor of Serine Proteases: Diisopropyl Fluorophosphate

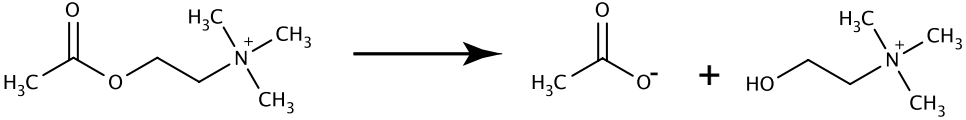


- Ser -

DIFP

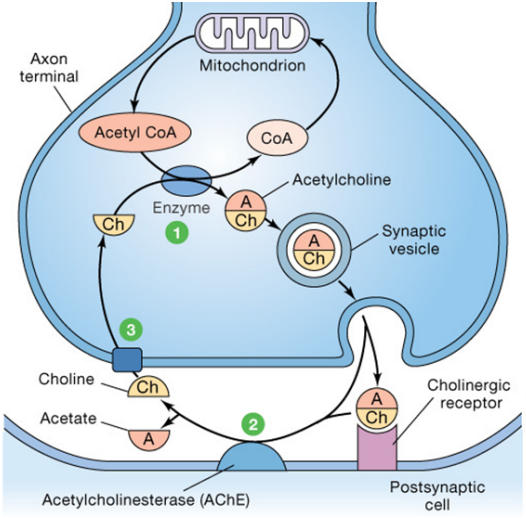
- First synthesized in the 1930's as a potential chemical weapon with neurotoxic effects.
- Found to inhibit esterases and proteases.
- Used as pesticide.
- Reported to be among chemical agents found in Syria in 2013.

Acetylcholine Esterase



- Reaction is very similar to peptide hydrolysis.
- Enzyme uses a catalytic triad (Ser-His-Glu).
- Enzyme is inhibited by DIFP and other serine-reactive agents.

A Cholinergic Synapse

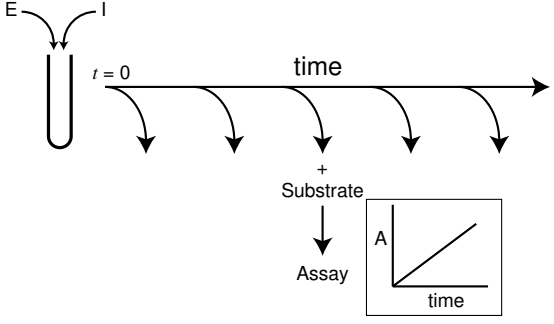


1. **Acetylcholine** (ACh) is made from choline and acetyl CoA.
2. In the synaptic cleft ACh is rapidly broken down by the enzyme **acetylcholinesterase.**
3. Choline is transported back into the axon terminal and is used to make more ACh.

- Irreversible inhibition of acetylcholine esterase is lethal.
- Mild reversible inhibition may be therapeutic.
- AEBSF is **much** less toxic than DIFP.

Experimental Protocol for Studying Irreversible Inhibition

- Follow the reaction by measuring enzymatic activity at increasing times after mixing enzyme and inhibitor.



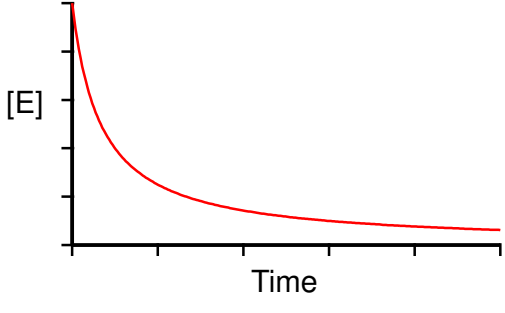
- For each sample withdrawn, measure reaction velocity.
- $V \propto$ concentration of uninhibited enzyme.
- Time for assay must be short relative to time of inactivation.

Kinetics of Irreversible Inactivation

- E + I → E-I
- Second-order kinetics:

$$\frac{d[\mathrm{E}]}{dt} = \frac{d[\mathrm{I}]}{dt} = -k_2[\mathrm{I}][\mathrm{E}]$$

[E] versus time:



- Why does the rate of inactivation decrease with time?

Pseudo First-Order Kinetics

- If [I] $\gg$ [E], [I] will remain approximately constant during the reaction.

$$\frac{d[\mathrm{E}]}{dt} = -\underbrace{k_2[\mathrm{I}]}_{\text{constant}} \cdot [\mathrm{E}]$$

- Define a pseudo first-order rate constant: $k_{app} = k_2[\mathrm{I}]$

$$\frac{d[\mathrm{E}]}{dt} = -k_{app}[\mathrm{E}]$$

- Rearrange and integrate the rate expression:

$$\int_{[\mathrm{E}]_0}^{[\mathrm{E}]} \frac{d[\mathrm{E}]}{[\mathrm{E}]} = \int_{t=0}^{t} -k_{app}\,dt$$

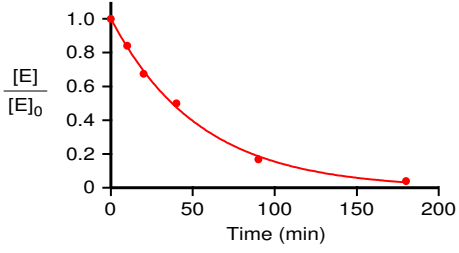
$[\mathrm{E}]_0$ = Initial enzyme concentration.

Pseudo First-Order Kinetics

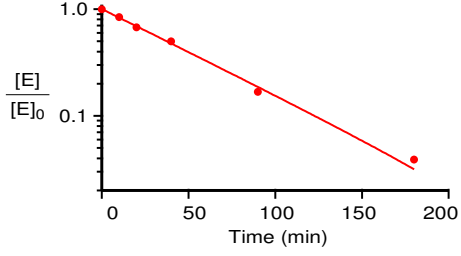
- Integrated rate expression:

$$\ln\left(\frac{[E]}{[E]_0}\right) = -k_{app}t, \qquad \frac{[E]}{[E]_0} = e^{-k_{app}t}$$

Standard plot:



Semi-logarithmic plot:



Data Interpretation

- Estimate k_{app} from fit of $[E]/[E]_0$ versus time.
- Calculate second-order rate constant, k_2, from k_{app}

$$k_2 = k_{app}/[I]$$

- Can use estimate of k_2 to predict kinetics of inactivation at other inhibitor concentrations.