Biological Chemistry Laboratory
Biology 3515/Chemistry 3515
Spring 2023

Lecture 17:

Irreversible Serine Protease Inhibitors

14 March 2023

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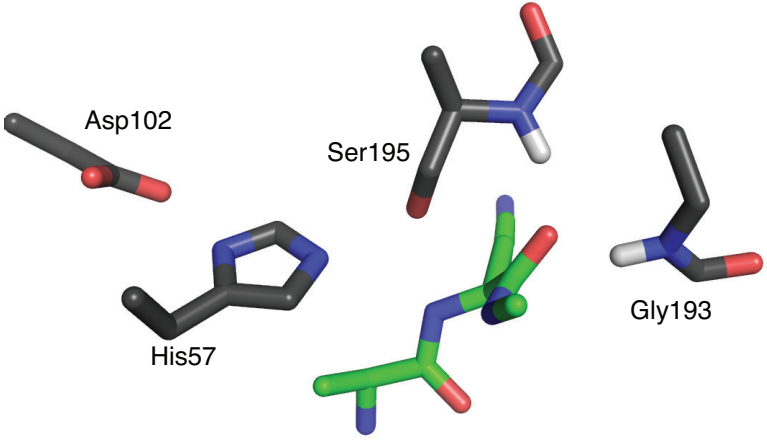
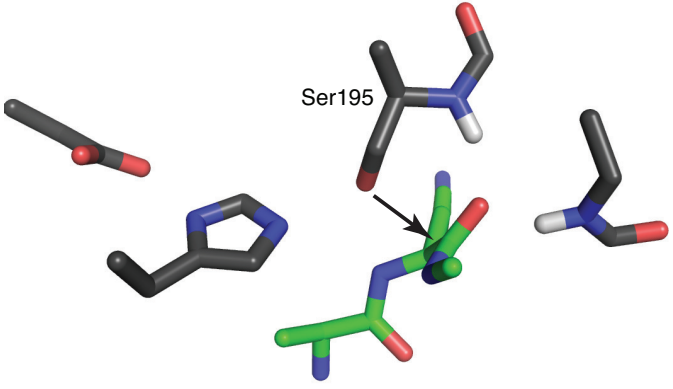
The Trypsin Active Site



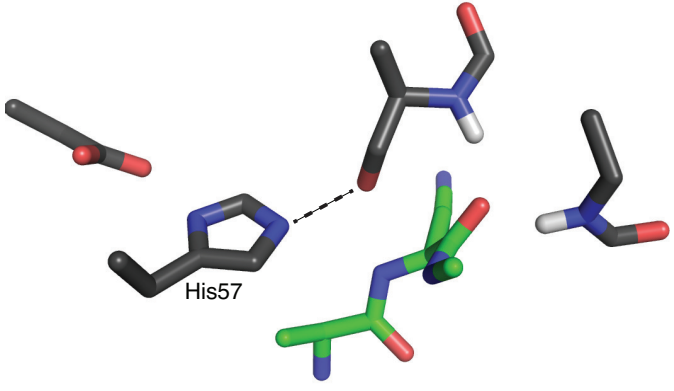
Clicker Question #1

Which residue carries out nucleophilic attack on the substrate?



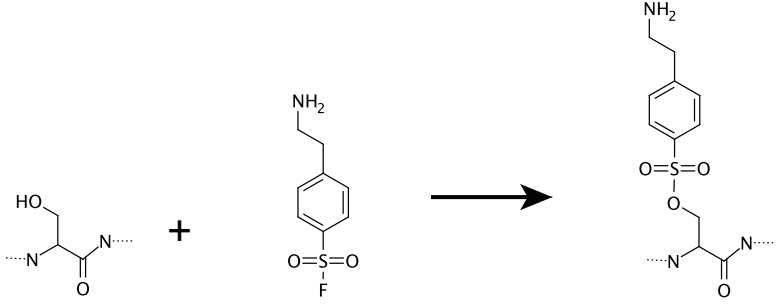
Clicker Question #2

Which residue deprotonates the nucleophilic oxygen atom?



Irreversible Inhibition of Trypsin by AEBSF

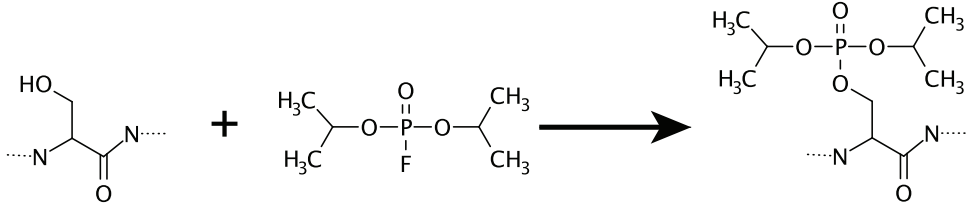
4-(2-aminoethyl)-benzenesulfonyl fluoride



-Ser- AEBSF

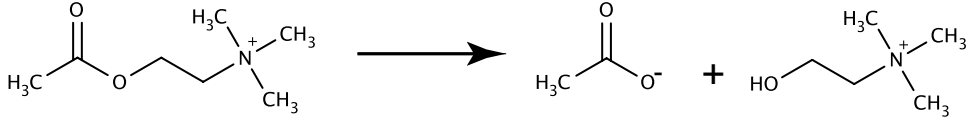
- Reaction is specific for the catalytic Ser residue.
- Reaction is irreversible.

An Earlier Irreversible Inhibitor of Serine Proteases: Diisopropyl Fluorophosphate



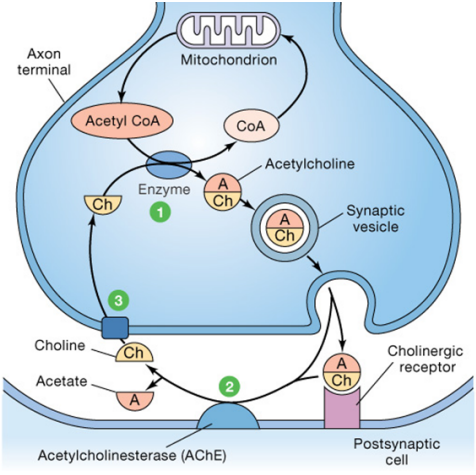
- First synthesized in the 1930's as a potential chemical weapon with neurotoxic effects.
- Found to inhibit esterases and proteases.
- Still widely used as a pesticide.

Acetylcholine Esterase



- Acetylcholine is a major neurotransmitter in vertebrates, insects and other animals.
- 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

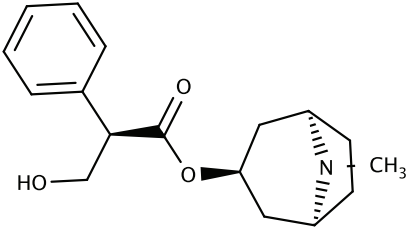


- Irreversible inhibition of acetylcholine esterase is lethal.
- Mild reversible inhibition may be therapeutic.
- AEBSF is **much** less toxic than DIFP.

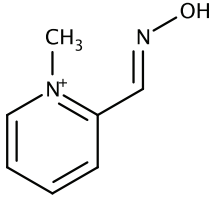
Figure from http://peaknootropics.com/acetylcholinesterase-memory-problems/

Two Antidotes for Organophosphate Poisoning

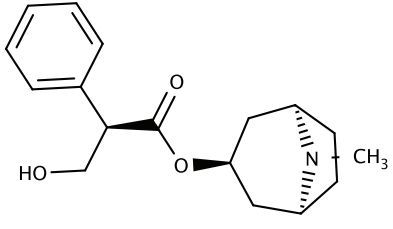
Atropine



Pralidoxime

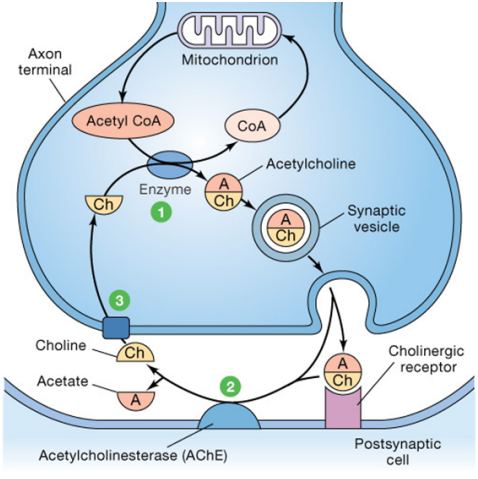


Atropine



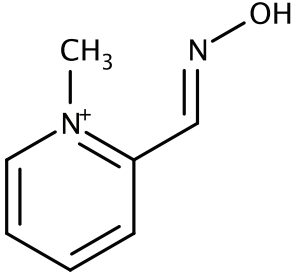
- Isolated from *Atropa belladonna*, “deadly nightshade”.
- Inhibitor of acetylcholine receptors.
- Causes many symptoms, including dilation of pupils.
- ”Bella dona” is Italian for “beautiful lady”. Plant extracts were used by women to dilate their eyes.
- Shorter acting drugs are used by ophthalmologists for dilation.

A Cholinergic Synapse



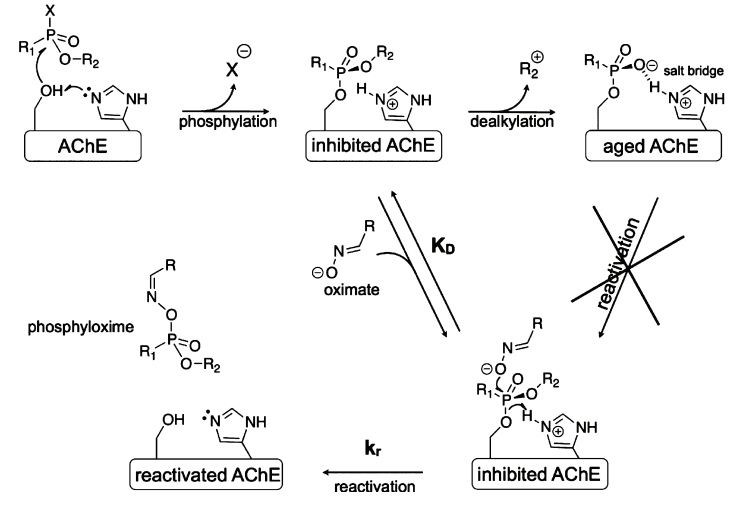
- Atropine competes with acetylcholine for binding to acetylcholine receptors.
- Atropine is an acetylcholine antagonist; binds to receptor without activating.
- Blocks synaptic transmission; better than continuous activation.

Pralidoxime



- Reactivates inhibited acetylcholine esterase.
- What makes this molecule special?
- Hydroxyl has unusually low $\mathrm{p}K_\mathrm{a}$, ≈ 8.
- Oxygen is especially reactive.

Inactivation and Reactivation of Acetylcholine Esterase



Mercey, G., Verdelet, T., Renaou, J., Kiliachynai, M., Baatli, R., Nachon, F., Jean, L. & Renard, P.-Y. (2012). Reactivators of acetylcholinesterase inhibited by organophosphorous nerve agents. *Acc. Chem. Res.*, 45, 756–766. http://dx.doi.org/10.1021/ar2002864

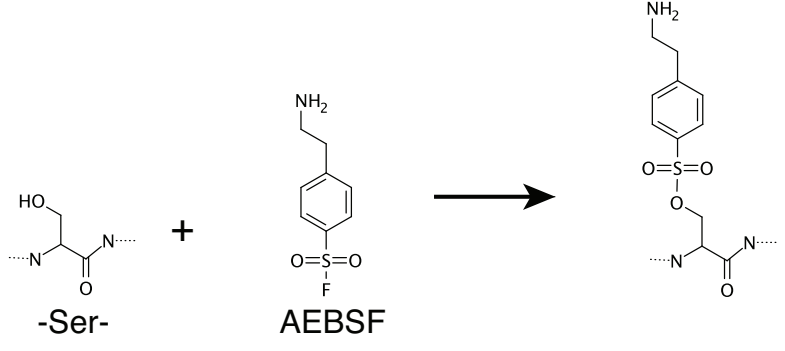
Organophosphorous Poisoning and Treatment

- $\sim$200,000 deaths per year by self-administered organophosphorous pesticides in rural Asia.
- Muscineric acetylcholine antagonists (*e.g.,* atropine) are generally the initial treatment.
- Oxime reactivators (*e.g.,* pralidoxime) are often used in conjunction with atropine.
  - Oximes are not very general. Different oximes are specific for different organophosphorous toxins.
  - Efficacy of oximes is debated.

Eddleston, M., Buckley, N. A., Eyer, P. & Dawson, A. H. (2008). Management of acute organophosphorous pesticide poisoning. *The Lancet*, 371, 16–22. https://doi.org/10.1016/S0140-6736(07)61202-1

Back to Irreversible Inhibition of Trypsin by AEBSF

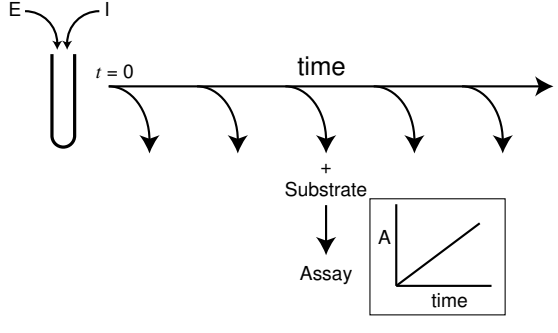
4-(2-aminoethyl)-benzenesulfonyl fluoride



-Ser- AEBSF

Experimental Protocol for Studying Irreversible Inhibition

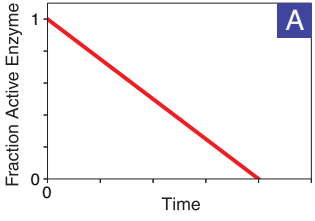
- Follow the reaction by measuring enzymatic activity at increasing times after mixing enzyme and inhibitor.

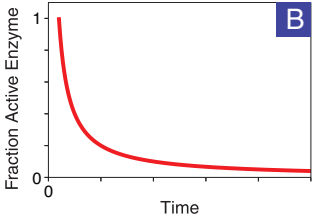


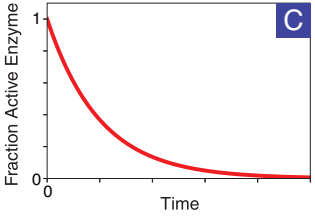
- For each sample withdrawn, measure reaction velocity.
- $V \propto$ concentration of uninhibited enzyme.
- Time for assay must be short relative to time of inactivation.

Clicker Question #3

How does the concentration of active enzyme change with time?







$$\frac{[E]}{[E]_0} = 1 - kt$$

$$\frac{[E]}{[E]_0} = \frac{1}{kt}$$

$$\frac{[E]}{[E]_0} = e^{-kt}$$

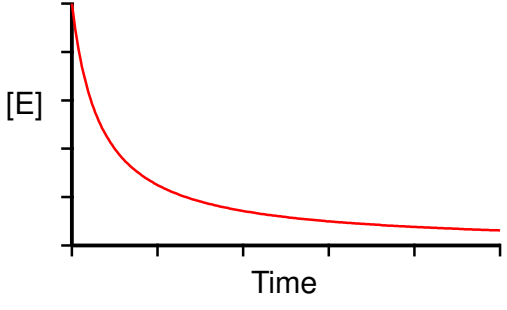
All answers count for now.

Kinetics of Irreversible Inactivation

- E + I $\rightarrow$ E-I
- Second-order kinetics:

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

- If initial concentrations of enzyme and inhibitor are equal:



- This is *not* an exponential decay function!

  Both [I] and [E] decrease with time, and both decreases contribute to reduced rate with time.

Pseudo First-Order Kinetics

- If $[\mathrm{I}] \gg [\mathrm{E}]$, $[\mathrm{I}]$ 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: $k_{\text{app}} = k_2[\mathrm{I}]$

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

- Rearrange and integrate the rate expression:

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

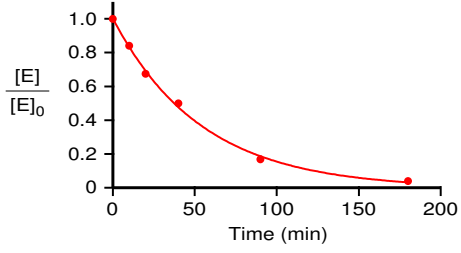
$[\mathrm{E}]_0$ = Initial enzyme concentration.

Pseudo First-Order Kinetics

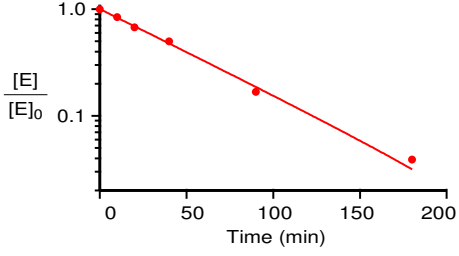
- Integrated rate expression:

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

Standard plot:



Semi-logarithmic plot:



Why Do We See Exponentials and Logarithms Everywhere?

- The exponential function, $y = e^x$, is its own derivative:

$$\frac{de^x}{dx} = e^x$$

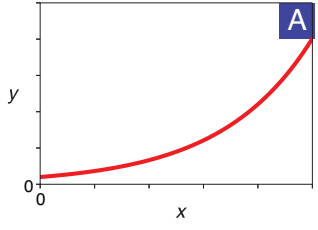
- More generally, if k is a constant:

$$\frac{de^{kx}}{dx} = ke^{kx}$$

- Why is this important?
- There are many physical and biological processes for which the rate of change is proportional to the quantity that is changing!

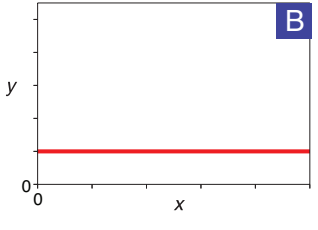
Clicker Question #4
(Multiple answers possible!)

Which of these represents an exponential function: $y = e^{kx}$?



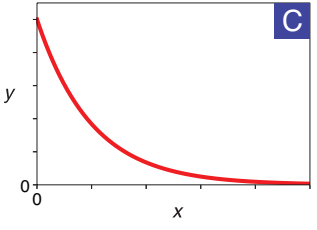
$k > 0$

$\frac{dy}{dx} = ke^{kx} > 0$



$k = 0$

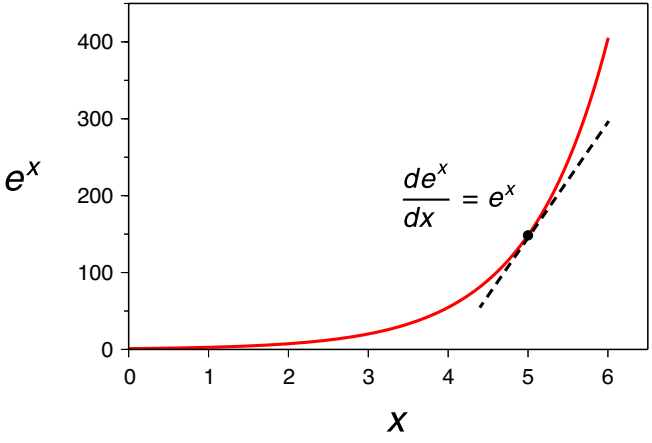
$\frac{dy}{dx} = ke^{kx} = 0$



$k < 0$

$\frac{dy}{dx} = ke^{kx} < 0$

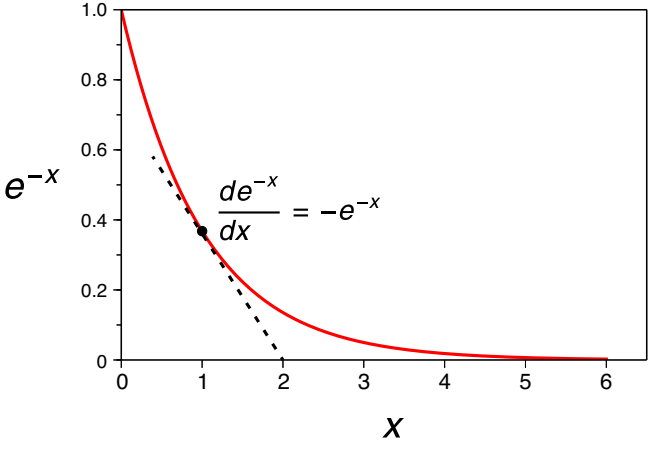
Exponential Growth



Example: Growth of bacteria:

- Each bacterium has an equal probability of dividing during a given time period.
- The number of new bacteria in a short time period is proportional to the number already present.

Exponential Decay



Example: Radioactive decay:

- Each nucleus has an equal probability of decaying during a given time period.
- The number of nuclei that decay in a short time period is proportional to the number of nuclei present.

Back to Pseudo First-Order Kinetics

- Second-order kinetics:

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

- If [I] does not change significantly, we can define an apparent rate constant:

$$k_{\mathrm{app}} = k_2[\mathrm{I}]$$

- Pseudo first-order kinetics

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

- Integrated rate expression:

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

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.