Enzyme Kinetics

http://courses.umassmed.edu/GSBS/Graduate_Core_Curriculum/2004_2005/Block_1/index.cfm

http://glutxi.umassmed.edu/default.html
Goals

1. Understanding reaction order and rate constants
2. Analysis of substrate/enzyme interactions
3. Analysis of Michaelis-Menten kinetics
4. Understand the significance of $K_m$, $K_s$ and $V_{\text{max}}$
5. Analysis of reaction rates
6. A method for developing solutions to new schema
7. Analysis of enzyme inhibitions
Rates of Reactions and Reaction Order

The order of a reaction is determined by the power of the concentration terms in the rate equation.

In a simple isomerization reaction

\[ A \xrightarrow{k_1} B \]

\[ \frac{d[B]}{dt} = k_1[A] \]

The units for this first order reaction are derived from moles of product formed per second per mole of reactant or,

\[ \text{molarity per sec} = k_1 \text{.molarity} \]

\[ \frac{\text{molarity per sec}}{\text{molarity}} = k_1 = \text{per sec} \]
In a reaction of the type

\[ \text{E} + \text{S} \overset{k_1}{\rightarrow} \text{ES} \]

This reaction is second order as one single step is involved in the reaction. The rate of the reaction is proportional to \([E][S]\)

\[ \frac{d[ES]}{dt} = k_1[E][S] \]

The units of this second order reaction are derived from moles of product formed per second per mol^2 of reactants or

\[
\text{molarity per sec} = k_1 \text{ (molarity)}^2
\]

\[
\frac{\text{molarity per sec}}{\text{molarity}^2} = k_1 = \text{per mole per sec}
\]
Some Definitions
Rate constants, Michaelis constants and Dissociation constants

Throughout this handout you will see the multiple use of terms including upper- and lower-case “k”. When used in the lower case (e.g. $k_1$, $k_{-1}$, $k_p$) these terms refer to individual steps or rate constants in a reaction. e.g.

$$E + S \xrightleftharpoons{k_1}{k_{-1}} ES \xrightarrow{k_p} E + P$$

When used in the upper case (e.g. $K_m$, $K_S$, $K_d$) these terms refer to Michaelis ($K_m$) or dissociation ($K_S$, $K_d$) constants - terms that group individual rate constants. e.g.

$$K_m = \frac{k_p + k_{-1}}{k_1} \quad K_S = \frac{k_{-1}}{k_1} = \frac{[E][S]}{[ES]}$$
CATALYTIC PRINCIPLES

A key step in enzyme function is the formation of the enzyme (E)/substrate (S) complex, ES.

A number of data point to the existence of ES prior to the release of product (P) and free E. These are:

1. ES complexes have been directly visualized by EM + X-ray crystallography.

2. The physical properties of an enzyme can change upon binding S.

3. The spectroscopic characteristics of E and S can change upon ES formation.

4. High specificity for ES formation is observed.

5. The ES complex may be isolated in pure form.
6. At constant [E], increasing [S] results in increased product formation to a point where product formation no longer increases. This saturation is presumed to reflect the fact that all E is now in the form ES. This is illustrated below.

\[ V_{\text{max}} \]

\[ K_m \]

\[ 0.5 V_{\text{max}} \]

\[ [S] \text{ mM} \]

\[ v, \text{ rate of reaction} \]

\[ V_{\text{max}} \text{ is a theoretical, maximum value for } v.\]

\[ K_m \text{ is that concentration of } [S] \text{ producing a } v \text{ of } V_{\text{max}}/2.\]
The reaction velocity curve is a section of a rectangular hyperbola which in this instance takes the generic form

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

This equation is called the Michaelis-Menten equation.

How can this phenomenon be rationalized in terms of enzyme-mediated catalysis?
Thermodynamics of enzyme-mediated catalysis

Let us examine an enzyme-mediated reaction.

Here enzyme E reacts with substrate S to form the complex ES. ES is then converted to EP which breaks down to E and product (P). The rate constants $k_1$, $k_{-1}$, $k_2$, $k_{-2}$, $k_3$ and $k_{-3}$ describe the rates of the various steps.

What does this reaction pathway mean in physical terms? Consider the energy diagram for this reaction:
\[ \Delta 1: \quad \text{E+S} \rightarrow \text{E+P} \text{ (or S} \rightarrow \text{P)} \text{ overall energy difference between unactivated product and unactivated substrate. } \Delta 1 \text{ is independent of reaction pathway (i.e. unchanged by presence of enzyme).} \]

\[ \Delta 2: \quad \text{energy difference between ES and E+S. } K_S = K_m \text{ in rapid equilibrium systems and lies in the range of } 10^{-6} \text{ to } 10^{-2} \text{ M. } \Delta 2 (\Delta G) \approx -2700 \text{ to } -8200 \text{ cal/mol.} \]

\[ \Delta 3: \quad \text{energy required to form activated } (\text{E...S})^\dagger \text{ complex. } k_1 \text{ values range from } 10^6 \text{ to } 10^9 \text{ M}^{-1}.\text{s}^{-1}. \Delta 2 + \Delta 3 = \text{energy required to raise ES to transition state for dissociation.} \]

\[ \Delta 4: \quad \text{bond breaking/making step. If this step is rate limiting, a plot of log } V_{\text{max}} \text{ versus } 1/T \text{ (Arrhenius Plot) gives the activation energy } (E_a) \text{ for this step. In absence of the enzyme, } E_a \text{ would be much greater.} \]

\[ \Delta 5: \quad \text{ES} \rightarrow \text{EP} \text{ energy difference between the two central complexes. Cannot be measured directly.} \]

\[ \Delta 6: \quad \text{energy required to raise EP to a transition state for dissociation. } k_3 \text{ is in the order of } 10^4 \text{ to } 10^7 \text{ per sec.} \]

\[ \Delta 7: \quad \text{energy difference between EP and E + P} \]
The solution (equation describing the rate of reaction in terms of substrate and product levels and rate constants) is complex and difficult to derive. However, we can make a number of simplifying assumptions in order to more readily obtain a solution to this scheme. How do we do this?

1. **Assume that the reverse reaction (P→S) is negligible.** While this reaction is thermodynamically feasible and thus occurs, we (acting as the biochemists working with this enzyme) can establish experimental conditions that preclude or minimize the reverse reaction. e.g. adding an additional enzyme which converts P into another species Q which cannot react with our enzyme. Or, we can measure the rate of reaction at very early time points where the reverse reaction is insignificant.

2. **Assume only a single central complex (ES) exists.** i.e. ES breaks down directly to E + P.
The overall scheme is visualized as follows.

There are two parts to this reaction:

1) Formation of ES
2) ES breakdown to product P and free enzyme E

We will consider ES formation first.
Enzyme/substrate interactions ≡ Receptor/ligand interactions

The first step in enzyme function is the formation of the enzyme (E)/substrate (S) complex, ES.

Consider the reaction

\[ E + S \xrightleftharpoons[k_2]{k_1} ES \]

The rate of ES formation is given by:

\[ \frac{+d[ES]}{dt} = k_1 [E][S] \]

The rate of ES breakdown is given by:

\[ \frac{-d[ES]}{dt} = k_2 [ES] \]

At equilibrium:

\[ \frac{+d[ES]}{dt} = \frac{-d[ES]}{dt} \quad \therefore k_1 [E][S] = k_2 [ES] \]

Thus:

\[ [ES] = \frac{k_1}{k_2} [E][S] \]
We are interested in how much ES is formed (how much substrate is bound) at any given [S] and [E]. We can express [ES] as as a fraction of total enzyme [E_\text{t}] as:

\[
\frac{[ES]}{[E_\text{t}]} = \frac{[ES]}{[E] + [ES]}
\]

and, substituting for [ES],

\[
\frac{[ES]}{[E_\text{t}]} = \frac{k_1 [E][S]}{[E] + \frac{k_1}{k_2} [E][S]}
\]

Canceling [E] thus gives us:

\[
\frac{[ES]}{[E_\text{t}]} = \frac{k_1 [S]}{1 + \frac{k_1}{k_2} [S]}
\]

thus

\[
\frac{[ES]}{[E_\text{t}]} = \frac{k_2}{k_1} [S] + [S]
\]

what is \(k_2/k_1\)?
The ratio $k_2/k_1$ has units of:

$$\frac{\text{per sec}}{\text{per M per sec}} = \text{M}$$

When $[S] = k_2/k_1$,

$$\frac{[\text{ES}]}{[E_t]} = \frac{[S]}{k_2/k_1 + [S]} = \frac{[S]}{2[S]} = 0.5$$

This means that one-half of $[E_t] = [\text{ES}]$ when $[S] = k_2/k_1$.
What is the significance of $k_2/k_1$?

$k_1/k_2 = K_{eq} = \text{equilibrium constant or association constant for E-S interaction.}$

$k_2/k_1 = 1/K_{eq} = K_S \text{ or } K_d = \text{dissociation constant for the ES complex}$
If $K_S$ is 1 µM, 50% of $E_t = ES$ when $[S] = 1$ µM.

If $K_S$ is 1 nM, 50% of $E_t = ES$ when $[S] = 1$ nM.

A low value for $K_S$ means that the ES complex is more stable (less dissociates to E + S) thus at any $[S]$, there is a higher probability that ES is formed. Less S is required to occupy one-half of the available binding sites. The enzyme shows high affinity for S.
How do you measure $K_S$ or $K_d$? - 1) Lineweaver Burk method

\[
\frac{[ES]}{[E_t]} = \frac{[S]}{k_2 + [S]k_1} \quad \therefore \quad [S_b] = [ES] = [E_t] \frac{[S]}{k_2 + [S]k_1} = \frac{[E_t][S]}{K_S + [S]}
\]

Taking the reciprocal of this expression:

\[
\frac{1}{[S_b]} = \frac{K_S}{[E_t][S]} + \frac{[S]}{[S][E_t]} = \frac{K_S}{[E_t][S]} + \frac{1}{[E_t]}
\]
The expression,

\[
\frac{1}{[S_b]} = \frac{K_S}{[E_t][S]} + \frac{1}{[E_t]}
\]

Is a familiar linear expression of the type

\[y = \text{slope} \times x + \text{y-intercept}.\] Thus
When \([S] = -K_S\) (a theoretical state because in life, \([S] \geq 0\)),

\[
\frac{1}{[S_b]} = \frac{K_S}{[E_t] - K_S} + \frac{1}{[E_t]} = -\frac{1}{[E_t]} + \frac{1}{[E_t]} = 0
\]

Thus the x-intercept = \(1/-K_S\)
\[
\frac{1}{[S_b]} = \frac{K_S}{[E_t][S]} + \frac{1}{[E_t]}
\]
What do this analysis assume?

• 1 molecule of E can bind 1 molecule of S

Thus $E_t = B_{\text{max}}$ (the maximum binding capacity of the enzyme).

If $[E_t]$ were 2 $\mu$M, $B_{\text{max}} = 2 \mu$M.

If 2 molecules of S bound to each molecule of E,

$$2E_t = B_{\text{max}}$$

If 1 molecule of S bound to only 2 molecules of E (the active enzyme complex is an E dimer),

$$0.5 E_t = B_{\text{max}}$$
How do you measure $K_S$ or $K_d$? - 2) Hanes Woolf method

\[
\frac{1}{[S_b]} = \frac{K_S}{[E_t][S]} + \frac{1}{[E_t]}
\]

Multiply by $[S]$

\[
\frac{[S]}{[S_b]} = \frac{K_S [S]}{[E_t][S]} + \frac{[S]}{[E_t]} = \frac{K_S}{[E_t]} + [S] \frac{1}{[E_t]}
\]

$y = y$-cept + $x$ * slope

When $[S] = -K_S$

\[
\frac{[S]}{[S_b]} = \frac{K_S}{[E_t]} + \frac{-K_S}{[E_t]} = 0
\]

Thus the x-intercept = $-K_S$
\[
\frac{[S]}{[S_b]} = \frac{K_S}{[E_t]} + [S] \frac{1}{[E_t]}
\]

Hanes-Woolf analysis

\[
y = 20 + 1 \times
\]
How do you measure $K_S$ or $K_d$? - 3) Scatchard Analysis

\[ [S_b] = \frac{[E_t][S]}{K_S + [S]} \]

\[ \therefore \frac{[S_b][K_S + [S]]}{[S]} = \left[ E_t \right] = \frac{[S_b]K_S}{[S]} + [S_b] \]

\[ \therefore \frac{[E_t]}{K_S} = \frac{[S_b]}{[S]} + \frac{[S_b]}{K_S} \]

\[ \frac{[S_b]}{[S]} = \frac{[E_t]}{K_S} - \frac{[S_b]}{K_S} = \frac{[E_t]}{K_S} - \frac{1}{K_S} [S_b] \]

y = y-cept - slope \times x

When $[S_b] = [E_t]$, \[ \frac{[S_b]}{[S]} = \frac{[E_t]}{K_S} - \frac{[E_t]}{K_S} = 0 \]
\[
\frac{[S_b]}{[S]} = \frac{[E_t]}{K_s} - \frac{1}{K_s}[S_b]
\]

Scatchard Analysis

\[y = 0.04 - 0.04x\]

slope = \(-1/K_s\)
GluT1 $+$ $^{[3H]}$-CCB $\leftrightarrow$ GluT1.$^{[3H]}$-CCB

- Purified GluT1 proteoliposomes
- $^{[3H]}$-CCB
- Sample suspension and count total [CCB]
- Sample supernatent and count free [CCB]
- Sediment membranes by centrifugation
\[
\begin{align*}
\text{BG} &= 30.92 \\
[C\text{CB}]_{\text{starting}} \text{ nM} & \quad \text{dpm total} \quad \text{dpm Free} \\
50 & \\
6186.77 & 680.80 \\
6095.22 & 634.28 \\
5840.72 & 666.96 \\
5757.74 & 644.11 \\
5917.63 & 656.39 \\
4870.16 & 572.09 \\
100 & \\
5456.44 & 590.19 \\
7045.35 & 715.15 \\
5645.22 & 605.38 \\
6229.24 & 709.15 \\
5781.79 & 694.02 \\
5936.64 & 715.58 \\
200 & \\
5423.40 & 642.58 \\
5536.15 & 757.46 \\
5978.32 & 818.24 \\
6364.50 & 800.62 \\
6315.15 & 764.64 \\
5353.64 & 766.76 \\
300 & \\
5825.84 & 657.92 \\
6023.38 & 823.33 \\
5547.10 & 806.92 \\
5503.19 & 863.50 \\
5986.15 & 912.01 \\
6291.65 & 783.19 \\
400 & \\
4751.94 & 733.07 \\
5496.05 & 784.33 \\
5875.57 & 749.64 \\
6278.16 & 847.04 \\
6585.83 & 793.28 \\
5267.55 & 858.30 \\
500 & \\
4988.30 & 785.90 \\
5669.91 & 863.30 \\
5383.14 & 858.37 \\
5978.96 & 903.62 \\
5894.62 & 907.20 \\
5565.45 & 857.34 \\
1250 & \\
5572.24 & 1304.99 \\
5551.38 & 1508.94 \\
5991.47 & 1528.28 \\
5714.77 & 1545.37 \\
6291.51 & 1629.74 \\
5717.34 & 1619.39 \\
2500 & \\
4799.73 & 2739.76 \\
5409.11 & 2847.25 \\
5928.61 & 3425.78 \\
5658.20 & 3169.53 \\
5391.90 & 3080.38 \\
6225.29 & 3375.45
\end{align*}
\]

\[
[C\text{CB}]_{\text{free}} = \frac{[C\text{CB}]_{\text{starting}} \text{ dpm}_{\text{free}}}{2 \text{ dpm}_{\text{total}}}
\]

\[
[C\text{CB}]_{\text{bound}} = [C\text{CB}]_{\text{free}} \frac{\text{dpm}_{\text{total}} - \text{dpm}_{\text{free}}}{\text{dpm}_{\text{free}}}
\]
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<td>0.0018</td>
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Chi squared = 9.4274e-7
Parameters:  
  slope = a[1] = 0.1142  
  y-intercept = a[2] = 1.6919e-3  
  Standard deviations:  
  Δa[1] = 1.1728e-3  
  Δa[2] = 1.7939e-4  
K_d = 0.1142/0.001692 = 67.5 nM  
B_max = 1/0.001692 = 591 nM
Hanes-Woolf analysis of CCB data

\[ \frac{[CCB]_f}{[CCB]_b} = \text{lin_regression}(x) \]

Chi squared = 9.8288e-4
Parameters:
- \( a[1] = 1.6289e-3 \)
- \( a[2] = 0.1124 \)
Standard deviations:
- \( \Delta a[1] = 2.0356e-5 \)
- \( \Delta a[2] = 5.1429e-3 \)

\( B_{\max} = \frac{1}{0.001689} = 592 \text{ nM} \)
\( K_d = \frac{0.1124}{0.001629} = 69 \text{ nM} \)
Scatchard Analysis of CCB data

Chi squared = 0.8427
Parameters:
slope = a[1] = -1.3382e-2
y-intercept = a[2] = 8.5761

Standard deviations:
\[\Delta a[1] = 7.2229e-4\]
\[\Delta a[2] = 0.2020\]

\[K_d = 1 / 0.013382 = 75 \text{ nM}\]
\[B_{max} = 8.576 / 0.013382 = 641 \text{ nM}\]
Nonlinear Regression analysis of CCB binding assuming Michaelis-Menten kinetics

Chi squared = 1059.8577
Parameters:

$B_{\text{max}} = 627 \pm 16$ nM
$K_d = 69.8 \pm 5.1$ nM
Let us now return to consider the breakdown of ES to form regenerated E and product, P.

The overall scheme is visualized as follows.
The rate of product formation, \( v \) is given by

\[
v = k_3 [ES]
\]  

(1)

We must again express [ES] in terms of known quantities.

Rate of [ES] formation

\[
+\frac{d[ES]}{dt} = k_1 [E][S]
\]  

(2)

Rate of breakdown of [ES]

\[
-\frac{d[ES]}{dt} = k_2[ES] + k_3 [ES]
\]

or

\[
-\frac{d[ES]}{dt} = (k_2 + k_3) [ES]
\]  

(3)
In the "steady state" the concentrations of intermediates (e.g. ES) are unchanged, whereas [S] + [P] can change. If we limit measurements of \( v \) to early stages, [ES] does not change (there is no reverse reaction)

\[
d[\text{ES}]/dt = 0
\]

i.e.

\[
k_1 [E] [S] = (k_2 + k_3) [\text{ES}]
\]

hence

\[
[\text{ES}] = \frac{[E][S]k_1}{k_2 + k_3}
\]

\[
[\text{ES}] = \frac{[E][S]}{(k_2 + k_3)k_1}
\]
The following steps are algebraic tricks

\[ [E_t] = [E] + [ES] \]  

(8)

where \( E_t \) is total enzyme

if we divide the velocity equation (1 by \( E_t \) we obtain

\[
\frac{v}{[E_t]} = \frac{k_3[ES]}{[E] + [ES]}
\]

(9)
We can rearrange this to

\[
\frac{v}{[E_t]k_3} = \frac{[ES]}{[E] + [ES]}
\]  

(10)

then substitute for \([ES]\) from equation (6 to give

\[
\frac{v}{[E_t]k_3} = \frac{k_1}{k_2 + k_3} \frac{[E][S]}{[E] + \frac{k_1}{k_2 + k_3} [E][S]} = \frac{k_1}{k_2 + k_3} \left( \frac{[S]}{1 + \frac{k_1}{k_2 + k_3} [S]} \right)
\]  

(11)
Define $V_m$ as

$$[E_t]k_3 = V_m \quad (12)$$

$$K_m = \frac{k_2 + k_3}{k_1} \quad (13)$$

$$\frac{v}{V_m} = \frac{[S]}{K_m \left(1 + \frac{[S]}{K_m}\right)} \quad (14)$$

or

$$v = \frac{V_m[S]}{K_m + [S]} \quad (15)$$
Very important

Note

\[ K_m = \frac{k_2 + k_3}{k_1} \]

If \( k_3 \) is very small compared to \( k_2 \) this means that

\[ K_m = \frac{k_2}{k_1} = K_S \]

where \( K_S \) is the dissociation constant for \( S \) interaction with \( E \).

Thus in some reactions where \( k_3 \ll k_2 \), \( K_m = K_S \) while in other enzyme mediated reactions where \( k_3 \geq k_2 \), \( K_m > K_S \).
\[ K_m = \frac{k_2 + k_3}{k_1} \]

*The units of \( K_m \) are:* \( \text{per sec/ per molarity per sec} = \text{molarity} \)

\[ V_m = [E_t]k_3 \]

*and the units of \( V_{max} \) are:* \( \text{molarity per sec} \)
Properties of the Michaelis Menten Equation

At low [S],
\[ v \approx \frac{V_{\text{max}}[S]}{K_m} \]

i.e. \( v \) increases linearly with [S]

At very high [S],
\[ v \approx V_m \]

i.e. \( v \) does not increase with increasing [S]

However, \( V_{\text{max}} \) is rarely measurable because in many instances it is not possible to add sufficient quantities of substrate to saturate the enzyme. You may then ask, if \( V_m \) is not measurable, how does one determine \( V_m \) and \( K_m \) for a reaction?
A simple, algebraic trick solves this problem! The reciprocal of the Michaelis-Menten equation is

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

This is a linear equation with the familiar form,

\[y = \text{slope} \times x + \text{intercept}\]

thus making: \(1/v = y\) and, \(1/[S] = x\)

a plot of \(1/v\) *versus* \(1/[S]\) will produce a straight line of:

positive slope = \(K_m/V_{\text{max}}\) and, y-intercept \(= 1/V_{\text{max}}\)
When \([S] = -K_m\) (a theoretical condition because \([S] \geq 0\) in the real world), this means that

\[
\frac{1}{\nu} = \frac{K_m}{V_{max}} \frac{1}{-K_m} + \frac{1}{V_{max}} = \frac{1}{\nu} = \frac{1}{-V_{max}} + \frac{1}{V_{max}} = 0
\]  

(9)

thus when \([S] = -K_m\), \(1/\nu = 0\). The plot known as a **Lineweaver-Burk** plot looks like

\[
y = 0.1 + 2.5x
\]
The Hanes-Woolf Plot

If we multiply the reciprocal form of the MM equation by \([S]\) we obtain

\[
\frac{[S]}{v} = \frac{1}{V_m} [S] + \frac{K_m}{V_m}
\]

Thus a plot of \([S]/v\) versus \([S]\) will produce a single straight line with slope \(1/V_m\) and y-intercept of \(K_m/V_m\).

When \([S] = -K_m\), the equation becomes

\[
\frac{[S]}{v} = \frac{-K_m}{V_m} + \frac{K_m}{V_m} = 0
\]

Thus the x-intercept = \(-K_m\)
Significance of Km and Vmax

$K_m$ is the concentration of [S] at which 1/2 of the active sites are filled with substrate. The fraction of filled sites $f_{ES}$

$$f_{ES} = \frac{[S]}{[S] + K_m} \quad (10)$$

Thus when $[S] = K_m$,

$$f_{ES} = \frac{K_m}{2K_m} = 0.5$$

Thus one-half of the enzyme is in the form of ES and the rate of the reaction, $v$, is

$$0.5[E_t] k3 \text{ or } v = 0.5 V_{\text{max}}$$
We also saw above that

\[ K_m = \frac{(k_2 + k_3)}{k_1} \]

Thus if \( k_2 \gg k_3 \)

\[ K_m = \frac{k_2}{k_1} \]

i.e. \( K_m \) is the dissociation constant (reverse of the equilibrium constant) for ES formation and is a measure of the stability of the complex.

A high \( K_m \) implies weak binding (it takes more substrate to fill substrate binding sites).

A low \( K_m \) implies strong binding (it takes less substrate to fill substrate binding sites).

\( V_{max} \) reveals the turnover number (\( k_{cat} \)) of an enzyme if \([E_t] \) is known because

\[ V_{max} = k_{cat} [E_t] \]

if \([E_t] = 1 \mu M \) and \( V_{max} = 600 \) mmols/L/sec,

\[ k_{cat} = 6 \times 10^5 \text{ sec}^{-1} \] - Each round of catalysis is \( 1/k_{cat} = 1.7 \mu \text{sec} \).
general significance of $k_{cat}$

**The $k_{cat}/K_m$ Criterion**

We saw earlier that when $[S] \ll K_m$, $v = \frac{V_{max}[S]}{K_m}$

We also know that $V_{max} = k_{cat} [E_t] = k_3 [E_t]$  ($k_{cat} = k_3$)

since $V_{max} = k_3 [E_t]$, this means

$$v = \frac{k_3 [S][E_t]}{K_m}$$  (11)

$v$ is therefore directly proportional to $\frac{k_3}{K_m}$ and $[S]$ at fixed $[E_t]$
Are there limits upon $k_{cat}/K_m$?

\[
\frac{k_{cat}}{K_m} = \frac{k_3 k_1}{k_2 + k_3}
\] (12)

Examination of eqn 12 indicates that $k_1$ is limiting.

When $k_2 \ll k_3$,

\[
\frac{k_3 k_1}{k_2 + k_3} \approx \frac{k_3 k_1}{k_3}
\]

The highest value that $k_3/K_m$ can achieve is $k_1$.

$k_1$ is the second order rate constant that describes association of E and S to form the ES complex. $k_1$ includes terms that describe the collisional frequency of E and S.
For the case of uncharged molecules in solution, the encounter rate constant, $k_e$ can be calculated from

$$k_e = \frac{4\pi N(D_A + D_B)(r_A + r_B)}{1000} \text{ M}^{-1} \text{ sec}^{-1}$$

**note**, $D_A = \frac{kT}{6\pi r_A \eta}$

where $D$ and $r$ refer to the diffusion constants and reaction radii of molecules $A$ and $B$ and $N$ is Avagadro’s number. For a system such as an enzyme molecule with a radius of about 30 Å and a substrate molecule with a radius of about 5 Å, the encounter rate constant comes to approximately $10^9 \text{ M}^{-1} \text{ sec}^{-1}$. 
Thus diffusion tends to limit the rate of encounters of E and S and an upper limit of $k_3/K_m$ is therefore $10^9 \text{M}^{-1} \text{sec}^{-1}$. Even this requires that the substrate can encounter the enzyme surface in any orientation.

$k_{cat}/K_m$ ratios of some enzymes, e.g. acetylcholinesterase and carbonic anhydrase are between $10^8 - 10^9 \text{M}^{-1} \text{sec}^{-1}$ indicating they have achieved kinetic perfection. Their activity is limited only by the rate at which they encounter substrate in solution. Any further gain can only be achieved by decreasing diffusion times. This can be achieved by sequestering substrates and products in the confined volume of a multienzyme complex, e.g. mitochondria.
Measuring rates of reactions

Consider the following time course of substrate consumption by an enzyme.

The reaction is fast and data were collected at 1 second intervals for the first 10 sec and thereafter at 5 sec intervals. How do we obtain the most accurate measure of the rate of the reaction?

The rate at 1 sec (slope a) is greater than at 5 sec (slope b) or at 8 sec (slope c).

How do we know which measure is most relevant?
Consider our reaction once again

\[
\begin{array}{c}
  \text{E+S} \quad \xrightarrow{k1} \quad \text{ES} \\
  \quad \xleftarrow{k2} \\
  \quad \xrightarrow{k3} \quad \text{E+P}
\end{array}
\]

The rate of product formation, \( v \) is given by

\[ v = k_3 [ES] \]

Since \( k_3 \) is invariant, when we observe the rate of substrate consumption falling (slopes a, b and c last page), this means that \([ES]\) is also decreasing ([S] falls and the product \( k_1 [E][S] \) must also fall). This also means that our assumption of “\( d[ES]/dt = 0 \)” is invalid.

Any analysis we perform using data collected under conditions where \( d[ES]/dt \neq 0 \) (e.g. if we use slopes b or c as our rate measurements) invalidates our assumption of steady-state (internal equilibrium) and renders the data less than useful.
For a given reaction, the rate of product formation is given by the Michaelis-Menten equation:

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

Knowing \( v \) at each \([S]\) used, let’s make a simplifying assumption that

\[ v = \frac{V_{\text{max}}[S]}{K_m + [S]} = k(\text{app}) [S] \]

For each \([S]\), we can calculate an apparent rate constant, \( k(\text{app}) \) as

\[ k(\text{app}) = \frac{v}{[S]} \]

You can see for this simple example (\( V_{\text{max}} = 100 \) mmol/L/min and \( K_m = 5 \) mM), \( k(\text{app}) \) falls with increasing \([S]\) having a maximum value of \( V_{\text{max}}/K_m \) at very low \([S]\) approaching zero at very high \([S]\).
This consideration illustrates the two major problems that experimentalists face in determining rates of reactions:

1. At low [S], the reaction (time course of product formation) is very rapid relative to [S].
2. At high [S], the reaction rate is very low relative to [S].

Why are these problems?

Point 1  When the rate of a reaction is rapid, the experimentalist may need to employ rapid sampling procedures because his/her goal is to measure v at a given [S]. If [S] falls significantly during the sampling period, which [S] does he/she use: [S]₀ or [S]ₜ?

Point 2  If the experimentalist cannot measure product directly, but rather must measure product as [S]₀ - [S]ₜ, then in order to determine v, a significant portion of S must be consumed. Again, which [S] does the experimentalist use: [S]₀ or [S]ₜ? Moreover, at high [S], the reverse reaction may become significant thus the rate of the reaction may be underestimated.
**Solutions to these problems**

The most practical approach is to attempt to design a system in which product is very rapidly and irreversibly converted into another species that can no longer participate in the reaction. This obviates the problem of reverse reactions and may allow rapid measurements.

In practice, however, this is not always possible. Thus a number of approaches have been developed.

**Approach 1 (First order kinetics)**

We saw above that when \([S] << K_m\), then \(v = V_{\text{max}} [S] / K_m\).

The rate constant, \(k_{(\text{app})} = V_{\text{max}} / K_m\) and is independent of \([S]\).

This first order rate constant (units of per unit time) is analogous to constants describing radioactive decay.
Thus if one were to measure the loss of substrate as a function of time, the following would be observed.

Substrate levels fall exponentially with time and this fall is characterized by a half-time ($t_{1/2}$) of $\approx 2$ sec for our simple enzyme.
How can one rationalize this phenomenon? The rate of substrate consumption is given by:

\[
\frac{-d[S]}{dt} = v = k_{(app)} [S]
\]
or

\[
\frac{-d[S]}{[S]} = k_{(app)} dt
\]

Integrating between \([S]_o\) at \(t = 0\) and \([S]\) at any other time \(t\) we obtain

\[
2.303 \log \left( \frac{[S]_o}{[S]} \right) = k_{(app)}.t
\]
or

\[
[S] = [S]_o e^{-k_{(app)} t}
\]
or

\[
\log[S] = \frac{-k_{(app)} t}{2.303} + \log[S]_o
\]
Thus a plot of log[S] versus time will produce a single straight line with slope \(-k_{\text{app}}/2.303\) and y-intercept = log \([S]_o\).
The half-time ($t_{1/2}$) is that time required for the amount of substrate $S$ to be reduced by one-half. $t_{1/2}$ can be related to $k_{(app)}$ in the following manner:

$$2.303 \log \frac{[S]_0}{[S]} = k_{(app)} t \Rightarrow 2.303 \log \frac{1}{0.5} = k_{(app)} \cdot t_{1/2}$$

Thus for our simple enzyme, $t_{1/2} \approx 2$ sec, hence $k_{(app)} \approx 0.693/2$ per sec $\approx 20$ per min. The predicted first order rate constant is given by

$$k_{(app)} = \frac{V_{\text{max}}}{K_m} = \frac{100 \text{ mM/L/min}}{5 \text{ mM}} = 20 \text{ per min}$$
A similar approach may be used when measuring product formation which is given by

\[ 2.303 \log \frac{[P_\infty] - [P_t]}{[P_\infty]} = -k_{(app)} t \]

Hence a plot of \( \log \frac{[P_\infty] - [P_t]}{[P_\infty]} \) versus \( t \)

will produce a single straight line with slope = \(-k_{(app)}/2.303\).
Approach 2: Reaction rate is independent of time (initial rate method)

If we reexamine the reactions we described above, we see at very early time points, the rate of the reaction is very nearly independent of time. That is, substrate consumption and product formation increase linearly with time. **This means that our theoretical assumption that** \( \frac{d[E]}{dt} = 0 \) **is valid.**

![Graph showing the linear relationship between substrate consumption [S] or product formation [P] and time.](Image)

This also means that the rate of the reaction can be estimated from the very early time points as the slope of the plot. Namely

\[
v = \frac{\text{Product formed}}{\text{time}} = \frac{\text{substrate consumed}}{\text{time}}
\]
This can be applied over a wide range of [S]. In other words, the constraint of first order kinetics ([S] \ll K_m) need not be applied. Indeed this is the method of choice of most experimentalists. However, there is a single problem. Since substrate is being consumed, which concentration of S does one apply the measured velocity to?

It can be shown that the following correction works very well.

If the measured rate of a reaction is \( v \) when starting [S] is \([S]_0\) and ending [S] is \([S]_t\) then an average [S], \([S]_{\text{avg}}\) may be calculated as

\[
[S]_{\text{avg}} = \frac{[S]_0 + [S]_t}{2}
\]

By calculating \([S]_{\text{avg}}\), the errors that result from using this parameter instead of \([S]_0\) are greatly reduced.
Rapid equilibrium approach to solving kinetic schema

Earlier we saw that

\[ K_m = \frac{k_2 + k_3}{k_1} \]

If \( k_3 \ll k_2 \) then

\[ K_m = \frac{k_2}{k_1} = K_s \]

\( K_s \) is the dissociation constant for S interaction with E.

In the reaction

\[ E + S \xrightleftharpoons[k_2]{k_1} ES \]

\( K_s \) is given by \( \frac{[E][S]}{[ES]} \) at equilibrium

At equilibrium, \( k_1[E][S] = k_2[ES] \), thus

\[ \frac{[E][S]}{[ES]} = \frac{k_2}{k_1} \]

Using the rapid equilibrium approach to solving kinetic schema we assume that the rate-limiting step in the reaction is the breakdown of ES to E and P and that ES is in equilibrium with E and S.
Consider the following hypothetical reaction

Here, $k_p$, $k_q$ and $k_r$ are the rate limiting steps and the constants $K_A$, $K_B$ and $K_S$ are the various dissociation constants for binding of substrates to enzyme.

The velocity equation for this and other reactions is simply obtained using the rapid equilibrium approach.
Step 1  Write the velocity equation in terms of intermediates

\[ v = kp[ES] + kq[EAS] + kr[EABS] \ldots \]

Step 2  Divide both sides of the equation by \([E]_t\)

i.e. \([E]_t = [E] + [EA] + [EAB] + [ES] + [EAS] + [EABS] \ldots \]

\[
\frac{v}{[E]_t} = \frac{kp[ES] + kq[EAS] + kr[EABS] \ldots}{[E] + [EA] + [EAB] + [ES] + [EAS] + [EABS] \ldots}
\]

Step 3  Express the concentrations of each species in terms of E

\[
[ES] = \frac{[S]}{K_S} \quad [EAS] = \frac{[A][S]}{K_A K_S} \quad [EABS] = \frac{[A][B][S]}{K_A K_B K_S}
\]
thus

\[
\frac{v}{[E_t]} = \frac{kp[S] + kq[S][A] + kr[S][A][B]}{K_S + K_{SA} + K_{SB}}
\]

\[
= \frac{1}{K_S} + \frac{[S]}{K_S} + \frac{[A]}{K_A} + \frac{[A][S]}{K_{AS}} + \frac{[A][B]}{K_{AB}} + \frac{[S][A][B]}{K_{SAB}}
\]

**Step 4** Simplify algebraically to produce an analog of the Michaelis-Menten equation. We will see examples of this below.

We will use this approach to examine some interesting examples of enzyme inhibition.
ENZYMES ARE INHIBITED BY SPECIFIC MOLECULES

Reversible Inhibitors - May occur naturally or may be synthesized as drugs.

Competitive Inhibition

A competitive inhibitor, I, competes for binding with S. I is not transformed into product. The inhibitor, I, resembles the substrate, S. I reduces the rate of reaction + S by reducing the proportion of enzyme in the form ES.
Noncompetitive Inhibition

S and I are not mutually exclusive but ESI is catalytically inactive. When S binds, the enzyme undergoes a conformational change which aligns the catalytic center, C, with the susceptible bonds of S; I interferes with the conformational change, but has no effect on S binding.
Uncompetitive Inhibition

I binds only to the ES complex. When S binds, a conformational change occurs in the enzyme which forms or unmasks the I site. The resulting ESI complex is catalytically inactive; C represents the catalytic center of the enzyme.

These 3 types of inhibition are readily distinguishable by experimentation.
**Competitive inhibition**

S and I compete for binding to the same site

\[
\frac{v}{[E_t]} = \frac{k_3[S]}{K_s} \frac{1}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i}} = \frac{k_3[S]}{K_s + [S] + \frac{K_s[I]}{K_i}}
\]

\[
v = \frac{[E_t]k_3[S]}{K_s \left(1 + \frac{[I]}{K_i}\right) + [S]} = \frac{V_{mapp}[S]}{K_{m(app)} + [S]}
\]

i.e. \([I]\) increases

\[
K_{m(app)} = K_s(1 + [I]/K_i)
\]

\[
V_{mapp} = (k_3[E_t]) \text{ and is thus unaffected.}
\]
**Competitive inhibition**

\[ V_{\text{max}} \]

\[ K_m \] and \[ K_{m(\text{app})} \]

- **Control**
- **+ inhibitor**

\[ V_m \text{ is unaffected but } K_{m(\text{app})} \text{ is increased.} \]

\[ K_{m(\text{app})} = K_S (1 + [I]/K_i) \]
Noncompetitive inhibition

\[
\frac{v}{k_3[E_t]} = \frac{[S]}{K_s} \frac{1}{1 + \frac{[S]}{K_s} + \frac{[I]}{K_i} + \frac{[S][I]}{K_s K_i}} = K_s \left\{1 + \frac{[I]}{K_i}\right\} + [S] \left\{1 + \frac{[I]}{K_i}\right\}
\]

or

\[
\frac{v}{k_3[E_t]} = \frac{[S]}{K_s + [S]} \left\{1 + \frac{[I]}{K_i}\right\}
\]

or

\[
v = \frac{V_{\text{mapp}}[S]}{K_{m(\text{app})} + [S]} \quad \text{where} \quad V_{\text{mapp}} = \frac{k_3[E_t]}{1 + \frac{[I]}{K_i}} \quad \text{and} \quad K_{m(\text{app})} = K_s
\]

i.e. I reduces \(V_m\) but has no effect on \(K_s\)
Noncompetitive inhibition

\[ V_{\text{max(app)}} = \frac{k_3}{1 + [I]/K_i} \]

where \( K_i \) is the dissociation constant for the EI complex, \( K_i = [E][I]/[EI] \).
Noncompetitive inhibition is a common theme in feedback inhibition. For example, the biosynthesis of isoleucine from threonine in bacteria involves 4 steps mediated by 4 different enzymes.

The first reaction is catalyzed by threonine deaminase (TD). This enzyme is noncompetitively inhibited by isoleucine. Thus as product increases, the first step in the reaction decreases. As P falls due to shut down of TD, so TD is released from inhibition due to dissociation of P from the TD.P complex and the reaction cycle proceeds again.
Uncompetitive inhibition

Defining $V_m = k_3 [E_t]$

$$\frac{v}{V_m} = \frac{[S]}{K_s} \frac{1}{1 + \frac{[S]}{K_s} + \frac{[S][I]}{K_s K_i}} = \frac{[S]}{K_s + [S]} \left\{1 + \frac{[I]}{K_i}\right\}$$

Divide both denominators by $\left\{1 + \frac{[I]}{K_i}\right\}$

$$\frac{v}{V_m} = \frac{[S]}{K_s} + [S] \quad \therefore v = \frac{V_{mapp}[S]}{K_{m(app)} + [S]}$$

Where $V_{mapp} = \frac{[E_t] k_3}{\left\{1 + \frac{[I]}{K_i}\right\}}$ and $K_{m(app)} = \frac{K_s}{\left\{1 + \frac{[I]}{K_i}\right\}}$

Thus both $V_m$ and $K_m$ are reduced by the uncompetitive inhibitor
An example of uncompetitive inhibition is the effect of intracellular Mg.ATP on human erythrocyte sugar transport protein activity. ATP reduces Km and Vm for sugar uptake by red cells.
Summary

1. Making simplifying solutions we derived the Michaelis-Menten equation and expressed $K_m$ and $V_{\text{max}}$ in terms of rate constants and $[E_{\text{total}}]$.

2. We discussed the significance of $K_m$, $K_s$ and $V_{\text{max}}$

\[
K_m = \frac{k_2 + k_3}{k_1}
\]

\[
K_s = \frac{k_2}{k_1}
\]

\[
V_{\text{max}} = k_3 [E]_t
\]

3. Reaction rates:
   a. When $S \ll K_m$, $v = k[S]$ and log plots are employed to calculate $k$.
   b. When $[S] \gg K_m$, $v = V_{\text{max}}$ and the rate of reaction is linear with time because [substrate] remains saturating.
   c. When $[S] \approx K_m$, the initial rate approach is used to measure $v$ at early time points where $v$ is independent of sample time.
4. We discussed the rapid equilibrium approach \((K_m = K_s)\) for producing solutions to kinetic schema.

5. Using this approach we examined enzyme inhibitions (Competitive, noncompetitive and uncompetitive) and showed that these are distinguishable.