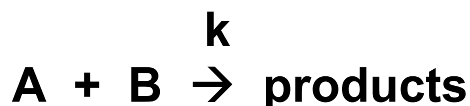
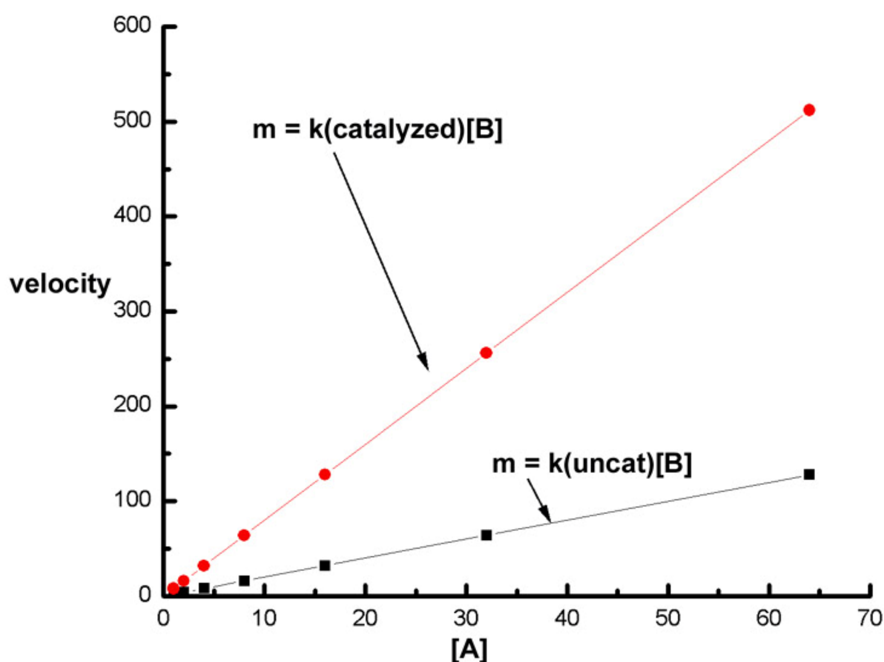
BIOC 463A
Enzyme Steady-State Kinetics and Competitive Inhibition

Steady-State Kinetics: Determination of K_m, V_{max}, and k_{cat}

Objectives: We will emphasis the methods and techniques employed in steady-state kinetic analysis of AP. We will not cover Transition State Theory in this class. Rather we will try to fully understand how data is obtained and analyzed in the context of the Michaelis-Menten equation.

Historical Perspective:
In a typical chemical reaction:





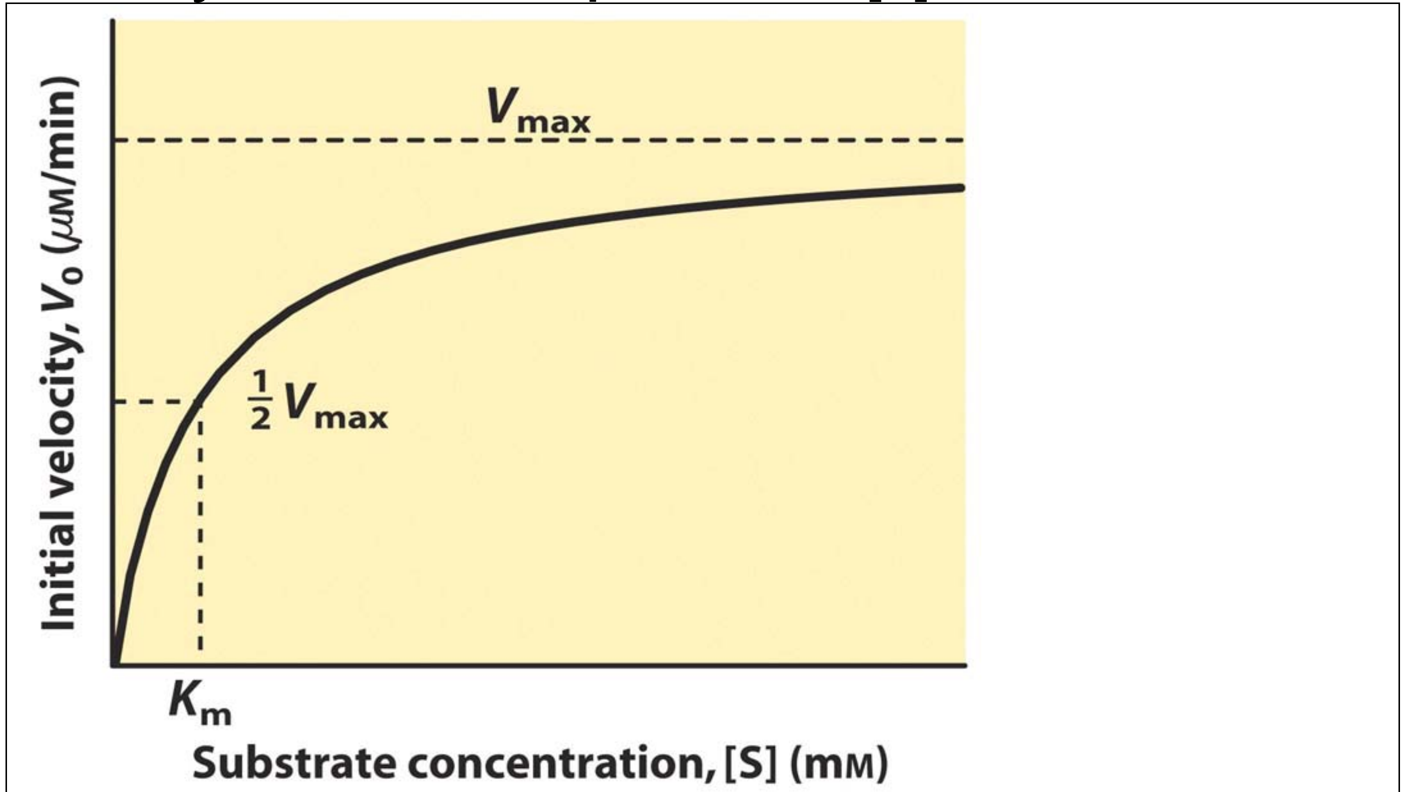
Velocity of reaction vs. [A] ([B] being held constant) is linear. If a catalyst is added, the velocity increases, but plot remains linear.

So, rate of reaction: $v = k_{obs}[A]$, where $k_{obs} = k[B]$.

And, $k_{uncatalyzed} << k_{catalyzed}$.

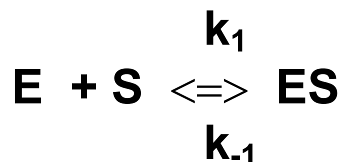
At beginning of 1900's it was understood that enzymes were catalysts involved in biological reactions.

For enzymes, a different plot of v vs. [S] was observed:



Michaelis and Menten suggested that the approach of v to a maximal level (V_{max}) meant E and S were forming a complex of distinct lifetime.

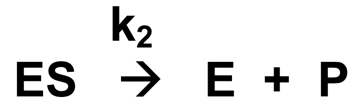
Assumption 1: E and S react rapidly and are in equilibrium:



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

and $K_s = k_{-1}/k_1 = [E][S]/[ES]$ = dissociation constant for ES!

Assumption 2: Product is formed in a 2nd DISTINCT first order step:

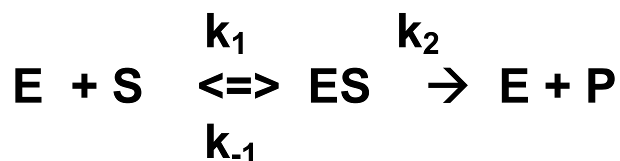


$$ES \xrightarrow{k_2} E + P$$

rate = k_2[ES]

Assumption 3: free E, created by this step can bind new S.

Taken together the Michaelis-Menten mechanism:



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

Briggs and Haldane then derived the M-M eqn we know today making the following assumptions:

- **[ES] rapidly reaches a constant level that does not change during "steady-state".**

$$\frac{d[ES]}{dt} = 0$$

- **This means:**

  rate of ES formation = rate of ES breakdown

  $k_1[E][S] = k_{-1}[ES] + k_2[ES]$

  (dissociation) (to product)

  $k_1[E][S] = (k_{-1} + k_2)[ES]$

- **B&H defined K_m:**

$$K_m = \frac{(k_{-1} + k_2)}{k_1} = \frac{[E][S]}{[ES]}$$ (look familiar?)

- **Initial Velocity Assumption:**

  at t = 0, [P] = 0

  this does two things:

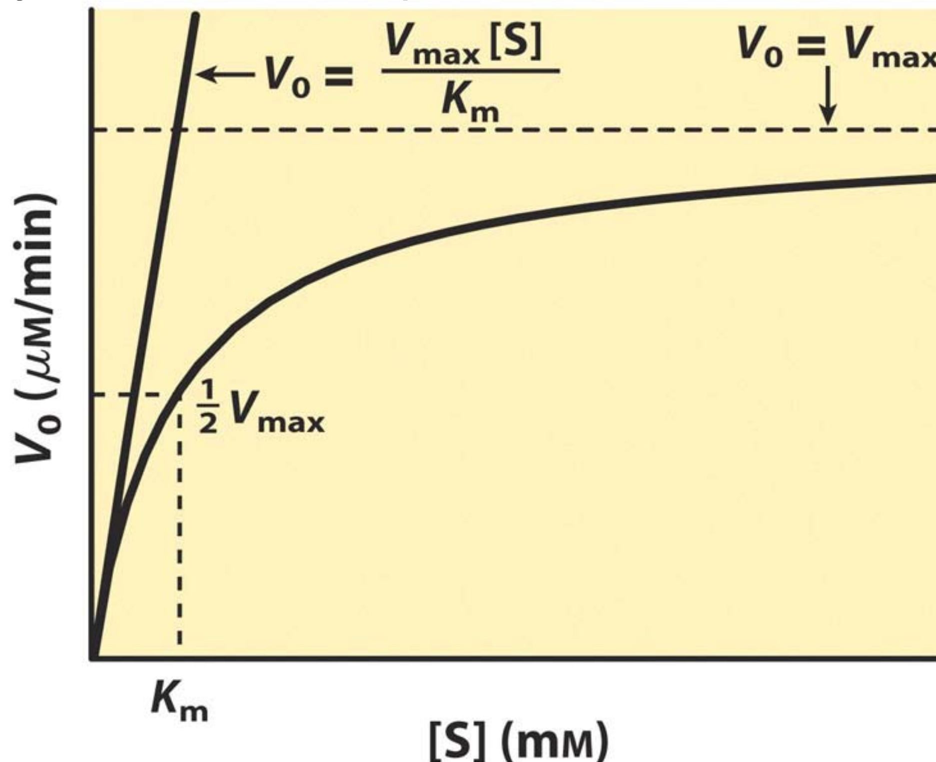
Since many P are very effective competitive inhibitors, eliminates math complications due to the back reaction: E + P → ES

Taking ALL of these assumptions into consideration, B&H derived the beloved M-M eqn:

$$v_0 = \frac{V_{max}[S]}{K_m + [S]} \quad \text{where } V_{max} = k_{cat}[E_t]$$

There are three [S] conditions to consider:

[S]	v_0
$\ll K_m$	$\frac{V_{max}[S]}{K_m} = \frac{k_{cat}[E]_t[S]}{K_m}$
$= K_m$	$\frac{V_{max}}{2}$
$\gg K_m$	$V_{max} = k_{cat}[E]_t$



Two Important Definitions of K_m:

1. $K_m = (k_{-1} + k_2)/k_1$
2. K_m is the [S] that gives $v_0 = V_{max}/2$!

The units of K_m are CONCENTRATION. K_m is a CONCENTRATION!!!!!!! (Sound familiar?)

K_m is primarily a measure of S binding, with a little catalysis thrown in (if k_2 is large relative to k_{-1}).

V_{max} is a measure of catalysis only (k_{cat} and $[E]_t$).

Usually, when people compare K_m values, it is implied they are comparing the binding of different substrates.

K_m and V_{max} are defined for an E and S under set conditions:

- **pH**
- **Ionic Strength**
- **Temp, pressure, etc.**

Significance of k_{cat}/K_m!

- **Under physiological conditions (in vivo):**

$$0.01 < [S]/K_m < 1 \qquad \text{(i.e. } [S] << K_m\text{)}$$

$$\text{so } [E]_{total} \sim [E]_{free} \text{ and } v_o = \frac{V_{max}[S]}{K_m}$$

$$= \frac{k_{cat}[E]_t[S]}{K_m}$$

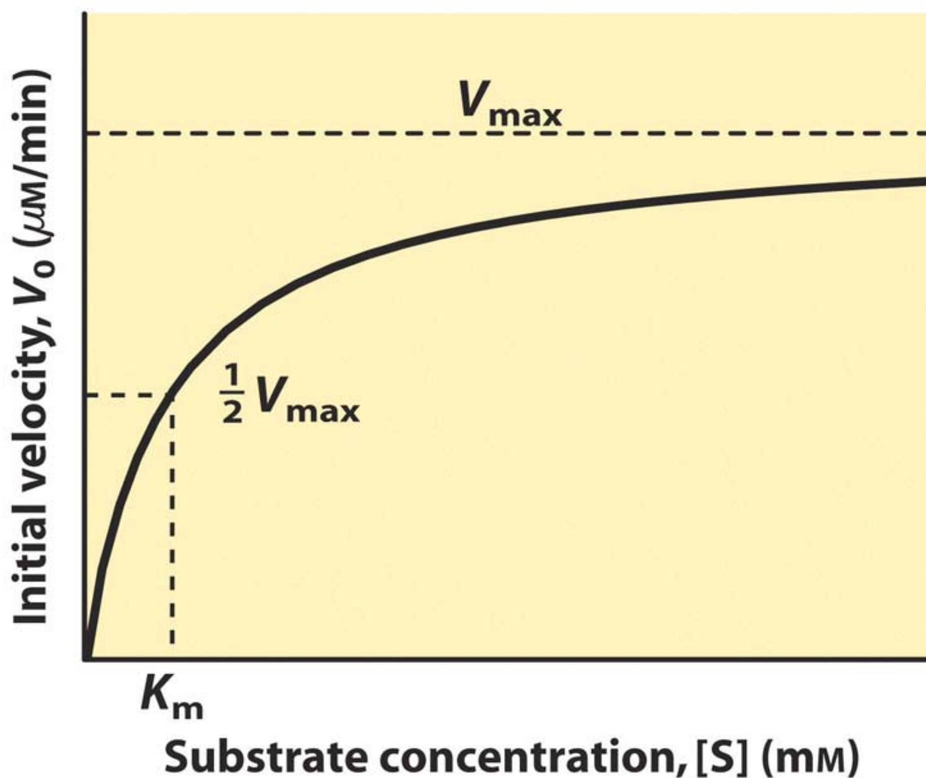
- **Thus, k_{cat}/K_m ($M^{-1}s^{-1}$) is a second-order rate constant for the reaction:**

Maximum value ~ 10^9 $M^{-1}s^{-1}$, signifying diffusion control limits for molecules the size of proteins and substrates.

For many metabolic pathways, the rate of flux is controlled by rate of the above reaction, thus reflects k_{cat}/K_m (catalytic efficiency) , [E] (via gene expression), and [S].

How are Steady-State experiments performed:

Typically, you have S and buffer in a cuvette, you add some E and follow an absorbance change at some wavelength for either decrease in [S] or increase in [P]. Find the slope of the line tangential to data as close to t = 0 as possible. This gives a v_o value for that [S]. Then you repeat using a different [S].



Data analysis: problem here is same as seen in ligand binding:

1. Extrapolate to infinite [S].
2. Determine V_{max}.
3. Determine K_m.

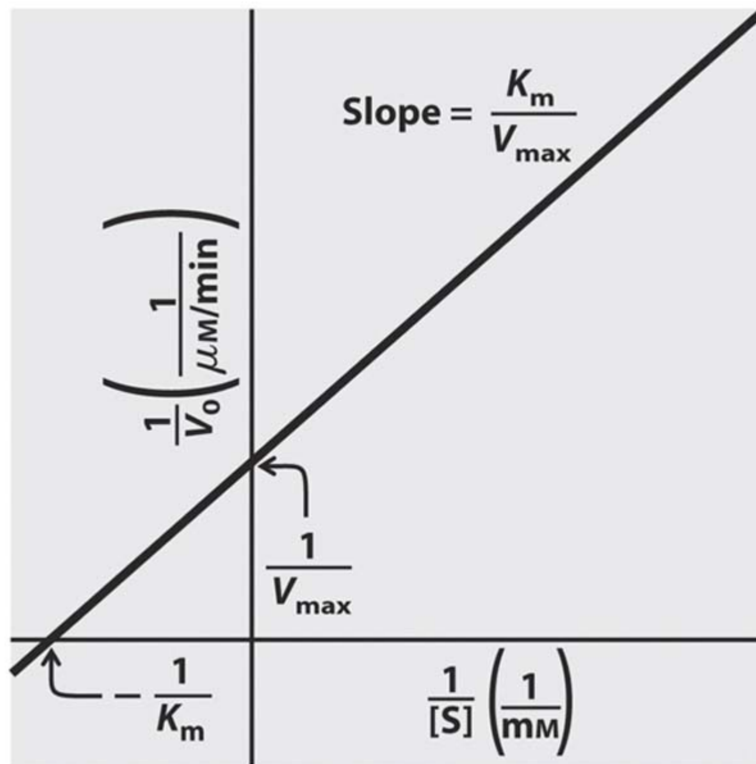
Linear Transform: Lineweaver-Burk Plot.

Starting with:

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

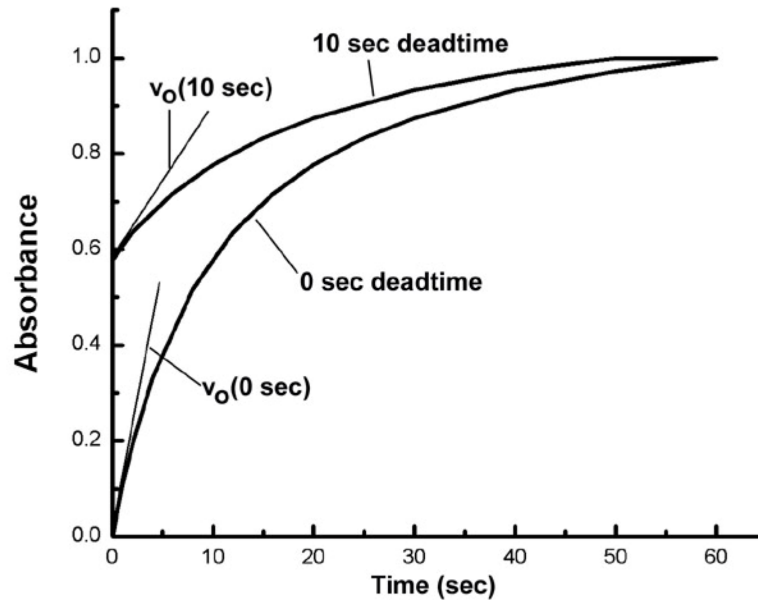
Take inverse of both sides:

$$\frac{1}{v_o} = \frac{K_m}{V_{max}}\frac{(1)}{([S])} + \frac{(1)}{(V_{max})}$$



Some practical problems with doing steady-state studies:

- **getting accurate v_o values at low [S]: if the reaction is fast, sometimes a lot of data is lost in the dead (mixing) time. In that case, you miss the true v_o and the one you observe is < the true value. This influences the Michaelis-Menten plot, giving higher K_m values.**



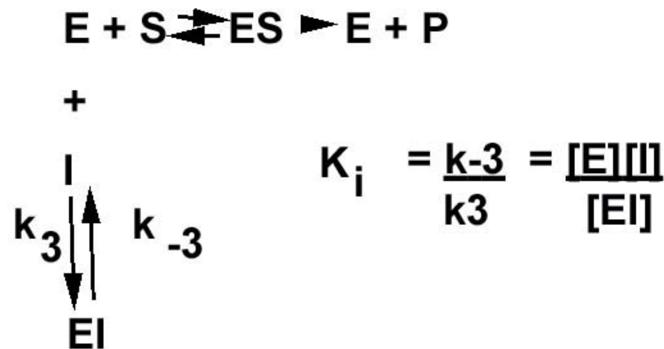
- **P inhibition: again if the reaction is fast, [P] will increase rapidly. If [P] is a strong inhibitor of E (K_i<K_m), then the observed v_o will be smaller.**

How do you avoid these problems: Simple, try to mix things together as fast as possible!

It is important to monitor how much of the ∆Absorbance you are missing in the dead time. Ideally it should be < 10% total ΔA_{total}.

Competitive Inhibitors: Determination of K_i.

Inhibitor binds to active site of E:



- **Effect can be overcome at high [S] by mass action, so V_{max} is the same.**
- **K_m, however, is APPARENTLY changed:**

$$K_{m,app} > K_m$$

$$K_{m,app} = K_m \left(1 + \frac{[I]}{K_i}\right)$$

Note, K_m is not actually changed! K_m is a property of the binding of S to E, and that is not altered by I !!!!!!

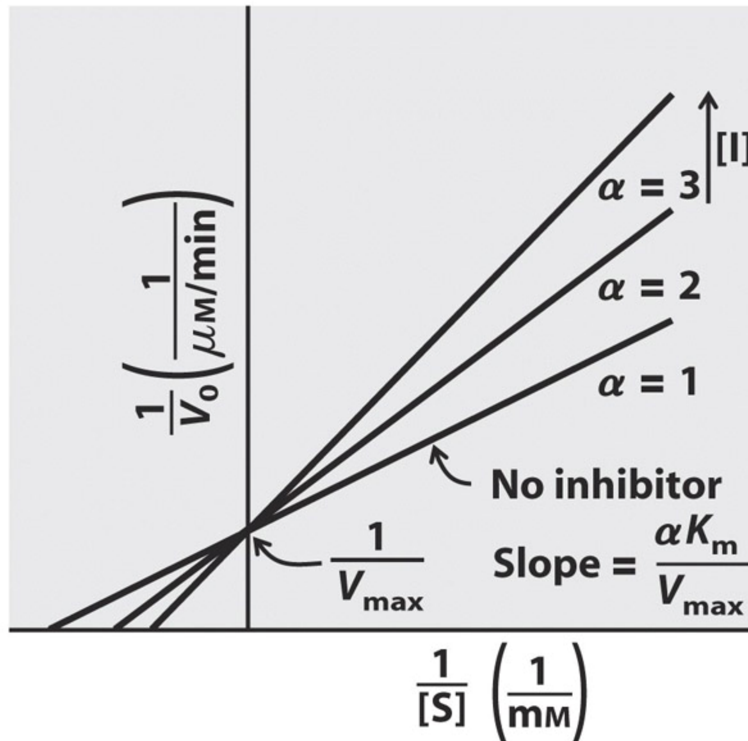
M-M eqn for Competitive Inhibitor:

$$v_o = \frac{V_{max}[S]}{K_m(1 + [I]/K_i) + [S]}$$

Lineweaver-Burk form of eqn:

$$\frac{1}{v_o} = \frac{K_m\,(1 + [I]/K_i)}{V_{max}}\,\frac{(1)}{[S]} + \frac{(1)}{V_{max}}$$

(As [I] increases or K_i decreases, how will slope change?)



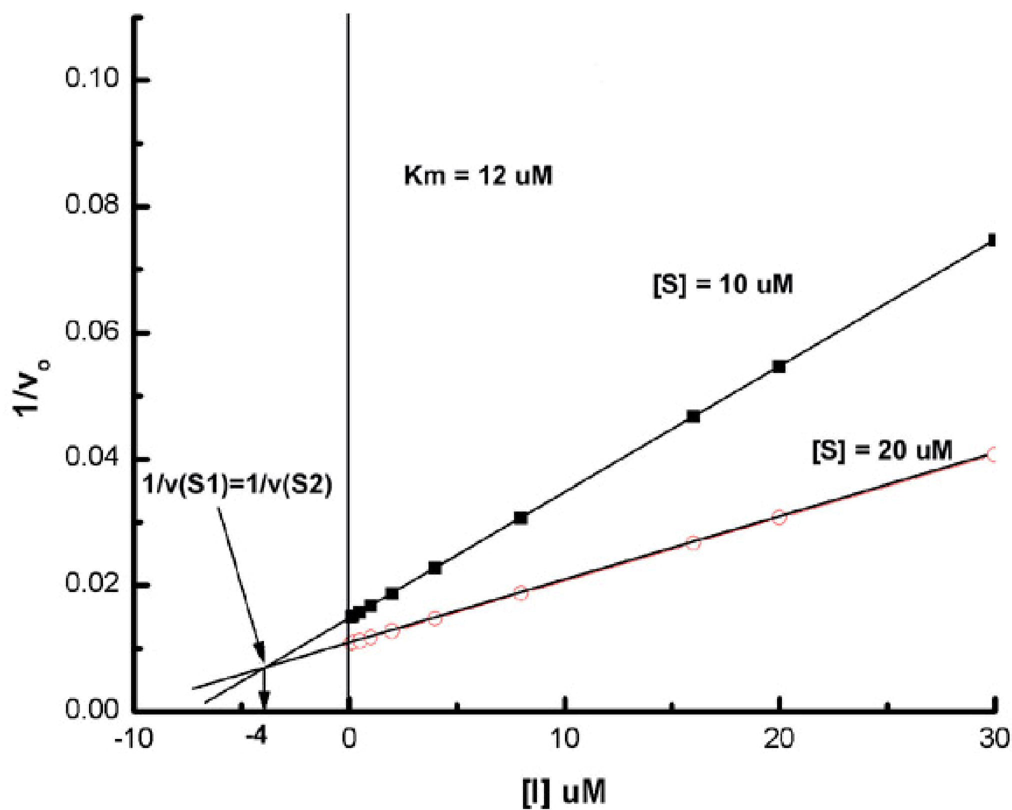
It is important to note that [I], K_i, V_{max}, and K_m are all CONSTANTS. 1/[S] is the VARIABLE. This is not only true for the plot, it is also true for the way the experiment is done. [I] is held constant at ALL [S] throughout experiment!

This is a Lineweaver-Burk METHOD of studying Inhibitors!

A second method and way of plotting data is called the Dixon Method/Plot.

- **[S] is now held constant, and [I] is varied.**
- **this requires a rearrangement of the Lineweaver-Burk eqn.**

$$\frac{1}{v} = \frac{(K_m)}{V_{max}K_i[S]}[I] + \frac{1}{V_{max}}(K_m/[S]+1)$$



In a Dixon expt, you do a set of reactions at first [S] (S1), then repeat at a second constant [S] (S2).

What is significance of point of intersection of two lines?

1/v for S1 = 1/v for S2

$$\frac{(K_m)}{(V_{max}K_i[S1])}[I] + \frac{1}{V_{max}}(K_m/[S1]+1) = \frac{(K_m)}{(V_{max}K_i[S2])}[I] + \frac{1}{V_{max}}(K_m/[S2]+1)$$

rearranging and eliminating terms:

$$\frac{K_i + [I]}{[S1]} = \frac{K_i + [I]}{[S2]}$$

If S1 ≠ S2, how can this equation be true?

Answer:It is true only if K_i = - [I] ! So the pt of intersection represents – K_i !

$$K_i = 4 \text{ uM}$$

In Summary:

Method	Constant	Variable
L-B	[I]	[S]
Dixon	[S]	[I]