Enzyme Kinetics

CHEM 420 – Principles of Biochemistry
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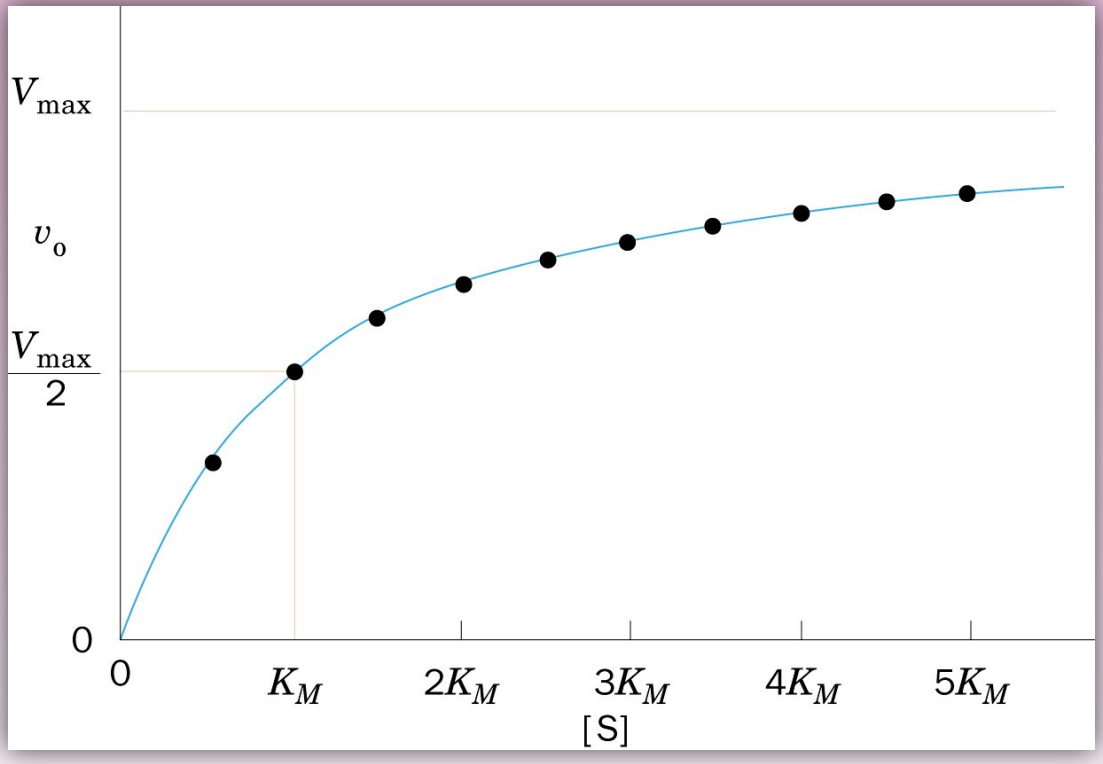
Chapter 14: Voet/Voet, *Biochemistry*, 2011
Fall 2015

October 5 & 7

$$E + S \longrightarrow ES \longrightarrow E + P$$



Plot of the initial velocity v_o of a simple Michaelis–Menten reaction *vs* substrate concentration [S], showing saturation kinetics; K_m = [S] at 1/2 V_{max}; enzyme saturation ($[E_{total}] = [ES]$) is achieved at [S] > 10 K_m

$[E_t]$ is fixed
[S] is varied

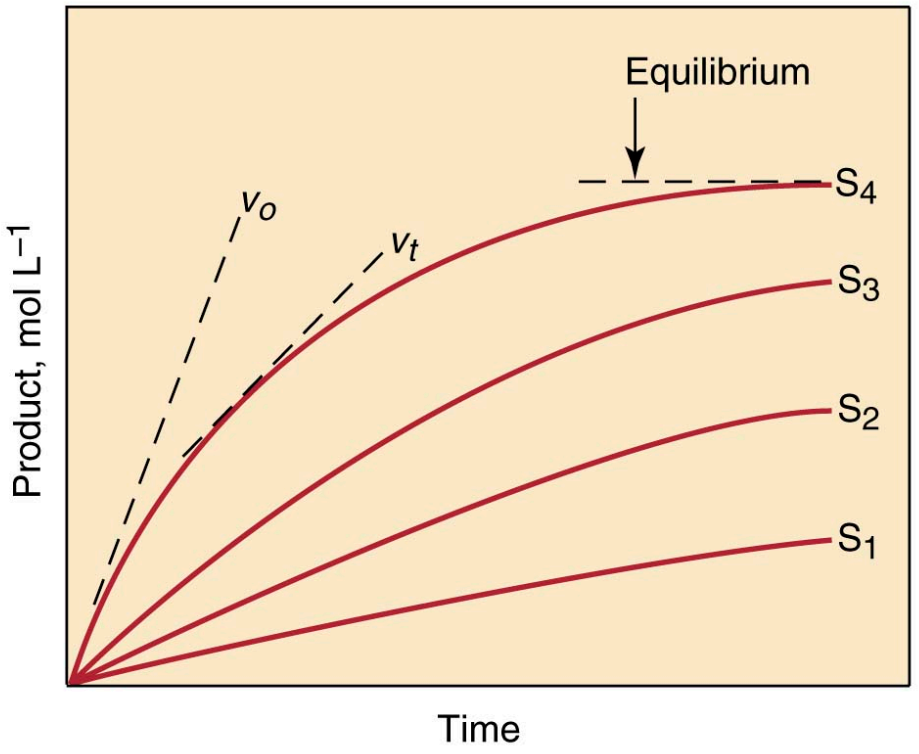


Figure 10.46. Progress curves for an enzyme-catalyzed reaction.

Plots of product formation *vs* time for an enzyme-catalyzed reaction as a function of the initial concentration of S; note the determination of initial velocities (v_o) from the initial slopes of the tangents to the reaction curves

[S] is fixed
[E] is varied

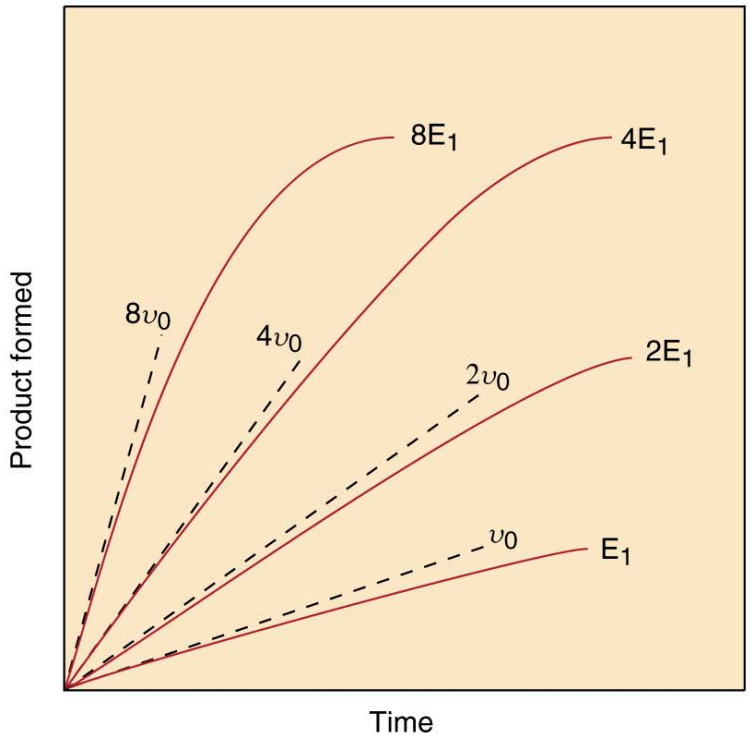


Figure 10.48. Progress curves at variable concentrations of enzyme and saturating concentrations of substrate.

Plots showing the dependence of initial velocities on [E]; initial rates are directly proportional to [E] under saturating [S].
Note that these are the conditions used to determine the specific activity of an enzyme preparation (see later slides).

More realistic kinetic models of a single-substrate enzyme-catalyzed reaction

$$E + S \rightleftharpoons ES \rightleftharpoons EP \rightleftharpoons E + P$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

Assumptions:

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

fast, reversible (binding step)

$$ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

slow, rate-determining (catalytic step); *i.e.*, the reaction rate is proportional to [ES]; when $[E_f]$ is small, rate is maximal (saturating conditions)

Kinetics of the single-substrate enzyme-catalyzed reaction: Michaelis-Menten kinetics

The Michaelis-Menten equation describes the relationship between [S] and enzyme reaction rate:

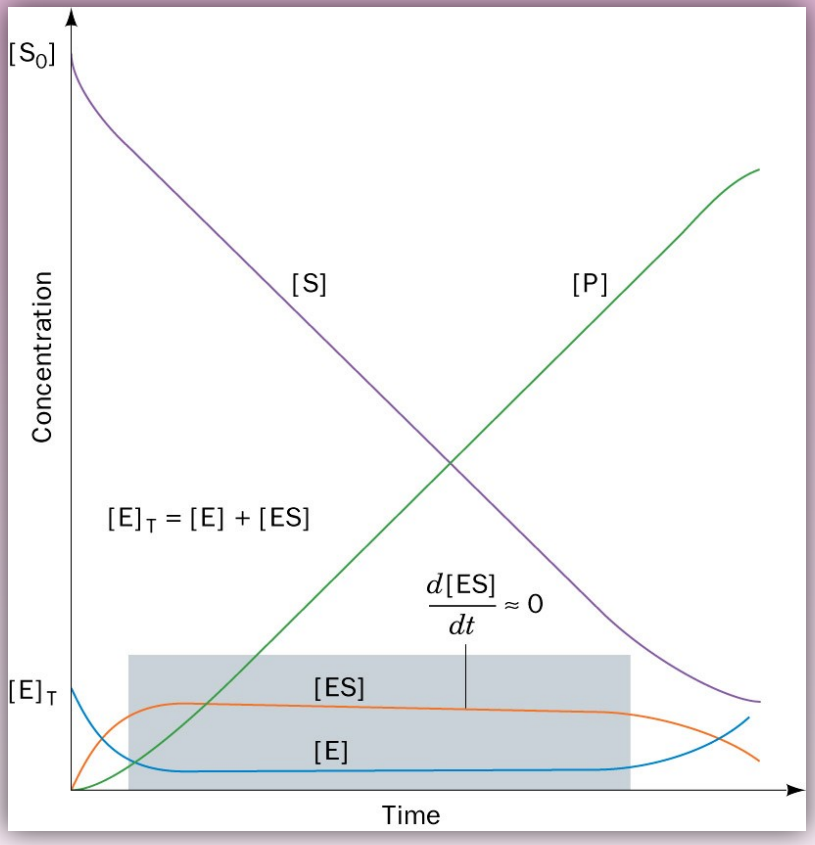
$$\text{initial rate or velocity} = v_o = \frac{V_{max}[S]}{[S] + K_m}$$

describes a hyperbolic, saturation kinetics curve

V_{max} = maximum initial rate

K_m = Michaelis constant = [S] at which 1/2 V_{max} is observed

[S] = concentration of free S



Progress curves for the components of a single substrate/single product Michaelis–Menten enzyme reaction, illustrating the steady-state principle (d[ES]/dt = 0)

Deriving the Michaelis-Menten equation

$$\text{initial rate or velocity} = v_o = \frac{V_{max}[S]}{[S] + K_m}$$

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P$$

Let:

- $[E_t]$ = total enzyme concentration
- $[ES]$ = concentration of the ES complex
- $[E_t] - [ES]$ = concentration of free enzyme
- $[S]$ = concentration of total substrate; since $[S] >> [E_t]$, then $[S_t] = [S_f]$

Furthermore, we will measure *initial rates*: thus, [P] will be very small and we can thus ignore the contributions made by the back-reaction (k_{-2})

To derive the MM equation, we make another important assumption: [ES] over the course of the measurements remains constant (steady-state approximation). Invoking this assumption:

$$\text{Rate of formation of ES} = k_1([E_t] - [ES])[S]$$
$$\text{Rate of breakdown of ES} = k_{-1}[ES] + k_2[ES]$$

For [ES] to remain constant, these two rates must be identical.

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

Solving for [ES]:

$$[ES] = \frac{[E_t][S]}{[S] + \frac{k_2 + k_{-1}}{k_1}}$$

Initial velocity, $v_o = k_2[ES]$; so, multiplying both sides by k_2:

$$v_o = k_2\,[ES] = \frac{k_2\,[E_t][S]}{[S] + \dfrac{k_2 + k_{-1}}{k_1}}$$

Since $k_2[E_t] = V_{max}$ and $\dfrac{k_2 + k_{-1}}{k_1} = K_m$

$$v_o = k_2\,[ES] = \frac{V_{max}\,[S]}{[S] + K_m}$$ **MM equation**

Note that if 1/2 V_{max} is substituted for v_o on the left-hand side of the equation, it can be shown that K_m = [S] under these conditions.

On the relationship between K_m and K_d for formation of the ES complex

$$\frac{k_2 + k_{-1}}{k_1} = K_m$$

Michaelis constant; obtained from kinetics measurements

$$\frac{k_{-1}}{k_1} = K_d$$

dissociation constant; obtained from Scatchard or related plots

The K_d is the formal measure of enzyme affinity for S. While K_m is commonly interpreted as a measure of this affinity, it can only be a reasonable estimate of the K_d when $k_2 << k_{-1}$.

The ratio, k_2/K_m, is a measure of catalytic efficiency.

If $[S] << K_m$, then $[E_f] = [E_t]$ (little ES forms).
Under these conditions:

$$v_o = \frac{k_2 [E_t][S]}{[S] + K_m}$$

or

$$v_o = \frac{k_2 [E_t][S]}{K_m}$$

or

$$v_o = \frac{k_2}{K_m} [E_t][S]$$

$k_2/K_m = k_{cat}/K_m$ = second order rate constant; maximal when k_2 is large and K_m is small; a measure of catalytic efficiency (considers both the binding and catalytic components to enzyme action)

Is there an upper limit on catalytic efficiency, k_{cat}/K_m?

$$\frac{k_{cat}}{K_m} = \frac{k_2}{K_m} = \frac{k_1 k_2}{k_{-1} + k_2}$$

The maximal ratio is reached when $k_2 >> k_{-1}$. Then…

$k_{cat}/K_m = k_1$ = second order rate constant for formation of the ES complex

k_1 can only be as fast as the rate of diffusion; the diffusion controlled limit in solution is estimated at **10^8 - 10^9 $M^{-1}s^{-1}$**

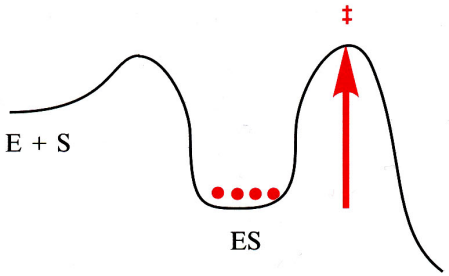
Enzyme	Substrate	K_M (M)	k_{cat} (s^{-1})	k_{cat}/K_M ($M^{-1} \cdot s^{-1}$)
Acetylcholinesterase	Acetylcholine	9.5×10^{-5}	1.4×10^{4}	1.5×10^{8}
Carbonic anhydrase	CO_2	1.2×10^{-2}	1.0×10^{6}	8.3×10^{7}
	HCO_3^-	2.6×10^{-2}	4.0×10^{5}	1.5×10^{7}
Catalase	H_2O_2	2.5×10^{-2}	1.0×10^{7}	4.0×10^{8}
Chymotrypsin	*N*-Acetylglycine ethyl ester	4.4×10^{-1}	5.1×10^{-2}	1.2×10^{-1}
	N-Acetylvaline ethyl ester	8.8×10^{-2}	1.7×10^{-1}	1.9
	N-Acetyltyrosine ethyl ester	6.6×10^{-4}	1.9×10^{2}	2.9×10^{5}
Fumarase	Fumarate	5.0×10^{-6}	8.0×10^{2}	1.6×10^{8}
	Malate	2.5×10^{-5}	9.0×10^{2}	3.6×10^{7}
Superoxide dismutase	Superoxide ion ($O_2^{-\cdot}$)	3.6×10^{-4}	1.0×10^{6}	2.8×10^{9}
Urease	Urea	2.5×10^{-2}	1.0×10^{4}	4.0×10^{5}

Values of K_m, k_{cat}, and k_{cat}/K_m for some enzymes and their substrates

$[S] >> K_M$

$v = k_{cat}[E_0]$

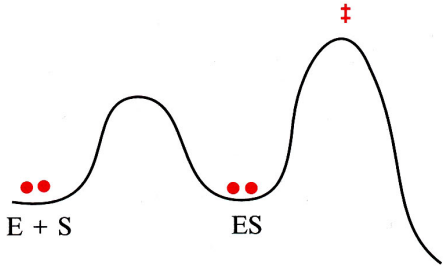
Enzyme saturated with substrate



$[S] = K_M$

$v = \frac{k_{cat}[E_0]}{2}$

Enzyme 50% saturated with substrate



$[S] << K_M$

$v = \frac{k_{cat}[E_0][S]}{K_M}$

Free enzyme + substrate

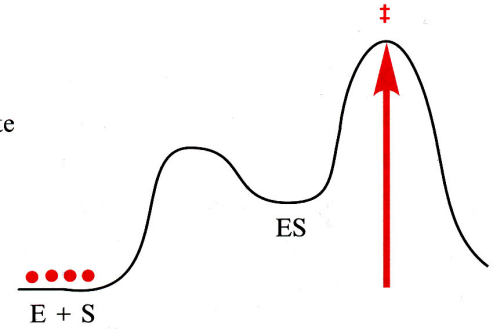


Figure 4.4 Significance of K_M, k_{cat}. Plots are of free energy versus reaction co-ordinate. Red dots indicate the population of enzyme molecules present.

Linearizing the Michaelis-Menten Equation

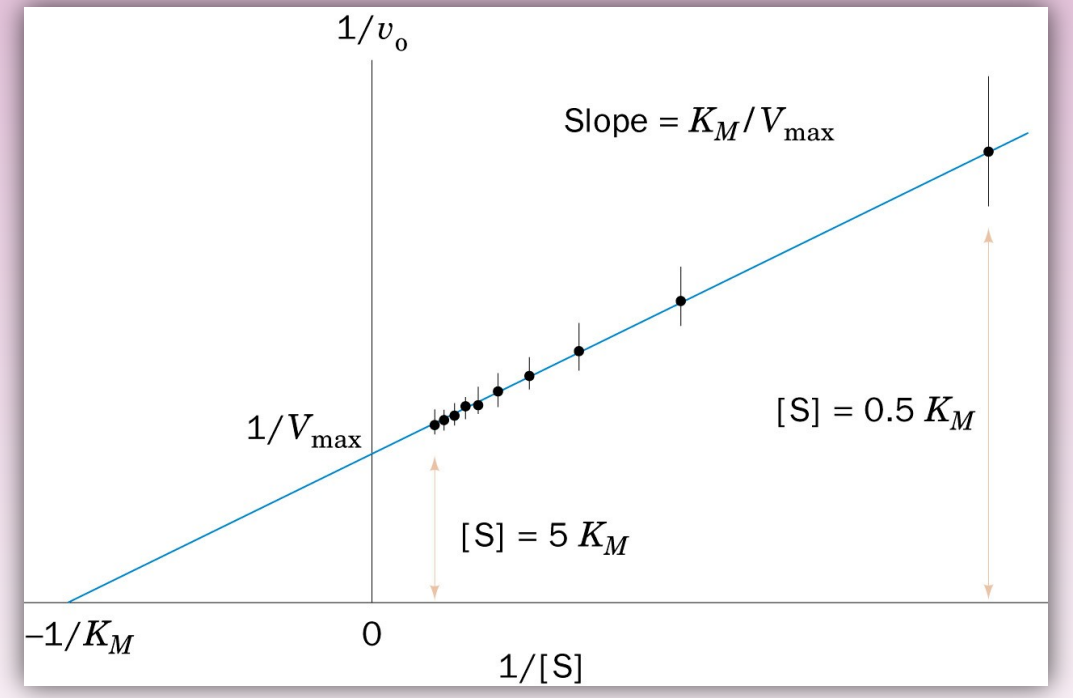
Experimental determination of K_m and V_{max}

a) The Lineweaver-Burk equation/plot (double-reciprocal):
(obtained from algebraic manipulation of the MM equation)

$$\frac{1}{v_o} = \frac{1}{[S]}\left[\frac{K_m}{V_{max}}\right] + \frac{1}{V_{max}}$$

Plot $1/v_o$ *vs* $1/[S]$: slope = K_m/V_{max};
y-intercept = $1/V_{max}$; *x*-intercept = $-1/K_m$

A double reciprocal (Lineweaver–Burk) plot



Note the large extrapolations to obtain the *y*- and *x*-intercepts, more so for the latter; solubility limitations of S determine the smallest achievable experimental value of 1/[S]

b) Eadie-Hofstee equations/plots (single reciprocal)

(obtained from algebraic manipulation of the MM equation)

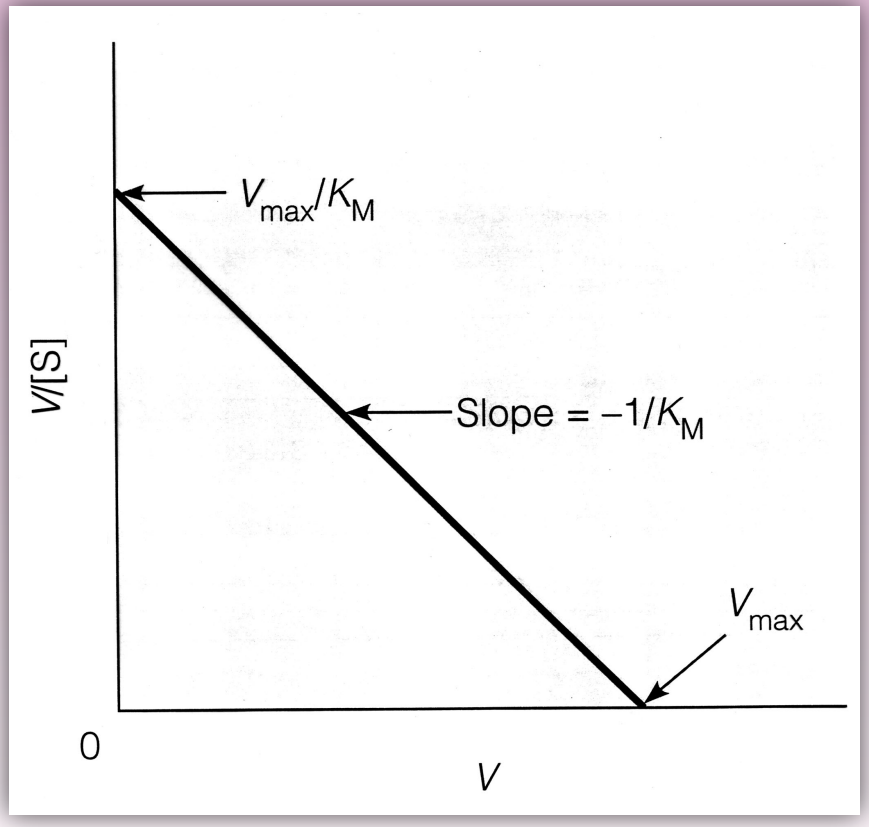
$$\frac{v_o}{[S]} = -\frac{1}{K_m} v_o + \frac{V_{max}}{K_m}$$ **form 1**

$$v_o = -K_m \left[\frac{v_o}{[S]}\right] + V_{max}$$ **form 2**

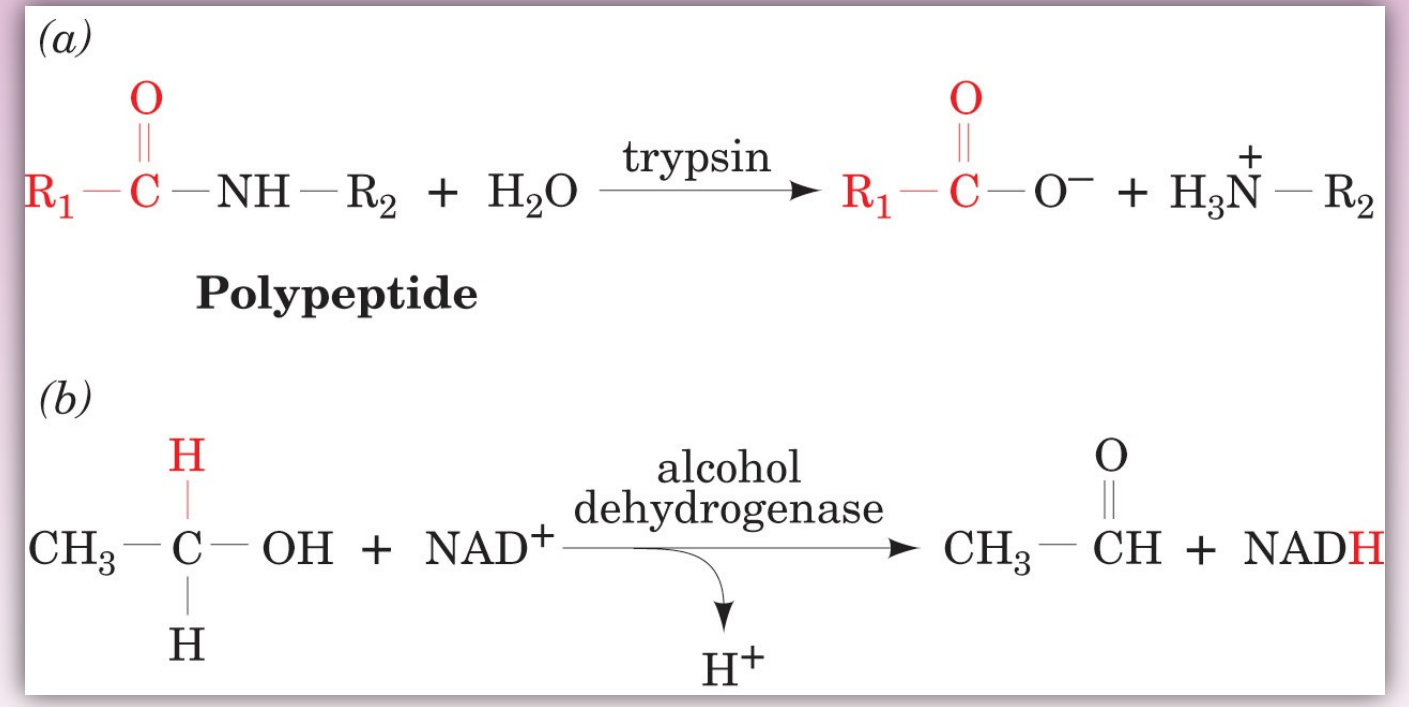
For form 1: plot $v_o/[S]$ *vs* v_o: slope = $-1/K_m$;

x-intercept = V_{max}; *y*-intercept = V_{max}/K_m

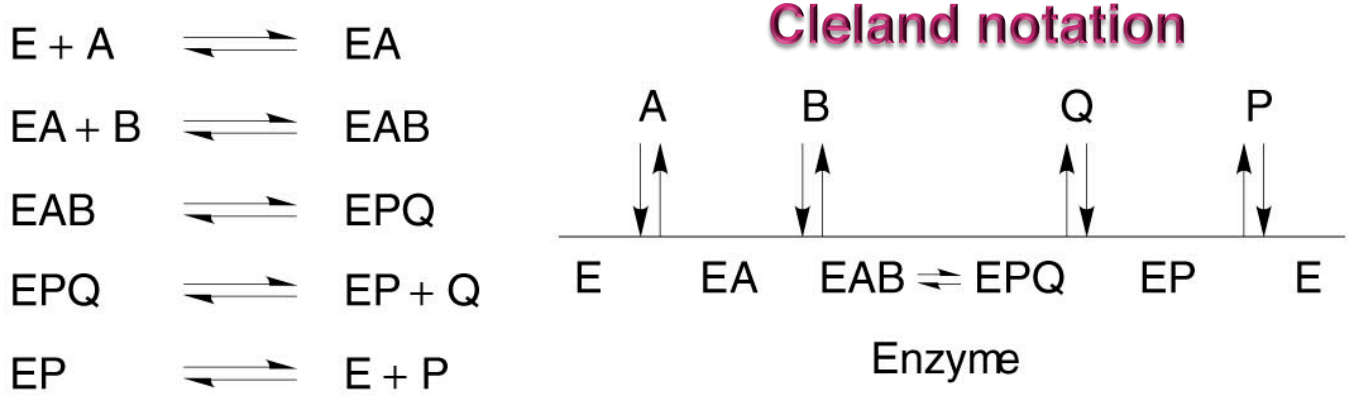
A single reciprocal (Eadie-Hofstee) plot



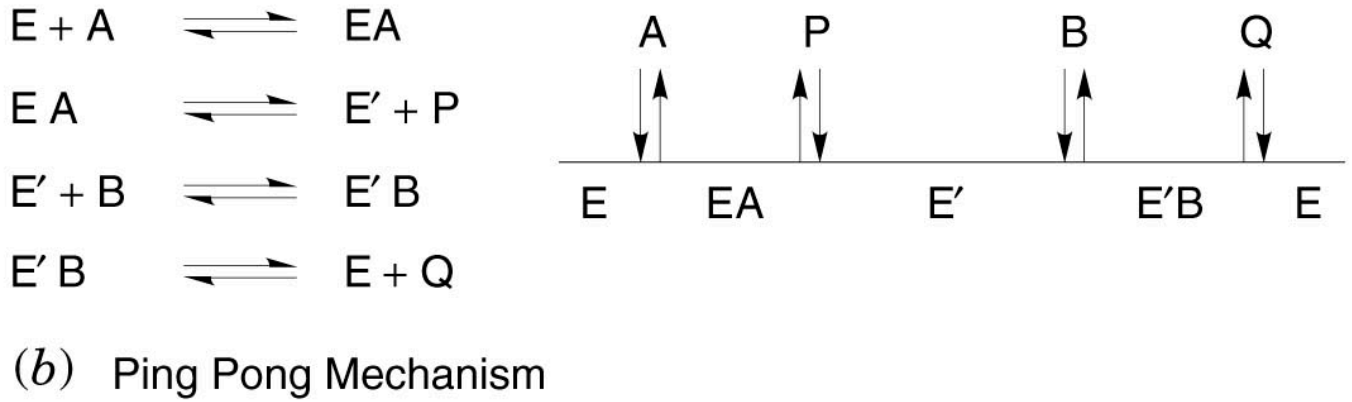
Two-substrate (bi-substrate) reactions. Peptide hydrolysis catalyzed by trypsin, and alcohol dehydrogenase



Cleland notation



(a) Sequential Mechanism

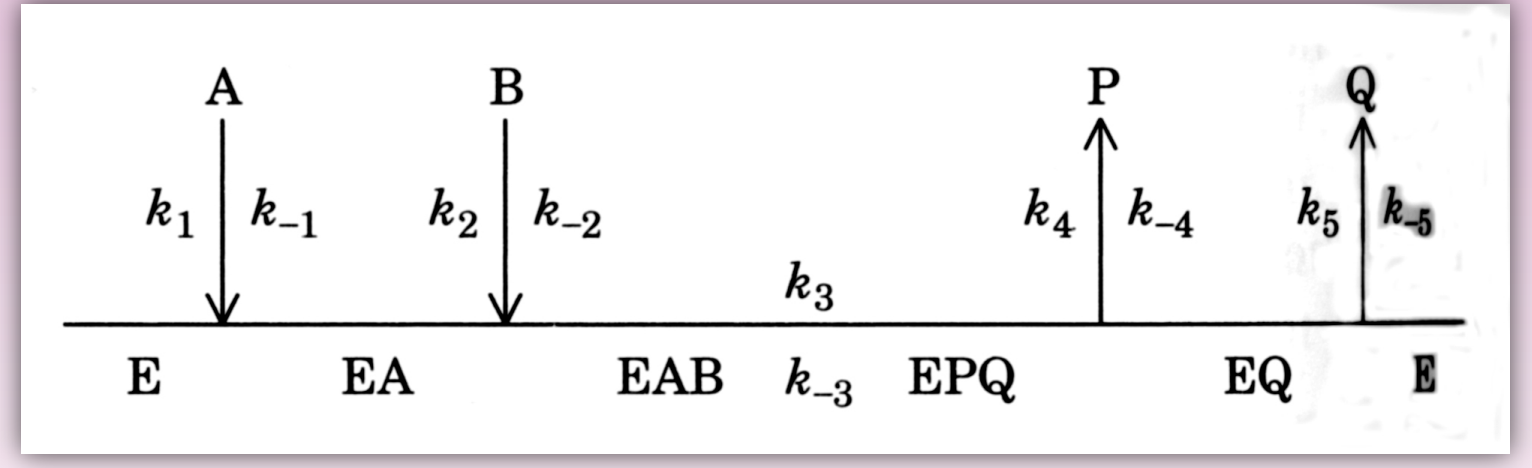


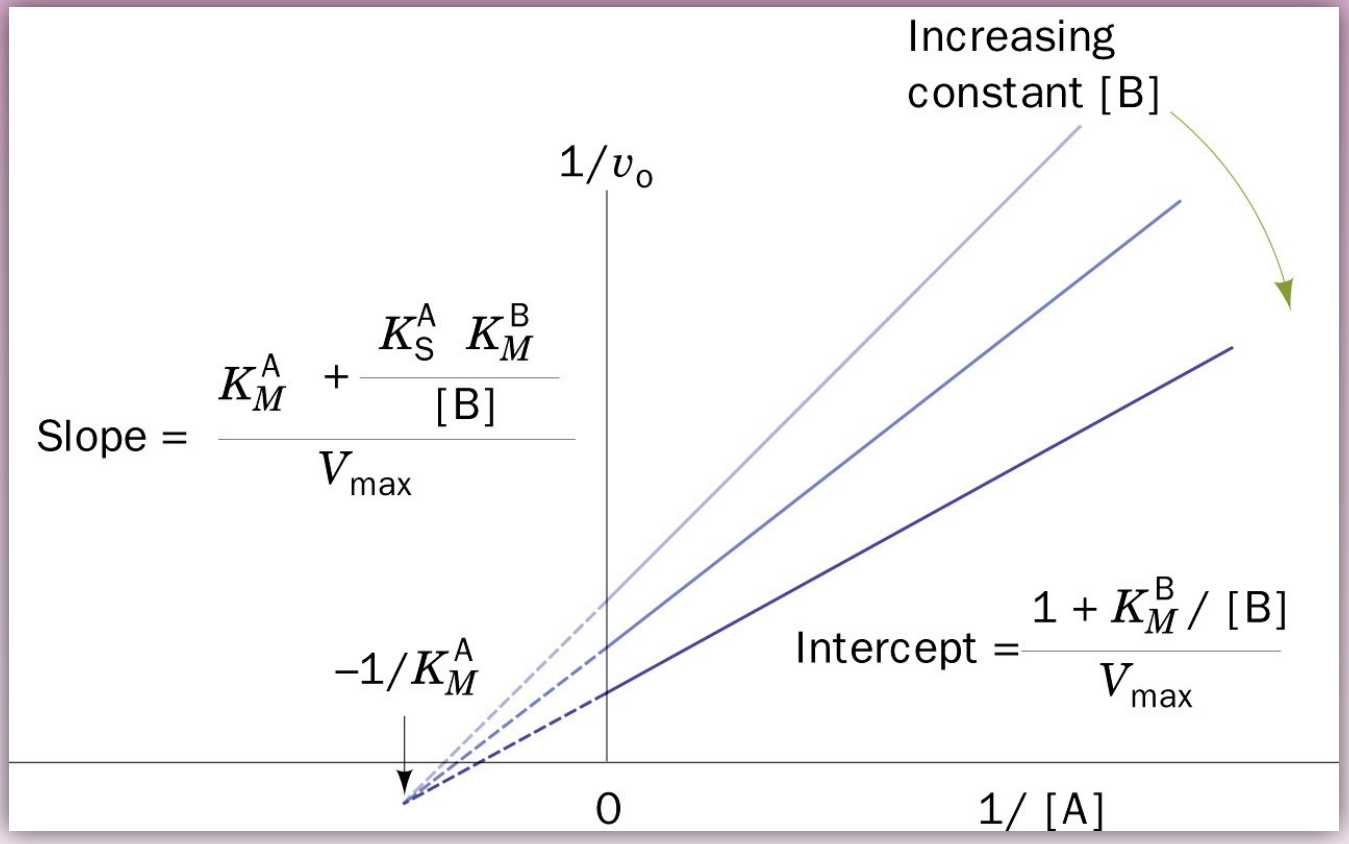
(b) Ping Pong Mechanism

Figure 10.53. Mechanisms of interaction for two substrate reactions.

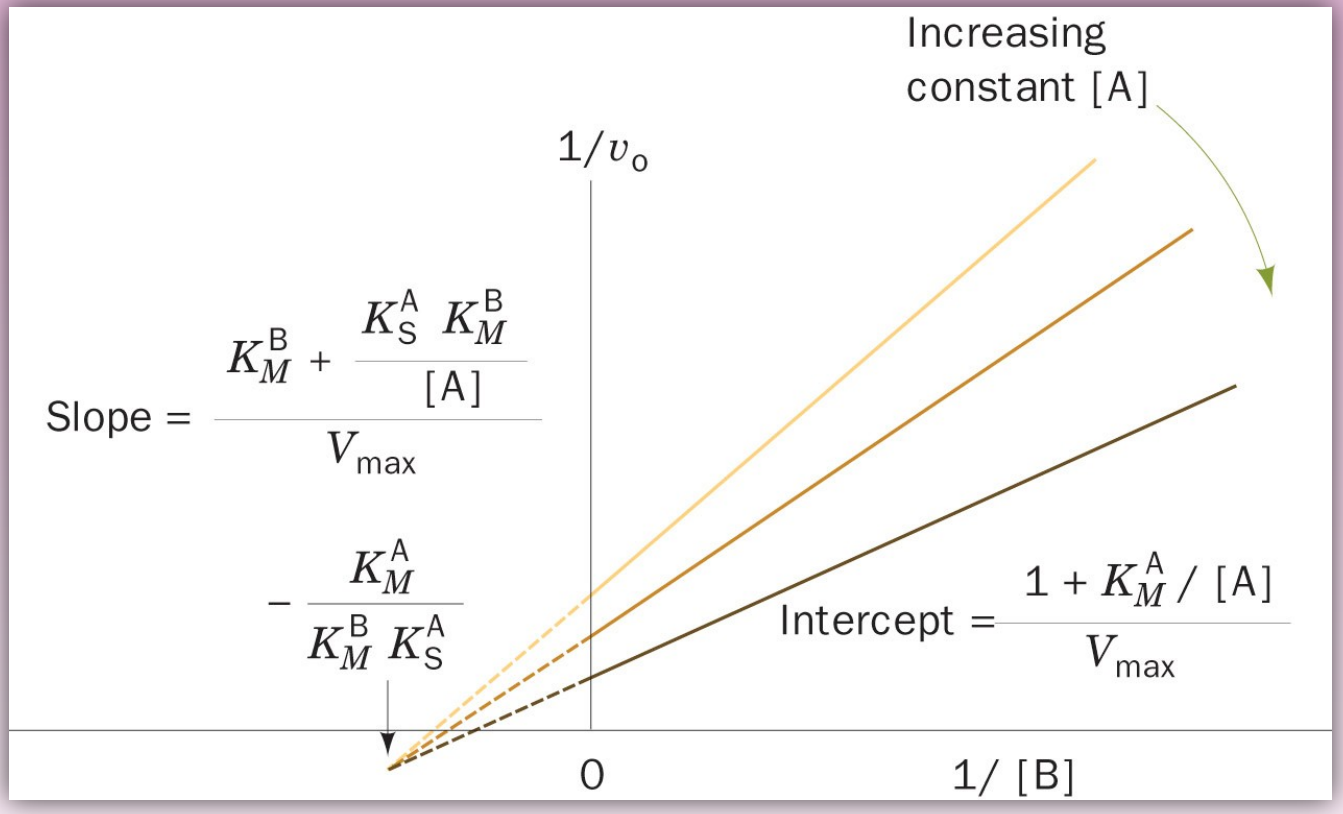
Sequential Reactions = Single Displacement Reactions

An **Ordered Bi Bi** enzyme reaction





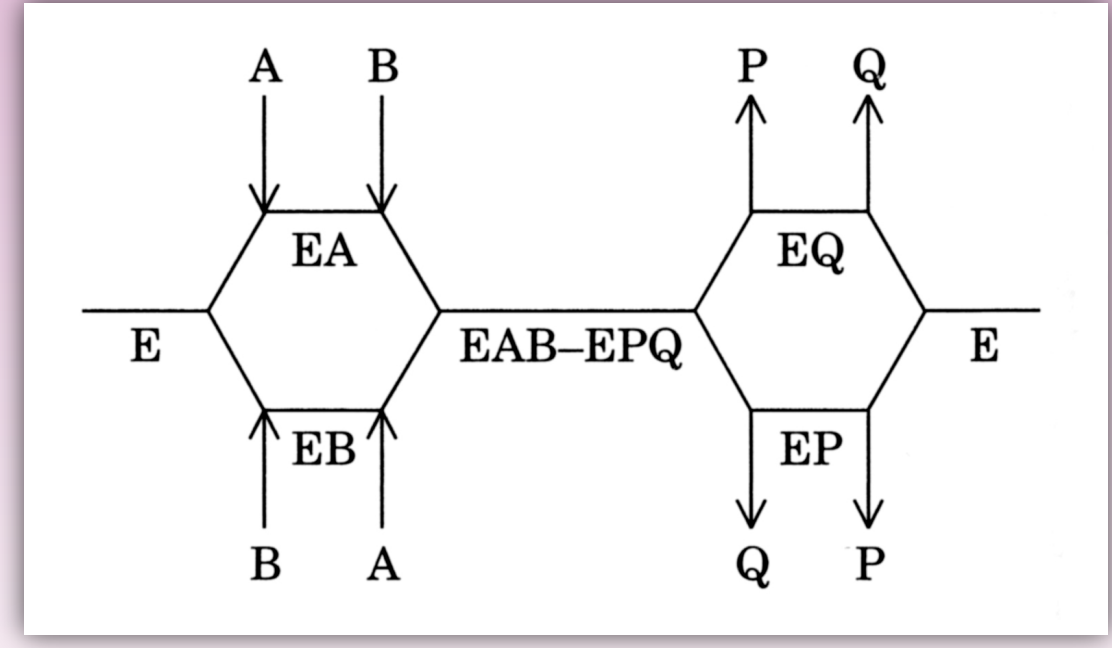
Double-reciprocal plots of an enzymatic reaction with a **Sequential Bi Bi** mechanism. Plots of $1/v_o$ versus 1/[A] at various constant concentrations of B.



Double-reciprocal plots of an enzymatic reaction with a **Sequential Bi Bi** mechanism. Plots of $1/v_o$ versus 1/[B] at various constant concentrations of A.

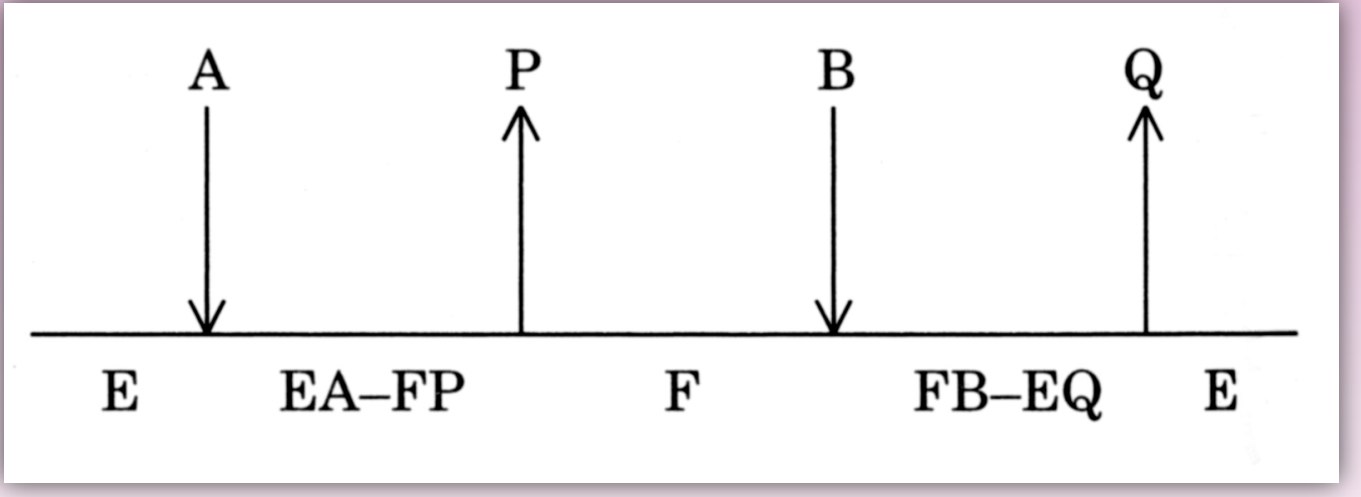
Sequential Reactions = Single Displacement Reactions

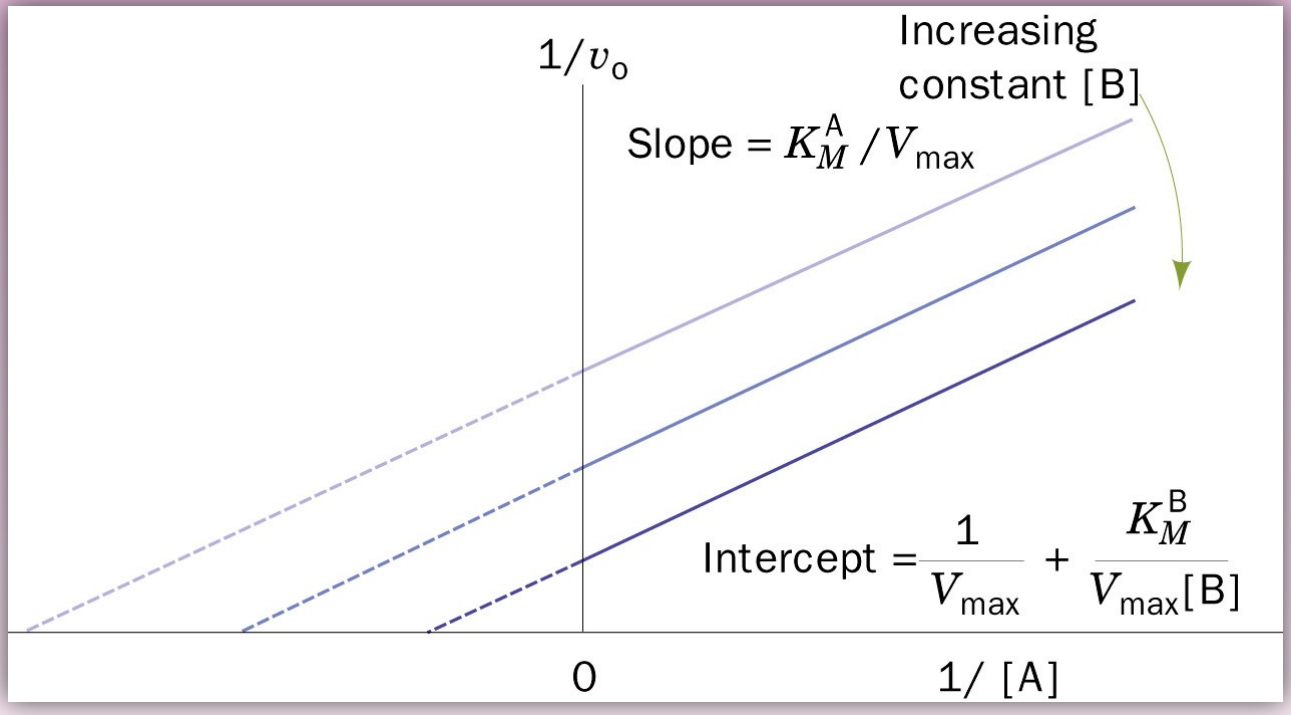
A **Random Bi Bi** enzyme reaction



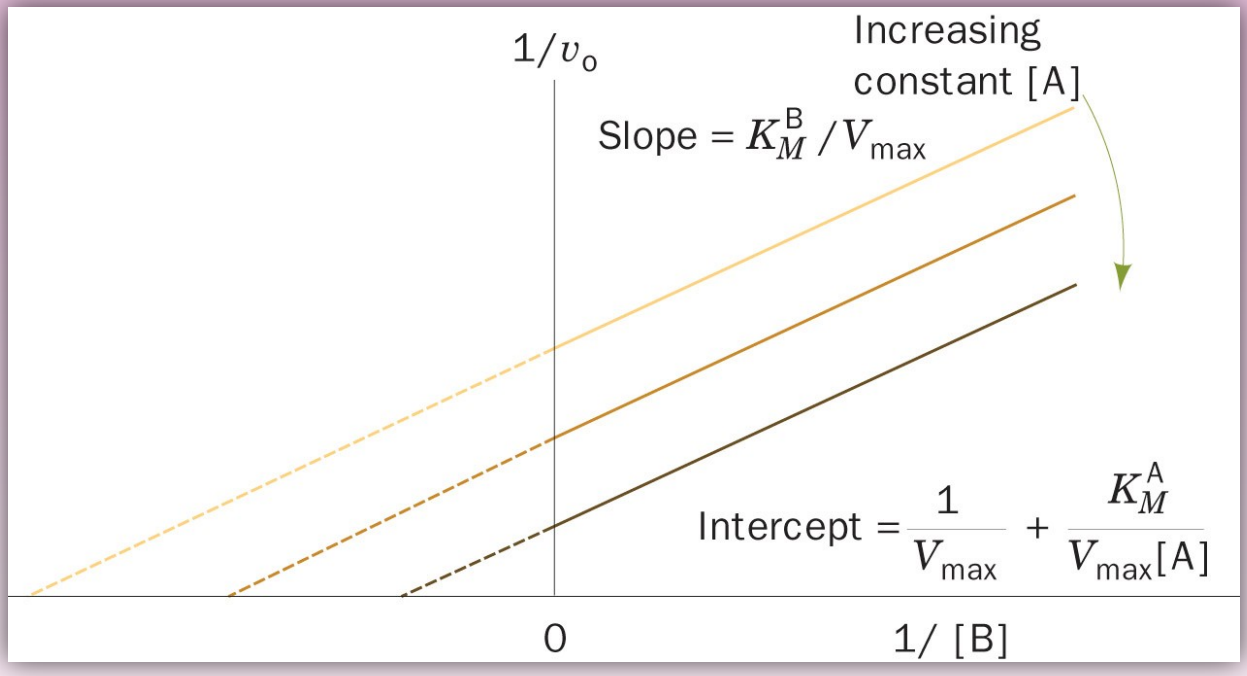
Double Displacement Reactions

A **Ping-Pong Bi Bi** enzyme reaction





Double reciprocal plots for an enzymatic reaction with a **Ping Pong Bi Bi** mechanism



Double reciprocal plots for an enzymatic reaction with a **Ping-Pong Bi Bi** mechanism

Assaying an enzyme (how much enzyme?)

A purification protocol to isolate/purify rat liver glucokinase

Stage	Specific Activity (nkat · g^{-1})[a]	Yield (%)	Fold[b] Purification
Scheme A: A "traditional" chromatographic procedure			
1. Liver supernatant	0.17	100	1
2. $(NH_4)_2SO_4$ precipitate	*c*	*c*	*c*
3. DEAE-Sephadex chromatography by stepwise elution with KCl	4.9	52	29
4. DEAE-Sephadex chromatography by linear gradient elution with KCl	23	45	140
5. DEAE-cellulose chromatography by linear gradient elution with KCl	44	33	260
6. Concentration by stepwise KCl elution from DEAE-Sephadex	80	15	480
7. Bio-Gel P-225 chromatography	130	15	780
Scheme B: An affinity chromatography procedure			
1. Liver supernatant	0.092	100	1
2. DEAE-cellulose chromatography by stepwise elution with KCl	20.1	104	220
3. Affinity chromatography[d]	**420**	**83**	**4500**

[a] A **katal** (abbreviation **kat**) is the amount of enzyme that catalyzes the transformation of 1 mol of substrate per second under standard conditions. One nanokatal (nkat) is 10^{-9} kat.

[b] Calculated from specific activity; the first step is arbitrarily assigned unity.

[c] The activity could not be accurately measured at this stage because of uncertainty in correcting for contamination by other enzymes.

[d] The affinity chromatography material was made by linking glucosamine (an inhibitor of glucokinase) through a 6-aminohexanoyl spacer arm to NCBr-activated agarose.

Source: Cornish-Bowden, A., *Fundamentals of Enzyme Kinetics, p.* 48, Butterworth (1979), as adapted from Parry, M.J. and Walker, D.G., *Biochem. J.* **99,** 266 (1966) for Scheme A and from Holroyde, M.J., Allen, B.M., Storer, A.C., Warsey, A.S., Chesher, J.M.E., Trayer, I.P., Cornish-Bowden, A., and Walker, D.G., *Biochem. J.* **153,** 363 (1976) for Scheme B.

1. need to know the equation for the reaction
2. need an experimental method to measure rate of disappearance of reactants(s) and/or rate of appearance of product(s)
3. need to know cofactor requirements (coenzymes, metal ions?)
4. need to know K_m of substrate(s)
5. need to know pH optimum

Procedure:

1. measure initial rates v_o at differing $[E_t]$ with [S] at saturating levels (>10 x K_m)
2. plot v_o against $[E_t]$
3. define one (1) unit of activity: *e.g.*, the amount of enzyme causing the transformation of 1.0 μmol of substrate per minute at 25 °C under optimal conditions

specific activity = units of activity/mg protein

turnover number = number of substrate molecules transformed per unit time by one enzyme molecule = $k_{cat} = V_{max}/[E_t]$

[S] is fixed
[E] is varied

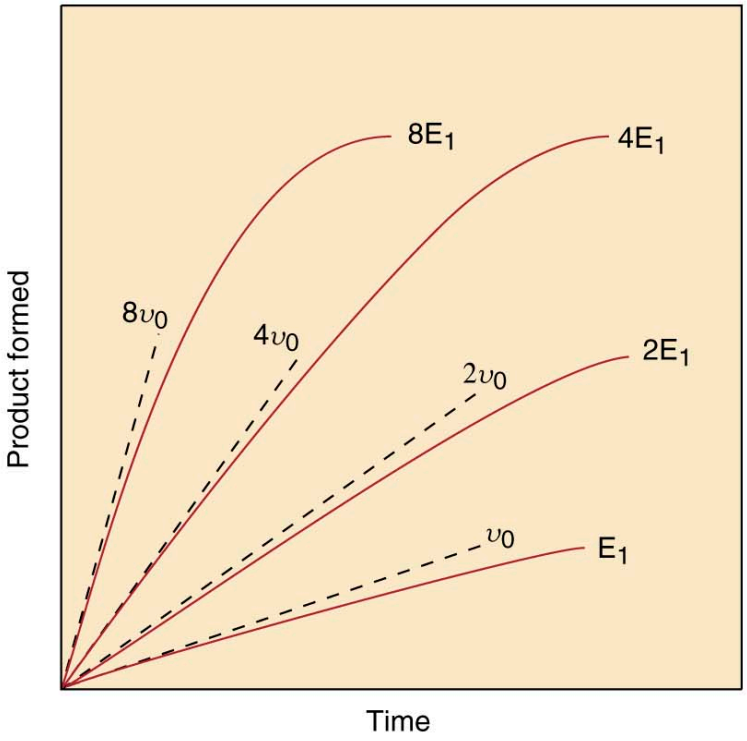
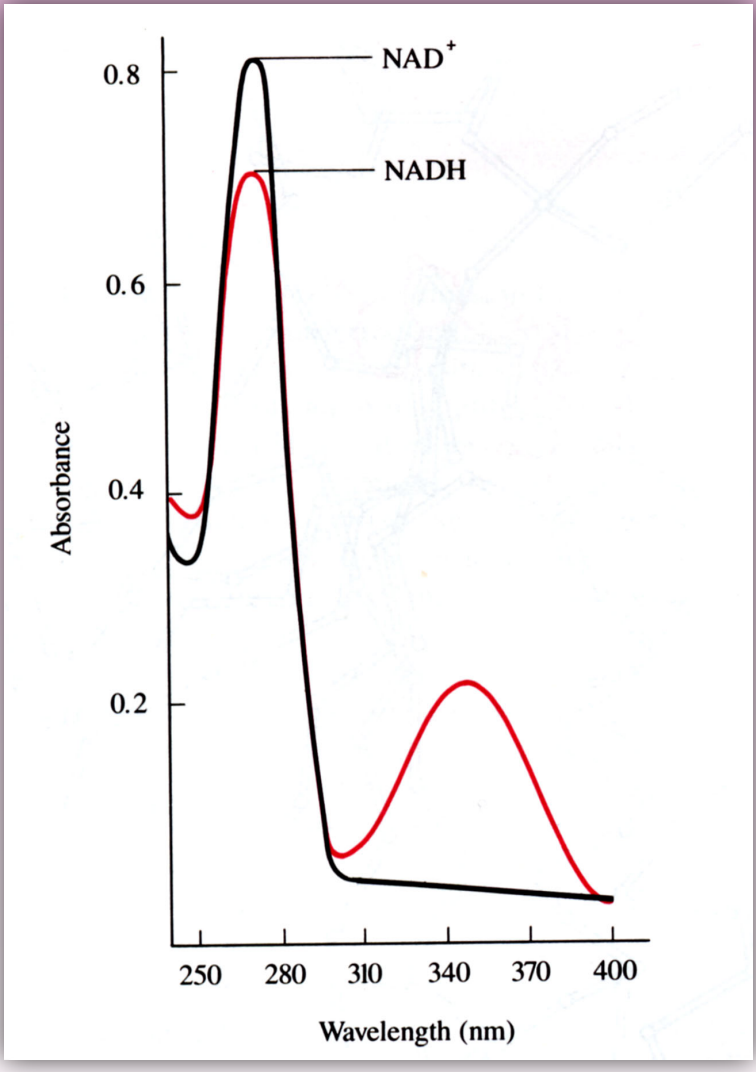


Figure 10.48. Progress curves at variable concentrations of enzyme and saturating concentrations of substrate.

Plots showing the dependence of initial velocities on [E]; initial rates are directly proportional to [E] under saturating [S].
These are the conditions used to determine the specific activity of an enzyme preparation.

The coupled-enzyme assay

(blackboard discussion)



Spectrophotometric absorption curves for NAD^+ and NADH. Note the distinct absorption by NADH at 340 nm.

Other experimental approaches to enzyme assays

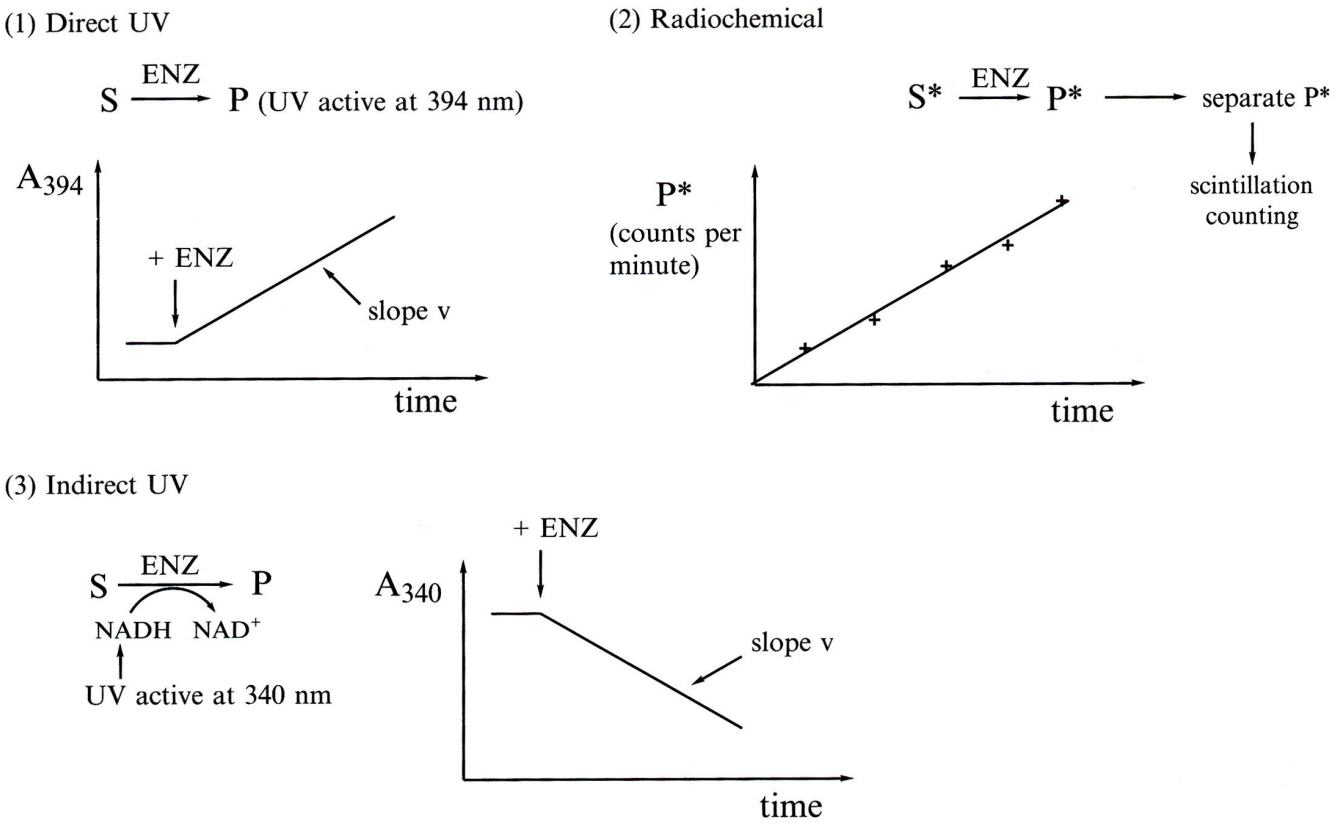
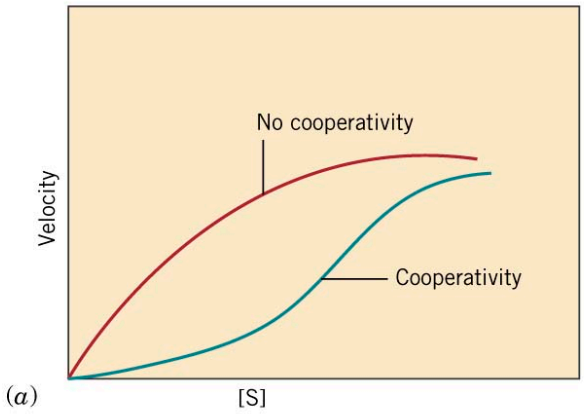


Figure 4.1 Types of enzyme assays. A_{340}, ultraviolet (UV) absorbance at 340 nm; A_{394}, UV absorbance at 394 nm; ENZ, enzyme; NADH, nicotinamide adenine dinucleotide, P, product; Q, product of coupling enzyme; S, substrate.



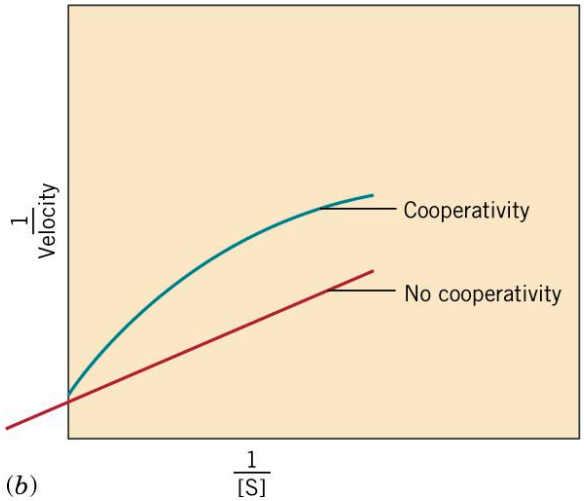


Figure 10.66 Plots for enzymes that exhibit cooperativity are atypical.

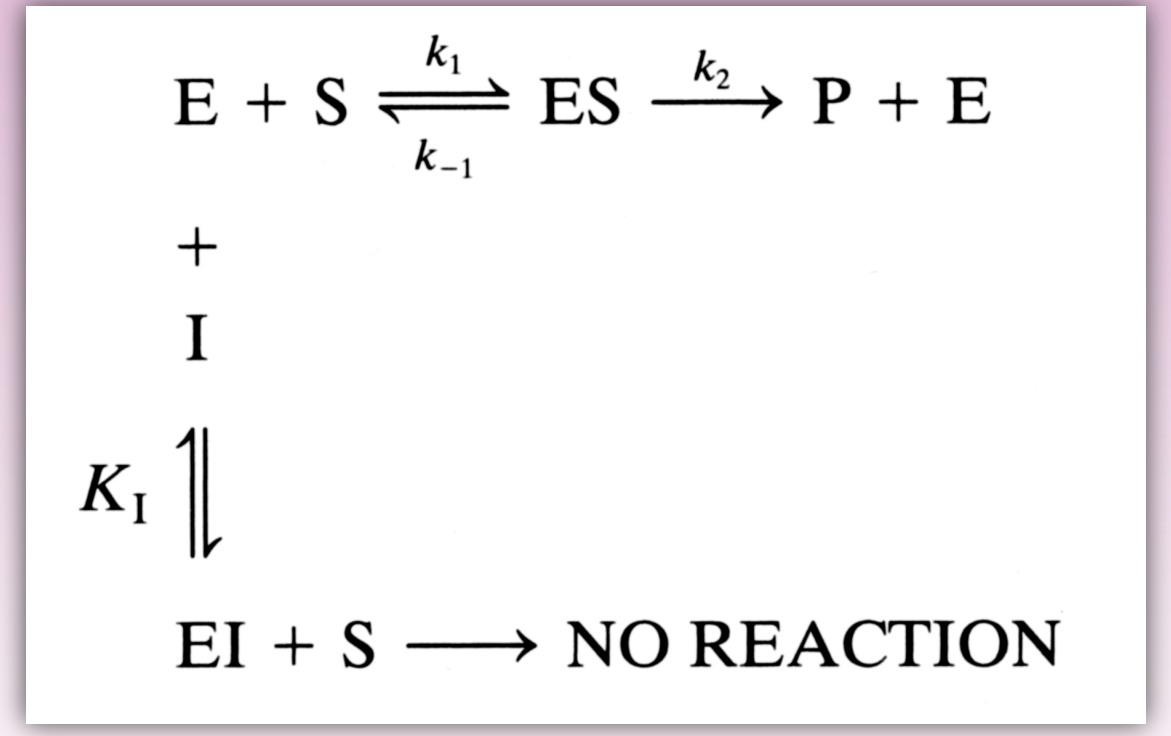
A reminder of the differences in v_o *vs* [S], and $1/v_o$ *vs* 1/[S] plots for enzymes exhibiting no cooperativity and for allosteric enzymes. The former plot is sigmoidal, and the latter plot non-linear, for enzymes displaying cooperativity.

Types of enzyme inhibition

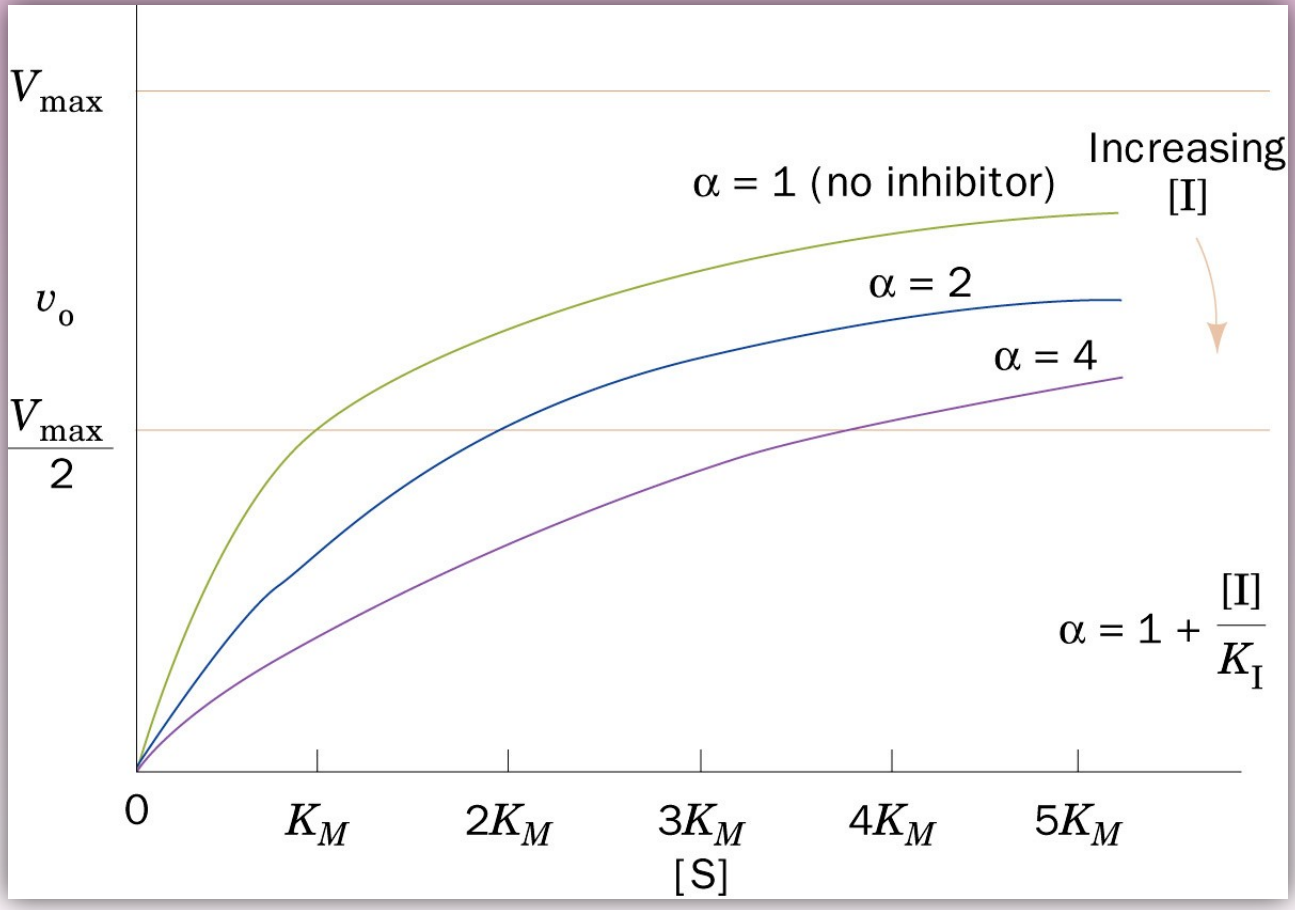
a) irreversible (covalent modification)

b) reversible
   1. competitive
   2. uncompetitive
   3. noncompetitive (mixed)

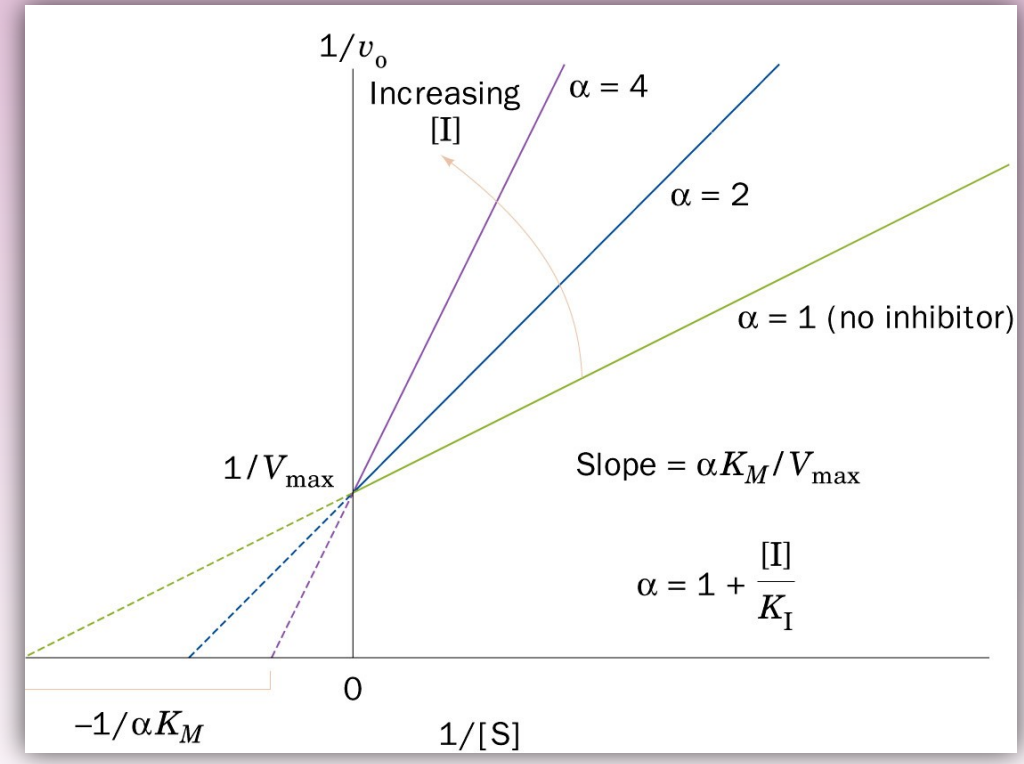
Model of competitive enzyme inhibition



Initial velocity *vs* [S] plots for <u>competitive inhibition</u> of an enzyme



Lineweaver–Burk plot of a competitively inhibited Michaelis–Menten enzyme



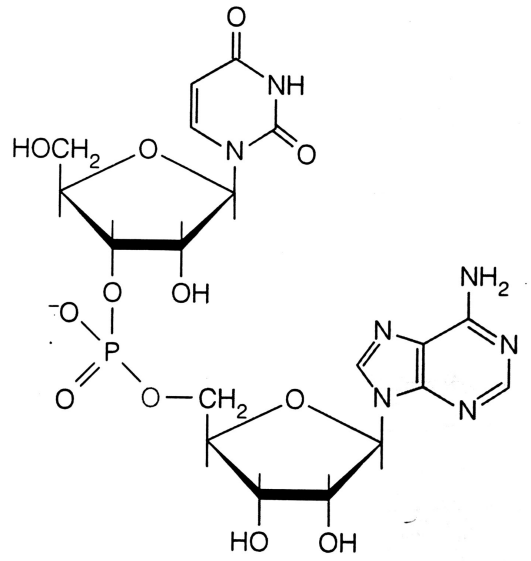
$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \xrightarrow{k_2} P + E$$

$$+$$

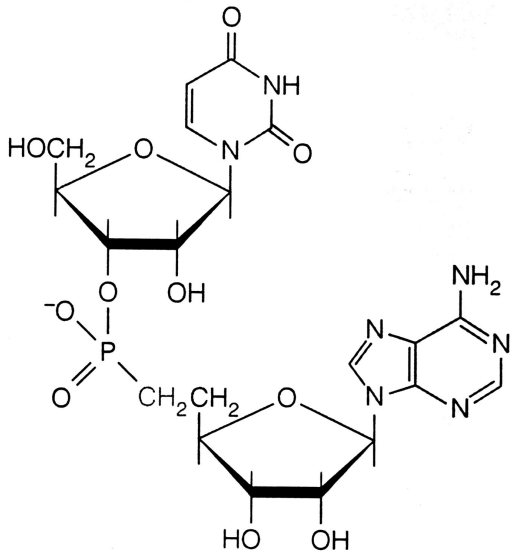
$$I$$

$$K_I \Updownarrow$$

$$EI + S \longrightarrow \text{NO REACTION}$$

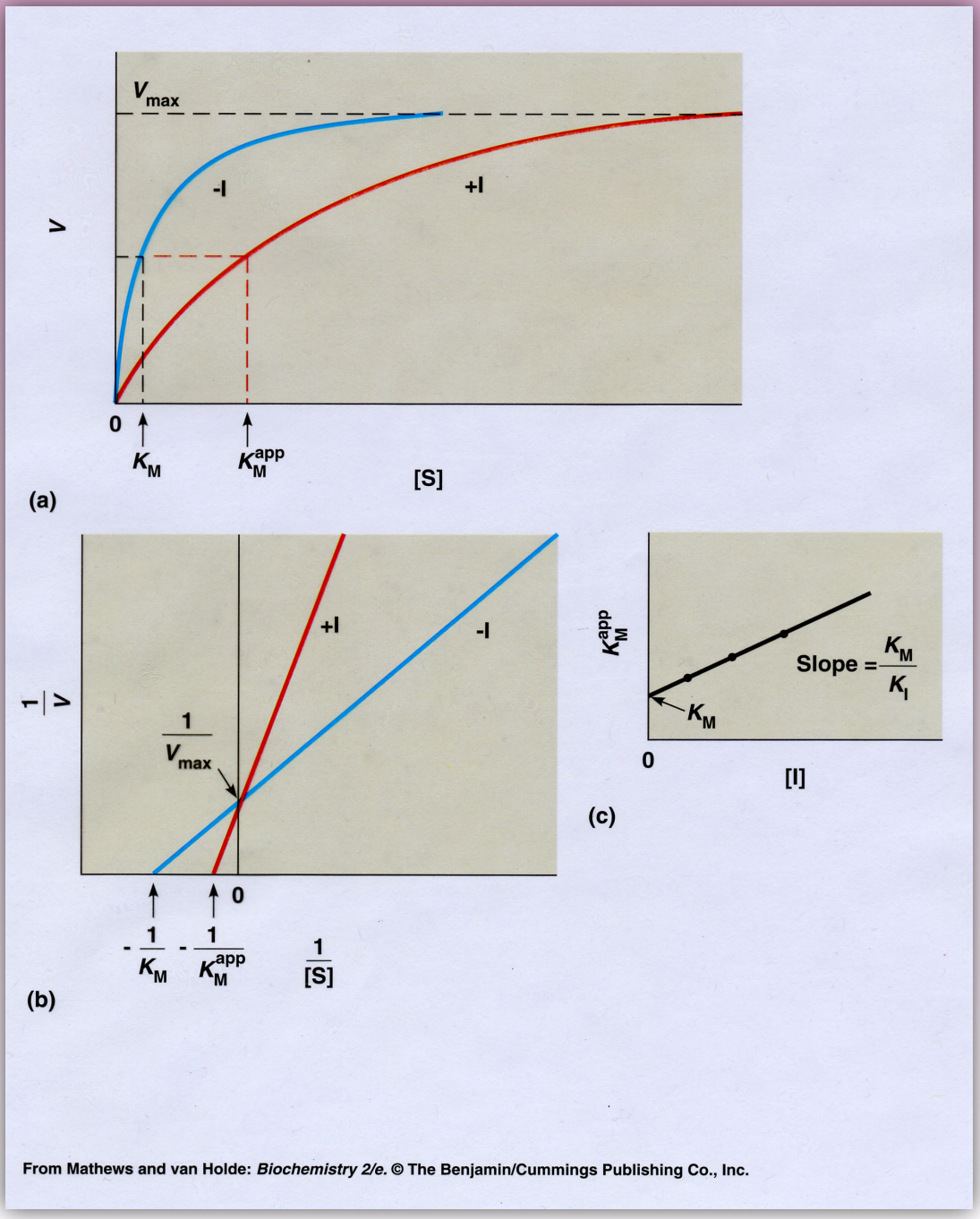


UpA: ribonuclease substrate



UpcA: competitive inhibitor of ribonuclease

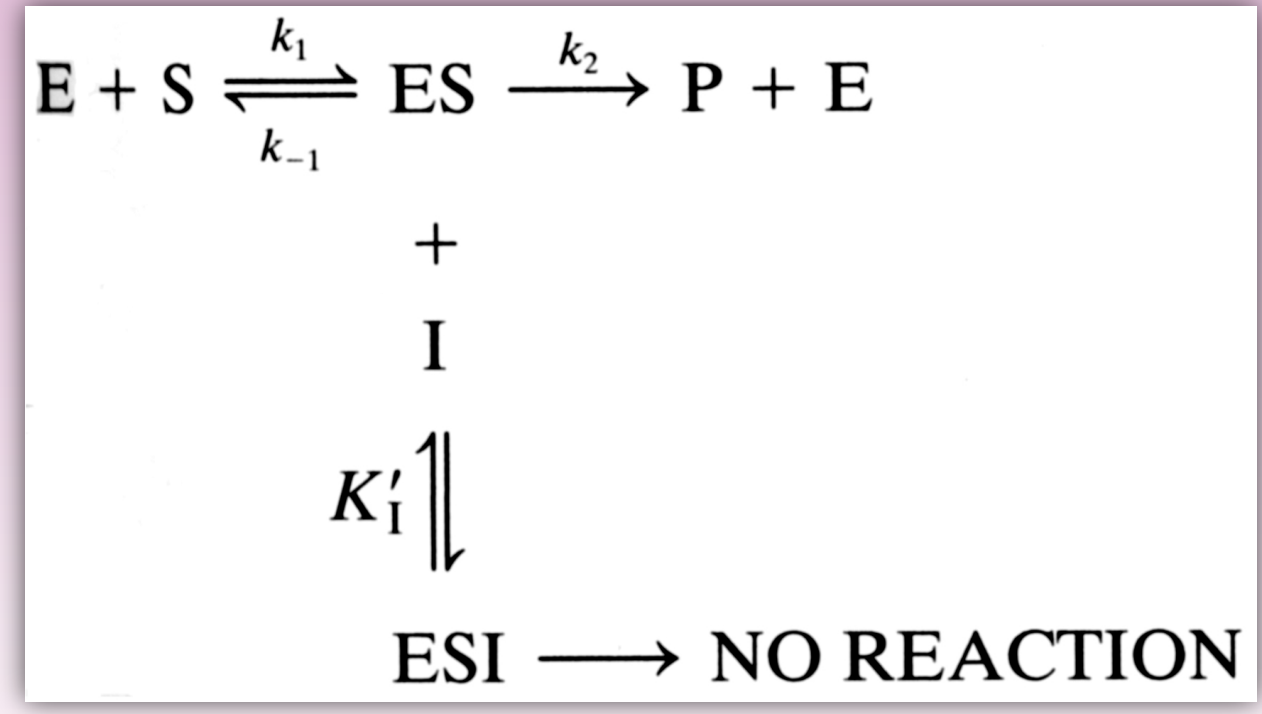
Compounds that behave as competitive inhibitors often resemble the substrate of the enzyme they inhibit. This is not surprising since both molecules bind to the same site on the enzyme.



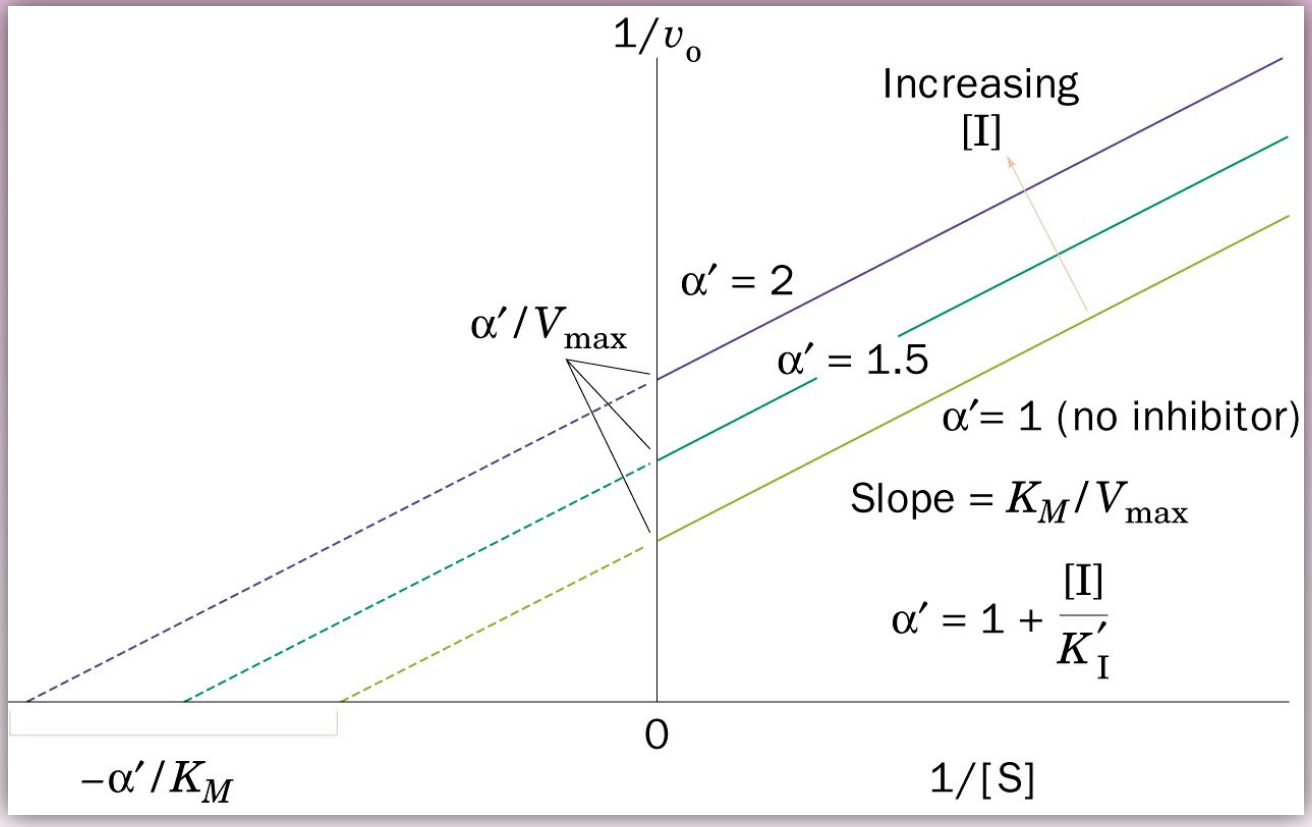
Experimental method to determine the value of K_I (inhibitor binding constant, a dissociation constant) for a <u>competitive inhibitor</u>

Smaller K_I values imply stronger binding: $K_I = [E][I]/[EI]$ (K_I is a specific type of K_d)

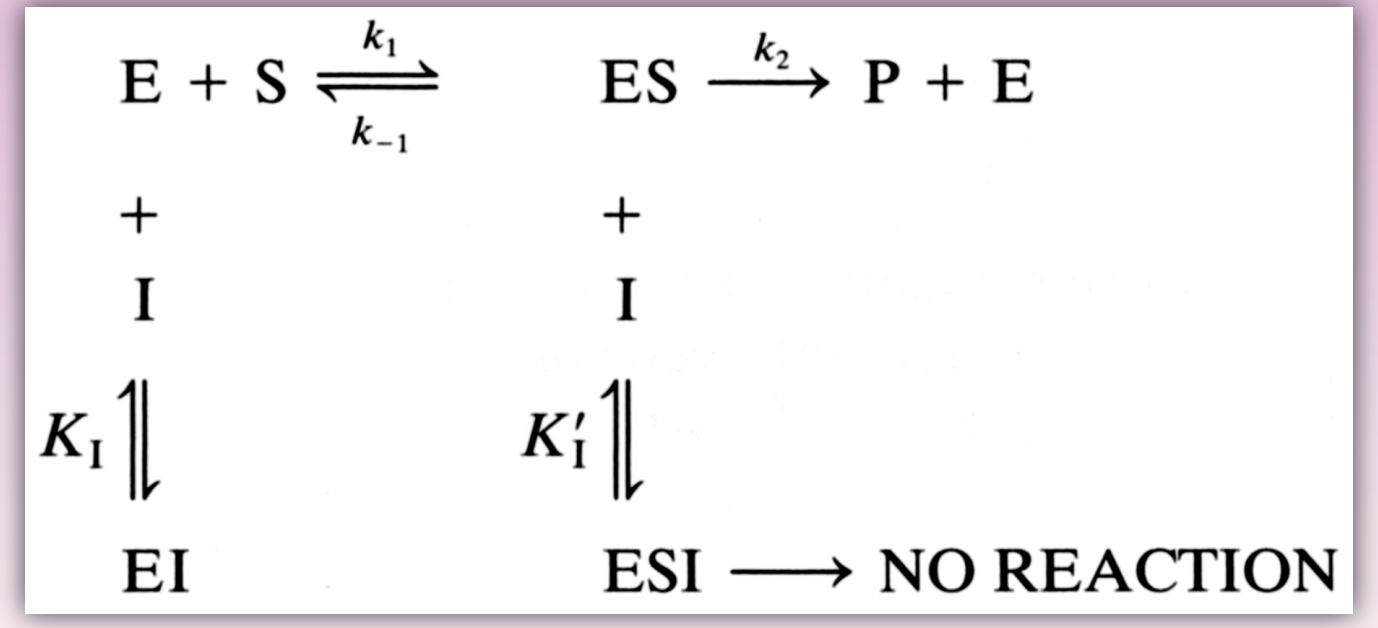
Model of uncompetitive inhibition



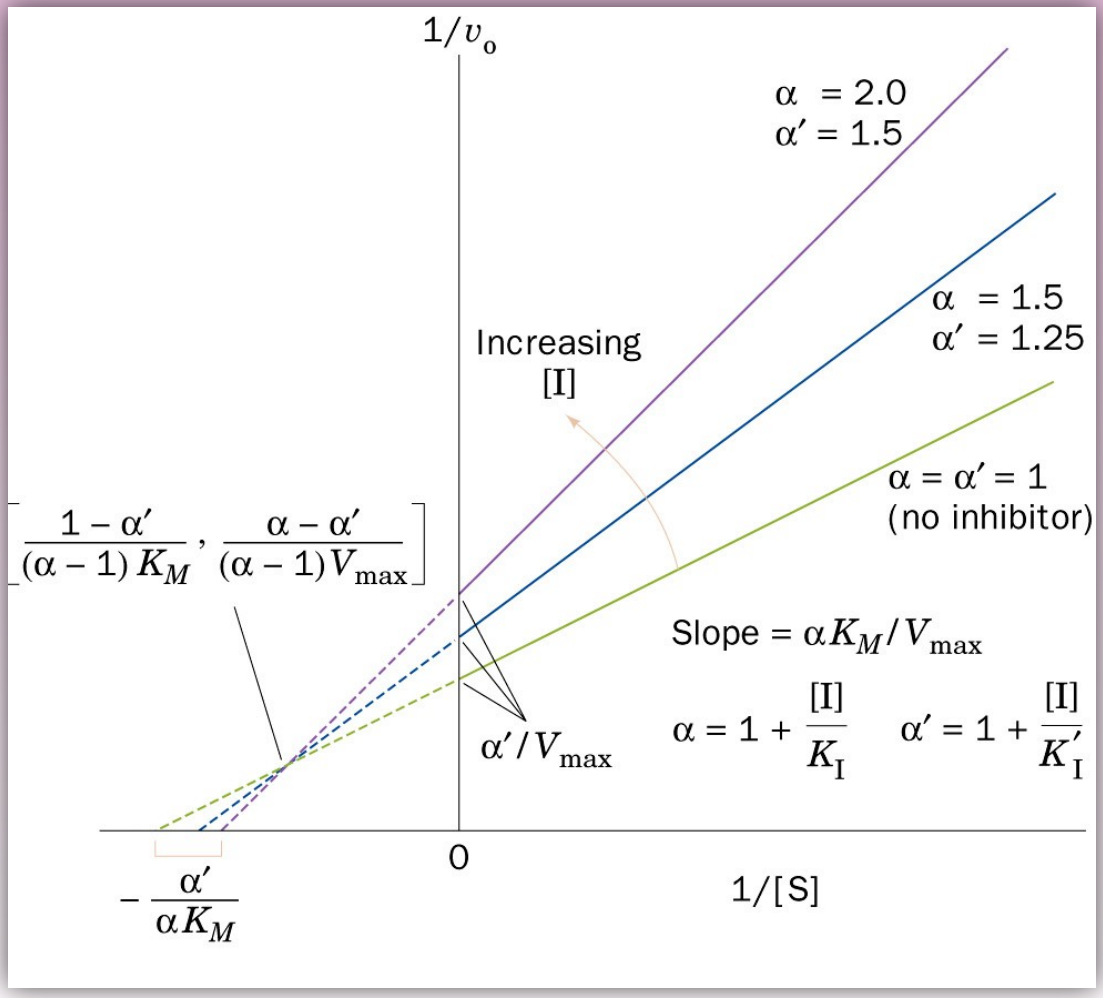
Lineweaver–Burk plot of a simple Michaelis–Menten enzyme in the presence of an uncompetitive inhibitor

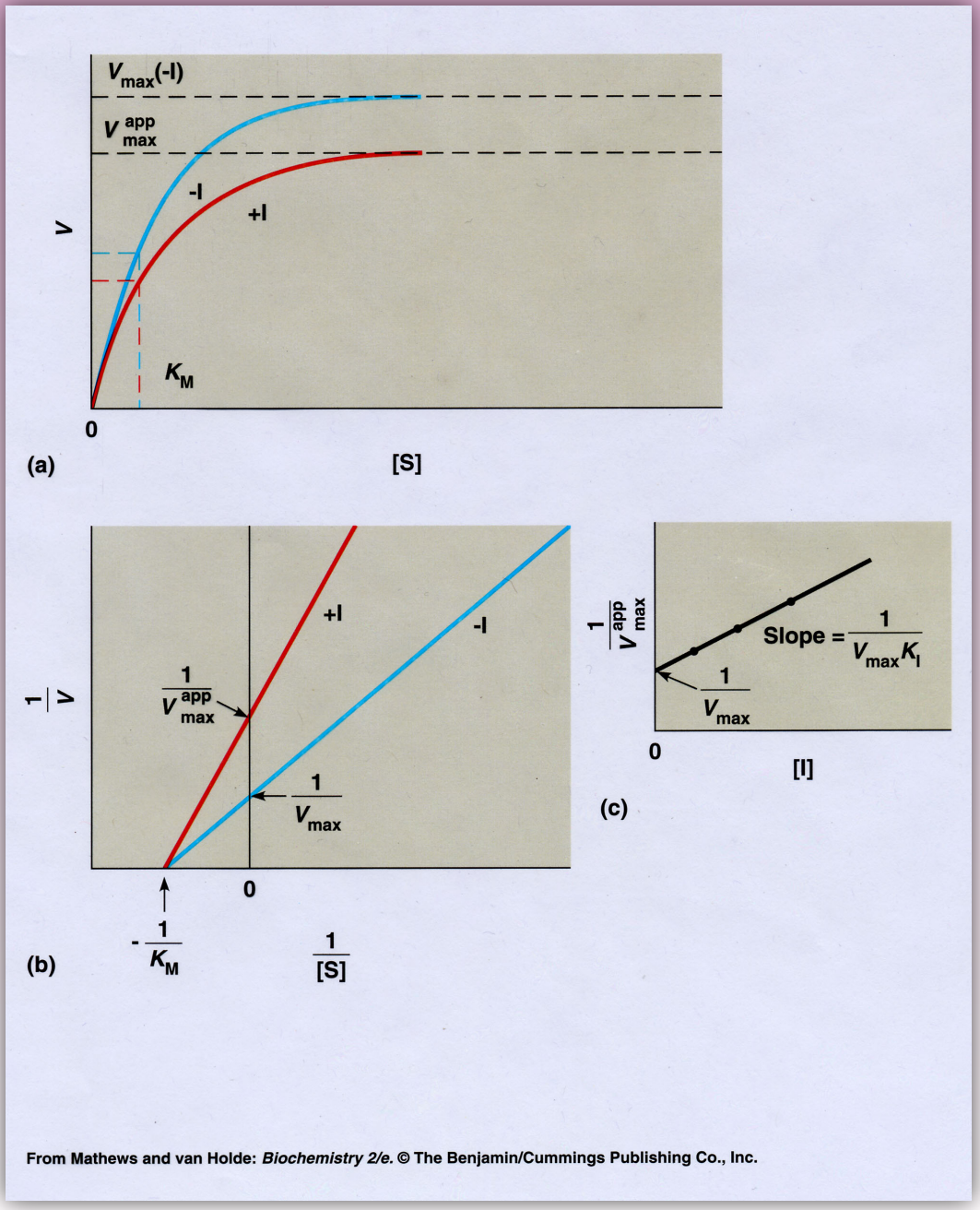


Model of mixed (noncompetitive) inhibition



Lineweaver–Burk plot of a simple Michaelis-Menten enzyme in the presence of a mixed inhibitor





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Experimental method to determine the value of K_I (inhibitor binding constant, a dissociation constant) for a noncompetitive (mixed) inhibitor

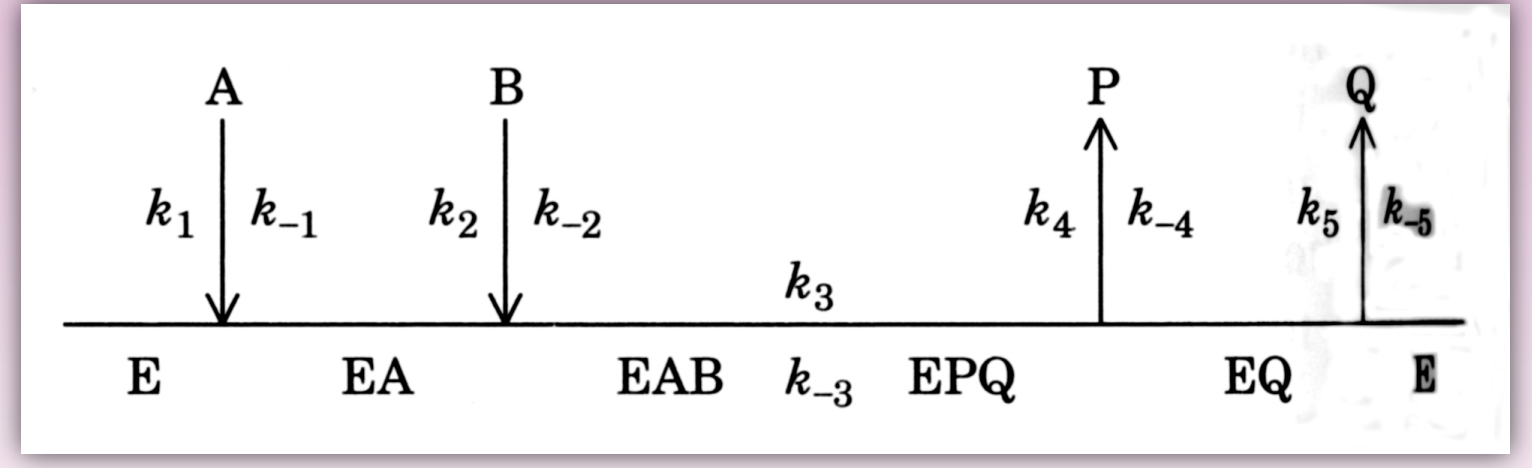
Effects of inhibitors on the parameters of the Michaelis–Menten equation

Type of Inhibition	V_{max}^{app}	K_M^{app}
None	V_{max}	K_M
Competitive	V_{max}	αK_M
Uncompetitive	V_{max}/α'	K_M/α'
Mixed	V_{max}/α'	$\alpha K_M/\alpha'$

[a] $\alpha = 1 + \frac{[I]}{K_I}$ and $\alpha' = 1 + \frac{[I]}{K_I'}$.

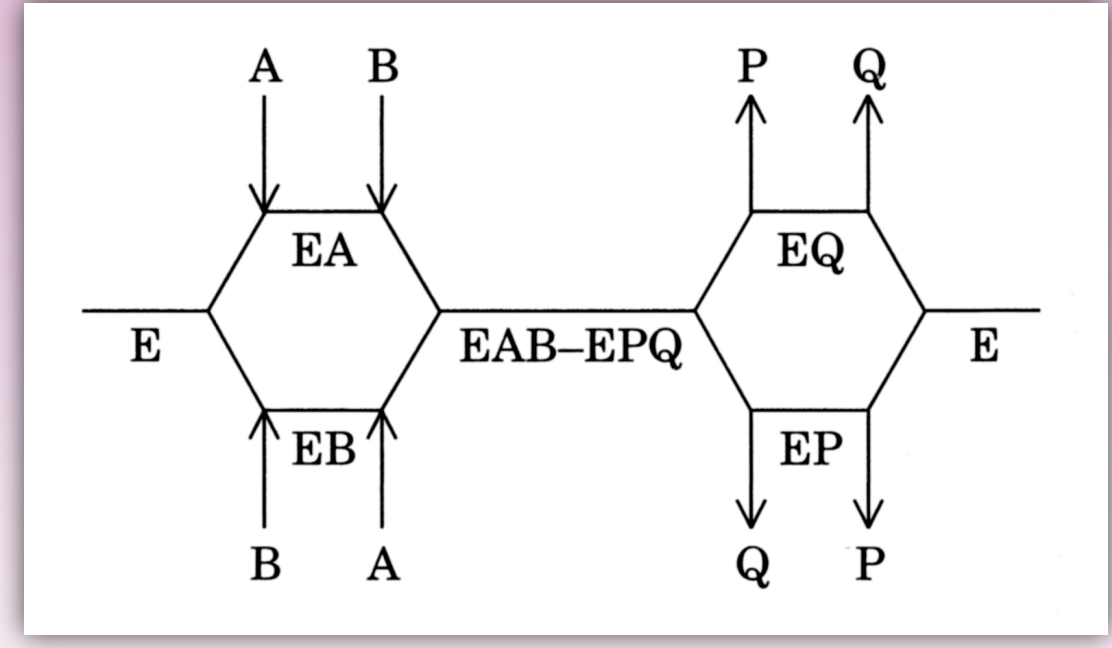
Sequential Reactions = Single Displacement Reactions

An **Ordered Bi Bi** enzyme reaction



Sequential Reactions = Single Displacement Reactions

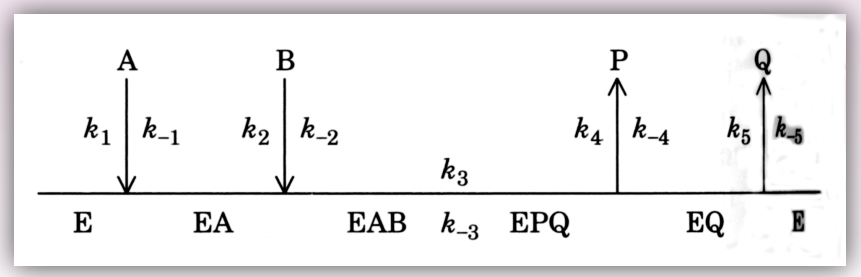
A **Random Bi Bi** enzyme reaction

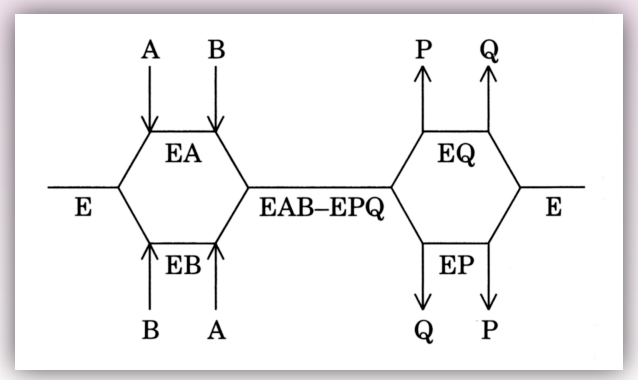


How to distinguish between random and ordered sequential enzyme mechanisms

Patterns of product inhibition for sequential two-substrate mechanisms

Mechanism	Product Inhibitor	[A] Variable	[B] Variable
Ordered Bi Bi	P	Mixed	Mixed
	Q	Competitive	Mixed
Rapid Equilibrium Random Bi Bi	P	Competitive	Competitive
	Q	Competitive	Competitive





Effect of temperature on enzyme-catalyzed reactions: Complicating factors

(a) Above a certain temperature, the enzyme unfolds; the rate at which this denaturation process occurs depends on solution pH, the concentrations of substrates and other ligands, ionic strength, and other factors. Denaturation is rapid for most enzymes at or above 323 K.

(b) For a multi-step enzyme-catalyzed reaction with different activation energies, a change in the rate-determining step may occur with temperature.

(c) The enzyme may exist in two interconvertible active forms that possess different activation energies.

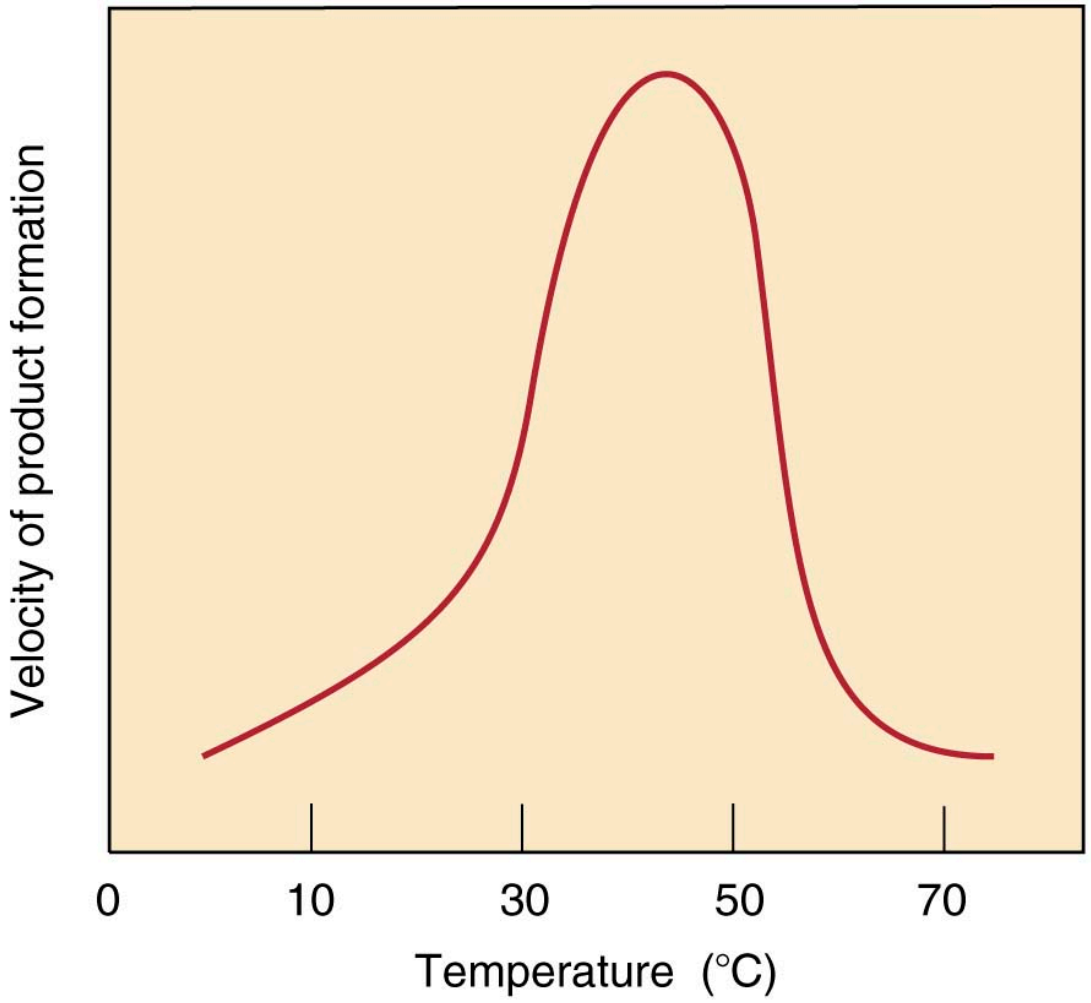
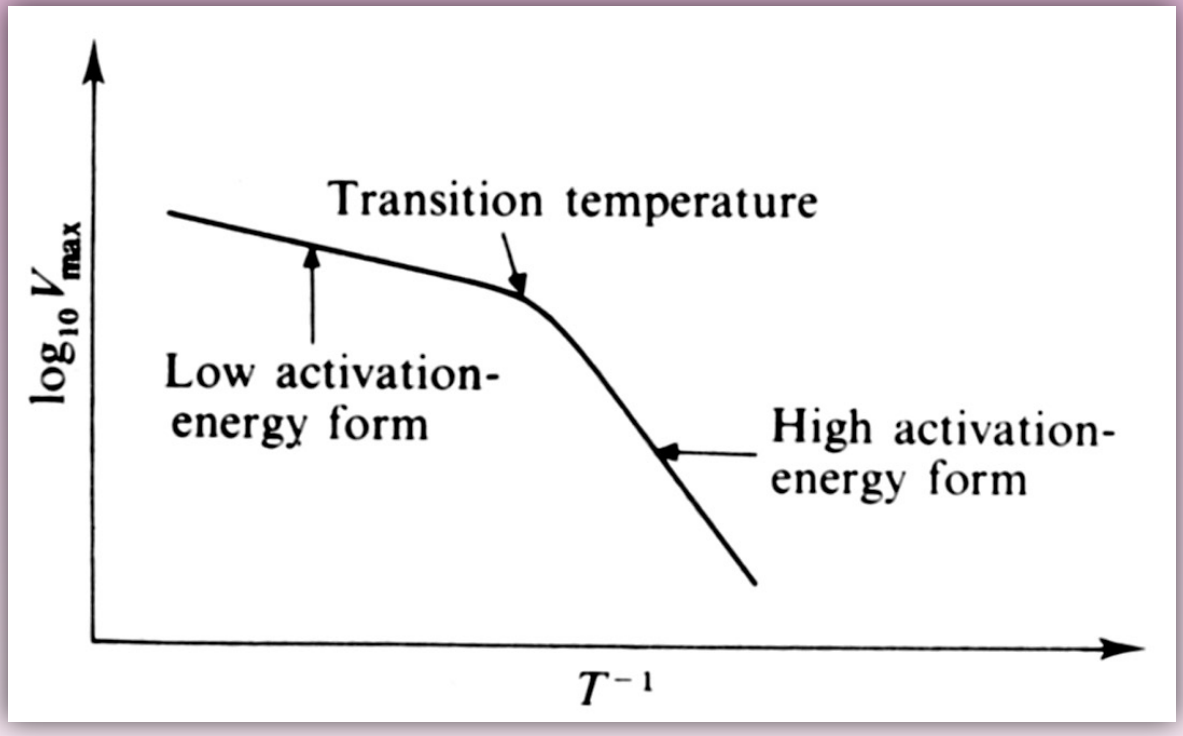


Figure 10.51. Temperature dependence of a typical mammalian enzyme.



An Arrhenius plot for an enzyme-catalyzed reaction, where the enzyme exists in two interconvertible forms