Chapter 10

Enzymes and Metabolic Reactions

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10-1. Introduction

In this chapter we consider the elements of metabolism, that is, the nature of a chemical reaction and its energetics, the facilitation of reactions by protein catalysts, enzymes, and the limitations to their enhancement of reactions, and several variations on the mechanisms for substrate-enzyme interactions. There are a variety of ways of computing the reaction rates or fluxes of substrate to form product. The simplest of these, the first-order Michaelis-Menten reaction, preserves the correct stoichiometry of the reaction but approximates the details, omitting steps in the binding and unbinding of substrate or product by assuming equilibrium binding, and omitting consideration of the quantity of substrate bound to the enzyme, while adjusting the reaction rate to give the observed rate of product formation in the steady state. Recognizing that all reactions are reversible, however slowly, the forward and reverse reaction rates are governed by thermodynamic constraints, namely that the free energies at equilibrium are determined by the

chemical potentials of the reactant-product set in accord with the Haldane relationships, and are not influenced by the presence of the enzyme-catalyst. More complete sets of equations will be developed to describe more general cases, where binding and unbinding occur at finite rates, and the buffering of the concentrations of substrate or product by binding to enzyme can be accounted for in a complete mass balance.

The chapter provides not only an introduction to enzyme-facilitated reactions and their regulation, but illustrates how to develop the equations for sets of reactions in metabolic systems. The systems of equations can then be used to explore the behavior of chains of reactions or of small networks. The historical idea has been that the flux through a reaction chain is determined by the slowest reaction in the sequence, and that a particular reaction can usually be designated "the rate-limiting step". The more modern concept allows this but makes more of the point that whatever one does to speed up a particular reaction is to little avail since another reaction in the sequence becomes "the rate-limiting step". In reality, different reactions in a sequence assume that role under different physiological conditions, sometimes because the fluxes through different parts of a sequence may change when there are two or more entry points into the chain, and sometimes because the enzyme kinetics are changed by modifications of the enzyme, such as by phosphorylation.

The chapter emphasizes kinetics, not molecular dynamics or conformational states of proteins or the physical nature of the site of substrate-enzyme binding. These are well described in modern text books (Stephen White et al., 1994) and are a major aspect of proteomic studies. The kinetics of the individual reactions are the key to developing full descriptions of network behavior but do not predict network dynamics; the whole is greater than the sum of the parts. Enzyme kinetics are better covered in earlier biochemistry texts (White, Handler et al 1978; Mahler and Cordes, 1971)For the most part, fluxes facilitated by enzymes increase monotonically to a maximum as substrate concentrations increase, and the maximum slope of the relationship is low, usually with a Hill coefficient of unity. In special cases, however, even a single enzyme reaction can behave chaotically (Section 10-4.1).

10-2. Chemical reactions, enzymatic and otherwise

Chemical molecules react only upon collision, so for each reaction there are a minimum of two mechanisms to consider: the convection and diffusion or electrical forces bringing a pair of molecules into contact, and the reaction process that may then ensue. For two solutes in a solution the probability of contact depends on their respective concentrations, on their mobility by free diffusion, and on any other forces directing the molecules so that they come into contact. Although we will ignore these forces in this chapter, they do come into play in a good many situations: electric charge (like charges repel, opposite charges attract), convection (e.g., the delivery in the blood of antibodies to a surface antigen on endothelial cells), gravity or centrifugal forces (concentrating proteins at high gravitational stress), mechanical forces (as experienced by mechanoreceptors in initiating signalling responses). Under idealized average concentrations, neither highly dilute nor highly concentrated, the likelihood of contact of solute and enzyme is proportional to the product of concentration times mobility, [S] times $D_s/\Delta x$, where D_s is the diffusion coefficient in the solution and Δx is the distance the molecule moves. $D_s/\Delta x$ is a velocity. The use of D_s, as if for the solute alone, assumes that solute moves far faster than enzyme. Enzymes are usually large proteins, and are often associated with membranes, so this is often true.

Diffusion is a random process, and requires thermal energy; the lack of directionality means that diffusion over long distances is slow.
When a substrate molecule collides with an enzyme it may merely bounce off; binding requires precise orientation of substrate to enzyme, and that the substrate come in close proximity to the site and reorient apporpriately for binding. Many collisions may occur before there is actual binding. A prerequisite for binding is that the site be available; large molecules "breath" or undergo relatively slow conformational changes of state that may be necessary to open up a passage to the active site. A fine review of such events is provided by Garcia-Viloca et al (2004 #7703). In most instances the binding is stereo-specific, for example, hexokinase phosphorylates D-glucose but not L-glucose.

10-2.1. Uncatalyzed reaction of substrate *S* to from product *P*

The simplest uncatalyzed reaction is the interconversion of one molecular form to another, without loss or addition of atoms in the translation:

$$S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P \quad , \qquad \text{Diagram 10-1}$$

where S is substrate and P is product, terms which are completely arbitrary in this case. The rate constants, k_1 and k_{-1}, both have units of inverse time, meaning that the probability of transformation is on the average a specific number per unit time. The ratio of rates gives also the ratio of concentrations at equilibrium and defines a dimensionless equilibrium constant, k_{eq}:

$$\frac{k_{-1}}{k_1} = \frac{[S]}{[P]} = k_{eq}. \qquad (10\text{-}1)$$

Equilibrium occurs at the concentration ratio where energy is minimal and entropy maximal, for the reactant pair. A system which supplies S and consumes P will deviate from the equilibrium ratio to a degree depending on the rate of utilization of P compared to the rates of transformation. A steady state in which P is consumed slowly, relative to the rates *S-P* interconversion, will be in a *near-equilibrium* state.

10-2.2. Uncatalyzed reactions of substrates S_i and S_2 to form a product *P*

For a pair of reactants, S_1 and S_2, to form a product, P, there is an energy barrier to overcome: one can think of it as requiring a collision energetic enough to overcome the free energy of activation of the reaction, after which there may or may not be energy released from the reaction. The reaction, or *mass action*,

$$S_1 + S_2 \rightarrow P, \qquad \text{Diagram 10-2}$$

can be described by a differential equation defining the rate of product formation:

$$d[\mathrm{P}]/dt = k[\mathrm{S}_1] \cdot [\mathrm{S}_2]. \qquad (10\text{-}2)$$

The rate parameter k describes the combined rates of S_1 and S_2 coming into contact and then reacting to form product P. It is easy to see that this k might not be always a constant, for at very low concentrations any delay in the diffusion of a reactant contacting the other can only retard the apparent reaction rate. Further, when concentrations are low and there are few molecules, the concentrations S_1 and S_2 cannot be looked upon as continuous variables, as represented in the equations. Reactions occur when molecules collide and dock with their appropriate parts interacting, so they may collide a number of times before reacting. At low temperatures thermal motion is reduced and viscosity increases. At high concentrations increased steric hindrance and increased viscosity result in diminished efficiency of the reaction.

At all reaction rates the rate of production of P is exactly the rate of utilization of S_1 and S_2.The stoichiometry of the reaction is 1 S_1 and 1 S_2 react to produce 1 P. Stoichiometries are integer. This turns out to be useful in characterizing networks of reactions, for it allows accounting for large numbers of solutes in a reaction mixture simply by the numbers of molecules used or produced. Note also the implications of a reaction on osmolarity: that two molecules react to form one indicates a diminution in total osmolarity.

All chemical reactions are in principle reversible, with a finite probability that P will breakdown to S_1 and S_2, so the reaction is bidirectional:

$$S_1 + S_2 \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} P, \qquad \text{Diagram 10-3}$$

where the rate constants k_1 $M^{-1} \cdot s^{-1}$ and k_{-1} s^{-1} are the forward and backward reaction rates. This reaction will tend to go to equilibrium, where the forward and backward fluxes are equal. The differential equation for S_1 is

$$d[S_1]/dt = k_{-1}[P] - k_1[S_1] \cdot [S_2]. \qquad (10\text{-}3)$$

(If all P were to be continuously instantaneously removed or k_{-1} is very low, the reverse reaction could be ignored, and at any constant S_2 the forward flux to form P, dS_1/dt, is a linear function of S_1.) When this reaction goes to equilibrium so that $d[S_1]/dt = 0$, then $k_{-1}[P] = k_1[S_1] \cdot [S_2]$, from which we define the equilibrium dissociation constant K_{eq}, molar:

$$K_{eq} = k_{-1}/k_1 = [S_1] \cdot [S_2]/[P]. \qquad (10\text{-}4)$$

Low values of K_{eq} indicate high affinity of S_1 for S_2 and high likelihood of reaction to form product P. If no other reactions involve the three solutes then from the initial concentrations of one of the substrates, together with the conservation statement, $[S_1] + [P] = [S_T]$, a constant, the equilibrium concentrations can be calculated:

$$[P] = [S_T] \cdot [S_2]/(K_{eq} + [S_2]). \qquad (10\text{-}5)$$

From this one can see that when $[S_2] \gg K_{eq}$ and $[S_2] \gg [S_1]$, then the reaction uses all S_1 to form P so that $[P] = [S_T]$, and when $[S_2] = K_{eq}$, then $[P] = [S_T]/2$ or $[P] = [S_1]$.

Most biochemical reactions are more complex than this. Exactly similar reactions can surely occur inside cells, but tend not to, simply because the biochemical systems have evolved to utilize enzymes to catalyze the reaction, i.e., to lower its energy of activation and to enhance the rate of product formation. Since the enzymatically facilitated reaction normally has a much lower activation energy than the unaided chemical reaction, the enzymatic reaction dominates. Even so, in experimental situations there will be many reminders that the non-enzymatic reactions continue, for example, in the test tube after the enzyme is blocked or destroyed.

10-2.3. Energy of activation in reaction kinetics

The formation of substrate from product requires overcoming an energy barrier. Eyring's expression for this general idea incorporated Arrhenius' expression for the effects of temperature on reaction rates, giving the reaction rate constant, k,

$$k = Ae^{-(E_a/RT)}, \tag{10-6}$$

where the parenthetic exponent is the Boltzmann relationship with E_a the activation energy and RT the gas constant times temperature (Kelvin). The A is a scalar accounting for dependence on such things as the time for molecular rotation for precise orientation. The k is proportional to the fraction of reactant molecules with sufficient energy to reach the transition state, as suggested by Fig. 10-1.

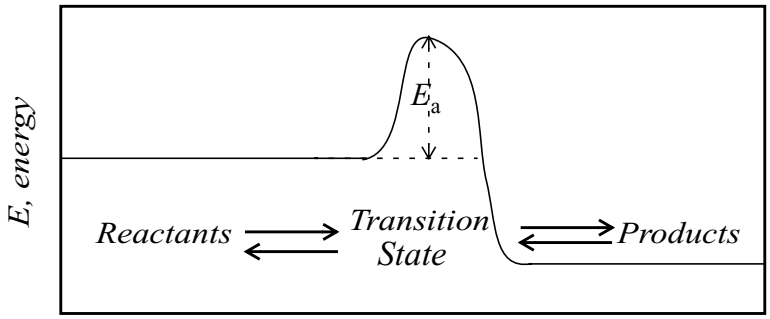

Figure 10-1: Energy barrier to a reaction. For a reaction involving higher energy reactant than products the net change in energy is a loss, but in principal all are reversible. The likelihood of reaction depends on the height of the barrier ΔE_a.

From Arrhenius relationships one is attuned to the idea that increasing the temperature by 10 degrees increases the rate of reaction: the ratio of the rate at temperature $T + 10$ to that at temperature T is the Q_{10}. When E_a is 8000 cal/mol, for example, and the temperature rises from $T_1 = 25$ to $T_2 = 35$ degrees Celsius, then the Q_{10} is the ratio of the rate constants k_2/k_1, at T_2 to that at T_1:

$$Q_{10} = \frac{k(T=35\text{ C})}{k(T=25\text{ C})} = e^{-E_a(1/RT_2 - 1/RT_1)} = 1.55 \quad . \tag{10-7}$$

Q_{10}'s of less than 2.0 are typical of temperature-controlled events such as diffusion, and for transporters not requiring ATP. However, consider the effect of lowering E_a directly from 8000 to 4000 cal/mol as might occur with an enzyme-facilitated reaction without changing temperature:

$$\frac{k_2}{k_1} = e^{-(\Delta E_a / RT)} = e^{4000/(1.99 \cdot 298)} = e^{6.7} = 880. \qquad (10\text{-}8)$$

Increases in reaction velocities may be considerably higher even than this example. Reactions involving a high-energy phosphate group with a potential energy of 10 to 12 kcal/mol seldom yield more than 8 kcal/mol. These reactions do tend to have much higher Q_{10}'s also, presumably a reflection not only of enhanced thermal motion of the reactants but also due to increased rates of conformational state of the enzyme-substrate complex. At the temperature used for Eq. 10-8, 25°C, a reduction in E_a by 1000 cal/mol from 8000 to 7000 kcal/mol gives a 5.45-fold increase in reaction rate, so that for $\Delta E_a = 4000$ cal/mol the ratio $k_2/k_1 = (5.45)^4 = 880$. Some enzymatic reactions bring about a 10 kcal/mol reduction, giving a ratio of catalyzed over uncatalyzed rates of 23 million, illustrating the immense gain in efficiency with catalysis.

This magnificent reduction in activation energy occurs as Linus Pauling predicted in 1946, namely that enzymes accelerate rates because they bind the transition state better than the substrate. The transition state is the conformational and energetic state of the reactant molecule or its components as it goes to form product; this is different from the substrate itself. As Schowen put it in 1978, " ...the entire and sole source of catalytic power is the stabilization of the transition state...". This is accomplished mainly through a reduction in E_a, though there are smaller effects on the transmission coefficient (generalizations of the rate constant multiplying the basic rate $k_B T/h$ of about 6 per picosecond for reactions in gasses or solutions (Garcia-Viloca et al, 2004).

10-2.4. Enzymatic facilitation of the reaction of substrate *S* to form product *P*

Enzymes are proteins which catalyze reactions among small solutes and among proteins. They are typically large proteins with parts of their structures suited to the high affinity binding of a small set of substrate molecules of a particular geometry. By binding a substrate, or bringing together a pair of substrate molecules, the enzyme facilitates a reaction, speeding it up by orders of magnitude. Enzymes are generally in low concentration relative to that of the solutes whose reactions they enhance. When the number of enzyme molecules is limited and solute concentration relatively high, the fraction of solute bound to enzyme cannot be proportional to the solute concentration. Only when solute concentrations are much less than the dissociation constant K_{eq} for substrate–enzyme binding is the bound fraction proportional to [S].

Consider first the enzymatic conversion of $S \rightarrow P$, a single substrate forming a single product. The nature of the reaction is more complex: first a binding of solute to the enzyme, forming the enzyme–substrate complex, then a reaction step resulting in an enzyme–product complex, and finally a release of the product from the complex:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \leftrightarrow \mathrm{EP} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + E. \qquad \text{Diagram 10-4}$$

This is usually simplified on the basis that the reaction step and the release step are combined, which makes sense if the reaction step is slow compared to the reaction binding *S* and slow compared to the release of *P,* and if the reaction is considered irreversible:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \overset{k_2}{\rightarrow} P + E \quad . \qquad \text{Diagram 10-5}$$

When k_2 is very slow compared to k_1 and k_{-1} then there is time to approach equilibration between *S* and ES: the equilibrium concentration ratio is analogous to that in Eq. 10-4 when the loss of ES to form *P* can be ignored:

$$[\mathrm{S}] \cdot [\mathrm{E}]/[\mathrm{ES}] = k_{-1}/k_1 = K_S \ . \qquad (10\text{-}9a)$$

Thus K_S, the dissociation constant for the ES complex, is k_{-1}/k_1. The forward reaction flux is:

$$k_2[\mathrm{ES}] = \frac{k_2 k_1}{k_{-1}} \cdot [\mathrm{E}] \cdot [\mathrm{S}] = \frac{k_2}{K_S}[\mathrm{E}] \cdot [\mathrm{S}] \quad . \qquad (10\text{-}9b)$$

Assuming a quasi-equilibrium between *S* and *E*, disturbed only to a minor extent by the breakdown of ES to release *P,* this is a good approximation for many reactions.

While we ignored reversibility, which is risky since virtually all reactions are reversible, the approximation is good when [P] is low and when the energetics are such that the reverse reaction is unlikely. Many experiments on transport or reaction are designed so that data are acquired so as to obtain the data from the forward reaction, i.e., the *initial velocity* of reaction when [P] ≪ [S]. This is the same strategy as used for estimating transporter parameters in Chapter 8. We shall return to this for a couple of reasons: one is that it is important to consider that almost all reactions are really reversible, and a second is that accounting for the reversibility accounts properly for the energetics, and makes for greater stability in computing the reactions in a network of biochemical reactions (Hofmeyr and Cornish-Bowden, 2000; Bassingthwaighte, 2001).

10-2.5. Differential equations for the enzymatic reactions

For the *S*, *P* system, still considering the concentration of *P* to be negligible, the equations are

$$d[\mathrm{S}]/dt = k_{-1}[\mathrm{ES}] - k_1[\mathrm{S}] \cdot [\mathrm{E}], \qquad (10\text{-}10)$$

$$d[\mathrm{ES}]/dt = k_1[\mathrm{S}] \cdot [\mathrm{E}] - (k_{-1} + k_2) \cdot [\mathrm{ES}], \qquad (10\text{-}11)$$

$$d[\mathrm{P}]/dt = k_2[\mathrm{ES}], \text{ and} \qquad (10\text{-}12)$$

$$[\mathrm{ES}] + [E] = [\mathrm{E_{tot}}]. \qquad (10\text{-}13)$$

Because E_{tot}, the total amount of enzyme in all forms, is constant, and $d[\mathrm{E}]/dt$ equals $-d[\mathrm{ES}]/dt$, the number of differential equations can be reduced. In steady state, $d[\mathrm{P}]/dt = -d[\mathrm{S}]/dt$. Following

the reasoning of Mahler and Cordes (1971), there are two ways to reduce the complexity of the equations. The first is by assuming equilibrium binding between substrate and enzyme, as we did to derive Eq. 10-9b. The second is not to assume equilibrium but to make a more tenable assumption, which is to assume a quasi-steady state, based on the idea that some reactions in the system are relatively fast, and so can be considered as in a steady state, even while the slower changes are occurring.

Substrate-to-product reaction with near-equilibrium binding between substrate and enzyme. The assumption made by Michaelis and Menten (1913) was that substrate, enzyme and their binding complex, ES, were in equilibrium. This classic paper defined the difference between uncatalyzed and catalyzed reactions. Using K_S from Eq. 10-9a to define the equilibrium dissociation of *S* and *E*:

$$k_1[\mathrm{S}] \cdot [\mathrm{E}] = k_{-1}[\mathrm{ES}], \tag{10-14}$$

and with [ES] + [E] = E_{tot}, then

$$[\mathrm{ES}] = E_{tot} \cdot [\mathrm{S}] / (k_{eq} + [\mathrm{S}]). \tag{10-15}$$

The rate of product formation, which is the velocity of the reaction, *V*, is proportional to the concentration of the complex [ES]:

$$V = d[\mathrm{P}]/dt = k_2[\mathrm{ES}] = k_2 E_{tot} \cdot [\mathrm{S}] / (K_S + [\mathrm{S}]) = V_{max}[\mathrm{S}] / (K_S + [\mathrm{S}]). \tag{10-16}$$

This defines V_{max} as $k_2\, E_{tot}$.In Fiug 10-1 the reaction velosity V is plotted versus S; at high [S] V goes to Vmax: in this high [S] region the reaction is *zero order*, that is the reaction rate is independent of [S]. When [S] = K_S then $V = V_{max}/2$, as one can see by substituting for [S] in Eq. 10-16. This equation is a good approximation when $k_2 \ll k_{-1}$ and k_1, but it is not so easy to evaluate its accuracy without analyzing a situation where the assumption of equilibrium is removed. (Note that $V_{max} = k_2 \cdot E_{tot}$ is based on the fact that <u>all</u> of the enzyme present, E_{tot}, is available to form ES, whereas with the transporters (Chapter 8) the V_{max} used $T_{tot}/2$ since half of it faced the other side of the membrane.) The form of the relationship is shown in Fig. 10-2, where the left panel shows a linear plot of V/Vmax versus [S] and the right panel shows the same thing on a logarithmic abscissa. On the logarithmic scale it is easier to see that when $V = V_{max}/2$ then [S] = K_m.

10-2.6. Single substrate, single product in quasi-steady-state reaction between *S* and *E*

Using dimensional analysis one can see that this approach does not require assuming equilibrium between *S* and ES, but relaxes the assumption to a less demanding one, namely that ES forms and dissociates at approximately equal rates at all times, so that *d*[ES]/*dt* is approximately zero. (This is momentarily false when there are sudden large jumps in [S], requiring that time be taken to load or unload ES, but is otherwise good. This development, from Cleland, 1970, the descriptions of Mahler and Cordes, 1971, and of Keener and Sneyd, 1999, shows that the equilibrium assumption does not hold when enzyme and substrate are first put together, but is so soon satisfied that the assumption has become standard.) The three differential equations above (Eq. 10-10 to Eq. 10-12)

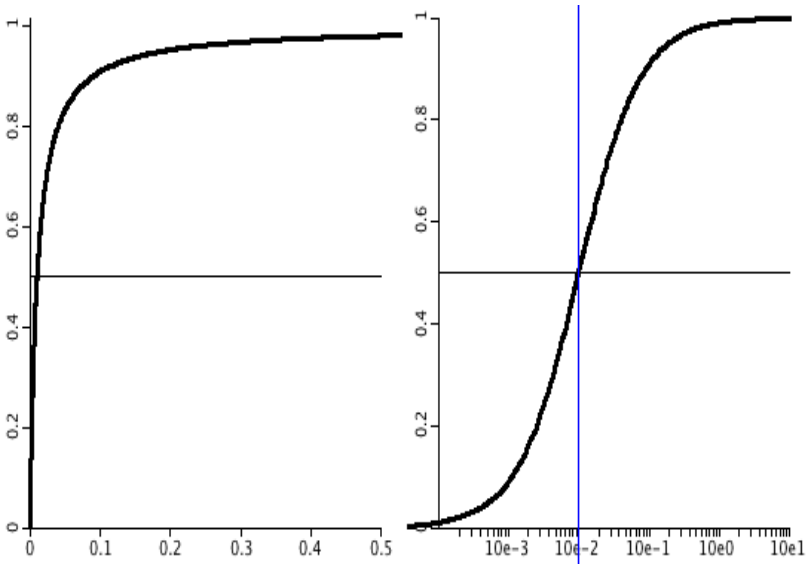

Figure 10-2: An enzymatic reaction is saturable, being first order with respct to [S] at low concentrations and zero order (plateaued) at high [S]. Curves are for Eq. 10-16 *Left panel*: Linear plot. *Right panel*: Logarithmic abscissa to show that reaction velocity is $V_{max}/2$ at [S] = K_m.

can be reduced to two by using the conservation of enzyme ([ES] + [E] = E_{tot}), and assuming that *S* is lost only by conversion to *P*. First define the apparent Michaelis constant K_m:

$$K_m = (k_{-1} + k_2)/k_1 . \tag{10-17}$$

The properties of the two resultant equations may be more readily understood if they are rewritten in dimensionless form, choosing a set of transformations designed to let us examine how d[ES]/dt can be regarded as unchanging over the time of an experiment. The equations for *S* and ES, Eq. 10-10 and Eq. 10-12, with the substitutions, σ = [S]/[S(t = 0)], χ = [ES]/E_{tot}, $K = (k_{-1} + k_2)/(k_1 \cdot [S(t = 0)])$, $\varepsilon = E_{tot}/[S(t = 0)]$, and $\alpha = k_{-1}/(k_1 \cdot [S(t = 0)])$ and $\tau = k_1 E_{tot} \cdot t$, become the dimensionless expressions:

$$d\sigma/d\tau = -\sigma + \chi(\sigma + \alpha), \tag{10-18}$$

$$\text{and} \quad \varepsilon d\chi/d\tau = \sigma - \chi(\sigma + K). \tag{10-19}$$

The quasi-steady-state approximation is $\varepsilon d\chi/d\tau = 0$; it is a stronger statement than $d\chi/d\tau = 0$, and safer, for it implies only that dES/dt = 0, at least momentarily, and that the changes in *S* and *P* occur while the condition dES/dt = 0 remains satisfied. Thus χ is changing while the right hand side of Eq. 10-19, $\sigma - \chi(\sigma + K)$, remains close to zero. For this to hold, E_{tot}/[S] must remain small, hopefully < 10^{-4} and $d\chi/d\tau$ is of order 1, but it becomes obvious that as S is consumed to produce P the ratio E_{tot}/[S] eventually becomes large unless S is supplied.

From the steady-state approximation, using $\sigma - \chi(\sigma + K) = 0$, or $\chi = \sigma/(\sigma + K)$ we translate back into experimental variables:

$$[ES]/E_{tot} = ([S]/S_0)/([S]/S_0 + K_m/S_0), \text{ where } S_0 = [S(t=0)] \ . \qquad (10\text{-}20)$$

This definition of S_0 assumes that [ES] is negligible compared to [S] or [P]. Thus $dP/dt = -dS/dt$ after the transient. The forward flux, $dP/dt = k_2 \cdot [ES]$, is the ratio $[ES]/E_{tot}$ and gives the flux as a fraction of the maximum reaction velocity, $[ES]/E_{tot} = V/V_{max}$:

$$V = dP/dt = k_2 \cdot [ES] = k_2 \cdot E_{tot}[S]/([S] + K_m) = V_{max} \cdot [S]/([S] + K_m), \qquad (10\text{-}21)$$

which is the classical form given by Michaelis and Menten but now using the more proper K_m of Eq. 10-17 instead of K_S. This is the Briggs-Haldane version of the Michaelis-Menten expression; it is interesting that their work anointed what we now call the Michaelis-Menten K_m.

A complete program for these reactions is provided in Table 10-1. The parameters given in the code are those used for Fig. 10-4. Included also in the program is the term $k_{-2} \cdot E \cdot P$ in teh eaquations for *dES/dt* and *dE/dt*, which allows reversibility to form S from P. The value chosen for K_P makes this reaction essentially irreversible..

Table 10-1: JSim code for a reversible first order enzymatic reaction:

```
JSim v1.1
import nsrunit;   unit conversion on;
math   e1_rev  {   // enzymatic facilitation of reversible reaction S <-> P

//                      k1->      k2->
// Reaction is: S + E <----> ES <----> P + E
//                      <-k_1     <-k_2

// DEFINE INDEPENDENT VARIABLE t seconds
   realDomain t s; t.min=0; t.max=800; t.delta= 0.2;
// PARAMETERS
   real k1  = 100 uM^(-1)*s^(-1), // forward reaction for binding S to E
        Ks  = 1e-8 uM,  // Equil.dissoc for S binding to E, = kbs/kfs
        k_1 = Ks*k1,    // backward reaction rate to release S from ES, 1/s
        k2  = 0.1 1/s,  // forward reaction for ES -> P
        Kp  = 1e6 uM,   // Equil dissoc. for binding P to E, = k2/k_2
        k_2 = k2/Kp, // 1/(mM*s) backward reactn -> EP (or ES) from E and P
        Km  = (k_1 + k2)/k1, //mM, apparent Km for reaction S -> P
        Etot= 0.1 uM;
// DEFINE VARIABLE FUNCTIONS OF TIME
      real S(t) uM, P(t) uM, E(t) uM, ES(t) uM;
// INITIAL CONDITIONS
      when(t=t.min) { S = 1 ; ES = 0 ; P = 0 ; }
// ODEs
      E     = Etot   - ES;                      // enzyme conservation
      S:t   = k_1*ES - k1*S*E;                  // S:t denotes dS/dt
      ES:t  = k1*S*E - k_1*ES - k2*ES +k_2*E*P; // Net rate of ES formation
      P:t   = k2*ES  - k_2*E*P;                 // Net rate of P formation
} // End of program
```

The shape of the relationship between flux and substrate concentration for a single-substrate, single-enzyme reaction (first order Michaelis-Menten) has the same form as does the flux across

the membrane via facilitated transport. This is shown in Figure 10-3. The effectiveness of the

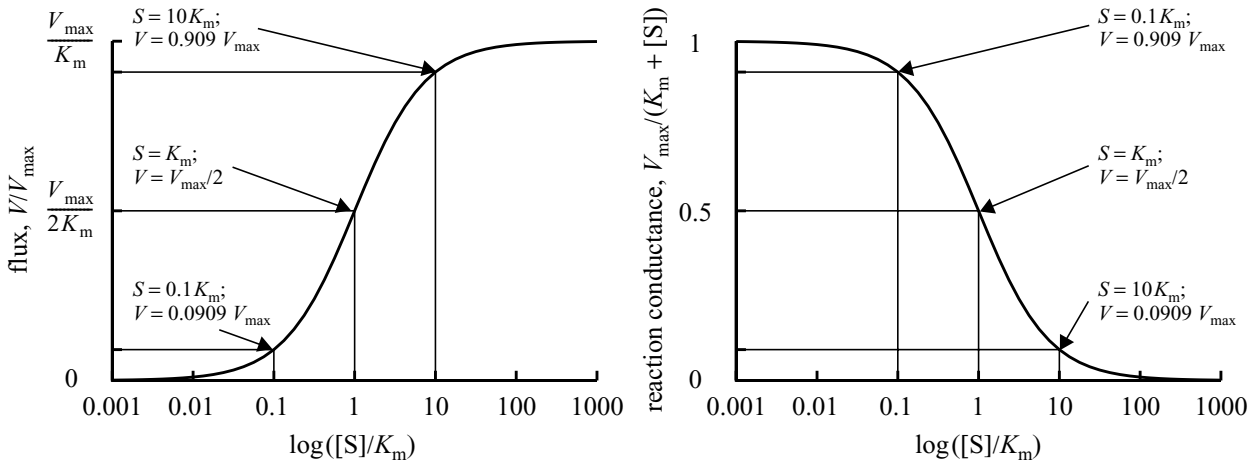

Figure 10-3: A saturable Michaelis-Menten reaction. *Left panel:* Flux $d[P]/dt$ relative to V_{max} versus $\log([S]/K_m)$. With $[S] = K_m$, $V = V_{max}/2$. At $[S] = 0.1K_m$, $V/V_{max} = 1/11$. At $[S] = 10K_m$, $V/V_{max} = 10/11$. *Right Panel*: Conductance for the reaction, $V_{max}/([S] + K_m)$ versus $\log([S]/K_m)$. When $[S] \ll K_m$ the conductance is constant at V_{max}/K_m.

enzyme, its relative conductance (right panel) declines as it becomes saturated. This contrasts with a passive or linear reaction; because V_{max} for enzymatic reactions is many orders of magnitude greater than the uncatalyzed reaction, enzymatic reactions are dominant at all concentrations.

Conditions for linearity: The range over which the reaction can be said to be linear is defined relative to the dissociation constant, k_S, for substrate binding: when [S] is low relative to K_S the reaction rate is proportional to [S] just as in the uncatalyzed mass action. Restating this, with $[S] \ll K_S$ and $k_2 \ll k_1$ or k_{-1}, then $d[P]/dt = (k_2[E]/K_S)\ [S]$, a linear reaction since [E] is essentially constant, close to the total enzyme concentration, E_{tot}, despite changes in [S]. Linearity is almost exact for $[S] << K_S$, the deviation being less than 1% percent up to $[S] = 0.1\ K_S$.

10-2.7. Progress curves: Solving the differential equations to show the progress of the reaction

Consider a well-stirred beaker experiment giving the results portrayed in Fig. 10-4, left panel: Substrate *S* is in the beaker at concentration $[S(t = 0)]$. At $t = 0$ enzyme is added to bring its concentration to E_{tot} instantly in the rapidly stirred solution. For Case 1, equilibrium binding, there is an instantaneous drop (arrow in left panel of Fig. 10-3) in [S] from its initial value of 1.0 to $[S(t = 0)] - [ES]$ where is $E_{tot} \cdot [S]/(K_S + [S])$. This is due to the loading of the enzyme with *S*, and is most evident when the initial substrate concentration $[S(t = 0)]$ is similar to E_{tot}. When $[S(t = 0)] \gg E_{tot}$ the sudden drop is not noticeable. After this the progress of the reaction reduces [S] and increases [P]. Note that [P] rises linearly at first. This is the basis of using the initial velocity of the reaction to estimate the K_m.

When equilibration is slower the conditions for Eq. 10-21 are not immediately fulfilled. Instead the initial phase of loading up the enzyme with the substrate takes finite time and [ES] reaches a quasi-steady state only after the loading phase is over (Fig. 10-4, right panel). [S] and

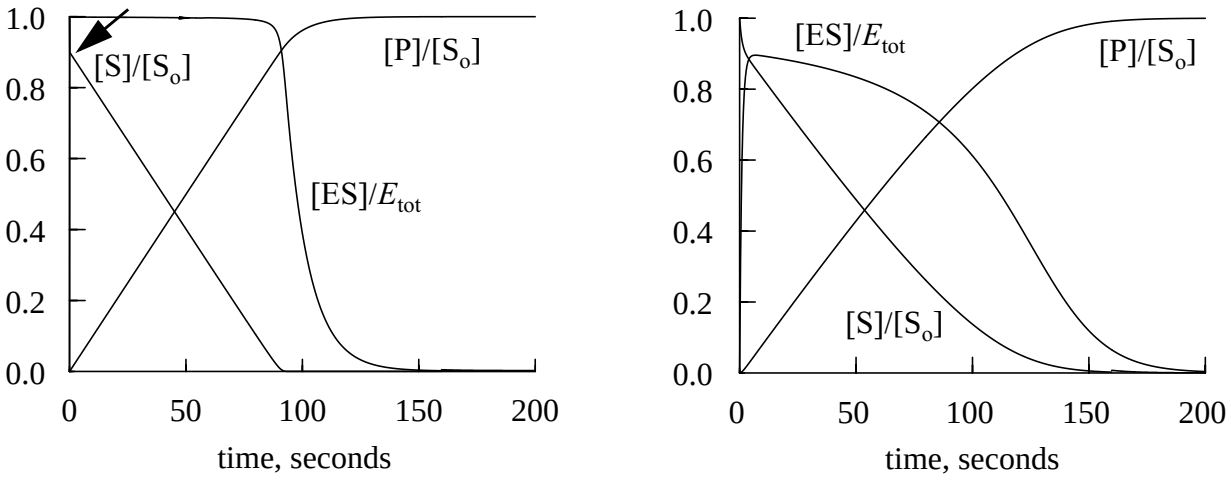

Figure 10-4: Progress curves for the "irreversible" reaction $S + E \leftrightarrow \mathrm{ES} \rightarrow P$.for which the code is in Table 10-1. *Left panel:* Near-equilibrium binding of substrate to enzyme. Note the sudden diminution in [S] at t > 0 due to buffering as S combines with enzyme added at $t = 0$, and [ES] jumps quickly to its highest value. [P] rises smoothly from zero, continuing to increase until S is used up and $[\mathrm{ES}] \rightarrow 0$. Parameters were $[\mathrm{S}(t = 0)] = 1\ \mu\mathrm{M}$, $[\mathrm{P}(t = 0)] = 0\ \mu\mathrm{M}$, $E_{tot} = 0.1\ \mu\mathrm{M}$; $K_S = k_1/k_1 = 10^{-8}\ \mu\mathrm{M}$, $k_1 = 100\ \mu\mathrm{M}^{-1}\mathrm{s}^{-1}$, $k_2 = 0.1\ \mathrm{s}^{-1}$, $K_P = k_2/k_{-2} = 10^6$ mm. It is the high value chosen for K_P that makes this essentially an irreversible reaction. *Right panel:* One hundred times slower binding and unbinding of substrate to enzyme, $k_1 = 1\ \mu\mathrm{M}^{-1}\mathrm{s}^{-1}$. Initial velocity of $d\mathrm{P}/dt$ is lower; the peak velocity, proportional to [ES], is reached only after 5 seconds and is < 90% of that in the left panel.

[ES] change in complementary fashion, as [ES] ramps up to its quasi-steady-state level. The big difference is in the initial rate of production of P: the steady-state "near initial" rate, $d[\mathrm{P}]/dt$, is not reached until [ES] reaches its maximum, so the rise of [P] is slightly delayed and the slope $d[\mathrm{P}]/dt$ remains lower than in the left panel.

10-2.8. Initial velocity experiments

The progress curves exhibit their maximum fluxes when [ES] is maximal. The traditional initial velocity experiment, taking its cue from Michaelis and Menten (1913) and assuming that binding is fast, was based on the assumption that the first observable measures of [P] versus time, to determine a set of initial velocities, each at a different starting concentration of S, were unimpeded by the reverse reaction. (This was an important strategy for them: the "invertase" reaction catalyzing sucrose to glucose and fructose is subject to strong product inhibition.) The measures made were of [P(t)], when it was small compared to [S] and therefore one didn't normally have observations [S] at early times to see whether or not $d[\mathrm{S}]/dt$ was the same as $d[\mathrm{P}]/dt$ or whether or not there was a sudden drop in [S] as E was added to the solution. An exemplary experiment is shown in Fig. 10-5 using the program in Table 10-1; the results are similar in style to those shown for a transporter (Chapter 8, Figure 8-2).

Estimating parameters from the data sets. Some shortcuts to estimate K_m or K_S evolved over the years, using algebraic manipulation of the equations to obtain linearization of the relationships. The Lineweaver-Burk (1934) plot, or double reciprocal plot shown in

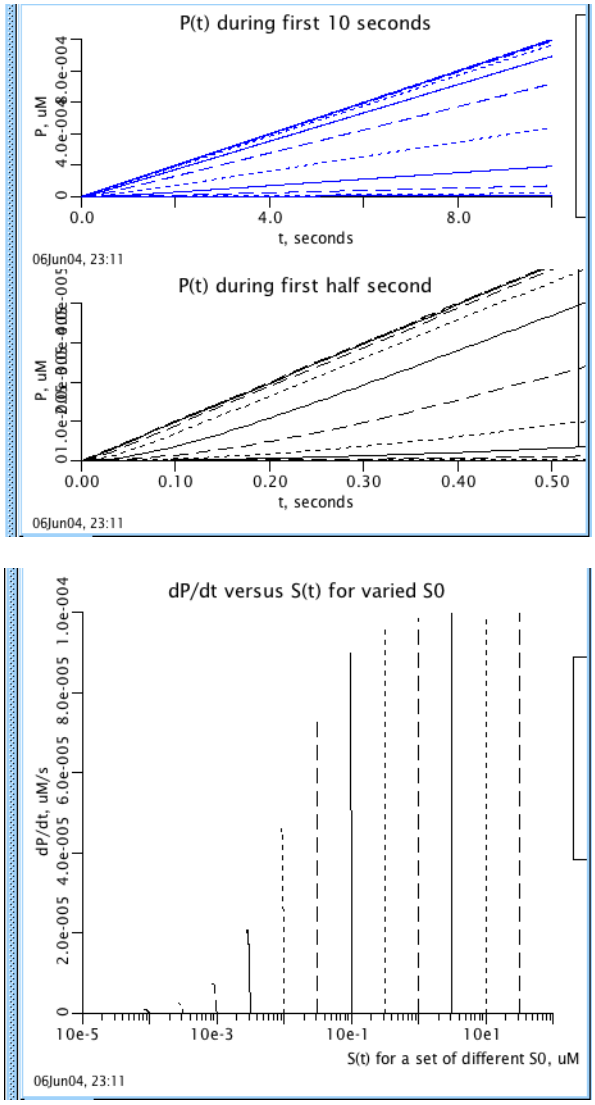

Figure 10-5: Initial velocity experiments. *Left Panel:* The time course of production of P with three different starting concentrations of S. The "data" are from solutions of the same model as used in Fig. 10-4. Parameters were: [S($t = 0$)] from 0.1 nM to 31 µM in steps of the square root of 10, [P($t = 0$)] = 0 µM, $E_{tot} = 0.1$ µM; $K_S = k_{-1}/k_1 = 10^{-8}$ µM, $k_1 = 100$ µM^{-1}s^{-1}, $k_2 = 0.1$ s^{-1}, $K_P = k_2/k_{-2} = 10^6$ nM. the same model parameters with initial concentrations [S(t=0)] = 1 µM (saturating the enzyme), 0.01 µM (half saturating) and 0.001 µM (nearly first-order).

Fig. 10-6, plots $1/V$ versus 1/[S]. This plot is useful when the binding/unbinding rates to form ES are fast. Then commonly, in an erroneous fashion, a linear regression analysis is applied to obtain the slope K_m/V_{max} and the intercept, $1/V_{max}$. The error is that the nature of the variation in the individual points is incorrectly represented; this method assumes that variation is Gaussian in $1/V$ but the actual variation in the original observations of the reaction velocity V was Gaussian (or other) in V, not $1/V$. The Lineweaver-Burk method puts overly large weight on the values of $1/V$ at small concentrations, just where the errors tend to be greatest.

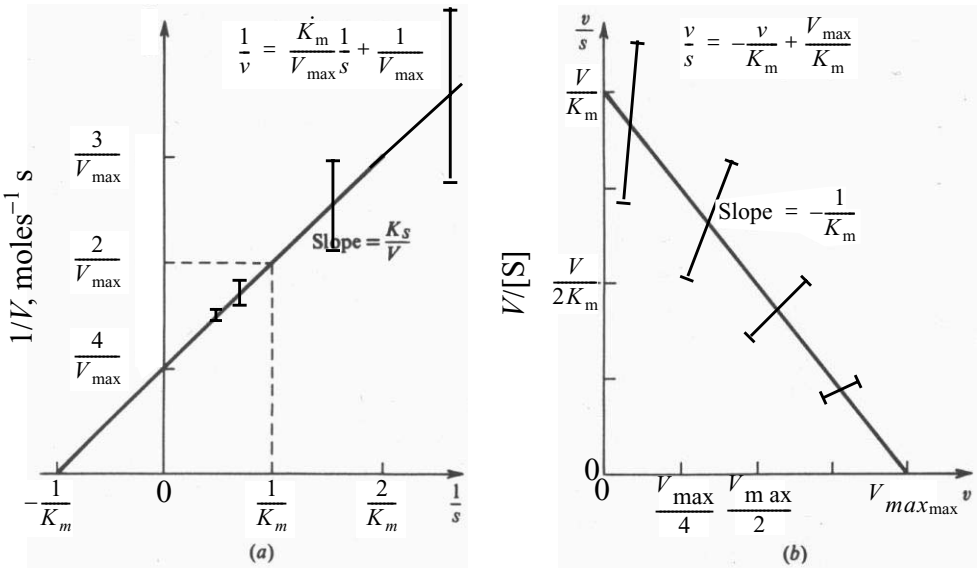

Figure 10-6: Linearizations of the Michaelis-Menten relationship. *Left panel*: The Lineweaver-Burk double reciprocal plot. This type of plot overemphasizes the influence of data obtained at low [S] and biases the position of the line. The regression assumes that error is only in the velocity measurement, with no error in concentration, and that the errors in V were of the same relative magnitude at all concentrations. *Right panel*: Eadie Hofstee plot. The plot, with V on both axes, has error in V directed toward and away from the origin. Any bias introduced by error in measured V is less than with the Lineweaver-Burk plot.

The Eadie Hofstee relationship, the right panel of Fig. 10-6, is better. Firstly it attempts a normalization, using the flux per unit concentration, V/[S], versus V, so that the slope is $-1/K_m$; the X intercept is V_{max} and the Y intercept is V_{max}/K_m, which is the conductance for the reaction at very low S and is the maximum flux per unit concentration. As with the Lineweaver-Burk plot, using standard linear regression analysis (minimizing the ordinate distances squared), introduces bias into the intercepts.

Perhaps the preferred linearization is one from Woolf (described by Cornish-Bowden, 1995; Woolf did not publish it), which is to plot [S] / V versus [S] on the abscissa. This gives more uniform error. The ordinate intercept is K_m/V_{max} and the slope is $1/V_{max}$.

The best method for parameter estimation is to fit the acquired data without a transformation so that the errors are distributed as they are experimentally, most likely Gaussian; this is accomplished using nonlinear regression analysis, and optimizing the fit of the model solution, Eq. 10-21, so that it fits most closely to the data using the least square criterion.

10-2.9. Reversible reactions

A critical requirement in formulating models of networks of reactions is to appreciate that almost all reactions are reversible: before worrying about competitors and inhibitors, take into account that high product concentrations push the reaction backward, producing substrate. This holds for the ionic pumps: driving the sarcoplasmic reticulum's CaATPase backwards produces ATP when ADP is available as substrate (Makinose and Hasselbach, 1971).

The equation for a single-substrate, reversible Michaelis-Menten reaction, Eq. 10-26 below, can be analyzed relatively simply. Even when there is a second reactant whose concentration is either very high relative to [S] or is well buffered and therefore doesn't change, the same equations apply to the substrate reaction velocities for the first reactant whose concentration is low. The reversible reaction is commonly written as

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + E\,. \qquad \text{Diagram 10-6}$$

The differential equations for this system, taking ES and EP to be indistinguishable, are

$$d[\mathrm{S}]/dt = k_{-1}[\mathrm{ES}] - k_1[\mathrm{S}]\cdot[\mathrm{E}], \qquad (10\text{-}22)$$

$$d[\mathrm{E}]/dt = (k_{-1} + k_2)\cdot[\mathrm{ES}] - [\mathrm{E}]\cdot k_1[\mathrm{S}] + k_{-2}[\mathrm{P}], \qquad (10\text{-}23)$$

$$d[\mathrm{ES}]/dt = [\mathrm{E}]\cdot(k_1[\mathrm{S}] + k_{-2}[\mathrm{P}]) - (k_{-1} + k_2)\cdot\mathrm{ES}, \qquad (10\text{-}24)$$

$$d[\mathrm{P}]/dt = k_2[\mathrm{ES}] - k_{-2}[\mathrm{P}]\cdot[\mathrm{E}]. \qquad (10\text{-}25)$$

The total [ES] + [E] = E_{tot}, allowing the equations to be reduced. Further, in the steady state, using the same logical deduction as given for Eq. 10-21, the net forward reaction rate v_{fnet} is:

$$v_{fnet} = -\frac{d[\mathrm{S}]}{dt} = \frac{d[\mathrm{P}]}{dt} = \frac{(k_1k_2[\mathrm{S}] - k_{-1}k_{-2}[\mathrm{P}])E_{\mathrm{tot}}}{(k_{-1} + k_2) + k_1[\mathrm{S}] + k_{-2}[\mathrm{P}]}\quad . \qquad (10\text{-}26)$$

Eq. 10-26, for a reversible Michaelis-Menten flux from substrate to product, may be simplified by dividing each term by (k_{-1}+k_2) and then reducing by assuming that the binding reactions are fast compared to the transformation reactions and omitting the slower rate constant:

$$v'_{fnet} = \frac{V_{\mathrm{f\,max}}S/K_{\mathrm{S}} - V_{\mathrm{r\,max}}P/K_{\mathrm{P}}}{1 + S/K_{\mathrm{S}} + P/K_{\mathrm{P}}}, \qquad (10\text{-}27)$$

where S and P are substrate and product concentrations, v_{fnet} is the net forward reaction, which may be negative or positive, $V_{\mathrm{f\,max}}$ is the maximum rate of the forward reaction, $V_{\mathrm{r\,max}}$ is the maximum reverse or backward reaction and K_{S} and K_{P} are the equilibrium dissociation rate constants for enzyme binding of substrate and product. Note that $V_{\mathrm{f\,max}}$ cannot quite equal $k_2 \cdot E_{\mathrm{tot}}$ and that $V_{\mathrm{r\,max}}$ cannot equal $k_{-1} \cdot E_{\mathrm{tot}}$, because the former is based on the approximation that k_{-1} and k_1 are both $\gg k_2$, and the latter is based on the approximation that $k_2 \gg k_{-1}$. This forces recognition that there must be an intermediate step if there is to be equilibrium binding for both substrate and product:

$$S + E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \underset{k_r}{\overset{k_f}{\rightleftharpoons}} EP \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + E, \qquad \text{Diagram 10-7}$$

where k_f and k_r are the forward and reverse reaction rates. Then, given that k_1 and k_{-1} are both $\gg k_f$ and that k_1 and k_{-1} are both $\gg k_r$, then k_S and k_P, the equilibrium dissociation constants, are appropriate approximations to the Michaelis constants, that is, k_S is very close to $(k_{-1} + k_f)/k_1$, and k_P is very close to $(k_r + k_2)/k_{-2}$. as can be seen by dividing the numerator and denominator of Eq. 10-26 by $(k_{-1}+k_2)$. The essence of the Briggs-Haldane definition of K_m (Eq. 10-17) is that [EP] remains very low, meaning that the k_2 degrading EP is fast. When both S and P are present the maximum reaction velocities cannot be reached, because both [ES] and [EP] must be less than E_{tot}, and both reactions are inhibited by the presence of product binding to the enzyme. A useful next step is to write Eq. 10-27 as

$$v_{fnet} = \left(1 - \frac{\Gamma}{\kappa}\right)\frac{V_{f\,max}(S/K_S)}{1 + S/K_S + P/K_P} \quad , \qquad (10\text{-}28)$$

where V_{fmax}, the *maximum* rate of the forward reaction, equals $k_f E_{tot}$, while V_{rmax}, the *maximum* rate of the reverse or backward reaction, equals $k_r E_{tot}$, κ is the ratio of [P] to [S] at equilibrium and equals $(V_{fmax}/K_S)/(V_{rmax}/K_P)$, and Γ is the ratio of [P] to [S] at any time t. When $\Gamma = \kappa$ the reaction is at equilibrium. Equilibrium means that the forward and backward reaction velocities are equal, a situation defined by the Haldane relationship. At equilibrium:

$$\kappa = \frac{P}{S} = \frac{(V_{f\,max}/K_S)}{(V_{r\,max}/K_P)} = \frac{k_1 k_2}{(k_{-1}k_{-2})} \; . \qquad (10\text{-}29)$$

When P is zero Eq. 10-28 reduces to the standard M-M relationship for the forward reaction $S \rightarrow P$, but using K_S rather than K_m.To repeat: it assumes equilibrium dissociation of both substrate and product in their binding with enzyme, and that the reaction step in either direction is slow compared to the association/dissociation rates. The virtue of using the reversible reaction compared to the standard forward M-M equation is that networks of such reactions are more stable than those assuming irreversibility: concentrations do not go to zero. Furthermore, the reversibility imposes a thermodynamic constraint that limits the effective range of possible values for the kinetics.

The original M-M and the Briggs-Haldane expressions are not necessarily good approximations when applied to the reaction of S, E, and P even when existence of ES and EP are accounted for. Atkinson et al. (1987) give an alternative but more complex expression for the effective K_A in the expression $v = V_{f\,max}[S]/(K_A + [S])$, assuming k_{-2} is negligible:

$$K_A = \frac{k_{-1}(k_r + k_2) + k_f k_2}{k_1(k_f + k_r + k_2)} \; . \qquad (10\text{-}30)$$

The $V_{f\,max}$ for this reaction $V_{f\,max} = k_2 E_{tot}$, as in general, the Michaelis-Menten model (Eq. 10-16) would lead one to believe. But since there are intermediate conformational states such as EP in

the reaction sequence not all of the enzyme will be bound in the ES form; consequently it is best to say that $V_{f\,max} \leq k_2\, E_{tot}$, for it is not readily known how much of the enzyme may be in other forms.

Asymmetry in reversible reactions: For this example it is necessary to carefully distinguish the dissociation constants $K_S = k_{-1} / k_1$ and $K_P = k_2 / k_{-2}$ from the apparent Michaelis constants $K_{mS} = (k_{-1} + k_2) / k_1$.and $K_{mP} = (k_{-1} + k_2) / k_{-2}$ To portray the asymmetry consider an equilibrium to occur with [P]/[S] = 1, for the sake of simplicity, which means that $K_P = K_S$. This means that $(v_f / K_S) / (v_r / K_P) = \kappa = 1$. The reaction rates as functions of [P]/[S] will be symmetrical when rates of binding and unbinding are equal, i.e. $K_{mS} = K_{mP}$ but asymmetrical otherwise when the rates differ even when keeping the dissociation constants the same. As shown in Figure 10-7, when the binding affinity for S is much less than for P , i.e. when $K_{mS} \gg K_{mP}$ the flux v_{fnet} versus log ([P]/[S]) is large at small values of [P]/[S] . The net forward reaction velocity v_{fnet} goes to zero at [P]/[S] = 1, and then at values of [P]/[S] > 1 is reversed but the flux is small. That is, at low [P] the reaction rate is strongly forward, from $S \rightarrow P$, but at high P the rate is slow from $P \rightarrow$ S. This asymmetry does not disturb the equilibrium point, only the velocities of reaction below and above it.

Likewise if $K_{mS} \ll K_{mP}$, meaning that the affinity of E for P is lower than the affinity for S, the opposite set of events occur. The flux v_{fnet} is small at values of [P]/[S] < 1, v_{fnet} goes to zero at [P]/[S] = 1, and v_{fnet} is large and negative at [P]/[S] > 1 where the reaction is much faster in the reverse direction.

In this situation it is easier and more insightful to use the K_m's to sort out the events than to use the dissociation constants, K_d's. The K_m's give information on the breakdown of ES in both forward and backward directions when these are comparable to one another, whereas the k_d's do not, and are therefore best used only when the on and off rates are very high compared to the reaction rates.

10-2.9.1. Examine Problem 1 to Problem 9

1. Define the conditions under which K_A in Eq. 10-30 reduces to k_{-1}/k_1, the original Michaelis constant.
2. Define the conditions under which K_A reduces to $(k_{-1} + k_2)/k_1$, the Briggs-Haldane version of the Michaelis constant.
3. Find a set of conditions in which k_{-1}/k_1, $(k_{-1} + k_2)/k_1$, and K_A are all similar. (Hints: Use values of 1.0 or 0.01; initially keep $k_{-1} = k_1$, and so on, in pairs. Atkinson et al., 1987, use a spreadsheet for this kind of calculation.)
4. Derive the expression for K_A.
5. What condition for Eq. 10-28 is violated when k_{-2} is neglected?
6. Can you derive a similar expression to Eq. 10-30 for K_A while including k_{-2}?
7. Graph v versus log([P]/[S]) for $K_P = 0.01\, K_S$. Describe the asymmetry of the reaction velocity at low versus high values of log([P]/[S]).
8. All reactions should be regarded as stochastic at the level of the individual molecule. Outline the processes involved in enzymatic facilitation of a reaction. (Reviewed by Garcia-Viloca et al., 2004 #7703.)
9. Following the ideas of Eq. 10-31, compute the rate of formation of P as a function of S for various levels of P and compare these with the rates obtained using Eqs. 10-22 to Eq. 10-25.

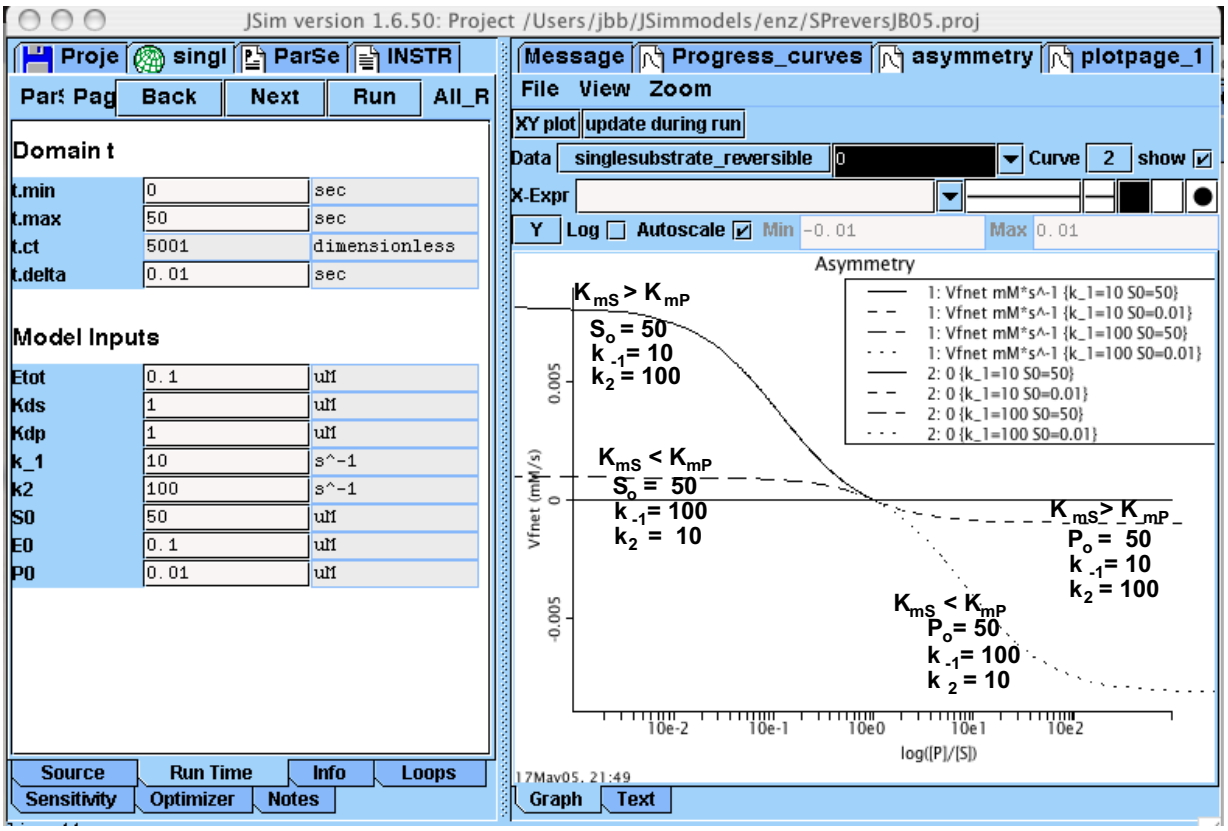

Figure 10-7: Asymmetry of net forward flux when $K_{MS} \neq K_{MP}$, but the dissociation constants are equal, $K_S = K_P$. With $K_{mS} \gg K_{mP}$ the forward flux is large and positive (*Solid line*) when [P]/[S] is < 1.0, but negative for [P]/[S] > 1.0 (*medium dashes*). With $K_{mS} \ll K_{mP}$ the forward flux is small and positive (*long dashes*) when [P]/[S] is small but is large and negative for [P]/[S] > 1.0 (*short dashes*).

10-2.9.2. Competition for substrate binding at the catalytic site

Product Inhibition: Considering the diagrammed reaction 10-6 from substrate to product, and Eq. 10-27 whose denominator depends on both [S] and [P], illustrates how competition from product limits a forward reaction velocity and why $V_{max} < k_2 E_{tot}$ in the situation where product remains bound to enzyme for any significant time. The **fraction of the enzyme bound in the form ES** is what governs the unidirectional forward reaction velocity, k_2 ES:

$$\frac{[ES]}{E_{tot}} = \frac{[S]/K_S}{1 + [S]/K_S + [P]/K_P} . \qquad (10\text{-}31)$$

Competing substrates and inhibitors: Competition may also come from another substrate that reacts to form a different product, or one that doesn't react at all. This is competitive inhibition of the forward reaction by substrate S_2 and, again assuming rapid equilibration, the fraction bound as ES_2 depends on its dissociation constant, K_{S_2}, thereby increasing the denominator:

$$\frac{[ES]}{E_{tot}} = \frac{[S]/k_S}{1 + \frac{[S]}{K_S} + \frac{[S_2]}{K_{S_2}} + \frac{[P]}{K_P}} . \tag{10-32}$$

The equation is the same if an inhibitor, *I*, that undergoes no reaction, binds with the catalytic site:

$$\frac{[ES]}{E_{tot}} = \frac{[S]/K_S}{1 + [S]/K_S + [I]/K_I} . \tag{10-33}$$

On a Lineweaver-Burk double reciprocal plot the presence of competitor slows the forward reaction and $v = k_2$ ES, and steepens the slope of $1/v$ versus $1/[S]$ from K_S/v_f to $(K_S/v_f)(1 + [I]/K_I)$, but does not change the apparent K_m. This can be derived from Eq. 10-33 using different constant values of [I]. Plots of v_{fmax}/v versus $K_S/[S]$ are shown for [I] = 0, 0.316 K_I, K_I, and 3.16 K_I in Fig. 10-8.

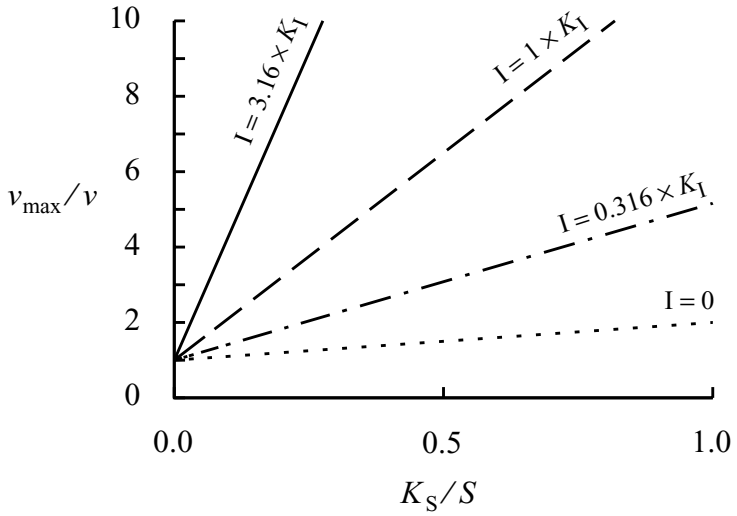

Figure 10-8: Normalized plots for v_{max}/v versus K_S /[S] at various concentrations [I] of inhibitor.

["To this section, add some of the variations of non-competitive versus competitive inhibition."]

10-3. Order of the reaction

An enzyme often binds more than one substrate molecule at a time,

$$E + nS \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} \mathrm{ES_n} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} P + E\,, \qquad \text{Diagram 10-8}$$

where n is the number of molecules of S binding to E. The stoichiometry of the forward reaction to form P is n molecules of S to form one molecule P. The reaction order is **n**; the flux to form P is therefore nonlinear when **n** > 1, as in Fig. 10-9. With **n** = 2, the reaction is "bimolecular", and when 3 is trimolecular.

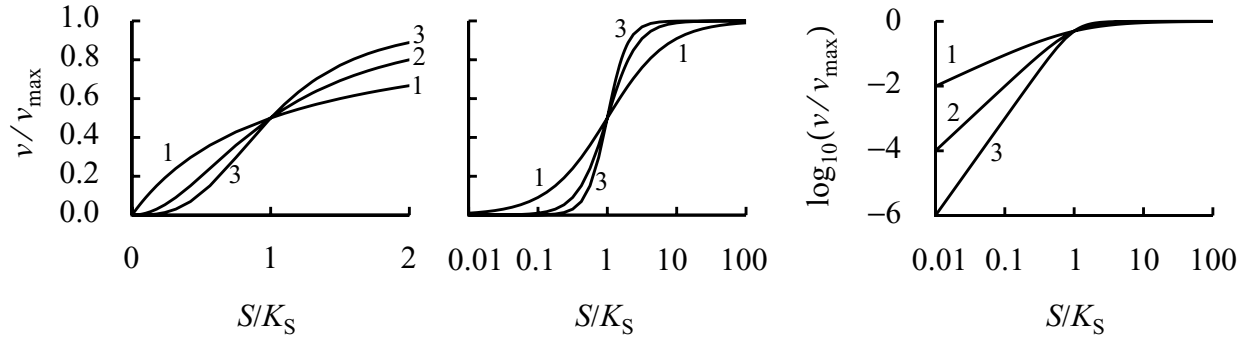

Figure 10-9: Enzyme catalyzed reactions of orders 1 to 3. *Left Panel*: Linear plot of reactions of orders 1 to 3 forming P from S. *Middle Panel*: Reaction velocity versus [S] over a wide range on logarithmic abscissa for concentration. All curves plateau at $v/v_{max} = 1.0$. Note the steepness of the slopes in the neighborhood of the K_S. *Right panel*: Log-log plots for reaction velocity with $[S] \ll K_S$ give straight lines of slope ***n*** at low [S].

In this situation the reaction velocity v is given by

$$\frac{v}{v_{max}} = \text{fractional binding} = \frac{[S]^n}{K_s + [S]^n} \qquad (10\text{-}34)$$

Here it is assumed that all of the substrate molecules bind to all of the sites simultaneously so that the enzyme is occupied by either **n***S* or none, an assumption which is not realistic but serves to illustrate the estimation of order of the enzyme-catalyzed reaction; this is called the *momentary kinetic order*. The order is calculated from the slope of the reaction velocities versus [S]. By using a calculation of the local slope on a log scale the apparent order can be estimated, as in the right panel of Fig. 10-9. Note from the right panel that the slopes should be measured where $S/K_s \ll 1$.

The Hill Equation: A variant on this expression was used by AV Hill (1910 #7387) to describe the relationship between the fractional saturation S of hemoglobin by oxygen and the pO_2, the partial pressure of oxygen in the solution:

$$\text{fractional saturation, } S = \frac{[\mathrm{pO_2}]^{\mathrm{nH}}}{[\mathrm{pO_2}]^{\mathrm{nH}} + P_{50}{}^{\mathrm{nH}}}, \tag{10-35}$$

where nH is the Hill exponent or coefficient. With $nH = 2.7$ and $P_{50} = 26$ mmHg partial pressure of oxygen at 50% saturation, Hill found that this gave a good quantitative description of his observations. This empirical expression is compared later, in Figure 10-11, with a formal expression for cooperative binding of oxygen to hemoglobin. This is commonly written as the "Hill Equation":

$$\log \frac{S}{1-S} = nH \cdot \log \mathrm{pO_2} - \text{const}, \tag{10-36}$$

and is useful in providing an estimate of nH from the slope of a log-log plot. With $nH = 2.7$, as for oxygen, the cooperativity effect is striking, for there is only a 4.8-fold change in $\mathrm{pO_2}$ needed to raise the saturation from 10% to 90%, whereas with a single site (first order) Michaelis-Menten reaction an 81-fold increase is needed.

10-3.0.1. Examine Problem 11 to Problem 13

10. Write the differential equations for the reactions of orders 1 to 3 in Diagram 10-8.
11. Use the definition of the derivative of $d \log v / d \log S$, where $v = k_2$ [ES], to calculate n from Eq. 10-33.
12. From Eq. 10-34 what are the estimates of the slopes, $d(v/v_{\max})/dS$, when $n = 1$, 2, and 3, with [S] = 0.1 k_S? When [S] = k_S?

10-3.1. Sequential binding of substrate at more than one site on the enzyme

Consider the sequential binding of n substrate molecules one at a time to one enzyme. The binding of the first may inhibit or enhance the binding of the second and so on. This is quite different from the all-or-nothing fashion considered in the previous section.

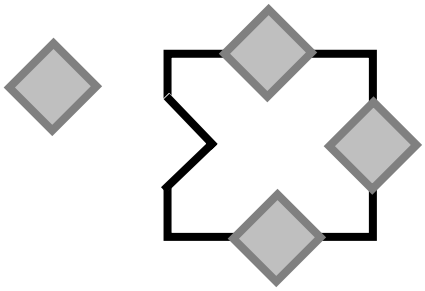

Figure 10-10: Diagram of a protein with 4 binding sites, with three occupied. The sites may be independent or interdependent with cooperativity in binding mediated through changes in conformational state of the protein.

10-3.1.1. Four identical but non-interacting sites without cooperativity

$$E + S \rightleftharpoons ES + S \rightleftharpoons ESS + S \rightleftharpoons ESSS + \ldots \qquad \text{Diagram 10-9}$$

We will consider first the situation where the binding of one S to the protein diagrammed in Figure 10-10 has no influence on the binding of the second, and so on. Later, we will look at cases with cooperativity, either positive or negative.

An enzyme with four sites has five forms so the total concentration E_{tot} is

$$E_{tot} = [E] + [ES] + [ES_2] + [ES_3] + [ES_4], \tag{10-37}$$

where it is assumed that all forms carrying two or more S are indistinguishable from one another physically and in their rates of the forward reaction to form product. We can calculate how much is in each form for any given [S] at equilibrium from the dissociation constants for each successive site, K_1, K_2, K_3, and K_4:

$$[ES] = \frac{[E][S]}{K_1}, \tag{10-38a}$$

$$[ES_2] = \frac{[E][S]^2}{K_1K_2}, \tag{10-38b}$$

$$[ES_3] = \frac{[E][S]^3}{K_1K_2K_3}, \tag{10-38c}$$

$$[ES_4] = \frac{[E][S]^4}{K_1K_2K_3K_4}. \tag{10-38d}$$

Substituting these into Eq. 10-37 yields

$$E_{tot} = [E]\left(1 + \frac{[S]}{K_1} + \frac{[S]^2}{K_1K_2} + \frac{[S]^3}{K_1K_2K_3} + \frac{[S]^4}{K_1K_2K_3K_4}\right), \tag{10-39}$$

from which one calculates $[E]/E_{tot}$ when [S] and the dissociation constants are known, and then derives the fractional or relative concentrations of ES, ES_2, ES_3, and ES_4.

K_1, K_2, K_3, and K_4 are not identical, even though the sites are identical. For a given site the intrinsic dissociation constant is $K_{eq} = k_{-1}/k_1$, Eq. 10-9a. When there are four free sites on E the rate of binding is 4 k_1, but the rate of dissociation is unchanged, so the effective dissociation constant K_1 is $k_{-1}/4k_1$, which is to say that the apparent affinity is four times higher than for the single site. When there is one site occupied, then the second S binds with a rate 3 k_1, but there are twice as many to dissociate, so K_2 is $2k_{-1}/3k_1$, and so on. Likewise for sites 3 and 4, giving

$$K_1 = K_{eq}/4, \tag{10-40a}$$

$$K_2 = 2K_{eq}/3, \tag{10-40b}$$

$$K_3 = 3K_{eq}/2, \tag{10-40c}$$

$$K_4 = 4K_{eq}. \tag{10-40d}$$

The forward reaction to form product is assumed to have the same rate constant, k_f, for each occupied site, independent of position, so that, substituting in from Eq. 10-38a to Eq. 10-38d and Eq. 10-40a to Eq. 10-40d, the forward flux v_f is

$$v_f = k_f([ES] + 2[ES_2] + 3[ES_3] + 4[ES_4]), \tag{10-41a}$$

$$v_f = [E]k_f\left(\frac{[S]}{K_{eq}/4} + 2\frac{[S]^2}{\left(\frac{K_{eq}}{4}\right)\left(\frac{2K_{eq}}{3}\right)} + 3\frac{[S]^3}{\left(\frac{K_{eq}}{4}\right)\left(\frac{2K_{eq}}{3}\right)\left(\frac{3K_{eq}}{2}\right)} + 4\frac{[S]^4}{K_1K_2K_3K_4}\right), \tag{10-41b}$$

$$v_f = [E]k_f\left(\frac{4[S]}{K_{eq}} + \frac{12[S]^2}{K_{eq}^2} + \frac{12[S]^3}{K_{eq}^3} + \frac{4[S]^4}{K_{eq}^4}\right), \tag{10-41c}$$

and, finally, substituting for [E] using Eq. 10-39 gives the result for the forward flux:

$$v_f = [E_{tot}]k_f\frac{(4K_{eq}^3[S] + 12K_{eq}^2[S]^2 + 12K_{eq}[S]^3 + 4[S]^4)}{(K_{eq}^4 + 4K_{eq}^3[S] + 6K_{eq}^2[S]^2 + 4K_{eq}[S]^3 + 1[S]^4)}. \tag{10-42}$$

Note that both the numerator and denominator show symmetry in the coefficients; the 1,4,6,4,1 sequence comes from $(x + y)^4$; the 4,12,12,4 sequence comes from $4(x + y)^3$. When $[S] \gg K_{eq}$, where $K_{eq} = k_{-1}/k_1$ (Eq. 10-9a), i.e., that for the first site occupied, the higher-order terms dominate, but when $[S] \ll K_{eq}$, the single site binding dominates and closely approximates the standard M-M equation except that the apparent K_m is $K_{eq}/4$, thus reflecting the four-fold increase in available catalytic sites. The nature of this non-cooperative system is quickly revealed by taking the algebra a step further. Replacing the ratio, $[S]/K_{eq}$ with G, one gets

$$\frac{v_f}{v_{f\max}} = \frac{4G(1 + G)^3}{(1 + G)^4} = \frac{4G}{1 + G}, \tag{10-43}$$

but v_{fmax} is nevertheless four times that for a single-site enzyme, i.e., $v_{fmax} = 4[E_{tot}]k_f$.

10-3.1.2. Examine Problem 13 to Problem 15

13. For abd enzyme with four non-interacting, independent sites, calculate the fraction of E_{tot} in each form when $[S] = K_{eq}$, when it is $K_{eq}/10$, and when it is $10\ K_{eq}$. A spreadsheet can be used for this.
14. The maximum rate for Eq. 10-42 occurs when all 4 sites are occupied. Graph the ratio of the rates for this four-site enzyme compared to that of a one-site enzyme with the same K_{eq} over at least four orders of magnitude of [S].

15. Calculate for the four-independent-site enzyme the apparent order of the reaction as a function of [S]. Do this analytically and numerically. At high [S] it goes to zero order, of course.

10-3.2. Four interacting sites with cooperativity in multisite binding

When there is more than one binding site and there is positive cooperativity, the presence of one substrate molecule on the enzyme causes an increase in the affinity of binding a second, and so on, if there are more than two sites. An enzyme with four cooperative catalytic sites has a higher rate of product formation, and, we will see later, sets up a situation by which regulation is more effective.

The system is the saamme as that diagrammed in Figure 10-10. For this particular section we assume that "cooperativity" applies to the binding process, not to the catalysis step, and therefore that all sites have the same catalysis rate. We assume further that the binding of the first molecule of *S* enhances the rate of binding of the second by a factor α, and that the binding of the second *S* enhances the rate of binding of the third α^2, and the fourth by α^3, that is, the cooperativity is by the same degree of enhancement at each successive binding event. The rationale is that the binding of each molecule of substrate affects the free energy of binding of all other binding sites identically. The change in ΔG_{bind}, the free energy of binding, is $-\text{RT} \ln(\alpha)$.

For positive cooperativity, if the binding of a first *S,* with affinity $1/K_{\text{eq}}$, reduces the free energy of binding by ***a*** kilocalories, increasing the rate to α times k_1, where a is a ratio of the rate of binding at the second compared to the firsrt site. Then the binding of the second *S* reduces the free energy requirement by 2***a*** kcal, increasing the binding rate for the third site to 2α times to k_1, and to 3α times to k_1 for the last site. Thus the equilibrium binding constants for the four sites are reduced from those given in Eq. 10-40a to Eq. 10-40d to $K_1 = K_{\text{eq}}/4$, $K_2 = 2K_{\text{eq}}/3\alpha$, $K_3 = 3K_{\text{eq}}/2\alpha^2$, and $K_4 = 4K_{\text{eq}}/\alpha^3$. The concentration at which the sites are half occupied, $S_{1/2}$, now is shifted. The shift is to the right for $\alpha > 1$, positive cooperativity, and to the left for $\alpha < 1$, negative cooperativity:

$$S_{1/2} = (K_1 K_2 K_3 K_4)^{1/4}, \tag{10-44a}$$

$$S_{1/2} = [(K_{\text{eq}}/4)(2K_{\text{eq}}/3\alpha)(3K_{\text{eq}}/2\alpha^2)(4K_{\text{eq}}/\alpha^3)]^{1/4}, \tag{10-44b}$$

$$S_{1/2} = K_{\text{eq}}/\alpha^{1.5}. \tag{10-44c}$$

From this one can see from an experiment the intrinsic binding constant, K_{eq}, can be calculated from the observed values of $S_{1/2}$ and α, the latter being determined from the log-log slope at $S_{1/2}$:

$$K_{\text{eq}} = \alpha^{1.5} \cdot S_{1/2}. \tag{10-45}$$

At this point we do not have an expression for $[\text{ES}]/E_{\text{tot}}$ as a function of [S], which we need in order to calculate $v/v_{\max} = k_{\text{f}}[\text{ES}]/E_{\text{tot}}$ and to compare the forward fluxes with the case for independent binding, Eq. 10-42.

Now, going back to the general expression for E_{tot} in Eq. 10-39, and recognizing that a rate of relative forward flux is equal to the fractional occupancy of binding sites times k_f. Assuming that all sites have the same forward reaction rate:

$$\frac{[ES]}{[E_{tot}]} = \frac{[E]\left(\frac{[S]}{K_1} + \frac{2[S]^2}{K_1K_2} + \frac{3[S]^3}{K_1K_2K_3} + \frac{4[S]^4}{K_1K_2K_3K_4}\right)}{[E]\left(1 + \frac{[S]}{K_1} + \frac{[S]^2}{K_1K_2} + \frac{[S]^3}{K_1K_2K_3} + \frac{[S]^4}{K_1K_2K_3K_4}\right)}. \qquad (10\text{-}46)$$

(This same equation serves for the fractional occupancy of hemoglobin's four oxygen-binding sites, given specific values for the K_i's.) For cooperativity with the same α for each site, subsittuting for the k_i's and using $G = [S]/K_{eq}$, the equation reduces to

$$\text{Fractional occupancy, } \frac{[ES]}{[E_{tot}]} = \frac{G + 3\alpha G^2 + 3\alpha^3 G^3 + \alpha^6 G^4}{1 + 4G + 6\alpha G^2 + 4\alpha^3 G^3 + \alpha^6 G^4}, \qquad (10\text{-}47)$$

where $G = [S]/K_{eq}$. The derivation uses the definitions for the K_i's defined in the paragraph above Eq. 10-44a into Eq. 10-46 to get Eq. 10-47. Obviously, if K_{eq} is held constant, the effective $S_{1/2}$ changes with α. But for a fixed $S_{1/2}$, e.g., 26.8 mmHg for the P_{50} for oxygen binding to hemoglobin, the cooperativity shows in the change of the slope at the P_{50}, the point where the slope is steepest, as shown in Fig. 10-11.

Since there are four sites, with the same k_f at each, then $v_f/v_{fmax} = k_f[ES]/E_{tot}$ or

$$v_f = \frac{k_f \cdot E_{tot} \cdot 4(G + 3\alpha G^2 + 3\alpha^3 G^3 + \alpha^6 G^4)}{1 + 4G + 6\alpha G^2 + 4\alpha^3 G^3 + \alpha^6 G^4}. \qquad (10\text{-}48)$$

This system for cooperative or anti-cooperative binding can work for any number of sites. With $\alpha = 1$ the reactions are uncooperative, and Eq. 10-48 becomes identical to Eq. 10-42.

The influence of slow binding on apparent affinity and cooperativity: When the on- and off-rates are slow, there is an influence on the shapes of the oxygen dissociation curve for Hb whenever there is a change in oxygen concentration. To represent this situation the P_{O2} was modeled as rising at constant rates of 0.1, 1.0 or 10 mmHg/ sec. and the on-rate constant for binding was set to 1 sec^{-1} with the result, shown in Fig. , that the apparent P_{50} for half saturation is shifted to the right. The shift is greater at higher rates of change of P_{O2}. Although there is a suggestion that the lower part of the saturation curve is shifted more than the upper part, there is no real change in the slope or any measurable increase in apparent cooperativity. The form of this shift is similar to that for the effect of slow permeation on an enzymatic reaction shifting its apparent K_m upward, but contrasts from the curves of Fig. 10-15 in that the apparent cooperativity is not changed.

10-3.2.1. Monod-Wyman-Changeux cooperativity: An asymmetric, allosteric system

The cooperativity is not necessarily the same with each successive binding, meaning that α is not a constant. In the 4-site case (Eq. 10-48) instead of α^2 we would use $\alpha_1\alpha_2$, and instead of α^3

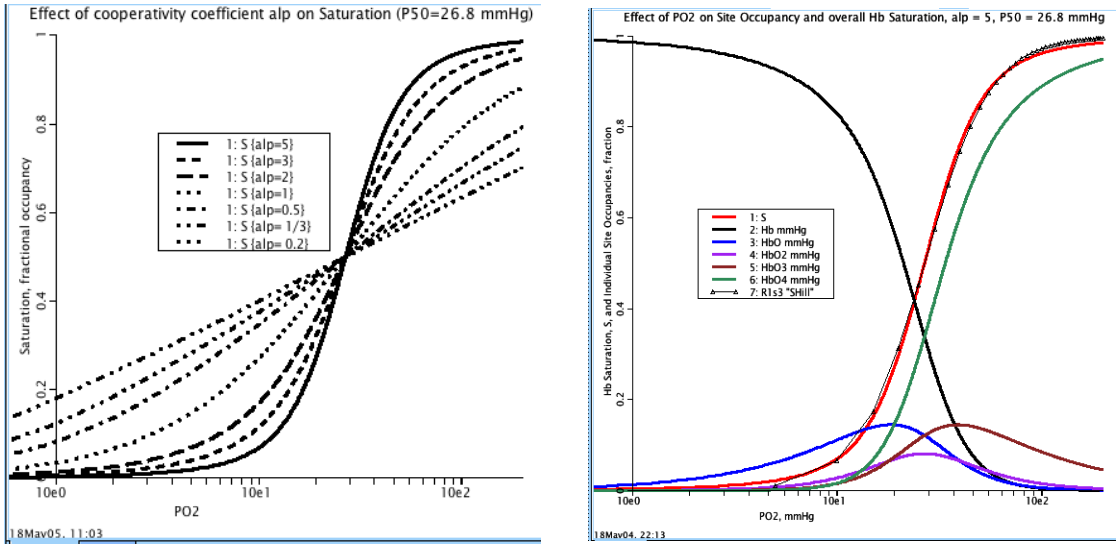

Figure 10-11: Fractional saturation curves for a protein with four binding sites with constant cooperativity α. *Left panel:* Varied α's: $\alpha < 1$ is negative cooperativity where binding a first site raises the activation energy to fill subsequent sites, and $\alpha > 1$ is positive cooperativity. *Right panel:* Comparison of Eq. 10-47 with the Hill Equation, using $S_{1/2} = 26.8$ mmHg, the P_{50} for oxygen on hemoglobin, and $n_H = 2.7$. The parameters for the four-site cooperativity were $\alpha = 5.0$ and $K_{eq} = S_{1/2}\alpha^{1.5} = 299.6$ mmHg. The curves for occupancy of 1 site only on the hemoglobin diminishes monotonically with less than half of Hb molecules being in this form at the P_{50} of 26.8 mmHg; at this P_{O2} 34% is Hb, 12.1% is $Hb(O_2)$, 8.1% is $Hb(O_2)_2$, 12.1% is $Hb(O_2)_3$, and 33.7% in $Hb(O_2)_4$, because of the high cooperativity.(hbcoop.proj)

we would use $\alpha_1\alpha_2\alpha_3$. Using individual values for α at each site increases flexibility in fitting observed data. More importantly, using distinct values for each site recognizes that most molecules are not symmetrical and cannot be expected to have exactly the same cooperativity for filling successive sites. **[This section to be completed by additional text plus figures. Applications to hemoglobin will be mentioned here and spelled out in Chapter ??]**

10-3.2.2. Problems

16. Determine α from a plot of $v/(v_{fmax} - v)$ versus [S] (using the relationship $\log(v/(v_{max} - v)) = \log(\text{Const}) + n \log([S])$, where n is the Hill exponent or coefficient) to obtain an estimate of K_{eq}. Is the estimate of K_{eq} affected by a change of α?
17. If the enzyme were hemoglobin, with four sites to bind up to four oxygen molecules, O_2, and no subsequent reaction step, (a) what is the K_{eq} if the oxygen partial pressure for 50% of sites occupied is 26.8 mmHg (the P_{50}) and the Hill coefficient is 2.7, and (b) at the P_{50}, what fraction of the hemoglobin has just three sites occupied by oxygen?
18. Compare the fractional site occupancies at varied [S] for a four-site enzyme exhibiting cooperativity with an enzyme showing no cooperativity. (Map the field of the ratio of fraction of enzyme in each of the five forms of E over a range of [S] for the enzyme with $\alpha = 0.5$ (negative cooperativity) divided by with $\alpha = 1$. Use a range of [S] from 0.01 $S_{1/2}$ to

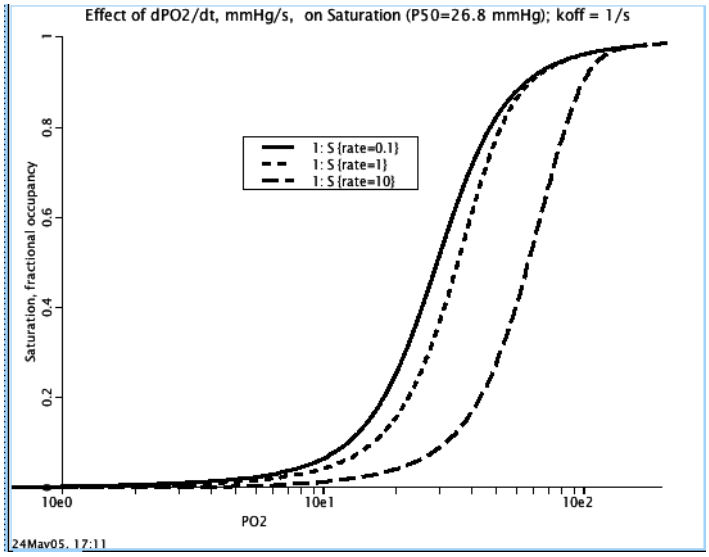

Figure 10-12: Apparent increase in the P_{50} resulting from rapid changes in P_{O2}: the half saturation point is shifted to higher P_{O2}s when the rate of change of P_{O2} is increased. (50% saturation is at P_{O2} = 27.4 mmHg with P_{O2} increasing at 0.1 mmHg/s, 32.9 mmHg at 1 mmHg/s and 61.5 mmHg at 10 mmHg/s.) There is no significant change in slope or apparent Hill coefficient. Diffusional delays or low RBC membrane permeability would have similar effects

100 $S_{1/2}$.) You might do the same for forward fluxes, but just from occupancy, where do you think the maximum sensitivity is to changes in [S]?

10-3.3. Form of reversible cooperative Michaelis-Menten reactions

Consideration needs to be given to the irreversibility or reversibility of reactions which show cooperativity, and for the same reasons as for single-site enzymes. Mathematically, this was more difficult to handle until Hofmeyr and Cornish-Bowden (1997) worked out a minimally complex version of the reversible Hill equation:

$$J_{S,P} = \frac{(v_f[S]/K_S)\left(1 - \frac{\Gamma}{\kappa}\right)([S]/K_S + [P]/K_P)^{nH-1}}{1 + ([S]/K_S + [P]/K_P)^{nH}}, \qquad (10\text{-}49)$$

where κ is the ratio of P to S at equilibrium and equals $(v_f/K_S)/(v_r/K_P)$, and Γ is the ratio of [P] to [S] at any time t.

The Hill exponent nH is a function of the degree of cooperativity in multisite binding, greater than 1 for positive cooperativity. The exponent cannot take values as high as the maximum number of catalytic sites available on the enzyme. For example, nH is only about 2.7 when there are four binding sites, as for oxygen binding to hemoglobin.

10-3.3.1. Problem

19. For a 4-site enzyme with constant cooperativity ratio α what is the relationship between α and $\boldsymbol{n^H}$? Figure out the relationship from Section 10-3.2.

10-3.4. Modifiers of enzymatic reactions

The considerations which follow come into play when another substance, a modifier, binds to a target enzyme. Many modifiers affect the affinity of substrate binding, and others affect the V_{max}. **[This section is incomplete.]**

10-4. Single-enzyme behavior in special situations

Enzymatically facilitated fluxes are normally low-gain processes such that they do not oscillate and merely increase monotonically as substrate concentrations increase. The Hill coefficient is normally unity, as for the common Michaelis -Menten reaction. Increases in gain can occur even without cooperativity in substrate binding and reaction. The examples which follow include higher-order behavior due to combinations of substrate variation and enzyme variation, and simpler mechanisms which change the gain by some form of competition. The traditional mechanism considered is branch point competition, where two enzymes compete for binding a single substrate. By sequestering an enzyme gain increases similarly simply by limiting the rate of access of substrate to the enzyme. The combination of access limitation and competition from another enzyme can result in a very high-gain process. By any of these mechanisms giving high gain to a reaction, a biochemical network can become more susceptible to instability.

10-4.1. Enzyme systems with high Hill exponents

There are three basic mechanisms for increasing the apparent gain of a reaction: (1) cooperativity in multisite binding as discussed above in Section 10-3.1, (2) enzyme competition or branch-point behavior, and (3) enzyme sequestering. With enzyme competition there is steepening of the slope of flux versus [S] because each enzyme steals away substrate from the other. With enzyme sequestering, there is retardation of access to the enzyme so that the forward flux keeps the [S] lowered in the region of the enzyme. The combination of branch point competition and enzyme sequestration gives a very high slope in the neighborhood of the K_m, so much so that it is termed “switch-like” behavior.

10-4.1.1. Branch point competition

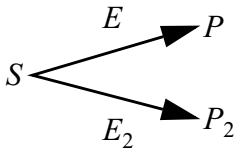

Figure 10-13: Branch point competition: S is consumed via reactions facilitated by enzymes E and E_2 in competition with affinities K_m and K_{m2} (*left panel*). The reaction with the higher affinity enzyme steals substrate away from the lower affinity enzyme, shifts the curve of reaction velocity for the lower enzyme to the right, and steepens the slope of [ES]/[E_{tot}], the reaction velocity divided by V_{max} (*right panel ADD PANEL*).

For a two enzyme system (Fig. 10-13), the presence of a competing enzyme E_2 affects the reaction rate through enzyme E when the affinity of E_2 for S is higher than that of E for S (Koshland, 1987 #6584). When $K_{m2} < K_m$ the preferential reaction is to form P_2, diminishing the rate of production of P at low concentrations of S. But when E_2 is saturated at higher [S], P is formed. The slope of the relative reaction rate, [ES]/[E_{tot}] versus [S] is thereby steepened as the curve is shifted to the right; an apparent K_m' at 50% V_{max} is greater than the true K_m for the enzyme, the degree of shift depending upon the ratio K_m/K_{m2}. This increase in effective gain for the $S \rightarrow P$ reaction occurs at concentration of S well above K_{m2}.

10-4.1.2. Increase in gain due to enzyme sequestration.

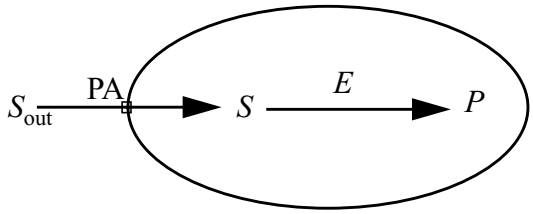

Figure 10-14: Product formation from a sequestered enzyme. The membrane peermeability-surface area product is PA. Sout it the concentration of substrate outside of the vesicle; S is concentration inside, E is enzyme and P is product.

An example of an enzyme within a vesicle such as the endoplasmic reticulum is shown in Figure 10-14. With high vesicle permeability the behavior is that of a simple M-M reaction. At reduced permeability the gain (slope) increases, as shown in Fig. 10-15.

We first consider the condition where the outside volume is much larger than V, the sequestered volume, so that S_{out} is almost unchanged by substrate transport into the vesicle, and where the enzyme is present in much smaller quantities than the substrate, so that a pseudo steady-state is achieved for some time, with [S] and [ES] reaching plateau concentrations so that the two derivatives of [S] and [ES] are essentially zero. Introducing the scaled variable for the fraction of the enzyme which is complexed, x = [ES]/[E_{tot}] where [E_{tot}] = [ES]+[E], and using $K_m = (k_{cat} + k_r)/k_f$ reduces at pseudo-equilibrium to

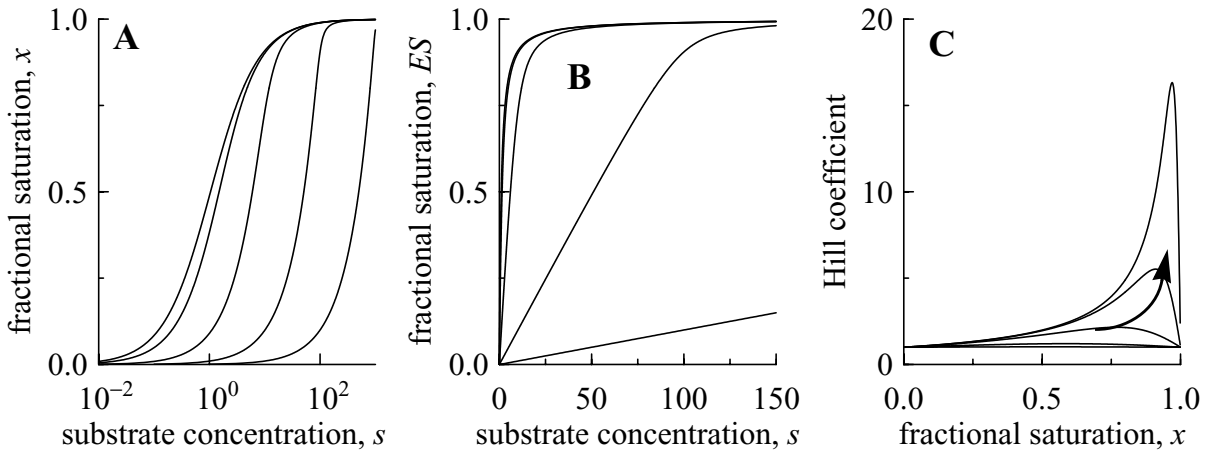

Figure 10-15: The sequestered enzyme. Solutions to Eq. 10-52. Switch-like enzyme behavior: (A) shows the half-log plot and (B) the linear plot, of the fractional saturation of enzyme vs. substrate concentration. In panels A and B, from left to right, $\alpha = 0.1$, 1, 10, 100, and 1000, where α is proportional to the catalytic rate divided by the permeation rate of S into the vesicle. (C) The Hill coefficient is graphed against the fractional saturation of the enzyme. α increases as before, from the bottom curve to top curve where α is 1000.

$$[\mathrm{S}] = K_\mathrm{m} \cdot \frac{x}{1-x}. \tag{10-50}$$

Similarly, by introducing $s = [\mathrm{S_{out}}]/K_\mathrm{m}$ as a dimensionless substrate concentration and defining $k_\mathrm{t} = \mathrm{PA}/V$ as a transport rate constant (in sec^{-1}) and V is the vesicle volume, and assuming pseudo-equilibrium the situation reduces to:

$$s = \frac{x}{1-x} + \frac{k_\mathrm{cat} \cdot [\mathrm{E_{tot}}]}{k_\mathrm{t} \cdot K_\mathrm{m}} \cdot x. \tag{10-51}$$

This equation suggests that the relationship between the fraction of enzyme complexed and the normalized substrate concentrations are dependent on a single parameter α, defined as the utilization of substrate by the enzyme relative to transport: $\alpha = (k_\mathrm{cat} \cdot [\mathrm{E_{tot}}])/(k_\mathrm{t} \cdot K_\mathrm{m})$. Then,

$$s = \frac{x}{1-x} + \alpha \cdot x. \tag{10-52}$$

Enzyme sequestration gives apparent cooperativity. While it is conceptually more straightforward to think of x as a function of s, it is mathematically simpler to express the relationship with s as a function of x. Since the curve increases monotonically, nothing is lost by this choice. Several things become apparent from the equation. First, if there is infinite transport, then $\alpha = 0$ and the equation is the usual Michaelis-Menten first order reaction equation, $s = x/(1-x)$, which reduces to $[\mathrm{S}] \cdot [\mathrm{E}]/[\mathrm{ES}] = K_\mathrm{m}$. Figure 10-15 shows the relative rate of product formation, $[\mathrm{ES}]/[\mathrm{E_{tot}}]$, or enzyme activity relative to changes in normalized substrate concentration, $[\mathrm{S_{out}}]/K_\mathrm{m}$, for several values of the parameter α. The half-log curve (left panel)

steepens as α becomes greater than 1, but when α is greater than about 10, the primary effect is to shift the curve towards higher substrate concentrations, increasing the apparent K_m. Thus, the membrane effects an ultrasensitive (more switch-like) response, but requires higher concentrations of substrate to reach the switch.

The shape of the response curve becomes increasingly asymmetric with higher α: at low enzyme saturation, e.g., $x < 1/2$ (the bottom half of the curve, panel A), the shape of the curve of x versus log s changes little with α, while at $x > 1/2$ (the top half of the curve, panel A), the curve steepens with increasing α. A linear graph (Fig. 10-15B) shows that slightly reduced influx (low α) has minimal effects at low external concentrations, s. The curves are hyperbolic at all α. Although the Hill coefficient [the slope of $\log(x/(1-x))$ vs. $\log(s)$] gets very high for high α, it is only high at high levels of enzyme saturation (Fig. 10-15C). Near $x = 1/2$, when changes may have the most physiological implications, the Hill coefficient remains under 2. Because of this asymmetry, the largest effect of increased membrane resistance ($\alpha = 1000$) is to reduce the concentration change needed to go from 10% to 90% maximum activity from 81-fold to 9-fold, even though the Hill coefficient exceeds 15. Thus, with a single, encapsulated enzyme, this high gain, switch-like behavior occurs only over a narrow range of S and only at over 80% enzyme saturation.

10-4.1.3. Combined branch point competition and enzyme sequestration

Here suppose a two enzyme system with substrate S such that enzyme E converts S to P and a higher affinity enzyme E_2 converts S to P_2, as diagrammed in Figure 10-16. The situation is that the two enzymes compete for the substrate S whose diffusion to the binding sites is restricted in some fashion such as by the need to permeate, with rate PA, across the vesicular membrane. The enzyme E_2, by virtue of its higher affinity, wins the competition for substrate when [S] is low, and P_2 is the main product formed. But when this higher affinity enzyme E_2 is saturated, then substrate binds to enzyme E, and P is produced in addition. The relative rate of production of P as a function of [S] is shown in Fig. 10-17.

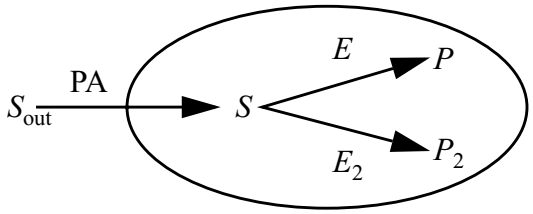

Figure 10-16: Enzyme sequestration combined with branch point competition gives switch-like kinetics

Using $s = [S]/K_m$, the substrate concentration can be expressed in terms of the fractional saturation of enzyme E, where $x = [ES]/E_{tot}$, as follows:

$$s = \frac{x}{1-x} + \frac{k_{cat} \cdot E_{tot}}{k_t \cdot K_m} \cdot x + \frac{k_{cat2} \cdot E_{2tot}}{k_t \cdot K_{m2}} \cdot \frac{x}{1 - x + \kappa x}. \qquad (10\text{-}53)$$

This can be simplified, as before, to an equation with only three parameters: α is the utilization of substrate by enzyme 1 relative to transport, as before: $\alpha = (k_{cat} \cdot E_{tot})/(k_t \cdot K_m)$, and ρ

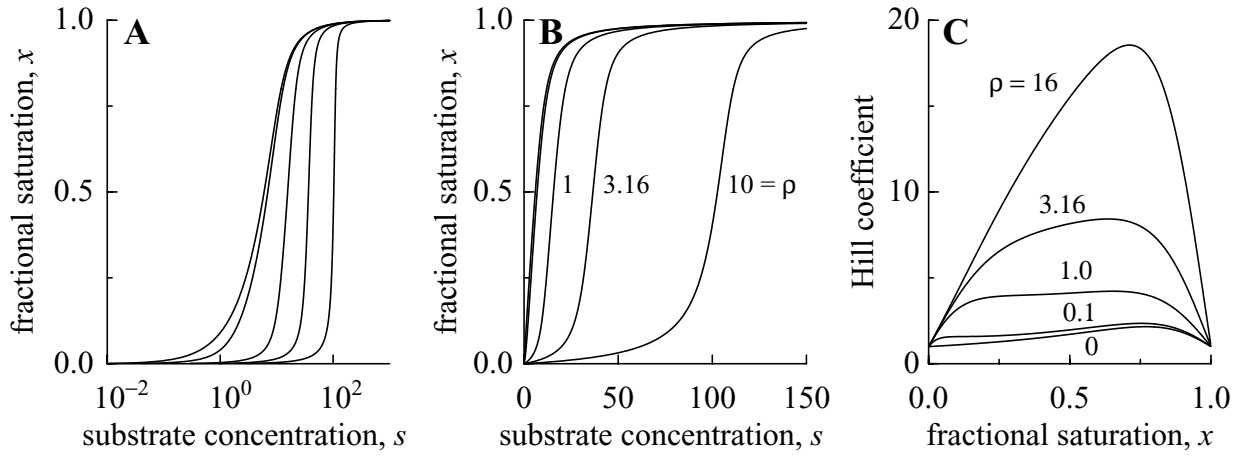

Figure 10-17: Fluxes with two enzymes sequestered in a vesicle. The fractional saturation, [ES] / E_{tot}, which is proportional to the rate of product formation is plotted on the ordinate versus $s =$ [S]/K_m (abscissa) for the two-enzyme model (Equation 10-54). (A) shows the half-log plot and (B) the linear plot, of fractional saturation of enzyme E vs. substrate concentration. (C) The Hill coefficient, the exponent of the power law measure of the local slope in is graphed against the fractional saturation of the enzyme. In all three plots, $\alpha = 10$, and $K_m / K_{m2} = \kappa = 30$ for all curves, while ρ, the ratio of V_{max} for E_2 to that for E, goes from $\rho = 0$, 0.1, 1, 3.16, to 10, going from left to right in panels A and B, and from bottom to top in panel C.

is the ratio of maximum flux for enzyme E_2 to that for enzyme E, $\rho = (k_{cat2} \cdot E_{2tot})/(k_{cat} \cdot E_{tot})$, with $\kappa = K_m/K_{m2}$, resulting in

$$s = \frac{x}{1-x} + \alpha x + \alpha\rho\kappa \cdot \frac{x}{1-x+\kappa x}. \qquad (10\text{-}54)$$

If there is no second enzyme ($\rho = 0$), then Equation 10-54 reduces to Equation 10-52. Similarly, if the second enzyme has a much higher K_m, (κ approaches 0), or the same K_m, ($\kappa = 1$) then Equation 10-54 still reduces to the same general form: $s = x/(1-x) + (\alpha + \alpha\rho\kappa) \cdot x$, or $s = x/(1-x) + (\alpha + \rho) \cdot x$, respectively. Hence, the second enzyme affects the behavior of the first only when it has a lower K_m, a higher affinity, for the substrate ($\kappa > 1$).

Enhanced ultrasensitivity when $K_{m2} < K_m$: When κ is high and when the substrate is transported slowly relative to the activity of enzyme 1 (e.g., $\alpha = 10$), the action of the second enzyme greatly steepens the response curve to produce P if the flux through E_2S is higher than that through ES (i.e., $\rho > 1$), at low concentration of S, as shown in Figure 10-17; the second enzyme starts to make a difference by $\rho = 1$ (middle curve on each plot panel).

As each of the three plots in Figure 10-17 shows, the competing, lower K_m enzyme E_2 has the effect of selectively reducing the flux to form P (via enzyme E) when [S] is low. The Hill coefficient is raised over the whole range of $x = [ES]/E_{tot}$ except at the extremes, 0 and 1 (Figure 10-17C). The response is sigmoidal for $\rho > 1$ on the linear plot (Figure 10-17B), and these are nearly symmetrical on the half-log plot (Figure 10-17A). Thus, introducing a competing enzyme into the vesicle converts the response of the first enzyme into a more switch-like response, making it into a very high gain process with Hill coefficients >5 over most of two

decades, $0.1 < x < 10$, when $\rho > 2$. Under these conditions, the switch, almost on-off, occurs at external concentration levels well above the K_m.

The ultrasensitivity induced by the membrane barrier is a form of kinetic cooperativity. Kinetic mechanisms which produce apparent cooperativity keep the system in a nonequilibrium state as a result of the conversion of substrate to product (Neet, 1995). Thus cooperativity is not observed in equilibrium binding measurements where there is no net conversion of substrate to product (Neet, 1995). This was the result shown in Figure 10-12 for oxygen binding to hemoglobin. In the encapsulated enzyme case, the non-equilibrium state is that S, the substrate concentration inside the vesicle, does not come into equilibrium with the extravesicular concentration S_{out}, because the enzyme is consuming it. As usual with kinetic mechanisms which lead to cooperativity, the shape of the linear/log curve is not symmetrical (Neet and Ainslie, 1980). This mechanism is, however, different from most of the kinetic mechanisms described so far in the literature. A ligand-induced slow transition (LIST, or hysteretic mechanism) has been shown to result in cooperative kinetics as a result of a slow transition involving the enzyme itself after exposure to substrate (Ainslie et al. 1972; Ricard et al., 1974; Ricard, 1977; and Frieden, 1970). Similarly, a two-substrate random addition kinetic mechanism has been suggested as a mechanism for cooperative kinetics which depends upon the individual rate constants for each substrate before and after the other substrate binds (Ferdinand, 1966; Pettersson, 1986). In contrast, the membrane effect which we have observed involves slow transport of the substrate, independent of the enzyme. Because of this, a membrane can affect cooperativity independently of the nature of the enzyme itself, even with near-equilibrium for substrate-enzyme association, as long as the parameters for membrane permeability and compartment size and therefore the rate constant for influx is small compared to the enzyme's forward reaction rate. These conditions keep the ratio S/S_{out} low, a necessary condition for achieving high gain.

A competing enzyme within the vesicle adds branch-point ultrasensitivity (Figure 10-16). When both enzymes consume a substrate delivered across the membrane at a limited rate, the lower K_m enzyme, E_2, consumes most of it at low S. At higher delivery rates E_2 becoming saturated allows S to rise so that it is consumed by the higher K_m enzyme (Koshland 1987, Walsh and Koshland, 1985, LaPorte 1984). Thus, the higher K_m branch responds as nearly a switch relative to the rate of production of substrate. In this case, however, it is slow transport through the membrane together with consumption by a competing enzyme in the branched pathway which creates the nonequilibrium conditions necessary for the ultrasensitivity. This mechanism could also be compared to the effect of chelators on any protein responding to free calcium concentration: the chelator eliminates most of the free calcium until it is saturated, after which time free calcium rises rapidly, and the responding enzymes switch on.

The conditions under which a membrane barrier can affect the behavior of an enzyme in a system are two: (1) the rate constant for the enzyme at low S, V_{max}/K_m or, $k_{cat}E_{tot}/K_m$ (in sec^{-1}) must be greater than the scaled transport rate, k_t or PA/V (in sec^{-1}), and (2) for a second enzyme to augment the apparent cooperativity it should have $K_{m2} < K_m$ and a V_{max2} not more than V_{max}.

An example of a sequestered enzyme is hepatic glucose-6-phosphatase, trapped within the endoplasmic reticulum, ER (Arion et al., 1980), where it serves as a key link in gluconeogenesis. Entry of substrate and exit of products across the ER membrane require specialized transporters. It is not known how this affects the dynamics of glucose kinetics, but the situation is appropriate for producing cycling or even chaotic behavior (Bassingthwaighte et al., 1994; Goldbeter, 1996).

Section summary: This section has covered several phenomena which can give rise to dynamical behavior in a network of equations. One can consider "homeostasis" to represent not "stasis" or a

static state, but a dynamic state wherein there is continuous fluctuation normally and in which fluctuations outside of the system may even be amplified within. But these systems, even when chaotic, have a limited dynamic range of concentrations, and in fact should be regarded as having remarkable stability. They settle into the "basin of attraction" and the system is "homeodynamic" not homeostatic.

10-4.2. Dynamical systems behavior with a single enzyme

The effect of steepening the flux-concentration relationships is to approach switch-like kinetics in parts of metabolic networks. Such behavior is also becoming evident in systems controlling gene expression. The existence of delays in feedback loops and sharply demarcated or steep response curves both contribute to instability in metabolic systems. Though Glass and Malta (1990) came to the conclusion that metabolic systems should not become chaotic, there is some evidence that period-doubling oscillations and even chaotic behavior can occur. One example is the glucose-insulin-ATP system studied by Selkov (1968), Goldbeter and Lefever (1972), and Markus and Hess (1985). Thus the expressions for individual reactions should describe nature as closely as is possible.

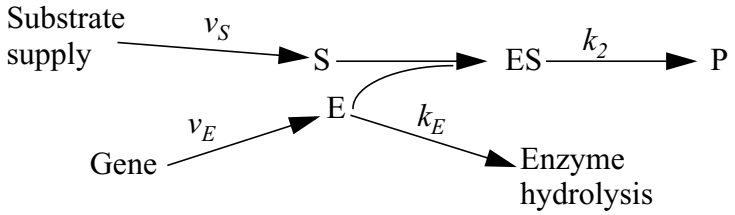

Figure 10-18: Diagram of an unstable system in which the reaction velocity k_2 [ES] varies widely and unpredictably.

A simple system exhibiting adaptive behavior, taken from Reich and Sel'kov (1981), is the enzymatic reaction shown in Fig. 10-18 . *S* is supplied at a rate, v_s, and *P* is removed, so there is no accumulation. Enzyme is synthesized at a constant rate, v_e, and degraded by a first-order process, but only when it is in the free uncomplexed form. [It is commonly the case that binding to substrate reduces the normal proteolysis of enzymes (Grisolia, 1964).] This allows adaptive behavior in that as [S] rises more enzyme accumulates in the [ES] form and E_{tot} rises, allowing more substrate utilization. This is adaptation to supply, not demand, and is a type of induction, that is, has characteristics of an electrical inductor. The equations, similar to Eq. 10-10 and following except for the influx of *S* and the production and degradation of *E*, are

$$d[\mathrm{S}]/dt = v_S + k_{-1}[\mathrm{ES}] - k_1[\mathrm{S}]\cdot[\mathrm{E}], \tag{10-55}$$

$$d[\mathrm{E}]/dt = v_E + (k_{-1} + k_2)\cdot[\mathrm{ES}] - k_1[\mathrm{S}]\cdot[\mathrm{E}] - k_E\cdot[\mathrm{E}], \tag{10-56}$$

$$d[\mathrm{ES}]/dt = [\mathrm{E}]\cdot k_1[\mathrm{S}] - (k_{-1} + k_2)\cdot \mathrm{ES}, \text{ and} \tag{10-57}$$

$$E_{\mathrm{tot}} = [\mathrm{ES}] + [\mathrm{E}]. \tag{10-58}$$

Reich and Sel'kov (1981, p. 41) note that this system demonstrates resonance: small diurnal oscillations in v_s give rise to dramatic oscillations in E_{tot}. This behavior is shown in Fig. 10-19.

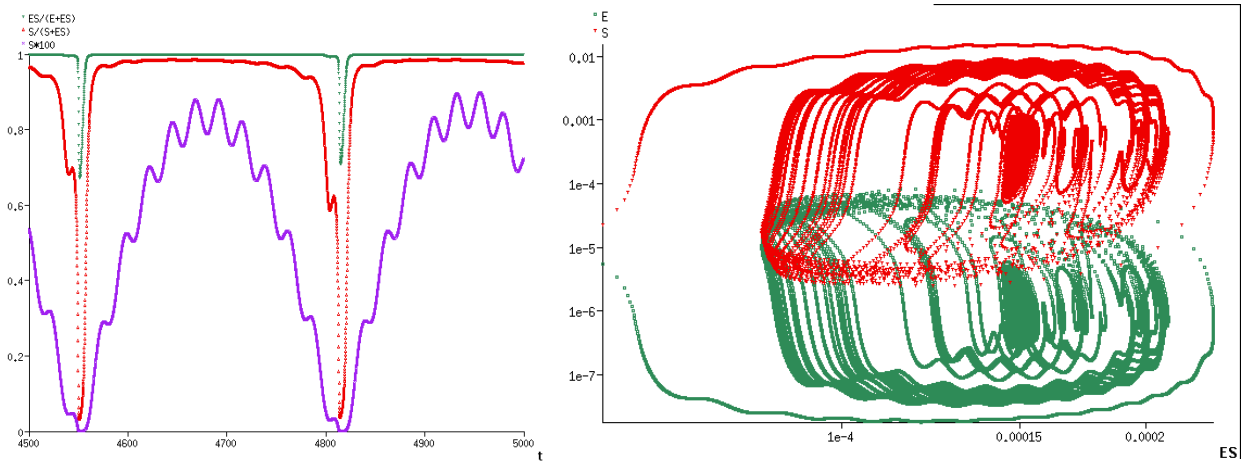

Figure 10-19: Chaotic behavior is a simple enzyme system. Enzyme induction: A small periodic oscillation in [S] gives rise to large oscillation in the E_{tot}, [ES], and the rate of production of P, which is k_2[ES]. Parameters are: $v_s = 3e\text{-}4 \cdot (0.435+\sin(2\pi t/24))$ mM/hr, $v_E = 5.51e\text{-}7$ mM/hr, $k_E = 0.6$ hr^{-1}, $k_1 = 1e6$ mM^{-1} · hr^{-1}, $k_2 = 2$ hr^{-1}, and $k_{eq} = k_{-1}/k_1 = 1e\text{-}7$ mM. Initial conditions were: [S] = 2.216e–5, [E] = 5.542e–6, [ES] = 5.8e-5 and [P] = 0. *Left panel:* Time course. The two cycles shown are different. *Right panel:* Phase plane showing chaotic attractor. The trajectory is highly sensitive to v_E. (JSim: degrad.proj, degrad.9period2.par)

The periodic response, Fig. 10-19, left panel, shows that the excursions in [ES] are proportionately larger than those of the sinusoidal input of $S(t)$. The system is like that of an electrical inductor giving resonance or amplification in the system. Further increases in the amplitude of input $S(t)$ result in larger excursions in [ES] and in the rate of formation of P, and induce a qualitative change in behavior, "period-doubling", meaning that there is alteration in the heights of the concentrations, so that the cycle (of pairs of waves) repeats with a basic period of twice the length of the period of the input function. Increasing the amplitude further doubles the period again so that it is four times the length. This is characteristic of a nonlinear dynamical system with "period-doubling on the route to chaos" (Goldbeter, 1996; Bassingthwaighte, Liebovitch and West, 1994; Kaplan and Glass, 1995). In our example, chaotic behavior is shown to occur with only a single enzyme and a single substrate and without a reverse reaction. One can easily imagine the potentiality for chaos in large networks of biochemical reactions; in fact, chaotic behavior is not common, as Glass and Malta (1990) figured for networks of Michaelis-Menten reactions, simply because the gain (the slope of reaction rate versus substrate concentration) of the reactions is low. However, looking at it from the point of view of stability, "homeostasis" can be chaotic and still fulfill the definition of maintaining constancy of the "milieu interieure" (Bernard, 1878), because a chaotic system will be constrained to lie in a "basin of attraction" limiting the extent of the variations.

10-4.2.1. A problem:

Does Fig. 10-19 assure you that the system is chaotic? Define chaos. Explain how the figure supports the notion. Explain where the figure fails to provide proof.

10-5. Enzyme induction and activation

All reactions, fundamentally, are reversible; likewise enzymatic reactions are reversible. The Haldane conditions for equilibrium (Section 10-2) are not changed by enzymatic facilitation. Consequently, net forward reaction rates must inevitably be reduced by any build-up of product. Further, all enzymes are subject to proteolysis and must be replaced by new protein generated through stimulation of transcription and translation. The fact that proteolysis is commonly inhibited by the binding of substrate to the enzyme has the effect of reducing the rate of proteolysis when there is abundance of substrate and allowing the enzyme concentration to decay when there is little substrate for it is only a quantitative detail. We think of membranes as being impermeable to proteins, but remind ourselves that newly translated proteins are carried along the Golgi strands by kinesin to their sites of action on or inserted through membranes, and others are carried in the opposite direction by dynein and penetrate the nuclear membrane. How the signaling proteins work to regulate expression, how helper proteins work to insert transporters into membranes, and how integral proteins are pushed into place is not yet known in any detail. Suffice it to say that these complex activities give credence to the notion that proteins can get anywhere, but like substrate molecules, will ordinarily need special mechanisms to traverse membranes.

In their standard locations, whether this is cytosolic, attached to a membrane or inserted into a membrane, enzymes are further modified in the course of their daily work. Changes in activity come about through modification of the active site either by changing the affinity for substrate or by modifying the rate of formation of product from the enzyme-substrate complex, or simply by inactivation the enzyme. Some enzymes requires metals to function (iron in heme groups, zinc, copper) and others are permanently inactivated by metals like thallium and mercury. The inactivation is not always complete, for example mercurials inhibit aquaporin channel conductance, making one suspect that sulfhydryl groups are prominent at the active site, but the effect appears to be graded and reversible (ref??).

10-6. Deriving steady-state flux equations

Steady-state expressions were derived for the Michaelis-Menten single enzyme reaction of $S \rightarrow P$ in Eq. 10-16 and Eq. 10-21, which wasn't difficult for the simple situation, or even for the more complicated reactions in Eq. 10-29, 10-43, and 10-49. When the reactions are more complex then it is useful to use the King-Altman approach, a general approach based on the inversion of the matrix of equations involved. Their method (King and Altman, 1956) has been described well by Cleland (1963) and Cornish-Bowden (1995); here we will use the result of the derivation, and will provide only the algebraic method. Keep in mind Cornish-Bowden's warning that the algebra is only a servant to the studies of enzymatic reactions, not the master, and that the method is only reasonable when the mechanisms assumed for the reactions are reasonably correct.

[MISSING: King Altman]

10-7. Metabolic networks

Biochemical systems are composed of myriads of interconnecting sets of biochemical, enzymatically facilitated reactions, each subject to regulatory control by various means. The control may be at the genetic level, the rate of transcription of the enzyme, or more often at the metabolic level, through inhibitors, pH, enzyme modification by phosphorylation, glycosylation, of other reaction inducing a conformational state, or directly by metabolites, as in feed-forward activation of pyruvate kinase by phosphofructokinase (Bali and Thomas, 2001). The consequence is that even in a simple reaction series such as glycolysis there are many complicated equations required to characterize the situation.

The field of metabolic control analysis can be looked upon as systems analysis at various levels of complexity. These levels are ordered with respect to applicability to describe real systems. Given a large network of equations, how does one go about trying to find the likely "solution space", the region in parameter space in which the values of the parameters and of the concentrations (the variables) are most reasonable or probable. The simplest approach is *flux balance analysis*: the network stoichiometry is provided for the matrix of reactions for which some of the fluxes have been measured, or for which the output function, such as energy or protein mass produced, can be observed. Flux balance analysis requires the least information, and is the least accurate, but is still useful because it can be so readily applied in situations where there is a paucity of data. More advanced, more accurately predictive approaches are being developed.

There are now four levels of approach, beginning with flux balance analysis:

1. Steady-state fluxes and flux balance analysis: Kirchoff's **current laws** suffice for determining flux balance. Linear analysis provides a self-consistent set of *fluxes constrained only by the fixed stoichiometry* of the reactions. Optimization of the fluxes to provide a maximum in chosen fluxes does not yield a unique solution but a broad almost unconstrained range of fluxes and concentrations.

2. Steady-state fluxes and thermodynamic balance: Adding Kirchoff's **voltage laws** to the current laws, still allowing linear systems analysis, provides *thermodynamically constrained models accounting for energetics and reversibility.* These account for the free energy of the reactants and products = voltage potential. The range of variable concentrations in state space is strikingly narrowed by this constraint. Since the equilibrium binding constants for most of the reactions are known, the constrained result has greatly improved predictive value.

3. Transient analysis and mass balance: Non-stationary, nonlinear systems require accounting for **capacitance** and thereby allow *accounting for mass balance of substrates* in their volumes of distribution and for their buffering by their binding to enzymes. The systems have constant parameters and tend toward stability. This extends the current and voltage relationships by accounting for capacitance; all of these are required for time-varying states, and are also required for the interpretation of tracer transients in a chemical steady state situation.

4. Transients analysis and amplification: Nonlinear, nonstationary systems with **induction** of enzymes *provide amplification of periodic driving functions*. Variations in fluxes result from changes in flux governing parameters such as induction of enzyme production or proteolysis. These systems also, without parametric changes but with changes in inputs can provide a full variety of behaviors, from periodic cycling through period doubling to fully chaotic behavior, but usually with a strictly limited range for the attractor in state space.

Metabolic control analysis of networks of reactions helps to understand system behavior while ignoring the details of the reactions. The first of the methods, steady-state linear analysis in accord with Kirchoff's current laws, has a long and illustrious history (Kacser and Burns,1973, Savageau, 1976), and its development continues (Fell, 1997; Hofmeyr and Westerhoff, 2002). Dependence upon its simplicity has not been abandoned, but now, with ever faster computers, it is no longer quite so important to simplify computation to gain speed, and the greater availability of information means that reactions can be correctly and explicitly described. However, the rate of acquisition of evidence of the complexity of interactions is increasing faster than are computational speeds: with increasing numbers of identified genes and a ten-fold ratio of proteins per gene, plus a combinatorial factor representing identified protein-protein interactions, all contribute to the need for more refined analysis. Gene-based targeting for therapeutics is an example where understanding the interactions among multiple proteins will be required for consistent success.

The first stage of the relationship of gene to function lies in the identification of the functions of the proteins derived from that gene or gene combination. The Predictome, a statistical approach to figuring out function proposed by Mellor et al. (2002), relies on putting together four different types of statistical inference to define putative links between any two proteins: (1) chromosomal proximity, a measure of closeness of two genes along the genome sequence, (2) phylogenetic linkage, sharing the same evolutionary pattern, (3) "fusion-linked", if in another species they are encoded into a multi-domain protein, and (4) experimental evidence of physical interactions between them, e.g., by yeast two-hybrid analysis.

There is a misconception that the gene to protein link is simple. But the one gene, one protein idea of Beedle and Tatum (1941) is wrong. The protein/gene ratio is 3/2 in E. coli, about three in yeast and perhaps ten in humans. Nor is it so simple to track a given substrate through a reaction sequence to a specific product. As genomes for more species come on line, it is sometimes observed that a species has the same beginning and ending reactions in a sequence as found in a previously characterized species, but some of the expected proteins cannot be found. This leads to a search for either an alternative pathway or for a protein that is quite different though it serves the same enzymatic function. Studies of flux balance analysis in E. coli by Schilling et al. (1999, 2000) revealed the continuance of product formation despite knockouts for two or even three enzymes in a sequence. Likewise fluxes through pathways around particular blocks occur with pharmaceutic inhibition of a protein, rendering the drug ineffective.

10-8. Summary

Simple enzymatically-facilitated reactions are the basis for virtually all cellular kinetics for transport, metabolism, signaling, and for gene regulation. The region of linearity, or first-order reaction, occurs at substrate concentrations well below the K_m. Saturation or zero-order response occurs at concentrations greater than 100 times the K_m. The region of maximum sensitivity and control occurs at concentrations around the K_m; most biochemical systems operate with substrate concentrations ranging around the K_m, so it is a good general rule to expect the K_m for an enzyme to be in the range of the normal concentration of its most important substrate. Saturation means there is no control.

Reversibility of reactions is universal, in accord with the thermodynamics. Thermodynamic constraints are useful in attempting to parameterize data. In biochemical systems the existence of the constraints tends to stabilize networks since concentrations cannot go quite to zero.

10-9. Problems

1. Define the conditions under which K_A reduces to k_{-1}/k_1, the original Michaelis constant.
2. Define the conditions under which K_A reduces to $(k_{-1} + k_2)/k_1$, the Briggs-Haldane version of the Michaelis constant.
3. Find a set of conditions in which k_{-1}/k_1, $(k_{-1} + k_2)/k_1$, and K_A are all similar. (Hints: Use values of 1.0 or 0.01; initially keep $k_{-1} = k_1$, and so on, in pairs. Atkinson et al., 1987, use a spreadsheet for this kind of calculation.)
4. Derive the expression for K_A.
5. What condition for Eq. 10-28 is violated when k_{-2} is neglected?
6. Can you derive an expression similar to Eq. 10-28 including k_{-2}?
7. Graph V versus log ([P]/[S]) for $K_P = 0.01\ K_S$. Describe the asymmetry of the reaction velocity at low versus high values of log ([P]/[S]).
8. All reactions should be regarded as stochastic at the level of the individual molecule. Outline the processes involved in enzymatic facilitation of a reaction.
9.
10. Use the definition of the derivative of $d \log v/d \log S$ to calculate n.
11. What are the estimates of the relative fluxes, v/V_{max}, for the system of equations when $\boldsymbol{n}$ = 1, 2, and 3, with [S] = 0.1 K_S? When [S] = K_S?
12. **[Problems set out in preceding text will be compiled here.]**

10-10. Further readings **[to be revised]**

Modern biochemistry texts should be consulted with respect to the relationships between biochemistry and molecular biology and genomic. However for kinetics, older sources are often better. The classic biochemistry texts of Mahler and Cordes (1971), and Lehninger et al. (2000) gave substantial attention to mechanisms of enzyme-facilitated reactions. With respect to biochemical systems analysis Fell (1997) presents a view of standard flux balance analysis, while Voit (2002) presents a another view using powerlaw approaches, but both explore only steady-state situations. Likewise, the straightforward book of Cornish-Bowden (1995) on the fundamentals of enzyme kinetics caters to those with steady-state applications. The modern trend is to examine enzymes, like other proteins, in the light of their genetic heritage, so databases like WIT (http://wit.mcs.anl.gov/WIT2/) and ECOcyc (http://ecocyc.org/) are ordered along the lines of bacterial and other genomes. Manipulation of the genome to enhance selected protein production is described by Palsson in his book (Palsson, 2006). A book on unsteady-state analysis of biochemical systems is not altogether lacking for not only is there excellent treatment of transients in Reich and Sel'kov (1981), but there are extensive mathematical systems analyses in Westerhoff and van Dam (1987).

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