Biophysical Journal Volume 67 October 1994 1414–1428

Role of Substrate Inhibition Kinetics in Enzymatic Chemical Oscillations

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ABSTRACT Two chemical kinetic models are investigated using standard nonlinear dynamics techniques to determine the conditions under which substrate inhibition kinetics can lead to oscillations. The first model is a classical substrate inhibition scheme based on Michaelis-Menten kinetics and involves a single substrate. Only when this reaction takes place in a flow reactor (i.e., both substrate and product are taken to follow reversible flow terms) are oscillations observed; however, the range of parameter values over which such oscillations occur is so narrow it is experimentally unobservable. A second model based on a general mechanism applied to the kinetics of many pH-dependent enzymes is also studied. This second model includes both substrate inhibition kinetics as well as autocatalysis through the activation of the enzyme by hydrogen ion. We find that it is the autocatalysis that is always responsible for oscillatory behavior in this scheme. The substrate inhibition terms affect the steady-state behavior but do not lead to oscillations unless product inhibition *or* multiple substrates are present; this is a general conclusion we can draw from our studies of both the classical substrate inhibition scheme and the pH-dependent enzyme mechanism. Finally, an analysis of the nullclines for these two models allows us to prove that the nullcline slopes must have a negative value for oscillatory behavior to exist; this proof can explain our results. From our analysis, we conclude with a brief discussion of other enzymes that might be expected to produce oscillatory behavior based on a pH-dependent substrate inhibition mechanism.

INTRODUCTION

Oscillatory behavior has been observed in several enzyme reaction systems (Field and Györgyi); its chemical kinetic source has been generally attributed to an autocatalytic mechanism. The question arises whether autocatalysis is a *necessary* condition for oscillations or whether it is merely *sufficient*; might other types of kinetics explain oscillatory behavior? A number of early studies suggested that substrate inhibition kinetics could also be a source of oscillatory behavior in chemical systems, particularly enzyme reactions. Spangler and Snell (1961, 1967) studied a two-enzyme model system in which the product of one enzyme-catalyzed reaction acted as an inhibitor for the other enzyme; this two-enzyme model was shown to exhibit bistability and sustained oscillations. Sel'kov (1968) investigated a single enzyme model involving both substrate inhibition and product activation (dynamically equivalent to autocatalysis) and observed oscillatory behavior. The oscillations observed were attributed to the substrate inhibition kinetics, but no proof of this assertion was given. Seelig (1976) investigated a model involving a single enzyme with substrate inhibition kinetics only (no product activation) and observed oscillations; however, this model involves two substrates, only one of which is an inhibitor. It is not clear whether the existence of multiple substrates is a necessary condition for oscillatory behavior in a system governed by substrate inhibition. Several examples exist in the literature (Degn and Harrison, 1969; Thomas, 1976; Lengyel et al., 1990), but they comprise only evidence of *sufficient* conditions for oscillatory behavior, but not *necessary* conditions.

The question remains, then, whether substrate inhibition involving only a single substrate can lead to oscillatory behavior. In this paper, we show that simple substrate inhibition is insufficient for oscillatory behavior to arise, contrary to the previous assertions. We further investigate a general model of an enzyme reaction, applicable to a large number of common enzyme-catalyzed reactions, which involves both substrate inhibition and autocatalysis. Our careful investigation shows that although oscillatory behavior is observed in this system, it is caused by either (1) the autocatalytic properties of the mechanism or (2) substrate inhibition coupled with product inhibition. Substrate inhibition alone is insufficient for oscillatory behavior in this mechanism. We apply the results of our simulations to the acetylcholinesterase enzyme for which oscillatory behavior has been reported when the enzyme is immobilized in an inert matrix (Friboulet and Thomas, 1982). Our calculations raise a number of questions regarding the validity of these previous experimental investigations. We conclude by suggesting experiments involving other enzymes that should be more likely to exhibit oscillatory behavior according to our calculations with this well accepted enzyme kinetic model.

CLASSICAL SUBSTRATE INHIBITION

We begin by considering a definition of the term "substrate inhibition." Any reactant (substrate) that causes a *decrease* in the rate of production of product as its concentration *increases* will lead to a reaction that displays substrate inhibition kinetics. In enzyme reactions, a mechanism can often be written in which an explicit binding of the substrate to the enzyme catalyst takes place, rendering the enzyme temporarily unavailable for production of product. A modified

Received for publication 14 March 1994 and in final form 6 June 1994.

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0006-3495/94/10/1414/15 $2.00

Michaelis-Menten scheme is the simplest example and will be discussed below. Algebraically, a substrate inhibition mechanism will lead to a term in the equation for the rate of formation of product that is inversely related to the substrate in question in the following way:

$$\frac{dP}{dt} = \frac{f_1}{f_2 + S^n} + \cdots,$$

where P is the product, S is the substrate, f_1 and f_2 are functions that may involve other reactants and products, and n is a number *larger than one*. Examples in the literature with terms of this form in the kinetic equations, then, are sometimes attributed to "substrate inhibition" (Lengyel et al., 1990) even if no binding process that ties up a catalyst is ever identified. We will consider both definitions in this paper.

The simplest enzyme kinetic model involving substrate inhibition is the Michaelis-Menten mechanism modified by adding a simple substrate-binding equilibrium:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad ES \xrightarrow{k_2} E + P \qquad ES + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES_2. \tag{1}$$

Degn (1968) studied this classical substrate inhibition mechanism graphically and showed that when the substrate S is allowed to flow into the system from a reservoir of constant concentration S_o,

$$S_o \overset{K}{\rightleftharpoons} S \tag{2}$$

that bistability could exist in this system (see Fig. 1). The rate expressions given in the figure are those found using the standard quasi-steady-state (QSS) approximation; because bistability involves the steady-state solutions of Eqs. 1 and 2, the QSS approximation becomes exact for this case. Aguda and Clarke (1987) analyzed the dynamics of the classical substrate inhibition mechanism using stoichiometric network analysis (Clarke, 1980) and found that the flow step, Eq. 2, is a critical feature of the network for the existence of bistability; without this feature (i.e., without a flow term), bistability is not seen in the classical substrate inhibition scheme.

We have continued the study of this simple model to determine whether oscillatory behavior can be supported by it. Using the QSS approximation, the variables $[ES]$ and $[ES_2]$ can be eliminated from the system of rate equations. Taking the total enzyme concentration to be constant, i.e., $[E_o] = [E] + [ES] + [ES_2]$, we can eliminate $[E]$ as well. Thus, in the QSS approximation the classical substrate inhibition scheme has only one dynamical variable, $[S]$, and would not be expected to produce oscillations.

To determine whether the QSS prediction holds for the full system, we carried out a standard linear stability analysis (Gray and Scott, 1990) of the three steady states found by Degn. The full set of rate equations corresponding to Eqs. 1 and 2 can be written in the usual way using mass-action kinetics. Taking the total enzyme concentration to be a constant, $[E_o]$, we again eliminate one of the enzyme variables (here we choose to eliminate $[ES]$). Furthermore, the system can be simplified by de-dimensionalizing the rate equations using the following definitions:

$$\tau = k_1[E_o]t, \qquad s_o = \frac{[S_o]}{[E_o]}, \qquad s = \frac{[S]}{[E_o]}, \qquad e = \frac{[E]}{[E_o]}$$

$$es_2 = \frac{[ES_2]}{[E_o]}, \qquad es = 1 - e - es_2, \qquad a = \frac{K}{k_1[E_o]},$$

$$b = \frac{k_2}{k_1[E_o]}, \qquad c = \frac{k_{-3}}{k_1[E_o]}, \qquad p = \frac{k_3}{k_1}, \qquad f = \frac{k_{-1}}{k_1[E_o]}$$

The de-dimensionalized rate equations then become

$$\begin{aligned}
\frac{ds}{d\tau} &= a(s_o - s) - se - ps(1 - e - es_2) + ces_2 \\
&\quad + f(1 - e - es_2) \\
\frac{de}{d\tau} &= -se + b(1 - e - es_2) + f(1 - e - es_2) \\
\frac{des_2}{d\tau} &= p(1 - e - es_2)s - ces_2.
\end{aligned} \tag{3}$$

The steady-state solutions of Eq. 3 are easily found by setting the right-hand sides equal to zero and simultaneously solving the resulting system for s, e, and es_2. The stability of the steady states are determined by linearizing the system 3 and finding the

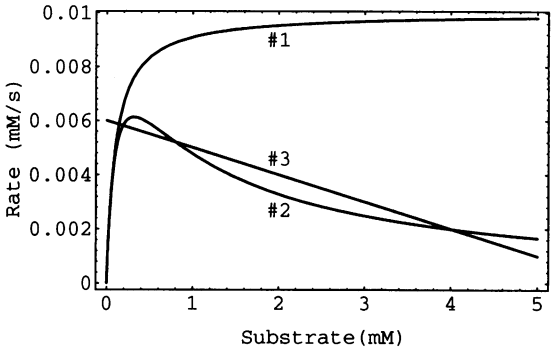

FIGURE 1 Enzyme reaction rate in a bistable system. (curve #1) Rate of Michaelis-Menten mechanism alone. (curve #2) Rate of Michaelis-Menten mechanism plus substrate inhibition from Eq. 1. (curve #3) Substrate flow rate into reaction system. Bistability will exist when substrate inhibition is combined with a flow term (i.e., where curves #2 and #3 intersect; the middle intersection is unstable). The three rate expressions are given below:

$$\text{Rate } (\#1) = \frac{k_2 E_0 S}{K_m + S} \quad \text{(Michaelis-Menten alone)}$$

$$\text{Rate } (\#2) = \frac{k_2 E_0 S}{K_m + S + (k_3/k_{-3})S^{-2}} \quad \text{(Michaelis-Menten + Substrate inhibition}$$

$$\text{Rate } (\#3) = K(S_0 - S) \quad \text{(Flow Rate)}$$

where

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

eigenvalues of the Jacobian matrix evaluated at one of the steady states. The resulting characteristic equation is

$$\lambda^3 + b_1\lambda^2 + c_1\lambda + d_1 = 0. \tag{4}$$

where the coefficients are defined as

$$b_1 = a + b + c + e + esp + s + ps + f$$

$$c_1 = ab + ac + bc + be + ce + besp + as + cs + aps + 2eps + esps + ps^2 + af + cf + 2esfp$$

$$d_1 = abc + bce + acs - besps + aps^2 + acf.$$

The presence of a Hopf bifurcation in this system can be most easily detected by re-writing the characteristic Eq. 4 in the following form:

$$(\lambda^2 + \omega^2)(\lambda + a_1) = 0. \tag{5}$$

The three eigenvalues, then, will be

$$\lambda_{1,2} = \pm i\omega, \qquad \lambda_3 = -a_1, \tag{6}$$

because a Hopf bifurcation occurs when the real part of a complex conjugate pair of eigenvalues changes sign; hence, the existence of eigenvalues $\lambda_{1,2}$ in the form above is a necessary condition for oscillatory behavior. Comparing the two forms of the characteristic Eqs. 4 and 5 allows the following identifications:

$$b_1 = a_1, \qquad c_1 = \omega^2, \qquad d_1 = a_1\omega^2.$$

Hence, a condition that guarantees the existence of a Hopf bifurcation in this system is

$$b_1c_1 - d_1 = 0. \tag{7}$$

By substituting the preceding expressions for b_1, c_1 and d_1, it is easy to show that every term in the resulting expression for Eq. 7 is positive regardless of the value of the steady state; hence, none of the steady states will ever exhibit a Hopf bifurcation, regardless of parameter values. These calculations prove that although the classical substrate inhibition mechanism is sufficient for bistability, oscillatory behavior cannot be sustained by such a mechanism. The results of Seelig (1976) and others (Degn and Harrison, 1969; Thomas, 1976; Lengyel et al., 1990) indicate that a more complex scheme is necessary; in particular, multiple substrates in a system with substrate inhibition kinetics can lead to oscillatory behavior.

SUBSTRATE INHIBITION WITH REVERSIBLE PRODUCT FORMATION

From the above results, it is clear that the classical substrate inhibition scheme does not support oscillations because it is inherently a one-variable system. By making the second step of scheme 1 reversible, however, we will introduce a second quantity (the product, P) into the system as a dynamical variable and will also provide, in essence, a second substrate for the enzyme:

$$E + S \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} ES \qquad ES \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E + P \qquad ES + S \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} ES_2. \tag{8}$$

The QSS expression for the rate of reaction, R, for the reversible mechanism (Eq. 8) is

$$R = \frac{E_{02}(S - K_1K_2P)}{K_m + (1 + S/K_3)(S + K_{n2}P)}, \tag{9}$$

where

$$K_1 = \frac{k_{-1}}{k_1}, \qquad K_2 = \frac{k_{-2}}{k_2}, \qquad K_3 = \frac{k_{-3}}{k_3},$$

$$K_m = \frac{k_{-1} + k_2}{k_1}, \qquad K_{n2} = \frac{k_{-2}}{k_1}, \qquad \text{and} \qquad E_{02} = k_2E_0.$$

As for the classical substrate inhibition scheme of the previous section, we consider an open system in which the dynamical variables are allowed to enter and/or leave the system via diffusion. To simplify our presentation, we consider first the case in which the substrate only *diffuses in* from a reservoir of constant concentration S_o, whereas the product only *diffuses out*; i.e., the following pseudo-reactions are included with mechanism 8.

$$S_0 \overset{k_0}{\rightarrow} S \qquad P \overset{k_p}{\rightarrow} P_0 \tag{10}$$

From QSS theory, the rate equations for the two dynamical variables substrate (S) and product (P) can be written

$$\frac{dS}{dt} = k_0S_0 - R \qquad \frac{dP}{dt} = k_pP + R, \tag{11}$$

where R is given by Eq. 9. An analysis of the steady-state solutions of Eq. 11 will indicate whether oscillatory behavior is possible for the reversible system. Again, a linear stability analysis was carried out in which the Jacobian matrix J is given by

$$J = \begin{pmatrix} -\frac{\partial R}{\partial S} & -\frac{\partial R}{\partial P} \\ \frac{\partial R}{\partial S} & -k_p + \frac{\partial R}{\partial P} \end{pmatrix}. \tag{12}$$

The eigenvalues of J, λ, satisfy the characteristic equation

$$\lambda^2 - \mathbf{tr}\lambda + \mathbf{det} = 0. \tag{13}$$

where $\mathbf{tr} = -\partial R/\partial S - k_p + \partial R/\partial P$ is the trace of J and $\mathbf{det} = k_p\,\partial R/\partial S$ is its determinant.

Oscillatory behavior may arise only if the steady states of the system are unstable foci or unstable nodes, which occurs when **tr** and **det** are both greater than zero. One can show that

$$\frac{\partial R}{\partial P} = -\frac{E_{02}K_3(\mathcal{A})}{(K_mK_3 + K_{n2}K_3P + K_3S + K_{n2}PS + S^2)^2} < 0,$$

where

$$\mathcal{A} = K_mK_1K_2K_3 + K_{n2}K_3S + K_1K_2K_3S + K_{n2}S^2 + K_1K_2S^2$$

which is always less than zero; therefore, $\partial R/\partial S$ must also be less than zero for $\mathbf{tr} > 0$. However, if $\partial R/\partial S < 0$, **det** will be less than

zero, too. Therefore, there is no way for the **tr** and **det** to both be larger than zero at the same time and we can conclude that no oscillations will occur for the system (Eqs. 8–10). Hence, simply adding the reversible product formation is insufficient to render the classical substrate inhibition scheme oscillatory.

To determine whether the restrictions on diffusion through the pseudo-reactions Eq. 10 are responsible for these results, we consider what changes will occur if we allow the diffusion steps in Eq. 10 to be reversible:

$$S_0 \overset{k_0}{\rightleftharpoons} S \qquad P \overset{k_P}{\rightleftharpoons} P_0. \tag{14}$$

The rate equations for S and P will become

$$\frac{dS}{dt} = k_0 (S_0 - S) - R \qquad \frac{dP}{dt} = k_P(P_0 - P) + R. \tag{15}$$

We de-dimensionalize Eq. 15 by setting $u = k_P/k_0$, $\tau = tk_0$, $E_{02} = k_2E_0/k_0$, and $\mathbf{R} = R/k_0$:

$$\frac{dS}{d\tau} = S_0 - S - \mathbf{R} \qquad \frac{dP}{d\tau} = u(P_0 - P) + \mathbf{R}. \tag{16}$$

The Jacobian matrix of Eq. 16 is

$$J = \begin{pmatrix} -1 - \dfrac{\partial R}{\partial S} & -\dfrac{\partial R}{\partial P} \\ \dfrac{\partial R}{\partial S} & -u + \dfrac{\partial R}{\partial P} \end{pmatrix}. \tag{17}$$

For the matrix J given by Eq. 17, the trace is $\mathbf{tr} = -1 - u - \partial\mathbf{R}/\partial S + \partial\mathbf{R}/\partial P$, and the determinant is $\mathbf{det} = u - \partial\mathbf{R}/\partial P + u\,\partial\mathbf{R}/\partial S$.

As before, the conditions for oscillation are that both **tr** and **det** are greater than zero. Now, however, there are more terms in **det** than just $\partial\mathbf{R}/\partial S$ alone. Because $\partial\mathbf{R}/\partial P$ is always less than zero, it is required that $\partial\mathbf{R}/\partial S$ be also always less than zero to ensure that $\mathbf{tr} > 0$. A visual inspection of the QSS reaction rate R versus substrate concentration S curve (Fig. 2) reveals that this requirement is the same as saying that the substrate concentration must fall to the right of the maximum; this range of values of S is the range in which substrate inhibition dominates the rate. Also, starting from the conditions that the **tr** and **det** are both larger than zero, one can derive that u must be less than 1, meaning that $k_0 > k_p$, i.e., the diffusion rate of substrate into the system must be greater than the rate of product diffusion out. So, oscillatory behavior does become possible for the classical substrate inhibition scheme when the product formation is made reversible and both substrate and product are taken to follow reversible pseudo-reactions for diffusion. However, we will show below that the range of parameter values over which oscillatory behavior can occur is so narrow it is essentially undetectable.

For certain ranges of parameter values, the system also exhibits bistability. Fig. 3 shows the steady-state values of S as a function of S_0 for different E_{02} (equal to k_2E_0/k_0) values. At higher enzyme concentration, the system exhibits multiple steady states, i.e., bistability. The region of parameter space in which oscillations are possible (i.e., for which $\mathbf{tr} > 0$ and $\mathbf{det} > 0$) is shown in terms of the parameters S_0 and E_{02} in Fig. 4. The oscillatory region is extremely narrow, and it is doubtful that any oscillations could ever be observed experimentally in such a system. Oscillations calculated via computer simulations are shown in Fig. 5. Sustained oscillations occurred over a very narrow range of E_{02} values (from 0.0433 to 0.0434 M, see Fig. 5 for other values of parameters) as predicted from the linear stability analysis.

SUBSTRATE INHIBITION + AUTOCATALYSIS

As reviewed in the introduction, an early study by Sel'kov (1968) of a mechanism involving both substrate inhibition and product activation was found to exhibit oscillatory behavior; although Sel'kov attributed this behavior to the substrate inhibition nature of the mechanism, the results of the previous section make it more likely that the product activation kinetics (which is essentially the same as autocatalysis) is actually the key mechanistic feature. In a chemical

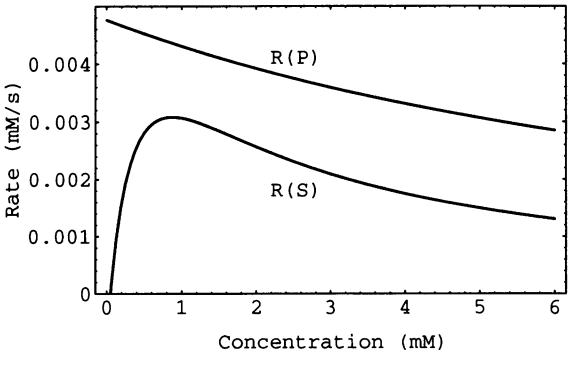

FIGURE 2 Enzyme reaction rate as a function of substrate concentration (curve $R(S)$) and product concentration (curve $R(P)$) calculated from Eq. 9. Parameter values are: $E_{02} = 0.01\ s^{-1}$, $K_m = 0.1$ mM, $K_{12} = K_1K_2 = 0.01$, $K_3 = 1.0$ mM, and $k_{n2} = 0.1$. For Curve $R(S)$, $P = 5.0$ mM; for $R(P)$, $S = 1.0$ mM.

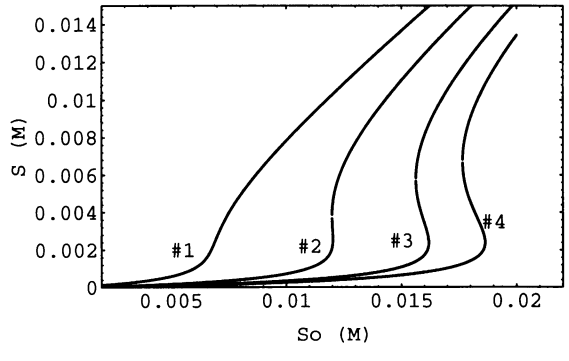

FIGURE 3 Steady-state values of substrate concentration (S) as a function of parameter S_0, the substrate concentration in the reservoir, for different values of $E_{02} = k_2E_0/k_0$. #1, #2, #3, and #4 correspond to $E_{02} = 0.02, 0.05$, 0.08, and 0.1, respectively. Bistability occurs for $E_{02} > 0.05$. Other parameter values are: $K_m = 1.0$ mM, $K_{12} = K_1K_2 = 0.0001$, $u = 0.1$, $P_0 = 0$, $K_3 = 1.0$ mM, and $k_{n2} = 0.01$.

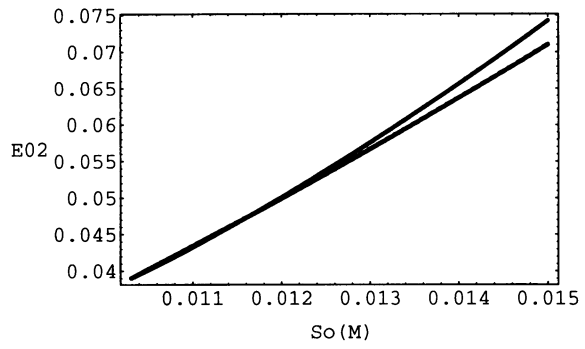

FIGURE 4 The oscillatory region in parameter (S_0 − E_{02}) space. Oscillations exist for S_0 and E_{02} values that fall within (not between) the narrow black lines; the oscillatory region is calculated from the conditions **tr** > 0 and **det** > 0. Other parameter values are: K_m = 1.0 mM, $K_{12} = K_1K_2$ = 0.0001, u = 0.1, P_0 = 0, K_3 = 1.0 mM, and k_{n2} = 0.01.

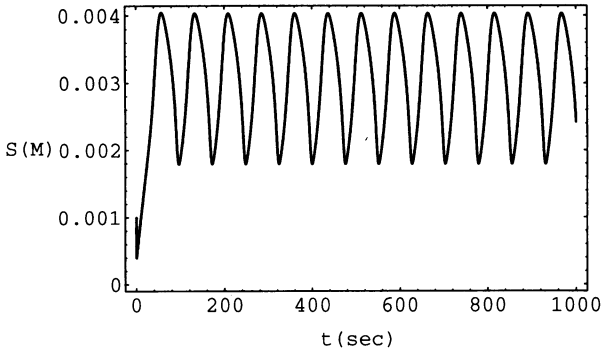

FIGURE 5 Oscillations in substrate concentration calculated from computer simulation of Eq. 16. Parameter values are: S_0 = 0.011 M, E_{02} = 0.0433 M, K_m = 1.0 mM, $K_{12} = K_1K_2$ = 0.0001, u = 0.1, P_0 = 0, K_3 = 1.0 mM, and k_{n2} = 0.01. The oscillations occur only in a very narrow parameter region; for example, if S_0 is fixed at 0.011 M, sustained oscillations only occur at E_{02} values from 0.0433 to 0.0434 M (see Fig. 4).

mechanism, key kinetic features cannot be totally separated. The *combination* of the effects of substrate inhibition and autocatalysis may lead to dynamical behavior that goes beyond the well understood effects of autocatalysis alone. Rather than re-investigating the Sel'kov mechanism, we chose to look at a more experimentally meaningful but similar mechanism. The model we study in this section has been used to describe quantitatively the kinetics of many enzyme systems (Ableles et al., 1992), among these acetylcholinesterase (AChE) and papain. In these enzyme systems, one of the products is H^+ and the enzymes are pH-dependent; the mechanism is slightly more complex than that considered in the first section:

$$\begin{aligned} E + S &\overset{K_s}{\rightleftharpoons} ES \qquad ES \overset{k_2}{\rightarrow} E^* + P_1 \\ E^* &\overset{k_3}{\rightarrow} E + P_2 + H^+ \qquad E^* + S \overset{K_{ss}}{\rightleftharpoons} E^*S. \end{aligned} \tag{18}$$

Here, two products (P_1 and P_2) are formed in subsequent steps from two forms of the enzyme-substrate intermediate complex (ES and E^*). The second of these, E^*, is involved in the substrate inhibition step (the last step, taken to be reversible). For AChE, the following species identifications can be made:

$$E = \text{AChE}, \qquad S = \text{ACh}^+ \text{ (acetylcholine)},$$

$$P_1 = \text{choline}, \qquad P_2 = \text{acetate anion}.$$

The form E^* is known as the acetyl enzyme species. Using the QSS approximation, the following reaction rate law is observed to hold for this system (Chay and Zabusky, 1983):

$$\text{Rate} = \frac{V_{max}S}{K_{sa} + S + S^2/K_{ssa}}, \tag{19}$$

where

$$V_{max} = \frac{k_2k_3E_0}{k_2 + k_3}, \qquad K_{sa} = \frac{K_2k_3}{k_2 + k_3},$$

$$K_{ssa} = \frac{K_{ss}(k_2 + k_3)}{k_2}.$$

The dependence of the rate expression (Eq. 19) on the substrate concentration is qualitatively the same as that in the classical substrate inhibition model (see rate expressions in Fig. 1), i.e., substrate inhibition is characterized by a *higher than linear* dependence on substrate in the denominator of the QSS expression. Hence, the substrate S inhibits the reaction in the same way S does in the classical substrate inhibition scheme. Indeed, plots of Eq. 19 are qualitatively similar to curve #2 labeled "M-M + Sub Inh" in Fig. 1 (see Fig. 6 *b*). Hence, we might expect that this mechanism would exhibit bistability but not oscillations.

Zabusky and others have shown (Zabusky and Hardin, 1973; Caplan et al., 1973) via numerical simulations of a model of this form that limit cycle oscillations do occur. The papain enzyme was modeled in these studies, i.e., the parameter values used in these simulations are those for papain. It is important to note that for papain K_{SS} = 0, i.e., papain does not exhibit substrate inhibition. However, papain (as well as all the other enzymes in this class) have pH-dependent rate constants, k_2 and k_3, that yield a bell-shaped enzyme activity curve as shown in Fig. 6 *a*. This diagram shows the rate (from Eq. 19) as a function of pH for a fixed substrate concentration using the pH-dependent rate constants that apply to AChE. To the right of the maximum in the curve (i.e., for pH > 8.5), the reaction is actually autocatalytic in H^+. H^+ is a product of the reaction, so as the reaction proceeds, one moves toward the *left* in Fig. 6 *a*. If the initial pH is very basic (pH > 8.5), the movement toward the left leads to an increasing rate and, hence, autocatalysis. So, although the reaction may be inhibited by substrate, it is simultaneously activated by one of the products (H^+). This feature is known (Hahn et al., 1974) to lead to oscillations in the absence of substrate inhibition effects.

In contrast with these theoretical predictions, no oscillations have ever been reported for the AChE system (also described by Eq. 18), except when the enzyme is immobilized in a membrane (Friboulet and Thomas, 1974); furthermore, we have been unable to reproduce the immobilized

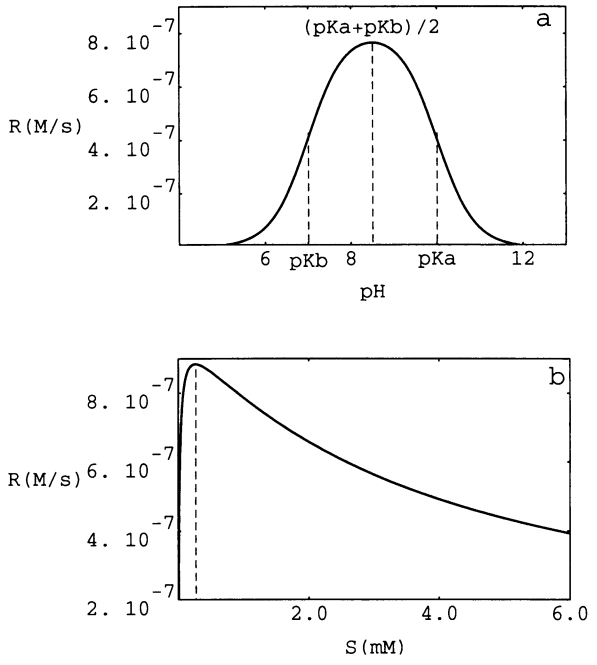

FIGURE 6 The rate of the pH-dependent enzyme reaction as a function of pH and substrate concentration. The rate R is given by Eq. 21. Parameter values for these plots are taken from data for AChE: $K_w = 10^{-14}$ M^2, $u = 0.1$, $b_1 = 3.865 \times 10^{-3}$ M, $b_2 = 1.786 \times 10^{-5}$ M, $K_a = 10^{-10}$ M, $K_b = 10^{-7}$ M, $K_h = 0.001$ s^{-1}, $\alpha = 8.571 \times 10^3$ s^{-1}, $H_0 = 10^{-10.8}$ M, and $E_0 = 1.3 \times 10^{-10}$ M. (*a*) Dependence of the enzyme reaction rate on pH. The maximum R value occurs when pH is equal to the average of the two equilibrium constants for the enzyme, pK_a and pK_b; for example, for AChE, the peak is at pH = 8.5. For high pH (to the right of the maximum), the reaction follows an autocatalytic mechanism because production of the product H^+ will increase the rate. For low pH (to the left of the maximum), the reaction is characterized by product inhibition. For this plot, parameter values are as given above with the following addition: $S = 5.0 \times 10^{-5}$ M. (*b*) Dependence of the enzyme reaction rate on S. The maximum R value occurs when S is equal to the square root of b_1 times b_2. To the right of the maximum, the reaction is characterized by substrate inhibition because increases in S will decrease the rate. For this plot, parameter values are given above with the following addition: $H = 10^{-9}$ M.

AChE experiments in which oscillations were reported. In addition, for the case of immobilized AChE, oscillatory behavior was reported at a pH of 7.5 for a substrate concentration, which corresponds to a pH optimum of approximately 8.0. (The pH optimum shifts toward the acid when this enzyme is immobilized, an effect that is well understood (Goldstein, 1974). The magnitude of the shift is dependent on the substrate concentration and is approximately 0.5 pH units at the substrate concentrations at which oscillations were reported.) Because the reported pH (at 7.5) at which oscillations were observed lies to the left of the pH optimum of 8.0, we would not expect oscillatory behavior to occur in this system on qualitative theoretical grounds using the argument that oscillations might occur if autocatalysis is dominant. However, because substrate inhibition also exists in this mechanism, it is possible that a complicated interaction might arise between the two important dynamic elements of substrate inhibition and autocatalysis in this system and that autocatalysis alone cannot fully explain the dynamical behavior of this enzyme. A second possibility is that the reported observations of oscillatory behavior in this system are in error (because, indeed, these experimental results have never been confirmed by other investigators).

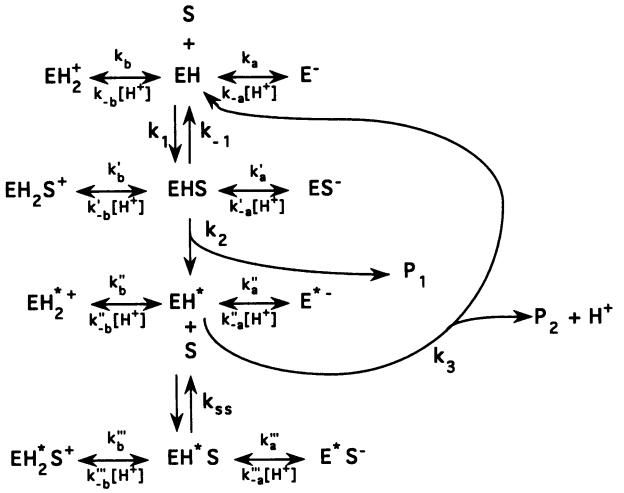

FIGURE 7 The full mechanism for the pH-dependent enzyme reaction model. The main reaction route occurs in the vertical direction. Each of the active enzyme species exists in equilibrium with inactive protonated and de-protonated forms. The equilibria are driven by the pH of the system. EH is the active form of the enzyme; EH_2 and E^- are the protonated and de-protonated inactive enzyme forms. EHS and EH* are enzyme intermediate complexes. The substrate can combine with EH* to form another complex EH*S that cannot react further to give product. So substrate inhibition is involved in this mechanism. P_1, P_2, and H^+ are the products of the reaction. The rate law for this mechanism is given in Eq. 20 with the following definitions:

$$K_a = \frac{k_a}{k_{-a}}, \quad K_a' = \frac{k_a'}{k_{-a}'}, \quad K_a'' = \frac{k_a''}{k_{-a}''}, \quad K_a''' = \frac{k_a'''}{k_{-a}''},$$

$$K_b = \frac{k_b}{k_{-b}}, \quad K_b' = \frac{k_b'}{k_{-b}'}, \quad K_b'' = \frac{k_b''}{k_{-b}''}, \quad K_b''' = \frac{k_b'''}{k_{-b}''}.$$

Because many enzymes obey the same type of kinetics as AChE, our investigation also has the general goal of determining under what set of conditions oscillatory behavior might be expected to occur in this entire class of enzymes. In the next section we derive expressions for the Hopf bifurcation conditions that allow us to highlight the dependence on the parameter values that characterize each individual enzyme in this class. With these results, we show that oscillatory behavior in AChE would be very difficult to observe experimentally (a result similar to that for the reversible classical substrate inhibition scheme); other enzymes in this class may have a broader range of oscillatory behavior that is much more amenable to experimental investigation. Such enzymes may be identifiable from the formulae we derive below.

PH-DEPENDENT MECHANISM

Fig. 7 shows a detailed mechanism that explains the source of the pH dependence of the rate constants in model 18. As can be seen, each enzyme species can exist in a protonated

or de-protonated form. This results in an apparently enormous number of variables to keep track of; however, in most cases, the protonation and de-protonation reactions occur very rapidly and are usually taken to exist in a pseudo-equilibrium state (Ableles, 1992). The equilibrium constants consisting of ratios of forward and reverse rate constants for the protonation/de-protonation equilibria are given in Fig. 7. This pseudo-equilibrium approximation will eliminate all of the enzyme species except *EH*, *EHS*, *EH**, and *EHS** as variables; these are analogous to *E*, *ES*, *E**, and *E*S* of the preceding section. If, then, the QSS approximation is made, the following rate law can be derived:

$$R = \frac{k_2 E H_o S}{\mathcal{B}}, \tag{20}$$

where

$$\mathcal{B} = K_m\left(1 + \frac{H}{K_b} + \frac{K_a}{H}\right) + S\left(1 + \frac{H}{K'_b} + \frac{K'_a}{H}\right) + \frac{k_2}{k_3}S\left(1 + \frac{H}{K''_b} + \frac{K''_a}{H}\right) + \frac{k_2 S^2}{k_3 K_{SS}}\left(1 + \frac{H}{K'''_b} + \frac{K'''_a}{H}\right)$$

where $H = [H^+]$, $K_m = (k_{-1} + k_2)/k_1$, and EH_o is the total enzyme concentration. If we make the simplifying assumption that all of the protonated and deprotonated forms of enzymes have the same equilibrium constant (i.e., that $K_a = K'_a = K''_a = K'''_a$ and $K_b = K'_b = K''_b = K'''_b$, but $K_a \neq K_b$), this expression can be simplified somewhat:

$$R = \frac{k_2 E H_o S}{\left(1 + \frac{H}{K_b} + \frac{K_a}{H}\right)\left(K_m + S\left(\frac{k_2 + k_3}{k_3}\right) + \frac{k_2 S^2}{k_3 K_{ss}}\right)}. \tag{21}$$

The resulting rate expression (Eq. 21) is similar to that used by other investigators (Chay and Zabusky, 1983) to fit experimental kinetic data and derive values of the rate constants k_2, k_3, etc.

TWO-VARIABLE MODEL

We consider an open system in which the reactions of Fig. 7 (under the simplifying assumptions described in the previous section) are supplemented by flow terms:

$$S_o \overset{K_o}{\rightleftharpoons} S \qquad [H^+]_o \overset{K_h}{\rightleftharpoons} [H^+] \qquad [OH^-]_o \overset{K_{oh}}{\rightleftharpoons} [OH^-]. \tag{22}$$

The reaction is thus taken to occur within a membrane that is fed by reservoirs of substrate at constant concentration and fixed pH (see Fig. 8). Taking the same approach as Zabusky (1973) (i.e., including the water equilibrium $H^+ + OH^- \rightleftharpoons H_2O$), we use the QSS approximation, which reduces the model to a pair of coupled ordinary differential equations:

$$\frac{dS}{dt} = K_o(S_o \cdot S) - R \tag{23a}$$

$$\frac{d\Delta}{dt} = K_h(\Delta_o - \Delta) + R, \tag{23b}$$

where R is given by Eq. 21 and $\Delta = [H^+] - [OH^-] = H - K_w/H$. We also assume that the transfer rate of H^+ is the same as that of OH^- ($K_h = K_{oh}$). Using the following definitions, Eq. 23 can be more easily analyzed:

$$\beta(S) = 1 + \frac{S}{b_1} + \frac{b_2}{S} \tag{24a}$$

$$\gamma(H) = 1 + \frac{H}{K_b} + \frac{K_a}{H} \tag{24b}$$

$$\alpha = \frac{k_2 k_3}{k_2 + k_3}, \tag{24c}$$

where the b_1 and b_2 coefficients are related to the parameters of the preceding section in the following way:

$$b_1 = \frac{(k_2 + k_3)K_{SS}}{k_2}, \qquad b_2 = \frac{k_3 K_m}{k_2 + k_3}.$$

Substituting the expressions 24 and the definition of Δ into Eq. 23 yields the following forms of the rate equations:

$$\frac{dS}{dt} = K_0(S_0 - S) - \frac{\alpha E_0}{\beta(S)\gamma(H)} \tag{25a}$$

$$\frac{dH}{dt} = \frac{H^2}{H^2 + K_w} \times \left[K_h\left(H_0 - \frac{K_w}{H_0} - H + \frac{K_w}{H}\right) + \frac{\alpha E_0}{\beta(S)\gamma(H)}\right]. \tag{25b}$$

We wish to determine whether the steady-state solutions of Eq. 25 undergo a Hopf bifurcation. Setting $dS/dt = dH/dt = 0$, we can eliminate S from the resulting pair of algebraic equations and derive the following 8th-order polynomial for H:

$$h_8H^8 + h_7H^7 + h_6H^6 + h_5H^5 + h_4H^4 + h_3H^3 + h_2H^2 + h_1H + h_0 = 0. \tag{26}$$

The roots of this polynomial corresponding to positive values of both S and H can then be determined using a standard root-finding algorithm; we used *FindRoots* in Mathematica (Wolfram Research).

To find the Hopf bifurcations in this system, we form the Jacobian matrix and evaluate it at one of the steady states found from the solution of Eq. 26:

$$\mathbf{J} = \begin{pmatrix} -K_0 + R\frac{\beta}{\beta'} & R\frac{\gamma}{\gamma'} \\ -\frac{H^2}{H^2 + K_w}R\frac{\beta}{\beta'} & -K_h - \frac{H^2}{H^2 + K_w}R\frac{\gamma}{\gamma'} \end{pmatrix}, \tag{27}$$

where, from Eq. 24, we see that β' and γ' are the following derivatives:

$$\beta' \equiv \frac{d\beta}{dS} = \frac{1}{b_1} - \frac{b_2}{S^2} \qquad \gamma' \equiv \frac{d\gamma}{dH} = \frac{1}{K_b} - \frac{K_a}{H^2} \tag{28}$$

The eigenvalues of Eq. 27 will thus satisfy the following

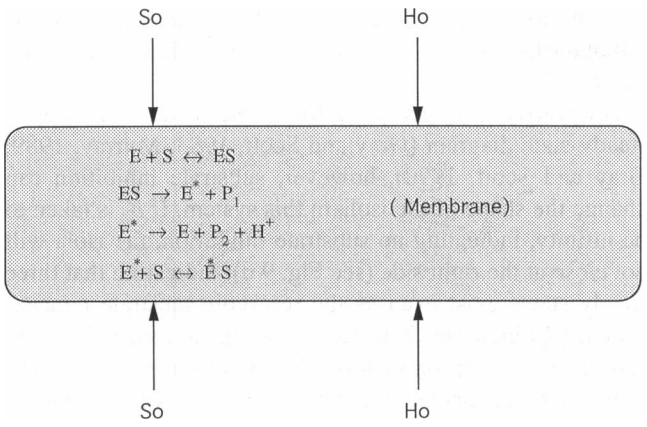

FIGURE 8 Schematic drawing of enzyme reaction occurring in open membrane system. The membrane is taken to be homogeneous with uniform enzyme distribution. The enzyme reaction takes place only inside the membrane. A reservoir at fixed pH_0 and constant substrate concentration, S_0, provides continuous feed of substrate and H^+ or OH^-. Diffusion and electric charges in the membrane are not considered in this model.

quadratic characteristic equation:

$$\lambda^2 - p\lambda + q = 0, \tag{29}$$

and the eigenvalues will take the following form:

$$\lambda_{\pm} = \frac{p}{2} \pm \frac{1}{2}\sqrt{p^2 - 4q} = \frac{p}{2} \pm \frac{1}{2}\sqrt{r}, \tag{30}$$

where

$$p = J_{11} + J_{22} = -(K_0 + K_h) + R\left(\frac{\beta'}{\beta} - \frac{H^2}{H^2 + K_w}\frac{\gamma'}{\gamma}\right) \tag{31a}$$

$$q = J_{11}J_{22} - J_{12}J_{21} = K_0K_h + R\left(\frac{H^2}{H^2 + K_w}K_0\frac{\gamma'}{\gamma} - K_h\frac{\beta'}{\beta}\right). \tag{31b}$$

Hopf bifurcations will occur when $r < 0$ while $p = 0$. Two possibilities exist that depend on the relative magnitudes of K_h and K_o. For the case $K_h > K_o$ (i.e., where H^+ has a higher transport coefficient than the substrate), the conditions for a Hopf bifurcation reduce to the conditions for the existence of a spiral focus ($r < 0$):

$$S < \sqrt{b_1 b_2} \tag{32}$$

$$H < \sqrt{K_a K_b}, \tag{33}$$

coupled with the condition that this spiral become unstable ($p > 0$). The latter condition results in the following expression, which S and H must satisfy:

$$R_{SS}\frac{\beta'}{\beta} - R_{SS}\frac{H^2}{H^2 + K_w}\frac{\gamma'}{\gamma} > (K_o + K_h), \tag{34}$$

where R_{SS} is the steady-state value of the enzyme reaction rate. Combining condition 32 with the condition that all concentrations are positive, we may use the steady-state equations to rewrite this result as an allowed range of reservoir concentrations:

$$\frac{R_{SS}}{K_0} < S_0 < \sqrt{b_1 b_2} + \frac{R_{SS}}{K_0} \tag{35}$$

where we note that R_{SS} is still a function of H (the concentration of H^+ within the membrane) and H_o (the concentration in the reservoir). Conditions 34 and 35, then, are the conditions for the existence of a Hopf bifurcation. The values of H that are allowed in this result are given by Eq. 33.

For the case described here (i.e., for $K_h > K_0$), we find that the allowed values of S fall to the *left* of the maximum in Fig. 6 *b*, whereas the allowed values of H fall to the *right* of the maximum in Fig. 6 *a*. Hence, the rate is *not* inhibited by substrate but *is* activated by product, i.e., the reaction is autocatalytic in H^+. Therefore, any oscillations found for this case are purely caused by autocatalysis.

Another case exists for which $K_h < K_0$. The conditions for the existence of a spiral focus ($r < 0$) for this case are

$$S > \sqrt{b_1 b_2} \tag{36a}$$

and

$$H > \sqrt{K_a K_b} \quad \text{or} \quad pH < \tfrac{1}{2}(pK_a + pK_b), \tag{36b}$$

which coupled with condition 34 (for $p > 0$) can yield a Hopf bifurcation. The conditions 36 correspond to the substrate inhibition range in Fig. 6 *b* and the product inhibition range in Fig. 6 *a* (i.e., no autocatalysis). Therefore, we find that oscillations can, in principle, occur when substrate inhibition is coupled with negative feedback (product inhibition) in this type of enzyme reaction.

EXAMPLE: ACETYLCHOLINESTERASE (ACHE)

To illustrate the general results derived in the preceding section, we have chosen the acetylcholinesterase enzyme, AChE, as an example. Rate constant data derived from experiment for this enzyme are given in Table 1 in the form of equilibrium and rate constants and values of b_1 and b_2 defined in Eq. 24. With these numerical values, the only free parameters that remain in the equations derived in the preceding section are S_o and H_o (the reservoir concentrations) and E_o (the total concentration of enzyme). We find that oscillatory behavior arises only through the autocatalytic process (i.e., K_h must be larger than K_0) for AChE.

TABLE 1 Parameter values for AChE (Chay and Zabusky, 1983)

K_a (M)	K_b (M)	K_m (M)	k_2 (s^{-1})	k_3 (s^{-1})
1×10^{-10}	1×10^{-7}	1.25×10^{-4}	6×10^4	1×10^4
b_1 (M)	b_2 (M)	K_{ss} (M)	K_h (s^{-1})	K_o (s^{-1})
3.865×10^{-3}	1.786×10^{-5}	3.3×10^{-3}	0.001	1×10^{-4}

Choosing chemically reasonable values for S_0, pH_0, and E_0, the steady-state Eq. 25 can be solved. Fig. 9 gives the steady-state dependence on pH_0 (Fig. 9 *a*) and on S_0 (Fig. 9 *b–d*). Fig. 9 *a* shows steady-state values of pH, and S and has a rough "S" shaped dependence on pH_0, meaning that multiple steady states are possible. The solid line is the stable node region, the dashed line represents a saddle point, and the unstable spiral region is indicated by very small empty circles below the saddle point region.

The steady-state dependence on S_0 is shown in Fig. 9 *b–d* and reveals the existence of an isola, another form of bistability. Isolas have been observed in some inorganic reactions (Gray and Scott, 1983; Murray, 1989; Gray and Scott, 1990), but none have been reported in biochemical or biological systems. The isola state only exists within a certain range of pH_0 and E_0 values. For example, if pH_0 is fixed at 10.8, the isola appears when the value of E_0 is between 0.8×10^{-10} M and 1.8×10^{-10} M. When E_0 is less than 0.8×10^{-10} M, the isola will shrink and disappear; when E_0 is larger than 1.8×10^{-10} M, the isola will connect to the upper steady-state line and become a mushroom. Furthermore, it appears that the AChE system will display sustained oscillations only when the parameter values are such that the isola exists. The range of S_0 values that can produce sustained oscillations is small (0.0059–0.0061 M) and located to the left of the isola (*empty circles*) next to the saddle point region (*dashed lines*).

Autocatalysis is generally the cause of an isola-shaped steady-state diagram (Gray and Scott, 1983; Murray, 1989; Gray and Scott, 1990); however, substrate inhibition can change the shape of the isola in this system. If b_1 is taken to be infinity, indicating no substrate inhibition, the isola will not close at the right side (see Fig. 9 d), indicating that three steady states exist even as the reservoir substrate concentration (S_0) increases beyond the biological range. But if b_1 is decreased to approximately 10^{-3} M, indicating strong substrate inhibition, another multiple steady-state region at low S_0 will appear (a very small "S" shape in Fig. 9 *c*). We conclude that substrate inhibition kinetics increases the complexity of the system beyond that due to autocatalysis alone.

The range of (S_o, pH_o, E_o) values over which Hopf bifurcations occur is shown in Fig. 10. Note that the lowest external pH values for which oscillatory behavior is observed is $pH_o = 10.2$. This result is in sharp contrast to that predicted from the qualitative autocatalytic kinetics for this system. As shown in Fig. 6 *a*, we would expect oscillatory behavior whenever autocatalysis is dominating, which corresponds to pH values greater than or equal to 8.5 (or 8.0 in the immo-

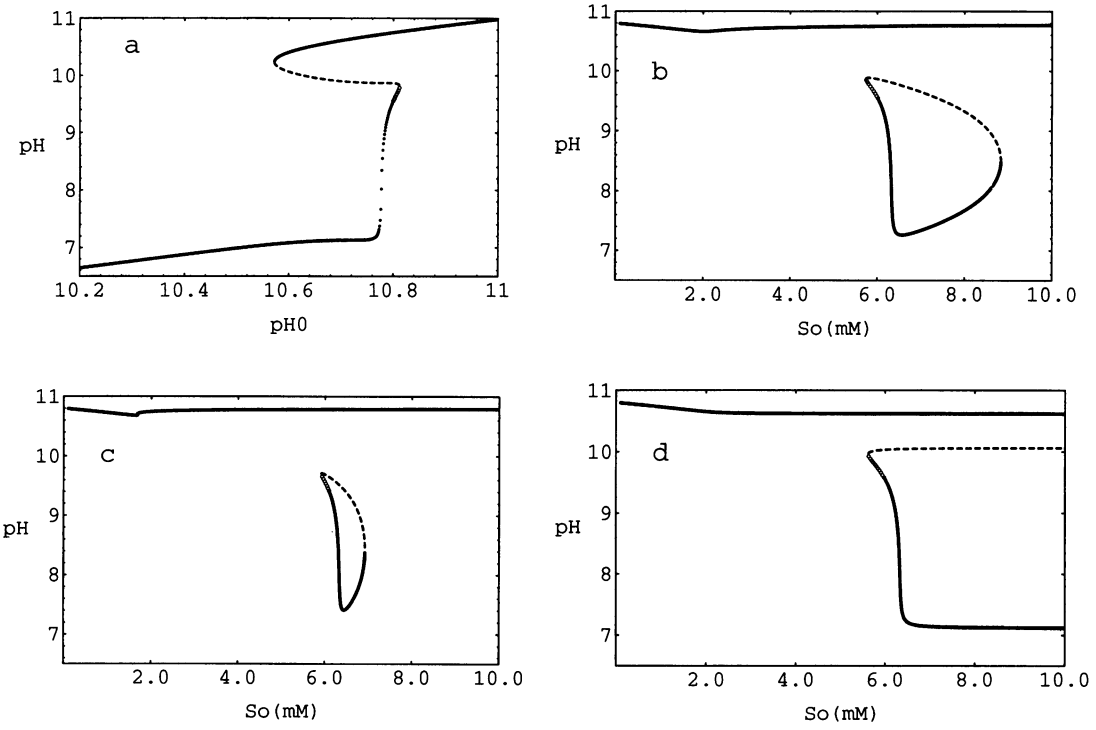

FIGURE 9 Steady-state pH value inside membrane as a function of control parameters pH_0 and S_0 for AChE. Other parameter values are: $K_w = 10^{-14}$ M^2, $u = 0.1$, $b_1 = 3.865 \times 10^{-3}$ M, $b_2 = 1.786 \times 10^{-5}$ M, $K_a = 10^{-10}$ M, $K_b = 10^{-7}$ M, $K_h = 0.001$ s^{-1}, $\alpha = 8.571 \times 10^3$ s^{-1}. For parts *a–d* in a certain range of parameters, the pH shows multiple steady states. The solid line or points represent the stable region (stable nodes and stable foci), the dashed line represents a saddle point, and the empty circles represent either unstable focus or unstable node; for the latter regions, we can expect oscillatory behavior. (*a*) Steady-state pH dependence on pH_0 is a common "S" shaped curve. Here $S_0 = 0.006$ M and $E_0 = 1.3 \times 10^{-10}$ M. (*b*) Steady-state pH as a function of S_0 gives an isola shape at certain parameter ranges. Here $pH_0 = 10.8$ and $E_0 = 1.3 \times 10^{-10}$ M. (*c*) The effect of increasing substrate inhibition. Here $b_1 = 1.0 \times 10^{-3}$ M. For smaller values of b_1, substrate inhibition increases. (*d*) No substrate inhibition. Here b_1 is taken to infinity, indicating no substrate inhibition.

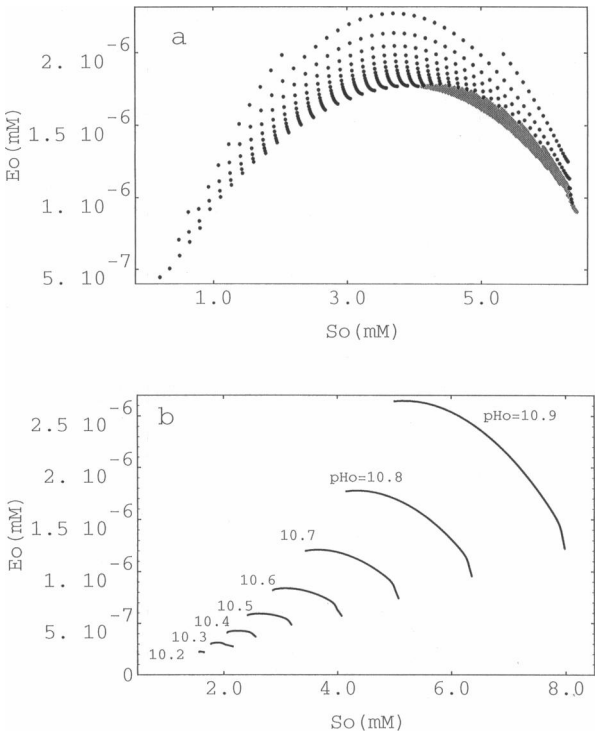

FIGURE 10 Oscillation and bifurcation regions in $S_0 - E_0$ space for the AChE reaction system. Parameter values are: $K_w = 10^{-14}$ M^2, $u = 0.1$, $b_1 = 3.865 \times 10^{-3}$ M, $b_2 = 1.786 \times 10^{-5}$ M, $K_a = 10^{-10}$ M, $K_b = 10^{-7}$ M, $K_h = 0.001$ s^{-1}, $\alpha = 8.571 \times 10^3$ s^{-1}. (*a*) Oscillation region in $S_0 - E_0$ parameter space at $pH_0 = 10.8$. The dotted area corresponds to damped oscillations, i.e., the region in which the steady state is a stable focus; the shaded area corresponds to possible limit cycle oscillations, i.e., an unstable focus or unstable node. (*b*) The bifurcation region in $S_0 - E_0$ parameter space for different values of pH_0.

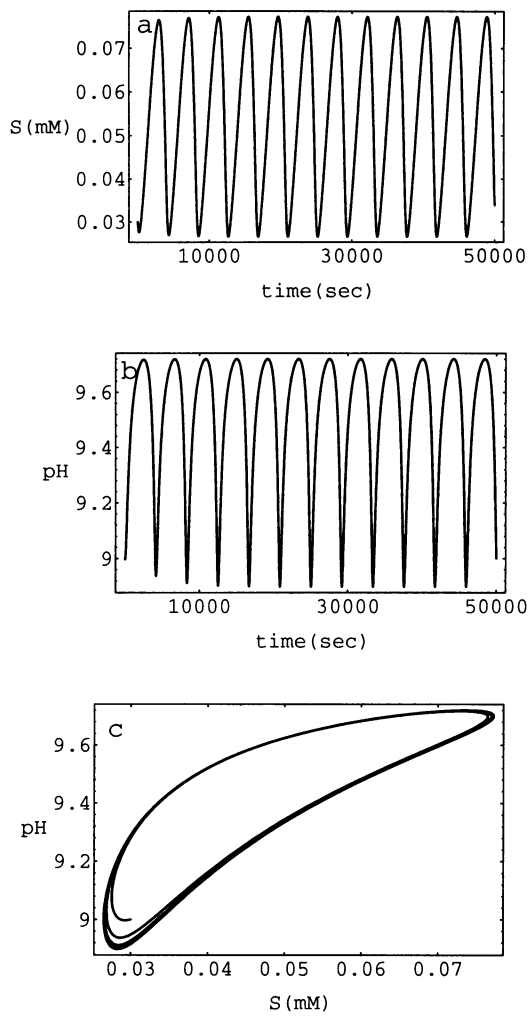

FIGURE 11 Simulated oscillations in AChE reaction system. Parameter values are: $K_w = 10^{-14}$ M^2, $u = 0.1$, $b_1 = 3.865 \times 10^{-3}$ M, $b_2 = 1.786 \times 10^{-5}$ M, $K_a = 10^{-10}$ M, $K_b = 10^{-7}$ M, $K_h = 0.001$ s^{-1}, $\alpha = 8.571 \times 10^3$ s^{-1}, $S_0 = 0.006$ M, $E_0 = 1.3 \times 10^{-10}$ M, and $pH_0 = 10.8$. (*a*) Oscillations in substrate concentration inside the membrane. (*b*) Oscillations in pH inside the membrane. (*c*) Limit cycle in S - pH space.

bilized enzyme case); the discrepancy between the qualitative prediction and the calculated cutoff amounts to approximately two orders of magnitude in the reservoir $[H^+]_0$.

Because of this discrepancy between qualitative prediction and calculation, we checked the linear stability analysis results by carrying out numerical simulations in the range for which oscillatory behavior is predicted from the linear analysis. Fig. 11 shows the oscillations, which are indeed observed only within the range calculated from the linear stability analysis. The parameter region in which these simulated oscillations occur is extremely small. In addition, the period of the oscillations is quite long (nearly 0.5 h), so it is doubtful that such oscillations would even be detected experimentally. (It should be noted here that the oscillations observed by Friboulet and Thomas for the immobilized AChE case were oscillations in the transmembrane potential. Because our analysis does not include any electrical effects, we are unable to calculate the expected response of the electrical potential to these concentration oscillations. We hope to extend our analysis in the future to include these electrical effects.)

We have studied further the general conditions that lead to oscillatory behavior for the case $K_h > K_0$. Recall that the conditions for the existence of a Hopf bifurcation in this case reduce to: (1) H^+ concentrations within the autocatalytic range, i.e., $pH > (pK_a + pK_b)/2$; and (2) S concentrations in the range for which substrate inhibition is inoperative, i.e., $S < S_{max}$, where S_{max} is the value of S at which R has a maximum. Beyond this, we can also conclude that oscillations will only occur if the enzyme has a small enough value of K_a, i.e., that $pK_a > 8$. Both papain ($pK_a = 8.2$) and AChE ($pK_a = 10.0$) fall in this category, then, and any oscillatory behavior observed experimentally for these enzymes can be explained by a mechanism that relies on the effects of autocatalysis only.

EXAMPLE: A PROTEASE ENZYME TO ILLUSTRATE SUBSTRATE INHIBITION

Oscillatory behavior can also arise via substrate inhibition coupling with product inhibition in these pH-dependent enzyme reactions. An imaginary protease enzyme with rate

TABLE 2 Parameter values for a protease enzyme

K_a (M)	K_b (M)	K_m (M)	k_2 (s^{-1})	k_3 (s^{-1})
1×10^{-7}	1×10^{-4}	2.0×10^{-5}	1×10^{2}	1×10^{2}
b_1 (M)	b_2 (M)	K_{ss} (M)	K_h (s^{-1})	K_o (s^{-1})
1.0×10^{-4}	1.0×10^{-5}	5.0×10^{-5}	0.001	0.004

constant parameters as shown in Table 2 was chosen to illustrate the substrate inhibition route that could lead to oscillations. These parameter values do not correspond to any known enzyme, but they are not chemically unreasonable. The predictions we calculate here illustrate the behavior of any real enzymes that might, in the future, be found to follow predominately substrate inhibition kinetics in this form. Substrate inhibition is known to lead to oscillations in a two-substrate system; here we have two dynamical variables, one substrate and one product (H^+). The steady-state values as a function of pH_o are shown in Fig. 12. Both a single steady-state region (Fig. 12 *a*) and the multiple steady-state region (Fig. 12 *b* and *c*) are found to possess a Hopf bifurcation, so this system may possibly exhibit oscillatory behavior. The oscillatory region in the $S_o - E_o$ parameter plane is shown in Fig. 13. At low E_o, one narrow region exists; at high E_o, two narrow regions appear. Oscillations are found when the parameter values are in these narrow oscillatory regions; Fig. 14 shows some typical oscillations that are computed in this range.

As in the previous section, we have further studied the general conditions that lead to the existence of a Hopf bifurcation for the case $K_h < K_o$. Again, the conditions for the existence of a Hopf bifurcation in this case reduce to: (1) H^+ concentrations in the range for which this product inhibits the reaction, i.e., $pH < (pK_a + pK_b)/2$; and (2) S concentrations in the range for which substrate inhibition is effective, i.e., $S > S_{max}$, where S_{max} is the value of S at which R has a maximum. Beyond this, we have also determined two more requirements for oscillatory behavior here: (1) a large value of K_b is required, i.e., $pK_b < 6$; (2) substrate inhibition must be relatively strong, i.e., a small value of b_1, $b_1 < 10^{-3}$ is necessary. Our search of the literature has not yet turned up any enzymes that fall in this category, but the conditions do not seem to be unreasonable. Indeed, AChE itself is very close to this situation with $pK_b = 6.3$ and $b_1 = 3.865 \times 10^{-3}$, although it is not in this class of substrate inhibition-driven oscillators. Further searching may reveal enzymes that might be expected to yield oscillatory behavior by the mechanism described for this imaginary protease enzyme.

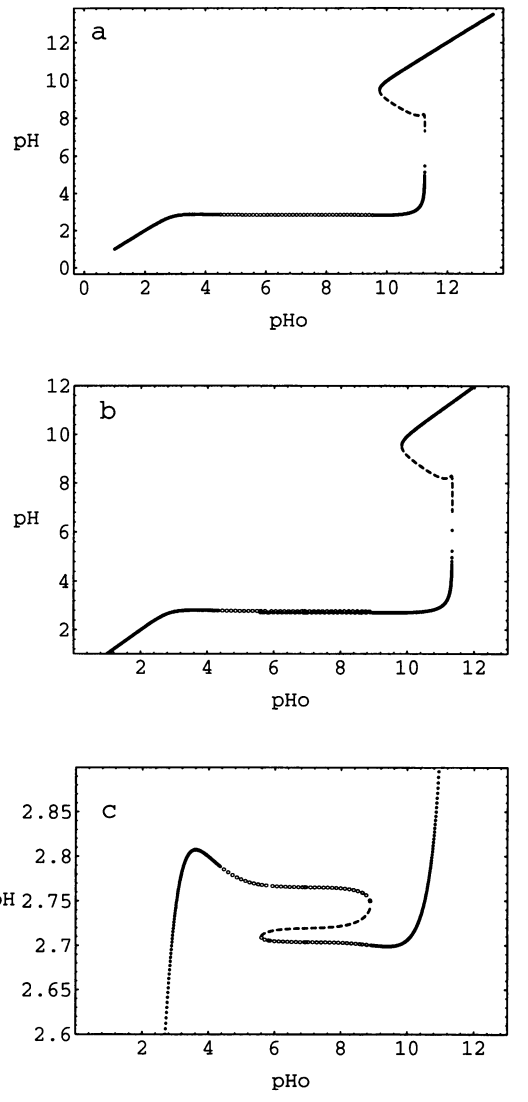

FIGURE 12 Steady-state pH as a function of pH_0 for a protease enzyme system. The solid line or points represent the stable region (stable nodes and stable focus), the dashed line represents unstable saddle points, and the empty circles are unstable focus regions that can give rise to oscillatory behavior. Parameter values are: $K_w = 10^{-14}$ M^2, $u = 4.0$, $b_1 = 1.0 \times 10^{-4}$ M, $b_2 = 1.0 \times 10^{-5}$ M, $K_a = 10^{-7}$ M, $K_b = 10^{-4}$ M, $K_h = 0.001$ s^{-1} and $\alpha = 100.0$ s^{-1} (*a*) $S_0 = 4.5 \times 10^{-4}$ M, and $E_0 = 4.5 \times 10^{-7}$ M. The unstable focus is in the single steady-state region. (*b*) When E_0 is increased to 7.0×10^{-7} M and $S_0 = 5.45 \times 10^{-4}$ M, the unstable focus region corresponds to multiple steady states. (*c*) Blow-up of unstable focus region in *b*.

NULLCLINE ANALYSIS OF OSCILLATORY BEHAVIOR

To analyze the origin of sustained oscillations and excitability, it is particularly illuminating to examine the two nullclines, defined as $dH/dt = 0$ and $dS/dt = 0$, of the dynamical system. First, consider a plot of the nullclines in the phase plane spanned by H and S. The intersections of the two nullclines define the steady states. In both the classical substrate inhibition case and the pH-dependent enzyme model, multiple steady states will arise (normally three steady states). A graphical analysis of the flow (i.e., the vector field) in the phase plane around these intersections gives qualitative information regarding the existence of oscillations. We have carried the qualitative approach further and are able to show that only the steady states at which both nullclines have a negative slope can develop limit cycle oscillations. We use the pH-dependent enzyme system as an example of our proof. The two nullclines are given by

$$\frac{dH}{dt} = \frac{H^2}{H^2 + K_w}\left[K_h\left(H_0 - \frac{K_w}{H_0} - H + \frac{K_w}{H}\right) + R\right] = 0 \quad (37a)$$

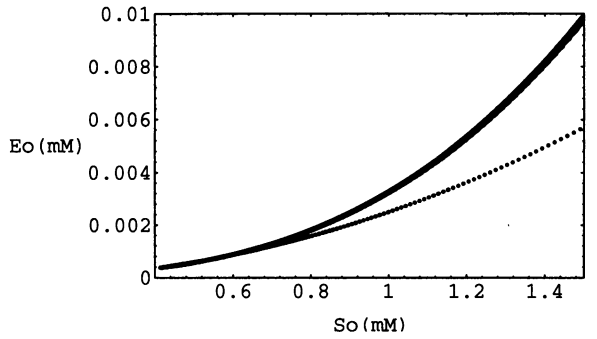

FIGURE 13 Oscillatory region in $S_0 - E_0$ parameter space for the protease reaction system at $pH_0 = 7.0$. The black region corresponds to an unstable focus for the following parameter values: $K_w = 10^{-14}$ M^2, $u = 4.0$, $b_1 = 1.0 \times 10^{-4}$ M, $b_2 = 1.0 \times 10^{-5}$ M, $K_a = 10^{-7}$ M, $K_b = 10^{-4}$ M, $K_h = 0.001$ s^{-1}, and $\alpha = 100.0$ s^{-1}.

$$\frac{dS}{dt} = K_0 (S_0 - S) - R = 0. \tag{37b}$$

We note that the derivative with respect to S of Eq. 37a and 37b evaluated at the steady state yields two slopes:

$$\frac{dS}{dH} = \frac{K_h\left(1 + \frac{K_w}{H^2}\right) - \partial R/\partial H}{\partial R/\partial S} \tag{38a}$$

$$\frac{dS}{dH} = -\frac{\partial R/\partial H}{K_0 + \partial R/\partial S} \tag{38b}$$

The conditions for the system to display sustained oscillations are that the real part of the eigenvalues are larger than zero; this means that p in Eq. 31a is larger than zero. This condition can be written as

$$-(K_0 + K_h) + \frac{H^2}{H^2 + K_w}\frac{\partial R}{\partial H} - \frac{\partial R}{\partial S} > 0. \tag{39}$$

Combining the condition, Eq. 39, with Eq. 38, a and b yields

$$\frac{dS}{dH} < -\left(1 + \frac{K_0}{\partial R/\partial S}\right)\left(1 + \frac{K_w}{H^2}\right) \tag{40a}$$

$$\frac{dS}{dH} < -\frac{K_0 + K_h + \partial R/\partial S}{K_0 + \partial R/\partial S}\left(1 + \frac{K_w}{H^2}\right) \tag{40b}$$

The slope of the two nullclines must satisfy the conditions 40 to give rise to a limit cycle. For the autocatalytic case, $\partial R/\partial S$ and $\partial R/\partial H$ are both larger than zero, so the slopes in condition 40 a and b are both negative for this case. For the substrate inhibition case, $\partial R/\partial S$ and $\partial R/\partial H$ are both less than zero; however, from condition 39 we can write

$$\left|\frac{\partial R}{\partial S}\right| > K_0 + K_h + \frac{H^2}{H^2 + K_w}\left|\frac{\partial R}{\partial H}\right|. \tag{41}$$

Eq. 41 is the condition for the existence of a Hopf bifurcation. When it is substituted into Eq. 40, a and b, we can easily see that the right-hand sides of the equation will both be negative. It is clear from these results that for the steady state to go through a Hopf bifurcation, the slopes of the nullclines must both be negative.

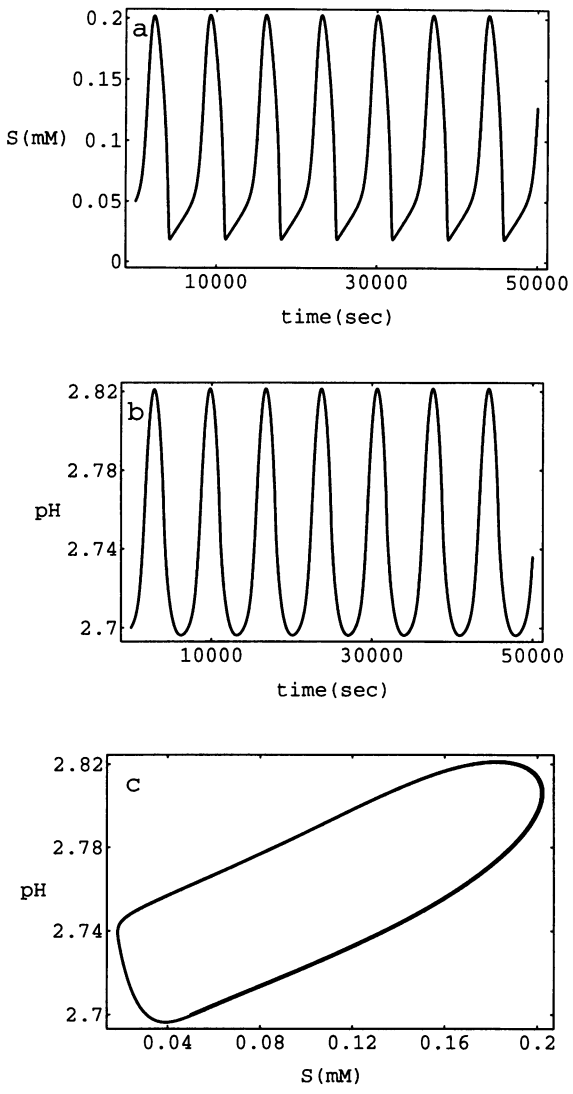

FIGURE 14 Simulated oscillations for the protease reaction system. Parameter values are: $K_w = 10^{-14}$ M^2, $u = 4.0$, $b_1 = 1.0 \times 10^{-4}$ M, $b_2 = 1.0 \times 10^{-5}$ M, $K_a = 10^{-7}$ M, $K_b = 10^{-4}$ M, $K_h = 0.001$ s^{-1}, $\alpha = 100.0$ s^{-1}, $S_0 = 5.45 \times 10^{-4}$ M, $E_0 = 7.0 \times 10^{-7}$ M, and $pH_0 = 7.0$. (*a*) Oscillations in substrate concentration inside membrane. (*b*) Oscillations in pH inside the membrane. (*c*) Limit cycle in S - pH space.

For the AChE case, Fig. 15 *a* shows the two nullclines ($dH/dt = 0$ and $dS/dt = 0$) in S-log[H] space. The nullcline $dH/dt = 0$ has an "isola" shape with a closed curve for certain ranges of parameter values. The appearance of this isola-type nullcline depends on the values of pH_0 and E_0. At $pH_0 = 10.8$, the closed curve nullcline shrinks and finally disappears as E_0 decreases to 0.8×10^{-10} M; it grows to become a mushroom as E_0 increases to 2.0×10^{-10} M. The observed oscillatory behavior always occurs in conjunction with the "isola" shape of this nullcline. The system will display sustained oscillations only when the H nullcline has this shape, which occurs when 0.8×10^{-10}M $< E_0 < 2.0 \times 10^{-10}$M for $pH_0 = 10.8$, for example. Oscillations arise only when the

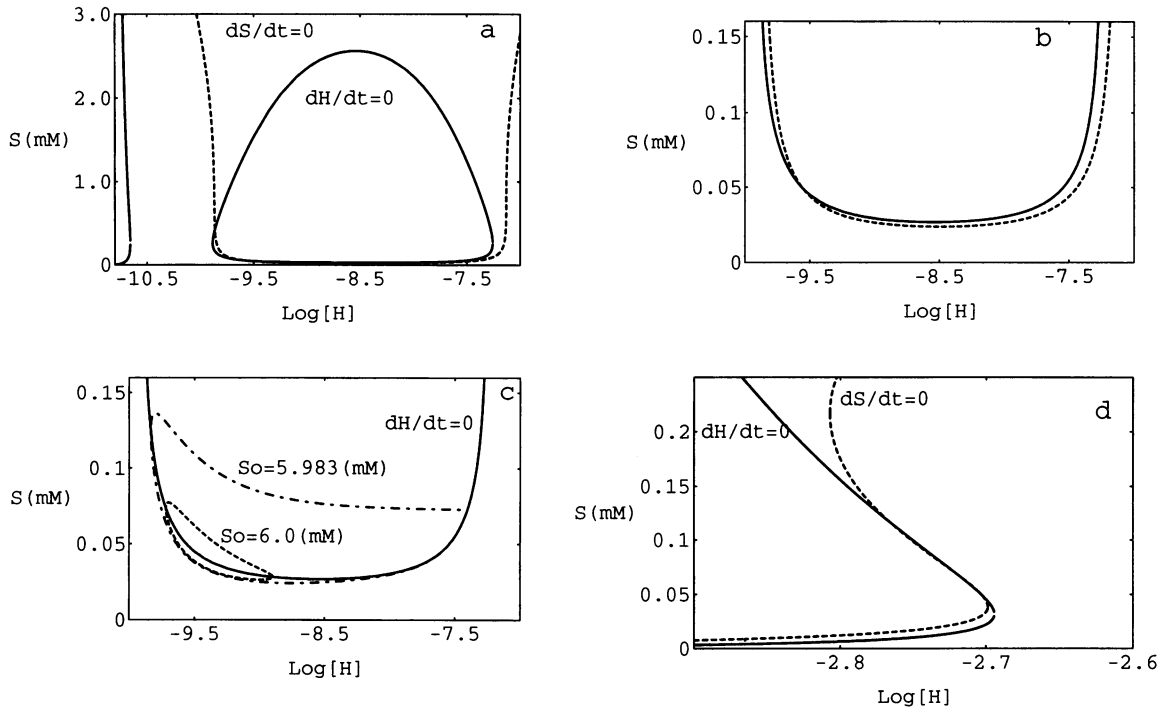

FIGURE 15 The two nullclines ($dH/dt = 0$ and $dS/dt = 0$) in Log[H] − S space. (*a*) The two nullclines for the AChE reaction system. The solid line represents $dH/dt = 0$, the dashed line represents $dS/dt = 0$. The closed curve of $dH/dt = 0$ appears only under certain parameter values. (*b*) Blow up of the region in *a* where the two nullclines cross at an unstable steady state. Parameter values for *a* and *b* are: $K_w = 10^{-14}$ M^2, $u = 0.1$, $b_1 = 3.865 \times 10^{-3}$ M, $b_2 = 1.786 \times 10^{-5}$ M, $K_a = 10^{-10}$ M, $K_b = 10^{-7}$ M, $K_h = 0.001$ s^{-1}, $\alpha = 8.571 \times 10^3$ s^{-1}, $S_0 = 0.006$ M, $E_0 = 1.3 \times 10^{-10}$ M, and $pH_0 = 10.8$. (*c*) The two limit cycles surround the unstable steady state; they are shown superimposed on the nullcline $dH/dt = 0$. Here $S_0 = 5.983$ and 6.00 mM for the two limit cycles. (*d*) The two nullclines superimposed on the computed limit cycle for the protease reaction system. Parameter values are: $K_w = 10^{-14}$ M^2, $u = 4.0$, $b_1 = 1.0 \times 10^{-4}$ M, $b_2 = 1.0 \times 10^{-5}$ M, $K_a = 10^{-7}$ M, $K_b = 10^{-4}$ M, $K_h = 0.001$ s^{-1}, $\alpha = 100.0$ s^{-1}, $S_0 = 5.45 \times 10^{-4}$ M, $E_0 = 7.0 \times 10^{-7}$ M, and $pH_0 = 7.0$.

point at which the two nullclines cross (the steady-state value) is located on the negative slope of the H nullcline with Log[H] < −8.5 (pH > 8.5, the condition for autocatalysis); this occurs only in a very small range of parameter values. The S nullcline can be chosen by changing S_0 so that it will cross the H nullcline at a region of negative slope and in the pH > 8.5 range (see Fig. 15 *b*). The appropriate S_0 value is very small when these conditions are satisfied. Fig. 15 *c* shows the limit cycle near the steady state at the H nullcline. The large cycle corresponds to $S_0 = 0.005983$ M and the small one to $S_0 = 0.006$ M. The amplitude of the oscillations decreases rapidly with a small changes of S_0. Fig. 15 *d* shows the nullclines and the limit cycle around the steady state for the imaginary protease case. This is qualitatively similar to the AChE example.

DISCUSSION AND CONCLUSION

We have continued the study of the classic substrate inhibition scheme originally studied by Degn (1968) to determine whether oscillatory behavior can be supported by it. Our calculations prove that although the classic substrate inhibition mechanism is sufficient for bistability, oscillatory behavior cannot be sustained by such a mechanism, because it is inherently a one variable system. By making the second step of scheme 1 reversible, however, we introduce a second species (the product, P) into the system as a dynamical variable and also provide, in essence, a second substrate for the enzyme, a feature that had been known to result in oscillations via substrate inhibition. However, we find that simply adding reversible product formation is insufficient to render the classical substrate inhibition scheme oscillatory. Only when the product formation is made reversible and both substrate and product are taken to follow reversible pseudo-reactions for diffusion (i.e., the reaction is taken to occur in a flow-through reactor) does oscillatory behavior become possible for the classical substrate inhibition scheme. However, even here, the range of parameter values over which oscillatory behavior can occur is so narrow it is essentially undetectable experimentally.

From these results, we can draw the following conclusions. Oscillatory behavior observed experimentally cannot be caused by substrate inhibition kinetics alone unless other conditions are met. One condition, known since 1976 (Seelig, 1976), occurs when multiple substrates are involved. This makes sense because a single substrate scheme is inherently a one-variable problem that renders oscillations impossible (these results also show that the QSS approximation is a valid indicator of the inherent dynamics in these systems). Multiple substrates merely allows for two or more variables and provides a mechanism by which the reactant that causes inhibition can be coupled to another reactant.

Even then, the multiple substrate condition is not sufficient to produce oscillatory behavior; we have shown that this requirement must be coupled with an additional requirement for the reaction to occur in a flow reactor. The Thomas model (Thomas, 1976) is a well studied example that includes both of these effects; it is a two-variable model for the uricase enzyme in which the two variables are the two substrates uric acid and oxygen; it also contains flow terms for both substrates. From our careful study of the classical substrate inhibition mechanism with reversible flow terms, we would conclude that it is the presence of the flow terms that is crucial for oscillatory behavior in a substrate inhibition mechanism with two substrates.

In the second model system studied, which included both autocatalytic effects as well as substrate inhibition, oscillatory behavior was observed from autocatalytic or substrate inhibition mechanisms. However, combination of these two effects cannot lead to oscillations. The mechanism of the first example is autocatalytic because of the pH dependence of the enzyme reaction rate, an effect well known to lead to oscillatory behavior (Zabusky and Hardin, 1973; Hahn et al., 1974; Chay, 1981). In the second example, substrate inhibition leads to oscillations when it is coupled with product inhibition.

We can further conclude that AChE, an enzyme that follows the kinetics of the second model, is not the best choice for studying oscillatory behavior in enzyme systems because the kinetic parameters for this enzyme reduce the range of values over which oscillations are expected; again, the range of values over which we calculate oscillatory behavior is so narrow it is essentially experimentally undetectable. Papain is a better case, as has already been observed in previous simulation studies; however, papain does not involve substrate inhibition at all, so these oscillations are caused by autocatalysis.

Our results also allow us to predict from a general class of enzymes those that might be good candidates for the generation of oscillatory behavior. From our analysis, we find that pepsin and chymotrypsin have a pH dependence similar to that of AChE and papain (see Table 3). The bell-shaped pH dependence of the enzyme activity for these two enzymes indicates that two ionizing groups near the active site of the enzyme with different pK_a values play key roles in the catalysis mechanism. If the substrate of the enzyme reaction is an ester, H^+ will be the product of the reaction. So for high pH conditions, the reaction will be the autocatalytic and might produce oscillations. Chymotrypsin, in particular, which has a pK_a value greater than 8, might be expected to produce oscillations via the autocatalysis route. Chymotrypsin is a serine hydrolase enzyme, and it is likely that other enzymes in this class might also produce oscillatory behavior.

TABLE 3 pK_a and pK_b values for common pH-dependent enzymes

Enzyme	pK_b	pK_a
Chymotrypsin (Ableles et al., 1992)	7.0	9.0
Acetylcholinesterase (Chay and Zabusky, 1983)	6.3	10.0
Papain (Ableles et al., 1992)	4.2	8.2
Pepsin (Ableles et al., 1992)	1.3	4.4

Pepsin might fall in the second category exemplified by the imaginary protease enzyme studied in our simulations because its pK_b value is <6. The second criterion for oscillatory behavior of enzymes in this category is that the parameter b_1 in our model be small enough ($<10^{-3}$). This parameter depends on the substrate of the reaction (through K_{SS}), and a thorough search of the literature has not revealed any substrates that would provide pepsin with the proper kinetic parameters to yield oscillations. It is possible that other substrates for pepsin might produce oscillatory behavior. Pepsin is an aspartyl protease, and other enzymes in this class or in the sulfhydryl hydrolase class (characteristic of papain) might be good candidates as well for oscillatory behavior.

The enzyme reaction is the most basic dynamic element in the biological world. Our model studies of oscillatory conditions for a pH-dependent enzyme in the presence of substrate inhibition have been analyzed in detail when the reaction takes place in an open system. However, in the real biological world, most enzymes are associated with cell membranes that have fixed electrical charges. The local concentration of substrate and product can be influenced by these charges, which are in turn affected by the local pH and ionic strength. So, buffer and salt solutions will also affect the enzyme reaction if the enzyme is immobilized in a charged membrane.

Others have shown that the oscillatory behavior of an enzyme reaction in a charged membrane may be influenced by buffer and salt concentration (Chay and Zabusky, 1983; Chay, 1980). The pH and ionic strength influence the dynamics of the system through their effect on the fixed charges in the membrane. For example, AChE has been experimentally studied immobilized in a BSA membrane, which has an isoelectric point of 5.0. At pH = 5.0 or above, the membrane is negatively charged and the transfer rate of the substrate ACh^+, a cation, is large at high pH and is dependent on the charge of the membrane. As the AChE-catalyzed reaction proceeds, H^+ is produced and the membrane local pH is decreased; hence, the local charge on the membrane is also decreased, influencing the transfer rate of ACh^+ into the membrane. High ionic strength of the solution will also eliminate the high surface charge on the membrane. The transfer rates K_o and K_h in the model for substrate and H^+ from reservoir to the membrane are then functions of reaction product (H^+), buffer, and ionic strength instead of constants, as we have taken them to be in the present study. It is our plan to continue our investigation to include the effects of charge and associated properties such as pH and ionic strength.

The authors would like to acknowledge support of this work by the donors of the Petroleum Research Fund administered by the American Chemical Society and by the National Science Foundation (Grant CHE-9307549).

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