pH oscillations and bistability predicted by a model of a trienzymatic system that produces an anti-depressant

Senior Thesis

Presented to
The Faculty of the School of Arts and Sciences
Brandeis University
Department of Biochemistry
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April 2017
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Acknowledgements

I would like to first thank Dr. Irving Epstein for giving me priceless opportunity to work in his lab. His wisdom and advice enabled me to conduct this research project and finish my senior honor thesis. I would also like to thank my mentor Dr. Zulma Jiménez for her patience and support. She provided me opportunity to start this project and guided me throughout the process. I would also like to thank all the lab members in the Nonlinear Dynamics group at Brandeis University for providing me help and friendly environment for the past three years. Finally, I would like to thank my family and friends for their trusts and love.
Abstract

pH oscillations and bistability predicted by a model of a trienzymatic system that produces an anti-depressant

A thesis presented to the Department of Biochemistry
Brandeis University
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A controlled drug delivery system is typically designed to deliver the drug at a controlled rate; its benefits including improve patients’ compliance compare to traditional formulations as well as not disrupting circadian rhythms if set on a 24-hour period. Some delivery systems exhibit the potential of producing drug in-situ without pre-loading the drug. Because of the availability of methylated creatine in human muscle, we use creatine-creatinase system as the source of an anti-depressant sarcosine. Sarcosine (N-methylglycine) is an exogenous amino acid that acts as a glycine transporter inhibitor. It can enhance glycine concentration around NMDA receptors; as a result, modulates glutamatergic transmission function, which is usually found impaired in patients with depressant and schizophrenia. Here we aim to establish the basis for a pH Sensitive Drug Delivery System (PSDDS) with characteristics of both biocompatibility and rhythmic delivery of the antidepressant. The system will have an enzymatic network as building block which is capable of inducing pH oscillations and sarcosine production. In this work, we carry out numerical simulations using a model based on the kinetics of the enzymatic reactions. The results of the simulations generate sets of the initial conditions necessary for the system to produce the rhythmic behavior mentioned above. Our enzymatic network is composed of creatinase-urease-sarcosine oxidase. Urea produced by
substrate-enzyme creatine-creatase will be hydrolyzed by urease to produce ammonia which participates in the pH oscillation. Furthermore, the concentration of sarcosine is controlled by the addition of sarcosine oxidase. Our results show that pH oscillations with a pH variation of 5 units are obtained by introducing an inhibitory effect on the enzyme urease. Urease inhibition adds a negative feedback which depends on the concentration of $\text{OH}^-$. We also found that the magnitude of the pH variation is related to the concentration of the enzymes and substrates added to the reaction through of a constant influx.
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1. Introduction

Designing pH oscillators is of particular interest as it can provide both insights into complex dynamic behaviors and can be coupled with a pH sensitive hydrogel as a drug delivery device. Typical pH oscillators usually are composed of inorganic species such as halogen and sulfites which are too toxic to incorporate with hydrogels. In order to generate a more biocompatible pH oscillator, enzymes are obvious candidates as they participate many biological oscillating reactions.

Here, we want to build an enzyme-based pH oscillator that generates and delivers a drug at a controlled rate. Sarcosine is a newer drug to treat patients with depression, which can be synthesized by the enzyme creatinase. The latter produces also urea, which combined with urease can be used as the base for the pH oscillator. Since creatine can be found in human muscles, human muscle will be considered as a free source of substrate for the enzyme creatinase. In this project, we study the dynamical behavior of the enzymatic system by simulating the kinetics of a system composed of creatine-creatinase-urease-sarcosine oxidase. We look for the initial conditions required for the enzymatic system to produce sarcosine and to induce temporal fluctuation of the pH. We hope this study can provide new insights in designing enzymatic oscillating systems.

In section 2, we offer background on enzymatic pH oscillators, including a brief review of oscillating chemistry history and how to use empirical methods to find pH oscillators. Section 3 discusses the computational method we used here, as well as the differential equations solved. Tables of enzyme constants and other conditions can be found as well. Results of numerical simulations are discussed in section 4. In this section, also the effect of the addition of an inhibitor for the enzyme urease is discussed. In the last chapter, conclusions and suggestions for future work are offered.
2. Background

2.1 Oscillations in Nature

2.1.1 Biological rhythms

Physiological rhythms are results of nonlinear dynamics. They are central to our lives as they constitute many of our bodily processes; for instance, heartbeats and sleeping cycles that occur daily keep us alive. Figure 2.1 shows some examples of complex physiological rhythms. These physiological rhythms are not strictly periodic but show some fluctuations in their dynamics. Disruption of physiological rhythms can lead to diseases; however, extremely regular rhythms are sometimes also considered pathological. (1)

Diseases associated with dysregulation of physiological rhythms are termed ‘dynamical diseases’, examples are tumors, asthma, hypertension, osteoarthritis, which are hard to tackle with traditional drug formulations. (2)

As one representative of physiological rhythms, circadian rhythms are molecular circuits that allow organisms to temporally coordinate a plethora of processes, with a rhythm close to 24h, optimizing cellular function in synchrony with daily environmental cycles. Circadian rhythms have been widely observed in prokaryotes, fungi, algae, plants and mammals. (1) It is critical for organisms to have such oscillating molecular mechanisms, for the purpose of maintaining homeostasis as well as adaption to environmental changes. (3) In mammals, circadian

Figure 2.1 Physiological rhythms. a). White blood count of a patient with cyclical neutropenia. b). Heartbeat of a subject at high altitude. c). Stride time of a patient with Huntington’s disease. (Adapted from Ref. 1)
Clocks coordinate with clusters of 20,000 neurons named as the suprachiasmatic nucleus (SCN), located in the hypothalamus of our brain. (Figure 2.2) Although circadian rhythm is endogenous in the living system, they can be adjusted by entraining agents: daily light/dark cues or Zeitgebers (German: “time giver”) which include temperature and redox cycles. (2) A freerunning circadian rhythm can be disrupted by genetic or environmental perturbations and results in metabolic dysregulation. (3) Such abnormal dynamics can be associated with the onset of disease. Previous studies (Wehr and Goodwin 1979) have shown that brain activity of patients with depression exhibits a decrease in dynamical complexity and circadian clock alternation has also been observed. (5) Therefore, in order not to disrupt the physiological rhythms including circadian clock, the simplest approach to design an implanted medical device is to set drug delivery period as a 24-hour cycle. (1)

Physiological functions in our body do not only depend on rhythmicity of a single cell, but are derivatives of interactions between them. (1) These complex integrated networks are indeed individual chemical reactions happening in living organisms. Because most of the chemical reactions happening in biological systems are catalyzed by enzymes, we call these oscillatory behaviors enzymatic oscillations. (6) The first enzymatic oscillating system discovered in history is the process of glycolysis, which is a periodic conversion of sugar into pyruvate. The enzyme phosphofructokinase (PFK) catalyzes the reaction of phosphorylation of fructose-6-phosphate to fructose-1,6-bisphosphate. (Figure 2.3) While energy (ATP) is
consumed during the process, more energy is being produced. In order to regulate the formation of ATP, PFK activity is inhibited by ATP concentration and activated by ADP, which means when there is sufficient amount of ATP, such conversion will stop. (7) Other classic examples of enzymatic oscillations include the periodic cyclic AMP (cAMP) pulses in Dictyostelium cells (8), Ca²⁺ oscillation and oscillation and waves in neutrophils (9).

Some common features of oscillations as well as where to find oscillations in enzymatic systems will be discussed in detail in the following section 2.2.

2.1.2 Chemical oscillations

Oscillating chemical reactions are characterized as temporally periodic, or nearly periodic, variation of the concentrations of one or more species in a reaction (10). When the idea was first proposed in the 1970s, chemists were skeptical because it seemed to contradict the Second Law of Thermodynamics. They made an analogy between an oscillating reaction and a pendulum and understood that each oscillation

![Chemical Oscillations Diagram](image-url)

Figure 2.3. Phosphofructokinase (PFK) catalyzes the conversion of sugar to alcohol. PFK is activated by ADP and inhibited by ATP. F6P is provided continuously from hexoses and product FBP is transformed into pyruvate. (Adapted from Ref. 7)

Figure 2.4 Two false interpretations of chemical oscillations in schematic drawing. a). Oscillations are around equilibrium. b). Oscillations are toward equilibrium. (Adapted from Ref. 10)
cycle passes through its equilibrium point. (Fig 2.4a) Though this idea was incorrect, they further concluded that chemical oscillations needed an input energy for the system to oscillate as shown in figure 2.4b. The system doesn’t pass through equilibrium during the oscillatory period, which contradicts the Second Law of Thermodynamics. Just like all other chemical reactions, the driving force for reaction completion in oscillations is the decrease in free energy, and yet not all chemical reactions become oscillatory. In general, there are three key components for the occurrence of chemical oscillations: 1) chemical oscillation is far from equilibrium and obey the laws of non-equilibrium thermodynamics; 2) at least two different pathways can happen after energy-releasing step; 3) an intermediate from one of the pathways is the ‘trigger’ for the two pathways to switch. (11) One example of chemical oscillators besides living organisms is the Belousov-Zhabotinsky (BZ) reaction. The BZ reaction is complicated and its mechanism involves eighteen chemical reactions. The oscillating species of the system are some of the intermediate species of the reaction and the metal catalyst, i.e., Br⁻ and ceric ions. The metal catalyst changes its oxidation state from Ce³⁺ to Ce⁴⁺ causing temporal color changes from colorless to yellow of the solution. (12) The overall equation for BZ reactions is:

\[
5\text{CH}_2\(\text{CO}_2\text{H}\)_2 + 3\text{BrO}_3^- + \text{H}^+ \rightarrow 3\text{BrCH(COOH)}_2 + 2\text{HCOOH} + 4\text{CO}_2 + 5\text{H}_2\text{O}
\] 

(2.1)

Another example of oscillating chemical reactions is the Briggs-Rauscher reaction, which is also known as ‘the oscillating clock’ because of its impressive color changing characteristics. (13) The approximate overall reaction of this system is:

\[
\text{IO}_3^- + 2\text{H}_2\text{O}_2 + \text{CH}_2(\text{COOH})_2 + \text{H}^+ \rightarrow \text{ICH(COOH)}_2 + 2\text{O}_2 + 3\text{H}_2\text{O}
\] 

(2.2)

The chemical oscillation family includes reactions of bromate, chlorite, bromite, oxygen, sulfur, Cu(II)-catalyzed, manganese-based and pH oscillators. (14) pH oscillator is of particular interest of our study, and the topic will be expanded in section 2.3.
2.2 Characteristics of Oscillations

2.2.1 Excitable System

The first chemical oscillator was discovered serendipitously by some chemists who were looking for other phenomena. Because of its interesting oscillating behaviors, it can be compared intuitively with a spring; however, it is more difficult to understand why some reactions can be oscillatory and some cannot. In order to find a way to design chemical oscillators, a simple model with two variables (two is the smallest number of variables required in the system for the system to oscillate) was proposed. (10) This model is described by non-linear differential equations:

\[ \frac{du}{dt} = \left(\frac{1}{\varepsilon}\right) f(u, v) \]  \hspace{1cm} (2.3)

\[ \frac{dv}{dt} = g(u, v) \]  \hspace{1cm} (2.4)

The functions \( f \) and \( g \) depend on the concentrations of the chemical species \( u \) and \( v \); which have nonnegative values. \( \varepsilon \) is a number smaller than one, and acts as a scaling factor in the model which indicates \( u \) is changing much faster than \( v \). When the concentrations of \( u \) and \( v \) are no longer changing, the differential equations are set to equal to 0 and we call the resulting curves null clines. One way to represent the dynamical behavior exhibited by the system is to use the phase plane diagram, also called response-response plot. This is a two-dimensional or multi-dimensional space whose coordinates are the values of the variables. The curves are called the trajectory of the system. (15) The intersections(s) of the two null clines is a steady state (ss) of the system. (Fig 2.5a) The null clines divide the phase plane into different regions. As we cross the null clines, the sign of time derivative of each variable changes. Based on the signs, we may able to predict the concentration evolvement of each variable over time.

A system is said to be excitable if it returns to its steady state quickly after being perturbed by a small amount but returns to the steady state only after a larger amplitude excursion if it is given a large perturbation. (Fig 2.5b, 2.6) Excitability is important in some biological events, i.e., organized structure in the cortex of egg cells and forest fires. It has two important characteristics: threshold and refractory period. Threshold is the minimum perturbation required to cause the system to shift away from its steady state; and
refractory period is the time the system needs to return to its steady state. (15) Take forest fire as an example, when one tree in the forest is on fire, it is said in excited state; and this is not going to affect the forest unless the fire is larger than some threshold. After all trees are burnt, the time that forest takes to grow new “burning material” is called the refractory period.

![Diagram](image1)

Fig 2.5 Perturbation diagram of system with excitability. Dashed arrows are the path of the system. (a) A small perturbation is applied to the system, and the system returns to its steady state with a shorter path. (b) A larger perturbation means the system need more time to rest. (Adapted from Ref. 10)

![Diagram](image2)

Fig 2.6 Small perturbation corresponds to shorter refractory period; larger perturbation corresponds to longer refractory period. (Adapted from Ref. 10)

### 2.2.2 Bistability and Oscillations

A two-variable model suggested by Boissonade and De Kepper in 1981 facilitated the search for real chemical oscillators. (16) Based on equations (2.1) and (2.2), they gave a new model with two variables x and y.

\[
\frac{dx}{dt} = -(x^3 - \mu x + \lambda) - ky
\]  
(2.5)

\[
\frac{dy}{dt} = (x - y)/\tau
\]  
(2.6)
where \( \mu \) is greater than 0, suggesting that in equation (2.5) \( x \) is produced in an autocatalytic fashion. The term \(-ky\) provides a negative feedback in equation (2.5) of variable \( y \), and \( \tau \) in equation (2.6) characterizes the time scale of this feedback. Here, feedback means the concentration of species \( y \) affects the kinetics of production of \( x \) negatively. (10) The key requirement for the system to oscillate is that the change in concentration of \( x \) is much faster than that of \( y \), therefore \( \tau \) needs to be much greater than \( 1/\mu \). Now, it is important to describe the linear stability analysis for two-variable model, because it gives the behavior of the system around its steady state. First finding the null cline for \( y \): set equation (2.6) to 0, it is easy to get \( x = y \). Equation (2.5) then becomes:

\[
q(x) = x^3 - (\mu - k)x + \lambda = 0
\]  

(2.7)

If \( \mu \) and \( k \) are constants, equation (2.7) can be plotted with different values of \( \lambda \). (Fig 2.7) When the cubic equation is tangent to the \( x \) axis, \( \lambda \) has two special values, \( \lambda_1 \) and \( \lambda_2 \). When \( \lambda_1 < \lambda < \lambda_2 \), equation (2.7) has three solutions or three states. The number of intercepts of the line and \( X \)-axis in figure 2.7 denotes the number of steady states in the system.

The Jacobian matrix can be calculated for equations (2.5) and (2.6) as an indicator of the stability of each steady state. We are going to skip the calculation of eigenvalues as well as its analysis here, but it
is important to point out that based on Poincaré-Bendixson theory we need all three steady states to be unstable for the system to oscillate. From the experimental results, a cross-shaped phase diagram can be constructed. (Fig 2.8) It holds for many autocatalytic chemical reactions carried out in a continuous flow stirred-tank reactor (CSTR), and can served as guidance for design of chemical oscillators. Depending on the initial conditions of the system (concentration of reactants, inflow rate etc.), some systems can evolve to different steady states. Such a property is called bistability and when we are looking for oscillation we first need to find bistability of the system. On the y axis of Figure 2.8, critical values of $\lambda$ are plotted with fixed values of $\mu$ and $\tau$. Four different dynamical behaviors are shown: bistability, two steady states, and oscillations. Bistability lies between two transition, and whether the system resides in steady state one or two depends on the “history” of the system, i.e., the different initial conditions of the system. (17) This is called hysteresis, which in steady state is a function of $\lambda$, and can be found in many natural processes; for example, a bistable circuit is at the core of the cell cycle which it prevents the cycle form slipping back to interphase. (18) Each state changing will pass the system’s critical point, if the system has two critical

![Figure 2.8](image-url)
points, it is reversible; if it has only one, it is an irreversible hysteresis. In figure 2.9 the solid lines are two steady states of the system, and the dashed line is its unsteady state. The characteristics of a bistable system make us believe the system has ‘memory’, which unlike a non-hysteretic system, which returns to its original state if the input change is removed, a bistable system will remain in the new state. Bistability is common in nature, for instance, transcription factors OCT4, SOX2, and NANOG are responsible for stem cell determination, regulate each other through feedback loops.

Besides oscillation, steady state and bistability; chemical systems can exhibit other dynamical behaviors. Chaos or aperiodic oscillation is a more complicated behavior, where its formation requires the system to possess more than two variables and multiple feedback loops. One example is the BZ system.

2.3 pH Oscillators

2.3.1 Definition and Features

As introduced in section 2.2, a chemical oscillator is created by combining positive with negative feedback. In this section, we are going to discuss chemical oscillators in which their oscillatory behaviors are mainly driven by the change in concentration of hydrogen ions. This type of chemical oscillators is defined as pH oscillators. The periodic changes of pH occur in unbuffered solutions, with a
pH variation as large as 6 units. If the pH oscillator is placed in a buffered solution, the amplitude of the pH variation will be suppressed and then dead.

Since early 1980s, there was a blossoming of chemical oscillators and to date, about 20 types of pH oscillators have been discovered. (20) Almost all of them can only function in a flow reactor (CSTR) (14) and most pH oscillators are composed of redox reactions (20). The continuous replenishment of reagents through the CSTR allows the periodic cycle of pH to repeat. Each pH oscillator is composed of two chemical reactions, as one reaction produces H⁺ in an autocatalytic fashion ( + feedback) and the other reaction consumes H⁺ ( − feedback) at a slower rate, such that there will be an appropriate time lag between the two reactions and pH oscillations can therefore be established. Among the two chemical reactions there is an oxidant and either one or two other components (reducing agents). Based on the number of reducing agents in the system, a pH oscillator can be categorized as “one-substrate pH oscillator” when there is one reductant or “two-substrate pH oscillator” if there are two. (20)

![One-substrate pH oscillator model](image)

Figure 2.10 One-substrate pH oscillator model. (Adapted from Ref. 20)

In the one-substrate pH oscillator model, oxidation of reductant happens in two separate steps which depend on the initial pH and the initial ratio of oxidant to reductant. (20) As shown in figure 2.10, the first pathway is partial oxidation of reductant. This step consumes H⁺. As reaction goes on, pH of the system keeps rising and therefore this reaction will slow down and then become self-inhibited. The second pathway is total oxidation and it starts when the pH of the system increases to a certain high extent that
partial oxidation completely shuts off. Total oxidation produces H⁺ in an autocatalytic manner and restores the pH of the system to the state before reactions begin. In some particular cases, a metal ion (Cu²⁺ or Mn²⁺) will serve as a catalyst of the reaction in order to tune the rate of feedbacks. (20)

\[ [\text{Ox}] > [S_1] + [S_2] \]

Figure 2.11 Two-substrate pH oscillator model. (Adapted from Ref. 20)

Both substrates in the two-substrate model undergo oxidation reactions. (Figure 2.11) In step 1, S1 will undergo total oxidation and this reaction is autocatalytic in hydrogen ion. Production of H⁺ decreases the pH of the system and such low pH environment will trigger the initiation of step 2. H⁺ is consumed in step 2 and results in an increase in pH of the system. The increase and decrease in pH (oscillation) can last as long as reactants are kept flowing into the system. (20)

Although most pH oscillating systems are composed of redox reactions, there are some non-redox ions that can also participate in chemical oscillations. For instance, Ca²⁺ and Na⁺ have only one stable oxidation state but are essential in some periodic biological events. Producing pulses of the non-redox ions can be accomplished by incorporating such species into a periodic system such as a pH oscillator. The latter must have fast complexation or precipitation equilibria. These types of systems have the potential of being used as biological probes. The BrO₃⁻-SO₃²⁻-Fe(CN)₆³⁻ oscillator with addition of CaEDTA is an example of oscillatory pulses of Ca²⁺. (Fig 2.12) The pH oscillator creates periodic change of pH in the system; when
the concentration of H⁺ is high, Ca-EDTA complex forms and the system is low in [Ca²⁺]; and when pH is basic, Ca²⁺ will be released as free ions in the system. (20) Another interesting example is the Methylene Glycol-Sulfite-Gluconolactone (MGSG) reaction, which is the first proposed organic-based pH oscillator. Unlike the traditional pH oscillators which contain toxic species like bromate, this system is established based on the formaldehyde-sulfite organic pH clock reaction, which is less aggressive and more biocompatible when coupling with a pH sensitive gel. (21) This study stimulates interest in designing organic-based pH oscillators as they are ideal devices for drug delivery.

2.3.2 Applications of pH Oscillators in Drug Delivery

Among all types of chemical oscillators, the design of pH oscillators is of particular interest. (22) Because of the ubiquity of hydrogen ion in chemical and biological processes, development of pH oscillators can provide both insights of complex dynamic behaviors and possibility of being coupled with a pH sensitive polymer as a real-world application. (20) The particular usage of a combination of pH oscillators and pH sensitive gel to deliver drugs is referred as pH Sensitive Drug Delivery System (PSDDS) or controlled release drug delivery system (CRDDS). In comparison with conventional drug delivery
system such as oral drugs or i.v., PSDDS can sustain drug delivery for a relative long period with a controlled rate varied in different patients. These features enable PSDDS to overcome shortcomings of overdosing and short half-life of conventional drugs. In Fig 2.13, the red dashed line represents conventional formulation. It is obvious that over an extended time period, plasma drug concentration can no longer sustain and patients need to take capsules or tablets more frequently than zero order controlled release drug. On the other hand, the zero order controlled release which is shown in green dashed line, has the most stable plasma drug concentration compare to other formulations. In addition to addressing patients’ compliance problems, PSDDS is both site-specific targeting and temporally modulating, therefore it has advances in treating diseases associated with dynamical dysregulation such as rhythmic heart disorders or peptic ulcer. (21, 23) Other advantages of PSDDS include lower drug costs for patients, improved drug utilization and decreased side effects.

Siegel and Gauri (24) reported the use of a chemical pH-oscillator to drive multipulse drug permeation across membranes. In their study, they showed that by choosing a suitable pH-oscillator and using a very small concentration of drug, a prototype system for drug to permeate across lipophilic membranes was achieved. The pH of the solution is changing remarkably relative to the pKa of the drug, the drug can therefore be charged and uncharged; only the uncharged species can cross the membranes.

Figure 2.13 Schematic drawing of therapeutic effects for conventional formulation and controlled release drug delivery. MSC is maximum safe concentration, MEC is minimum effective concentration. (Adapted from Ref. 26)
Bell et al. in 1996 proposed a hydrogel-based drug delivery system, using a biodegradable material named poly (methacrylic acid-g-ethylene glycol), which exhibits pH sensitivity. (25) Its pH sensitivity enables it to couple with a pH oscillator and its biocompatibility enlarges its range of applications. Similar materials include dextrin. (26)

2.4 Enzymes

Enzyme activity is pH dependent, and its typical bell-shaped curve indicates its autocatalytic effect. (9) When coupled with a production of acid/base, enzyme can also exhibit feedback-driven behaviors. (27) Both properties (autocatalysis and feedback) are essential for pH driven chemical oscillators, therefore enzymes are perfect candidates for the design of enzymatic pH oscillators. The biocompatibility of enzymes ensures they can be coupled with pH sensitive gel and compose PSDDS. Although there is an increasing need for using enzymatic oscillators for real-life applications, to date, the number of real examples remains small. (28) Just like the previous examples of producing Ca^{2+} pulses, many investigators have tried to incorporate enzymes into existing pH oscillators. However, those successful trials are not examples of real enzymatic oscillators, in which enzymes play the major role in driving the reactions and pH oscillations. (9)

In 2010, Taylor et al. designed a urea-urease pH oscillator that exploited the bell-shaped activity curve of urease (Fig 2.14) and its base-catalyzed feedback. Products of urease acting on urea include ammonia and carbon dioxide.

![Figure 2.14 Typical bell-shaped graph of urease: its optimal pH is around pH 7. (Adapted from Ref. 27)](image-url)
CO(NH₂)₂ + H₂O → 2NH₃ + CO₂  \hspace{1cm} (2.8)

Both products can affect the pH of the system:

\[ \text{NH}_4^+ \rightleftharpoons \text{NH}_3 + \text{H}^+ \hspace{0.5cm} \text{pK}_a = 9.25 \]  \hspace{1cm} (2.9)

\[ \text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \hspace{0.5cm} \text{pK}_a = 6.5 \]  \hspace{1cm} (2.10)

\[ \text{HCO}_3^- \rightleftharpoons \text{CO}_3^{2-} + \text{H}^+ \hspace{0.5cm} \text{pK}_a = 10.25 \]  \hspace{1cm} (2.11)

\[ \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{OH}^- \]  \hspace{1cm} (2.12)

In the CSTR, they had an initial inflow of urea, urease, acetic acid or sulfuric acid with a specific inflow rate \( k_0 \). Acid is used to adjust the initial pH of the system, and they found that using different acids the nonlinear dynamical behaviors of the system varied: sulfuric acid gave a broader bistability region and hysteresis, while with acetic acid, aperiodic oscillations were observed experimentally. However, the oscillations are not reproducible or explained by the model. A possible answer is that experimental fluctuations caused the oscillations. The rate equation for acid is:

\[ \text{HA} \rightleftharpoons \text{A}^- + \text{H}^+ \]  \hspace{1cm} (2.13)

The Michaelis-Menten kinetics for the enzyme is:

\[ v_1 = \frac{k_1 E_1 U}{(K_M + U) \left( 1 + \frac{U}{K_{u}} \right) \left( 1 + \frac{NH_4^+}{K_P} \right) \left( 1 + \frac{K_{eg2}}{H^+ + K_{es1}} \right)}; \]  \hspace{1cm} (2.14)

where \( v_1 \) is the rate of reaction of urease. The different terms of equation 2.14 will be described in chapter 3.
Unfortunately, they didn’t find oscillatory behavior in their numerical simulations. In our study, which is a continuation of Taylor’s work; we will use the enzymes: urease, creatinase, sarcosine oxidase and a hexokinase. For this reason, the next paragraphs describe some aspects of each enzyme. (Fig 2.15)

![Figure 2.15 Enzymatic reactions](image)

Creatinase (EC 3.5.3.3) is an important enzyme that acts on creatine and controls the creatine concentration in biology. (29) Another product of the creatine/creatinase reaction is sarcosine, a natural amino acid found in human muscles and other organs. Sarcosine has been demonstrated to help patients with obsessive-compulsive disorder (OCD). Its antidepressant activity comes from enhancing the activity of NMDA receptors in the brain, which helps the brain adapt and is involved in memory. (30) We also introduce sarcosine oxidase to the system for the purpose of controlling the sarcosine concentration.

Sarcosine oxidase (SOX, EC 1.5.3.1) is an enzyme that catalyzes the oxidative methylation of sarcosine and forms formaldehyde, glycine and hydrogen peroxide. Intramolecular sarcosine oxidase appears in many organisms, including mammals, bacteria and plants. Together with the creatine/creatinase reaction, the concentration of final product hydrogen peroxide is used to estimate creatine concentration, which is important in determining kidney function in clinics. (31)

Hexokinase is an enzyme that phosphorylates six-carbon sugars (hexoses) to hexose phosphates. The most important substrate of hexokinase in most tissues and organs is glucose, which hexokinase
catalyzes transfer of a phosphoryl group from ATP to glucose and forms glucose 6-phosphate. The optimal pH of hexokinase from *Saccharomyces cerevisiae* (EC 2.7.1.1) is around 7.5-9, the enzyme also experiences much less inhibition by ATP at pH around 8.5 than pH at around 7.0, indicating hexokinase works well in basic pH and therefore can be a source of protons in our system. (32)
3. Methods and Models

We have mentioned the linear stability analysis in section 2.2.1, which by solving the nonlinear differential equations, we can find the real value of inflow rate in CSTR to give rise to oscillations and bistability in the system. Unfortunately, the model we are interested in contains too many variables, and it is unrealistic to conduct linear stability analysis here. Instead of using LSA, we look for bistability by solving numerically differential equations based on chemical reactions of the system with an in-house MATLAB script.

Therefore, in this thesis we present the numerical analysis of the kinetics of an enzymatic network involving: creatinase, urease and sarcosine oxidase in the presence of sulfuric or acetic acid. In this chapter, we introduce the chemical and biochemical equations, the differential equations based on the reaction rates, and the initial conditions for the simulations of the system. We divide our study in three sections. In the first one, we include the substrate-enzyme urea-urease in the presence of the acid and also a urease inhibitor. In the second section, we add to that system the substrate-enzyme creatine-creatinase and remove urea as a substrate; this is because urea is being produced by the creatinase. In the third section, we add to the creatine-creatinase-urease system the sarcosine oxidase enzyme.

3.1 Model

3.1.1 Urea-urease system

The first step is to reproduce the work reported by Taylor et.al in 2010. (27) We used their enzymatic model to find the bistability region in the urea-urease/acid system. However, we point out that we use a different constant $k_1$ for urease (see appendix A). The reason for this modification is that their calculation of the constant was not clear and the author could not clarify its origin.

$$\text{CO(NH}_2\text{)}_2 + \text{H}_2\text{O} \xrightarrow{k_1} \text{urease} \quad \text{2NH}_3 + \text{CO}_2$$ (3.1)
Besides enzymatic reaction that affects the pH, pH of the system is also determined by following equilibria:

$$\text{NH}_3 + \text{H}^+ \rightleftharpoons \text{NH}_4^+ \quad (3.2)$$

$$\text{CO}_2 + \text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{HCO}_3^- \quad (3.3)$$

$$\text{HCO}_3^- \rightleftharpoons \text{H}^+ + \text{CO}_3^{2-} \quad (3.4)$$

$$\text{H}_2\text{O} \rightleftharpoons \text{H}^+ + \text{OH}^- \quad (3.5)$$

$$\text{HA} \rightleftharpoons \text{H}^+ + \text{A}^- \quad (3.6)$$

The rate equations for the system are shown below

$$v2 = k_2 * \text{NH}_4$$

$$v2r = k_{-2} * \text{NH}_4$$

$$v3 = k_3 * \text{CO}_2$$

$$v3r = k_{-3} * \text{HCO}_3^- * \text{H}^+$$

$$v4 = k_4 * \text{HCO}_3^-$$

$$v4r = k_{-4} * \text{CO}_3^{2-} * \text{H}^+$$

$$v5 = k_5$$

$$v5r = k_{-5} * \text{OH}^- * \text{H}^+$$

$$v6 = k_6 * \text{HA}$$

$$v6r = k_{-6} * \text{A}^- * \text{H}^+$$
where \( v_i \) is the forward reaction, and \( v_i r \) is the reverse reaction, \( k_i \) is the rate constant for the forward reaction, and \( k_i \) is the rate constant for the reverse reaction (for \( i = 1, 2, \ldots, 6 \)). Each chemical species in the rate equation represents the concentration of that species.

For the urease, the enzyme rate \( v l \) is written based on the Michaelis-Menten kinetics and is given by the following expression:

\[
v1 = \frac{k_{1+E1+U}}{\left(\frac{K_M+U}{K_U}\right)\left(1+\frac{NH_3^+}{K_P}\right)\left(1+\frac{K_{es2}+H^+}{K_{es1}}\right)};
\]  

(3.17)

In this equation, \( E1 \) is urease concentration, \( U \) is urea concentration, \( K_M \) is the Michaelis constant for urease, \( K_U \) is the equilibrium constant of the uncompetitive substrate inhibition (urea), and \( K_P \) is the equilibrium constant for the noncompetitive product inhibition. The enzyme rate is also affected by formation of an active protonated enzyme-substrate couple (\( K_{es2} \)) and an inactive biprotontated form (\( K_{es1} \)).

The model consists of the following differential equations:

\[
\frac{dH^+}{dt} = v2 - v2r + v3 - v3r + v4 - v4r + v5 - v5r;
\]  

(3.18)

\[
\frac{dNH_4^+}{dt} = -v2 + v2r;
\]  

(3.19)

\[
\frac{dNH_3}{dt} = 2v1 + v2 - v2r;
\]  

(3.20)

\[
\frac{dCO_2}{dt} = +v1 - v3 + v3r;
\]  

(3.21)

\[
\frac{dHCO_3^-}{dt} = +v3 - v3r - v4 + v4r;
\]  

(3.22)

\[
\frac{dCO_3^{2-}}{dt} = +v4 - v4r;
\]  

(3.23)

\[
\frac{dOH^-}{dt} = +v5 - v5r;
\]  

(3.24)
\[
\frac{dA^-}{dt} = +v5 - v5r; \\
\frac{dHA}{dt} = -v6 + v6r; \\
\frac{dU}{dt} = -v1;
\]

where the variables corresponding to concentrations are as follows: \(HA = [\text{CH}_3\text{COOH}]\) or \([\text{H}_2\text{SO}_4]\) (depends on which acid we are using), \(A^- = [\text{CH}_3\text{COO}^-]\) or \([\text{HSO}_4^-]\), and \(U = [\text{urea}]\) in solution.

<table>
<thead>
<tr>
<th>Rate Constants (^{27})</th>
<th>(k_2/\text{M}^-\text{s}^{-1})</th>
<th>(k_{-2}/\text{s}^{-1})</th>
<th>(k_3/\text{s}^{-1})</th>
<th>(k_{-3}/\text{M}^-\text{s}^{-1})</th>
<th>(k_4/\text{s}^{-1})</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>24</td>
<td>(4.3 \times 10^{10})</td>
<td>0.037</td>
<td>(7.9 \times 10^4)</td>
<td>2.8</td>
</tr>
<tr>
<td>Rate Constants (^{27})</td>
<td>(k_{-4}/\text{M}^-\text{s}^{-1})</td>
<td>(k_3/\text{M}^-\text{s}^{-1})</td>
<td>(k_{-5}/\text{M}^-\text{s}^{-1})</td>
<td>(k_6/\text{s}^{-1})</td>
<td>(k_{-6}/\text{M}^-\text{s}^{-1})</td>
</tr>
<tr>
<td></td>
<td>(5 \times 10^{10})</td>
<td>(1 \times 10^{-3})</td>
<td>(1 \times 10^{11})</td>
<td>a. (1.2 \times 10^9)</td>
<td>b. (7.8 \times 10^5)</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>a. (1 \times 10^{11})</td>
<td>b. (4.5 \times 10^{10})</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme Constants (^{27})</th>
<th>(k_1/\text{unit}^{-1})</th>
<th>(K_\text{v}/\text{M})</th>
<th>(K_{\text{es}1})</th>
<th>(K_{\text{es}2})</th>
<th>(K_\text{u})</th>
<th>(K_\text{P})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td>(4.02 \times 10^{-5})</td>
<td>(3 \times 10^{-3})</td>
<td>(5 \times 10^{-6})</td>
<td>(2 \times 10^{-9})</td>
<td>3</td>
<td>(2 \times 10^{-3})</td>
</tr>
</tbody>
</table>

Table 1. Rate constants for urea-urease reactions. a. \(\text{HSO}_4^-\). b. \(\text{CH}_3\text{COOH}\). * see Appendix 1.

In table 1, rate constants and enzyme parameters of above equations are summarized. Rate constants for (5) - (9) are adopted from literature. (27)

In order to find the oscillatory regime, we add an inhibitor for the urease, whose activity as inhibitor depends on the concentration of hydroxide ions. This inhibitor acts like a sink for the urease concentration as the solution becomes more basic. In the simulations, the concentration of urease \((EI)\) is not a constant and its concentration changes in time.

\[
v7 = k_7 \ast OH^- \\
\frac{dE1}{dt} = -v7;
\]

and its corresponding differential equation:
For the purpose of finding the oscillatory behavior of the system, we manipulate the inhibitory rate by varying the value for the rate constant \( k_7 \).

### 3.1.2 Creatine-creatinase-urease system

The second enzyme creatinase is introduced to system. It catalyzes the transformation of creatine into urea and sarcosine.

\[
\text{Creatine} + H_2O \xrightarrow{k_1 C} \text{Sarcosine} + \text{CO(NH}_2\text{)}_2
\]  

(3.30)

Creatine, creatinase and sarcosine are added to the model as three new varying species. Differential equation (10) is modified, and two new differential equations are added:

\[
\frac{dU}{dt} = -v1 + v1C; \quad (3.27b)
\]

\[
\frac{dCr}{dt} = -v1C; \quad (3.31)
\]

\[
\frac{dSr}{dt} = v1C; \quad (3.32)
\]

where \( U \) = [urea] in solution, \( Cr \) = [creatine] in solution, and \( Sr \) = [sarcosine] in solution.

Michaelis-Menten kinetics for creatinase is defined as following:

\[
v1C = \frac{k_1 C \cdot E2 \cdot Cr}{((K_{MC} \cdot (1 + \frac{Sr}{K_{IC}}) + Cr) \cdot (1 + \frac{K_{es2C}}{H^+} + \frac{H^+}{K_{es1C}}))}; \quad (3.33)
\]

where \( E2 \) represents the concentration of creatinase, \( K_{MC} \) is the Michaelis constant, \( k_{IC} \) is the equilibrium constant for noncompetitive product (sarcosine) inhibition. The calculation for the value of \( k_1 C \) is presented in appendix A.
Table 2. Rate constants for creatinase.

Simulations including creatine-creatine-hydrolyase were carried out in the presence and in the absence of the urease inhibitor.

### 3.1.3 Creatine-creatine-urease-sarcosine oxidase system

Sarcosine oxidase is the third enzyme of the system, and it is introduced for the purpose of controlling the concentration of sarcosine in the solution.

\[
\text{Sarosine} + \text{H}_2\text{O} + \text{O}_2 \xrightarrow{k_1S} \text{CH}_2\text{O} + \text{H}_2\text{O}_2 + \text{Glycine}
\]

The Michaelis-Menten form of enzyme rate is:

\[
v1S = \frac{k_1S \cdot E3 \cdot Sr}{(K_{MS} \cdot \left(1 + \frac{K_{es2S}}{H^+} + \frac{H^+}{K_{es1S}}\right))}
\]

where \( E3 \) represents the concentration of sarcosine oxidase, \( K_{MS} \) is the Michaelis constant, \( K_{es2} \) is the formation of an active protonated enzyme-substrate couple and \( K_{es1} \) is an inactive biprototated form.

Differential equations (13) is modified as following:

\[
\frac{dSr}{dt} = v1C - v1S;
\]

Table 3. Rate constants for sarcosine oxidase.

Simulations including creatine-creatine-urease-sarcosine oxidase were carried out in the presence and in the absence of the urease inhibitor.
3.2 Simulations

The set of differential equation was solved by using a MATLAB function-solver called odes15s, which the latter is the abbreviation of the *ordinary differential equation* that can solve a dependent variable with respect to a single independent variable *t* (time for the most cases). Figure 3.1 shows an example of the usage of odes15s. Here, a time step, a relative tolerance, and an absolute tolerance were set to be 0.01, $1 \times 10^{-12}$, $1 \times 10^{-13}$ respectively.

```matlab
%% settings
startofsim = 0;           %start of simulation
duration = 3600;          %duration of simulation
relative_tolerance = 1e-12;    %relative tolerance of time step
absolute_Tolerance = 1e-12; %absolute tolerance of time step
non_negative = 1:1:length(ic); %index variables which cannot go below zero

% bundling settings into a cell array
options = odeset('RelTol', relative_tolerance, ... 'AbsTol', absolute_Tolerance,... 'NonNegative', non_negative);

% solver returns T series and Y (variable values in a row vector) series
[Tm,Y] = odes15s(@(t,y) odesUK7(t, y, flow, k6, k7value), [startofsim startofsim+duration], ic, options);
merged = array2table([Tm, Y], 'VariableNames', {'t', 'h', 'NH4+', 'NH3', 'CO2', 'HCO3-', 'CO3^2-', 'OH-', 'A^-', 'HA', 'U', 'U1'});
```

Figure 3.1 An example of odes15s used in our simulations.

Simulations of reactions are assumed to be performed in a CSTR. The flow is the set of initial conditions (ics) that we chose. In our study, there are two sets of initial conditions that give the system either low or high initial pH; this is before reactants are input into CSTR. The ics are given in table 4.

<table>
<thead>
<tr>
<th>Initial Condition</th>
<th>[H+]</th>
<th>[NH₄⁺]</th>
<th>[NH₃]</th>
<th>[CO₂]</th>
<th>[HCO₃⁻]</th>
<th>[CO₃²⁻]</th>
<th>[OH⁻]</th>
<th>[A⁻]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Higher pH</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Lower pH</td>
<td>$5 \times 10^{-9}$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Table 4. Initial conditions (ics) of the system. *Creatine* and *Sarcosine* concentration only applies for creatine-urea-urease and sarcosine oxidase models.
In Table 5, we show the inflow concentration of each substrate and enzyme. When performing simulations, we used a range of concentrations; however, we will present values with best bistability and oscillation behaviors in the results and discussion section.

<table>
<thead>
<tr>
<th></th>
<th>Urea-urease</th>
<th>Creatine-creatinase-urease</th>
<th>Creatine-creatinase-urease-SOX</th>
</tr>
</thead>
<tbody>
<tr>
<td>[urea]₀ (M)</td>
<td>5 × 10⁻³</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[creatinine]₀ (M)</td>
<td>0</td>
<td>0 – 1</td>
<td>0 – 1</td>
</tr>
<tr>
<td>[sarcosine]₀ (M)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>[HA]₀ (M)</td>
<td>0 – 1×10⁻³</td>
<td>0 – 1×10⁻³</td>
<td>0 – 1×10⁻³</td>
</tr>
<tr>
<td>[urease] (units mL⁻¹)</td>
<td>1.4</td>
<td>1.4</td>
<td>1.4</td>
</tr>
<tr>
<td>[creatinase] (units mL⁻¹)</td>
<td>0</td>
<td>0 – 10</td>
<td>0 – 10</td>
</tr>
<tr>
<td>[sarcosine oxidase] (units mL⁻¹)</td>
<td>0</td>
<td>0</td>
<td>0 – 1×10⁴</td>
</tr>
</tbody>
</table>

Table 5. Inflow concentration of substrates and enzymes in three different enzymatic models.
4. Results and Discussion

The overall idea of the project is to design a network of enzymes that rhythmically produces a drug in situ, in this case the anti-depressant sarcosine, by consuming substrates found in the body. A periodic dosing of the antidepressant could maintain its concentration within a therapeutic window, avoiding valleys where the therapeutic properties vanish or peaks where the patient may show adverse effects such as irritability (35).

The specific goals for the project presented here are a) to identify a group of enzymes capable of producing the antidepressant sarcosine by using substrates found in human muscle and b) to find the initial conditions required for the enzymatic system to produce sarcosine rhythmically and to induce the temporal fluctuation of the pH of the solution. Because finding these conditions experimentally by trial and error could consume large amounts of time and resources, here we use numerical simulations based on the kinetics of the enzymatic reaction and of the species in solution that affect the pH.

Sarcosine is a product of the enzymatic action of creatinase on creatine. This enzyme exists mainly in microorganisms and it has been also detected in human skeletal muscle; however, its confirmation and physiological relevance in humans are still pending. (36) In mammals, phosphorylated creatine exists mainly in muscles and is the substrate of creatine kinase, an enzyme that converts it into creatine and cellular energy ATP. (29) Because of the availability of creatine in muscles, in this project we will use the creatine-creatinate system as the source of the antidepressant sarcosine.

With the aim of delivering sarcosine in a self-sustained and oscillatory manner; we will combine a pH oscillator and the enzyme sarcosine oxidase with the enzymatic reaction mentioned above. Oscillations in the pH will be used to couple the enzymatic system proposed here with a pH sensitive gel; however, this coupling is out of the scope of the research presented here. Sarcosine oxidase is used here as the controller for the concentration of the antidepressant.
Figure 4.1 shows a schematic representation of the enzymatic system proposed. Here, the pH oscillator is built by the production of ammonia resulting from the action of urease on urea; and the production of hydronium ions as a product of the enzymatic reaction of a hexokinase.

The results reported in this thesis are the results of the simulations using three enzymes creatinase-urease-sarcosine oxidase, the substrate creatine, an acid as the source of H⁺, and an inhibitor for the urease to induce pH oscillations. Future work will include the addition of the hexokinase as a replacement of the acid used in this work.

![Figure 4.1 Schematic drawing of the enzymatic system: Creatine-creatinase-urease-sarcosine oxidase with hexokinase which might be incorporated in the future.](image)

**4.1 Urea-Urease system**

Based on the bell-shaped graph of urease activity (see section 2.4), at a low pH, the urease reaction rate is slow; the rate gradually accelerates as the pH of system increases because of the production of ammonia. Urease activity reaches its maximum at pH around 7 and gradually falls to 0 when pH continues to increase; this is an example of negative feedback. Taylor et al. in 2010 proposed a model using urea-urease reaction to generate a pH oscillator. (27) They tuned the initial low pH of the system with sulfuric...
acid and acetic acid. Because of the buffering effect acetic acid has at around pH 4 to 5, a weaker feedback is expected from this acid for its weaker ability in producing H⁺ ions.

**Bistability**

Taylor et al. simulated the urea-urease reaction under open conditions with inflow of urease, acid and urea, where inflow concentrations are \([\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}, [\text{urease}]_0 = 1.4 \text{ units mL}^{-1}\). With an inflow of acetic acid, a phase diagram of inflow acid concentration versus inflow rate is plotted. (Figure 4.1) Three regions of steady states were observed: TS represents single thermodynamics steady state, FS is single flow state, and BS is bistability which two states coexist. Because the existence of bistability is one of the properties desired in a system when looking for oscillatory behaviors, Taylor’s system is the basis for the tri-enzymatic system introduced here. For this reason, we use the same initial and flow conditions to run our numerical simulations. However, we use a different rate constant for urease (as mentioned in section 3.1.1).

![Figure 4.2 Simulated phase diagram of BS (bistability), TS (high pH) and FS (low pH) with inflow of acetic acid. (Adapted from Ref. 27)](image_url)

The phase diagram we obtained in our simulations for acetic acid (Figure 4.2) is similar to their results (Fig 4.3). The concentration of acetic acid varies from 0 to 1mM, and flow rate changes from 0 to 0.1 s⁻¹. Each steady state has similar shape and area to theirs; however, the bistability region is shifted both
upward and rightward. For instance, we don’t observe bistability when [CH₃COOH] is around 0.1mM and when k₀ is about 0.01.

Figure 4.3 Simulated phase diagram under open conditions with inflow concentration: [CO(NH₂)₂]₀ = 5mM, [urease]₀ = 1.4 units mL⁻¹. Inflow rates and acetic acid concentration vary.

Figure 4.4 Simulated diagrams with inflow of sulfuric acid. (a). bifurcation diagram shows bistability of the system. (b) phase diagram. (Adapted from Ref. 27)

With an inflow of sulfuric acid, they showed a bifurcation diagram where the system was stable at both low pH (around 4) and high pH (around 9) when k₀ was between 0.007 and 0.35. (Figure 4.4a) The pH plotted here is the final pH of the system. Compared to acetic acid, sulfuric acid in their model generated bistability over a larger range of flow rates. (Figure 4.4b) However, our model shows that sulfuric acid, instead of giving more bistability with respect to acetic acid, makes the bistability region shrinks. In Figure 4.5a, bistability is only observed over an acid range from 0.2mM to 0.55mM, and at each acid concentration, bistability can only be sustained across a small range of k₀. This might be attributed to the different urease
rate constant used in our simulations; which has a value of \(4.02 \times 10^{-5}\) unit\(^{-1}\) mL M\(^{-1}\) s\(^{-1}\). Compared to their value \(3.7 \times 10^{-6}\) unit\(^{-1}\) mL M\(^{-1}\) s\(^{-1}\), our value is 10 times greater, which results in a faster production of the base (ammonia). This causes damping of the effect of the base-catalyzed positive feedback which occurs before the optimum enzyme pH; this results in the loss of bistability area. Bistability only appears when either sulfuric acid concentration is high and inflow is slow or sulfuric acid concentration is low and inflow is fast, indicating there is a threshold for total amount of sulfuric acid in the system to produce bistability.

Figure 4.5 Simulated phase diagrams with inflow of sulfuric acid with rate constants we modified. (a) phase diagram with inflow concentration: \([\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}, [\text{urease}]_0 = 1.4\text{ units mL}^{-1}\). (b) bifurcation diagram, acid concentration is 0.122Mm.
Besides decrease in bistability, the TS region slightly expands. Because TS accounts for the basic steady state, this can resolve by the fact more ammonia is produced by urea-urease reaction in our system. The phase diagram agrees with the bifurcation diagram shown in figure 4.5b, in which bistability appears at the same range of $k_0$.

**Oscillations**

The urea-urease model does not show any oscillations for any parameters explored. In order to generate oscillations, we added a negative feedback reaction to the bistable system we already had. For this purpose, we include an inhibitory effect for the urease. In our model, the rate constant of the inhibitory reaction is a parameter that we can vary to explore the existence of oscillations. Based on the characteristics of the inhibitor needed to find oscillations, we could identify a real urease inhibitor for the experimental reproduction of the simulations outcome later. Here, we first introduce a small inhibitory effect in both strong/weak acid systems, and generate 3-dimensional phase diagram to illustrate the dynamical behavior of the system at each level of inhibition. The bistability region is observed as expected; however, there are no oscillations present with either of the acids systems for $k_7$ (inhibitory rate constant) values between $0 - 1 \times 10^5$ M$^{-1}$ s$^{-1}$. In figure 4.6, the green region (color bar No. 3) is stationary state 2 (high inflow rate), the blue region (color bar No. 5) is bistability and the yellow region (color bar No. 1) is stationary state 1 (low inflow rate). Other numbers on the color bar are mixed states and are defined as following: No. 3 is micro-oscillation, No. 4 is oscillation, No. 6 is micro-oscillation mixed with oscillation, No. 7 is oscillation mixed with steady state 1 or 2. With inflow of acetic acid, the bistability region is smaller than that found in inflow of sulfuric acid. (Figure 4.6 and 4.7, respectively) This is opposite to our results without inhibitory effect $k_7$, and the possible explanation is the inhibitory effect overcomes the divergence caused by the difference between urease rate constants; therefore, the system now displays the weaker destabilizing effect from acetic acid (smaller bistability region obtained). The red points on the phase diagram are stationary state
mixed with micro-oscillations (color bar No 8); however, they only appear when acid concentration is 0 and the inflow rate is nearly equal to 0. Therefore, we do not consider that oscillations appear in this case.

Figure 4.6 3D phase diagrams with inflow of acetic acid. $k_7$ from left to right: $1 \times 10^5$, $5 \times 10^3$ and 0. Flow rate $k_0$ on x axis, $k_7$ on y axis, and acid concentration on z axis. Inflow concentrations: $[\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}$, $[\text{urease}]_0 = 1.4$ units mL$^{-1}$.

Figure 4.7 3D phase diagram with inflow of sulfuric acid. Inflow concentrations: $[\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}$, $[\text{urease}]_0 = 1.4$ units mL$^{-1}$.
We then investigate the effect of larger inhibitory effect, where $k_7$ is greater or equal to $5 \times 10^5$ M$^{-1}$ s$^{-1}$. More mixed states have been generated, and there are no clear separate regions seen on the sulfuric acid 3D phase diagram. (Figure 4.8) But on the diagram of acetic acid, there is a clear band of red dots which is mixed modes of micro-oscillations and stationary states, and another clear blue band, which is the bistability region (Figure 4.8) Figure 4.8 and 4.9 also show the existence of steady states and oscillations.

![Figure 4.8 3D phase diagram with inflow of sulfuric acid. $k_7$ from left to right: $1 \times 10^7$, $5 \times 10^7$ and 0. Inflow concentrations: [CO(NH$_2$)$_2$] = 5mM, [urease]$_0$ = 1.4 units mL$^{-1}$.](image)

![Figure 4.9 3D phase diagram with inflow of acetic acid. $k_7$ from left to right: $5 \times 10^7$, $2.5 \times 10^7$ and 0. Inflow concentrations: [CO(NH$_2$)$_2$] = 5mM, [urease]$_0$ = 1.4 units mL$^{-1}$.](image)
in which the acid concentration is low. This corresponds to the fact that strong inhibition affects the production of ammonia, and therefore only little acid is needed to achieve pH oscillations.

In order to find the experimental condition where the system can produce oscillations, we further decreased the inflow acid concentration. The 3D phase diagrams of figure 4.10 and 4.11 give clearer bands of mixed oscillations, steady state 1 and steady state 2. With acetic acid, when \( k_7 \) is \( 5 \times 10^7 \) M\(^{-1}\) s\(^{-1}\), little mixed oscillations and stationary states are observed as seen in color bar 7. (Figure 4.10) The phase diagram looks like a typical cross-shaped diagram, in which mixed oscillation and bistability lie between two stationary states. When we increase or decrease \( k_7 \), mixed oscillations will reduce to bistability or steady states. Here, \( 5 \times 10^7 \) M\(^{-1}\) s\(^{-1}\) is a possible appropriate chemical feedback parameter under the initial condition of the system. Using a value beyond or below this number could make the negative feedback brought by urease inhibitor too weak to destabilize the steady states into the oscillatory region.

![Figure 4.10 3D phase diagram with inflow of acetic acid. \( k_7 \) from left to right: \( 1 \times 10^7 \), \( 5 \times 10^7 \) and 0. Inflow concentrations: \([\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}, [\text{urease}]_0 = 1.4 \text{ units mL}^{-1}\).](image)

In the system with sulfuric acid, color bar 7 (combination of oscillation and steady state 1) is the dominant state. As shown in figure 4.11, mixed oscillation produced with inflow of sulfuric acid appears at a much broader range of acid and \( k_0 \) than with acetic acid. To generate oscillations, the range of the inhibitory effect applied in the system with sulfuric acid is not as limited as the one compared to acetic acid.
The strength of the inhibitory effect used is weaker, and the area of oscillations generated is also bigger.

With only 3D phase diagrams, we cannot directly tell which sulfuric acid is more favorable over the acetic acid to find oscillations.

Figure 4.11 3D phase diagram with inflow of sulfuric acid. \( k_7 \) from left to right: \( 1 \times 10^5 \), \( 5 \times 10^3 \) and 0. Inflow concentrations: \([\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}, [\text{urease}]_0 = 1.4 \text{ units mL}^{-1}\).

Figure 4.12 Examples of oscillations found in the model with inflow of acetic acid. (a) Initial pH of the system is basic; (b) Initial pH of the system is acidic. Parameters: \( k_7 = 5 \times 10^7 \), \([\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}, [\text{urease}]_0 = 1.4 \text{ units mL}^{-1}\).
pH profiles and temporal behaviors of each species are shown in figure 4.12 with acetic acid and 4.13 with sulfuric acid. In contrast with 3D phase diagrams in which acetic acid generates a greater area of oscillations, kinetic profiles show that only sulfuric acid can produce oscillations under two different sets of initial conditions. With inflow of sulfuric acid, sustained oscillations are only found when the initial condition was slightly acidic. (Figure 4.12b) When the initial pH is basic, only one peak is generated and pH then becomes a constant. (Figure 4.12a) With inflow of sulfuric acid, sustained oscillations are found at both sets of initial conditions. (Figure 4.13) The pH variations discovered in both cases are close which is between 4.5 — 9.5. However, the periods of oscillation vary: 11.11 s when initial pH is basic and 270 s when initial pH is acetic. Oscillation also lasts a longer time as seen in figure 4.13b. Together with 3D
phase diagrams we obtained, we may conclude that sulfuric acid is more promising in generating oscillations.

4.2 Creatine-creatinalse-urease system

Bistability is demonstrated numerically in the urease system when a second enzyme, creatinase, is used to transform creatine into urea and sarcosine. In our simulations, the inflow of urea is removed because we only consider using the urea produced by creatine-creatinalse reaction. Creatine and creatinase are added to the system as new inflow parameters.

Both creatine/creatinalse ratio and urease/creatinalse ratio can control the existence and magnitude of the bistability region. With little amounts of creatinase and excess amount of creatine presented in the system, the bistability region was very small and can be controlled by increasing creatinase/urease ratio. When creatine/creatinalse ratio is about 1, bistability region becomes larger and expands in both higher acid concentration and higher flow rate direction. When creatine/creatinalse ratio is below 1, creatinase is in excess and creatine concentration can control the bistability region. We found the most bistability when urease/creatinalse ratio is 1 and creatine/creatinalse ratio is below 1. The rate constants $k_1$ for urease and $k_1C$ for creatinase are the same. ($4.05 \times 10^{-5}$ unit$^{-1}$ mL M$^{-1}$ s$^{-1}$ compare to $4.09 \times 10^{-5}$ unit$^{-1}$ mL M$^{-1}$ s$^{-1}$). Their rates of taking substrates are close; this means when creatinases/urease ratio is one, creatinase acts on creatine and produces ammonia, ammonia will be intake immediately by urease. Ammonia reacts with acid, and forms NH$_3$-NH$_4^+$ buffer in the solution at high pH. While creatinase will not be saturated, saturation of urease might be another important negative feedback in the system.

The 3D phase diagram for creatine-creatinalse-urease system are shown in figure 4.14 (with acetic acid) and 4.15 (with sulfuric acid). For each system the acid concentration we use for numerical simulations is up to $[0, 5 \times 10^{-4}]$ M, creatine concentration range from 0 to 0.1 M, and flow rate changes from 0 to 0.15.
Bistability, steady state 1 and steady state 2 are observed on the phase diagrams, but oscillations are not obtained. Bistability can be sustained on a wide range of flow rates. For acetic acid, a wide range of acid concentration over bistability is observed; for sulfuric acid, range of acid shrinks to half of acetic acid’s. With each acid, the gradual increase in creatine shifts a very slight change in bistability, which might suggest creatine is not the key species to tune the bistability of the system.

Figure 4.14 3D phase diagram with inflow of acetic acid. [creatinine] from left to right: 0.1, 0.05 and 0. Flow rate $k_0$ on x axis, [creatinine] on y axis, and acid concentration on z axis. Inflow concentrations: $[\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}, [\text{urease}]_0 = 1.4 \text{ units mL}^{-1}$, creatine/urease ratio = 1. (Color bar 5 is bistability)

Figure 4.15 Figure 4.14 3D phase diagram with inflow of sulfuric acid. [creatinine] from left to right: 0.1, 0.05 and 0. Inflow concentrations: $[\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}, [\text{urease}]_0 = 1.4 \text{ units mL}^{-1}$, creatine/urease ratio = 1.
The overall the shape of bistability we obtained here is similar to what we got in the urea-urease system; one difference is creatine-creatinase-urease system can reach bistability by adding small amount of acid (~ $1 \times 10^{-5}$ M), which urea-urease system cannot. Another difference is the species we use to control the bistability of the system. Here, we can control bistability by varying concentration of creatine: when [creatine] is smaller than 0.01M, increase its concentration can its bistability region; and when [creatine] is greater than this number, there will no change in bistability region.

**Inhibitory effect**

Just as the urea-urease system, the creatine-creatinase-urease system didn’t demonstrate any oscillation. Here again we introduced the inhibitory effect of urease to the creatine-creatinase-urease system and the value we applied are large $k7$ values. Initial conditions and inflow concentration of each substrate we used are numbers we found promising from the last section. In principle, if the amount of urea produced

![Figure 4.16 3D phase diagram with inflow of acetic acid. [creatine] from left to right: 2, 1 and 0.1. Parameters: $k7 = 1 \times 10^6$, $[\text{CO(NH}_2\text{)}_2]_0 = 5\text{mM}$, $[\text{urease}]_0 = 1.4$ units $\text{mL}^{-1}$, creatinase/urease = 1.](image)
by the creatine-creatinase reaction is close to the inflow concentration of urea used in the urea-urease system, similar oscillatory patterns are expected here.

Figure 4.17 3D phase diagram with inflow of sulfuric acid. [creatine] from left to right: 2, 1 and 0.1. Parameters: \( k_7 = 1 \times 10^6 \), \([\text{CO(NH}_2\text{)}_2]\)_0 = 5 mM, \([\text{urease}]_0 = 1.4 \text{ units mL}^{-1}\), creatinase/urease = 1.

In figure 4.16 and 4.17, 3D phase diagrams of creatine-creatinase-urease are plotted, with inflow of acetic acid and sulfuric acid respectively. Inhibitory effect \( k_7 \) is a constant for both figures, \( 5 \times 10^7 \text{ M}^{-1}\text{s}^{-1} \) for acetic acid and \( 5 \times 10^6 \text{ M}^{-1}\text{s}^{-1} \) for sulfuric acid. First, the system does not show oscillations with this condition even when we applied an inhibitory effect. We only obtained bistability, stationary state 1 and stationary state 2. Red dots are observed when acid concentration was 0, and can be considered as the noise of the simulations. Comparing figure 4.16 and 4.17, change in concentration of creatine didn’t change the bistability of the system if acetic acid was used, but increase in creatine concentration makes the bistability region in the system with sulfuric acid shrink. The shape of the bistability region is similar for both conditions, in which the bistability region is across a broad range of flow rate and acid concentrations. When comparing figures 4.14 and 4.16, the bistability region doesn’t shift, indicating the dynamical behavior of the system depends little if the initial pH of the system is adjusted by the weak acid. When
comparing figures 4.15 and 4.17, the bistability region expands a lot if the inhibitory effect is considered; this may suggest that as a strong acid, sulfuric acid enables the system to generate more dynamical behaviors with response to the inhibition of the enzyme. The distinctions in the bistability region between figure 4.14 and 4.15 are obvious, but these distinctions disappeared after the negative feedback was introduced to the system. This phenomenon is interesting, because in previous sections, we have seen how the behaviors of the system depend on the choice of acid to adjust the pH of the system. Here, no behavior differences observed indicates that with addition of the inhibitory effect, the system is less sensitive to the choice of acid, no matter it can form a buffer in the solution.

4.3 Creatine-creatinalse-urease-sarcosine oxidase system

Figure 4.18 Concentration of sarcosine vs. time. Inflow parameters [CH3COOH] = 5x10^-5M, [Creatine]₀ = 0.10M, [creatinalse/urease]₀ =1, [urease]₀ = 1.4 units mL⁻¹, [sarcosine oxidase]₀ = (a) 1, (b)10, (c) 100 units mL⁻¹.

Although we didn’t find temporal oscillating pH in the creatine-creatinalse-urease system, we still want to study how the enzyme sarcosine oxidase can change the dynamical profile of sarcosine. Sarcosine oxidase is added to the system to control sarcosine concentration. Because sarcosine oxidase only acts on
sarcosine and produces products irrelevant to H⁺ dynamics, its existence should not interrupt previously found pH oscillations. We ran numerical simulations with inflow of acetic acid, creatine, urease, creatinase and sarcosine oxidase, and plotted the change in sarcosine concentration versus time. (Figure 4.18) From left to right, sarcosine oxidase concentrations are 1, 10, 100 unit⁻¹ mL⁻¹ M⁻¹ s⁻¹ respectively. When sarcosine oxidase is 1 unit⁻¹ mL⁻¹ M⁻¹ s⁻¹, sarcosine is being produced and its concentration increases rapidly but remains a constant. When sarcosine oxidase is 10 unit⁻¹ mL⁻¹ M⁻¹ s⁻¹, a peak in [sarcosine] appears. The magnitude of the peak decreases with the increase in inflow rate. When we increase sarcosine oxidase to 100 unit⁻¹ mL⁻¹ M⁻¹ s⁻¹, a narrower peak is observed, and the magnitude of the peak increases in comparison with 4.18 (b).

When introducing the inhibitory effect to the creatine-creatinase-urease-sarcosine oxidase system, there still only one peak observed. (Figure 4.19) Comparing 4.19 with 4.18, the peak can be sustained longer and the magnitude of the peaks increases as well.
5. Conclusion

The key factor to generate a pH oscillator is creating a time lag between the positive and negative feedbacks in the system. In the biochemical system, positive and negative feedback can be reflected as the inhibition and activation of enzymes. In this study, we explored the dynamical behaviors of the enzymatic system creatine-creatine-urease-sarcosine oxidase, which is a basis of a pH oscillator that can produce an anti-depressant sarcosine in situ and rhythmically. Bell-shaped curve of urease give rise to base-catalyzed feedback. Substrate and product inhibition are also associated with nonlinearity occurring in the system. We looked for bistability and oscillations in 3 enzyme models and investigated the effect of urease inhibition on the dynamical behavior of the system.

In the urea-urease model, bistability is observed in the presence of a weak acid (acetic acid) and a strong acid (sulfuric acid). The bistability region we obtained roughly agreed with literature (27), but instead of having more bistability with sulfuric acid, the system with presence of acetic acid gave more bistability. We didn’t observe any oscillation until we introduced an inhibitory effect to the system which it inhibited urease irreversibly and its strength depended on the concentration of OH\(^-\). Inhibition of urease decreased the rate of producing ammonia, and therefore OH\(^-\) was produced less and caused less inhibition on urease. This negative feedback allowed us to observe oscillations with a pH variation from 4.5 to 9.5 if the initial pH of the system was adjusted by sulfuric acid. The large inhibitory constant \(k_7 \geq 5 \times 10^4 \text{ M}^{-1} \text{s}^{-1}\) we applied suggests urease needs to be inhibited strongly to produce oscillations.

In the creatine-creatine-urease model, we removed urea from the inflow because it can be produced by creatine-creatine reaction. We expected to see similar pH oscillation in this model if appropriate inhibitory effect is used and the amount of urea generated by the system is close to the concentration of replaced urea inflow. However, we didn’t observe any oscillation cross a wide range of inhibitory constant we used. The introduction of creatine-creatine reaction must lead to other dynamical
behaviors of the system which we didn’t foresee. For the future work to find oscillations, we can expand the map we explore and consider the effect of creatine-creatинase on the rate of urease. The bistability region showed little difference between system using acetic or sulfuric acid, indicating this model is less sensitive to the choice of acids compared to the urea-urease model.

Although no oscillations were found in creatine-creatинase-urease model, we still want to see the sarcosine concentration profile and to study the effect of the enzyme sarcosine oxidase and urease inhibition on this profile. With or without inhibitory effect, sarcosine concentration only generated one peak and then became a constant. The magnitude of the peak mainly depended on the concentration of sarcosine oxidase, and the inflow $k_0$ can control when the peak appears. When inhibition was introduced, the peak was sustained for a longer time, but then the concentration of sarcosine became nearly 0. We believe that there is a very subtle relationship between creatine/creatинase ratio and urease/creatинase ratio to give rise to oscillations in pH and sarcosine concentration. This relationship requires further work.

In the future, for the consideration of biocompatibility, we may substitute sulfuric acid with hexokinase, which is an enzyme that catalyzes the conversion of hexoses into hexophosphate and produces hydrogen ions. If the optimal pH of the hexokinase chosen is basic, a positive feedback now can participate in the autocatalysis of the system.
6. Appendix

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_{cat}/s^{-1}$</th>
<th>Specific Activity/µmol/min · mg pure enzyme</th>
<th>Impure Enzyme Activity/units/g impure enzyme</th>
<th>Molar mass/mol pure enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td>5913</td>
<td>2700</td>
<td>40100</td>
<td>54462</td>
</tr>
<tr>
<td>Creatinase</td>
<td>0.246</td>
<td>0.128</td>
<td>10-15</td>
<td>41000</td>
</tr>
<tr>
<td>Sarcosine Oxidase</td>
<td>458</td>
<td>44.8</td>
<td>25-50</td>
<td>42000</td>
</tr>
</tbody>
</table>

Table 6. Enzymes constants used for kinetic constants calculations.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>$K_M/M$</th>
<th>Rate constants/unit$^{-1}$ mL M$^{-1}$ s$^{-1}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urease</td>
<td>$2.9 \times 10^{-3}$</td>
<td>$4.02 \times 10^5$</td>
</tr>
<tr>
<td>Creatinase</td>
<td>$1.33 \times 10^{-3}$</td>
<td>$4.09 \times 10^5$</td>
</tr>
<tr>
<td>Sarcosine Oxidase</td>
<td>$3.6 \times 10^{-3}$</td>
<td>$2.42 \times 10^5$</td>
</tr>
</tbody>
</table>

Table 7. Calculated enzyme kinetic constants.

Sample calculation for urease rate constant:

Specificity activity changing units:

$$2700 \frac{\mu mol \text{ Urea}}{min \cdot mg \text{ pure enzyme}} \times \frac{1 \text{ min}}{60 \text{ s}} \times \frac{1000 \text{ mg}}{1 \text{ g}} \times \frac{54462 \text{ g}}{1 \text{ mol pure enzyme}} \times \frac{1 \text{ mol pure enzyme}}{1 \times 10^6 \mu mol \text{ Urea}}$$
= 2450.79 \( \frac{\text{umol Urea}}{s \cdot \text{umol pure enzyme}} \)

Impure enzyme activity changing units:

\[
\frac{40100 \text{ units}}{g \text{ impure enzyme}} \times \frac{\mu \text{mol NH}_3}{\mu \text{mol urea} \cdot 60s} = 668.33 \frac{\mu \text{mol NH}_3}{g \text{ impure enzyme} \cdot s}
\]

Ratio impure/pure enzyme:

\[
\frac{668.33}{2450.79} = 0.2727 \frac{\mu \text{mol pure enzyme}}{g \text{ impure enzyme}}
\]

Assume 1M solution used:

\[
1 \times 10^3 \frac{\mu \text{mol}}{mL} \times \frac{1g \text{ impure enzyme}}{0.2727 \mu \text{mol}} \times \frac{40100 \text{ units}}{g \text{ impure protein}} = 1.47 \times 10^8 \text{ units/mL}
\]

\( k_1 \) calculation:

\[
k_1 = \frac{5913 \text{ s}^{-1}}{1.47 \times 10^8 \frac{\text{units}}{mL} \cdot 1M} = 4.02 \times 10^{-5} \frac{mL}{\text{units} \cdot s \cdot M}
\]
7. References


