

# ENZYME KINETICS AND MECHANISM: INVESTIGATING SUBSTRATE PROTONATION BY ADENOSINE DEAMINASE

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## ABSTRACT

In this study we observed how adenosine deaminase catalyzes reactions with adenosine and 6-chloroadenosine at different pHs that ranged from 7.3 to 9.4. The two substrates differed in that 6-chloroadenosine has a Cl atom in the same location where adenosine has a  $\text{NH}_2$  group; this  $\text{NH}_2$  group needs to be protonated in order for the reaction to occur, whereas the Cl does not. The goal of the experiment was to see whether the structure of 6-chloroadenosine changed the pH dependence relative to that of adenosine. The samples with different concentrations of adenosine or 6-chloroadenosine and different pH buffers were passed through a spectrophotometer at wavelength 260 nm and 264 nm respectively. Using the absorbance of each sample over time, the  $k_2$  and  $K_m$  could be calculated using the Michaelis-Menten equation. We found that at higher pHs, such as 8.9 to 9.4, the 6-chloroadenosine was not as pH dependent as adenosine; however, at lower pHs, such as 7, the two substrates were relatively similar in pH dependence. The  $k_2$  decreased at lower pHs with the adenosine substrate than for 6-chloroadenosine. However, more samples need to be run in order to confirm that 6-chloroadenosine has an advantage at higher pHs. For future research, additional functional groups in adenosine deaminase mutants and a broader pH spectrum should be tested.

## INTRODUCTION

### Enzymes

Enzymes act as catalysts in thousands of the chemical reactions which constantly occur in all organisms, including humans. Without the help of enzymes, these reactions would not occur fast enough for life to exist. One such reaction involves the deamination of adenosine. Without enzyme catalysis, this reaction would have a half-life of 120 years. With the help of the enzyme adenosine deaminase (ADA), adenosine's half-life is reduced to less than  $1/370^{\text{th}}$  of a second [1].

Every reaction has activation energy, which is the minimum amount of energy needed to drive a reaction to completion. Enzymes act as catalysts in biochemical processes by lowering the activation energy. The complexity of their structures is characterized by polypeptide chains that fold into tertiary and even quaternary structures. Each enzyme has a location known as the active site where its specific substrate binds. This binding is analogous to a lock-and-key mechanism, in which only a specific size, shape, and orientation of a key (substrate) can fit into the keyhole (active site) of a particular lock (enzyme) [2]. Enzymes and substrates bind to form

enzyme-substrate (ES) complexes. These complexes facilitate quicker reactions by collecting and orientating substrates that would normally have a low probability of reacting. Furthermore, the complexes stabilize the transition state through the use of hydrogen bonds which weaken the intra-substrate bonds and speed up the reaction. Enzymes are very energetically efficient, since they are neither consumed nor altered during the reaction they catalyze. The enzyme does not change throughout the course of the reaction and is able to catalyze the reaction many times [2].

A key component of understanding enzyme kinetics and mechanisms involves studying the effect of substrate concentration on initial reaction rate ( $V_0$ ). Many enzymes exhibit a course of reaction rate that represents a rectangular hyperbolic curve (Figure 1). As substrate concentration increases, the initial velocity increases and approaches maximum velocity.

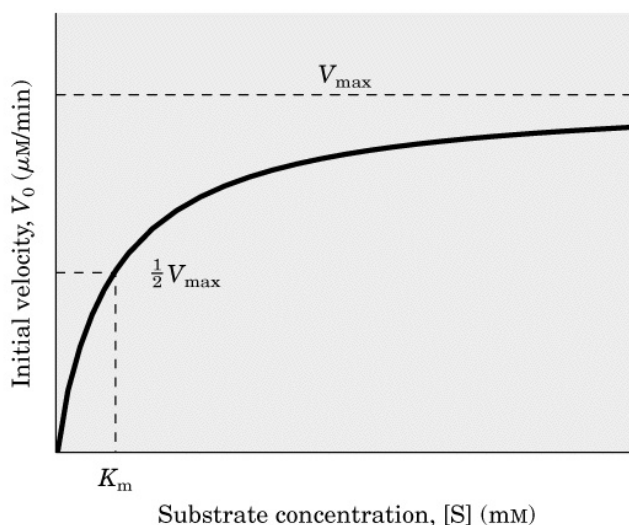
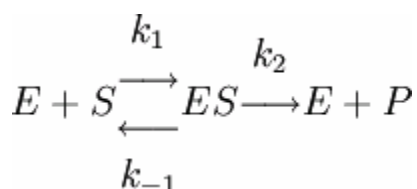


Figure 1: Graph of Substrate Concentration vs. Initial Velocity [3].

### Michaelis-Menten Enzyme Kinetics

In order to explain this phenomenon in enzyme kinetics, Leonor Michaelis and Maud Menten derived an algebraic equation that now bears their names. The derivation is based on the general scheme (see below) for an enzyme ( $E$ ) that catalyzes a reaction by binding to substrate ( $S$ ), forming an enzyme-substrate complex ( $E \cdot S$ ), and finally yielding product ( $P$ ). The rate constants  $k_1$ ,  $k_{-1}$ , and  $k_2$  represent, respectively, the association of substrate and enzyme, the dissociation of unaltered substrate from the enzyme-substrate complex, and the dissociation of altered substrate from the enzyme to form product [4].  $k_1$  is a constant for second-order processes, while  $k_2$  represents a first-order catalysis process and  $k_{-1}$  is a first-order rate constant governing substrate binding. Because this study of enzyme kinetics considers the initial rates of reaction, i.e. when the enzyme is first introduced to the substrate, it is assumed that  $P_0$  is equal to 0 and thus the theoretical reverse reaction, i.e.  $k_{-2}$ , does not occur.



In this scheme, the overall rate of reaction ( $V_0$ ) will be limited by both the process of  $E \cdot S \rightarrow E + P$  with rate constant  $k_2$  and the concentration of enzyme-substrate complex ( $[E \cdot S]$ ) available for reaction. The overall reaction is therefore

$$V_0 = k_2[E \cdot S] \quad \text{Equation 1}$$

It is furthermore assumed that there is an excess of substrate, i.e.  $[S] \gg [E]$ , and that the reaction system is in a steady state, i.e.  $[E \cdot S]$  is constant because the enzyme-substrate complex is formed and degraded at the same rate. This yields the following equation:

Rate of [ES] formation = Rate of [ES] breakdown + rate of [ES] dissociation

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

Rearrangements of this equation will produce the formula for the Michaelis-Menten constant ( $K_M$ ), shown below.

$$K_M = \frac{[E][S]}{[E \cdot S]} = \frac{k_2 + k_{-1}}{k_1} \quad \text{Equation 2}$$

This definition can be rewritten as

$$\frac{[E][S]}{K_M} = [E \cdot S] \quad \text{Equation 3}$$

Next, this study requires the knowledge of the total amount of enzyme ( $E_{total}$ ) present, whether as unbound enzyme ( $E$ ) or part of the enzyme-substrate complex ( $E \cdot S$ ). Therefore,

$$[E_{total}] = [E] + [E \cdot S]$$

This can be rearranged to read that the amount of unbound enzyme is equal to the total amount of enzyme minus the amount of enzyme bound to substrate.

$$[E] = [E_{total}] - [E \cdot S]$$

This definition of  $[E]$  can be substituted into equation 3 and manipulated to yield:

$$\frac{[E]_{total} \cdot [S]}{K_m + [S]} = [E \cdot S]$$

Equation 1 can be rewritten to represent the formula for the maximum rate ( $V_{max}$ ). This is theoretically achieved when all the enzymes are bound to substrates, i.e. when all  $E$  are in the form of  $E \cdot S$ . Thus,

$$V_{Max} = k_2[E_{total}]$$

The definitions of  $V_{max}$  and  $V_0$  can be substituted into the working derivation to yield

$$V_0 = \frac{[E]_{total} \cdot k_2 \cdot [S]}{K_M + [S]}$$
$$V_0 = \frac{V_{max} \cdot [S]}{K_M + [S]} \quad \text{Equation 4}$$

This final equation for  $V_0$  is known as the Michaelis-Menten equation. Using this equation is crucial to the understanding of enzyme kinetics because it incorporates several important values labeled in Figure 3:  $V_0$ ,  $V_{Max}$ ,  $K_M$ , and  $[S]$ .

### Biological Functions of Adenosine

Adenosine, a nucleoside made from adenine bonded to ribose via a  $\beta$ -N<sub>9</sub>-glycosidic bond, forms of the backbone of many biologically essential compounds. As part of adenosine triphosphate, it plays a key role in cellular metabolism, indirectly supplying energy for nearly all biological functions. It is also a primary component of nucleic acids, specifically deoxyadenosine. It may even trigger responses such as coronary vasodilation; reduction in heart rate and contractile force; inhibition of platelet aggregation; mast-cell degranulation; renal vasoconstriction; regulation of ion channel activity; membrane potential; and neurotransmitter and hormone release when bound to specific cellular receptors [5]. Adenosine constantly undergoes deamination, or removal of the amine group, producing inosine, a molecule that is structurally similar to adenosine. Inosine is significant to processes in the human body due to its neuroprotective properties, its presence in tRNA, and its role in proper genetic translation [9].

### Structure and Function of Adenosine Deaminase

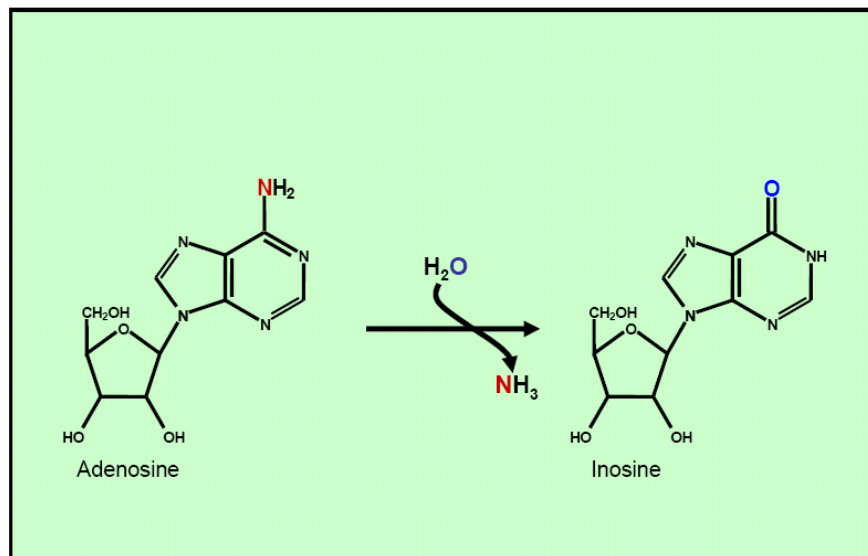


Figure 2: Conversion from Adenosine to Inosine [10].

Adenosine deaminase (ADA) is the enzyme involved in the degradation of adenosine into inosine (Figure 2). Furthermore, ADA converts 2'-deoxyadenosine into 2'-deoxyinosine. ADA is found primarily in the cytosol, though it also resides on the cell surface as ecto-ADA. Structurally, ADA consists of 363 amino acids with a high degree of sequence conservation amongst species. It is comprised of a parallel  $\alpha/\beta$ -barrel motif with central strands and peripheral helices (Figure 3) [5]. Its active site contains a zinc atom, which is essential to the deamination process. Chang et al. (1991) suggested that the catalytic functions of the enzyme are carried out by the amino acids Cys 262, Asp 295, Asp 296, and His 214, while the zinc ion is coordinated by His 15, His 17, His 214, and Asp 295 [6]. Mutations in these amino acids often have deleterious effects on ADA function. Bhaumik et al. (1993) showed that in vitro mutagenesis of the amino acids coordinating the zinc ion eliminated ADA activity [7]. Wilson et al. (1991) suggested that point mutations causing misalignments of the  $\beta$ -strands that partially line the active site pocket would also impair activity [8].

The availability of functioning ADA in a given area is affected by the region-specific typical level of expression. The highest activity of ADA in humans occurs in the thymus and other lymphoid tissues, though high levels are found in non-lymphoid tissues such as the villi of epithelial cells in the duodenum. Additionally, ADA activity has been shown in variable amounts in all cell types of the nervous system, with neural development being negatively affected by the absence of ADA [5]. ADA is also responsible for regulating the concentration of intracellular and extracellular adenosine. ADA-induced extracellular adenosine deprivation has been shown to result in the selection of differentiated cells. This deprivation may reduce tumor growth in the colon and stimulate proliferation of microglial cells in the brain [5]. Though the role of ADA in the body is varied, the most vital process is the metabolism of the purine adenosine and deoxyadenosine.

### Medical Implications of Abnormal Adenosine Deaminase Activity

Understanding the mechanisms by which ADA functions is of particular interest due to the possible ramifications on human health and disease. Though rare, ADA deficiency causes one-half of the autosomal recessive forms of severe combined immunodeficiency disease (SCID) [14]. In this disease, B- and T-lymphocytes are both dysfunctional, impairing cellular immunity and immunoglobulin production. The weakened immune system may be caused by susceptibility of immature lymphoid cells to toxic levels of ADA substrates, adenosine and deoxyadenosine, although the exact mechanisms by which this occurs have yet to be more thoroughly investigated. Additionally, a lack of ADA enzyme activity may result in mesangial sclerosis in renal tissues, pulmonary insufficiency, and liver abnormalities [5].

Because ADA is so vital to maintaining normal body function, deficiencies of the enzyme at the molecular level cause serious disorders when magnified to the level of the organism. ADA has been observed to be particularly active in lymphocyte development due to its association with the CD26 complex found on activated T cells [5]. Activity is also high in thymocytes of the thymic cortex. ADA-deficient patients have elevated accumulations of dAdo and dATP, the phosphorylated end-product of dAdo, in these areas especially [5]. The presence of these substances result in the inactivation of the enzyme *s*-adenosylhomocysteine hydrolase (SAHase),

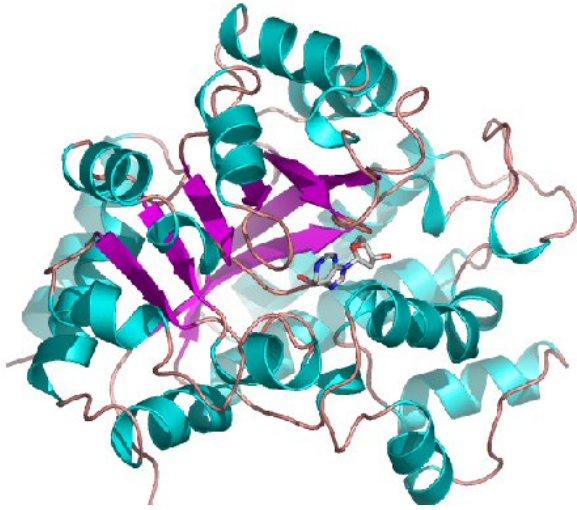


Figure 3: The quaternary structure of adenosine deaminase [12].

imbalances cause the apoptosis of developing thymocytes. This results in the loss of T-cell function and severely weakens the immune system.

Due to the essential role that ADA plays in lymphocyte development and function, abnormal enzyme activity can severely impair the body's defense system. ADA deficiency can manifest itself in varying degrees of weakened immune system functions, with the most deleterious phenotypic effect being severe combined immunodeficiency disease (SCID). The genetic form of SCID may be caused by hot-spot mutations in any of the 40 alleles identified to cause ADA deficiency [11]. This type of SCID is usually first detected in infants but sometimes may not manifest itself until late childhood and adulthood. In cases of complete ADA deficiency, the onset of SCID can be fatal, but, even in mild cases, T-cell function and antibody responses are so depressed that the individual remains significantly immunocompromised and is more susceptible to opportunistic infections. "Delayed onset" is first recognized during the first few years of childhood, while "late onset" shows symptoms in adults. These forms are characterized by an increased risk of chronic infections, commonly sinusitis and pulmonary insufficiency [13]. Additionally, ADA-deficient individuals may have autoimmune phenomena such as lymphopenias, anti-thyroid antibodies, allergies, and elevated serum concentration of IgE [13].

ADA deficiency can be treated by three main mechanisms. The first involves bone marrow or stem cell transplants from a haploidentical donor. This treatment, however, is limited because of the difficulty in finding haploidentical donors who are not ADA deficient themselves. Transplant cells contain the genes necessary to produce functional T- and B-cells, which facilitates proper immune system responses. Another route of treatment is enzyme therapy, which involves direct addition of missing ADA. Human or bovine ADA is covalently attached to polyethylene glycol (PEG) to protect from the body's own degradative enzymes. Weekly injections have been shown to reverse the primary effects of genetic ADA deficiency by establishing sufficient lymphocyte levels and effective antibody responses [11]. Additionally, enzyme therapy reduces toxic levels of dAdo and dATP. A third course of treatment is somatic gene therapy, which creates functional ADA+ T cells. Bone marrow stem cells and peripheral blood

which subsequently impairs some of the methylation processes of nucleic acids, proteins, and lipids [5]. Additionally, elevated dAdo levels inhibits ribonucleotide reductase, and this results in an imbalance of deoxynucleotides, which in turn impairs DNA synthesis and the repair of T-lymphocytes. Thymocytes are particularly affected by ADA deficiencies during the transitional  $CD8^{low}$  and  $CD4^{+}CD8^{+}$  stages of maturation in the thymic cortex [5]. Because of the imbalance of dNTPs, DNA cannot be normally repaired, resulting in DNA breaks in the cell's genome. dATP toxicity also impairs cell function because it depletes the cell of ATP and NAD, chemicals essential to providing energy for metabolism. The cumulative effects of dysfunctional processes and molecular

lymphocytes were transfected with vectors expressing human ADA cDNA in vitro. The results of this manipulation were increased levels of leukocytes and bone marrow progenitors following the incorporation of vector-derived DNA in PBLs [11]. Along the lines of gene therapy is direct T-cell gene modification. T cells are extracted, cultured, and transfected with human ADA cDNA with a retrovirus before being reintroduced into the patient. This technique is advantageous because it avoids injecting the patient with a potentially uncontrollable virus and furthermore increases the efficiency of gene transfer. 1% transfer, for example, increases proliferation by  $10^9$  to  $10^{10}$  new T cells [11].

Additionally, research has shown that the enzyme adenosine deaminase is connected to the formation of malignant lymphomas. In one case a deficiency of adenosine deaminase caused serious harm to a patient's immune system and precipitated the development of a lymphoma. Researchers have found that adenosine deaminase is expressed with the CD26 antigen [16]. Many believe that the CD26 antigen plays an active role in malignant lymphoma and it has been observed that in 42 cases of Hodgekin's lymphoma the CD26 antigen and the ADA antigen have been expressed [15].

### Mechanism of Deamination

The mechanism of the deamination process has been studied to a great degree, and many details of the process have been brought to light.

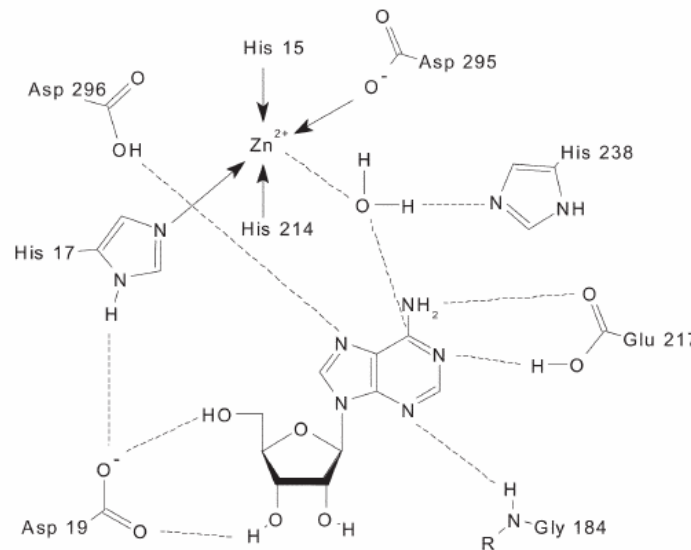


Figure 4: Deamination of adenosine [17].

The zinc ion, held by five atoms from different residues of the enzyme, activates the water molecule and puts it into its position (Figure 4). Histidine 238, or His-238, also plays a role in orienting the water molecule. The hydroxyl group of the water is then attached to carbon that previously formed a trigonal plane with nitrogen, another carbon, and the amine group,  $\text{NH}_2$  [17]. After the tetrahedral bonding pattern is achieved by the carbon, the leftover proton from water is attracted by a base in one of the residues, whose exact identity is of some dispute [18]. Next step

that leads to the intermediate of the activated complex is the transfer of proton from Glu-217 to the nitrogen next to NH<sub>2</sub>. There have been various proposals on the identity of the amine group's protonating agent. For example, a cysteine residue was suggested to be the donor of the proton. Soon afterwards, however, the claim was disproved for the cysteine was found to be too far from the active site, especially the sixth carbon that holds the amine group [17]. To this day, no one idea has gained dominance over the others, and the exact mechanism involving deamination of the amine group still remains as a question.

### Purpose of the Experiment

The objective of the experiment is to become more acquainted with the amine group of the adenosine compound. The unknown protonating agent is not identified through this study, but the reason for adenosine's pH dependency can possibly be found. In addition to adenosine, ADA can convert numerous alternative substrates to inosine. One such alternative substrate is 6-chloroadenosine. Instead of having NH<sub>2</sub> on the sixth carbon of the purine ring as adenosine does, 6-chloroadenosine has a chloride atom. Since the atom's ionic form can exist in nature without difficulty, it does not require protonation in order to be separated from 6-chloroadenosine. Also, ADA exhibits lower activity at higher pH values; the most probable cause for the phenomenon is the decrease in available protons for the substrate to be converted. Therefore, if the increase in pH does not affect the reaction rate of 6-chloroadenosine, it can be said that the protonating agent of NH<sub>2</sub> is the reason of adenosine's alkaline pH dependence. If the reaction rate of 6-chloroadenosine decreases in a similar degree as that of adenosine at higher pH, it can be concluded that the protonating agent of NH<sub>2</sub> is not the cause of pH dependence, since the agent is not in use for this particular reaction involving the chloride ion. In other words, the protonating agent of the amine group has a lesser effect on the reaction rate with relation to pH than originally believed. The pH dependence curve of  $k_2$  for adenosine deaminase with adenosine as a substrate is known, and the pH dependence curve for adenosine deaminase with 6-chloroadenosine will be compared to the adenosine deaminase/adenosine pH curve. Although it is known that there are three primary amino acids in the adenosine deaminase active site which interact with the substrate, it is not known what protonates the NH<sub>2</sub> on the adenine purine ring. This comparison of pH curves will provide insight into which acidic amino acid region protonates the NH<sub>2</sub> [1].

The experiment was carried out using a spectrophotometer, which records the absorbance (Abs) in absorbance units of light in a chemical reaction on the basis of Beer's Law. Beer's Law measures the amount of light absorbed by matter at a specific wavelength. The equation for Beer's Law is shown below, where A represents absorbance (cm<sup>-1</sup>), C concentration,  $\ell$  path length of the cuvette (1 cm), and  $\epsilon_\lambda$  (L·mol<sup>-1</sup>·cm<sup>-1</sup>) how much of the molecule absorbs at a given wavelength of light:

$$A = C\ell\epsilon_\lambda \qquad \text{Equation 5}$$

## PROCEDURE

### Materials

For this experiment 500 mM HEPES buffer at a pH of 7.29, 7.88, 7.48. 500mM Tris buffer at a pH of 8.08, 8.41, and 8.93, as well as 500 mM Ches buffer at a pH of 9.4, were also used. To make the correct dilutions, distilled water filtered with the Millipore water purification system was used. The enzyme adenosine deaminase had a concentration of 0.02 U/mL. All samples were run in a Beckman DU ® 530 spectrophotometer. 4.2E-07 M 6-choloroadenosine stock solution and 1mM adenosine stock solution were used. All chemicals were ordered from Sigma Company.

### Methods

#### *Spectrophotometer Analysis*

Spectrophotometer analysis required the preparation of differential solutions in order to derive initial data of absorbance vs. time for each substrate-pH-[substrate] set. Each 1000  $\mu$ L sample was unique and followed a basic recipe to yield a final composite of 25.0 mM buffer, a variable concentration of adenosine ranging from 5  $\mu$ M to 100  $\mu$ M, and a variable concentration of adenosine deaminase ranging from  $1.4 \times 10^{-10}$  M to  $2.9 \times 10^{-9}$  M, save for a blank, in which adenosine was omitted. HEPES buffers ranging from 7.29 to 8.93 were used to investigate the effects of pH environment on enzyme activity, with one set of varying adenosine solutions (5, 10, 20, 30, 40, 60, and 100  $\mu$ M) created for each pH tested. In all cases, the same concentration of ADA was used throughout the analysis of a substrate-pH set. Reactions were monitored by absorbance at 260 nm and 264 nm for adenosine and 6-chloroadenosine respectively.

After ADA was added to each sample, the differentially concentrated adenosine solutions within a common substrate-pH set were run consecutively in the spectrophotometer to test for absorbance at a wavelength of 260 nm. Data points were collected at consistent intervals for each substrate-pH-[substrate] set, though alterations were sometimes made between different substrate-pH sets in the interest of time. In general, the solutions were run for 10 minutes each with data points recorded at one-minute intervals, while some substrate-pH-[substrate] trials were run for 5 minutes with data collection occurring at 30-second intervals.

In the second part of the investigation, the aforementioned solution preparations were slightly altered to test a second hypothesis. 6-chloroadenosine was substituted for adenosine as an ADA substrate in all samples, with the same conditions for varying substrate concentration and pH. Another change in procedure was an increase in final ADA concentration for each prepared sample to  $4.2 \times 10^{-8}$  M. The prepared 6-Cl-Ado samples were all run with the spectrophotometer at a wavelength of 264 nm.

The resulting data points for each set were compiled into a graph of time (sec) vs. absorbance (absorbance units) for each particular substrate-pH-concentration sample. The graphs of all samples within the same substrate-pH set were then compressed into a single graph representing the linearization of the Michaelis-Menten equation for the substrate at a particular

pH. From this it was possible to derive substrate-pH specific Michaelis constant ( $K_M$ ) values as well as  $k_2$  values.

### *Derivation of $k_2$ values*

The amount of light absorbed during the course of the reaction is divided by the amount of time that the reaction runs in the spectrophotometer to get a value which is equal to the slope of the straight line seen on the spectrophotometer. Once the slope of the line is determined, the  $V_0$  for each substrate concentration can be calculated, using a linear regression in Microsoft Excel to fit the data. The absorbance of Adenosine subtracted from the absorbance of Inosine at a wavelength of 260nm is used in collaboration with the equation  $V_0 = \Delta[\text{Inosine}]/\Delta t$  to give the equation  $\Delta \text{Abs} = (\epsilon_{260I} - \epsilon_{260A})\Delta[\text{Inosine}]\ell$ .

From this equation, we can conclude that  $\Delta \text{absorbance}/\Delta t = \Delta \epsilon_{260} \Delta[\text{Inosine}]\ell/\Delta t$ . Through the substitution of  $V_0 \Delta[\text{Inosine}]/\Delta t$ , the slope can be determined.  $\text{Slope}/\Delta \epsilon_{260} \ell = \Delta[\text{Inosine}]/\Delta t = V_0$ , where  $\Delta \epsilon_{260} = -7800 \text{ abs/M}$  and  $\ell = 1 \text{ cm}$ .  $V_0$  therefore equals  $\text{slope}/(-7800 \text{ Abs/M})$ .

Rough data points from spectrophotometer analysis were inserted into an Excel spreadsheet to create a graph comparing substrate concentration ( $[S]$ ) with absorbance (Abs). A linear regression of the scatter plot yielded a line of best fit whose slope was a constant. This constant is used to derive  $V_0$  as described in Equation 4.

Based on the Michaelis-Menten equation, the equation  $V_0 = [E \cdot S]k_2$  shows that the steady state has a constant reaction rate. Through the derivation of these equations,  $\Delta[\text{Adenosine}]/\Delta \text{time}(t) = \Delta[\text{Inosine}]/\Delta t = \text{constant}$  ( $V_0$ ) and  $\Delta[\text{Abs}]/\Delta t = \text{constant}$ ,  $V_0$  is determined. By knowing the value of  $V_0$  and deriving the Lineweaver-Burk equation, the  $V_{\text{max}}$  and  $K_M$  may be derived.

$$\frac{1}{V_0} = \frac{V_{\text{max}}}{K_m} \cdot \frac{1}{[S]} + \frac{1}{V_{\text{max}}} \quad \text{Equation 6}$$

Lineweaver-Burk equation

The Lineweaver-Burk equation is derived by taking the reciprocal of each side of the Michaelis-Menten equation and graphing  $1/V_0$  vs.  $1/[S]$ . Since this graph is linear, it follows the form  $y = mx + b$ , where the following is true:

$$V_{\text{max}} = \frac{1}{y - \text{intercept}} \quad \text{Equation 7}$$

$$K_m = \frac{\text{slope}}{y - \text{intercept}} \quad \text{Equation 8}$$

Once  $V_0$  is determined,  $k_2$  can be calculated using the equations derived above.

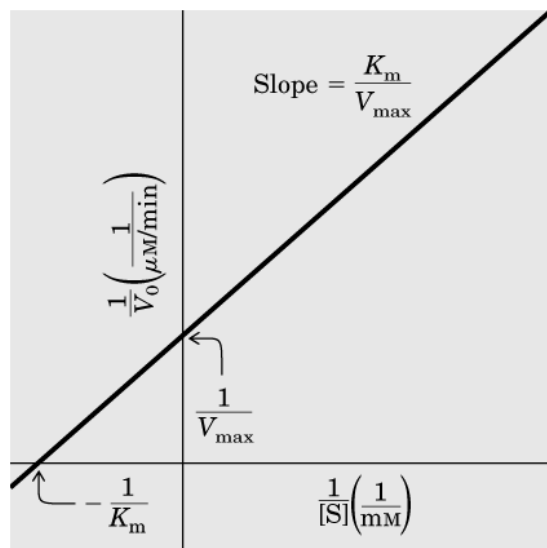


Figure 5: Lineweaver-Burk linearization [3].

Figure 1 shows how the substrate concentration and velocity are related. Initially, there is a large amount of available enzyme to catalyze the substrate reaction, which allows the reaction to proceed at a fast initial rate. After the reaction has continued for some time, the enzyme catalyzes the reaction at the maximum rate and cannot go any faster. Since the graph produced is logarithmic, it cannot be graphed in Microsoft Excel. The graph of  $V_0$  vs.  $[S]$  is plotted using the double reciprocal Lineweaver-Burk graph,  $1/V_0$  vs.  $1/[S]$ , in order to produce a straight line that can be represented on Microsoft Excel (Figure 5). After  $1/V_0$  vs.  $1/[S]$  is plotted, a linear regression may be used to calculate the y-intercept and the slope.  $V_{\text{max}}$  and  $K_M$  may be calculated through the derivations above.

This procedure was also used to calculate the conversion from 6-chloroadenosine to inosine. The spectrophotometer was set for a wavelength of 264 nm and Beer's Law was calculated using  $\Delta\epsilon_{264} = -4500 \text{ Abs/M}$ .  $V_0$  also equals  $\text{slope}/(-4500 \text{ Abs/M})$ .

## DATA ANALYSIS

The adenosine and 6-chloroadenosine samples were tested at a variety of pHs; the graphs and charts below show typical data for adenosine and 6-chloroadenosine at two of the pHs: 8.93 and 7.29 respectively. Data was collected regarding the change in absorbance of these substrates over time, and this data was graphed to determine the  $V_0$ ,  $V_{\text{max}}$ ,  $k_2$ , and  $K_M$  of these various reactions.

### Adenosine

Table 1 contains the amount of absorbance collected from the spectrophotometer over the course of the adenosine deaminase-adenosine reaction at a pH of 8.93. The initial substrate concentration of adenosine was 40  $\mu\text{M}$ . As the reaction proceeded and the amount of absorbed adenosine decreased, more adenosine was converted to inosine.

Table 1: The change in absorbance of adenosine over time, illustrating the decreasing enzyme and substrate as the reaction proceeds and more adenosine is converted into inosine.

Adenosine 40	
pH 8.93	uM
Time (s)	Absorbance Units
10	0.5056
20	0.5049
30	0.5043
40	0.5039
50	0.5034
60	0.5025
70	0.5020
80	0.5010
90	0.5006
100	0.4998
110	0.4991
120	0.4984
130	0.4978
140	0.4973
150	0.4964

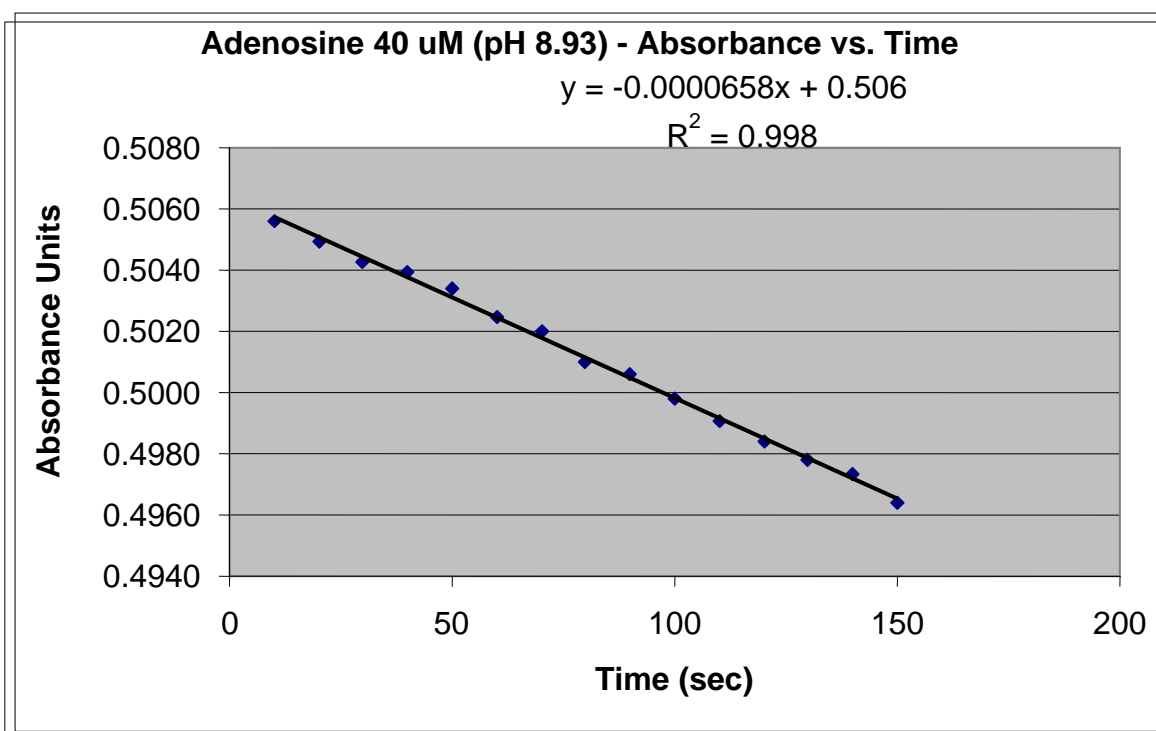


Figure 6: a linear plot of  $\Delta\text{Abs}_{\text{adenosine}}/\Delta t$ , illustrating the  $V_0$  for this concentration-specific reaction.

When absorbance units of adenosine were graphed against time, a distinct inverse linear trend is visible (Figure 6). Since the amount of adenosine decreases with a linear slope over time, the  $V_0$  of this reaction can be determined.  $V_0 = \text{Slope}/\Delta\epsilon_{260}l = -0.0000658/-7800 = 8.44\text{E-}09$ . This procedure was repeated for all adenosine concentrations at all pHs, as the path length of light and wavelength-specific adenosine absorption remains the same.

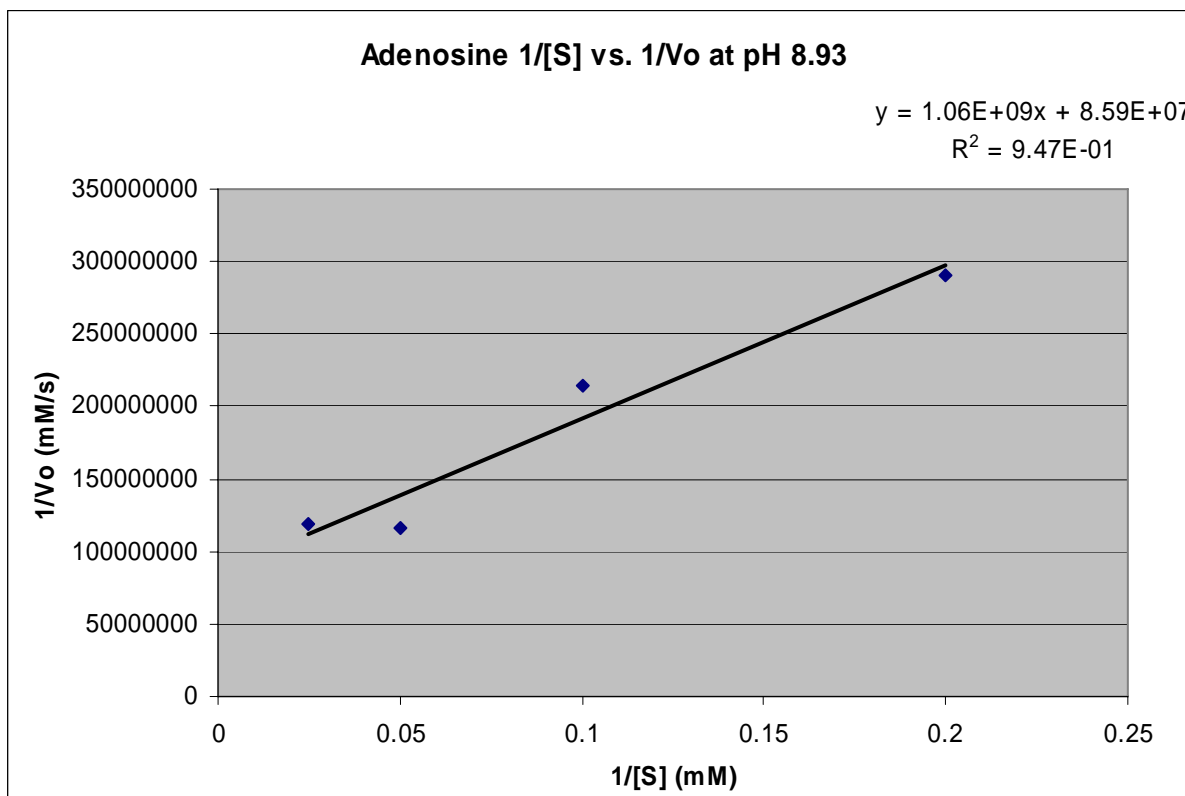


Figure 7: a Lineweaver-Burk plot of the double reciprocal of the concentration of adenosine at an initial rate and a pH of 8.93. It shows that the initial speed of the reaction increases as the substrate concentration increases. The high  $R^2$  value shows that this linear relationship is very strong.

For every given pH,  $1/V_0$  at each substrate was graphed against  $1/[S]$  to produce the Lineweaver-Burk graph as described in procedures (Figure 7). This enabled the  $V_{\max}$ ,  $k_2$ , and  $K_M$  to be derived. According to the Lineweaver-Burk derivations of the Michaelis-Menten equation,  $V_{\max} = 1/\text{y-intercept} = 1/8.59\text{E}07 = 1.16\text{-}08$ . Furthermore,  $K_M = \text{slope}/\text{y-intercept} = 1.06\text{E}09/8.59\text{E}07 = 12.3$ .  $k_2 = V_{\max}/[E]_{\text{total}} = 1.16\text{E-}08/2.252\text{E-}09 = 4.62$ .

### 6-Chloroadenosine

Table 2 provides the data for absorption of 6-chloroadenosine over time for pH 7.29. The trend in substrate concentration over time was the same as adenosine; as catalysis increased, the amount of absorbance of 6-chloroadenosine decreased.

Table 2: The change in absorbance of 6-chloroadenosine over time, illustrating the decreasing enzyme and substrate as the reaction proceeds.

**pH 7.29 40 microM 6-chloroadenosine**

Time (sec)	Absorbance (absorbance units)
0	0.3548
60	0.3494
120	0.3454
180	0.342
240	0.339
300	0.3364
360	0.3337
420	0.3315
480	0.33
540	0.3278
600	0.3258

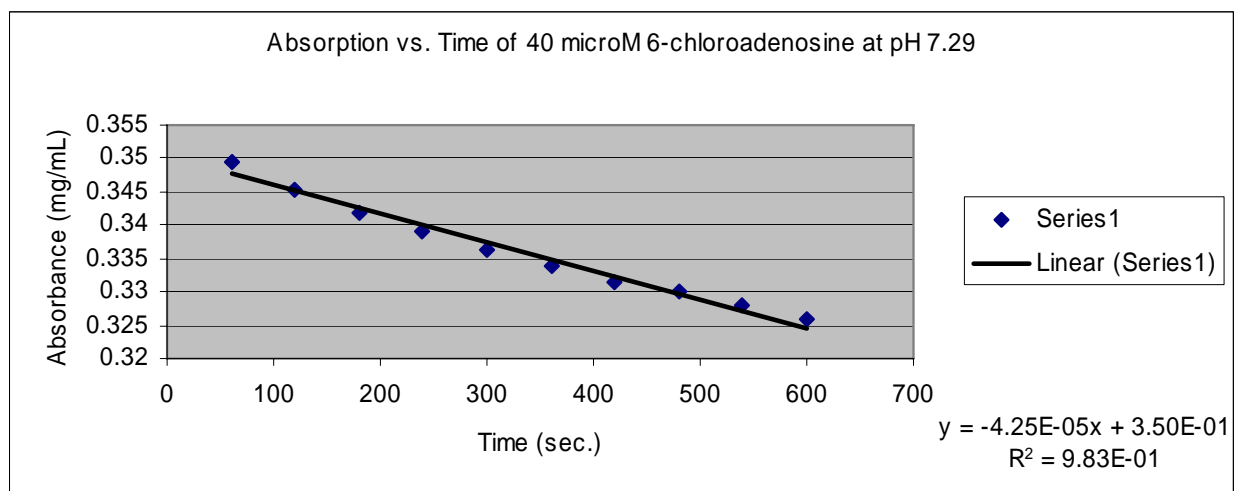


Figure 8: a linear plot of  $\Delta\text{Abs}_{\text{inosine}}/\Delta t$ , illustrating the  $V_0$  for this concentration-specific reaction.

The slope of this linear graph is equal to  $V_0$ , the initial rate of conversion from 6-chloroadenosine to inosine (Figure 8). According to Beer's law,  $V_0 = \text{Slope}/\Delta\epsilon\ell$ . However, the amount of 6-chloroadenosine that is absorbed at 264 nm is  $-4500 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ , as opposed to adenosine, which is absorbed at 260 nm with  $-7800 \text{ L}\cdot\text{mol}^{-1}\cdot\text{cm}^{-1}$ . Therefore, the  $V_0$  for 40 mM of 6-chloroadenosine is equal to  $-4.25E-05/-4500 = 5.45E-09$ .

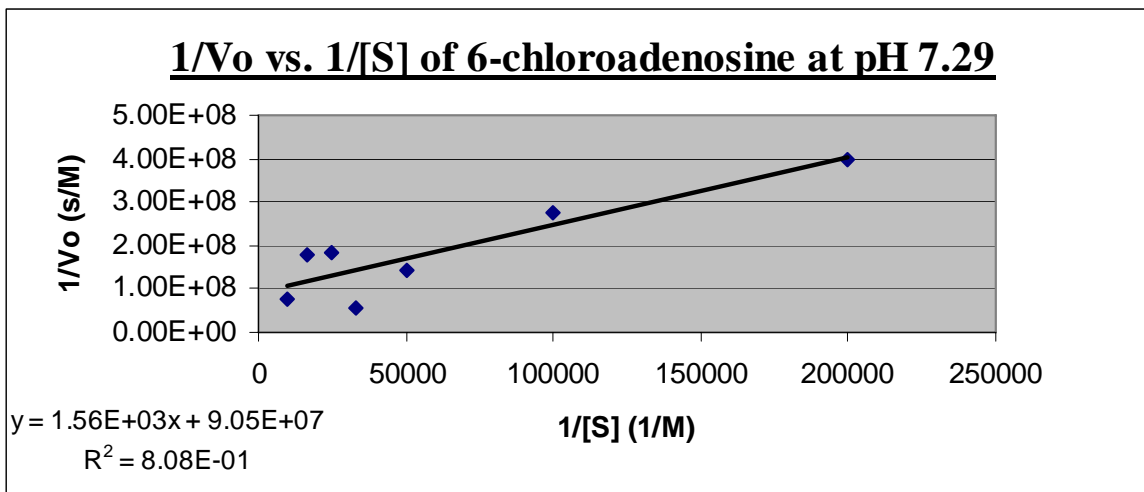


Figure 9: a Lineweaver-Burk plot of the double reciprocal of the concentration of 6-chloroadenosine at an initial rate and a pH of 7.29.

As with the procedure for deriving  $V_{max}$ ,  $k_2$ , and  $K_M$  for the 40 mM concentration of adenosine at pH 8.93, these values may be calculated from the Lineweaver-Burk graph of  $1/V_0$  vs.  $1/[S]$  (Figure 9). Therefore,  $V_{max} = 1/y\text{-intercept} = 1/9.05E07 = 1.10E-08$ .  $K_M = \text{slope}/y\text{-intercept} = 1.56E03/9.05E07 = 1.72E-05$ .  $k_2 = V_{max}/[E]_{total} = 1.16E-08/2.252E-09 = 0.262$ .

Similarly this procedure was replicated for all substrate concentrations of 6-chloroadenosine at all pHs.

Table 4: Chart of  $k_2$  values for adenosine and 6-chloroadenosine substrates at various pHs.

pH	k2(Ad)	pH	k2(Cl-Ad)
7.1	67.1		
7.3	68.3	7.3	0.26
7.5	62.5		
8.4	30.6	8.4	0.319
8.9	4.62	8.9	0.77
9.4	1.63	9.4	<0.08

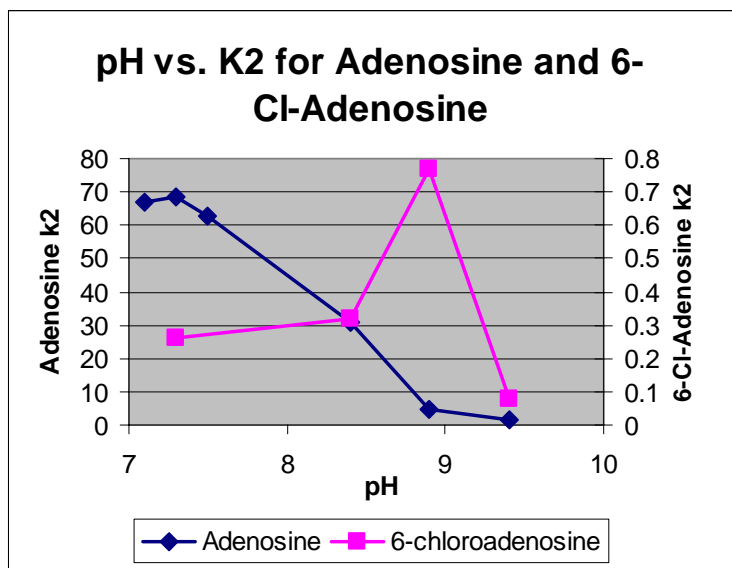


Figure 10: a plot of substrate-specific  $k_2$  values at various pHs with two y-axes, showing the relative rates of reaction.

The graph above shows the  $k_2$  calculated for adenosine and 6-chloroadenosine at different pHs. The graph has two y-axes which show the  $k_2$  at different scales depending on the substrate used. At lower pHs, the adenosine samples have a higher  $k_2$ , which represents a higher rate of reaction; as the pH gets higher, the  $k_2$  of adenosine conversion decreases. For lower pHs, the rate of 6-chloroadenosine conversion starts off lower and peaks around a pH of 9. The line representing the  $k_2$  for adenosine resembles a titration curve with one protonation; this reaction has a  $pK_a$  of about 8. The adenosine curve sharply decreases in rate at a pH of about 8.9. The 6-chloroadenosine curve gradually increases from a pH of 7 to one of 8.5, and then it sharply increases to a pH of 9. Finally, the rate decreases steeply at a pH of about 9.4.

The  $k_2$  of 6-chloroadenosine at 9.4 was indeterminable due to the fact that the reaction proceeded extremely slowly. Therefore, the value 0.08, highlighted dark in Table 4, was arbitrarily assigned.

## CONCLUSION

As illustrated in Figure 10, the adenosine and 6-chloroadenosine have very different pH dependence curves. This is due to the differences in rate of catalysis of each substrate as pH increases. One of our hypotheses was that the conversion of adenosine to inosine would be slower at higher pHs and the conversion of 6-chloroadenosine to inosine would be faster at higher pHs. It was proposed that this was due to the acidic nature of the protonating region. The preliminary conclusions suggest that our hypothesis was correct. The adenosine reaction went relatively slower at higher pHs, because the region that protonates the  $NH_2$  group on the adenosine became more basic. As the pH became more basic, the protonating region lost its ability to give off an  $H^+$  ion. On the other hand, since 6-chloroadenosine has the Cl atom instead

of the NH<sub>2</sub> group on the sixth carbon, it does not need to be protonated; its catalysis is unaffected by the higher pH.

Possible sources of error during the course of this study include lack of uniformity in the amount and concentration of adenosine deaminase used to catalyze the reaction. Furthermore, some data may have been misleading due to the lack of 6-chloroadenosine  $k_2$  values. Time restraints prevented additional 6-chloroadenosine trials from being tested; since the substrate 6-chloroadenosine has high pH specificity, it is recommended that a greater number of pHs from 8.4 to 9.4 should be tested in order to produce a more representative pH curve. It is also advised that future studies obtain absorption rate data in smaller time intervals in order to increase efficiency and quality of  $\Delta\text{Abs}/\Delta t$  graphs.

Due to the preliminary nature of this conclusion, further research is suggested. It is recommended that the  $k_2$  value be derived at various pHs by running more adenosine and 6-chloroadenosine concentrations under ideal conditions. This would yield more comprehensive data regarding the pH dependence curves for both substrates. New research may also be recommended regarding the different functional groups of the varying amino acids in the active site. If certain amino acids in the active site were replaced via mutagenesis by other amino acids, it would be beneficial to study the respective pH dependence curves of these adenosine deaminase mutants.

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