ENZYME KINETICS AND MECHANISM

Lina Carmona, Shuyi Chen, Janice Chou, Zhen Gong, Michael Hurley, Frances Jin, Stephanie Lo, Ramya Natarajan, Angela Wu, Derek Yee

Team Project Leader: Dr. Adam G. Cassano
Team Project Assistant: Jennifer Gomez

ABSTRACT

This study investigated various positions on the adenine ring of the adenosine molecule which, when altered, would inhibit the enzyme adenosine deaminase (ADA) and its reactions. Specifically, the carbons at positions 2 and 6 were modified in this experiment. For exploring position 6 on the ring, both 6-chloroadenosine and N-6-cyclohexyladenosine were used, while 2-chloroadenosine was used for similar consideration of position 2. Using the Michaelis-Menten equation, K_m and V_{max} values were determined for adenosine and then compared to that of 6-chloroadenosine, which appeared to act as a substrate rather than as an inhibitor of ADA. The V_{max} value for adenosine at 6.0×10^{-4} U/mL was found to be 3.6×10^{-9} μM/s and the K_m was calculated to be 5.5 μM. 2-chloroadenosine and N-6-cyclohexyladenosine seemed to act as inhibitors of ADA, as the rate of the deaminase reaction significantly decreased in their presence. 2-chloroadenosine appeared to act as the most effective inhibitor; thus, position 2 should be one possibility further studied for future identification and synthesis of ADA inhibitors.

INTRODUCTION

Enzyme Properties

An enzyme is a biological catalyst that expedites bodily reactions by binding a specific substrate in its active site and facilitating a reaction involving the substrate [1]. This enzymatic process occurs through two steps; the first is transition state stabilization, in which the activation energy (E_A) of the reaction is lowered (Figure 1). The second way is through substrate positioning, during which the reactant molecule is bound in a favorable orientation to promote reaction initiation [2]. Catalytic proficiencies of different enzymes vary greatly: some enzymes, such as orotic acid 5'-phosphate decarboxylase (ODCase), reduce the half-time of the reaction from 78 million years in neutral solution to 18 milliseconds [3].

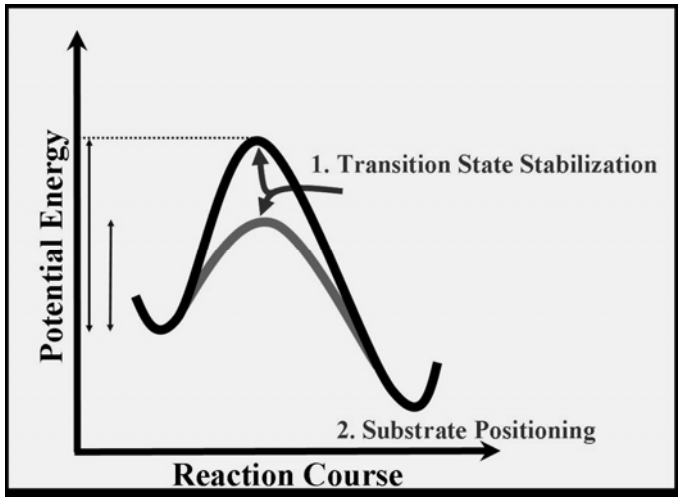

Figure 1: Activation Energy Curve

Many factors affect the activity of enzymes; these include the concentration of substrate ([S]), the temperature, the pH, and the presence/concentration of inhibitors. Of particular interest is the concentration of substrate; an increase in the concentration of substrate typically increases the rate at which enzyme molecules collide with and bind to the substrate molecules [1]. The presence of an inhibitor reduces the enzyme's efficiency. An important and well-known example is the inhibition of succinic dehydrogenase, an enzyme that plays an important role in cellular respiration [1]. The scientific study of an enzyme's rate of catalysis is called enzyme kinetics.

Enzyme Kinetics and the Michaelis-Menten Equation

Enzyme kinetics refers to the study of how experimental conditions, such as substrate and enzyme concentration, affect the rate of an enzyme-catalyzed chemical reaction [4]. The effect of substrate concentration on the initial rate of reaction is an essential part of enzyme kinetics. Numerous experiments have produced data that follow the trend shown on Figure 2, where initial rate V is plotted against the substrate concentration [S].

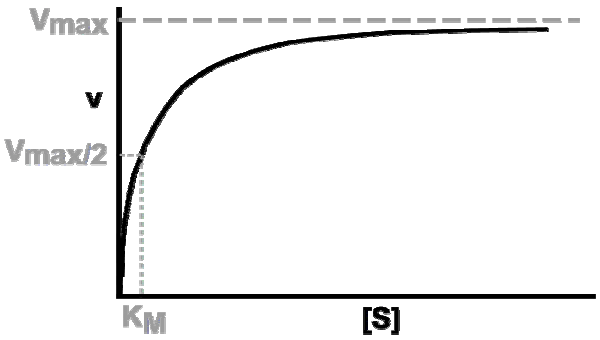

Figure 2: Initial Rate as a Function of [S] [5]

Leonor Michaelis and Maud Menten tried to explain this phenomenon by deriving a logical mathematic model to fit the experimental data. This model describing enzyme reaction rate with respect to substrate concentration is known as the Michaelis-Menten Equation [6].

Enzyme-catalyzed reactions often occur in two stages; the substrate first binds to the enzyme to form an enzyme-substrate (ES) complex, which subsequently breaks down into enzyme and product.

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

Equation 1: The enzyme-catalyzed reaction

The rate constants k_1, k_{-1} and k_2 are concerned respectively with the association of substrate and enzyme, the dissociation of unchanged substrate from the enzyme, and the dissociation of product from the enzyme. Although the reaction takes place in two elementary steps, the overall rate of the reaction depends on the how quickly the ES complex is converted to product and enzyme [6].

There are two important underlying assumptions that allow for this derivation. This first assumption is that that the reverse reaction is negligible, and the initial concentration of the product is approximately zero. The second assumption, also known as the "steady state approximation," states that when the concentration of the substrate is much higher than that of the enzyme, the ES complex is formed and broken down at the same rate, allowing [ES] to be a

constant. From the second assumption, an equation can be derived and after a series of algebraic arrangement and simplification, the final equation, the Michaelis-Menten equation becomes

$$V = \frac{V_{\max}[S]}{K_m + [S]} \quad \text{(Equation 2)}$$

in which $K_m = \frac{k_{-1} + k_2}{k_1}$ (Equation 2.1)

and $V_{max} = k_2[E_0]$ (Equation 2.2).

When graphing V_0 against [S], a graph resembling Figure 2 is obtained.

At low [S], the amount of [S] limits the overall rate of the reaction. Hence, as more substrate is added, a rapid increase in rate occurs until a saturation point is reached when all of the enzyme binding sites are occupied. Consequently, the addition of more substrate will not dramatically increase the reaction rate, as indicated by the plateau in Figure 2.

In addition, when the rate of reaction (V) is exactly half of V_{max}, it can be shown that [S] = K_m. Hence, K_m can also be defined as the substrate concentration at which V = 0.5 V_{max} [6]. From this definition, K_m becomes an indicator for measuring substrate affinity to a particular enzyme [4]. Low values indicate that the ES complex is bound together tightly and rarely dissociates to E and S, whereas high values denote an unstable ES complex [4]. However, because K_m consists of three different rate constants (k_1, k_{-1}, and k_{-2}), a mere increase or decrease in the K_m value cannot conclusively determine the strength of the ES complex without an analysis of possible changes in all three rate constants. In spite of this complication, since k_2 could be controlled in this particular study by paralleling the enzyme concentration and comparing V_{max} value, K_m is still a valuable indicator of the strength of the ES bond for the designated analogs.

Because V and [S] forms a non-linear relationship that is inconvenient to analyze, scientists frequently use another analysis technique known as the double reciprocal Lineweaver-Burk plot graphs $1/V_0$ against 1/[S]. This plot is linear, with an x-intercept of $1/K_m$, a y-intercept of $1/V_{max,}$ and a slope equal to K_m/V_{max}.

Adenosine Deaminase and its Effects

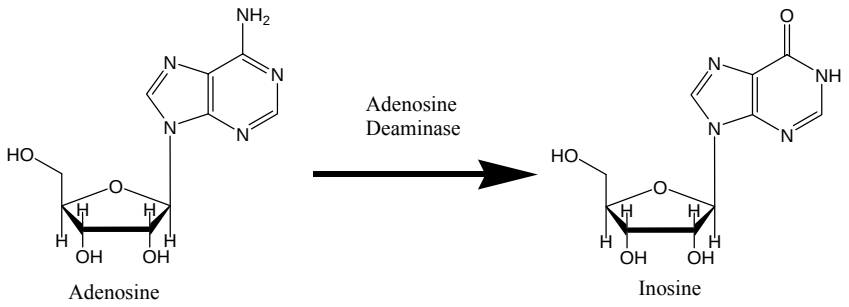

Figure 4: Adenosine conversion into Inosine

The enzyme adenosine deaminase (ADA) converts adenosine and deoxyadenosine into inosine and deoxyinosine, respectively (Figure 4). These reactions have been a subject of particular interest because of the vitality of the balance between substrate and product concentrations in biological systems. ADA is primarily found in the cytosol, where it catalyzes the deamination of adenosine [7]. However, research has shown that it also exists as ecto-ADA, a form which appears on the cell surface. It has been suggested that this form of ADA regulates cellular adenosine levels [1]. In humans, the highest concentration of this cellular ADA was found to be in the thymus and other lymphoid tissues, while the lowest was in the erythrocytes [7]. This suggests that the activity of ADA enzyme varies according to the degree of activity of the cell [8]. This enzyme has similar importance in other animals as well. For example, in the genus *Drosophila*, it has been found that adenosine acts as a tumor suppressor, which inhibits cell growth, in the absence of cellular ADA [1]. As such, it goes without saying that deactivation/inhibition or overproduction of ADA can lead to a myriad of consequences for the organism. In humans, it is shown that ADA deficiency can lead to SCID (or Severe Combined Immunodeficiency Disease), pulmonary insufficiency, and Mesangial sclerosis [7]. ADA-SCID, is caused by a lack of adenosine deaminase. ADA deficiency accounts for nearly one-half of all cases of SCID [7]. Often referred to as the “Bubble Boy” disease, SCID’s defining characteristic is a dysfunction of both the B and T lymphocytes [9]. As mentioned before, ADA degrades adenosine to inosine and/or deoxyadenosine to deoxyinosine.

With an ADA deficiency, however, there is a toxic buildup of these substrates which eventually can adversely affect the immune system [8]. Since deoxyadenosine is normally phosphorylated to dATP, a buildup of deoxyadenosine results in an excess of dATP. The dATP competes with ATP to bind to the enzyme APAF-1; when ATP is bound to the enzyme, it is turned on and gives a signal to make DNA [10]. However, when dATP is bound to the enzyme, it is turned off, and DNA replication is inhibited. This is feedback inhibition, the mechanism by which SCID develops because B & T lymphocytes die [11]. Furthermore, other complications can arise from unregulated levels of adenosine concentrations. Adenosine is a signaling nucleoside; elevated levels can cause pulmonary insufficiency and/or cell signaling disturbances [8]. In a study done by scholars in the University of Texas-Houston Medical School, high adenosine levels cause them to exhibit lung inflammation in mice[12]. In humans, excess adenosine has been found to play a role in the development of asthma. While the exact

mechanism by which elevated adenosine levels contribute to asthma is unknown, it is partly due to its ability to influence mediator release from mast cells [12].

Excessively high concentrations of adenosine deaminase also prove to be detrimental and even fatal. In humans, this causes a form of hereditary hemolytic anemia and also erythroblastosis fatalis in fetuses. This defect is inherited as an autosomal dominant trait [13]. Unlike in ADA deficiency where structural changes have been found on the enzyme molecule itself (e.g. single point mutations), in ADA excess, the ADA molecule not only appears to be structurally normal, but also exhibits an abnormality that can only be detected in erythroid elements [13]. As such, it has been suggested that increased ADA activity in erythrocytes is a result of increased translation of a form of an aberrant ADA mRNA [14]. This increased ADA enzyme activity causes a buildup of structurally normal ADA protein in erythrocytes. This results in an elevated catabolism of adenine ribonucleotide pools in erythrocytes and consequently a depletion of cellular ATP. Eventually, premature red blood cell destruction occurs [13]. However, in recent clinical trials, novel ADA inhibitors such as coformycin or deoxycoformycin have shown promising; patients showed enhanced recovery of left-ventricular end-diastolic and left-ventricular developed pressure [15].

These symptoms are characteristic of hemolytic anemia, in which the rate that red blood cells are destroyed is greater than that of the formation of bone marrow. If untreated, hemolytic anemia can lead to a variety of symptoms including fever, chills, fatigue, and eventually, even cardiovascular collapse [15]. Elevated activity of adenosine deaminase has also been found in the cells of patients with AIDS (acquired immunodeficiency syndrome); however, the exact mechanism by which increased levels of ADA is related to AIDS is still unknown [14]. Furthermore, individuals with elevated levels of adenosine deaminase have also been known to develop chronic lymphocytic leukemia (CLL). Studies have shown that the ADA level was 2.3-fold higher in T-Cells from patients with CLL than those of normal T-Cells. Symptoms of CLL include shortness of breath, weight loss, and frequent infections of the skin, lungs, kidneys, and other sites [16]. In addition, abnormally high concentrations of ADA have also been reported in tuberculosis, viral hepatitis, some leukemia diseases, hereditary hemolytic anemia, and Parkinson's disease [17].

Adenosine Deaminase Inhibitors

Inhibitors are defined as molecules that slow down or block enzyme-catalyzed reactions. In particular, adenosine deaminase inhibitors slow down the deamination of adenosine to inosine. Different enzymatic inhibition mechanisms include competitive inhibition, mixed inhibition, and non-competitive inhibition. The most common inhibition form, competitive inhibition, results in the competition between the substrate and inhibitor for the binding site, thus reducing the rate of substrate molecules binding to the enzyme. In mixed inhibition, the inhibitor binds directly to the enzyme or the ES complex. Lastly, in uncompetitive inhibition, the inhibitor only binds to the ES complex and prevents the formation of products [18].

As previously mentioned, adenosine deaminase plays vital roles in regulating the total amount of adenosine [18]. Intracellular adenosine participates in cellular energy production and purine metabolism, whereas extra-cellular adenosine acts as a signal receptor between cell

surface receptors in many body systems including the immune system [18]. As mentioned before, an abnormally high level of ADA has proven to be harmful [17]. In a recent study, adenosine has also come to be considered an important factor in alleviating inflammation. An ADA inhibitor may increase the concentration of adenosine at a specific inflamed site and serve as an anti-inflammatory drug with few side effects [18]. Besides the curing effect of this specific ADA inhibitor for inflammation, researchers have found and are attempting to find more ADA inhibitors in an attempt to alleviate diseases caused by an excess of the enzyme. Common adenosine deaminase inhibitors that have already been discovered and tested include coformycin, 1-deazaadenosine derivatives, EHNA compounds, and flavonoid [17].

MATERIALS AND METHODS

Beer's Law, or the concept that states that the absorption of a solution is equivalent to the concentration of substrate times the pathway length and the molar extinction coefficient, was used in order to detect the production of inosine [18].

$$E_0 = \frac{Absorbance}{Concentration\ (M) \bullet Cell\ length\ (cm)}$$

Equation 3: Beer's Law

All experiments were conducted using a Beckman DU® 530 UV-visible spectrophotometer (Figure 5).

Figure 5: Beckman DU® 530 spectrophotometer [19]

Part I: Determination of Wavelength

In order to properly utilize Beer's Law, a standard wavelength had to be established using a spectrophotometer which measures absorbance in order to establish the extinction coefficient for the control, adenosine. The target absorbance was between 0.10 and 1.00. Because adenosine and the other substrates absorb ultraviolet (UV) radiation differently than inosine, the absorption spectrum from 200 to 300 nm was taken, and the wavelength that demonstrated the greatest difference in absorbance for the two substances was used in the spectroscopic procedure for determining the rate of ADA's conversion of adenosine to inosine.

First, 1.0 mL of distilled water was placed in a plastic cuvette and used as a blank to calibrate the spectrophotometer. A 10 mM stock solution of adenosine, purchased from Sigma, was diluted 1:200.The same procedure was applied to the product of the reaction, 10 mM inosine. The absorbance spectra for the two substances were taken in the wavelength range of 200-300 nm. The peaks of both curves were recorded, and the highest difference in absorption between adenosine and inosine was observed at wavelength 263 nm, which became the standard wavelength used for the control. Using recorded absorbances and calculated concentrations with Beer's Law, the change in extinction coefficient from adenosine to inosine was determined.

Part II: Testing the Adenosine

Before performing the experiment with the three analogs, or modified adenosine molecules, a control was generated by determining the Michaelis constant (K_m) and maximum reaction rate (V_{max}) for the conversion of adenosine to inosine via ADA. This was done using spectroscopy and analyzing the results using the Microsoft Excel program to produce primary and secondary plots of the data.

The original 10 mM adenosine solution was diluted to a concentration of 1mM using distilled water. This 1 mM stock solution was then used in creating solutions of varying adenosine concentration (5 μM, 10 μM, 20 μM, 30 μM, 40 μM, 60 μM, 100 μM adenosine). Reaction solutions contained 500 μM of the100 mM Tris buffer (pH 7.4) and varying amounts of distilled water to make a 1 mL final volume. The resulting solution was mixed thoroughly and gently. A blank for the spectrophotometer was then created with 500 μM of the 100 mM Tris buffer, 6.0×10^{-4} U/mL ADA (a U is defined as the amount of ADA required to convert 1.0 μmole of adenosine to inosine in one minute) that was Type V from bovine spleen (Sigma-Aldrich) and kept on ice. The blank was mixed with enough water to make the total volume 1 mL.

After blanking, each concentration of adenosine mixture was tested consecutively in the spectrophotometer. The reactions were initiated by adding ADA to a concentration of 6.0×10^{-4} U/mL. The machine was set to take absorbance readings at wavelength 263 nm at 30-60 second intervals for a total of 600 seconds per trial. Each of the concentrations of adenosine was tested in three independent trials to ensure consistency.

Part III: Testing of the Analogs

After testing all prepared concentrations of adenosine for the control, the three analogs were subjected to the same procedure. Each analog was tested blindly and independently to preclude biased results. The spectrophotometer was blanked with the analog prior to testing.

Analog 1 was tested in a similar procedure to that of adenosine. The concentrations of the analog were tested at 20 μL, 35 μL, 50 μL, 70 μL, 80 μL, 100 μL, and 150 μL. Final solutions contained 2×10^{-2} U/mL ADA, 500 μM of the100 mM Tris buffer and varying volumes of water to keep the solution volume constant at 1 mL. Absorbances were recorded every 60 seconds at a wavelength of 266 nm for 10 minutes.

Analog 2 was tested as an inhibitor of adenosine deaminase since a reaction was not initially observed when the analog was subjected to the previous method and analysis. The adenosine concentration was kept constant at 80 μL of 1 mM stock, and six concentrations of the analog (0 μL, 10 μL, 20 μL, 40 μL, 60 μL, and 80 μL) were tested. 6.0×10^{-4} U/mL ADA was used as the enzyme, and like in previous experiments, solutions contained 50 mM of Tris buffer and different amounts of water. Absorbance was measured for 10 minutes in increments of either 30 or 60 seconds, and was read at either 263 nm or 266 nm.

Like Analog 2, Analog 3 was tested as an inhibitor of adenosine. The adenosine concentration was kept constant at 60 μL, and 6 concentrations of the analog (0 μL, 20 μL, 40 μL, 50 μL, and 80 μL) were tested. Final solutions contained 1.2×10^{-3} U/mL ADA as well as 50 mM of Tris buffer, and the volume of water was changed accordingly to keep the volume of solution at 1 mL. Absorbance was recorded every 60 seconds at a wavelength of 263 nm for 10 minutes.

Part IV: Analysis of Reaction Rates

The raw data from the previously described trials for the adenosine control was analyzed in Microsoft Excel. Assuming that the steady state approximation was valid, the primary plots consisted of graphing the linear relationship between the absorbance of the reaction mixtures and the passage of time to give V_0 (during the progression of the ADA catalysis reaction). The secondary plot (Lineweaver-Burke plot) was a double-reciprocal graph relating the reciprocal of V_0 (with respect to concentration) to the reciprocal of the substrate (adenosine) concentration. The relationship is shown in Equation 4, which demonstrates that slope of this graph is K_m/ V_{max} and the y-intercept is $1/ V_{max}$.

$$\frac{1}{V_0} = \left(\frac{K_m}{V_{\max}}\right)\frac{1}{[S]} + \frac{1}{V_{\max}}$$

Equation 4: Lineweaver-Burke equation. Note that it is simply a linear transformation of Equation 2, the Michaelis-Menten Equation.

Analysis of the graphs and comparative K_m and V_{max} would indicate the nature of the substrate binding and catalysis.

For each analog, graphs were prepared on Microsoft Excel showing the relationship between analog concentration versus the reaction rate, which had been determined by the change in absorbance over time. Graphs were examined to determine whether the analog had acted as an effective inhibitor.

RESULTS AND DISCUSSION

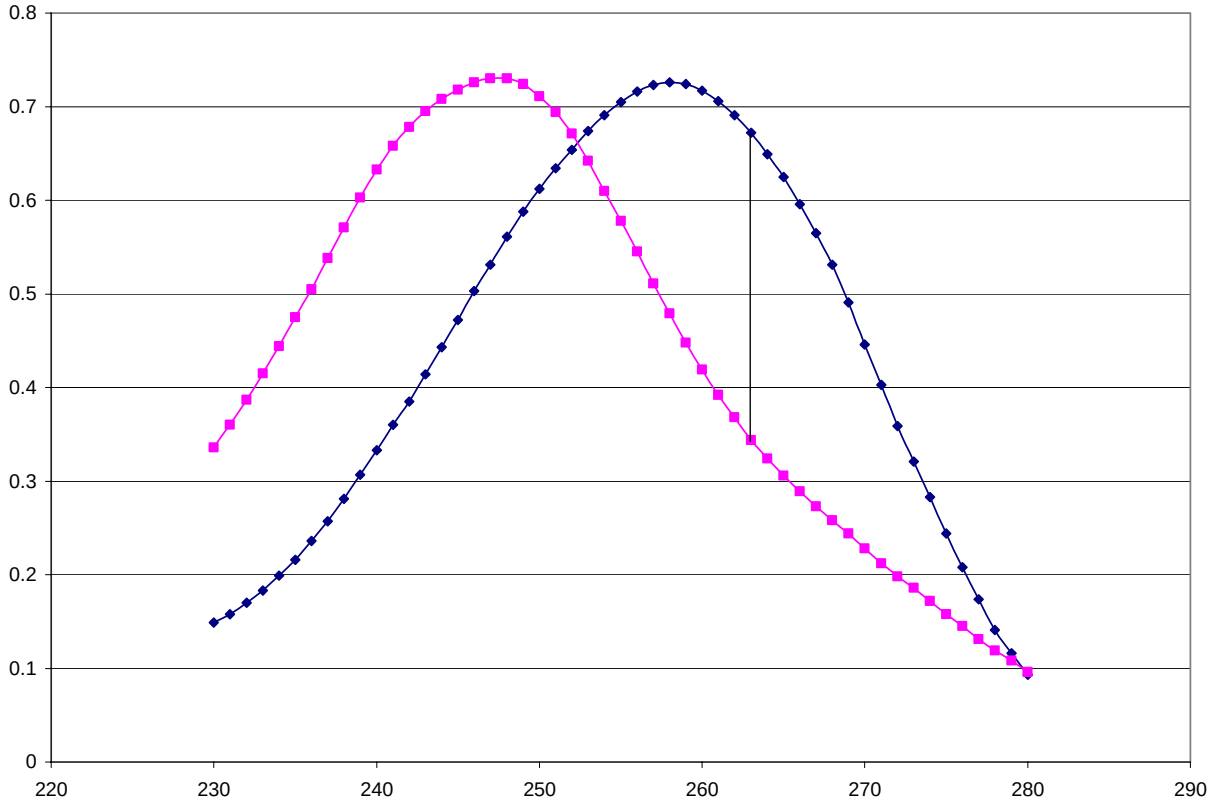

Figure 6: Absorbance of adenosine and inosine at varied wavelengths

The graph above shows the absorbance of adenosine (indicated by squares) and inosine (indicated by the triangles). The greatest absorbance difference occurred at $\lambda = 263$ nm.

In part I, it was determined that the most advantageous wavelength at which to monitor the reaction is 263 nm, which was calculated according to the greatest observed difference between the absorbance of inosine and adenosine (Figure 6). These results are within consistent range to those of Paul et al [16], who published the general monitoring absorbance for adenosine as 265 nm. Conversely, the reaction can be monitored to the increase of inosine at 235 nm.

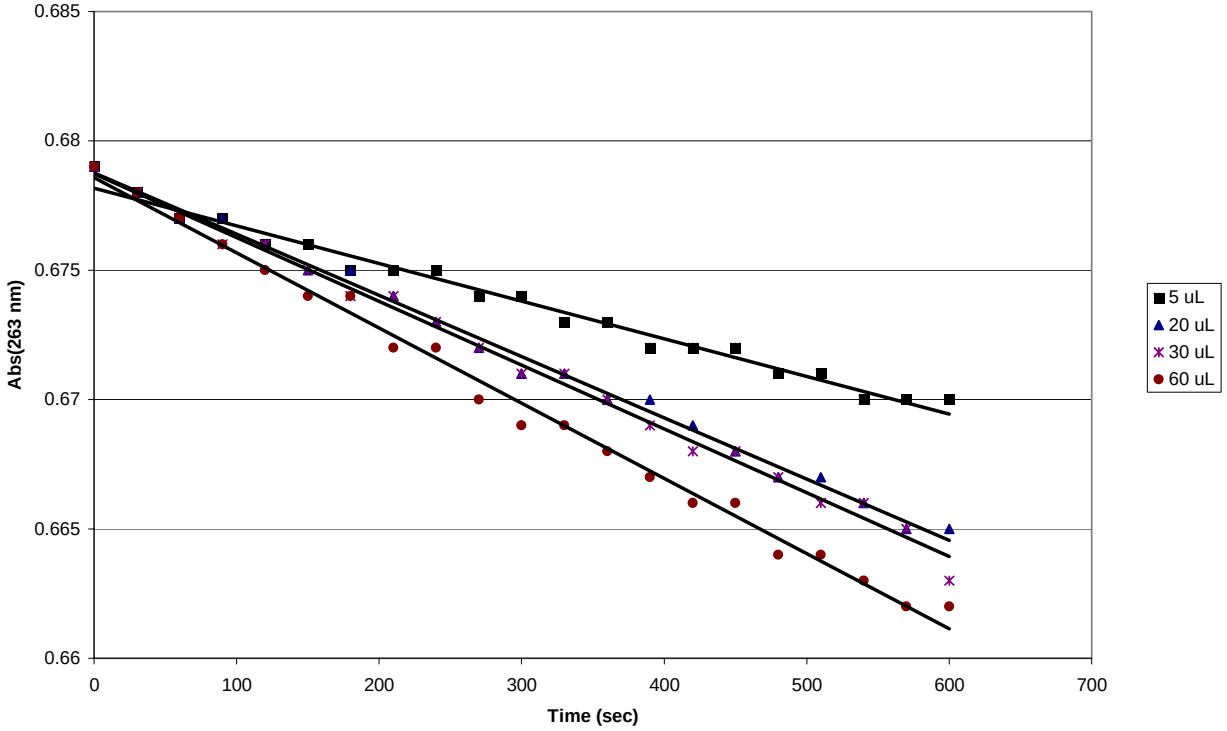

Figure 7: Normalized graph of varied concentrations of adenosine

The normalized graph above shows absorption of UV radiation at 263 nm versus time (s) of four different concentrations of adenosine: 5 µL, indicated by a square; 20 µL, indicated by a triangle; 30 µL, indicated by an asterisk; 60 µL, indicated by a circle.

Once the optimal wavelength at which to monitor the reaction was determined, the V_0 for the ADA-catalyzed reaction was determined for several concentrations of adenosine. As time passes, the absorption of radiation tends to decrease in a linear fashion. Higher concentrations of adenosine exhibit graphs with sleeper slopes, indicating that increasing amounts of adenosine cause a greater absorbance decrease in a given amount of time (Figure 7). The increasing magnitudes of the slopes, and correlating reaction rates, with increasing adenosine concentration is consistent with the Michaelis-Menten Model.

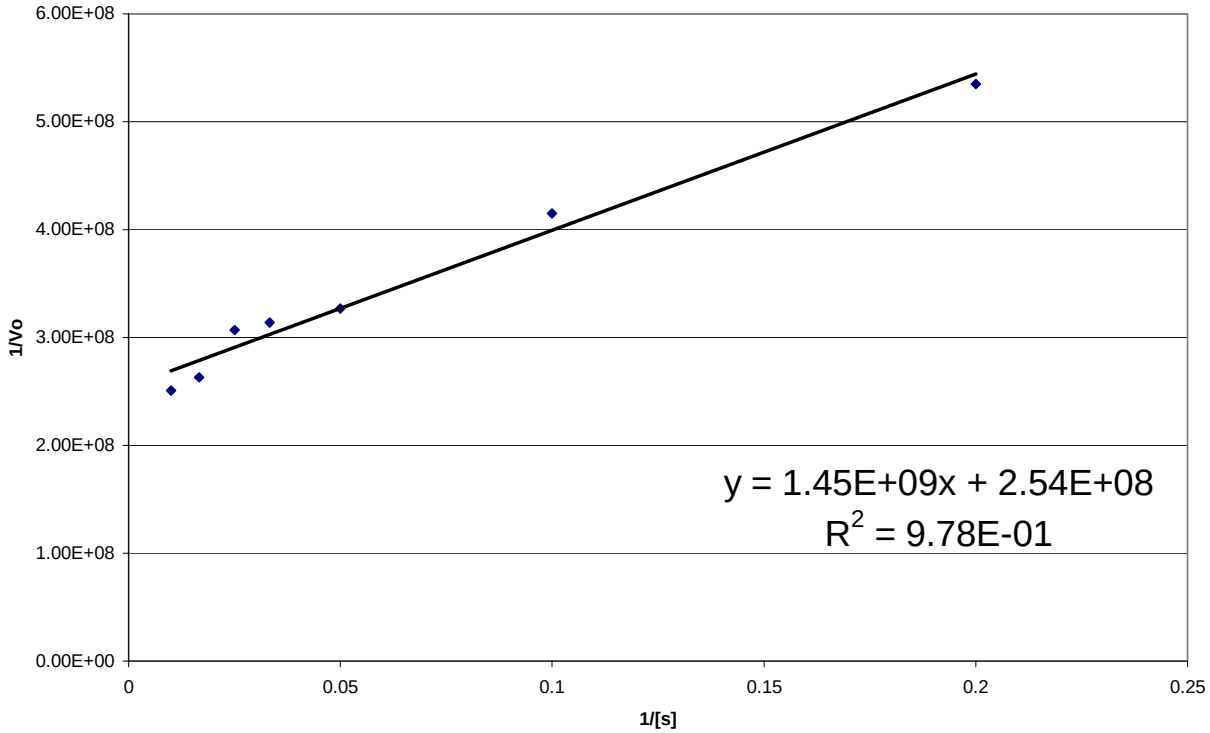

Figure 8: Double-Reciprocal Plot of $1/V_0$ vs. 1/[s]

The graph shows the inverse of the initial velocity of the reaction versus the inverse of seven different concentrations of adenosine (5 uL, 10 uL, 20 uL, 30 uL, 40 uL, 60 uL, and 100 uL). The r^2 value of .98 demonstrates a strong correlation between the inverses of concentration and initial velocities. From this plot, K_m was calculated to be 6 µM and V_{max} was calculated to be 3.6 x 10^{-9} µM/s.

The linear relationship between the reciprocals of concentration and initial velocities suggests that the Michaelis-Menten equation is a fitting model for examining ADA kinetics (Figure 8). The three groups calculated a K_m that averaged to 5.5 µM. The accepted value is 25 µM at 25 degrees Celsius [20]; however, the literature value is based on an experiment conducted at pH 7, whereas the current experiment was executed at pH 7.4. Given this variable, and because the three groups worked independently and found similar values, the results were not dismissed as invalid, and the difference was attributed to the pH change.

The objective of part III of the experiment was the determination of how well the three given analogs bind to ADA, if they bind at all. To explore the mechanisms of the reactions, the experiment was carried out in two steps: one with pure adenosine and the other with the analog. By comparing some key values, such as V_{max} and K_m, from these two experiments, one could determine the strength of the enzyme-substrate (ES) complex for the analog. If a reaction was not detected, trials were run with varied concentrations of analog while the concentration of the enzyme was kept constant in order to detect the degree of inhibition.

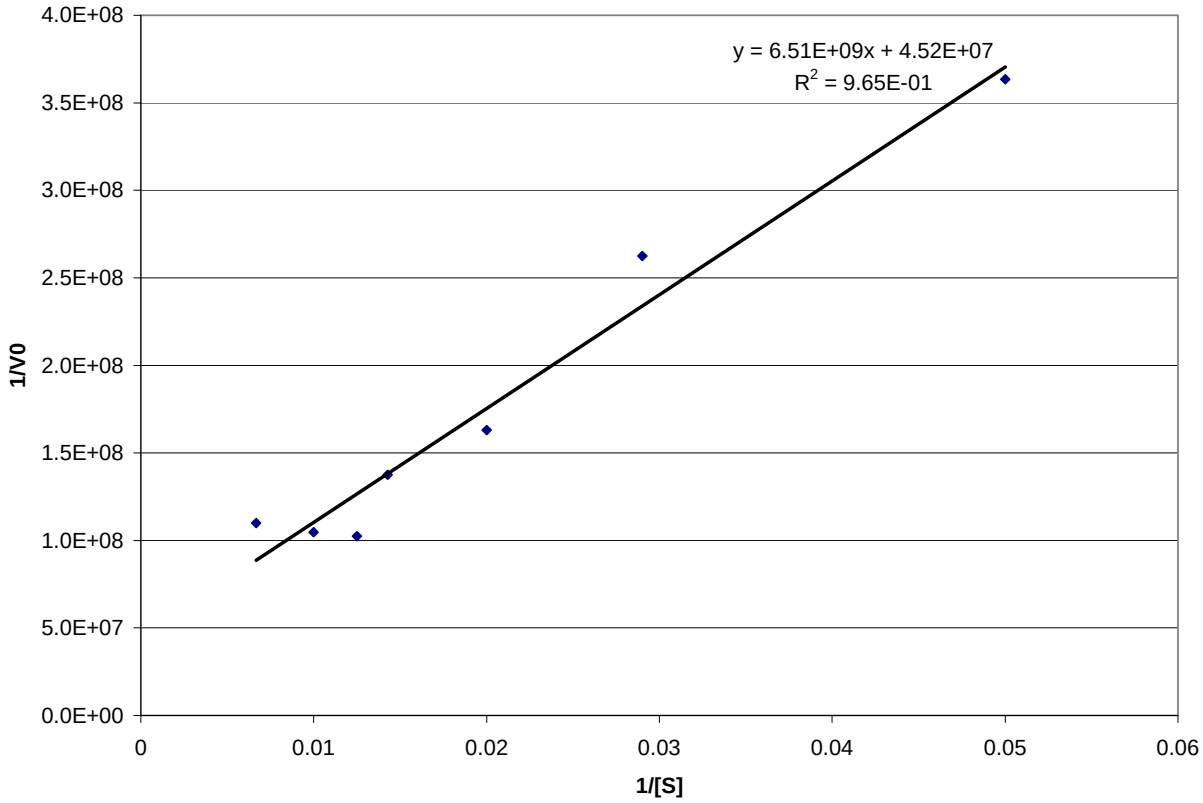

Figure 9: Double-reciprocal plot of $1/V_0$ vs. 1/[s] of 6-chloroadenosine

The substrate of 6-Chloroadenosine was tested following the previous method of testing adenosine. The graph above depicts the double reciprocal plot between inverse of initial rate and inverse of substrate concentration. From this plot, K_m was calculated to be 1.4 x 10^2 μM and V_{max} was found to be 2.2 x 10^{-8} μM/s.

The objective of this part of the experiment is to determine how well the analog binds to adenosine deaminase (ADA), if it binds at all. To achieve this goal, the experiment was carried out in two steps: one with pure adenosine and the other with 6-chloroadenosine. The 6-chloroadenosine analog was found to act as a substrate of ADA; therefore, K_m values of adenosine and 6-chloroadenosine could be directly compared. By comparing some key values, such as V_{max} and K_m, from these two experiments, one could determine the strength of the enzyme-substrate (ES) complex for the analog, 6-chloroadenosine.

The reaction between pure adenosine and ADA yields a K_m value of 5.5 μM and a V_{max} of 3.8 x 10^{-9} μM/s while the reaction between 6-chloroadenosine and ADA gives a K_m value of 140 μM and a V_{max} of 2.2 x 10^{-8} μM/s (Figure 9). However, because the enzyme concentrations in both reactions differ greatly (the first one 6.0 x 10^{-4} U/mL; the second one 2.0 x 10^{-2} U/mL), the two V_{max} values are incomparable until the two enzyme concentrations are calibrated to the same level. Because V_{max} is directly proportional to $[E_0]$, the total enzyme concentration, ($V_{max} = k_2[E_0]$), the V_{max} for the reaction with 6-chloroadenosine would hypothetically be 6.6 x 10^{-10} μM/s if the enzyme concentration were 6.0 x 10^{-4} U/mL which is much smaller than the V_{max} of the adenosine reaction, 3.8 x 10^{-9} μM/s. When the enzyme concentrations of both reactions are

calibrated to the same level, V_{max} also becomes directly proportional to k_2. Hence, k_2 for the reaction with 6-choloroadenosine is smaller than that of the reaction with pure adenosine.

Another important quantity describing the strength of the ES complex is the K_m value which yields ($\frac{k_{-1}+k_2}{k_1}$). The experiment demonstrates that the K_m of the 6-chloroadenosine, 140 μM, is much greater than that of the adenosine reaction, 5.5 μM. Since it has already been established that k_2 of the 6-chloroadenosine reaction is much smaller than that of the adenosine reaction, the greater K_m value in the 6-chloroadenosine reaction can only be attributed to an increase of $k_{-1,}$ or a decrease of $k_{1,}$ or a combination of both effects. The nearly 40 times increase of K_m indicates that the magnitude by which k_1 and k_{-1} change is also quite dramatic. Therefore, either an increase of k_{-1} or a decrease of k_1 suggest that the ES complex in the 6-chloroadenosine reaction is not nearly as strong as that in the adenosine reaction because the rate of dissociation of ES (indicated by k_{-1}) increases while the rate of formation (indicated by k_1) decreases (Refer to Equation 1).

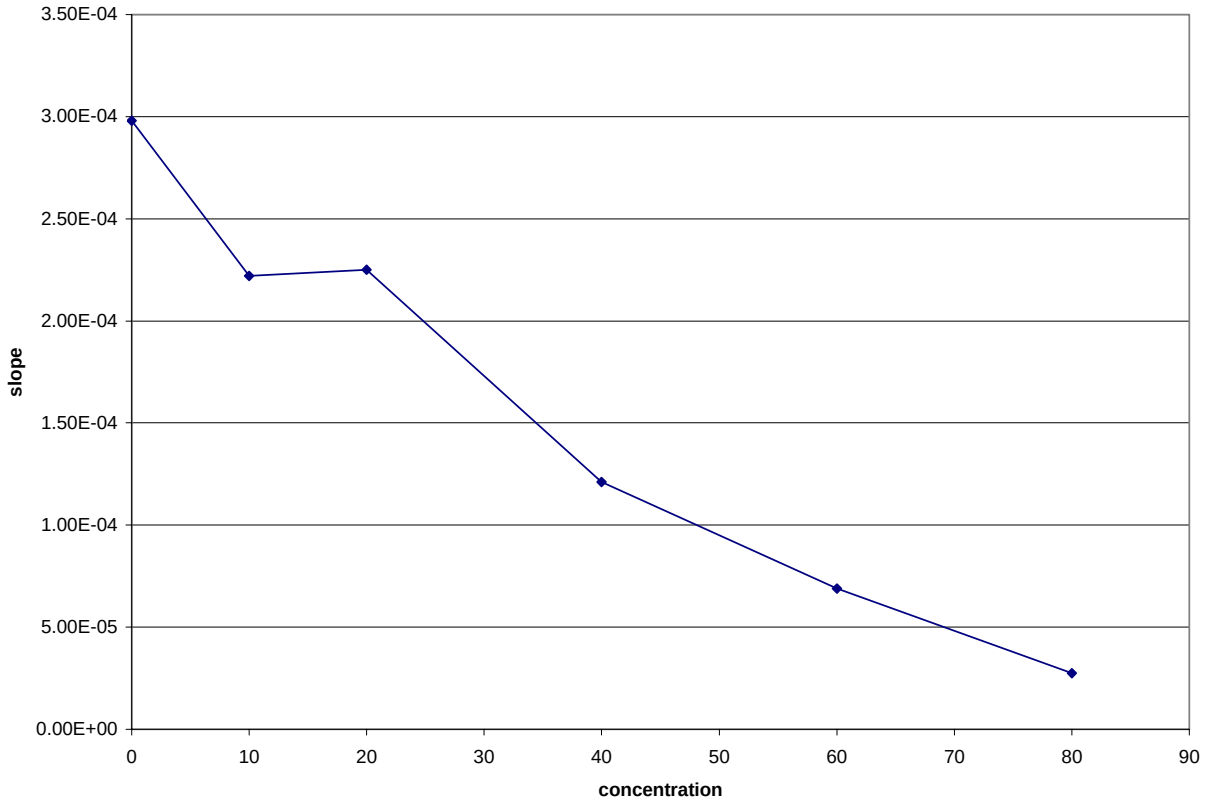

Figure 10: Rate of reaction vs. concentration of 2-chloroadenosine

This graph implies that analog 2, the 2-chloroadenosine, acted as an inhibitor for the active site of adenosine deaminase. A relatively linear relationship can be observed between the rate of the reaction and the concentration of the analog.

The second analog, 2-chloroadenosine, showed a definite trend that indicated that higher concentrations of the analog caused the rate of the reaction to decrease. The change in the rate of the reaction was nearly ten-fold, showing a drastic difference between the trial in which the

analog was not present and the trial in which the concentration of the analog was equal to the concentration of adenosine at 80 μM. Because an increase in the concentration of substrate reduces the effect of the inhibitor (and vice-versa), 2-chloroadenosine acted as an inhibitor for the reaction from adenosine to inosine. [21]

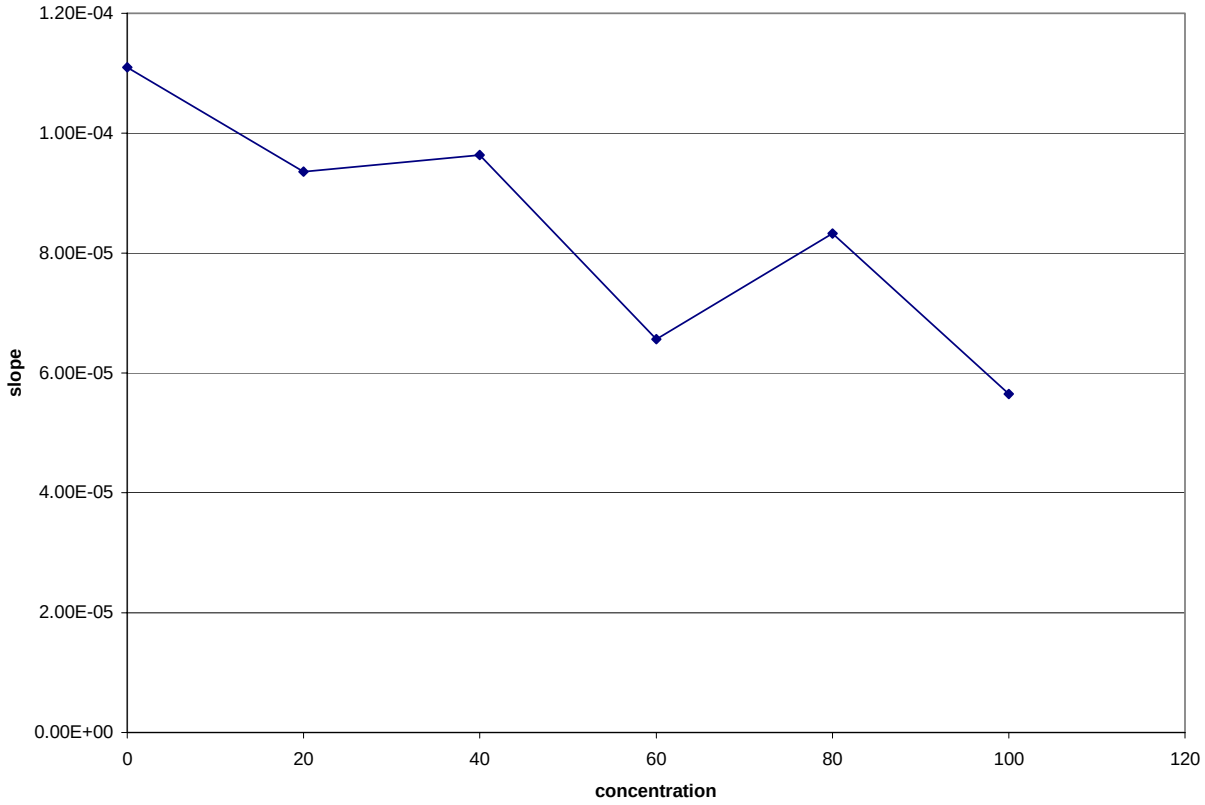

Figure 11: Rate of reaction vs. concentration of N-6-cyclohexyladenosine

The general trend in the plot of reaction rate versus analog concentration implies that while this analog is a weak inhibitor for this reaction, the change in reaction rate caused by even large amounts of analog (100μL of analog to 60μL of adenosine) only amounted to a 50% decrease in reaction rate from the control run containing only adenosine with no analog.

The third adenosine analog, N-6-cyclohexyladenosine, was not conclusively determined through experiments and data to be either a competitive or uncompetitive inhibitor. While the reaction rate of adenosine, which was catalyzed by ADA to form inosine, decreased as the concentration of the N-6-cyclohexyladenosine analog increased, the data was neither definitive nor accurate enough to make a positive assertion concerning the nature of inhibition (Figure 11). If N-6-cyclohexyladenosine is indeed a competitive inhibitor of ADA, the data plot of the reaction rate of catalysis versus the concentration of analog would signify that the binding of the analog molecule to the active site of ADA is poor. There is a significant overall decrease in catalysis as the concentration of analog increases, but the irregularities in the graph cannot be overlooked. These results are reasonable if N-6-cyclohexyladenosine is actually a competitive inhibitor because although the analog molecule resembles adenosine closely, the bulky aromatic group could result in steric hindrance that would prevent the molecule from binding tightly to the

active site. In fact, it was expected before the experiment was carried out that the steric hindrance caused by the large side group would prevent the molecule from binding at all. If competitive inhibition really occurred, this would imply that ADA's active site demonstrates a great deal of plasticity, and could probably accommodate many other molecules that are not as drastically altered in structure from adenosine's as N-6-cyclohexyladenosine.

A comparison of the results of 2-chloroadenosine and N-6-cyclohexyladenosine shows a wide contrast. N-6-cyclohexyladenosine showed a much less dramatic increase of inhibition as concentration of analog increased, indicating that it bound worse than did 2-chloroadenosine. Furthermore, this distinction between the results of the two groups is magnified because the 2-chloroadenosine was tested using 80 μM adenosine while N-6-cyclohexyladenosine was tested against 60 μM adenosine. Ultimately, the extreme difference between the degrees of inhibition between the two analogs implies that 2-chloroadenosine acts as a better inhibitor.

Our results may have been influenced by the following sources of error. Foremost, there was a fair amount of scatter in each set of data; for example, the plot of the N-6-cyclohexyladenosine data seemed to oscillate rather than adhere to the linear trendline. In addition, the spectrophotometer was limited in its measuring capacity to only three significant figures, which affected the precision of its measurements. Also, the experiments often dealt with concentrations outside of the limits of .100 and 1.00, so the results may not have been as accurate as possible. Lastly, we made the assumption that the reaction progressed linearly over a given duration of time; however, some graphs seemed to indicate a curved rather than a linear fit, which was not anticipated fully.

For further study, the specific types of inhibitor should be studied with more depth. With the current results, any conclusion on the inhibitor type is based on mere speculation and weak empirical evidence. To determine definitively the nature of the inhibitor, the group would test with different concentrations of analog and adenosine, allowing a recalculation of K_m as each variable changes. If the studied analog exhibited a K_m increase, it would serve as further evidence that the analog is a competitive inhibitor. In a noncompetitive inhibitor, experiments would produce a smaller V_{max}.

A number of scientists have already studied the kinetics and inhibition of adenosine deaminase. One group has concluded that the HPLC-based method (High Performance Liquid Chromatography) is preferable over the spectrophotometric method. This is based on the assertion that adenosine and inosine have extremely close λ_{max}, which makes it very difficult to quantitatively evaluate the product formation since both adenosine and inosine contribute at these wavelengths. The suggested HPLC-based assay system allows a clear distinction of peaks of the substrate, product, and inhibitor because the polarity of the molecules exhibits a difference in the migration time. Furthermore, the limit of detection was found to be in the nanomolar (nM) range in the HPLC method, allowing for greater accuracy than the spectrophotometric method, through which we only measured to micromolars (μM). For further studies of adenosine deaminase, we would consider the HPLC-based method due to its specific advantages over the spectrophotometric method; however, because of the time needed to prepare each specific sample, and given the time constraints of the experiment, such examination was not realistic [21].

CONCLUSION

The data in these experiments suggested that the K_m of adenosine at a pH of 7.4 was 5.5 μM, and the V_{max}, calculated with an ADA concentration of 6.0×10^{-4} U/mL, was 3.6×10^{-9} μM/s. Three independent trials produced relatively consistent results, suggesting that the techniques used in the experiment were fairly accurate. This result was used as a standard for comparison for Analog 1, 6-chloroadenosine, which acted as a substrate for the enzyme ADA. On the contrary, Analog 2, 2-chloroadenosine, and Analog 3, N-6-cyclohexyladenosine, did not exhibit evidence of binding as a substrate with ADA.

The objective was the alteration of the structure of the adenine ring to explore which sites could possibly act as effective inhibitors when modified. The limited data implied that 2-chloroadenosine was the most successful inhibitor among the three analogs tested. As 6-chloroadenosine acted as a substrate with less affinity for ADA than adenosine, it would not function as a satisfactory inhibitor. Additionally, it cannot be deduced whether N-6-cyclohexyladenosine binds to ADA; however, the data served as marginal evidence that if the analog was indeed a competitive inhibitor, it did not bind well. Thus, it can be concluded that the best modification site on the adenine ring is the carbon-2 position. Furthermore, the carbon-6 position does not appear to be an effective site for future alteration.

REFERENCES

1. Kimball JW. ; Enzyme Kinetics [Internet]. Cambridge, MA: Harvard University; 2003 July 11; Available from: http://users.rcn.com/jkimball.ma.ultranet/BiologyPages/E/EnzymeKinetics.html
2. UBC Department of Botany [Internet]. Vancouver, B.C. : University of British Columbia; 2003 March 28; Available from: http://www.botany.ubc.ca/teaching/biol201/e2201.htm
3. Miller BG., Wolfdenden R. Catalytic Proficiency: The Unusual case of OMP Decarboxylase. Annual Review of Biochemistry [serial online] 2002; 71(11). Available: http://arjournals.annualreviews.org/doi/abs/10.1146/annurev.biochem.71.110601.135446? via the INTERNET. Accessed 2005 Aug 06.
4. ENZYME BIOLOGY [monograph online]. MIT: Massachusetts Institute of Technology; 2005. Available from: http://web.mit.edu/esgbio/www/eb/ebdir.html. Accessed 2005 August 06.
5. Grant, CM. Introduction to Enzymology. Available: http://www.bi.umist.ac.uk/users/mjfsscmg/1SFM/enzymes.htm. Accessed 2005 August 06.
6. [UL] University of Leicester. 2003 May 22. Enzyme Kinetics and the Michaelis Menten Equation. Available: http://www.le.ac.uk/by/teach/biochemweb/tutorials/michment2print.html. Accessed 2005 August 06.
7. Doležal T. ; Adenosine Deaminase: A Review of Psychological Roles [Internet]. South Bohemia: University of South Bohemia; 2001; Available from: http://www.entu.cas.cz/fyziol/seminars/ada.html

8. INTRODUCTION TO ENZYME KINETICS [monograph online]. UK: Oxford University Press; 2003 April. Available from: Graphpad Software, http://www.curvefit.com/introduction63.htm. Accessed 2005 Aug 06.
9. Nelson D. ; Nucleotide Metabolism II [Internet]. Memphis, TN: University of Tennessee; 2001 Nov 1; Available from: http://drnelson.utmem.edu/nucbio2.html
10. Zhong H., Chunn JL., Volmer, *et al.* Adenosine-Mediated Mast Cell Degranulation in Adenosine Deaminase-Deficient Mice. Journal of Pharmacology and Experimental Therapeutics [serial online] 2001; 298(2). Available: http://jpet.aspetjournals.org/cgi/content/abstract/298/2/433 via the INTERNET. Accessed 2005 Aug 06.
11. McKusick VA. Baltimore, MA: Johns Hopkins University; 1986 June 16; Available from: http://www.ncbi.nlm.nih.gov/entrez/dispomim.cgi?cmd=entry&id=102730
12. Chottiner EG., Cloft HJ., Tartaglia AP. *et al.* Albany, NY: Albany Medical Center Hospital; 2005; Available from: http://www.pubmedcentral.nih.gov/picrender.fcgi?artid=424261&blobtype=pdf
13. MedlinePlus Medical Encyclopedia [Internet]. Bethesda, MD: U.S. National Library of Medicine; 2005 July 19; Available from: http://www.nlm.nih.gov/medlineplus/ency/article/000571.htm
14. Paul, Manash K., Vikas Grover, and Anup K. Mukhopadhyay. 2005. Merits of HPLC-based method over spectrophotometric method for assessing the kinetics and inhibition of mammalian adenosine deaminase. Journal of Chromatography; 822. Available from: http://www.sciencedirect.com/. Accessed 2005 July 27.
15. Cristalli G., Costanzi S., Lambertucci C., *et al.* Adenosine Deaminase: Functional Implications and Different Classes of Inhibitors [Internet]. John Wiley & Sons, Inc. [serial online] 2001; 21(2). Available: http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&list_uids=11223861&dopt=Abstract
16. Fink T. ; Enzyme Kinetics Continued [Internet]. Santa Cruz, CA: University of California; 1998; Available from: http://www.chemistry.ucsc.edu/~fink/231/lecture13.htm.
17. Kinoshita T., Nakanishi I., Terasaka T., *et al.* Structural Basis of Compound Recognition by Adenosine Deaminase [Internet]. ACS Publications [serial online] 2005; 44(31). Available: http://pubs.acs.org/cgi-bin/abstract.cgi/bichaw/2005/44/i31/abs/bi050529e.html
18. [SHU] Sheffield Hallam University School of Science and Mathematics. 2002 July. Beer's Law. Available: http://www.shu.ac.uk/schools/sci/chem/tutorials/molspec/beers1.htm. Accessed 2005 August 07.
19. Beckman Coulter. 2005. DU® Series 500 UV/Vis Spectrophotometer. Available from: http://www.beckman.com/products/instrument/analytical/uvvis/duseries500_inst_dcr.asp. Accessed 2005 August 09.
20. Thorn, James A. and Jarvis, Simon M. 1996. Adenosine Transporters. General Pharmacology: The Vascular System; Volume 27 (Issue 4): Pages 613-620. Available from: http://www.sciencedirect.com/. Accessed 2005 August 07.
21. Holland, James F. and Frei, Emil. 2003. Cancer Medicine [online]. Hamilton, London: BC Decker Inc. Available from: PubMed, http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=cmed6. Accessed 2005 August 06.