

**ENZYME KINETICS AND MECHANISM:  
THE EFFECTS OF ADENOSINE-NUCLEOTIDE DERIVATIVES ON  
ADENOSINE DEAMINASE**

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**ABSTRACT**

In this study we investigated the nature of the active site on the enzyme adenosine deaminase (ADA) in hopes of finding an analog structure to act as an inhibitor. Our research involved comparing the binding patterns of adenosine to several structural analogs by analyzing and comparing kinetic and spectroscopic data. We examined variations in positions 2 and 6 of the carbon ring to determine if either structural change may result in inhibition. 2-chloroadenosine was used to examine the 2-position, and similarly, 6-chloroadenosine and N-6-cyclohexyladenosine were used to consider the 6-position. The  $V_{\max}$  and  $K_M$  values were established from data analysis with the Michaelis-Menton equation, and were used to compare adenosine and 6-chloroadenosine, which is observed to act as a substrate. The  $K_M$  for adenosine was found to be 11.85  $\mu\text{M}$  and the  $V_{\max}$  was determined to be  $6.8 \times 10^{-9}$  M/s. Both 2-chloroadenosine and N-6-cyclohexyladenosine were found not to bind at all, suggesting that structural alterations at either location would not yield inhibition. However, the possibility exists of smaller alterations at the 6 position leading to ADA inhibition.

**INTRODUCTION**

Mechanisms of Enzymes

Enzymes are the essential catalysts of life. They allow slow-paced reactions to occur in seconds so that reagents do not build up within an organism. Every reaction has an activation energy, which is the minimum amount of energy the reactants must overcome in order to drive the reaction forward. Enzymes lower this activation energy barrier, speeding up a reaction [1]. Figure 1 demonstrates how the activation energy is decreased with enzyme addition. Like all catalysts, enzymes are not consumed in the reaction and can be used repeatedly.

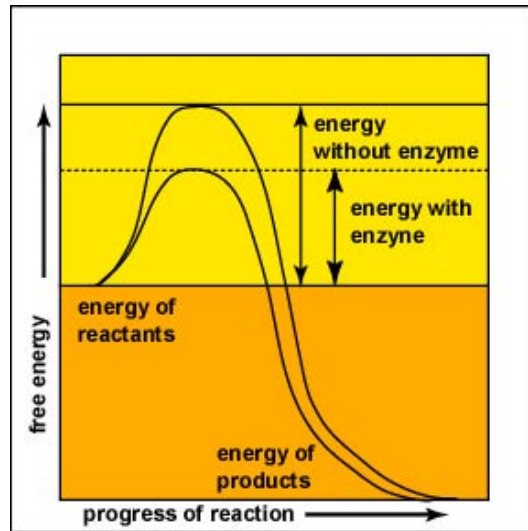


Figure 1: Lowering of Activation Energy [4]

Enzymes are proteins coded by DNA, and they consist of polypeptide chains, varying in length from hundreds to thousands of amino acids [3]. The intermolecular bonds and repulsions of the hydrophobic and hydrophilic parts of the amino acids bend the protein into unique 3-D configurations. Because each enzyme has a unique tertiary structure, the three-dimensional conformation, an enzyme binds only to specific substrates. This binding occurs at a specific site of the enzyme, called the active site. Since the enzyme is usually much larger than the substrate, the enzyme will only interact with its substrate within a small area, around ten amino acids in size [2].

### Catalysis and Michaelis-Menten Equation Derivatives

Chemical reactions that involve enzymatic pathways are often treated as two stage processes. In the first, the substrate binds to the enzyme to form an enzyme-substrate (ES) complex, and sequentially the enzyme changes the substrate and the complex breaks apart to form enzyme and product (Equation 1). According to this knowledge of enzyme catalysis, we used the following model to describe the action of the enzyme ADA on adenosine.



Equation 1: Enzyme-Substrate Reaction Model

The rate constants, displayed as  $k_1$ , etc. represent the rate constant of each reaction with regard to the concentration of the reactants in a proportional relationship. Thus the overall rate constant depends on  $k_1$ ,  $k_{-1}$ , and  $k_2$ .

Using this model and two important assumptions we can formulate the relationship of substrate concentration and the initial rate of reaction. The first of these assumptions is

that the reaction rate of the reverse reaction for the second step in the model is 0 by assuming that the  $[P] = 0$  at the start of the reaction. The second assumption is the steady state assumption, which claims that if  $[S]$  is much greater than  $[E_t]$ , then the rate of formation of enzyme-substrate complex is the same as the rate of decay. Therefore, the reaction runs to completion because the first step in the reaction mechanism is the only step that involves the steady state assumption. Also this allows us to compose the following equation based on the Michaelis-Menten equation (Equation 2).

$$V = \frac{V_{\max}[S]}{K_m + [S]}$$

Where:

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

Equation 2: The constants in this equation,  $K_M$  and  $V_{\max}$ , are defined so that  $V_{\max}$  = Maximum velocity catalyzed by a fixed  $[E]$  and  $K_M$  = the  $[S]$  which gives  $1/2 V_{\max}$ . [13]  $k_1$  is the constant of bonding,  $k_{-1}$  is the constant of dissociation, and  $k_2$  is the constant of catalysis.

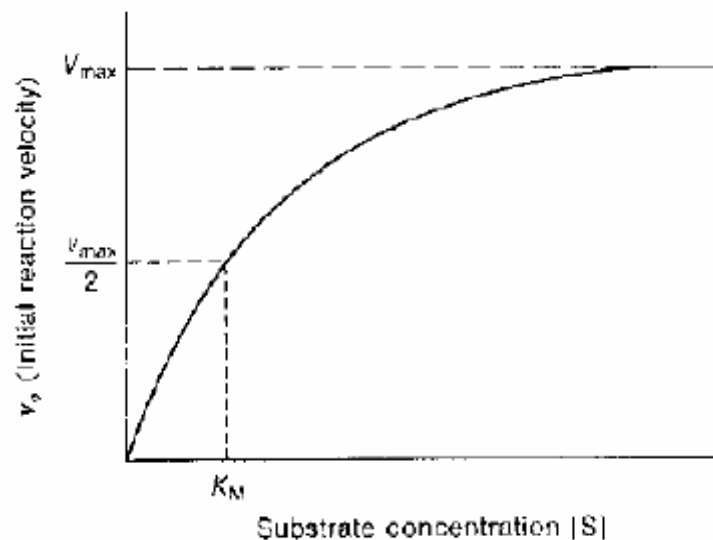


Figure 2: This figure shows the plot of  $V_o$  versus  $[S]$ , illustrating the operational definitions of  $K_M$  and  $V_{\max}$ . Therefore, the  $V_o$  versus  $[S]$  plot approaches the limit, which is the  $V_M$  [14]

$V_{\max}$  has asymptotic properties and is the maximum value of catalysis, however, it is a theoretical value and the reaction can never proceed at this highly accelerated rate. At the y value for  $V_{\max}/2$ , the  $K_M$  is the corresponding x value, as shown in Figure 2. In order to calculate the  $K_M$  and  $V_{\max}$ , the Michaelis-Menten equation is converted into a linear form by taking the reciprocal of both sides of the equation. This double reciprocal plot is called the Lineweaver-Burk equation, as shown in equation 3.

$$\frac{1}{v_0} = \frac{K_M}{V_{\max}} \frac{1}{[S]} + \frac{1}{V_{\max}}$$

Equation 3: Lineweaver-Burk equation [13]

From this new equation, it is possible to find a linear slope and to determine the  $V_{\max}$  and  $K_M$  of the substrate more efficiently. This equation follows the pattern of slope intercept form, where  $y = mx + b$ . The slope of the line represents the ratio of  $K_M$  to  $V_{\max}$ . The independent variable is equal to  $1/V_0$  and the dependent variable is  $1/[S]$ . The slope is equal to  $K_M/V_{\max}$ , while the y-intercept and equal to  $1/V_{\max}$ .

Through experimental analysis, we seek to determine the rate of this reaction, namely the velocity of the reaction ( $d[P]/dt$ ) defined by the elementary rate constants that govern the aforementioned simplified reaction. Additionally the velocity of the reaction will be measured through known concentrations of enzyme and substrate  $[E]$  and  $[S]$ .

In order to determine these values, we tested the inhibiting ability of certain adenosine-related molecules on the active site of the ADA enzyme. Using the known values for the  $K_M$  and  $V_{\max}$  from a control sample for ADA and adenosine in known concentrations, it is possible to compare the rate of reactions for alternative compounds. From the values, it will be possible to determine whether or not differing concentrations of adenosine related compounds will affect in any way the reaction of ADA and adenosine through competitive or noncompetitive inhibition.

The Michaelis-Menten Equation produces two numbers which are significant to our study:  $V_{\max}$  and  $K_m$ . These figures serve as quantitative indices of the activity of ADA in conjunction with the values of absorbency and  $\epsilon$ .

### Inhibition of Enzymes

Enzymes are designed to accommodate a particular substrate, but oftentimes various compounds can bind into the active site. These molecules, called inhibitors, slow down reactions by preventing the proper substrate from binding to the enzyme. Numerous types of inhibitors exist, including competitive and non-competitive inhibitors.

Competitive inhibitors bind at the same active site as the substrate because they have similar structures. Often, competitive inhibition can be overcome as long as the concentration of the substrate greatly exceeds that of the concentration of the inhibitor [2].

Non-competitive inhibitors bind to the enzyme at a site different from the one to which the substrate binds. Attaching at the allosteric site induces the enzyme to change its shape, altering the active site, preventing the substrate from binding to the enzyme (see Figure 3).

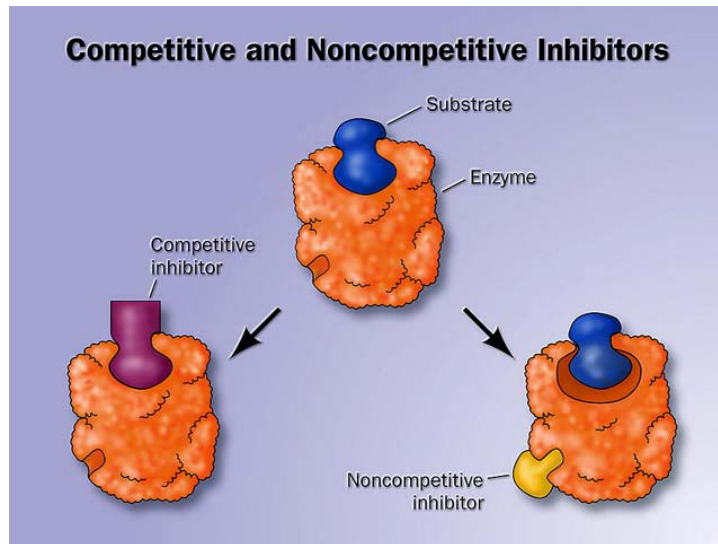


Figure 3: Competitive and Noncompetitive Inhibitors [5]

### The Adenosine Pathway

Adenosine is a nucleoside which serves as a metabolic messenger by sending extracellular-facing receptor reports on the intracellular metabolic state of a cell. Extracellular adenosine is removed in two ways. A portion of it is reabsorbed into the cell and the rest is changed into inosine by adenosine deaminase (ADA) when it enters the bloodstream, as shown in Figure 4 [7]. Adenosine is deaminated permanently by this enzyme through the removal of an amine group, in the reaction shown below:

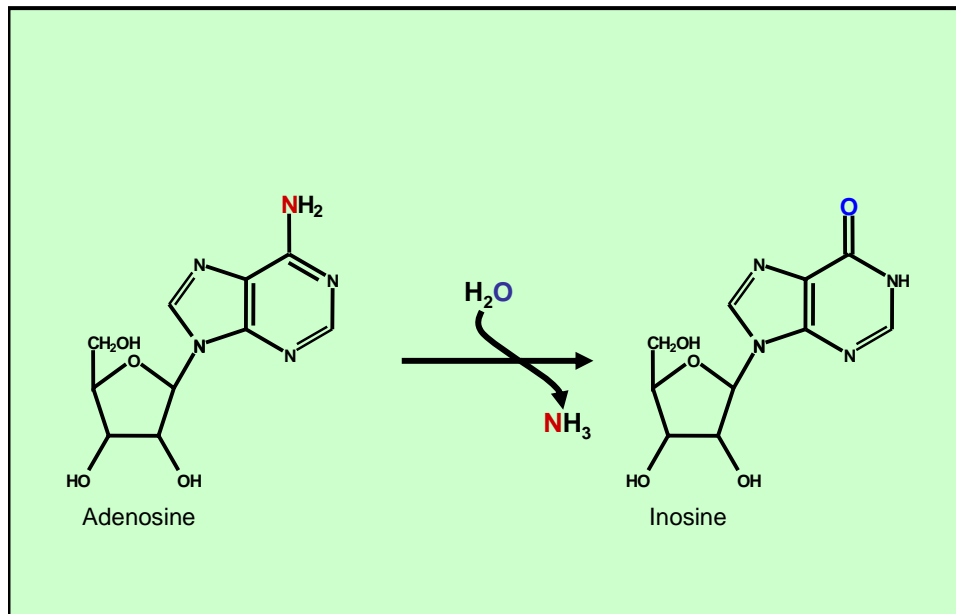


Figure 4: The Adenosine Pathway [7]

This process is essential for the turnover of nucleic acids in tissue and the removal of adenosine from food. It is also important in the production of inosine, which is used in the body for many functions. It is associated with the release of insulin, protein synthesis, and oxygen metabolism. Recently, research has also shown that inosine has neuroprotective qualities and can be helpful when administered after a stroke for axonal re-wiring. [8]

### Practical Applications of Adenosine Deaminase Research

ADA is found in high concentrations in the lymphatic system of the human body. The lymphatic system is highly responsible for the production of T-cells, which are the body's main line of defense. Thus, ADA deficiencies are associated with the lymphatic diseases. These include viral infections and immunoproliferative disorders, specifically tuberculosis, severe combined immunodeficiency syndrome (SCIDS), viral hepatitis, and acquired immunodeficiency syndrome (AIDS) and coronary artery disease (CAD) [9]. In CAD, ADA mediates part of the inflammation response, one of the main characteristics of CAD. This means that if ADA can be inhibited, the resulting process could become a treatment for CAD. The inflammation process is marked by the activity of lymphocytes and macrophages, which bring with them the ADA which causes maturation of lymphocytes [11].

The interaction between adenosine and ADA is an important balance that can alter a human state when not correct. By studying this interaction, we can hope to gain information that will help to improve the balance of these two essential compounds in the human body that cause disease [9].

It has been found that adenosine presence in cancer has some effect on the proliferation of cells. Studies have been made to test the pathway from enzyme activity in cells that cause adenosine-production, which activates other cells' receptors to signal for growth in blood vessels [12]. The process of growing new blood vessels is a key component in cancer growth. If the levels of adenosine and its signaling pathway can be understood and manipulated, angiogenesis may be controllable, leading to a more effective cancer treatment.

ADA deficiencies can cause selective toxicity of cells in lymphatic tissues, indicating that inhibitors of ADA may be administered as a selective chemotherapeutic drug. This would be limited to lymphatic malignancies. It also has potential to act as an immunosuppressive agent, meaning it could be helped to treat the diseases that plague the lymphatic system where many immunodeficiency diseases have major symptoms [9].

There are several hypotheses that all propose ADA inhibition in relation to some other step of the pathway in which adenosine is converted to inosine. [9]. These hypotheses include both competitive and feedback inhibition; competitive including an inhibitor from an outside source, such as a drug, and feedback inhibition meaning a product of the pathway binding to an allosteric site to prevent further catalyzation by the enzyme [9].

## The Consequences of Beer's Law

$$A = [\epsilon l]C$$

Equation 4: Beer's Law

Beer's Law relates a compound's concentration to the rate at which it absorbs electromagnetic radiation. "A" represents the amount of light absorbed at a specific wavelength. "ε" is the extinction coefficient that proportions the amount the substance absorbs (per mole) at a given wavelength of light. "l" is the length of the pathway. For the purposes of our study, this will always be 1 cm. "C" is the concentration in molarity. The value of ε and l, which are constants that can be determined from the substance under observation and the length of the cuvette used multiply to produce the constant of proportionality between the concentration of a substance and the amount of radiation it will absorb. Our study required us to utilize Beer's law to pinpoint the value of ε for adenosine, inosine, 6-chloroadenosine, 2-chloroadenosine, and N6-Cyclohexyladenosine. We also had to determine the change in the value of ε and the change in the absorbency as Adenosine is deaminated into inosine.

## **MATERIALS AND INSTRUMENTS**

All reactions conducted for this project were observed in a Beckman DU® 530 Spectrophotometer and with 10mM stock solutions of adenosine, inosine, and the adenosine nucleotide derivatives from Sigma-Aldrich. We diluted these solutions 1:10 with dH<sub>2</sub>O to produce stock solutions of 1mM. Because of the natural environment of these substances, we use 50 mM HEPES buffer (*N*-2-hydroxy-ethyl-piperazine-*N'*-2-ethanesulfonic acid), pH 7.5, in all solutions [15]. All calibrations of the spectrophotometer were performed with 1000 μL of buffer and dH<sub>2</sub>O, a blank control.

## **METHODS**

### Determining the Value of ε

Reactions were monitored at the wavelength yielding the largest change in ε. Each compound, however, had its own specific wavelength. Because the spectrophotometer gives more accurate readings at absorbance values of 0.10 - 1.00, we used a concentration 50μM of adenosine and inosine solutions. We blanked the spectrophotometer using 50μM inosine and ran a wavelength (λ) scan in the range of 220-300nm. The peak of the resulting graph represented the wavelength at which the change in absorbency between the substrate and product is greatest.

After procuring a wavelength to observe the activity of ADA, we divided the absorbency value (from wavelength scan) by the concentration of the adenosine in the solution to produce the value of ε. These values, the change in absorbency and the change in ε help assay the rate of change in the concentration of adenosine. This procedure was repeated to produce these values for 6-chloroadenosine, N6-cyclohexyladenosine, and 2-chloroadenosine

### Determination of $V_{\max}$ and $K_m$

Figure 2 suggests that saturation of a solution with substrate concentration will eventually result in a maximum speed of enzyme activity. Because of the asymptotic nature of  $V_{\max}$ , only creating highly concentrated solutions to observe through the spectrophotometer would be a poor indication as to the effectiveness of the enzyme. Instead, we chose to create several solutions of 3 $\mu$ M, 5 $\mu$ M, 8 $\mu$ M, 10 $\mu$ M, 12 $\mu$ M, 15 $\mu$ M, 16 $\mu$ M, 20 $\mu$ M, 30 $\mu$ M, 40 $\mu$ M, 60 $\mu$ M, and 100 $\mu$ M adenosine. All these solutions contained 0.0002 Units/mL ADA.

Each of the solutions underwent a kinetics/time scan for four minutes each, taking readings every 15 seconds. We then plotted the data from the spectrophotometer on Microsoft Excel®. The slope of the linear regression of each graph is equivalent to the change in absorbency. Using the relationship established by Beer's Law, we divided this number by the number obtained for  $\epsilon$  to find the  $V_o$  of each reaction at a different concentration of adenosine. Plotting the inverse of the concentration (in molar) by the inverse of the velocity of reaction gives the double reciprocal plot. This new regression shows the values of  $V_{\max}$  and  $K_m$ .

This procedure was repeated for 6-chloroadenosine using a kinetic time scan for ten minutes at one minute intervals.

### Testing the Analogs that did not Bind into the Enzyme

In order to test for inhibition, solutions containing 100  $\mu$ M adenosine and 0 M, 1 nM, 10 M, 0.1  $\mu$ M, 1  $\mu$ M, 10  $\mu$ M, 20  $\mu$ M, 30  $\mu$ M of the analog were created. These solutions underwent a kinetic/time scan for ten minutes at one minute intervals. If true inhibition is observed, the change in absorbency will steadily decline as the concentration of the analog increases.

## **RESULTS**

### Determining the Optimal Wavelength and Extinction Coefficient

In the first part of the experiment, the average of the three optimum experimental wavelengths at which the rate of the reaction could be observed was measured at 264 nm. At 264 nm, the difference in absorbency between adenosine and inosine was the greatest.



Table 1: A Comparison of Values for all Adenosine-Based Nucleotides

	$\lambda(\text{nm})$	<b>Abs</b>	$\epsilon$ (Abs/(M·cm))
<b>Adenosine</b>	264	0.315	6300
<b>6-Chloroadenosine</b>	269	0.105	2100
<b>N6-Cyclohexyladenosine</b>	274 (used 264)	0.409	8180
<b>2-Chloroadenosine</b>	264	0.289	2890

Table 1 shows the varying optimal wavelengths, absorbencies and extinction coefficients of adenosine and three analogs we tested.

Table 2: Rate of conversion of adenosine into inosine with different initial concentrations of adenosine (single trials)

<b>Concentration of adenosine</b>	<b><math>\mu\text{Molars/second}</math></b>
3 $\mu\text{M}$	5.429
5 $\mu\text{M}$	6.571
8 $\mu\text{M}$	11.43
12 $\mu\text{M}$	13.33
20 $\mu\text{M}$	17.14
30 $\mu\text{M}$	19.05

Once the average of the optimal wavelength was calculated, Beer's Law was applied to find the extinction coefficient,  $\epsilon$ . Knowing the wavelength, change in absorbance, and concentration of adenosine, the extinction coefficient was calculated for each group's data. The average of  $\epsilon$  was determined to be  $6.3 \times 10^3$  Abs/ (M · cm). The difference in extinction coefficient between the adenosine analogs and inosine are given in Table 1.

#### Maximum Reaction Rate and Rate Constant

Using the wavelength of 264 nm found in part one  $V_{\text{max}}$ , the maximum rate of the reaction, and  $K_M$ , the rate constant, were determined. Various concentrations of adenosine were mixed with a constant mixture of 0.0002 units/mL ADA. The change in absorbency at 264 nm, the slope of each set of graphed values, increased as concentration increased as expected, since greater substrate concentration would result in greater absorbency. These Rates are summarized in Table 2.

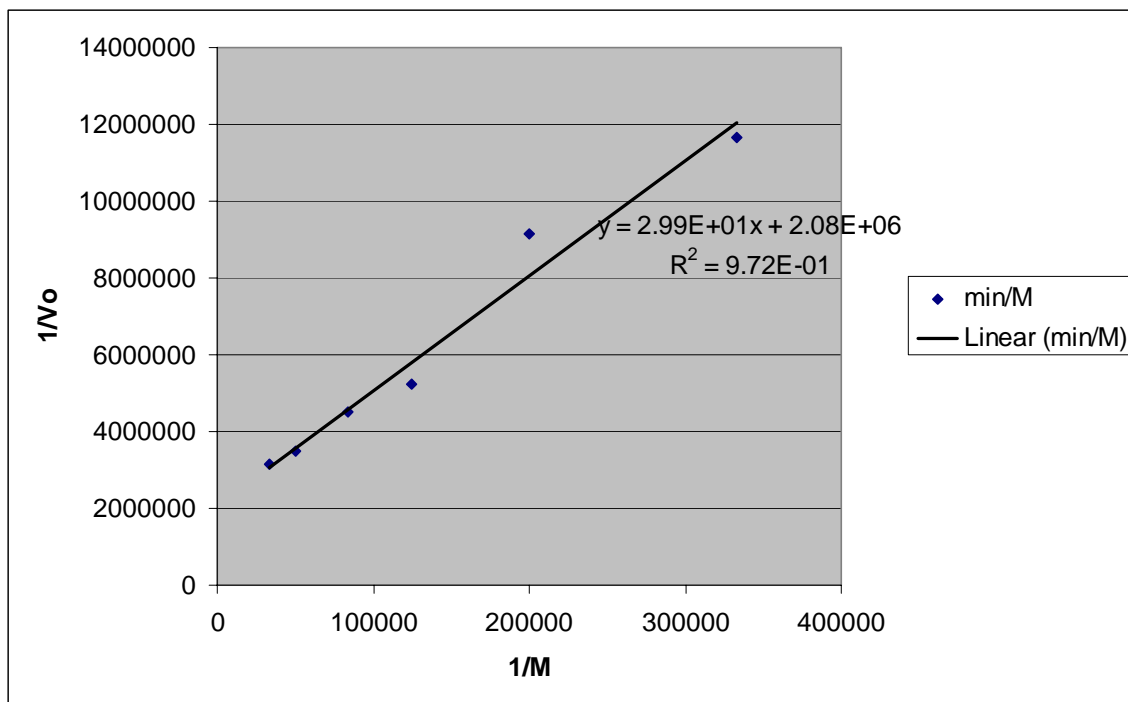


Figure 5: Lineweaver-Burk Double-Reciprocal Plot

Figure 5 displays the linear relationship between the reciprocals of molarity ( $1/M$ ) against the initial velocity of each reaction ( $1/V_o$ ). The  $R^2$  value of 0.972 proves that there is a strong linear relationship between the variables

Using the Lineweaver-Burk equation (Figure 5), the average maximum velocity was calculated to be  $6.8 \times 10^{-9}$  M/s, and the average rate constant was calculated to be 11.85. Thus, calculations for  $V_{max}$  and  $K_M$  consisted of the average of both trials.

### 6-Chloroadenosine

Table 3: Changes in Absorbency of 6-Chloroadenosine over Time

<b>Concentration of 6-Chloroadenosine</b>	<b>Rate (OD/second)</b>
3 $\mu$ M	-0.0001
5 $\mu$ M	-0.0002
8 $\mu$ M	-0.0007
12 $\mu$ M	-0.0001
16 $\mu$ M	-0.0002
30 $\mu$ M	-0.0002

Recall that the Michaelis-Menten Equation can be rewritten to create the Lineweaver-Burk Equation. This equation yields the  $V_{max}$  and  $K_m$  of the substrate. When we ran the 6-chloro-purine riboside (6-Chloroadenosine) solutions through the spectrophotometer, we determined that compound effectively bound into the active site of ADA. We came to this conclusion on the basis that because there was a change in absorption, the compound must bind and react with ADA (see Table 3). Because of the

catalysis,  $V_{\max}$  and  $K_m$  values of adenosine and its analog (6-chloroadenosine) can be compared.

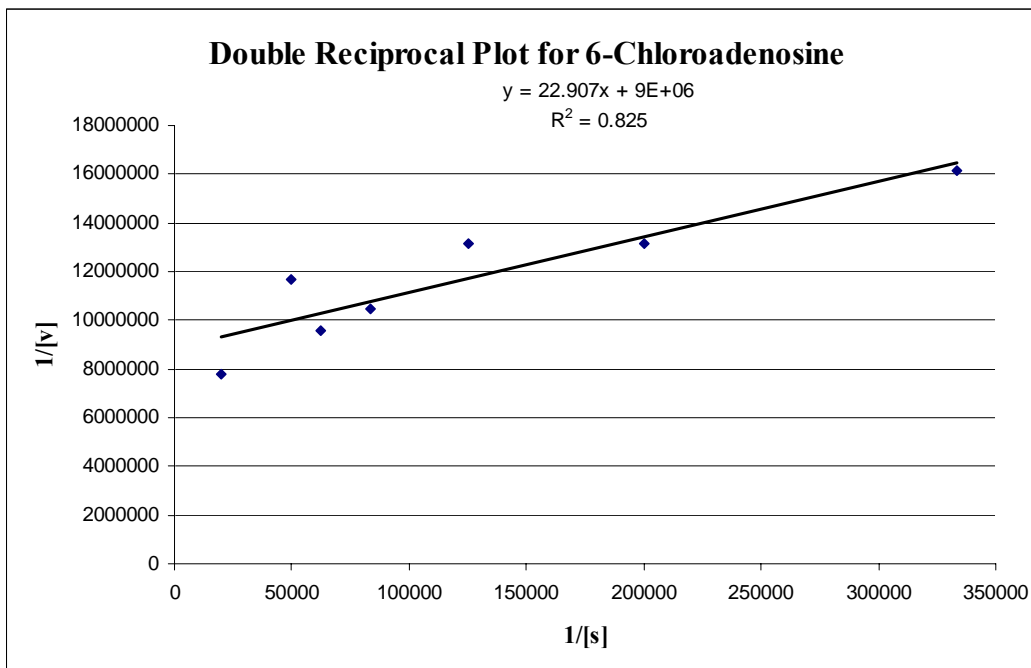


Figure 6: The Lineweaver-Burk Double Reciprocal Plot for 6-Chloroadenosine

The catalysis of adenosine yields a  $V_{\max}$  of  $6.8 \times 10^{-9}$   $\mu\text{M/s}$  and a  $K_m$  of  $11.85 \mu\text{M}$ , whereas the catalysis of 6-chloroadenosine yields a  $V_{\max}$  of  $1.85 \times 10^{-9}$   $\mu\text{M/s}$  and a  $K_m$  of  $2.5 \mu\text{M}$ . However, these numbers do not reflect the physical reality of the reaction mechanism; adenosine was placed in an ADA solution of  $2.0 \times 10^{-4}$  Units/ml, but 6-chloroadenosine was placed in an ADA solution of  $2.0 \times 10^{-3}$  Units/ml. We recalculated the  $V_{\max}$  of 6-chloroadenosine to account for this vast difference in concentration in enzyme.  $V_{\max} = k_2[E_o]$ , therefore we divided the  $V_{\max}$  of 6-chloroadenosine by 10 to yield comparable numbers [9].

From the Lineweaver-Burke plot (Figure 6), 6-chloroadenosine's  $K_m$  is roughly 4.7 times less than that of adenosine. A low  $K_m$  value is an indicator of either a slower catalysis or a tighter binding into the enzyme substrate complex or both. If the  $V_{\max}$  is same then the difference in  $K_m$  can be attributed to a tighter binding situation, however, if the  $V_{\max}$  is lower then a slower rate is more likely to explain a smaller  $K_m$ . Our data indicates that adenosine moves through the reaction mechanism 37 times faster than 6-chloroadenosine; its higher  $K_m$  suggests either a faster catalysis or a looser binding situation with ADA.

$V_{\max}$  is also proportional to  $k_2$ , therefore the  $k_2$  of 6-chloroadenosine is much lower than that of adenosine (see equation 2). In order for  $K_m$  to decrease, either  $k_2$  or  $k_{-1}$  must decrease or  $k_1$  must increase. This reasoning leads us to believe that the  $K_m$  of 6-chloroadenosine is lower than that of adenosine because comparatively, the analog catalyzes slower.

## N6-Cyclohexyladenosine

The N6 cyclohexyladenosine compound was tested for binding and inhibition as well. Initial tests did not produce a steady decrease in absorbance, suggesting no reaction was occurring. In order to find out if it bound, we tested N6 cyclohexyladenosine as an inhibitor. To do this, we compared various concentrations of N6 cyclohexyladenosine mixed with both adenosine and enzyme and compared these trials with blanks composed of the enzyme and the adenosine alone. Because of the set up of these trials, we searched for a change in  $K_M$  as the concentration of N6 cyclohexyladenosine increased in comparison to the constant concentration of adenosine and enzyme in the solution.

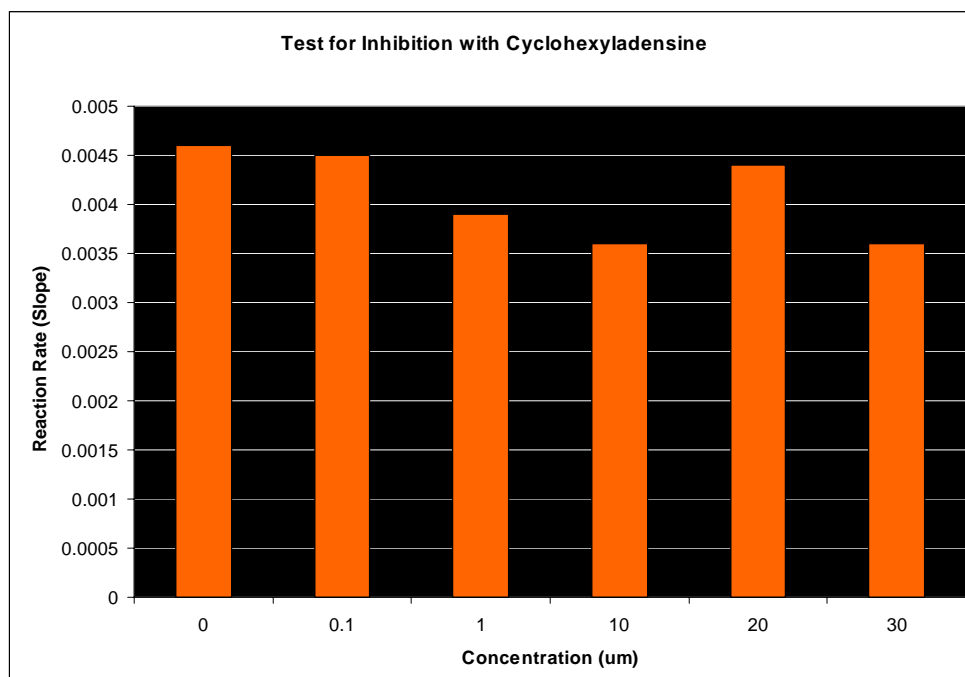


Figure 7: As you can see, there is no direct pattern across the increased concentration, but rather the rates are all very similar. If N6 cyclohexyladenosine acted as an inhibitor, the rates would get increasingly lower as concentration increased. Thus, it is clear that N6 cyclohexyladenosine does not bind or act as an inhibitor.

The results of the trials showed a very miniscule change in rate (~20%) in comparison to the greatly increasing concentration of N6 cyclohexyladenosine. Data shows that the rate for a solution with no N6 cyclohexyladenosine is nearly the same as the rate of a solution with an additional concentration of 20 $\mu$ M and very similar to the rates of other increased concentrations. Thus, we can conclude that N6 cyclohexyladenosine does not bind or act as an inhibitor. The slight difference among the different concentration could be a result of imperfections in data collection or cuvettes, but not because of inhibition. Overall, there is no direct or indirect competitive inhibition towards ADA with N6 cyclohexyladenosine.

## 2-Chloroadenosine

We tested 2-chloroadenosine's relationship to ADA by maintaining a constant concentration of adenosine,  $10\mu\text{M}$ , which we chose based on its proximity to our determined  $K_m$  of adenosine. Then we added a range of 2-chloroadenosine concentrations to the adenosine;  $5\mu\text{M}$ ,  $10\mu\text{M}$ ,  $20\mu\text{M}$ , and  $30\mu\text{M}$  to make 4 solutions. To each concentration we added  $10\mu\text{M}$  of ADA and monitored the absorbency of the reaction at a wavelength of  $264\text{nm}$  for three minutes. We ran three trials of the same concentrations and the results were consistent.

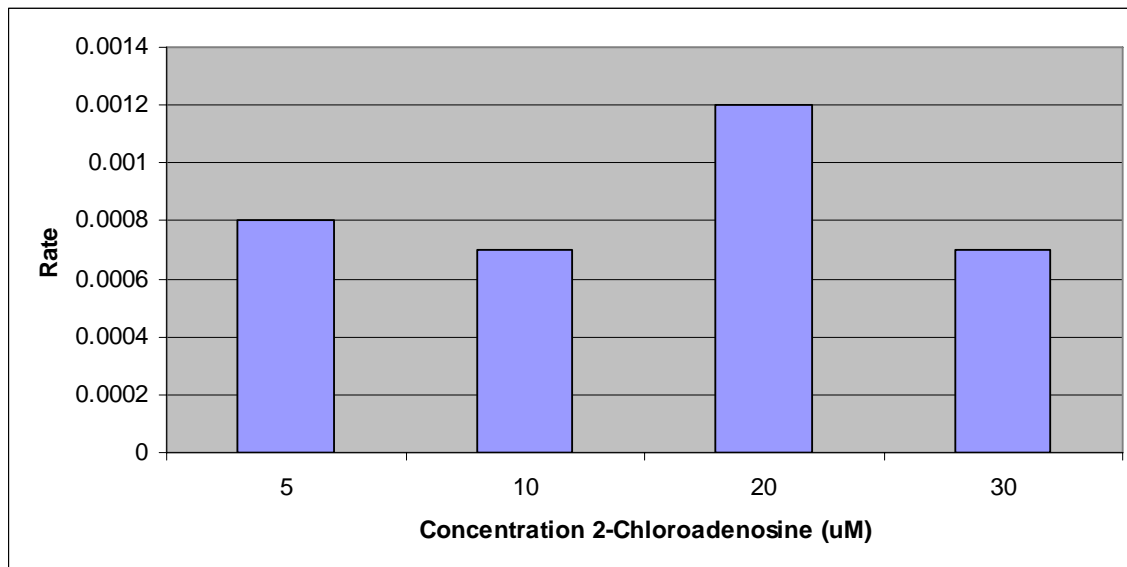


Figure 8: Rate of Reaction vs. Concentration Plot of 2-chloroadenosine  
The bars illustrate the rate of reaction for solutions with specific concentrations of chloroadenosine and  $10\mu\text{M}$  adenosine, reacted with  $10\mu\text{M}$  of ADA.

The rate of the reactions depicted in figure 8 show no trend; the reaction proceeded faster with  $30\mu\text{M}$  of 2-chloroadenosine than it did with  $5\mu\text{M}$ , yet it proceeded slower with  $40\mu\text{M}$  than  $30\mu\text{M}$ . The rates were scattered even after several trials, which allows us to conclude that 2-chloroadenosine neither inhibits nor binds with ADA.

## DISCUSSIONS

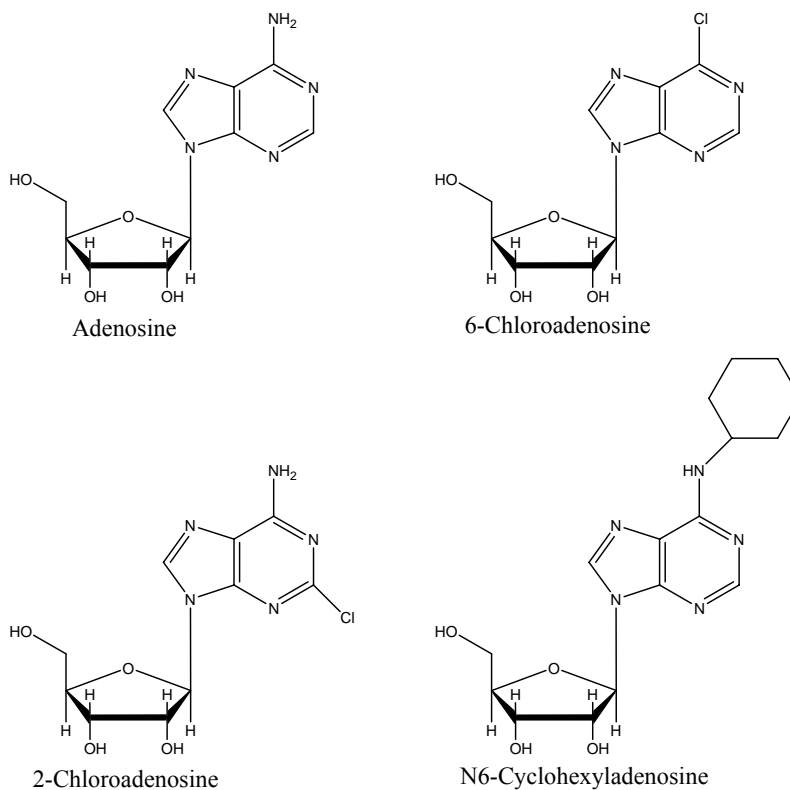


Figure 9: Adenosine and adenosine derivatives [18]

After determining the activity of ADA in the presence of adenosine, we compared its activity in the presence of 2-chloroadenosine, 6-chloroadenosine, and N6-cyclohexyladenosine. Even though these three are adenosine derivatives, they do not share the same wavelength of maximum absorbency with adenosine (e.g. 269nm for 6-chloroadenosine). Therefore, before analyzing the efficacy of these compounds as direct inhibitors, we repeated the process described in “Determining the Value of  $\epsilon$  for Adenosine.”

Once we had the wavelengths,  $\epsilon$ 's, and absorbencies of the three compounds, we had to compare the ADA's effect on the analogs (derivatives of a compound) relative to adenosine. First, we analyzed our data to determine if each analog bound. Catalysis of the hydrolysis reaction is this first test indicated binding of the analog to the enzyme. A substrate must bind before it is catalyzed. Thus a steady decrease in absorbance indicating a reaction was occurring would demonstrate binding of the analog in the active site. Analyzing our  $K_M$  and  $V_{max}$  values, we could determine how effectively the analog bound to the enzyme. By running a kinetic/time scan of 50  $\mu\text{M}$  analog with enzyme, we were able to determine whether the analogs that were found to bind reacted.

If the scan shows negligible to no change in the absorbency of the selected wavelength, then the enzyme is not catalyzing the reaction of the analog. The next

determination is whether the analog inhibits the conversion of adenosine to inosine. In order to test this, we created solutions containing both adenosine and the analog in the presence of ADA. Comparing the resulting  $V_o$  and  $K_{m:apparent}$  with the data collected from the runs of adenosine, we were able to determine if the presence of the analog diminished the conversion of adenosine to inosine. If the analog slowed down the reaction, then it effectively bound to the ADA and inhibited the enzyme.

If the scan suggested that the analog underwent a significant change in absorbency, then it probably bound and was converted into some derivative of inosine. To better understand the mechanics of ADA, we put any analog that appeared to react through the process described in "Determination of  $V_{max}$  and  $K_m$ ."

Once we obtained the values for  $V_{max}$  and  $K_m$  we were able to compare the reactivity of the derivative to that of the original adenosine. If the differences between the indices are significant, one much lower than the other, then the possibility exists that these slower reacting substances can function as a partial inhibitor of ADA.

## CONCLUSIONS

The data suggest that the  $K_m$  of adenosine in a 50% by volume solution of HEPES-buffer (pH = 7.4) is 11.85 and it has a  $V_{max}$  is  $6.8 \times 10^{-9}$  M/s for an ADA concentration of 0.0002 U/mL. This conclusion was used as a basis of comparison for the three analogs that we ran. Our objective was to learn about the plasticity of the active site on ADA. This dynamic active site is designed to best fit the transitional state between substrate and reactant. We designed this experiment to explore which alterations to the purine ring can bind into the active site. Specifically the 2 and 6 positions on the 6 carbon ring.

Our results show that 6-chloroadenosine effectively bound and reacted in the active site of ADA, but its  $K_m$  is 2.5 and its  $V_{max}$  is  $1.85 \times 10^{-10}$  M/s. Although this derivative reacts, it does so at a respectably slower pace than adenosine. A study of circular dichroism in adenosine derivatives by Daniel W. Miles suggests that the similarity in  $\lambda_{max}$  between adenosine and 6-chloroadenosine may be attributed to the pi-electrons of the compound [21]. Intermolecular forces in the active site determine the ability of a compound to bind into an enzyme. New research should be conducted to explore the use of alterations or small additions in the 6 position as potential inhibitors or substrates of ADA.

We would like to acknowledge the following sources of error which may have altered our results. There was a fair amount of scatter in the data, especially at the low concentrations and in particular with N-6-cyclohexyladenosine. This may have been due to difficulty in detecting changes in absorbency at these low concentrations or difficulty in binding to the active site of the compound. However, our trials mostly fell within the domain of 0.100-1.00 required by the spectrophotometer to produce reliable data. Furthermore, our trials on average produced the anticipated linear regressions, suggesting that our methodology was accurate.

Previous studies have shown 2-chloroadenosine to be a rather powerful inhibitor in brain tissue while producing insignificant changes in heart, kidney, and liver tissue. In

general, it works strongly in the tissue of the nervous system because of purinergic receptors and negligibly in the peripheral system due to the lack of purinergic receptors [22]. The Adenosine used in our experiment was of bovine spleen tissue, which is a component of the latter, showing consistency between our results and those of past experimentations.

This study was broader in scope than it was detailed; this topic should be studied in more depth to attain irrefutable results. We suggest that anyone interested in continuing this research should run samples of adenosine with an analog at various concentrations to derive a more continuous analysis of the kinetics behind the deamination of adenosine and the inhibition of ADA. A research conducted by Dr. Daniel W. Miles implemented a technique called High-Performance Liquid Chromatography (HPLC). The practicality of this method lies in the fact that adenosine and inosine have very similar  $\lambda_{\max}$  and therefore a comparison of absorbencies via spectrophotometers becomes limited. An HPLC assay method would allow a researcher to analyze catalysis of adenosine or an analog by “quenching” or halting the reaction at different times and measuring the amount of substrate and product. While this process yields far more accurate results, it is vastly more time consuming, limiting its efficacy for our short research time [20].

## REFERENCES

1. Farabee, MJ. 2001 *REACTIONS & ENZYMES*: Available from:  
<<http://www.emc.maricopa.edu/faculty/farabee/biobk/BioBookEnzym.html>>  
Accessed 2006 August 6.
2. Berg, Jeremy M., J.L. Tymoczko, L. Stryer. *Biochemistry*, 5 ed. “Enzymes Accelerate Reactions” <<http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=stryer.section.1031>>  
Accessed 2006 August 6.
3. Sharp, D. “Enzymes” *rae.org* <<http://www.rae.org/enzymes.html>> Accessed 2006 August 8
4. Kaiser, G.E.  
<<http://student.ccbcmd.edu/~gkaiser/biotutorials/proteins/images/u4fg3a.jpg>>  
Accessed 2006 August 6.
5. Petkovic, M.  
<<http://www.eccentrix.com/members/chempics/Slike/Enzyme/1Inhibitors.jpg>>  
Accessed 2006 August 6.
6. *Enzyme Kinetics Chapter 3 - Noncompetitive/mixed inhibitors*:  
<[http://www-biol.paisley.ac.uk/kinetics/Chapter\\_3/chapter3\\_3.html](http://www-biol.paisley.ac.uk/kinetics/Chapter_3/chapter3_3.html)> Accessed 2006 August 6.
7. Seigel G.J, B.W. Agranoff, S.K. Fisher, R.W. Albers, M.D. Uhler. *Basic Neurochemistry*. <<http://www.ncbi.nlm.nih.gov/books/bv.fcgi?rid=bnchm>>  
Accessed: 2006 August 1
8. Chou, D.S. “Protective Mechanisms of Inosine in Platelet Activation and Cerebral Ischemic Damage” <<http://lib.bioinfo.pl/auth:Chou,DS>> Accessed: 2006 August 1
9. Martin F, E. Siaw, B.S. Mitchell, C.A. Koller, M.S. Coleman, J.J. Hutton . *ATP Depletion as a Consequence of Adenosine Deaminase Inhibition in Man*.



- Medical sciences: Proc. Natl. Acad. Sci. USA. Vol 77, No. 10, pp. 6157-6161  
October 1980
10. Hamed S., S. Hayatshahi, P. Abdolmaleki, S. Safarian, K. Khajeh .*Non-linear Quantitative Structure-Activity Relationship for Adenosine Derivatives a Competitive Inhibitors of Adenosine Deaminase.*  
Biochemical and Biophysical Research Communications. Vol.338 (2005) 1137-1142.
  11. Tang R, Ma C, Dong J, Liu X, Liu X. *Does Adenosine Deaminase Play a Key Role in Coronary Artery Disease.* Medical hypotheses 2006: 67(2): 371-4.
  12. Adair, TH. *Growth Regulation of the Vascular System: an Emerging Role for Adenosine.*  
American journal of physiology: regulatory, integrative, and comparative physiology. Aug 2005. 289 (2): R283-R296.
  13. *Lineweaver-Burk.*  
<<http://www.chm.davidson.edu/erstevens/Lineweaver/Lineweaver.html>>. Accessed: 2006 August 5.
  14. Campbell W.H. *Enzyme Kinetics Part 1.*  
< <http://www.bio.mtu.edu/campbell/401lec12.pdf>> Accessed: 2006 August 5
  15. *Carbodiimide Coupling using EDC.* <<http://chem.ch.huji.ac.il/~eugeniik/edc.htm>>  
Accessed 2006 July 31.
  16. Qian H. *A Guide for Michaelis-Menten Kinetics.*  
<<http://nsr.bioeng.washington.edu/Software/DEMO/MICMEN/>> Accessed: 2006 August 8.
  17. Reisberg P. *Caffeine*  
<<http://www.wellesley.edu/Chemistry/Chem101/caffeine/caffeine.html>> Accessed 2006 August 2.
  18. Wilson, David K; Rudolph, Frederick B; Quioco, Florante A. *Atomic Structure of Adenosine Deaminase Complexed with a Transition-State Analog: Understanding Catalysis and Immunodeficiency Mutations.*  
Science; May 31, 1991; 252, 5010; Research Library Core  
pg. 1278-1284
  19. Dr. Adam Cassano. Monday July 31 2006. Hall of Sciences Drew University.
  20. 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.fcbi?rid=cmed6>>  
Accessed 2006 August 9.
  21. Daniel W. Miles; Morris J. Robins; Roland K. Robins; Henry Eyring. *Circular Dichroism of Nucleoside Derivatives, VI. The Optically Active Bands of Adenine Nucleoside Derivatives*  
Proceedings of the National Academy of Sciences of the United States of America, Vol. 62, No. 1. (Jan. 15, 1969), pp. 22-29.  
<<http://links.jstor.org/sici?sici=0027-8424%2819690115%2962%3A1%3C22%3ACDONDV%3E2.0.CO%3B2-T>>  
Accessed: 2006 August 8.
  22. Williams M; E.A. Risley . *Biochemical Characterization of Putative Central Purinergic Receptors by Using 2-chloro[<sup>3</sup>H]adenosine, a Stable Analog of*

*Adenosine*. Proceedings of the National Academy of Sciences of the United States of America, Vol. 77, No. 11, [Part 2: Biological Sciences]. (Nov., 1980), pp. 6892-6896. < <http://links.jstor.org/sici?sici=0027-8424%28198011%2977%3A11%3C6892%3ABCOPCP%3E2.0.CO%3B2-D>> Accessed August 6 2006.