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CHARACTERIZATION OF ADENOSINE NUCLEOSIDASE FROM *LUPINUS LUTEUS L.*

Nancy Cooper McDonald

A dissertation presented to the
Graduate Faculty of Middle Tennessee State University
in partial fulfillment of the requirements
for the degree Doctor of Arts
December, 1998

Characterization of Adenosine Nucleosidase from *Lupinus luteus L.*

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ABSTRACT

Characterization of Adenosine Nucleosidase from *Lupinus luteus L.*

Nancy Cooper McDonald

Adenosine nucleosidase (EC 3.2.2.7), a key enzyme in metabolism of purines and plant growth regulators, catalyzes the irreversible hydrolysis of adenosine to yield adenine and ribose. Adenosine nucleosidase had previously been isolated by Abusamhadneh and obtained in a high state of purity from yellow lupin seeds after four days of germination. A final purification step using an ω-aminohexyl agarose column resulted in an enzyme of sufficient purity for further characterization of the enzyme.

Adenosine nucleosidase was characterized utilizing several experimental approaches. One method was the measurement of kinetic isotope effects (KIEs) using stable isotopes of adenosine with the label in the ribose moiety. Kinetic isotope effects determined for [1'-^{13}C] and [1'-^{2}H] adenosine using gas chromatography/mass spectrometry (GC/MS) were 1.012 ± 0.045 and 1.081 ± 0.028 respectively. These KIEs are the average of three line pairs at 158/159, 187/188, and 217/218. The low values may indicate kinetic suppression. The BEBOVIB-IV program was used to calculate kinetic isotope effects to match the experimentally determined values. These calculations indicated an early transition state characterized by substantial bond order to the C1'-N9 bond.

The data from isotope trapping experiments using the pulse-chase method indicated a very low ratio of product formed to substrate released. This low commitment to catalysis indicates that the observed isotope effects are intrinsic. Substrate-trapping showed an initial burst followed by a slower rate of product formation.

Certain structural features in the substrate are crucial to activity. Purines lacking an exocyclic nitrogen in the 6-position, a nitrogen in the 7-position, or a hydroxyl group in the C3' position are poor substrates or do not react. The best substrate was 5'-deoxyadenosine.

The hydrolytic reaction can be reversed to give an experimental equilibrium constant of 263 M. Hydrolysis is preferred over synthesis.

ACKNOWLEDGEMENTS

I would like to express my appreciation to Dr. Paul Kline for his patience, expertise, and guidance during the course of this investigation. Also I would like to thank Dr. Gale Clark for his assistance with the instrumentation.

Finally, I would like to thank Joe for his constant love and support.

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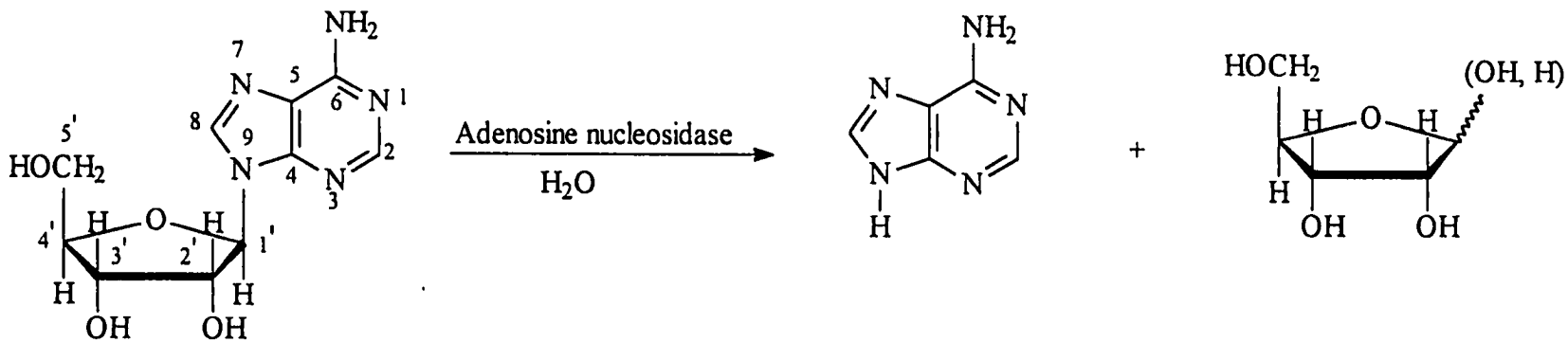
CHAPTER ONE

INTRODUCTION

Adenosine nucleosidase (EC 3.2.2.7) catalyzes the irreversible hydrolysis of adenosine to yield adenine and ribose. (1) (Fig. 1) Adenosine nucleosidase is a typical lytic enzyme. It acts in catabolizing tissue to mobilize adenine from cotyledons and to make this base available for transportation to, and reutilization in, the other organs of the growing seedling. It may also be possible that apart from its role in the purine salvage pathway, adenosine nucleosidase acts to quickly decompose the adenosine resulting from the nucleosidase and/or nonspecific phosphatase actions on AMP. It has been reported that perhaps at higher concentration, adenosine impairs the metabolism of plant cells. (1) Its effect on nucleolar morphology in animal cells has also been reported. (2) The nucleolus is a spherical body of the metabolic nucleus that is associated with a specific part of a chromosome and contains much ribosomal RNA. The nucleolus has three main components: a fibrillar component, a granular component, and nucleolar chromatin. In the normal nucleolus, the granular component and fibrillar material are often intermingled in rather coarse masses. As a result of the action of adenosine, the nucleolus swells, followed by segregation of the fibrillar material and the disappearance of most of the granular material. The swollen nucleolus becomes completely disorganized after prolonged treatment.

Figure 1: Hydrolysis of adenosine in the presence of adeosine nucleosidase yields adenine and ribose. It is not known if the initial ribose formed is the α or β anomer.

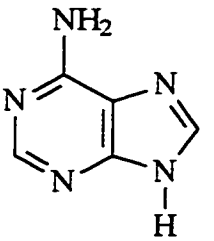


Adenosine Adenine Ribose

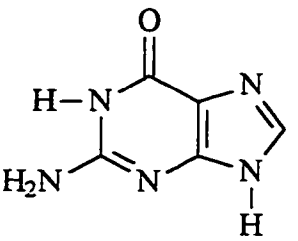
Purine derivatives constitute the most important group of compounds in all living organisms. (Fig. 2) They participate in an amazing array of biochemical reactions which range from energy metabolism to the transfer of biochemical specificity from parent to progeny. Purines may form nucleosides by a glycosyl condensation and nucleotides from the phosphate esters of the nucleosides. (3) Inosinic acid is the parent nucleotide in the sense that the other nucleotides are formed from it. (4) The degradation of purines leads in many plants to the formation of uric acid which is further degraded to form the ureides, allantoin and allantoic acid. Although not purines, the structural relationship of these two to the purines is obvious. (Fig. 3) In certain plants these ureides seem to play a role in storage and translocation. Adenine-8-^{14}C labels allantoic acid, allantoin, and urea when applied to leaves of *Acer saccharinum.* Hypoxanthine-8-^{14}C and glycine-2-^{14}C label allantoin when supplied to sterile root cultures of *Symphytum uplandicum* but little or no label is found in allantoic acid or urea. These results suggest glycine is incorporated into purine bases which are subsequently converted to allantoin.

Free nucleosides as well as free bases are considered as catabolic products of nucleic acids and are normally present in cells of higher plants in trace amounts. (3) Adenine is probably the most widespread followed by guanine. (5) Other purines and pyrimidines which are not components of nucleic acid have been found to occur free in some plants, although they are not widespread in the plant kingdom. (4) However, a number of methylated derivatives of purine, such as caffeine in tea and coffee and theobromine in cocoa beans, accumulate in considerable quantities in a number of plants.

Figure 2. Structures of purines that are components of all nucleic acids.

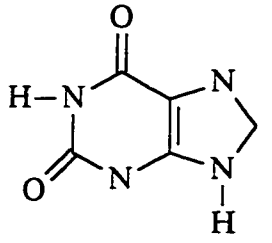


Adenine

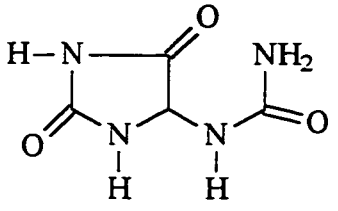


Guanine

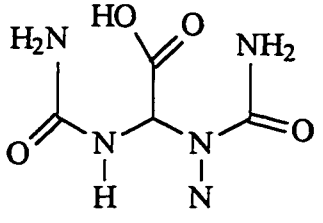
Figure 3: Degradation products of the purines.



Uric acid



Allantoin

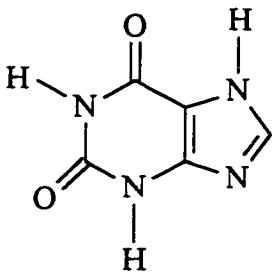


Allantoic acid

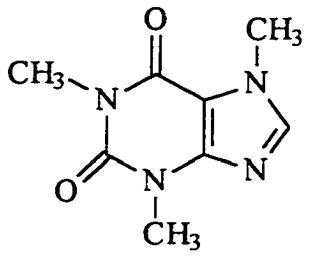
(Fig. 4) These compounds are derivatives of xanthine which also occurs in coffee beans. There are several nucleosides which also occur naturally in higher plants. Two of these are crotonoside from croton beans and vicine from vetch meal. On the other hand, a relatively large number of nucleosides have been isolated from fungi and sponges. Most of the ones isolated from fungi are antibiotics. The exact biological role which these nucleoside antibiotics play in organisms is yet unknown. Cordycepin and puromycin are among these purine-containing antibiotics.

The interconversion of purine ribotides, ribosides, and bases occurs through the action of a complex series of enzymes. (6) (Fig. 5) This scheme comprises data obtained from various plant species; therefore some details of the pathways may differ in different systematic groups. The set of enzymes involved in purine metabolism changes with the course of plant development. Some activities are not present in dry seeds and appear only in the developing seedlings. Adenosine nucleosidase plays a central role in purine metabolism. In addition to purines, many adenosine nucleosidases from a variety of plant sources also metabolize cytokinins. (Fig. 6) (7-9) Cytokinins are a group of phytohormones that are derivatives of an N-6 substituted adenine base and regulate cell division and differentiation. (10) The metabolism of cytokinins and unsubstituted purines such as adenosine are controlled by many of the same enzymes. Because of its central role in purine metabolism, adenosine nucleosidase is an attractive target for study, even though cytokinins are not a substrate for the enzyme isolated from *Lupinus luteus L.* that is the subject of this research. (11)

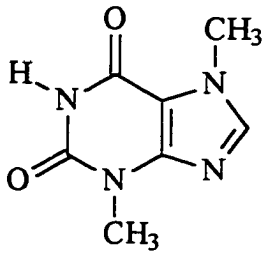
Figure 4. Some naturally occurring purines that are not components of nucleic acids



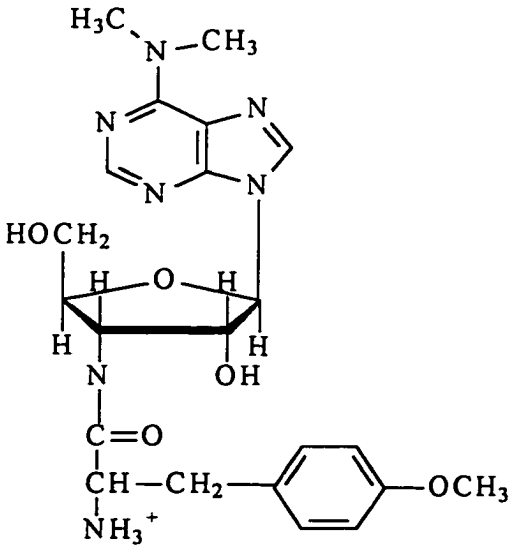
Xanthine



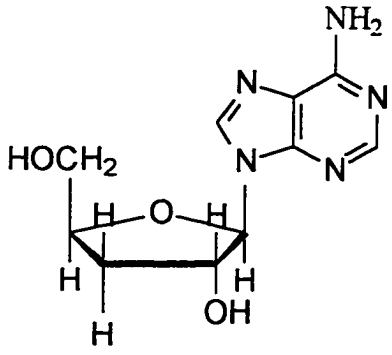
Caffeine



Theobromine



Puromycin



Cordycepin
3'-Deoxyadenosine

Figure 5. Purine salvage pathway in plants (Guranowski, A. *Plant Physiol.* **1982**, *70*, 344-349) (1) nonspecific acid phosphatase; (2) inosine nucleosidase; (3) xanthine dehydrogenase; (4) urate oxide; (5) guanine deaminase; (6) IMP dehydrogenase; (7) GMP synthetase; (8) adenylsuccinate synthetase; (9) adenylsuccinate lyase; (10) AMP deaminase; (11) adenylate kinase; (12) apyrase; **(13) adenosine nucleosidase;** (14) adenine deaminase; (15) adenosine kinase; (16) adenine phosphoribosyltransferase; (17) hypoxanthine-guanine phosphoribosyltransferase; (18) S-adenosylmethionine (SAM) synthetase; (19) methyltransferase; (20) S-adenosylhomocysteine (SAH) hydrolase; (21) S-adenosylmethionine decarboxylase; (22) aminopropyl transferase; (23) 5-methylthioadenosine (MTA) nucleosidase; (24) adenosine kinase

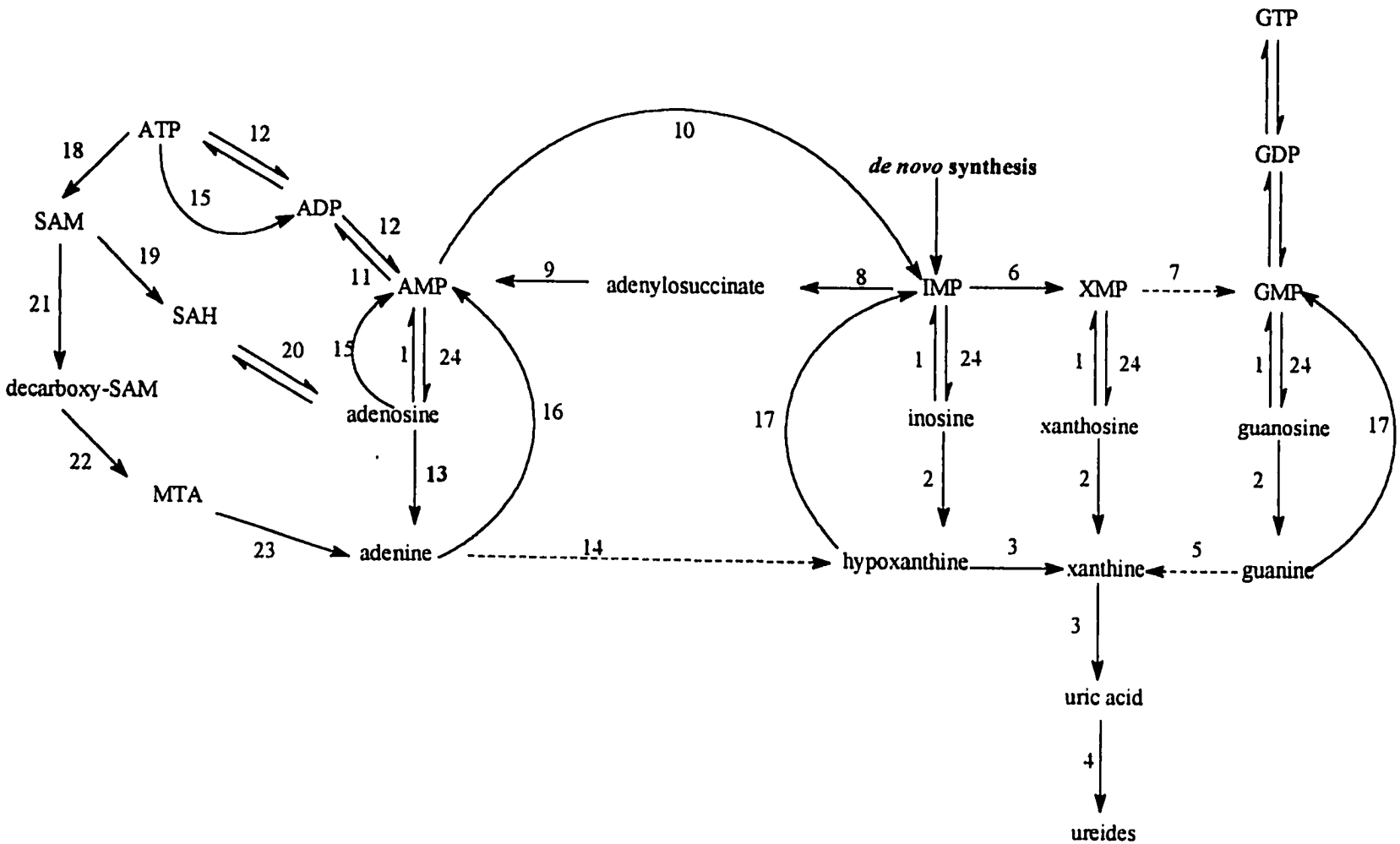
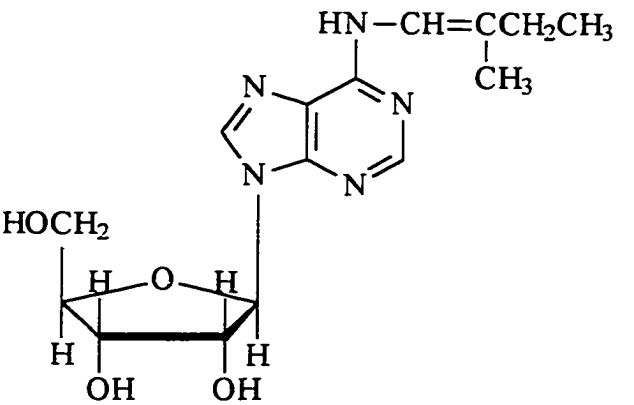
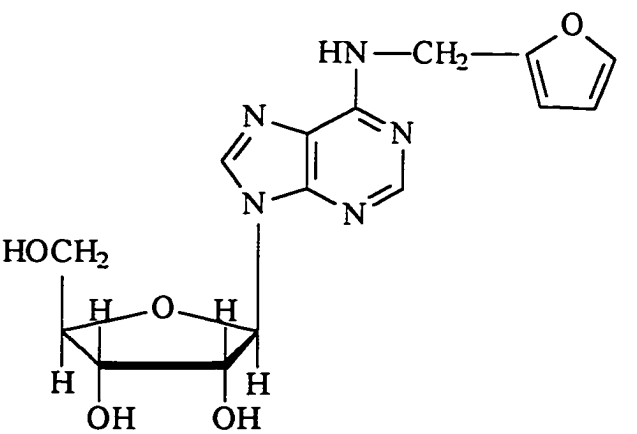


Figure 6. Some examples of cytokinins which are derivatives of adenosine.



Kinetin Riboside



Zeatin riboside

Leszczynska and coworkers have reported that four species of apple trees known for their high productivity, have levels of adenosine nucleosidase three times that of two wild species. (12) They also noted a similar difference in enzyme levels between wild and cultivated cherry trees. Work is continuing on the correlation, if any, between high productivity and adenosine reutilization.

Adenosine nucleosidase was first isolated by Miller and Evans from soy bean leaves (13), and its presence has since been reported in a variety of plants. (1, 7-9, 13-23). Adenosine nucleosidase has been isolated from spinach beet leaves by Poulton and Butt. (18) The enzyme had optimum activity at pH 4.5 and the K_m for adenosine was 11 μM. Comparable activity was also shown for adenosine N-oxide.

Guranowski and Schneider reported the isolation of adenosine nucleosidase from barley that showed pH optima of 4.7 and 5.4 with citrate and (N-morpholino)-ethanesulphonic acid (MES) buffers respectively. (19) The K_m for adenosine varied from 0.8 to 2.3 μM depending on temperature and buffer system. The enzyme also showed activity with 2'-deoxyadenosine as a substrate. The K_m for 2'-deoxyadenosine in MES buffer, pH 5.4, is much higher (120 μM) than for adenosine. Adenosine N-oxide and purine riboside were also substrates whereas cordycepin (3'-deoxyadenosine) was not hydrolyzed by the adenosine nucleosidase from barley leaves. The high level of the enzyme in seven day old seedlings, which still rely heavily on the limited energy and mineral supply from their seeds, suggests that adenosine nucleosidase in plants is a member of the purine (adenine) salvage pathway. The unusually high affinity of the

enzyme to the substrate found in two buffer systems ($K_m \approx 1$ μM) suggests very low concentrations of adenosine subject to enzymatic activity. The 3'-hydroxyl group of adenosine seems to be important for activity as evidenced by the lack of reactivity of cordycepin. The lack of a 2'-hydroxyl group in 2'-deoxyadenosine does not interfere with enzymatic activity.

Three adenosine nucleosidases were isolated from the extracts of tea leaves by Imagawa. (20) In the final purification steps (CM-cellulose and Sephadex G-100 column chromatography), three peaks having adenosine nucleosidase activity were detected and designated I, II, and III. The highest activity was observed at about pH 4.0 for enzymes I and III and at pH 4.5 for enzyme II. There was a remarkable similarity in the substrate specificity of the three adenosine nucleosidases. They hydrolyzed only adenosine, 2'-deoxyadenosine, and adenosine N-oxide. The cleavage rate of the N-glycosidic bond in 2'-deoxyadenosine was three or four times greater than that in adenosine. The hydrogens of the exocyclic amino group attached to the purine ring are necessary for hydrolysis by the enzyme, and the hydroxyl group at the 3'-position of the sugar is also indispensable. The absence of a hydroxyl group at the 2'-position increased the rate of hydrolysis.

Chen and Kristopeit reported the presence of an adenosine nucleosidase in wheat germ that showed activity for adenosine as well as for the cytokinin, N^6-(Δ^2-isopentenyl) adenosine. (7) K_m values of 1.43 μM for adenosine and 2.38 μM for the cytokinin were determined, and the pH optimum was 4.7.

Burch and Sturchbury reported the presence of two distinct forms of adenosine nucleosidase, R1 and R2, in tomato roots and one form, Lf, in tomato leaves. (22) R2 and Lf adenosine nucleosidase are very similar with a pH optimum of 6.0 and K_m for adenosine of 9 μM and 6 μM respectively. The R1 enzyme was found to have a pH optimum of 5.0 and K_m value for adenosine of 25 μM.

Guranowski and co-workers have detected activity for three different purine nucleosidases in yellow lupin seeds. (1, 6, 23) Two of these enzymes, inosine nucleosidase and 5'-methylthioadenosine nucleosidase, have been isolated, purified, and characterized. (6, 23) Adenosine nucleosidase activity was detected in the crude extract but the enzyme was not isolated. (1) Inosine nucleosidase activity is present both in dry seeds and seedlings. The pH optimum for inosine nucleosidase is 8.0. Purine riboside was a substrate for inosine nucleosidase but tubercidin, 7-deazaadenosine, was not. There is a strict requirement for the ribose moiety of inosine. Any change of substituents at the 2', 3', or 5' carbon of the nucleoside abolished its substrate property, whereas some alterations at the purine ring of inosine (at C-2, C-6, and C-8) were tolerated by the enzyme. (6) The second enzyme isolated was 5'-methylthioadenosine nucleosidase with a K_m of 0.41 μM for 5'-methylthioadenosine. Methylthioadenosine and its adenosyl analogs were better substrates than their deaminated (inosyl) counterparts. Again, the tubercidin moiety, 5'-methylthiotubercidin, was not a substrate, indicating the necessity of the nitrogen atom in the 7-position of the purine ring. (23) Adenosine nucleosidase activity

appeared only in cotyledons of two day seedlings reaching the highest activity on the fourth day of germination. (1)

Adenosine nucleosidase has recently been isolated and characterized by Abusamhadneh. (11) The enzyme was isolated from yellow lupin seeds four days after germination. The purification scheme included heat treatment, ammonium sulfate precipitation, ion exchange chromatography, and gel filtration chromatography. The apparent molecular weight was determined by G-200 Superdex gel filtration using coelution position of catalase, aldolase, chicken egg albumin, and myoglobin. The purified enzyme corresponded to a molecular weight of 177,000 daltons. In comparison, adenosine nucleosidases from other sources such as wheat germ (7), barley (19), tea leaves (20), tomato roots and leaves, (22) and tomato (9) have molecular weights of 59,000, 66,000, 68,000, 68,000, and 43,500 daltons respectively. Adenosine nucleosidase isolated from lupin seeds has approximately three times the molecular weight of these enzymes. The K_m for adenosine was 4.7 μM and V_{max} was 0.016 μmol/sec. The Michaelis constant from wheat germ was 1.43 μM (7), from barley leaves 0.8-2.3 μM (19), and from tomato leaves and roots, R_1 25 μM, R_2 9 μM and L_f 6 μM. (22) Using adenosine as a substrate and different buffers at different pH, the pH optimum was determined to be 7.5. This is in contrast to adenosine nucleosidases from other sources that showed pH optima in the range of 4.7 to 6.0. The substrates adenosine, 2'-deoxyadenosine, guanosine, cytidine, 6-benzyl amino purine riboside, inosine, and thymidine were tested for activity. Assigning a relative reactivity of 100 to

adenosine , 2'-deoxyadenosine at 65, guanosine at 27, and cytidine at 16 were the best substrates for adenosine nucleosidase. The exocyclic nitrogen, even in the absence of the purine ring, was shown to be essential to activity as evidenced by the lack of activity by the cytokinin, 6-benzyl amino purine riboside, and inosine. Cytodine, a pyrimidine with an exocyclic amino group was a better substrate than thymidine, a pyrimidine without the exocyclic amino group. 2'-Deoxyadenosine resulted in a 35% loss of activity showing that the ribose moiety is involved in binding the enzyme as well.

The aim of a purification procedure is to isolate the enzyme in a state of maximum possible purity with no other enzymes or large molecules. (24) Proteins are purified by fractionation procedures. In a series of independent steps, the various properties of the protein of interest are utilized to separate it progressively from other substances. The idea here is not necessarily to minimize the loss of the desired protein but rather to eliminate selectively the other components of the mixture such that only the desired substance remains. The operational criterion for establishing purity takes the form of the method of exhaustion: the demonstration, by all available methods, that the sample of interest consists of only one component. Experience has shown that when a sample of material previously thought to be pure is subjected to a new separation technique, it is occasionally found to be a mixture of several components. The development of an efficient procedure for the purification of a given protein is still an intellectually challenging and time consuming task.

Enzymes normally display highly specific interactions with their substrates and other ligands. Affinity separation methods take advantage of this specificity. (25) Many proteins have the ability to bind specific molecules tightly but non-covalently. This property can be used to purify such proteins by affinity chromatography. In this technique, a molecule known as a ligand, which is specifically bound by the protein of interest, is linked covalently to an inert matrix. The matrix must be chemically inert, have high porosity, and have large numbers of functional groups capable of forming covalent linkages to ligands. Of the few materials available that meet these criteria, agarose, which has numerous free hydroxyl groups, is by far, the most widely used. If the ligand has a primary amino group that is not essential for binding to the protein of interest, the ligand can be covalently linked to the agarose after treatment with cyanogen bromide. When a mixture is passed through this chromatographic material, the enzyme is retained and other enzymes and proteins are washed away. The desired protein can then be recovered by a pulse of substrate, which will compete for the binding sites on the enzyme, or by changing the pH or ionic strength in such a way that the protein is released from the chromatographic matrix. The great advantage of affinity chromatography is its ability to exploit the desired protein's unique biochemical properties rather than the small differences in physical and chemical properties between proteins that other chromatographic methods must utilize.

This simple method is, in principle, capable of purifying an enzyme in a single step from a crude extract. However, there are a number of problems associated with the

use of affinity chromatography in enzyme purifications. It is a difficult task to attach a suitable substrate analog to the matrix, and the reactions involved in coupling have not been completely characterized. Linking of the ligand to the matrix may interfere with the binding to the enzyme. For affinity chromatography to work successfully, the strength of the interaction between matrix-bound ligand and the enzyme must be in the correct range. If it is too weak, the enzyme will not be retarded by the column. If it is too strong, removal of the bound enzyme may require such harsh conditions that the enzyme is inactivated. Special problems are posed by enzymes that catalyze reactions involving more than one substrate. In spite of these problems, affinity chromatography has made a very significant contribution to the purification of enzymes.

In addition to the ligands which exploit the specificity of the enzyme, dye ligands have also been used successfully to purify a number of enzymes and proteins. The dye Cibracon Blue F3G-A was initially intended for use as a visible void volume marker for gel filtration, but it was soon discovered that the enzyme was binding to the dye and appearing in the void volume. When the dye marker was removed, the enzyme came out after the void volume. (26) The interactions between the dyes and proteins are complex and not well understood. The dyes generally contain aromatic rings, sometimes heterocyclic, often fused, and by definition of a dye, a long series of conjugated double bonds must be present to give a strong absorption of light in the visible spectrum. Additionally sulfonic acid groups are included to confer aqueous solubility; these groups

are negatively charged at all pH values above zero. Some dyes contain carboxylates, amino, chloride, or metal complexing groups. Most contain nitrogen both in and out of aromatic rings. Thus, interactions with proteins as a result of hydrophobic, electrostatic, and hydrogen bonding are all likely. There are many variables, pH, ionic strength, metal ions, and buffer composition that can influence the behavior of proteins on dye columns; and there are many different dye columns that have been developed since the early work on Cibracon Blue. Although the molecular basis for the apparent specificity has not been completely clarified, the interaction has proved to be a useful tool in protein purification. A Matrex™ Gel Red A column was used successfully in the final purification of nucleoside hydrolase from *Crithidia fasciculata.* (27)

Since enzyme activity is also affected by substances other than their substrates, which may include products, alternative substrates, substrate analogs, drugs, toxins, and allosteric effectors, it is necessary to map the active site by specificity studies. For a satisfactory investigation of the specificity of a given enzyme, the enzyme should be as pure as possible. The reference substrate, which is the most readily attacked biological substrate, should be identified and the optimum conditions worked out for it. The general procedure in investigating specificity is, having obtained an active substrate, to make small chemical modifications in every part of the molecule separately, and to determine the effect on affinity and reactivity. These compounds, called substrate analogs, are useful tools in specificity studies and may be used to determine structural requirements of the binding sites of enzymes. These compounds resemble the real substrate closely

enough to bind the active site but may or may not be chemically able to undergo reaction. The vast majority of specificity studies consist only of determination of the reaction velocity of the analog relative to that of the biological substrate using a series of analogs at constant concentration. From the results, it is possible to formulate the minimal structures necessary for combination and for reaction. (28)

A complete understanding of an enzyme requires knowledge of the chemical mechanism by which that enzyme carries out a reaction. The transition state conceptually is the most important geometry in characterizing the molecular distortions which lead to chemical reactions. (29) Recent advances in the analysis of enzymatic transition states make it possible to determine the structure of the transition state of an enzymatic reaction.

Although a number of kinetic probes are available for reactions in solution, application of these methods to enzyme systems is frequently limited by the conformational and specificity requirements of enzymes. (29) The use of kinetic isotope effects (KIEs) to determine transition state structures offers a unique perspective on enzymatic reactions. The substitution of hydrogen by a deuterium or tritium comes as close as possible to a non-interacting probe since vibrational force constants are independent of isotopic substitution. This fact together with the large number of enzyme systems which catalyze hydrogen abstraction reactions makes the application of primary hydrogen isotope effects to the study of enzyme transition state structure particularly attractive.

Steady state kinetics tell about the comings and goings of reactants during enzymatic turnover, but reveal little about events within the catalytic sequence. Transient-state kinetics are sensitive to catalytic events, but often the identity of the event detected is uncertain, and reference points on which to base quantitative analysis are lacking. Non-kinetic methods such as x-ray crystallography can reveal structural information about active sites of enzymes but fail to accurately portray the structures during catalysis because of their dependence upon static analogs. (30) While spectroscopic and crystallographic methods provide important information, it is information on the stable complexes which are next to the transition state on the reaction coordinate. Only kinetic isotope effects allow the experimental determination of the transition state geometry and provide a needed link between steady-state, transient-state, and transition-state processes.

The term isotope effect refers to the difference in a chemical or physical property between chemical species which differ only in their isotopic composition. Substitution of one isotope of an atom for another is the simplest and chemically most similar substitution that can be made in a chemical species. The resulting isotope effect reflects not only the change in relative mass, which is quite small except for the isotopes of hydrogen, but also the nature of the bonding to, and the chemical environment of, the labeled atom. Isotope effects are often small, of the order of a few per cent of the measured property value, the exception being substitution of hydrogen by deuterium or tritium. (31) This is to be expected since the masses of carbon-12 and carbon-14 differ by less than 17%,

whereas the mass of a deuterium atom is twice that of a hydrogen atom. Fortunately, modern analytical techniques allow determination of isotopically induced changes accurately and simply, even when these are of quite small magnitude. (32)

Chemical events susceptible to isotope effect study are changes in structural features. (33) These include:

1. alterations in bond angles at a reacting center.
2. the extension of a bond as it breaks.
3. the shortening of an inter-atomic distance as a bond forms.

The effect on vibrational zero-point energy emerges as the predominant source of isotope effects. Isotope effects can therefore be considered to be vibrational effects and to give structural information that is closely related to that obtainable by vibrational spectroscopy.

Rate isotope effects are expressed as ratios of specific rate constants. The ratio most often used is k/k* in which k is the reaction rate constant of the normal molecules, and k* is that of the isotopically substituted molecules. (32) A normal KIE is observed if the heavy substrate has a slower conversion to product, and an inverse KIE is observed if the heavy substrate has a faster conversion to product. The relative rates of product formation or substrate depletion are usually measured. (34)

Methods of determining KIEs may be divided into two types: those where radioisotopes are used and those where stable isotopes are used. In either case, the best

results are obtained when 15-50% of the substrate is converted to product. Following partial and complete conversion, the products must be completely separated from the substrate, and the method must provide complete recovery of the product. It is preferable that substrate bind to the column, and the product elutes completely. One method uses disposable charcoal columns with one column for each sample, although high-performance liquid chromatography (HPLC) and fast performance liquid chromatography (FPLC) have also been used successfully. Products from radio-labeled samples are counted in a multi-channel, liquid scintillation counter. The total ^{3}H and ^{14}C counts in product are calculated and used to establish $^{3}H/^{14}C$ ratios for the product near 30% conversion compared to ratios at 100% conversion. (33) The use of radio-labeled substrates to measure KIEs provides sufficient accuracy but is accompanied by some problems as well. Carbon-14 compounds must be synthesized and are very difficult and expensive to obtain. Carbon-14 is a weak beta emitter and has a relatively long half-life (5745 years), properties that necessitate caution in the use of radio-labeled compounds. Tritium, with a half-life of 12 years, is among the least toxic of the radio-nuclides because the low energy betas emitted cannot penetrate the outer dead layer of skin. The critical organ for tritium uptake is the whole body water. (35) The hazards of C-14 and tritium are negligible compared to most other radioisotopes, and precautions include preventing the radioactivity from entering the body through open wounds, by inhalation, or directly through the skin. At the level of radioactivity with which most laboratory work is done, C-14 and tritium compounds can be safely handled with rubber gloves. However, there is no lower limit of radioactivity,

or of transmutation of an element contained in living tissue, below which danger is considered to be impossible. (32)

Carbon-13 offers two practical advantages, cost and safety. It is a stable isotope and ^{13}C labeled compounds can be obtained at a relatively low cost at high enrichment. The disadvantage of carbon-13 is that the isotope effects are normally much smaller compared to carbon-14 which poses a problem in detection. Malonic acid is the classic example of an easily decarboxylated acid, and it was used in early work to compare the magnitudes of carbon-13 isotope effects with theoretical predictions and with the corresponding carbon-14 effect. Using malonic acid labeled in the carboxyl group, the rate ratio $^{12}C/^{14}C$ from the CO_2 evolved was found to be 1.12 ± 0.03 while the $^{12}C/^{13}C$ ratio was determined to be $1.019 \pm .001$. (36)

When an organic compound of carbon-13 is to be analyzed by mass spectrometry, the compound is usually degraded to a simpler gaseous compound of carbon, which is inserted in a mass spectrometer for determination of the $^{13}C/^{12}C$ ratio. Most often the simpler compound is carbon dioxide. In either case, an isotope-ratio mass spectrometer is the instrument preferred for the measurement of carbon-13. Carbon-13 isotope fractionation may occur when a carbon-13 compound is degraded for introduction into a mass spectrometer. If this fractionation occurs during the degradation, the carbon-13 concentration observed by the mass spectrometer is normally lower than the true concentration of carbon-13 in the compound being assayed. In most cases the magnitude of the error is difficult to assess. (32)

When the un-degraded compound is used, such isotope fractionation is avoided. While the experimental conditions and product separation are essentially the same for both radio-labeled and stable isotopes, analysis of the labeled/unlabeled ratio may be determined by the combination gas chromatography/mass spectrometry (GC/MS). (37)

Selective ion monitoring (SIM) and Scan are two ways of operating the GC/MS instrument. In the Scan mode, the entire spectrum is scanned, whereas in SIM, only a few selected ions are monitored. SIM mode is more sensitive than the scanning mode because more time is spent monitoring specific ionized fragments and accumulates a higher signal. This method utilizes multiple line pairs for isotope enrichment.

Single line pairs were previously used for calculating isotopic enrichment in ^{15}N-labeled amino acids. (38-40) Multiple line pair analysis has also been reported for measuring ^{15}N enrichment of amino acids. Multiple line pair analysis improves precision, allows evaluation of calibration curves for nonlinear behavior, and permits a degree of differentiation among the sites within an amino acid containing more than one nitrogen atom. (41) The integrated areas of each line pair are used to calculate the natural to heavy isotope ratio for the products and subsequently the KIE.

Rezaee has developed a method of measuring KIEs for the nucleoside hydrolase catalyzed hydrolysis of inosine to form hypoxanthine and ribose using substrates labeled with stable isotopes in conjunction with GC/MS. (42) A calibration curve of observed enrichment versus actual enrichment showed a linear relationship. The linearity of the calibration curve for both [1-^{13}C] and [1-^{2}H] ribose indicated that GC/MS is able to

respond to a small amount of sample, and that there is a predictable response in a peak based on changes in isotope composition.

This method gave results of sufficient accuracy to quantitate KIEs for [1'-^{13}C] and [1'-^{2}H] inosine. (42) Using inosine labeled in the ribose moiety, the product ribose was separated from the reaction mixture and converted to the high molecular weight compound, ribitol acetate. The labeled ribitol acetate was analyzed by GC/MS and the $^{12}C/^{13}C$ ratio was determined. The method was tested by comparing known KIEs of nucleoside hydrolase obtained from the trace label technique to those acquired by GC/MS. KIEs obtained for [1'-^{13}C] and [1'-^{2}H] inosine are 1.0320 ± 0.005 and 1.1140 ± 0.011 respectively. These KIEs are the average of three line pairs: 158/159, 187/188, and 217/218. KIEs for [1'-^{14}C] and [1'-^{3}H] obtained from trace label technique are 1.044 ± 0.004 and 1.150 ± 0.006 respectively. The close similarity between the data obtained by GC/MS using substrate labeled with stable isotopes, and the data obtained by the trace label technique provides sufficient evidence of accuracy of the GC/MS method.

Transition states are of interest for two reasons, one intellectual, the other practical. First, the intellectual motivation: the molecular structure of a transition state of a reaction is determined by the structural and electrostatic "pushes" needed to force a substrate over an energetic barrier toward conversion into the product. One view is that the transition state for a reaction is intrinsically determined by the molecular properties of the substrate and product, and that enzymes catalyze reactions by stabilizing transition states. Another view is that the micro-environment of an enzymatic active site not only

stabilizes transition states, but alters them. Very little experimental data exist to address the question of which view is correct.

The practical reason for being interested in transition state structures of enzymatic reactions is the fact that transition state analogs make very potent enzyme inhibitors and are important targets in drug design and discovery. To construct a transition state analog, the mechanism and transition state structure for the enzymatic reaction must be determined directly. As stated previously, the use of heavy atom isotope effects is a unique tool in determining transition state structures. Using computational methods, the heavy atom isotope effect data can be translated into a very detailed picture of the transition state of the reaction. (43) Ultimately this information can be used to design and synthesize potential transition state analogs which should be potent inhibitors of the enzyme.

A geometric model of the transition-state structure for the enzymatic hydrolysis of inosine by nucleoside hydrolase has been established by fitting a family of kinetic isotope effects in a vibrational analysis. The electrostatic potential surface of the geometric model was defined by molecular orbital calculations to detail the electronic nature of the transition state. (44) From this information, a transition state analog, 1(S)-phenyl-1,4-dideoxy-1,4-iminoribitol, which mimics the electrostatic features required for the nucleoside hydrolase transition-state, was synthesized and was shown to be a competitive inhibitor with respect to inosine. (45-46)

The transition-state for the hydrolysis of AMP by AMP deaminase has also been characterized by heavy atom kinetic isotope effects, and the electrostatic potential surface for the purine ring of the transition state has been determined by molecular orbital calculations. A comparison of the electrostatic potential surface for the purine ring of the transition state for AMP deaminase to that of the base of (R)-coformycin 5-monophosphate indicated a near match. The observed tight binding inhibition by (R)-coformycin analogs as transition state inhibitors results from the similarity of the partial charges on the inhibitors to that of the enzymatic transition state stabilized by AMP deaminase. (47)

Transition state geometry can be modeled using a bond energy, bond order vibrational analysis (BEBOVIB-IV) program available through the Quantum Chemistry Program Exchange. (48) The bond energy bond order vibrational approach to calculating kinetic isotope effects has been reviewed by Sims, Burton, and Lewis. (31) The program calculates a kinetic isotope effect given a ground state structure and proposed transition state structure. The ground state structure is taken from the crystal structure of the starting nucleoside. The transition state structure is varied systematically until the calculated isotope effects match the experimentally determined kinetic isotope effects. Different transition states can be generated by changing one or more structural features of the transition state. The principal variable of the transition state is its position along the reaction coordinate. For the purine hydrolases only, progress along the reaction coordinate is measured by the degree of reduction in the C1'-N9 bond order.

Transition state analysis is therefore a very powerful tool that promises to revolutionize science in many areas including the development of anti-bacterial drugs, green herbicides and the utilization of enzymes as synthetic machines. For example, the enzymes of aromatic amino acid synthesis are found only in plants making them ecologically sound targets for herbicides. Enzymes are also increasingly being used to manufacture drugs, since they can be used to produce chiral chemical precursors which are difficult to obtain in pure form by chemical synthesis.

CHAPTER TWO

MATERIALS AND METHODS

Equipment and Instrumentation

High performance liquid chromatography (HPLC) was carried out on a Hewlett Packard HP 1110 system equipped with diode array detector (DAD) and auto injector. Data collection and instrument control were performed using an HP Chemstation for LC Rev. A .04.01.

Ribitol acetate samples were analyzed on a Hewlett Packard (HP) 5890 capillary column gas chromatograph interfaced to an HP 5970 mass selective detector. Data collection and instrument control were performed using an HP 5890 MS chemstation (HP-series II).

Liquid scintillation counting was performed using a Wallac Winspectral™ 1414 Liquid Scintillation Counter.

Materials and Reagents

AMP sepharose, phenyl sepharose, GMP sepharose, ω-aminohexyl agarose, purine riboside, 5'-deoxyadenosine, cordycepin, and tubercidin were purchased from Sigma Chemical Co. Labeled [1'-^{13}C] adenosine was a gift from OMICRON Biochemical. Labeled [1'-^{2}H] adenosine was prepared according to the method of Kline and Serianni. (49) Labeled [2, 8, 5'-^{3}H] adenosine was purchased from NEN™ Life

Science Products, Inc. A Dyematrex™ Screening Kit containing the dye ligands Matrex Gel Blue A, Matrex Red A, Matrex Gel Orange A, Matrex Gel Green A, and Matrex Gel Blue B was purchased from Amicon. Norit™ decolorizing carbon, < 100 mesh, was purchased from Aldrich. Whatman cellulose CF11 was purchased from Fisher.

All solutions were prepared using purified water from a reagent grade water system (Modulab Polisher I HPLC, Continental Water Systems, Inc.). All other chemicals were reagent grade.

Assays for Enzyme Activity

Reducing Sugar Assay

Enzymatic activity was measured by HPLC or a reducing sugar assay. Catalytic activity was measured colorimetrically by the appearance of reducing sugar in a reaction mixture containing 1 mL of 1 mM substrate, which was usually adenosine, in Tris buffer pH 7.2. (50) The reaction was initiated by the addition of enzyme and incubated at 37 °C for one hour. The reaction was terminated by the addition of 300 μL copper reagent. The copper reagent consisted of 4% Na_2CO_3, 1.6% glycine, 0.045% $CuSO_4 \cdot 5H_2O$ in 400 mL distilled water. Color was developed by the addition of 300 μL neocuproine solution. The neocuproine solution consisted of 0.12% neocuproine (2,9-dimethyl-1-10-phenanthroline HCl) dissolved in 400 mL of distilled water with the pH adjusted to 3.0 with concentrated HCl. After addition of the reagents, the reaction mixture was incubated for seven minutes in boiling water, and the optical density was

measured at 450 nm. Reducing sugar concentration was determined by comparison to a standard curve constructed using ribose. This method works best when the enzyme has been partially purified, since most of the interfering substances have been removed by this point.

HPLC Assay

Activity was also measured by the appearance of a peak in the HPLC chromatogram for adenine along with the disappearance of the peak for adenosine. The reaction was initiated by the addition of the enzyme to 1 mL of 1 mM substrate, usually adenosine, in 50 mM Tris buffer pH 7.2. The samples (5 μL injections) were analyzed by reverse phase chromatography on a C_{18} column (100 x 2.1 mm) and eluted with 5% methanol in water at 0.350 mL/min flow rate. Retention times for adenine and adenosine were 3.30 and 9.02 minutes respectively. The presence of the purine ring was detected by absorbance at 254 nm using a photodiode array detector.

Preparation of Enzyme Extract

Adenosine nucleosidase activity was found to be present in four day old seedling cotyledons of *Lupinus luteus L.* (yellow lupin). The cotyledons were grown on moistened paper towels at room temperature and under natural light conditions. The cotyledons were disrupted in 50 mM HEPES buffer at pH 7.2 using a blender. The crude mixture was centrifuged, and activity was determined using fractions of the supernatant. The reaction mixture included 100 μL of 10 mM adenosine, 10, 20, or 40 μL of the crude

enzyme extract, 50 μL of 1 M Hepes buffer pH 7.2, and distilled water to make the final volume to 1 mL. The time was recorded upon addition of the enzyme extract. Adenosine nucleosidase activity was determined by the appearance of a peak in the HPLC chromatogram for adenine along with the disappearance of the peak for adenosine.

Final Purification Step of Adenosine Nucleosidase

Adenosine nucleosidase was purified using a modification of the method developed by Abusamhadneh. (11) The purification scheme included heat treatment, ammonium sulfate precipitation, ion exchange chromatography, and gel chromatography. These steps resulted in a protein of high purity, and some of the physical properties were characterized. (11) A final purification step was necessary for the study of kinetic isotope effects. A series of columns which have been used successfully to purify many proteins were tried to effect the separation of the adenosine nucleosidase from the few remaining proteins. (26)

Dyematrex™ Screening Kit

The kit contains five dye-ligand columns (9 x 32 mm), Matrex Gel Blue A, Red A, Orange A, Green A, and Blue B. Since very slow hydrolysis of the dye-agarose linkage occurs during storage of the columns, a regeneration step is necessary to remove the free dye as well as any tightly bound or precipitated protein remaining from previous runs. Twelve mL of 8 M urea were applied to the column and allowed to drain. The column was allowed to sit overnight with the bottom cap on and some 8 M urea solution above

the top frit. A second 12 mL were added and allowed to drain the following morning. While this step can be carried out at room temperature, the subsequent steps must be carried out in the cold room (4-8 °C). The upper reservoir and extension funnel were rinsed with buffer to remove residual regeneration solution. In the equilibration step, 12 mL of 20 mM Tris buffer pH 7.5 were applied to the column and allowed to drain. The sample, 20 μL of enzyme made to a final volume of 100 μL in 20 mM Tris pH 7.5, was applied to the column and allowed to drain. An additional 100 μL of the starting buffer was applied to the column and allowed to drain, and a no-flow time of 30 minutes allowed binding equilibration which otherwise would not occur due to the high column flow rate. Following this no-flow period, 10 mL of the 20 mM Tris pH 7.5 was applied to wash the column, and 1 mL fractions were collected. The column was eluted with 10 mL of 1.5 M KCl in 20 mM Tris pH 7.5, and 1mL fractions were collected. Elution time for each column was approximately 1.5 hours. A reducing sugar assay was done using a reaction mixture that contained 200 μL of each fraction in a final volume of 1 mL of 1 mM adenosine in 50 mM Tris buffer pH 7.5. Each of the five dye-ligand columns was tested in this manner.

Inosine Sepharose

Although adenosine is the best substrate for this enzyme, activity was also found for inosine and guanosine. Attempts were made to separate the enzyme on an Inosine Sepharose column. An Inosine Sepharose matrix was prepared from AMP Sepharose by suspending the gel in 50 mM Tris buffer pH 7.5 and 1mM $MgCl_2$ containing alkaline

phosphatase and incubating overnight in a micro-centrifuge tube to remove the phosphate group. Adenosine deaminase was added to convert the adenosine to inosine. These reagents were removed from the gel by centrifugation, and the resulting Inosine Sepharose was re-suspended in 50 mM Tris pH 7.5. A sample containing 10 μL of the enzyme was applied to this matrix on a 3 x 15 mm column, washed with 10 mL of 50 mM Tris buffer pH 7.5, and eluted with 10 mL of 1 mM adenosine. One mL fractions of each were collected, and 100 μL samples of each fraction were assayed for activity using the reducing sugar assay. The AMP was attached to the matrix at the ribose hydroxyls with an 11 atom spacer.

Adenosine Sepharose

An Adenosine Sepharose matrix was prepared from AMP Sepharose by treating the gel overnight with alkaline phosphatase in 1 mM $MgCl_2$. The gel was removed from the conversion mixture by centrifugation, washed and re-suspended in 50 mM Tris pH 7.5. A 2.5 μL sample of the enzyme was added directly to the suspended Adenosine Sepharose in the micro-centrifuge tube. Fractions of 250 μL were removed at one hour intervals and assayed for activity using the reducing sugar assay.

Guanosine Sepharose

GMP resin was treated in the same way as the AMP resin to remove the phosphate group and convert it to the Guanosine Sepharose. A stock solution of 5 μL enzyme in 1995 μL of 50 mM Tris pH 7.5 was made up, and 1 mL was applied to the resin in the micro-centrifuge tube and left for thirty minutes. The other mL of the stock solution

served as the control. A 200 μL fraction of both the resin treated enzyme and the control were added to 1 mL of 1 mM adenosine in 50 mM HEPES pH 7.5 and assayed using the HPLC. The GMP was also connected to the matrix by the ribose hydroxyls with an 11 atom spacer.

Phenyl Sepharose

A sample containing 2.5 μL of enzyme in 100 μL of 1 M NaCl was loaded on a 3 x 15 mm Phenyl Sepharose column. The column was washed with 10 mL of 50 mM Tris pH 7.5, and eluted with 10 mL of 1 M NaCl. One mL fractions were collected , and 100 μL samples of each fraction were assayed for activity using the reducing sugar assay. The phenyl group was attached to the matrix by an amino group with a 12 atom spacer.

ω-Aminohexyl Agarose

The enzyme was loaded onto an ω-aminohexyl agarose column, 3 x 60 mm, washed with 20 mL of 50 mM Tris pH 7.5 and eluted with a salt gradient from 300 mM NaCl to 1 M NaCl. From each 3 mL sample collected, 100 μL fractions were assayed using the reducing sugar assay. The enzyme bound to this column and was eluted with 300 mM NaCl. It was determined that this size column could bind 25 μL of the enzyme which has a concentration of 0.59 μg protein/μL. The ω-aminohexyl group was attached to the matrix with a 12 atom spacer.

Kinetic Isotope Experiments

For each kinetic isotope experiment, two reactions were run, one to 20-30% completion and the other to 100% completion. The 100% reaction provides the control

ratio of $^{12}C/^{13}C$ or $^{1}H/^{2}H$, and the 20-30% reaction provides the $^{12}C/^{13}C$ or $^{1}H/^{2}H$ ratio changed by the presence of a kinetic isotope effect. The experiment consisted of the following steps:

1. 10 mL of 1 mM adenosine solution in 50 mM Tris pH 7.5 were made using approximately a 50:50 ratio of labeled and unlabeled adenosine.
2. two separate samples of 2 mL were taken.
3. the reactions were initiated by the addition of 100 μL of enzyme.
4. the reactions were monitored by HPLC.
5. one reaction was stopped at approximately 30% conversion by submerging it in boiling water for five minutes.
6. the other reaction was allowed to go to 100% completion after which it was terminated by submerging it in boiling water for five minutes.
7. both reaction mixtures were chromatographed on separate charcoal columns to separate the product ribose from adenine and unreacted adenosine. The columns were eluted with 10 mL distilled water.
8. the eluate from each column was evaporated to dryness.
9. 100 μL of water was added to each dried sample.
10. ribose was derivatized to form ribitol acetate.
11. derivatized samples were analyzed by GC/MS.

Preparation of Ribitol Acetate

Ribose was reduced to ribitol, and its hydroxyl groups were acetylated. The

reduction was carried out by the addition of 1 mL of a $NaBH_4$ solution (1g of $NaBH_4$ dissolved in 50 mL of dimethyl sulfoxide at 100 °C) to the ribose from the enzymatic reaction dissolved in 100 μL of water and incubating it for 90 minutes at 40 °C. After reduction, the excess $NaBH_4$ was decomposed by the addition of 100 μL of glacial acetic acid. The ribitol was peracetylated according to the following procedure: (51)

1. 200 μL of 1-methylimidazole and 2 mL of acetic anhydride were added to the reaction mixture and stirred for 10 minutes at room temperature.
2. 5 mL of water were added to the reaction mixture to decompose the excess acetic anhydride.
3. when cool, 1 mL of methylene chloride was added and the reaction mixture was vortexed.
4. the bottom organic layer containing the ribitol acetate was removed and analyzed by GC/MS.

Gas Chromatography/Mass Spectrometry

The ribitol acetate samples were analyzed by Hewlett-Packard (HP) capillary gas chromatography. The gas chromatograph was operated at a temperature of 150 °C which was increased 10 °C/min to a final temperature of 250 °C. The injection port temperature was 300 °C. The injection volume was 1.5 μL. The split ratio was approximately 40:1, and the flow rate was 1.58 mL/min.

Determination of Isotopic Enrichment

The derivatized ribose samples were analyzed using the SIM mode set on line

pairs, m/z 158/159, 187/188, and 217/218. Extracted ion chromatograms (EIC) were prepared and integrated. This procedure minimizes potential errors in the enrichment calculations by minimizing the random contribution of the baseline to the ions monitored. The integrated peak areas for the selected ions were used to calculate the true per cent isotope enrichment, E, according to equation 1. (41)

$$E = \frac{I_{(P+1)} - I_P * f}{I_{(P+1)} - I_P * f + I_P} \qquad (1)$$

where f is a correction factor used to correct for the natural abundance of the isotopes. The correction factor, f, is calculated from the ratio of the integrated areas of P+1 line to the P line. An unlabeled standard of ribitol acetate was analyzed to obtain these data.

Calculation of Kinetic Isotope Effects

Kinetic isotope effects were measured by analyzing triplicate samples removed from the common reaction mixture containing labeled and unlabeled substrate. One set came from the 20-30% hydrolysis and the other from the 100% hydrolysis. The general equation for the kinetic isotope effect is: (52)

$$\text{observed isotope effect} = \frac{\dfrac{\text{natural isotope (20-30\% hydrolysis)}}{\text{heavy isotope}}}{\dfrac{\text{natural isotope (100\% hydrolysis)}}{\text{heavy isotope}}} \qquad (2)$$

The observed isotope effects are corrected to 0% hydrolysis according to equation 3. (52)

$$\text{actual isotope effect} = \frac{\ln[1-(\text{fraction hydrolyzed})*(\text{observed isotope effect})]}{\ln[1-\text{fraction hydrolyzed}]} \quad (3)$$

The fraction hydrolyzed is determined from integration of the adenine/adenosine peak areas of the HPLC chromatogram.

Commitment to Catalysis

Isotope trapping experiments were performed using the pulse-chase method described by Rose. (53) A stock solution of 1.5 μL labeled [2, 8, 5'-^{3}H] adenosine in 30 μL Tris pH 7.5 buffer was made up. One μL was added to 15 mL of scintillation cocktail and counted on the scintillation counter to determine the actual counts in each reaction. The remainder of the stock solution was divided among three reactions. In the first experiment, the pulse-chase, 20 μL of enzyme was added to 10 μL of the stock solution of labeled adenosine. After 10 s, 1 mL of the chase solution, 5 mM unlabeled adenosine, was added so that most of the radioactivity in the product ribose would be derived from any labeled adenosine held to the enzyme and only a small amount would come from turnover of the enzyme in the steady state that follows the completion of the reaction mixture. The reaction was terminated after one minute by the addition of 100 μL 1 M HCl. The product ribose was separated from the reaction mixture by charcoal column chromatography eluted with 10 mL of distilled water. One ml fractions were collected,

15 mL of scintillation fluid were added, and the fractions were analyzed for radioactivity by scintillation counting.

Control experiments were used to correct for the fraction of total adenosine reacted following the addition of the chase solution. This fraction was determined by including the labeled adenosine in the 1 mL chase solution before the enzyme was added. The reaction was terminated after 1 minute by the addition of 100 μL 1 M HCl and analyzed as above.

The other control experiment used 10 μL of the stock solution of labeled adenosine to which 20 μL of enzyme was added. The reaction was terminated after 10 s and the radioactivity analyzed. The amount of ribose formed in the control experiments was subtracted from the ribose formed in the pulse-chase experiment and the commitment to catalysis was calculated. In most enzyme catalyzed reactions, the second substrate is added in varying concentrations in the pulse reaction. For example, purine nucleoside phosphorylase catalyzes the arsenolysis of inosine to form hypoxanthine and ribose 1-arsenate, which spontaneously hydrolyzes to ribose and arsenate. (54) The concentration of the second substrate, arsenate, can be varied and extrapolated back to zero concentration. The second substrate in this reaction is water and its concentration cannot be varied, therefore the experiment had to be modified.

Substrate Trapping

A sample of 10 μL of the adenosine nucleosidase was added to 0.5 μL of [2, 8, 5'-^{3}H] adenosine in a micro-centrifuge tube. At approximately 10 s intervals, 2 μL

aliquots were removed and added to 50 μL of 1 M HCl to kill the reaction. These samples were applied to a charcoal/cellulose column and eluted with 10 mL distilled water. Three mL fractions were collected, 15 mL of scintillation fluid were added to each fraction, and the radioactivity analyzed on the scintillation counter. A graph of concentration of ribose in counts per minute (CPM) versus time was constructed to determine if the chemistry or product release from the enzyme was the rate-limiting step.

Substrate Specificity

The relative velocity for adenosine analogs was measured by determining the adenine or adenine analog released using HPLC monitoring. The reaction mixture consisted of 1 mL of 1 mM adenosine analog in 50 mM Tris pH 7.2 to which 100 μL of the adenosine nucleosidase containing 0.59 μg protein/μL was added to initiate the reaction. Analogs assayed were purine riboside, 5'-deoxyadenosine, cordycepin (3'-deoxyadenosine) , and tubercidin (7-deazaadenosine). Reaction velocities are expressed relative to adenosine (100%). An HPLC chromatogram of the adenosine analog was taken just prior to initiating the reaction, and another HPLC chromatogram of the same unreacted substrate was taken five days later to determine if any chemical hydrolysis of the substrate was occurring.

Equlibrium Constant

The equilibrium constant was determined by the equilibrium concentration of adenosine in the presence of relatively high concentrations of adenine and ribose according to the method of Parkin and Horenstein. (27) Four reaction mixtures were made up

according to Table I. The reaction was initiated by the addition of 50 μL of the adenosine nucleosidase. No enzyme was added to the control. The reaction mixtures were incubated at 37 °C for 24 hours at which time they had reached equilibrium. The reaction mixtures were then analyzed by HPLC for the appearance of adenosine.

Table I. Reaction Conditions for Determination of K_{eq} for Adenosine Nucleosidase

Sample	10 mM Adenine	Ribose	50 mM Tris	Enzyme
1.	500 μL	0.15 g	450 μL	50 μL
2.	500 μL	0.30 g	450 μL	50 μL
3.	500 μL	0.45 g	450 μL	50 μL
4. (control)	500 μL	0.30 g	500 μL	

BEBOVIB-IV Calculations

The BEBOVIB-IV program (Quantum Chemistry Program Exchange, No. 337) was used to model transition-state geometries which would provide kinetic isotope effects to match the experimentally determined values. Due to limitations on the maximum number of atoms employed in a BEBOVIB model, only the relevant atoms for the transition state structure were used. The constants for inosine were used in these calculations since the cut-off atoms in the purine ring of adenosine and inosine are identical. The cut-off model included all carbons and nitrogens of the ribosyl and

imidazole portions of inosine as well as all other atoms α and β to the C-N glycosidic bond. The incoming nucleophile, water, was modeled as an oxygen atom 180° from the breaking C1'-N9 bond. The attacking nucleophile was assigned a bond order value corresponding to its distance from the adenosine molecule, which was more than a bond length away. Bond orders were varied systematically for the C1'-N9 bond and the C1'-O4' bond while holding the bond order for the attacking nucleophile constant. The reaction coordinate was generated by coupling the stretching motion of the C1'-N9 bond with the forming C1'-O bond from the attacking oxygen. (54) KIEs were then calculated for each bond order, and a graph of bond order versus KIE was plotted.

CHAPTER THREE

RESULTS AND DISCUSSION

Final Purification Step

Adenosine nucleosidase activity was determined in the initial crude extract of the 4-day cotyledons by HPLC assay. The adenine concentration increased with time, and a change in enzyme concentration caused an increase in reaction rate. Activity was also found for guanosine and inosine.

While adenosine nucleosidase has been purified from *Lupinus luteus L.* to a high degree of purity, the electrophoresis gel showed that there is still about 5% of contaminating protein. (11) Affinity chromatography was used for the final purification step, and a number of different ligands were tried. A large number of affinity chromatography adsorbents based on group specific ligands coupled to a variety of carrier matrices are commercially available. Two of the most widely used adsorbents are 5'-AMP agarose and the biomimetic textile dye Cibracron Blue F3G-A coupled to agarose. The blue dye ligand is an analog of adenylyl-containing cofactors. Consequently the adsorbent can be used to purify a very wide range of enzymes and has been used for the isolation of several quite disparate proteins. A red dye, Procion Red HE-3B coupled to Sepharose has been used for the purification of a variety of $NADP^+$ dependent enzymes and a number of unrelated proteins suggesting that binding may depend not only on specific steric factors

but also on ionic and hydrophobic interactions. (55) Dye ligand chromatography has been used for many large scale purifications. In the large scale purification of glycerokinase from *Bacillus stearothermophilus*, a final step of dye affinity chromatography replaced three steps in the conventional small scale purification and gave excellent recovery of homogenous enzyme. (56) Procion Red HE3B (Matrex Gel Red A, Amicon) was unusually effective for isolation of nucleoside hydrolase from *Crithidia fasciculata.* The purification following the Red A columns resulted in enzyme which was typically >95% pure. (27) Based on this information, the following ligands were determined to be good candidates for further purification of adenosine nucleosidase.

Dyematrex™ Screening Kit

Most work to date has been done using the Blue A and the Red A dyes, but three new dye ligands, Orange A, Green A, and Blue B, have been introduced. Screening was done using the full kit, since it is still not possible to predict which, if any, ligand will be best for a particular protein. The enzyme was applied to each of the 5 different dye ligand columns (9 x 32 mm). The column was washed with 10 mL of 20 mM Tris pH 7.5 and eluted with 10 mL of 1.5 M KCl in 20 mM Tris pH 7.5. Ten one mL fractions were collected from both the buffer wash and the KCl elution. Even though dye-ligand chromatography has been used successfully in the purification of over two hundred enzymes and other proteins, the adenosine nucleosidase did not bind to any of these columns. Using the reducing sugar assay, all of the activity was found in the first fraction of the wash with Tris buffer.

Affinity chromatography describes the interaction between a protein's natural ligand binding site and the actual ligand itself. The enzyme shows specific activity for adenosine, but also shows lower activity for the nucleosides inosine and guanosine as well. If the enzyme binds to a ligand for which it has a low affinity, it can frequently be eluted with a much milder eluting agent preventing possible inactivation of the protein by high ionic strength of the eluant. Trials were carried out using a series of columns with various nucleosides as ligands.

Inosine Sepharose

The enzyme shows some activity for inosine as a substrate, so a trial was made using the Inosine Sepharose resin. A sample of 10 μL of the enzyme was applied to a column containing 1ml of Inosine Sepharose, washed with 10 mL of 50 mM Tris pH 7.5 and eluted with 10 mL of 1 mM adenosine. The reducing sugar assay again showed that all of the activity was found in the first fractions of the Tris buffer wash.

Adenosine Sepharose

Since we had such a small amount of the Adenosine Sepharose resin, 2.5 μL of the enzyme was applied directly to the ligand which was suspended in 1 mL of 50 mM Tris pH 7.5 in a micro-centrifuge tube. Fractions of 250 μL were removed at one hour intervals and assayed using the reducing sugar assay. Even though adenosine is the best substrate for the adenosine nucleosidase, an Adenosine Sepharose resin failed to bind the enzyme. This may be attributed in part to the fact that the adenosine is connected to the matrix by the ribosyl hydroxyls. The 2' and 3' hydroxyl groups, particularly the 3', have

been shown by substrate specificity studies to be essential for activity by the enzyme. However, the length of the spacer that attaches the ligand to the matrix is also important. If the spacer is not the appropriate length, the enzyme may not have enough space to fit around the ligand. No significant differences in the reducing sugar assays were noted for the resin-treated enzyme and the control.

Guanosine Sepharose

Again, because of the limited amount of column material, a batch rather than a column was prepared to determine if the enzyme would bind. A stock solution of 5 μL of the enzyme in 1995 μL of 50 mM Tris pH 7.5 was made up, and 1 mL of this solution was applied directly to the resin in a micro-centrifuge tube. The other 1 mL served as the control. After 30 minutes, 200 μL fractions of both the resin-treated enzyme and the control were assayed using the HPLC. Guanosine is also a substrate for the enzyme, but no significant difference in the HPLC chromatograms were noted for the resin-treated enzyme and the control.

The nucleoside ligands were not successful in binding the enzyme even though adenosine is the preferred substrate, and inosine and guanosine show some activity. Ligand binding is determined by the affinity of the protein to certain structural features of the ligand and by the length of the spacer attaching the ligand to the matrix. Specificity studies have shown that the exocyclic amino group on the purine, the hydroxyl groups in the 2' and 3' positions on ribose, and the lack of a hydroxyl group in the 5' position are necessary for enzyme activity. In the case of the nucleoside ligands, either the exocyclic

amino group in the 6-position was missing, as in inosine and guanosine, or the ribose hydroxyls were involved in linking the nucleoside to the matrix as was the case for all three ligands tested. Two possible explanations exist here. A group crucial to binding by the active site on the enzyme is not available or the distance between the matrix and ligand is too small to accommodate the binding. Hydrophobic columns were then tested to explore the possibility of a hydrophobic binding site.

Phenyl Sepharose

A sample of 2.5 μL of the enzyme in 100 μL of 1M NaCl was applied to the column followed by washing with 10 mL of 50 mM Tris pH 7.5 and elution with 1 M NaCl. One ml fractions were collected and assayed using the reducing sugar assay. No binding occurred since all activity was found in the first fraction of the Tris buffer wash.

ω-Aminohexyl Agarose

A 25 μL sample of the enzyme was loaded onto an ω-aminohexyl agarose column, washed with 20 mL of 50 mM Tris pH 7.5 and eluted with a salt gradient from 300 mM NaCl to 1 M NaCl. This column was found to bind the enzyme which was then eluted with 300 mM NaCl. Activity was measured both with the reducing sugar assay and HPLC. Fractions containing activity were dialysed against 50 mM Tris pH 7.5 followed by concentration on a collodion bag. Electrophoresis in polyacrylamide gels containing sodium dodecylsulfate showed a single band using silver nitrate staining procedure. The apparent molecular weight of the sub-unit was approximately 36,000 daltons. The specific activity was 6.16 μmol/min/mg.

The interactions between ligands and proteins are complex and not well understood when the ligand and the bound enzyme do not resemble each other chemically as in this case. However, the ω-aminohexyl ligand has the required free amino group. 5'-Deoxyadenosine also has been shown to be a much better substrate for the enzyme than adenosine. The replacement of the polar hydroxyl group with a non-polar group may indicate some hydrophobic point that is necessary for binding. It may be speculated that the combination of the terminal amino group and accessibility of the aliphatic side chain may explain the success of this particular column and the failure of the Phenyl Sepharose column with no amino group and an aromatic side chain.

Kinetic Isotope Effects

Derivatization of Ribose

Kinetic isotope effects of adenosine nucleosidase were calculated by measuring the ratio of stable isotopes in products using GC/MS. The product ribose had to be isolated from the reaction mixture and converted to ribitol acetate, a volatile derivative suitable for gas chromatography. (Fig. 7) The flow chart for the synthesis of ribitol acetate is shown in Figure 8 and was carried out according to the procedure of Blakeney and co-workers. (51) This procedure converted the ribose to a compound volatile enough to be detected by GC/MS and also to the open chain form which yields a single signal, eliminating signals for the α and β anomers of ribose. The ribitol acetate samples were analyzed under SIM

Figure 7. The structure of ribitol acetate with molecular weight 362. The * represents the labeled hydrogen atom and the # represents the labeled carbon atom.

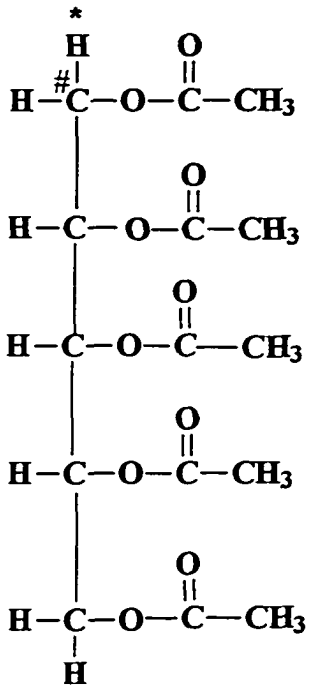
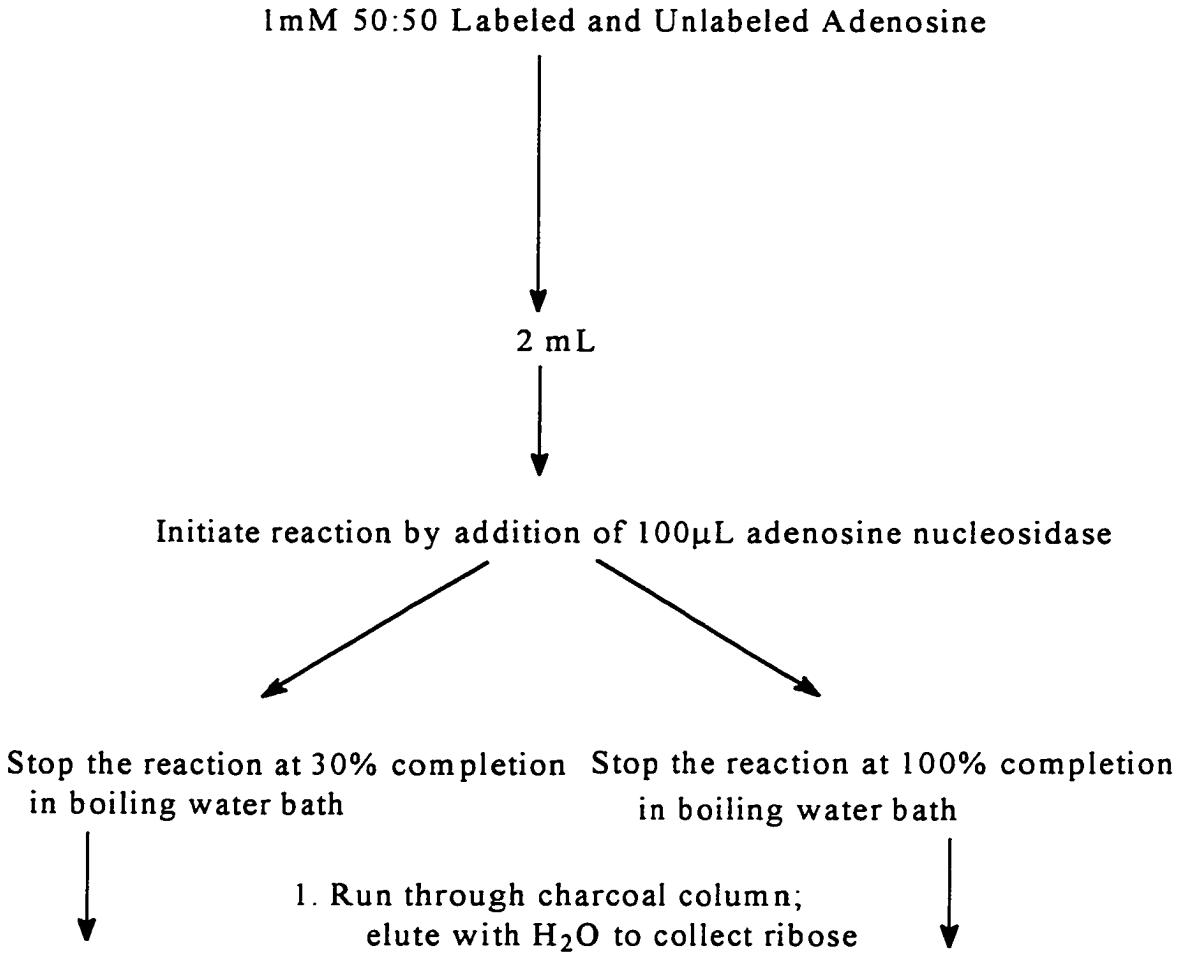


Figure 8. Flow chart for measuring kinetic isotope effects



KIE has occurred if $^{12}C/^{13}C$ (30-40%) completion $\neq$ $^{12}C/^{13}C$ (100%)

conditions. The method uses extracted ion chromatograms (EIC) which were integrated for each monitored m/z. A chromatogram of ribitol acetate is shown in Figure 9 with a retention time of 7.6 minutes while the total mass spectrum is shown in Figure 10. The line pairs used for the enrichment calculations were 158/159, 187/188, and 217/218 corresponding to fragments ($C_7H_{10}O_4^-$), ($C_7H_7O_6^-$), and ($C_9H_{13}O_6^-$) respectively. These lines are isotopically shifted and of significant intensity. Isotopic enrichments were calculated based on the integrated area of the EIC collected under the SIM mode. Figures 11 and 12 show the SIM mass spectra for the unlabeled ribitol acetate and the ribitol acetate formed from an approximate 50:50 mixture of labeled and unlabeled ribose.

Gas Chromatography/Mass Spectrometry

Since ribitol acetate is a symmetrical compound, fragment ions can be produced from either end; therefore the observed enrichment is approximately one half of the actual enrichment. To account for this, the calibration curves for [1-^{2}H] and [1-^{13}C] ribose that were previously constructed by Rezaee, were used to relate observed enrichment to actual enrichment. (Figures 13 and 14). (42)

Four injections were made for each derivatized ribose sample, and the KIE was calculated for each of the following line pairs, 158/159, 187/188, and 217/218. The average KIEs for [1'-^{2}H]-adenosine and [1'-^{13}C]-adenosine are 1.081 ± 0.028 and 1.012 ± 0.045 respectively. The observed KIE is adjusted to 0% conversion using Equation 3. The correction of observed to actual isotope effect at 0% conversion is due to the enrichment of the heavy isotope in the remaining substrate during the course of the

Figure 9. Chromatogram of ribitol acetate with retention time of 7.6 minutes analyzed on a Hewlett Packard (HP) 5890 capillary column gas chromatograph interfaced to an HP 5870 mass selective detector.

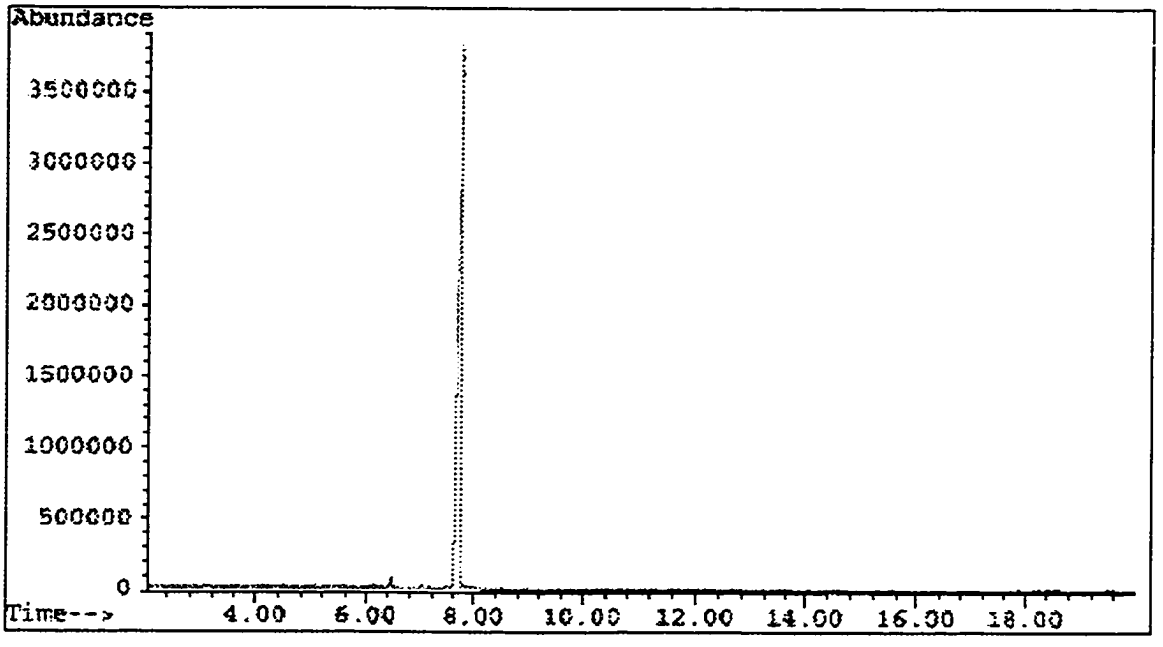


Figure 10. Total mass spectrum of ribitol acetate. The * denotes the mass fragments used for the line pair isotope enrichment studies.

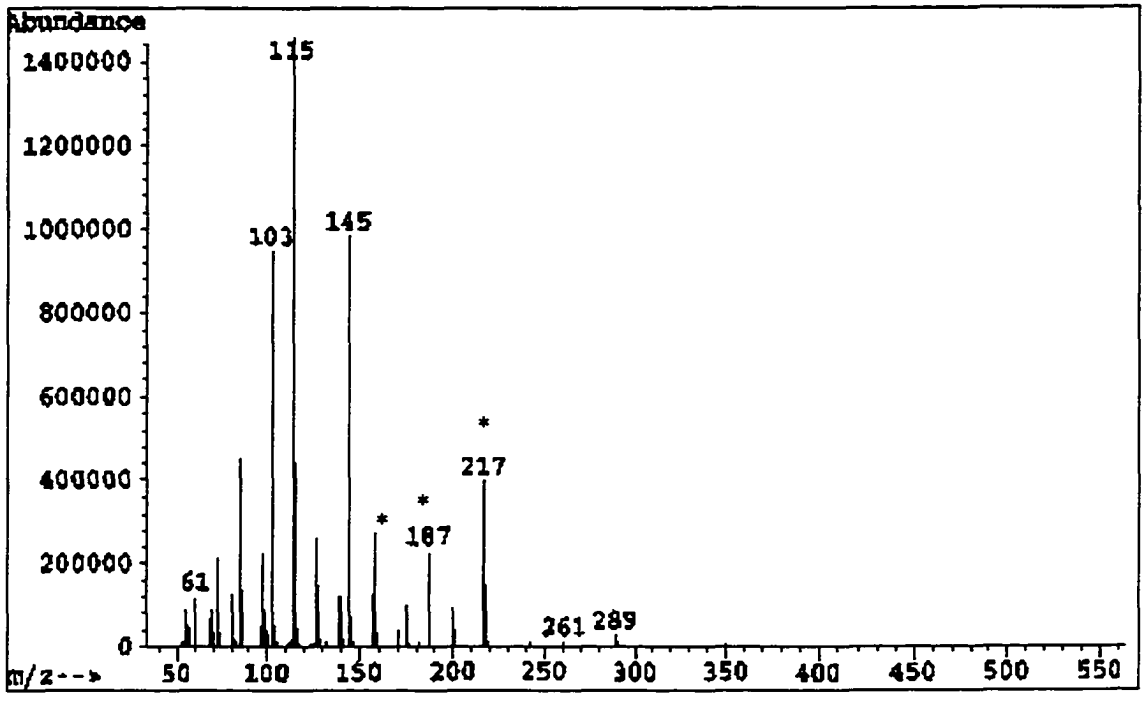


Figure 11. SIM mass spectrum of unlabeled ribitol acetate showing the line pairs used to calculate the kinetic isotope effects.

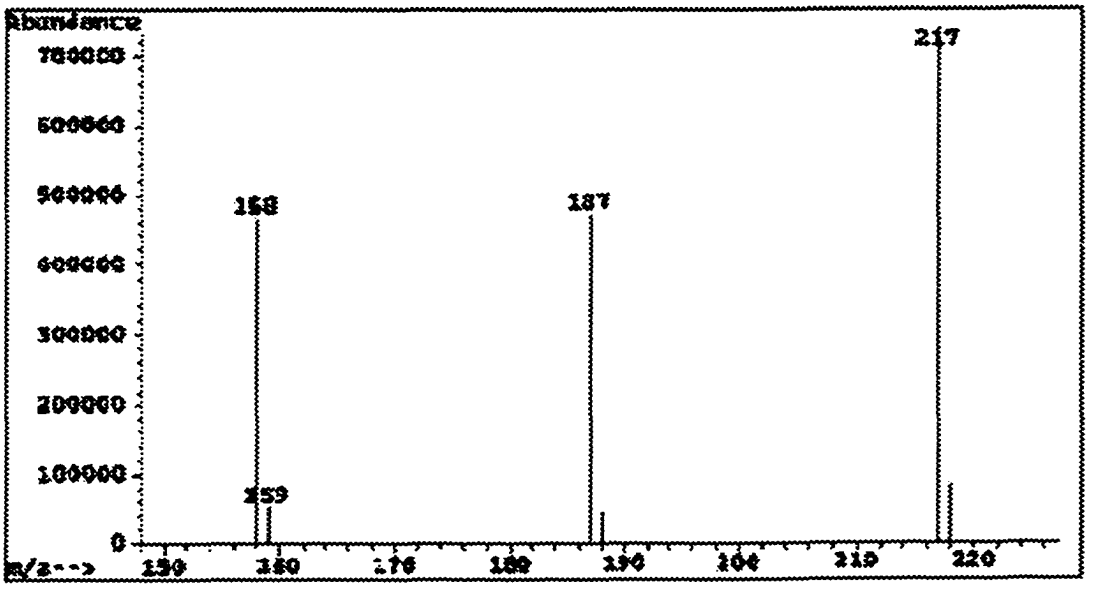


Figure 12. SIM mass spectrum of labeled ribitol acetate showing line pairs used for kinetic isotope effects. The ribitol acetate was formed from a mixture of approximately 50:50 labeled and unlabeled substrate.

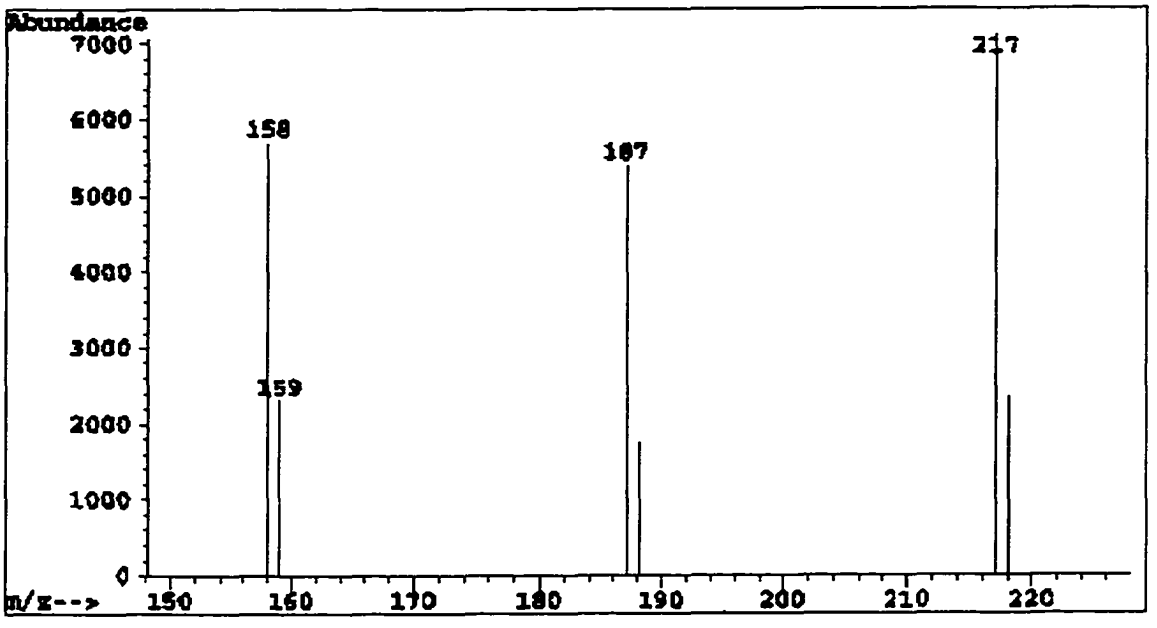


Figure 13. Calibration curve for [1-^{2}H]-ribose.(Rezaee, M. M. S. Thesis, Middle Tennessee State University, August, 1997)

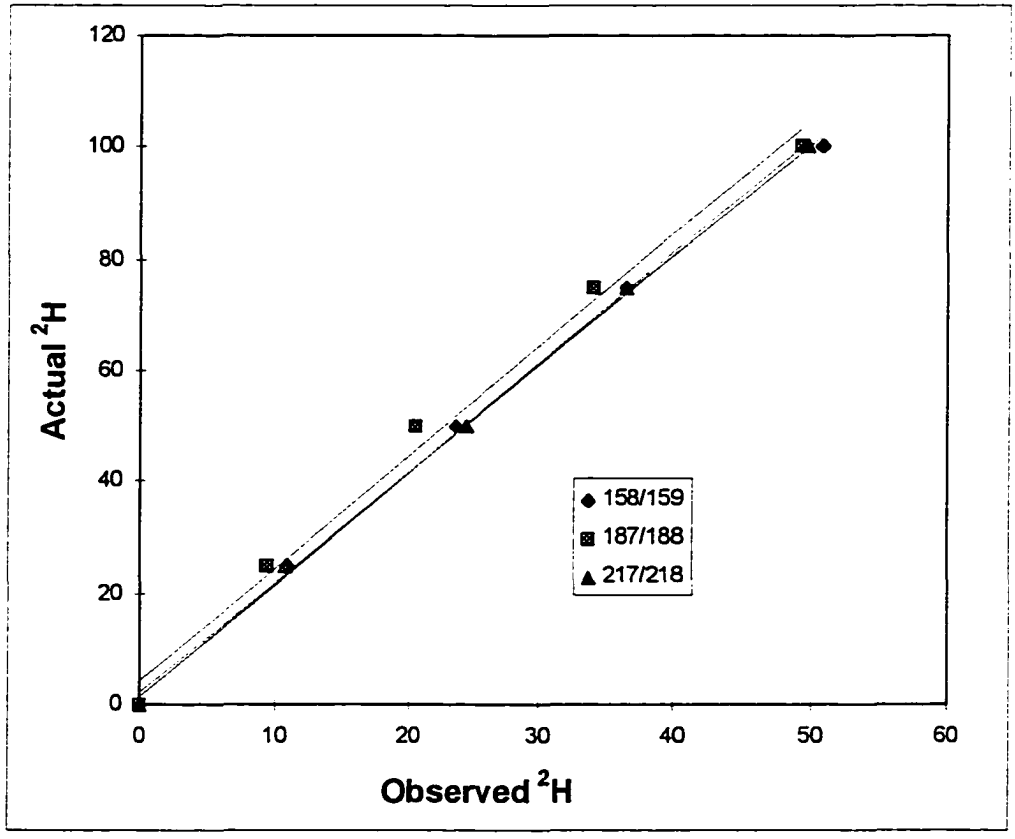
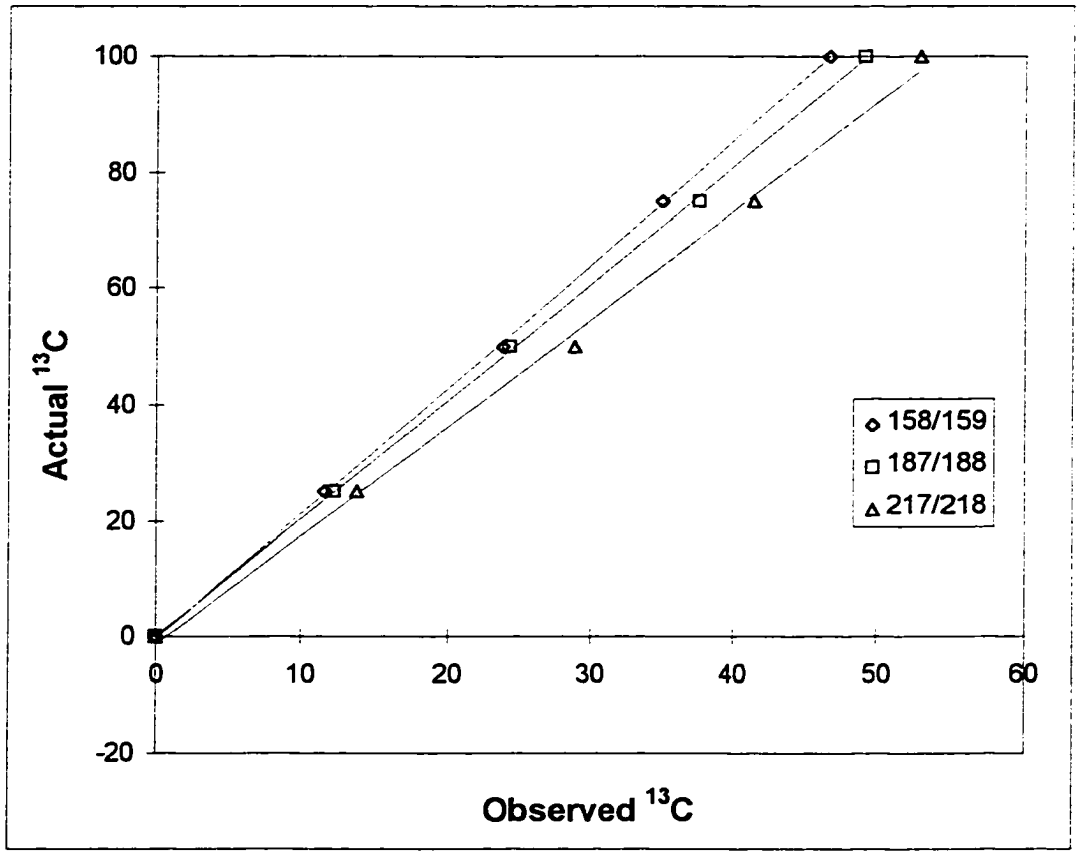


Figure 14. Calibration curve for [1-^{13}C]-ribose.(Rezaee, M. M. S. Thesis, Middle Tennessee State University, August, 1997)



reaction. (54) The stable isotope effects reported by Rezaee for nucleoside hydrolase were 1.1140 ± 0.011 and 1.0320 ± 0.005 for [1'-^{2}H]-inosine and [1'-^{13}C]- inosine respectively. (42)

BEBOVIB Calculations

The transition state geometry was modeled using the BEBOVIB-IV program, which is used to calculate kinetic isotope effects for a pre-selected transition state structure. Transition state structure was varied systematically to match the observed kinetic isotope effects.

From the BEBOVIB calculations, a plot was made of calculated KIE versus bond order in the transition state for the 1'-^{13}C and the 1'-^{2}H labeled adenosines. The plot in Figure 15 for the 1'-^{2}H labeled adenosine, shows that the calculated KIEs range from a low value of 0.939 with a bond order of 1.36 to a high value of 1.111 with an almost completely broken bond shown by a bond order of 0.26. The plot in Figure 16 for 1'-^{13}C labeled adenosine showed a KIE range from 0.994 with bond order of 1.36 to 1.025 with bond order of 0.26.

The transition state model used for the BEBOVIB calculations was determined from a family of KIEs for nucleoside hydrolase using labeled inosine as the substrate. These included KIEs for the [1'-^{3}H], [2'-^{3}H], [1'-^{14}C], [9-^{15}N], [4'-^{3}H], and [5'-^{3}H] labeled inosine compounds. (57) The transition state involves a protonated hypoxanthine leaving group with a C-N glycosidic bond elongated to approximately 2 Å. The ribose group contains substantial carbocationic character, unusually strong hyperconjugation of

Figure 15. Effect of altered C1'-N9 bond order on the calculated 1'-^{2}H KIE. The ⊗ indicates the experimental KIE.

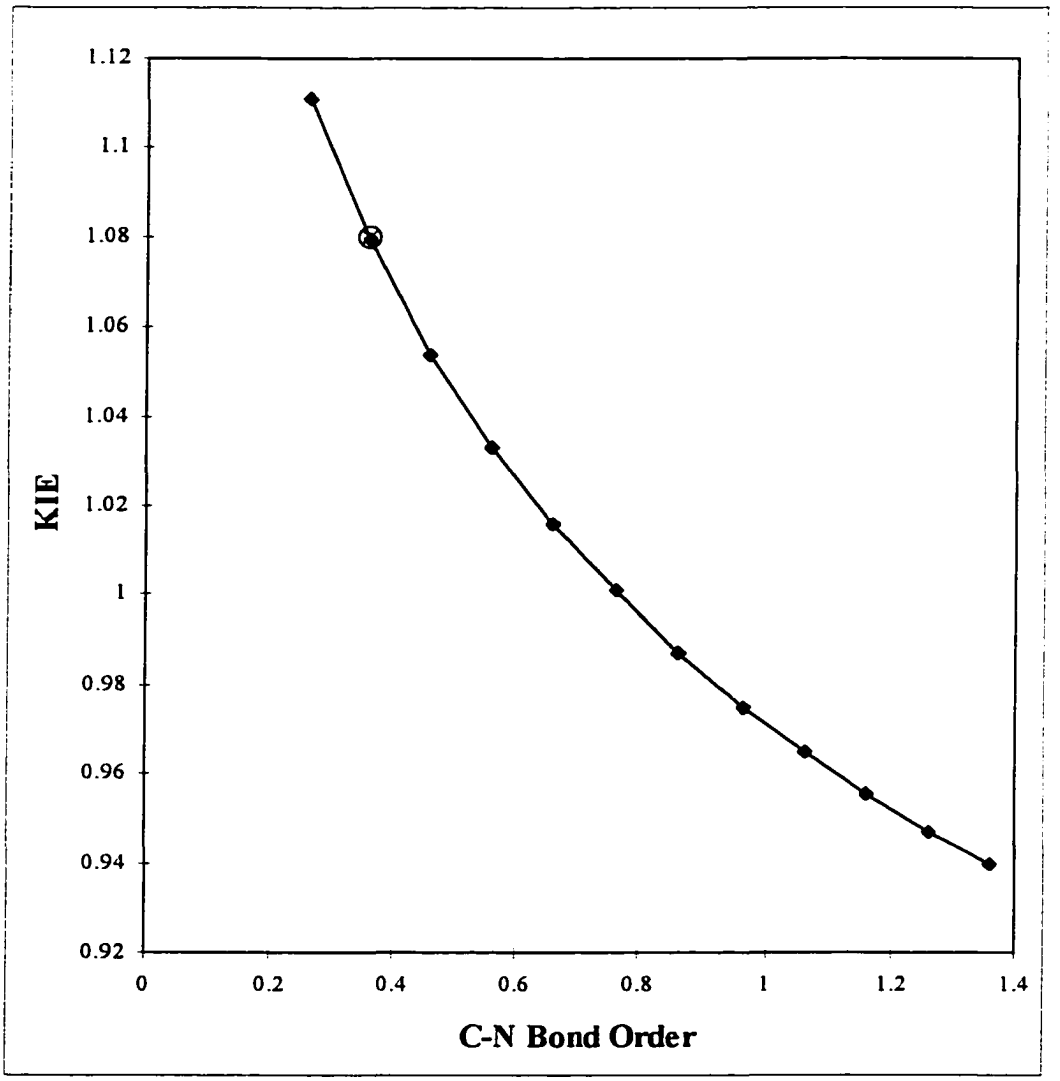
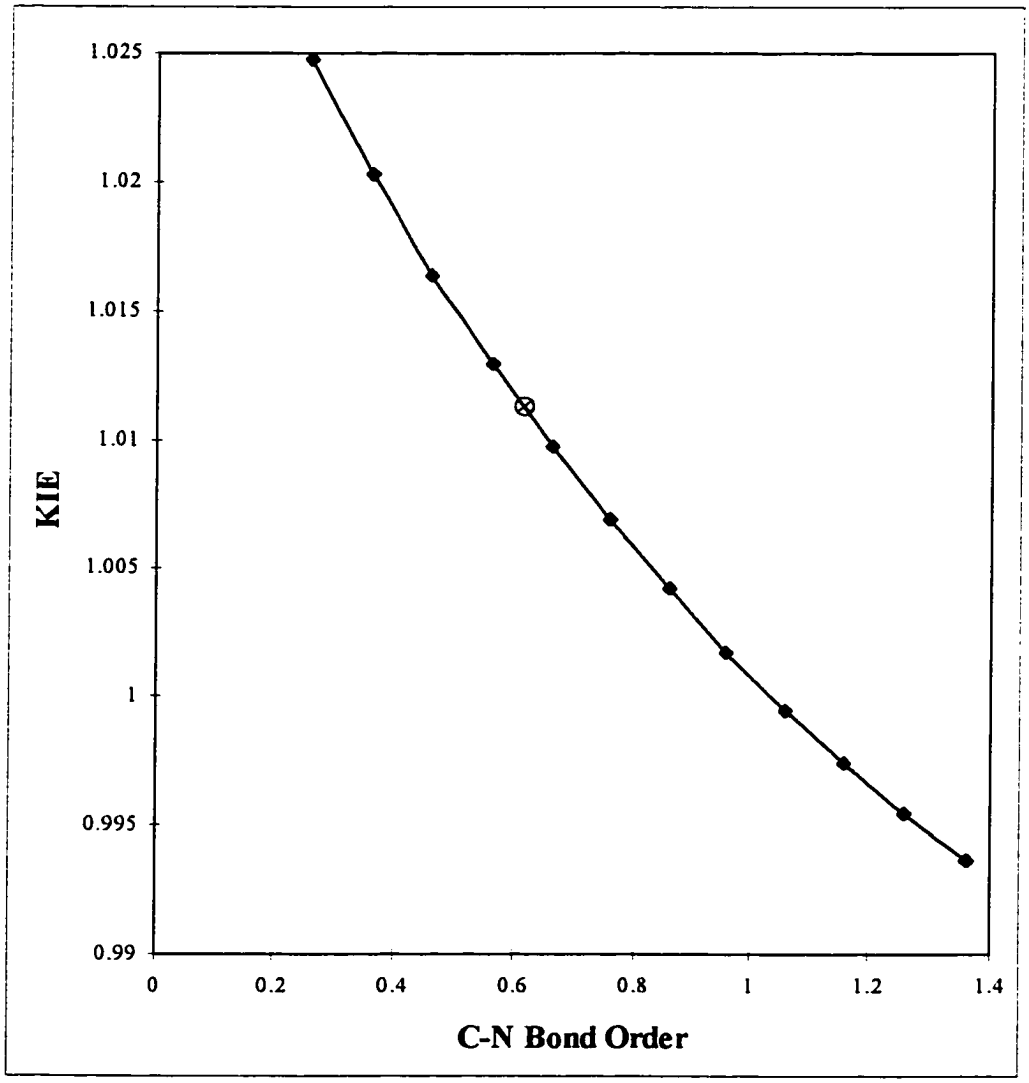


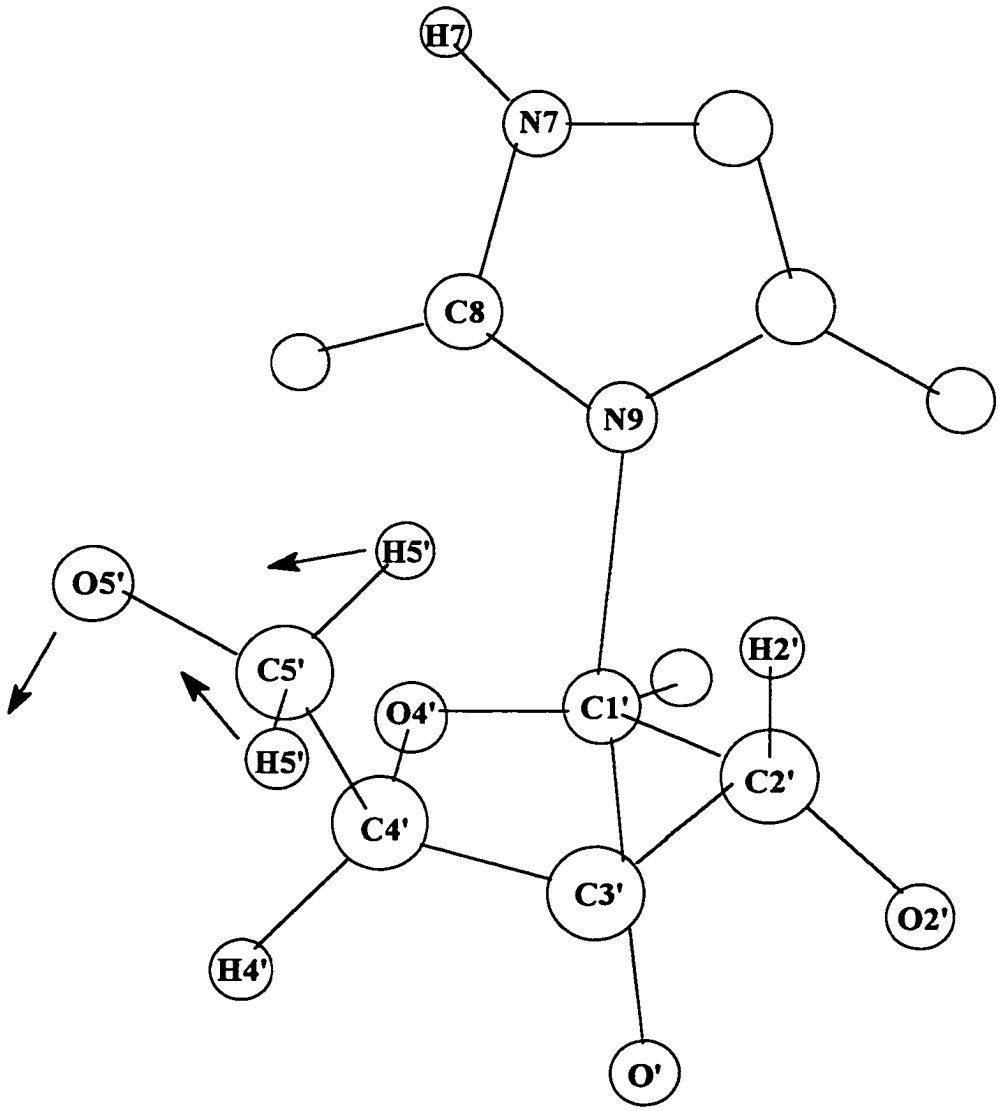
Figure 16. Effect of altered C1'-N9 bond order on the calculated 1'-^{13}C KIE. The ⊗ indicates the experimental KIE.



H-2', and a bond length of approximately 3 Å to the incoming nucleophile. The configuration of the ribose ring changes from 3'-*endo* to 3'-*exo* as C-1' rehybridizes as shown in Figure 17. The ribose portion of the nucleoside is held in a reactive conformation via interactions with its hydroxyl groups.

In order to completely characterize the transition state of an enzyme, a family of isotope effects must be determined which would include the 9-^{15}N and the 2'-^{2}H isotopes. These labeled adenosine compounds were not available commercially or by synthesis. Hence, the KIE data is of only qualitative significance. Kinetic isotope effects are small indicating kinetic suppression caused either by commitment factors or internal equilibria, or an early transition state. The relatively large bond orders of 0.36 calculated for the C1'-N9 bond of the [1'-^{2}H] labeled adenosine and of 0.65 for the C1'-N9 bond of the [1'-^{13}C] labeled compound indicate an early transition state. An early transition state was reported for purine nucleoside phosphorylase with bond order of 0.40 between C1'-N9 and very little bond order from the attacking nucleoplile. (58) In contrast, the nucleoside hydrolyase from *Crithidia fasciculata* has a C1'-N9 bond order of 0.27 at the transition state. (57) Again, the data for adenosine nucleosidase are of only qualitative significance since the family of isotope effects was not determined. If there is a high commitment to catalysis, the KIEs will be suppressed and the observed KIEs will need to be corrected for this commitment factor to determine the intrinsic isotope effects. To address this problem, a commitment to catalysis experiment was performed.

Figure 17. Transition state for nucleoside hydrolase showing using bond energy bond order vibrational analysis. The transition state involves a protonated hypoxanthine leaving group with a C-N bond elongated to approximately 2 Å. The ribose group contains substantial carbocationic character, unusually strong hyperconjugation of H2' and a bond length of approximately 3 Å to the incoming oxygen nucleophile, O'. The configuration of the ribose ring changes from 3'-endo to 3' *-exo* as C1' rehybridizes. (Horenstein, B. A.; Parkin, D. W.; Estupinan, B.; Schramm, V. L.; Biochem. **1991,** *30,* 10788-10795)



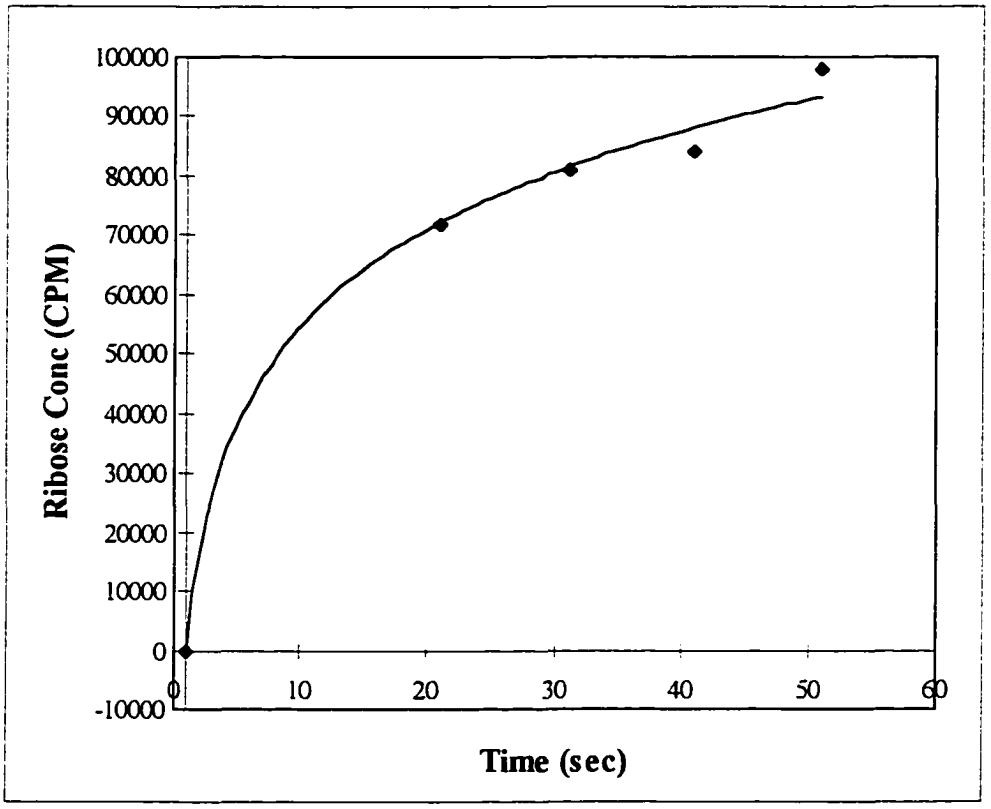
Commitment to Catalysis

The commitment of enzyme-bound adenosine to catalysis was determined using the pulse-chase method. (53,58) Catalytic commitment defines the probability with which enzyme bound substrate is converted to products relative to release as unchanged substrate. The adenosine nucleosidase has a K_m of 4.7 μM for adenosine. The adenosine concentration in the pulse solution was 333 μM or approximately 70 K_m. Under these conditions, the enzyme active sites are 0.98 saturated with adenosine. At an adenosine nucleosidase concentration of 1.92×10^{-5} μM, 2.30×10^{-7} μM of adenine was formed. The product ribose was isolated by charcoal column chromatography and quantitated by scintillation counting. Hydrolysis of adenosine gives equivalent concentrations of adenine and ribose. For each adenosine bound to adenosine nucleosidase, 0.01 adenine is formed and 0.99 adenosine is released. The commitment for bound adenosine is defined as the ratio of product formed to substrate released and is 0.01. This reveals that release of substrate from the enzyme is much faster than conversion of adenosine to products. A low commitment to catalysis normally results in fully expressed isotope effects. A substrate trapping experiment was then done to determine if the chemistry or the product release was the rate limiting step.

Substrate Trapping

The time-dependent formation of radio-labeled ribose is shown in Figure 18. A burst is shown initially, followed by a slower rate of product formation. This indicates that product release is the rate limiting step rather than hydrolysis of the adenosine. These

Figure 18. Time course of adenosine hydrolysis by adenosine nucleosidase



experiments show that a low concentration of bound substrate goes on to form products, and the product that is formed is released very slowly.

Substrate Specificity

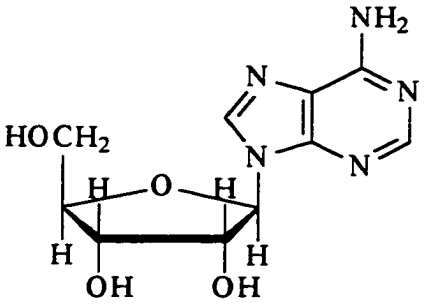
The relative velocities of various adenosine analogs, shown in Figure 19, are summarized in Table II. Information about requirements for binding in the active site of the enzyme can be determined by changing different moieties on the purine ring and the ribose ring.

Table II. Substrate Specificity

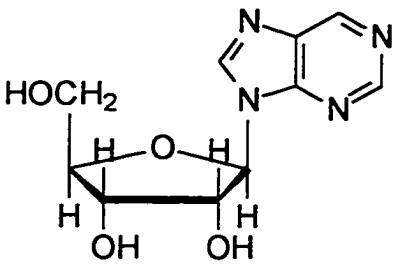
Compound	Relative Activity
Adenosine	100
5'-Deoxyadenosine	218
Purine riboside	4.6
Cordycepin (3'-deoxyadenosine)	1.5
Tubercidin (7-deazaadenosine)	0.0

The data show that adenosine and 5'-deoxyadenosine are the best substrates for adenosine nucleosidase isolated from yellow lupin cotyledons. The other substrates tested exhibited much lower activity, and the tubercidin was not a substrate for adenosine nucleosidase at all. HPLC chromatograms of the substrates after five days showed no change, indicating that the concentration of the adenine or adenine analog was due to the action of the adenosine nucleosidase rather than hydrolysis of the substrate. The relative

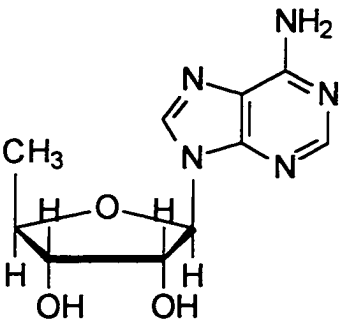
Figure 19. Structures of adenosine and adenosine analogs



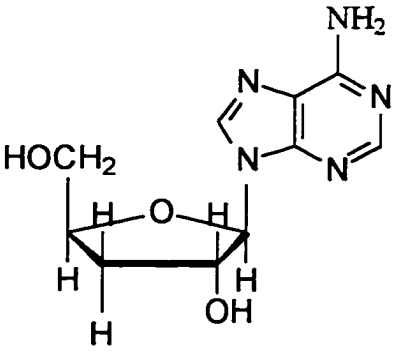
Adenosine



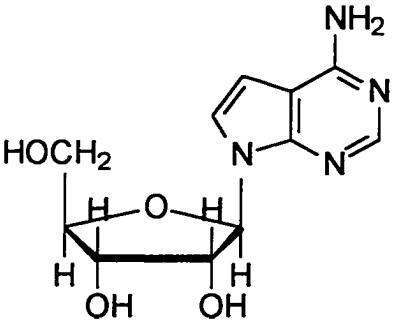
Purine riboside



5'-Deoxyadenosine



Cordycepin
3'-Deoxyadenosine



Tubercidin
7-Deazaadenosine

activities indicate that certain structural features affect the rate of reaction. The best substrate, 5'-deoxyadenosine, indicates that the hydroxyl group in the 5' position on ribose inhibits binding by the enzyme. This indicates that the enzyme has a hydrophobic group at one of the contact points in the active site and may also explain why the enzyme was bound on an affinity chromatography column by the aliphatic group of ω-aminohexyl agarose.

An exocyclic amino group in the 6-position of the purine ring is essential to bind in the active site of this enzyme. This may partially explain the lack of activity of cytokinins toward the adenosine nucleosidase isolated from yellow lupin. (11) The structure of the cytokinin molecules differs from adenosine only on the substitution of the exocyclic nitrogen. (Fig. 6) Substrates such as the cytokinins and purine riboside, that retain the purine ring but lack the free amino group, showed low activity. The lack of activity toward purine riboside also eliminated the possibility that steric factors were preventing the cytokinins, with their bulky N-substituted alkyl groups, from binding to the enzyme. It is the exocyclic amino group that is important.

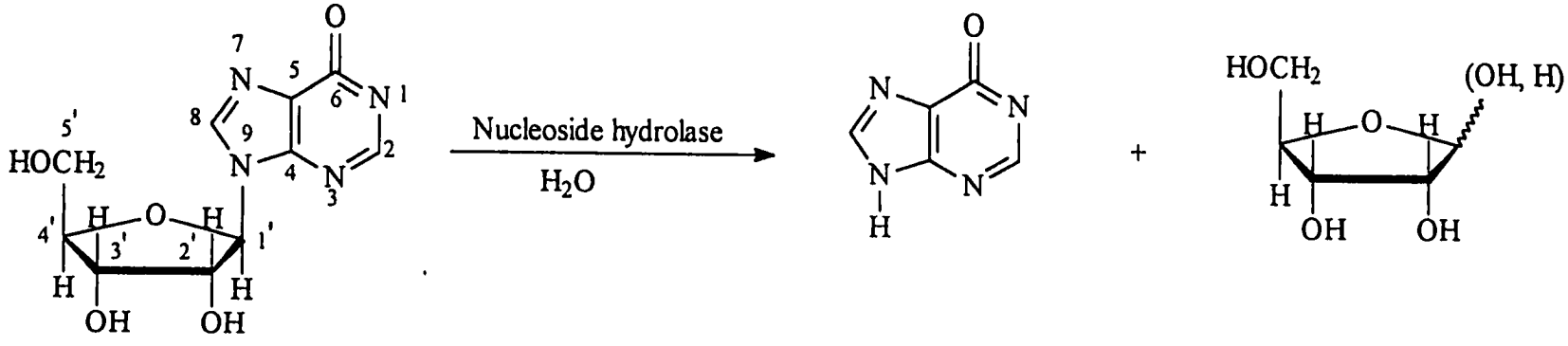
It has already been shown that using 2'-deoxyadenosine as substrate resulted in 35 % loss of activity. (11) The loss of the hydroxyl group in the 3' position of ribose as in cordycepin results in lowering the activity by 98%. Replacing the N-7 with a carbon atom completely destroys the activity. This confirms part of the validity of the transition state since the model for the BEBOVIB calculations included a protonated N7 in the transition state.

In summary, the structural features essential for binding by adenosine nucleosidase are the purine ring and free exocyclic amino group of adenosine and the hydroxyl group at the 3' position of the ribose moiety. A non-polar group in the 5'-position on ribose results in a substrate that is approximately twice as reactive as adenosine.

These results are in agreement with previous work on nucleoside hydrolases. (7,6,18-20,11,27) Adenosine nucleosidases from barley leaves and tea leaves also tolerate changes in the 2' position. (19-20) Adenosine nucleosidase from barley leaves and inosine nucleosidase from yellow lupin seeds were the only enzymes capable of utilizing purine riboside as a substrate. (6,19) The tubercidin moiety, 5'-methylthiotubercidin was not a substrate for 5'-methylthioadenosine nucleosidase from yellow lupin. Inosine activity correlates strongly to retention of the ribose moiety. Changes at the 2', 3', and 5' positions resulted in loss of activity for the inosine nucleosidase from yellow lupin and for the nucleoside hydrolase from *Crithidia fasciculata*. (6, 27)

Essential to all of the above hydrolases are the N-7 and the 3' hydroxyl groups. Presumably the N-7 permits protonation of the adenine converting it to a good leaving group. Horenstein, Parkin, Estupinan, and Schramm determined a small solvent deuterium isotope effect of 1.30 for the hydrolysis of inosine by nucleoside hydrolase. (Fig. 20) This is indicative of nearly complete transfer of at least one proton in the transition state. (57) The ground state structure for both nucleoside hydrolase (57) and for purine nucleoside phosphorylase (58) show that the configuration of the ribose ring is *endo* at the 3'

Figure 20. Hydrolysis of inosine in the presence of nucleoside hydrolase yields hypoxanthine and ribose. It is not known if the initial ribose formed is the α or β anomer.



Inosine

Hypoxanthine

Ribose

position. The configuration changes from the 3'-*endo* in the ground state to 3'-*exo* in the transition state as the C1' rehybridizes. The enzyme thus stabilizes a specific conformation of ribose at the transition state which requires contact at the 3' hydroxyl group.

Equilibrium Studies

The equilibrium constant for adenosine nucleosidase was established by the equilibrium formation of adenosine from relatively high concentrations of adenine and ribose as shown in Table III.

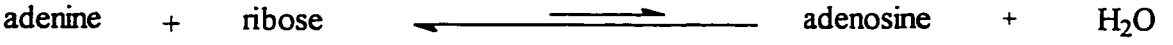


Table III Determination of the Equilibrium Constant for Adenosine Nucleosidase

[Ribose]	[Adenine]	[Adenosine]
1M	4.98 mM	0.02 mM
2 M	4.97 mM	0.03 mM
3 M	4.94 mM	0.06 mM

At 5 mM adenine and with ribose concentrations varying from 1 to 3 M, adenosine was detected and quantitated by HPLC. The concentration of adenosine increased relative to the increase in ribose concentration.

$$K_{eq} = \frac{[\text{Adenine}][\text{Ribose}]}{[\text{Adenosine}]} \qquad (4)$$

The hydrolytic reaction can be reversed to give an experimental K_{eq} of 263 M. This value is similar to that of 106 M obtained for nucleoside hydrolase and of 170 M obtained for AMP nucleosidase in similar experiments. (27, 59) Hydrolysis is highly favored as opposed to synthesis.

CONCLUSIONS

Adenosine nucleosidase has been purified from 4-day cotyledons of *Lupinus luteus L.* (yellow lupin). A final purification step on an ω-aminohexyl agarose column resulted in a protein of high purity as evidenced by a single band using gel electrophoresis and a silver nitrate staining procedure. The specific activity was 6.16 μmol/min/mg, and the molecular weight of the sub-unit was approximately 36,000 daltons.

Low kinetic isotope effects for [1'-^{2}H] and [1'-^{13}C] adenosine of 1.081 ± 0.028 and 1.012 ± 0.045 respectively show either kinetic suppression or a low intrinsic isotope effect with an early transition state. BEBOVIB calculations indicated an early transition state with substantial bond order of 0.36 between C1'-N9 for the [1'-^{2}H] compound and 0.65 for the [1'-^{13}C] compound. Thus the transition state appears to occur early in the reaction coordinate with little bond formation to the attacking nucleophile. However, the early transition state cannot be clearly concluded without the KIEs of the [2'-^{2}H] and the [9-^{15}N] compounds which were not available. Commitment to catalysis was excluded by isotopic trapping experiments. A low commitment factor of 0.01 does not influence KIEs in a significant way and would indicate that the isotopic effects are near intrinsic. Substrate trapping experiments show that the hydrolytic reaction includes a burst of product formation followed by a slow steady state reaction in which the rate determining step is the release of adenine. The KIEs are being suppressed by slow product release by the enzyme.

However, without the entire family of isotope effects, the transition state cannot be completely characterized. Additional studies would include the synthesis of the [2'-^{2}H] and [9-^{15}N] labeled substrates and the subsequent determination of their respective KIEs. The KIE for the [2'-^{2}H] labeled adenosine gives information on the geometry of the ribose ring. The KIE for the [9-^{15}N] compound will provide information on the extent of breakage of the N9-C1' bond as well as bonding changes within the purine ring. By a combination of a family of KIEs and BEBOVIB analysis, the transition state conformation of adenosine nucleosidase may be deduced.

Catalytic and substrate recognition forces are focused on both the ribosyl group and the purine ring. The best substrates for adenosine nucleosidase were 5'-deoxyadenosine and adenosine. The remaining substrates, purine riboside, cordycepin, and tubercidin, even though they are close structural mimics, bind poorly or not at all. Purine nucleosides lacking an exocyclic amino group such as purine riboside, lacking the N-7 such as tubercidin, or lacking the C3' hydroxyl group as in cordycepin were all poor substrates.

Information about substrate binding has been determined by modifying the substrate. A complementary experimental approach would be to modify the enzyme, eliminating certain enzyme-substrate interactions by replacing specific amino acids through site-directed mutagenesis.

Hydrolytic reactions are often experimentally irreversible because of the unfavorable concentration of water compared to substrate concentration. The equilibrium

position for adenosine nucleosidase was reached in the reverse reaction by the use of molar concentrations of ribose and saturating adenine and an experimental K_{eq} of 263 M was determined. Hydrolysis is preferred over synthesis.

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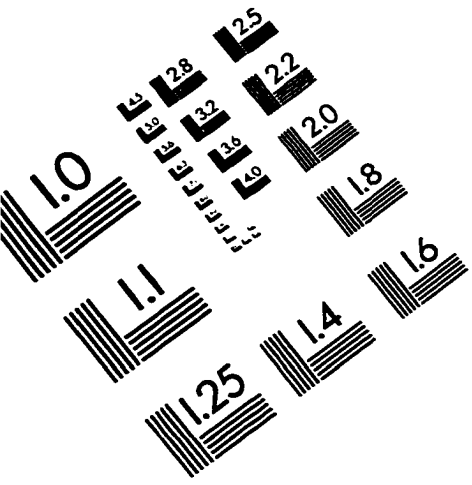
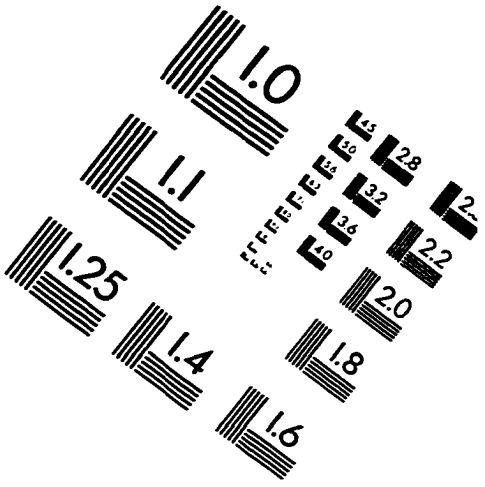
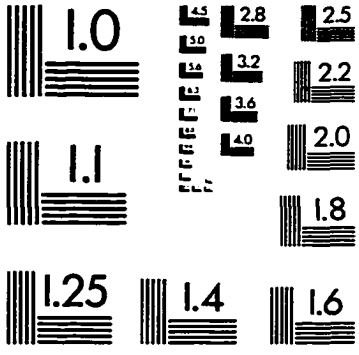
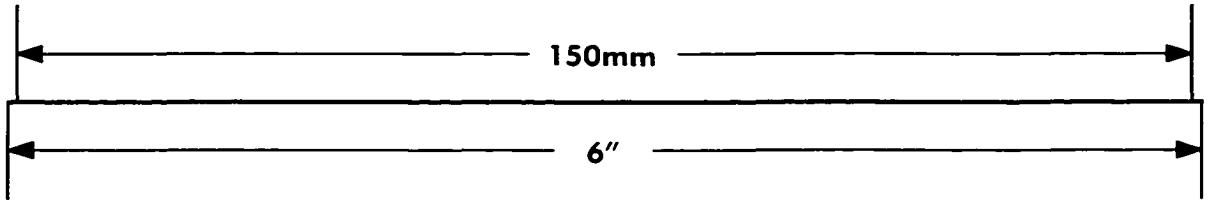
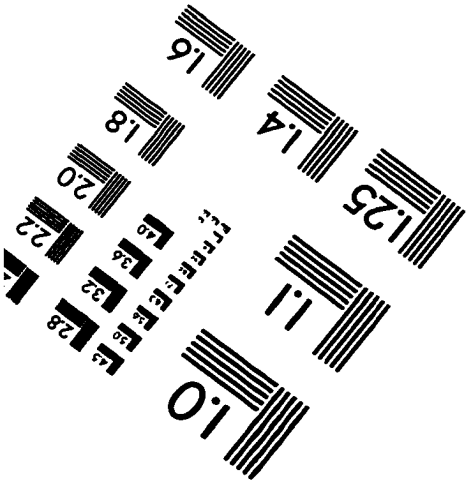
IMAGE EVALUATION TEST TARGET (QA-3)

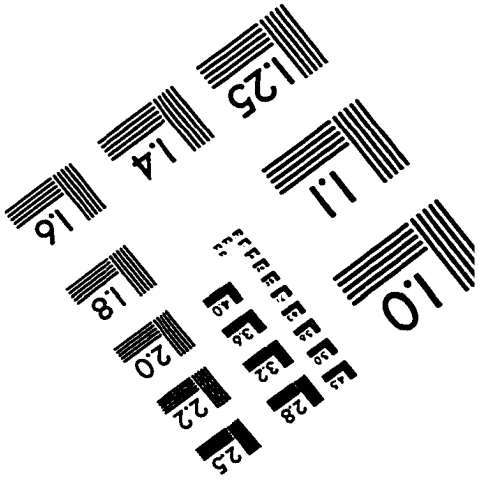












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