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Calf Intestine Adenosine Deaminase. Substrate Specificity*

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ABSTRACT: Substitutions can be made in the 8 position of the purine nucleus to produce compounds which still retain their ability to be bound to the "nonspecific" adenosine deaminase, isolated from calf intestine. Such substitutions must be of a limited nature. Thus, while 8-amino-2′-deoxyadenosine is deaminated, the 8-*N*-methylamino derivative is not bound to the enzyme. Furthermore, while both N^1-methyladenosine (IV) and 8-oxoadenosine (V) are substrates for the enzyme the latter is deaminated at a rate that is 3000 times slower than IV. 8-Aminoadenosine and 8-azaadenosine were also found to be substrates for the enzyme. The K_m values for these alternate substrates varied within a relatively narrow range (2.35–13.7 $\times$ 10^{-5}) while the V_{max} values varied over approximately a 6000-fold range. The results of these substrate studies are discussed in relation to the steric and electronic properties of the purine nucleoside analogs studied. All of the alternate substrates were also competitive inhibitors of the deamination of adenosine and in addition several of the 2-substituted adenosine and deoxyadenosine derivatives that were not deaminated were found to be competitive inhibitors of the enzyme. 2-Aminopurine riboside was the best inhibitor and had a K_i value of 4.0 $\times$ 10^{-6}.

The study of enzymes responsible for the deamination of purine and pyrimidine nucleosides has both a theoretical and practical interest. On the one hand, a great number of potentially useful chemotherapeutic agents have been synthesized which possess the nucleoside structure, only to find that while the compounds were effective antimetabolites *in vitro* or in tissue culture, they were rapidly inactivated by deamination *in vivo*. Cytosine arabinoside and adenine arabinoside have been found to have antitumor and antiviral activity (Privat de Garilhe and De Rudder, 1964; Buthala, 1964) but both are deaminated to relatively inactive compounds by the respective cytidine and adenosine deaminases (Pizer and Cohen, 1960; Hubert-Habart and Cohen, 1962). A thorough understanding of the effect of substrate modification on enzyme activity could afford the knowledge necessary to design effective chemotherapeutic agents that would either be resistant to, or inhibitors of the mammalian deaminases.

The availability of a mammalian enzyme in relatively pure form capable of deaminating adenosine as well as an active program in the synthesis of purine nucleoside antimetabolites of potential chemotherapeutic value, led us to study what effects ring modification of adenosine had on the substrate specificity of calf intestine adenosine deaminase.

Previous studies with this enzyme, have shown that various modifications can be made in the carbohydrate moiety of the adenosine molecule to produce analogs of adenosine that still retain either all or part of their ability to be bound to the enzyme (Bloch *et al.*, 1967; Frederiksen, 1966). A number of ring-modified compounds have been studied as substrates and/or inhibitors of the enzyme. It has been shown (Schaeffer and Odin, 1965; Schaeffer and Vince, 1968; Schaeffer and Bhargava, 1965; Schaeffer *et al.*, 1965) that replacement of the ribose moiety at the 9 position with various alkyl, hydroxyalkyl, and aralkyl functions produced compounds that would bind to the enzyme. Wolfenden has recently demonstrated that replacement of the ribose moiety at N-9 with H (adenine) does not completely abolish the ability of the compound to bind to the enzyme as a substrate (Wolfenden *et al.*, 1969). There is however a rather large diminution of the rate of reaction ($^1/_{30,000}$), although the K_m of adenine is not drastically different from adenosine's. Several workers have shown that various substitutions can be made in the 2 and 6 position of the purine nucleus while still maintaining the ability of the compounds to bind to the enzyme (Chassy and Suhadolnik, 1967; Cory and Suhadolnik, 1965; Baer *et al.*, 1968; Frederiksen, 1966). Further, 1-methyladenosine (Wolfenden *et al.*, 1969) and *N*-3-(β-D-ribofuranosyl)-6-aminopurine (Wolfenden *et al.*, 1969) are also deaminated by the enzyme.

It is the general purpose of our program to study how structural modifications within the purine nucleus of the

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TABLE I: Maximal Absorbancy Changes Resulting from Enzymic Hydrolysis.

Substrate	λ (mμ)	$\Delta\epsilon \times 10^{-3}$ M
Adenosine	265	−8.33
Deoxyadenosine	265	−5.1
8-Aminoadenosine	272	−5.37
8-Azaadenosine	280	−7.17
8-Hydroxyadenosine	272	−4.55
8-Amino-2′-deoxyadenosine	278	−8.1

adenosine molecule change its substrate specificity *vis a vis* enzymes of nucleoside metabolism. In this communication we will report on part of these investigations, which show that several adenosine (deoxyadenosine) derivatives, may serve as alternate substrates and/or inhibitors of the enzyme.

Experimental Procedures

Preparations of adenosine deaminase from calf intestine were obtained from Calbiochem and were found to have the stated specific activity of 255 units/mg.

All compounds were prepared by the indicated methods and tested for their ability to serve as substrates by the following procedure. Incubation mixtures were made up containing the test compound at 6.6×10^{-5} M concentration in phosphate buffer at pH 7.05. An ultraviolet absorption spectra was run using a Cary Model 15 or Perkin-Elmer Model 202 ultraviolet–visible recording spectrophotometer. The mixtures were then incubated with 200 times the normal amount of enzyme at 37° for approximately 240 min. Under standard conditions (normal amount of enzyme, at 25°) adenosine (6.6×10^{-5} M) is completely converted into inosine in less than 4 min. Spectra were examined over the range from 200 to 340 mμ for the appearance of new or disappearance of old absorption maxima. The change in optical density at the appropriate wavelength was recorded. Using this method we would have detected a reaction occurring at 0.000004 the rate of adenosine's deamination.

Compounds were tested for their ability to inhibit the deamination of adenosine in the following manner. A standard reaction mixture was prepared in which the concentration of adenosine was 2.2×10^{-5} M. The compound to be tested was added to both the solvent blank and reaction mixture at a concentration of 2.2×10^{-4} M. The rate of the reaction was determined after addition of enzyme and compared with the uninhibited reaction.

For the determination of kinetic parameters the reactions were followed using the Cary Model 15 recording spectrophotometer equipped with a synchronous drive motor which allowed recording optical density changes at a fixed wavelength over a period of time that could be accurately measured and recorded. Initial velocities were determined from optical density changes over the initial 5–20-sec reaction and were calculated in moles per minute using the change in extinction coefficients for deaminated products, $\Delta\epsilon_m$, as accurately determined for each compound (Table I). The best straight lines were obtained in a double-reciprocal plot ($1/V$ *vs.* $1/S$) using regression analysis. K_m, K_i, and V_{max} were calculated from the straight-line equations. Correlation coefficients for all lines were not less than 0.996.

Infrared spectra were determined with 5–10 mg of compound using the Nujol mull technique and a Perkin-Elmer infrared spectrophotometer Model 257.

TABLE II

Compound	Substrate	Inhibitor
Adenosine	+[a]	−[a]
2′-Deoxyadenosine	+	−
8-Aminoadenosine[b]	+	+
8-Azaadenosine[c]	+	+
8-Oxoadenosine[b]	+	+
8-Amino-2′-deoxyadenosine[d]	+	+
1-Methyladenosine[e]	+	+
2-Amino-9-β-D-ribofuranosylpurine[f]	−	+
2-Methoxy-2′-deoxyadenosine[g]	−	+
2-Chloro-β-D-2′-deoxyadenosine[g]	−	+
2-Chloro-α-D-2′-deoxyadenosine[g]	−	+
8-Methoxyadenosine[b]	−	−
8-Ethylaminoadenosine[d]	−	−
8-Thioadenosine[h]	−	−
8-Mercapto-*S*-(β-D-ribofuranosyl)-6-aminopurine[i]	−	−
8-Bromoadenosine[h]	−	−
Toyocamycin[j]	−	−
7-Methyl-8-oxoadenosine[k]	−	−
1-Methyl-8-oxoadenosine[k]	−	−
8-Methylamino-2′-deoxyadenosine	−	−

[a] + used as substrate or inhibitor; − not used as substrate or inhibitor. [b] Holmes and Robins (1965). [c] W. Hutzenlaub and R. K. Robins, to be published. [d] Long *et al.* (1967). [e] Jones and Robins (1963). [f] Zorbach and Tipson (1968). [g] L. Christenson, R. K. Robins, and M. J. Robins, to be published. [h] Holmes and Robins (1964). [i] Shuman *et al.* (1969). [j] Tolman *et al.* (1969). [k] Rizkalla *et al.* (1969). [l] R. A. Long, R. K. Robins, and L. B. Townsend, to be published.

Results

The results of an initial screen of the compounds used in this study are presented in Table II. Approximately one-half of the compounds studied were neither substrates nor inhibitors of the enzyme. For those compounds which were substrates, the initial rates of deamination by the enzyme followed normal Michaelis–Menten kinetics and competitive-type inhibition was observed for each of the inhibitors. Kinetic constants, K_m and V_{max}, obtained from the double-reciprocal plots for the alternate substrates are given in Table III. These values were obtained in 0.05 M potassium phosphate buffer at pH 7.05 and 25°. A K_m value of 2.9×10^{-5} was obtained from adenosine (I) and was well within the range obtained by other workers.

It has previously been shown (Bloch *et al.*, 1967) that 6-amino-8-mercapto-8-*S*-(β-D-ribofuranosyl)purine (II) was

TABLE III: Kinetic Constants for Alternate Substrates.

Substrate	$K_m \times 10^5$	Rel V_{max}
Adenosine	2.9	1.0
Deoxyadenosine	2.79	0.98
8-Aminoadenosine	13.7	0.037
8-Oxoadenosine	6.27	0.10
8-Amino-2′-deoxyadenosine	2.35	0.020
8-Azaadenosine	9.6	2.17
1-Methyladenosine	9.6	0.000014

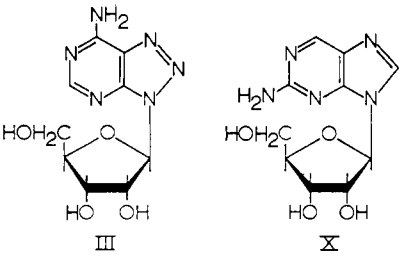
not a substrate or an inhibitor of adenosine deaminase from calf intestine. With this single exception, compounds in which the 8-hydrogen in adenosine has been replaced with other groupings have not been studied as inhibitors or substrates of adenosine deaminase. As can be seen from Table II several of the 8-substituted adenosine and deoxyadenosine derivatives were substrates for the enzyme. All of them had K_m values that were greater than adenosine. Thus substitution at the 8 position lowered the affinity of the compound for the enzyme in every instance even though in at least one case 8-azaadenosine (III) (see Chart I) the rate of the reaction, V_{max}, was over twice as great for the test compound, as it was for adenosine. The differences in affinity for the enzyme, K_m, did not vary as greatly as did the reaction rates, V_{max}, with substituent changes at the 8 position. Thus both 1-methyladenosine (IV) (Chart II) and 8-oxoadenosine (V) were substrates for the enzyme and had nearly identical K_m values; however, the 8-oxo derivative was deaminated at a rate that was 3000 times greater than 1-methyladenosine. Because of this finding, we studied the deamination of two different methyl-substituted 8-oxoadenosine (VII). Since tubercidin (7-deazaadenosine) and toyocamycin (7-cyano-7-deazaadenosine) were shown to be neither substrates nor inhibitors of the deaminase (Acs *et al.*, 1964), it seemed likely that changes at the 7 position of the adenosine nucleus would abolish the ability of the compound to be bound to the enzyme. It was thus not surprising to find that 7-methyl-8-oxoadenosine (VII) was not acted upon by the deaminase.

Not all compounds that were inactive as substrates were inactive as inhibitors of the enzyme; for this reason all

TABLE IV: K_i of Inhibitors of Adenosine Deaminase from Calf Intestine.

Substance	$K_i \times 10^{-5}$
8-Aminoadenosine	8.95
8-Oxoadenosine	12.3
8-Azaadenosine	14.5
2-Aminopurine-9-β-D-ribofuranosylpurine	0.417
2-Methoxy-2′-deoxyadenosine	5.88
8-Amino-2′-deoxyadenosine	2.91
2-Chloro-β-2′-deoxyadenosine	2.09
2-Chloro-α-D-2′-deoxyadenosine	6.04

CHART I

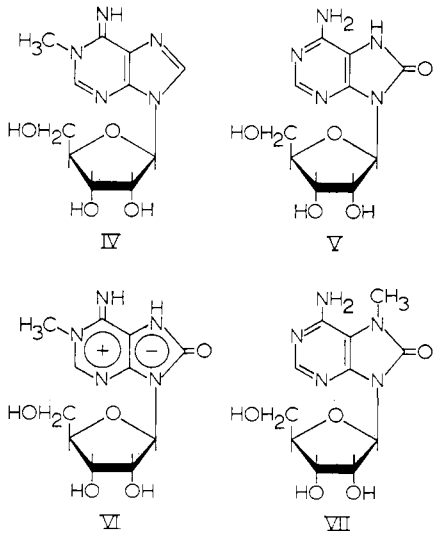


III X

compounds were also tested as inhibitors of the deaminase. Each of the alternate substrates were found to inhibit the deamination of adenosine. Thus, 8-hydroxy-, 8-amino-, and 8-azaadenosine as well as 8-amino-2′-deoxyadenosine were inhibitors of the deamination of adenosine. In addition, several compounds that were not substrates of the enzyme were relatively good inhibitors of adenosine deamination. In particular, 2-aminopurine riboside (X), a compound lacking a grouping at C-6 was the best competitive inhibitor we have found to date (Table IV).

Frederiksen has found that 2-amino-2′-deoxyribofuranosylpurine (XI) was an extremely good inhibitor of adenosine deamination and had a K_i value 1.9×10^{-6} (Frederiksen, 1966). All of the 2-substituted adenosine (deoxyadenosine) compounds that we have studied were competitive inhibitors although they did not serve as substrates for the enzyme and 2-aminoribofuranosylpurine was the best inhibitor having a K_i value of 4.0×10^{-6}. Thus both 2-chloro-6-amino-α-D-deoxyribofuranosylpurine and 2-chloro-6-amino-β-D-deoxyribofuranosylpurine were good inhibitors. The β derivative was three times better an inhibitor than the α derivative. In addition, purine riboside (nebularine) has also been shown to be an inhibitor of the enzyme although it obviously cannot be deaminated (Frederiksen, 1966). It thus appears that there seems to be a very low order of specificity with regard to the 2 position in the purine nucleus for binding to the enzyme and indeed 2-substituted compounds appear to be bound quite

CHART II



IV V VI VII

well. Deamination however, proceeds rarely or so slowly that it cannot be measured. Thus 2-fluoroadenosine was bound to the enzyme but the rate of reaction was so slow that neither V_{max} nor K_m could be measured (Frederiksen, 1966).

Discussion

The proposed mechanism of action of this enzyme is thought to involve a nucleophilic attack at C-6 of adenosine as a rate-limiting step (Wolfenden *et al.*, 1969). Thus the presence of an electron-withdrawing group in the 8 position might be expected to enhance such an attack. Compound VI is 1-methyladenosine in which a hydroxyl has been substituted for hydrogen at C-8 in the purine ring. The resulting compound however (1-methyl-8-oxoadenosine) (VI) served neither as a substrate nor an inhibitor of the deaminase. Two explanations seem plausible for the total lack of reactivity (VI) 1-methyl-8-oxoadenosine. First the presence of a substituent other than hydrogen at the 8 position, whether electron withdrawing or not, may sterically interfere with the binding of the substrate to the enzyme and/or the decomposition of the enzyme–substrate complex. That this may indeed be the case can be inferred from the summary in Table II. Thus, 8-oxoadenosine, 8-aminoadenosine, and 8-amino-2′-deoxyadenosine are substrates for the deaminase, but 8-thioadenosine, 8-methoxyadenosine, 8-ethylaminoadenosine, and 8-methylamino-2′-deoxyadenosine are devoid of activity. Since an activity trend according to electron-withdrawing ability has not been established, it can therefore be concluded that steric factors are important in determining activity, inasmuch as it is observed that those 8-substituted derivatives which are devoid of activity all possess greater steric requirements than those which have been demonstrated to be substrates for the enzyme. It is possible that the presence of substituents at the 1 and 8 positions of purine (*i.e.*, VI) contributes too much bulk to the molecule thus precluding its binding to the enzyme.

Secondly, the electronic configuration of some of the 8-substituted purine nucleosides may be drastically altered as compared with adenosine, thereby rendering the compound incapable of being bound to the enzyme. In this regard, examination of the infrared spectra of 1-methyl-8-oxoadenosine (VI) and derivatives revealed some interesting differences. 7-Methyl-8-oxoadenosine (VII) whose oxygen function at C-8 must be in the keto form shows a strong absorption band at 1715 cm^{-1}, which can be assigned to the carbonyl function. 8-Oxoadenosine (V) also shows a characteristically strong band in the 1735-cm^{-1} region that can be assigned to the carbonyl function thus indicating that V also exists in the keto form.

Investigation of 1-methyladenosine (IV) revealed no absorption maxima between 1700 and 1900 cm^{-1} and a sharp maximum at 1650 cm^{-1} characteristic of the imino function at C-6. 1-Methyl-8-oxoadenosine (VI) however possessed no absorption bands between 1700 and 1900 cm^{-1} and a moderately strong band at 1680 cm^{-1}. The absence of an absorption in the 1700–1900-cm^{-1} region of the infrared spectrum forces the assignment of the 1680-cm^{-1} band to carbonyl stretching mode, since there is no reported instance of an aromatic secondary amide (*i.e.*, positions 7 and 8 of VI) existing in the enol form. This appearance of the C-8 carbonyl absorption at unusually long wavelength is further evidence for the unique electronic configuration of (VI) proposed by Rizkalla *et al.* (1969). While it would be interesting to speculate that the electronic changes in this ring system are solely responsible for the lack of binding of 1-methyl-8-oxoadenosine (VI) to the enzyme, this cannot be done since we have presented some evidence to indicate that steric factors also play a role in the binding of 8-substituted adenosine derivatives to the enzyme. It would be of great interest to test the ability of 8-fluoroadenosine (VIII) to be bound to the deaminase since the 8-bromo derivative (IX) was devoid of activity. The fluorine atom would approximate the size of the H atom, but would still be more potent than bromine as an electron-withdrawing substituent.

In summary, we have shown that substitutions can be made of the 8-hydrogen in the purine nucleus and the resulting compounds still retain their ability to be bound to "non-specific" calf intestine adenosine deaminase. These substitutions are limited however to reasonably small groups. In addition, while both 1-methyladenosine and 8-oxoadenosine are substrates and/or inhibitors for the reaction, the presence of both substituents in the same molecule completely abolished the ability of the compound to be bound to the enzyme. The possible explanations for this have been discussed. Finally several 2-substituted adenosine and deoxyadenosine analogs are capable of being bound and were found to be competitive inhibitors of the enzyme. It thus appears possible to design antimetabolites that will not serve as substrates for deamination, *but* the important question as to whether they will retain their biological activity awaits further synthesis and testing.

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Molecular Interactions of Pyrimidines, Purines, and Some Other Heteroaromatic Compounds in Aqueous Media*

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ABSTRACT: Interactions of mixed purines, pyrimidines, and other heteroaromatic compounds (quinoxaline and chloroquine) were examined in aqueous media by the phase-solubility method. Association constants at 25° are reported for 18 adenines, 10 deoxyguanosines, and 5 other complexes. These data confirm the fact that there is no specific interaction of bases in aqueous media in the Watson–Crick sense, and support base stacking as being primarily responsible for the interaction. Although the base stacking appears to depend primarily upon the size of the π-electron systems, substituents modify the association tendency considerably. The present study suggests a possibility that some structural specificity does exist to discriminate the interaction of a certain pair of heteroaromatic compounds from that of another pair, although base stacking is generally considered to be nonspecific.

Specific hydrogen-bond formation between purines and pyrimidines is believed to be involved in the molecular basis of information transfer in biological systems. Much attention has, therefore, been focused on the molecular interaction of these heteroaromatic compounds. Several groups have been attempting to gain insight into the mechanism of the basic recognition processes as well as into the source of stabilizing energy for the structure of nucleic acids (Felsenfeld and Miles, 1967; Pullman, 1968).

Extensive studies on the molecular interactions of purine and pyrimidine derivatives have shown that these molecules interact with specific complementarity between purines and pyrimidines in such organic solvents as carbon tetrachloride (Küchler and Derkosch, 1966), chloroform (Hamlin *et al.*, 1965; Kyogoku *et al.*, 1966; Kyogoku *et al.*, 1967a,b; Miller and Sobell, 1967; Pitha *et al.*, 1966), and dimethyl sulfoxide (Katz and Penman, 1966; Shoup *et al.*, 1966; Newmark and Cantor, 1968). In aqueous solution, however, their interactions exhibit no such specificity (Ts'o 1968; Solie and Schellman, 1968; Lord and Thomas, 1967). These observations have led a number of investigators to consider a possibility that the basic recognition process may take place in a hydrophobic environment formed by exclusion of water in the presence of enzyme (Tinoco *et al.*, 1968). However, it is not known how enzymes, the polynucleotide back-bone structure, and other factors interplay to provide such an environment. In order to approach this problem, basic information about the association tendencies between mixed monomeric constituents of nucleic acids in aqueous media is needed so that effects of other factors can be individually ascertained.

Although a considerable amount of quantitative information is available for the self-association of monomers in aqueous solution (Ts'o, 1968), similar information regarding the interactions of mixed monomers is very limited (Solie and Schellman, 1968; Ts'o *et al.*, 1963). Little quantitative data concerning the association tendency of the guanine residue in water is available in the literature, although the association is often assumed to be substantial because of a strong tendency of guanosine monophosphate to form a gel in concentrated solutions under appropriate conditions (Gellert *et al.*, 1962).

We have, therefore, undertaken a quantitative study of the association tendencies of the adenine and guanine residues toward a wide variety of structurally modified purines and pyrimidines as well as toward some other heteroaromatic compounds. The phase-solubility method extensively used for studies of molecular interaction by Higuchi and his associates (for review, see Higuchi and Connors, 1965) was employed with the aid of radioactive materials for some systems. The same technique was used by Ts'o *et al.* (1963) for the systems similar to those investigated in the present study. Equilibrium association constants at 25° were calculated from solubility data on the assumption that the forma-

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