Ehrenberg, A. (1957), *Acta Chem. Scand. 11*, 1257.
Jacob, M. (1959), Thesis, Strasbourg.
Lehman, I. R., Roussos, G. G., and Pratt, E. A. (1962), *J. Biol. Chem. 237*, 829.
MacHattie, L., Bernardi, G., Thomas, C. A., Jr. (1963), *Science 141*, 59.
Martin, R. G., and Ames, B. N. (1961), *J. Biol. Chem. 236*, 1372.
Patchornik, A., Lawson, W., and Witkop, B. (1958), *J. Am. Chem. Soc. 80*, 4747.
Plummer, T. H., Jr., and Hirs, C. H. W. (1964), *J. Biol. Chem. 239*, 2530.
Schram, E., Moore, S., and Bigwood, E. J. (1954), *Biochem. J. 57*, 33.
Sjöquist, J. (1960), *Biochim. Biophys. Acta 41*, 20.
Smithies, O. (1955), *Biichem. J. 61*, 629.
Tristram, G. R., and Smith, R. H. (1963), *Advan. Protein Chem. 18*, 227.
Young, E. T., II, and Sinsheimer, R. L. (1965), *J. Biol. Chem. 240*, 1274.

Structural Requirements of Nucleosides for Binding by Adenosine Deaminase*

Joseph G. Cory and R. J. Suhadolnik†

ABSTRACT: The substrate specificity of adenosine deaminase has been studied in detail. It has been observed that a significant difference exists between the binding of those compounds altered in the 6 or 9 position of adenine. Substitutions in the 6 position (N^6-methyl, -hydrogen, or -mercapto) of adenosine result in compounds that are competitive inhibitors. Substitution of a chlorine atom for the amino group in the 6 position (6-chloropurine ribonucleoside) results in a nucleoside that is, in fact, a substrate for adenosine deaminase. Changes in the 9 substituent of adenine results in compounds that are either substrates (e.g., adenosine, 2′-deoxyadenosine, 3′-deoxyadenosine, 3′-amino-3′-deoxyadenosine, xylofuranosyladenine, and arabinofuranosyladenine) or inhibitors (e.g., 9-hexyladenine, 9-pentyladenine, 9-cyclopentanoladenine, and 9-cyclohexanoladenine).

Seven of the 9 position substituent analogs studied were not bound by the enzyme (adenine, psicofuranine, fructofuranosyladenine, 2′-adenylic acid, 3′-adenylic acid, 5′-deoxyadenylic acid, and 9-cyclohexyladenine). Based on these observations, it is concluded that the "binding site" of adenosine deaminase is more specific for the substituent on position 9 than for the substituent on position 6 of adenine.

It has been shown by several workers (Kalckar, 1947; Chilson and Fisher, 1963; Coddington, 1962) that, in addition to adenosine, 2′-deoxyadenosine, 2-fluoroadenosine, 2,6-diaminopurine riboside, and 3′-deoxyadenosine (cordycepin) are substrates for adenosine deaminase. N^6-Methyldeoxyadenosine is a competitive inhibitor of adenosine deaminase, while adenine, adenylic acid, nicotinamide–adenine dinucleotide, and cytidine are neither substrates nor inhibitors of this enzyme.

A number of adenosine analogs were surveyed as substrates or inhibitors of adenosine deaminase isolated from calf serum (Weinbaum *et al.*, 1964). The activity of the calf serum deaminase was too low to determine the nature of the inhibition experimentally. To overcome this difficulty, calf intestinal adenosine deaminase was used. With this enzyme, we have evaluated experimentally the nature of the inhibition and the inhibition constants. This study reports on the structural requirements necessary for compounds to bind at the "active site" as either substrates or competitive inhibitors of calf intestinal adenosine deaminase.

Materials and Methods

Adenosine deaminase from calf intestinal mucosa (type 1, 210 units/mg) was purchased from Sigma Chemical Co. Specific activity is expressed as μmoles of adenosine deaminated per minute/mg of protein. Compounds were tested as substrates by measuring the change in absorbance at 265 mμ, using cells with 5-mm path lengths, in a Beckman DU spectrophotometer. Reactions were carried out at 24–26°. Deaminase inhibition was determined by observing the decrease in

* From the Research Laboratories, Department of Biochemistry, Albert Einstein Medical Center, Philadelphia, Pa. *Received October 26, 1964; revised June 21, 1965.* This investigation was supported by a grant (GM08685-04) from the National Institutes of Health and a grant (G21239) from the National Science Foundation.

† Research Career Development Awardee of the National Institutes of Health (5-K3-GM-7100-04).

the rate of adenosine deamination. Assay mixtures consisted of substrate, 0.05 M sodium phosphate buffer, *p*H 7.0, and 0.021 enzyme unit in a total volume of 1.5 ml. Michaelis constants and inhibitor constants were determined graphically from plots of v versus $v/[s]$.

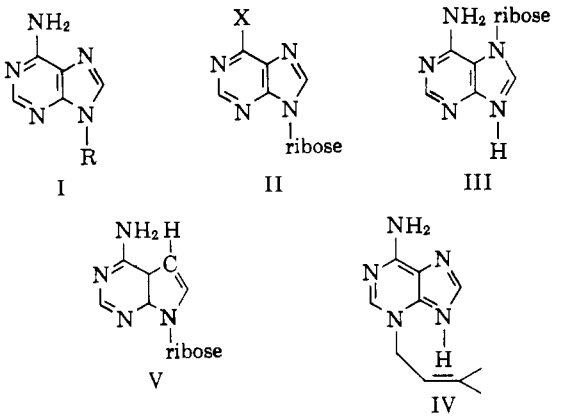
The reaction products were determined as follows: the reaction mixtures were evaporated to dryness with steam under nitrogen, and the residues were treated with warm ethanol and centrifuged. The ethanol supernatant was decanted, spotted on Whatman No. 1 filter paper, and subjected to descending chromatography with butanol–2% NH_3 (86:14) and ammonium sulfate–sodium phosphate buffer–1-propanol (60 g:100 ml, 0.1 M, *p*H 6.8:2.0 ml). The reaction products on the chromatograms were detected by ultraviolet light absorption.

We are grateful to Dr. J. Montgomery of Southern Research Institute for 9-(cyclohexyl)adenine, 9-(cyclopentyl)adenine, 9-(*n*-pentyl)adenine, 9-(*n*-hexyl)adenine, *cis*-2-[9-(6-aminopurinyl)]cyclopentanol, *cis*-2-[9-(6-aminopurinyl)]cyclohexanol, and 7-(β-D-ribofuranosyl)-adenine; to Dr. J. Fox of Sloan-Kettering Institute for N^6-methyladenosine; to Dr. G. M. Savage of Upjohn Co. for 9-(β-D-psicofuranosyl)adenine; to Dr. E. J. Reist of Stanford Research Institute for 9-(α- and -β-D-fructofuranosyl)adenine, 9-(β-D-xylofuranosyl)-adenine, and 9-(β-D-arabinofuranosyl)adenine; to Dr. C. A. Nichols of Roswell Memorial Park for tubercidin; and to Dr. N. Gerber for 3′-amino-3′-deoxyadenosine. Triacanthine was isolated from *Gleditsia triacanthos* (Leonard and Deyrup, 1962).

Results and Discussion

Adenosine analog models have been employed to ascertain the structural requirements for binding at the "active site" of calf intestinal adenosine deaminase. The models utilized in this present communication either maintain the integrity of the natural substituent at the 6 position of adenosine while the 9 position substituent was varied or vice versa. In this way the ambiguities involved in interpreting the results of previous studies on this enzyme where substitutions were made on the 6 and 9 positions simultaneously have been minimized (Schaeffer and Bhargava, 1965).

Seventeen position-9 analogs (Ia–q) and four position-6 analogs (IIa–d) were tested as either substrates or inhibitors of adenosine deaminase. The compounds studied were as follows:



Ia, R = D-ribose
b, R = H
c, R = 2′-deoxyribose
d, R = 3′-deoxyribose
e, R = 3′-amino-3′-deoxyribose
f, R = D-xylose
g, R = D-arabinose
h, R = D-psicose
i, R = β-fructofuranosyl
j, R = 2′-ribose phosphate
k, R = 3′-ribose phosphate
l, R = 5′-deoxyribose phosphate
m, R = *n*-hexyl
n, R = *n*-pentyl
o, R = *cis*-2-cyclopentanol
p, R = *cis*-2-cyclohexanol
q, R = cyclohexyl

IIa, X = $NHCH_3$
b, X = H
c, X = SH
d, X = Cl

A correlation of structure and activity of these compounds is shown in Table I. It is presumed that a compound which acts as a substrate or competitive inhibitor binds at the active site of the enzyme.

The data in Table I show that 9-purine ribonucleoside (compound IIb) is bound to the enzyme as an inhibitor, while adenine (compound Ib) is neither a substrate nor an inhibitor. Although adenine has the amino group in the 6 position, no binding to the enzyme occurs when the ribose moiety at the 9 position is absent. However, 9-purine ribonucleoside which has the ribose on the 9 position of the purine ring but no amino group at position 6 is bound to the enzyme as a strong competitive inhibitor ($K_i = 8.8 \times 10^{-6}$). When other changes were made on the 6 position [6-mercaptopurine ribonucleoside (compound IIc), N^6-methyladenosine (compound IIa)], these compounds were also competitive inhibitors. 6-Chloropurine ribonucleoside (compound IId) is even a substrate for adenosine deaminase. Inosine and chloride ions are the products of this reaction (Cory and Suhadolnik, 1965). These data, therefore, emphasize the importance of the 9-position substituent in bringing about binding of the substrate to the enzyme. It is of interest to note that Schaeffer *et al.* (1964) reported that the "isostearic nucleosides" with noncarbohydrate substituents at the 9 position but with a 6-hydrogen, 6-mercapto, 6-hydrazino, and 6-chloro substituent were noninhibitory. By altering the 6 and 9 positions simultaneously, Schaeffer and Bhargava (1965) concluded that the 6 position of the purine ring was critical for binding to the enzyme. Studies in our laboratory in which other model compounds were used (compounds IIa–d) provided data which lead to different conclusions. When compounds were used in which the substituent in the 9 position was ribose or deoxyribose, alteration of the 6 position substituent produced compounds that were bound to the enzyme and acted as competitive inhibitors or as a substrate in the case of the 6-chloropurine ribonucleoside. However, the 6-chloropurine compounds with the noncarbohydrate moiety as the 9 substituent were essentially noninhibitory (Schaeffer

TABLE I: Relative Initial Velocity of Adenosine Analogs and Per Cent Inhibition of Adenosine Deaminase.

	Compound	Relative Initial Velocity[a]	Inhibition[b] (%)	K_i $\times 10^5$
Ia	Adenosine	1.0	0	0
	9-Substituent change			
Ib	Adenine	0	0	0
Ic	9-(β-D-2-Deoxyribofuranosyl)adenine	1.2	0	0
Id	9-(β-D-3-Deoxyribofuranosyl)adenine	0.78	0	0
Ie	9-(β-D-3-Amino-3-deoxyribofuranosyl)adenine	0.46	0	0
If	9-(β-D-Xylofuranosyl)adenine	0.30	0	0
Ig	9-(β-D-Arabinofuranosyl)adenine	0.20	0	0
Ih	9-(β-D-Psicofuranosyl)adenine	0	0	0
Ii	9-(β-D-Fructofuranosyl)adenine	0	0	0
Ij	Adenosine 2′-monophosphate	0	0	0
Ik	Adenosine 3′-monophosphate	0	0	0
Il	Deoxyadenosine 5′-monophosphate	0	0	0
Im	9-(*n*-Hexyl)adenine	0	60	1.9
In	9-(*n*-Pentyl)adenine	0	45	5.0
Io	*cis*-2-[9-(6-Aminopurinyl)]cyclopentanol	0	20	
Ip	*cis*-2-[9-(6-Aminopurinyl)]cyclohexanol	0	6	
Iq	9-(Cyclohexyl)adenine	0	0	0
	6-Substituent change			
IIa	N^6-Methyladenosine	0	80	0.53
IIb	9-Purine ribonucleoside	0	78	0.88
IIc	6-Mercaptopurine ribonucleoside	0	12	37.0
IId	6-Chloropurine ribonucleoside	0.10		

[a] Reaction mixture contained substrate, 1.3×10^{-4} M; sodium phosphate buffer, *p*H 7.0, 0.05 M; and 0.021 unit of adenosine deaminase. [b] Assay mixtures contained adenosine, 1.3×10^{-4} M; inhibitor, 1.3×10^{-4} M; and 0.021 unit of adenosine deaminase in a final volume of 1.5 ml.

et al., 1964). These contrasting conclusions emphasize the importance of the proper choice of model compounds when studying enzyme-substrate binding.

Removal of hydroxy groups of the ribose of adenosine produced substrates that were deaminated at varying rates [2′-deoxyadenosine (compound Ic) > adenosine (compound Ia) > 3′-deoxyadenosine (compound Id) > 3′-amino-3′-deoxyadenosine (compound Ie)]. With monohydroxylated cyclopentyladenine or cyclohexyladenine derivatives, Schaeffer and Bhargava (1965) concluded that "the hydroxy group at C_2' makes a significant contribution to the binding, whereas the hydroxy group at C_3' and the hydroxymethyl group at C_4' make only a small contribution to the binding of this enzyme." The fact that 2′-deoxyadenosine is a better substrate than adenosine (Kalckar, 1947) tends to argue against the C_2' hydroxy group as being more important in the enzyme-substrate binding. Since 3′-deoxyadenosine and 3′-amino-3′-deoxyadenosine are both poorer substrates than either adenosine or 2′-deoxyadenosine, it is concluded that the C_3' hydroxy group plays an important role in the binding of the substrate to the enzyme.

In order to study the importance of the stereochemical configuration of the hydroxy groups on the furanosyl moiety on the 9 position of adenine, the rates of deamination of 9-(β-D-xylofuranosyl)adenine (compound If) and 9-(β-D-arabinofuranosyl)adenine (compound Ig) were compared with that of adenosine. With calf intestinal adenosine deaminase, xylofuranosyladenine was more rapidly deaminated than arabinofuranosyladenine (Table I). LePage and Junga (1965) recently reported that adenosine deaminase from transplanted tumors deaminated these two compounds at the same rate. The data as reported in Table I indicate that changes in the stereochemistry of the hydroxyl groups of the furanosyl ring result in compounds that are poorer substrates (compounds If, Ig) than those compounds in which the hydroxyl groups are removed and replaced by a hydrogen atom or an amino group (compounds Ic, Id, Ie). In all of the models studied, changes in the furanosyl ring substituted on the 9 position of adenine affect the rate of deamination by adenosine deaminase.

The importance of substituting an hydroxymethyl group for the hydrogen atom at the C_1' of the 9-position substituent for binding to the enzyme was studied by using the compounds psicofuranosyladenine (Ih) and β-fructofuranosyladenine (Ii). These compounds were neither substrates nor inhibitors of the

deaminase (Table I). These results indicate that the enzyme has a size specificity for the furanosyl ring at the 9 position of adenine. The β anomers are of interest here since this is the configuration needed for deamination of compounds by adenosine deaminase (Klenow, 1963).

To study the importance of the size and/or charge at the C_5' position of adenosine, adenosine and deoxyadenosine nucleotide monophosphates were studied (compounds Ij, Ik, Il). These three nucleotides were neither substrates nor inhibitors (Table I). These data show the importance of the size and/or charge at the C_5' substituent for binding at the enzyme site.

Since changes in the stereochemistry of the ribose moiety of adenosine produces substrates with varying rates of deamination by the enzyme, it was of interest to study the binding of those compounds that contain aliphatic substituents on position 9 of adenine. The models used in this study were the adenine derivatives having at the 9 position *n*-hexyl (Im), *n*-pentyl (In), cyclopentanol (Io), cyclohexanol (Ip), or cyclohexyl (Iq) (Table I). The results obtained with 9-cyclopentanoladenine and 9-cyclohexanoladenine are in agreement with the results of Schaeffer and Bhargava (1965). In addition, 9-*n*-hexyladenine and 9-*n*-pentyladenine were also competitive inhibitors; 9-cyclohexyladenine was neither a substrate nor an inhibitor. On the basis of the compounds studied (Im–Iq), it is difficult to predict a pattern of binding to adenosine deaminase for the noncarbohydrate substituents on adenine. For example, 9-cyclohexanoladenine (Ip) is an inhibitor, but 9-cyclohexyladenine (Iq) is neither a substrate nor an inhibitor. It cannot be that the hydroxy group is the only difference, since 9-*n*-hexyladenine (Im) and 9-*n*-pentyladenine (In) are inhibitors. It is possible that the solubility of these noncarbohydrate substituents in the bound water of the protein is also an important factor to consider.

The final three compounds employed in this study (III, IV, V) were included since they represent structures with substituents on the 3 and 7 positions of the purine ring as well as the 7-deazapurine ribonucleoside, tubercidin (compound V). These three compounds were neither substrates nor inhibitors for adenosine deaminase. Apparently the N^7 nitrogen of the adenine ring plays an important role in binding to the enzyme since substitution of a C–H for the N^7 nitrogen [tubercidin (4-amino-7β-D-ribofuranosyl-7*H*-pyrrolo[2,3-*d*]-pyrimidine) (V)] results in a compound that is not bound. That the substitution of a carbon atom for the nitrogen atom changes the chemical and physical properties of the purine ring have been shown by the differences in electrophoretic mobilities of tubercidin and adenosine (Acs *et al.*, 1964) as well as changes in the rate of hydrolysis of the *N*-riboside bond (Pike *et al.*, 1964). The fact that triacanthine (compound IV) and 7-ribofuranosyladenine (compound III) were neither substrates nor inhibitors might well be explained on the basis of the size of the 3 and 7 substituents (isopentenyl and ribofuranosyl) on the purine ring. However, it is more probable that a substituent is required at the 9 position. Evidence for this is the data obtained for adenine (compound Ib) (Table I).

A summary of the data in Table I indicates that a significant difference exists between the binding of those compounds altered in the two specific positions we have studied. Without exception, all of the 6-position substituents studied were bound to the enzyme (compounds IIa–d) either as competitive inhibitors or as substrates. However, it is important to note that seven of the analogs studied (Ib, Ih–l, Iq), where changes occur in the 9 position, were not bound to the enzyme. Hence, it is concluded that the binding of adenosine analogs by adenosine deaminase is more specific for the 9-position substituent than it is for the 6-position substituent. A question remaining is, "how must the 6-position substituent of adenosine be altered in order to prevent binding to the enzyme?" Additional 6-position analogs are currently being studied.

References

Acs, G., Reich, E., and Mori, M. (1964), *Proc. Natl. Acad. Sci. U.S. 52*, 493.

Chilson, O. P., and Fisher, J. R. (1963), *Arch. Biochem. Biophys. 102*, 77.

Coddington, A. (1962), *Biochim. Biophys. Acta 59*, 472.

Cory, J. G., and Suhadolnik, R. J. (1965), *Biochemistry 4*, 1733 (this issue; following paper).

Kalckar, H. M. (1947), *J. Biol. Chem. 167*, 462.

Klenow, H. (1963), *Biochim. Biophys. Acta 76*, 347.

Leonard, N. J., and Deyrup, J. A. (1962), *J. Am. Chem. Soc. 84*, 2148.

LePage, G. A., and Junga, I. G. (1965), *Cancer Res. 25*, 46.

Pike, J. E., Slechta, L., and Wiley, P. F. (1964), *J. Heterocyclic Chem. 1*, 159.

Schaeffer, H. J., and Bhargava, P. S. (1965), *Biochemistry 4*, 71.

Schaeffer, H. J., Marathe, S., and Alhs, V. (1964), *J. Pharm. Sci. 53*, 1368.

Weinbaum, G., Cory, J. G., Suhadolnik, R. J., Meyers, P., and Rich, M. A. (1964), *Proc. Intern. Congr. Biochem. 6th, New York, 1964, 4* (abstracts), 343.