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# COMPETITIVE INHIBITION OF ADENOSINE DEAMINASE BY UREA AND AMIDINE DERIVATIVES

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## SUMMARY

1. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) from calf intestinal mucosa is inhibited by urea and amidine derivatives.

2. The inhibition is reversible, competitive, and 1 or 2 molecules of inhibitor are involved in the inhibitory process.

3. The $K_i$ values for the *N*-alkyl-substituted ureas decrease with increasing chain length of the substituent, while the $K_i$ values for the amidine derivatives increase with their increasing $pK_a$. Only the uncharged form of the amidine derivatives inhibits the enzyme.

4. Kinetic evidence is reported which indicates that on adenosine deaminase there are two distinct sites, one of which binds the *N*-alkyl-substituted ureas, and the other one the uncharged form of the amidine derivatives.

5. The two sites are tentatively identified with the binding site of the purine moiety of the substrate and with an apolar region located near the binding site for the ribose moiety of adenosine.

6. The enzymes competitively inhibited by urea and amidine derivatives are proposed as models for the study of the specific binding between proteins and these denaturants.

## INTRODUCTION

Urea and guanidine at high concentrations are effective protein denaturants; the denaturation can be reversible or irreversible. Two general types of mechanisms were proposed for the denaturation of protein by these compounds. A possible action of the denaturants is through their effect on the solvent structure. The recent use of ultrasonic attenuation measurements as a probe of the microscopic solvent structure has provided strong evidence for the breakdown of the hydrogen-bonded structure of water by urea[1]. A second mode of action is through the specific binding of the denaturants to groups in the protein molecule, with a weakening of noncovalent bonds responsible for the maintaining of the native configuration[2].

Besides this denaturing effect, at relatively low concentrations urea and guanidine show a reversible inhibitory effect on many enzymes. The most extensive study on this subject is that of RAJAGOPALAN *et al.*[3] who studied a number of enzymes. According to their findings the inhibition is mostly of the competitive type for the enzymes which act on organic substrates, while the enzymes which act on inorganic substrates are noncompetitively inhibited. When the inhibition is competitive, 1 or 2 molecules of urea per molecule of enzyme are involved in the formation of the enzyme–urea complexes[3]. The competitive nature of the inhibition and the ratio of moles of inhibitor bound to moles of enzyme indicate that the inhibition results from interference with the formation of the appropriate enzyme–substrate complex. At this moment the exact nature of the interaction between enzyme and inhibitors and the relationship between the inhibition and the effectiveness of denaturation are unclear.

Adenosine deaminase from intestinal mucosa is irreversibly denatured by concentrations of urea greater than 6 M, while at nondenaturing concentrations urea and guanidine are competitive inhibitors with respect to adenosine[4,5]. It does not seem that the inhibition of adenosine deaminase by urea and guanidine can be directly correlated with their denaturing effect: in fact at pH 7.5 biuret and guanylurea, which are poor denaturing agents, are far more powerful competitive inhibitors[4]. To explain the differences observed in the inhibitive capacities of these compounds on adenosine deaminase the study was extended to other compounds of the urea–guanidine class.

The results obtained show that the inhibition constants for the *N*-alkyl-substituted ureas decrease with increasing chain length of the substituent. We have also seen that only the uncharged forms of the amidine derivative, guanidine, guanylurea, *O*-methylisourea, methyl- and aminoguanidine inhibit the enzyme, and the affinity of these compounds for the enzyme increases with the increase of their $pK_a$. Kinetic evidence was found which indicated that in or near the active site of adenosine deaminase there are two distinct sites, one of which binds the *N*-alkyl-substituted ureas, and the other one the uncharged form of the amidine derivatives.

## MATERIALS AND METHODS

Methyl-, 1,3-dimethyl-, ethyl-, propyl- and phenylurea, guanidine sulphate, methyl- and aminoguanidine sulphate, guanylurea sulphate and biuret were obtained from Eastman; urea, 1,1-dimethylurea and *O*-methylisourea sulphate were from Merck, Schuchardt and Aldrich Chemical Co., respectively. All the reagents were used without further purification. Adenosine deaminase (adenosine aminohydrolase, EC 3.5.4.4) was purified from calf intestinal mucosa. The enzymatic activity was determined spectrophotometrically by following the decrease of the absorbance at 265 m$\mu$ according to KALCKAR[6]. Enzyme, adenosine and inhibitors were dissolved in the appropriate buffer and the pH was corrected when necessary with KOH or HCl. Usually the enzyme activity was determined in 0.1 M potassium phosphate buffer (pH 7.5) at 20°. When the activity was followed as a function of pH, an acetate–phosphate–Tris buffer system was used in which all the substances were 0.1 M. The solutions of the inhibitors were prepared fresh daily.

## RESULTS

### *Reversibility of inhibition*

The reversibility of the inhibition by the reagents assayed was demonstrated by means of a procedure whereby the enzyme was exposed to a given concentration of inhibitor which was then lowered by dilution. Prolonged incubation (60 min) of adenosine deaminase, at pH 7.5 and 20°, in solutions containing the highest inhibitor concentration used, resulted in no detectable inactivation of the enzyme. When the inhibition was studied as a function of pH, the same procedure was used at each pH value tested. In no case was inactivation detected.

### *Inhibition by urea and N-substituted ureas*

The following N-substituted ureas were tested: urea, methyl-, 1,3-dimethyl-, 1,1-dimethyl-, ethyl-, propyl-, and phenylurea and semicarbazide. The effect of varying the adenosine and the inhibitor concentrations on the enzyme initial velocity was investigated. Lineweaver–Burk plots of the inhibition data indicated that all the compounds tested are formally competitive inhibitors with respect to adenosine. However when urea, methylurea and 1,3-dimethylurea inhibition data are formulated in a Dixon plot, curves are obtained instead of straight lines. A deviation of this type is observed when more than 1 molecule of inhibitor reacts with 1 molecule of enzyme[7]. It may also indicate a reaction between inhibitor and substrate; however if that was the case, a deviation from linearity in the Lineweaver–Burk plots[8] would be seen and this was not observed with the present data.

When the inhibition data are plotted according to JOHNSON *et al.*[9] the slopes of the lines obtained fall within the range 1.0 ± 0.1 for all the compounds with the exceptions of urea, methylurea and 1,3-dimethylurea, which give slopes of 1.85, 1.5 and 1.3, respectively. In Figs. 1 and 2 some of these plots are given.

The inhibition constants for the compounds tested were calculated graphically

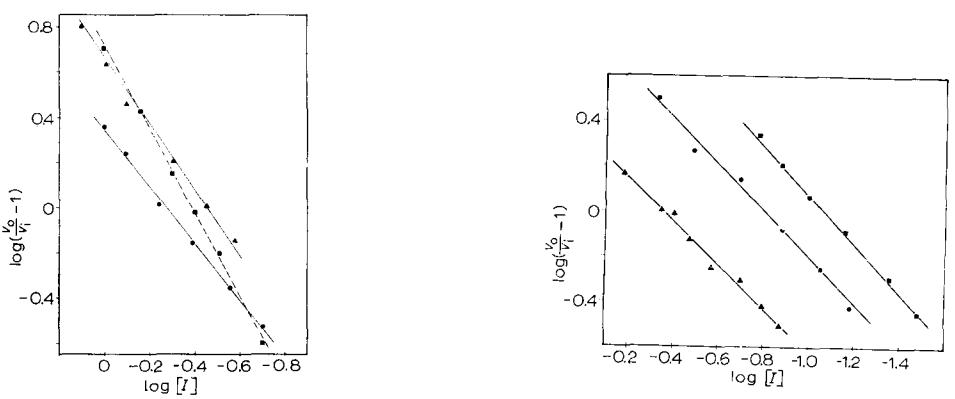



Fig. 1. JOHNSON–EYRING–WILLIAMS plot of urea (■), methylurea (▲) and 1,3-dimethylurea (●) inhibition data. The slopes of the lines are 1.85, 1.50 and 1.28 for urea, methylurea and 1,3-dimethylurea, respectively.

Fig. 2. JOHNSON–EYRING–WILLIAMS plot of 1,1-dimethylurea (▲), ethylurea (●) and propylurea (■) inhibition data. The slopes of the lines are 0.97, 1.0 and 1.05 for 1,1-dimethylurea, ethylurea and propylurea, respectively.

TABLE I

INHIBITION CONSTANTS FOR UREA DERIVATIVES

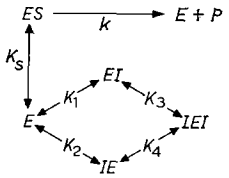
| *Urea derivative* | $1/K_i$* | $1/K_1K_3$** | $n$*** |
|---|---|---|---|
| Urea | 0.7 | 9.0 | 1.85 |
| Methylurea | 4.5 | 4.1 | 1.50 |
| 1,3-Dimethylurea | 3.5 | 1.2 | 1.30 |
| 1,1-Dimethylurea | 3.6 | — | 1 |
| Ethylurea | 13.0 | — | 1 |
| Propylurea | 22.0 | — | 1 |
| Phenylurea | 125.0 | — | 1 |
| Semicarbazide | 3.7 | — | 1 |

* For urea, methylurea and 1,3-dimethylurea the $1/K_1 + 1/K_2$ values as calculated from Eqn. 1 are reported. The reaction was carried out in 0.1 M potassium phosphate buffer (pH 7.5) at 20°.

** Calculated from Eqn. 1.

*** Slope of the JOHNSON–EYRING–WILLIAMS plot (see Figs. 1 and 2).

and are reported in Table I. The $K_i$ values for urea, methylurea and 1,3-dimethylurea as calculated in the Lineweaver–Burk plot, increase with increasing inhibitor concentrations[4]. In calculating the $K_i$ values for these compounds we have made the following assumptions: 2 molecules of the inhibitor bind adenosine deaminase, and only the enzyme with no inhibitor bound can form the enzyme–substrate complex. The following reaction scheme is proposed:




where $K_1K_3 = K_2K_4$.

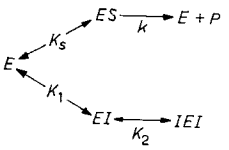
The general rate equation is

$$1/v = 1/V + \frac{K_m}{V} \cdot \frac{1}{[S]} \cdot \left(1 + \frac{[I]}{K_1} + \frac{[I]}{K_2} + \frac{[I]^2}{K_1K_3}\right) \quad (1)$$

Two particular cases of this general rate equation may be considered. If the binding of the first molecule of the inhibitor does not modify the affinity of the enzyme for the second one ($K_2 = K_3$ and $K_1 = K_4$), the rate equation becomes:

$$1/v = 1/V + \frac{K_m}{V} \cdot \frac{1}{[S]} \cdot \left(1 + \frac{[I]}{K_1} + \frac{[I]}{K_2} + \frac{[I]^2}{K_1K_2}\right) \quad (2)$$

If a compulsory order exists for the binding of the first and the second molecule of the inhibitor, the reaction scheme is:



and the rate equation becomes:

$$1/v = 1/V + \frac{K_m}{V} \cdot \frac{1}{[S]} \cdot \left(1 + \frac{[I]}{K_1} + \frac{[I]^2}{K_1 K_2}\right) \quad (3)$$

From Eqn. 1 only $1/K_1 + 1/K_2$ and $1/K_1K_3$ values can be calculated, while from Eqns. 2 and 3, $K_1$ and $K_2$ can be obtained. Owing to the impossibility of distinguishing among these three mechanisms in many cases, we prefer to report $1/K_1 + 1/K_2$ and $1/K_1K_3$ instead of $K_1$ and $K_2$ values (Table I). It is of interest to note that the $K_i$ values for urea cannot be calculated from Eqn. 2, while for methyl- and 1,3-dimethylurea two pairs of $K_i$ are obtained.

*Inhibition by amidine derivatives*

The following amidine derivatives were tested: guanidine, methylguanidine, aminoguanidine, guanylurea and *O*-methylisourea. All these compounds are competitive inhibitors with respect to adenosine, and the slopes of JOHNSON, EYRING AND WILLIAMS plots fall within the range of 1.0 ± 0.1. The study of the inhibition as a function of pH shows that the $K_i$ values for guanidine, guanylurea and *O*-methylisourea decrease with the increasing of pH (Fig. 3). The same phenomenon was observed

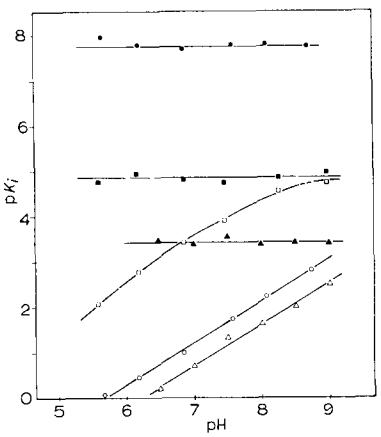



Fig. 3. p$K_i$ values for guanylurea (p$K_a$ 8.3), *O*-methylisourea (p$K_a$ 9.7) and guanidine (p$K_a$ 13.6) as a function of pH. The p$K_i$ values for guanylurea (□), *O*-methylisourea (△) and guanidine (○) are calculated by using total inhibitor concentration, while the p$K_i$ values for guanylurea (■) *O*-methylisourea (▲) and guanidine (●) are calculated by using the concentration of the deprotonated form.

for methylguanidine and aminoguanidine. The pH dependence of the inhibition constants suggests that only the uncharged form of the inhibitor can form the enzyme–inhibitor complex. A recalculation of $K_i$ based on the concentration of the uncharged form of the inhibitor instead of that based on the total inhibitor concentration shows that the $K_i$ values are essentially constant between pH 5 and 9 for guanidine, guanylurea and *O*-methylisourea (Fig. 3). The same is true for methyl- and aminoguanidine. The $K_i$ values obtained at pH 7.5 and those calculated on the concentration of the uncharged form are reported in Table II. In the same table the $K_i$ of biuret, 2,4,6-triaminopyrimidine and 2,6-diaminopurine are also reported. The

TABLE II

INHIBITION CONSTANTS FOR AMIDINE DERIVATIVES AND BIURET

| *Derivative* | $pK_a$* | $K'_i$ *(pH 7.5)*** *(M)* | $K_i$**** *(M)* |
|---|---|---|---|
| Guanidine | 13.6 | $2.0 \cdot 10^{-2}$ | $1.6 \cdot 10^{-8}$ |
| Methylguanidine | 13.4 | $3.6 \cdot 10^{-2}$ | $4.5 \cdot 10^{-8}$ |
| Aminoguanidine | 11.0 | $4.0 \cdot 10^{-2}$ | $1.3 \cdot 10^{-5}$ |
| *O*-methylisourea | 9.7 | $6.5 \cdot 10^{-2}$ | $4.1 \cdot 10^{-4}$ |
| Guanylurea | 8.3 | $1.4 \cdot 10^{-4}$ | $1.9 \cdot 10^{-5}$ |
| 2,4,6-Triaminopyrimidine | 6.8 | $4.0 \cdot 10^{-4}$ | $3.4 \cdot 10^{-4}$ |
| 2-Aminoadenine | 5.1 | $1.7 \cdot 10^{-4}$ | $1.7 \cdot 10^{-4}$ |
| Biuret | 4.0 | $3.7 \cdot 10^{-3}$ | $3.7 \cdot 10^{-3}$ |

* $pK_a$ of the most basic nitrogen (refs. 2 and 11).
** Calculated on the total inhibitor concentration.
*** Calculated on the concentration of the uncharged form of the inhibitor at pH 7.5.

$K_i$ of biuret, urea and N-substituted ureas, which have $pK_a$ values lower than 4.5, are constant between pH 5 and 9.

*Effect of ionic strength*

The effect of increasing the ionic strength on adenosine deaminase activity was also studied. In fact the ionic strength of the incubation mixtures increased when the amidine derivatives were tested. Potassium phosphate (pH 7.5), $Na_2SO_4$ and $NaNO_3$ at an ionic strength of 2 do not modify the $V$ and the $K_m$ for adenosine. Only the monovalent anions inhibit the adenosine deaminase in this order: $F^- > I^- > Br^- > Cl^-$ = acetate; the molar concentrations required for 50% inhibition with 0.06 mM adenosine are 0.12, 0.16, 1.1, 2.1 and 2.1 for $F^-$, $I^-$, $Br^-$, $Cl^-$ and acetate, respectively.

*Mixed inhibition studies*

From the data reported above the hypothesis that two different sites exist in or near the active site can be formulated. One site should have the highest affinity for the most basic compounds, the other one for the most hydrophobic compounds. To confirm this hypothesis mixed inhibition studies were carried out.

If two inhibitors (A and B) which combine reversibly with the free enzyme are simultaneously present, the slope of Lineweaver–Burk plots will increase by a factor of $(1 + [A]/K_{iA} + [B]/K_{iB})$ if both inhibitors act at the same site, and by a factor of $(1 + [A]/K_{iA}) \cdot (1 + [B]/K_{iB})$ if the two inhibitors act at independent sites[10].

For this kind of study we have chosen the urea and amidine derivatives which have the lowest molecular weight and slopes of 1 when plotted according to JOHNSON *et al.*[9]. The compounds used are 1,1-dimethylurea, ethylurea, guanidine and *O*-methylisourea.

When two inhibitors of the same class are simultaneously present (*O*-methylisourea and guanidine, Fig. 4, or 1,1-dimethylurea and ethylurea, Fig. 5), the slopes of the Lineweaver–Burk plots increased by a factor of $(1 + [A]/K_{iA} + [B]/K_{iB})$; when an amidine and an urea derivative are present simultaneously (Figs. 6 and 7)


Fig. 4. Mixed inhibition study with guanidine and *O*-methylisourea. Line A, no inhibitor; B, 0.022 M guanidine; C, 0.12 M *O*-methylisourea. To plot Line D, the points were obtained in the combined presence of 0.12 M guanidine and 0.022 M *O*-methylisourea, while the line was calculated from the slopes of Lines A, B and C on the basis of identical sites of action for guanidine and *O*-methylisourea. Line E was calculated on the basis of independent sites of action of the two inhibitors. The $K_i$ values for guanidine and *O*-methylisourea calculated from these data are $1.85 \cdot 16^{-2}$ M and $6.5 \cdot 10^{-2}$ M, respectively.

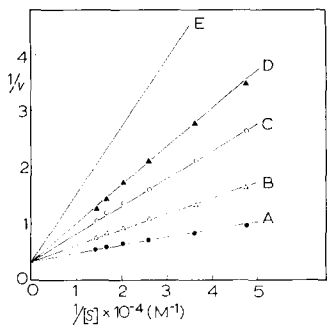
Fig. 5. Mixed inhibition study with ethylurea and 1,1-dimethylurea. Line A, no inhibitor; B (●), 0.2 M ethylurea; B (▲), 0.8 M 1,1-dimethylurea. Line C was calculated from the slopes of Lines A, B (●) and B (▲) on the basis of identical sites of action for ethylurea and 1,1-dimethylurea, while Line E was calculated on the basis of independent sites of action for the two inhibitors. Line D was obtained in the simultaneous presence of 0.2 M ethylurea and 0.8 M 1,1-dimethylurea. The $K_i$ values for ethylurea and 1,1-dimethylurea calculated from these data are 0.07 M and 0.27 M, respectively.


Fig. 6. Mixed inhibition study with 1,1-dimethylurea and guanidine. Line A, no inhibitor; B 0.65 M 1,1-dimethylurea; C, 0.07 M guanidine. Line D was calculated from the slopes of Lines A B and C on the basis of identical sites of action for 1,1-dimethylurea and guanidine while Line F was calculated on the basis of independent sites of action for the two inhibitors. Line E was obtained in the simultaneous presence of 0.65 M 1,1-dimethylurea and 0.07 M guanidine. The $K_i$ values for 1,1-dimethylurea and guanidine calculated from these data are 0.26 M and 0.02 M, respectively.

Fig. 7. Mixed inhibition study with *O*-methylisourea and 1,1-dimethylurea. Line A, no inhibitor; B, 0.4 M 1,1-dimethylurea; C, 0.28 M *O*-methylisourea. Line D was calculated from the slopes of Lines A, B, and C on the basis of identical sites of action for *O*-methylisourea and 1,1-dimethylurea. Line E points were obtained in the simultaneous presence of 0.28 M *O*-methylisourea and 0.4 M 1,1-dimethylurea, while the line was calculated on the basis of independent sites of action for the two inhibitors. The $K_i$ values for *O*-methylisourea and 1,1-dimethylurea calculated from these data are $6.7 \cdot 10^{-2}$ M and 0.28 M, respectively.

the slopes increased by a factor of $(1 + [A]/K_{iA}) \cdot (1 + [B]/K_{iB})$. These results indicate that the urea and amidine derivatives bind the enzyme at two independent sites.

These sites must be close to each other. In fact mixed inhibition studies using each inhibitor and 2,6-diaminopurine, which is a competitive inhibitor and a substrate analog, indicate that 2,6-diaminopurine and all the amidine and urea derivatives compete for the same site. Mixed inhibition studies with urea or guanylurea and 2,6-diaminopurine are reported in Figs. 8 and 9.



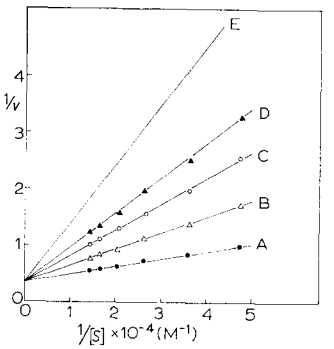




Fig. 8. Mixed inhibition study with 2,6-diaminopurine and urea. Line A, no inhibitor; B, 0.2 mM 2,6-diaminopurine; C, 0.5 M urea. To plot Line D, the points were obtained in the simultaneous presence of 0.2 mM 2,6-diaminopurine and 0.5 M urea, while the line was calculated from the slopes of Line A, B and C on the basis of identical sites of action for 2,6-diaminopurine and urea. Line E was calculated on the basis of independent sites of action of the two inhibitors. The $K_i$ value for 2,6-diaminopurine calculated from these data is $1.7 \cdot 10^{-4}$ M, while the apparent $K_i$ for urea is 0.18 M.

Fig. 9. Mixed inhibition study with 2,6-diaminopurine and guanylurea. Line A, no inhibitor; B, 0.2 mM 2,6-diaminopurine; C, 0.3 mM guanylurea. To plot Line D, the points were obtained in the simultaneous presence of 0.2 mM 2,6-diaminopurine and 0.3 mM guanylurea, while the line was calculated from the slopes of Line A, B and C on the basis of identical sites of action for 2,6-diaminopurine and guanylurea. Line E was calculated on the basis of independent sites of action of the two inhibitors. The $K_i$ values for 2,6-diaminopurine and for guanylurea calculated from these data are $1.7 \cdot 10^{-4}$ M and $1.25 \cdot 10^{-4}$ M, respectively.

## DISCUSSION

The different behaviors of the *N*-alkyl-substituted ureas and the amidine derivatives suggested that there are two different sites for the two inhibitor classes. The mixed inhibition studies confirmed this hypothesis. These two sites apparently are close to each other. We have tentatively identified these sites with the binding site of the purine moiety of the substrate and with an apolar region located near the binding site of the ribose moiety of adenosine.

The inhibition of adenosine deaminase by amidine derivatives shows a behavior which is similar in some respects to that observed with purine and pyrimidine bases. We have seen, in fact, that there is a relationship between the basicity of the N-1 atom of the purine nucleus and the affinity of adenosine deaminase for the purine bases and that the protonation of the N-1 atom of the purine ring and of the N-3 atom of pyrimidine ring results in noninhibitory compounds[11]. It is of interest to note that the $K_i$ values for some of the most basic amidine derivatives such as guanidine,

guanylurea, methyl- and aminoguanidine, which are structurally quite different from the substrate, are of the same order as or lower than those observed for the purine bases or for the ribosides[11]. However the basicity of the compounds does not seem to be the only factor responsible for the binding to this site, in fact other basic compounds such as $NH_3$, tetramethyl-ammonium chloride, glycine, arginine, lysine, piperidine ($K_i > 0.1$ M), piperazine ($K_i > 0.1$ M), ethylamine ($K_i = 0.7$ M) and imidazole ($pK_a$ 7; $K_i$ $2.5 \cdot 10^{-2}$ and $5 \cdot 10^{-2}$ $M$ at pH 8 and 7, respectively) do not inhibit or are weaker inhibitors than would be expected from their basicity. All the basic acyclic inhibitors, which have the highest affinity for the enzyme, are unsaturated, contain at least one $-NH_2$ and one $=NH$ group, and must be uncharged molecules.

The results obtained with *N*-alkyl-substituted ureas indicate that the affinity of these compounds for the enzyme increases with increasing chain length of the substituent. A similar behavior has been observed with aliphatic alcohols[12] and 9-alkyladenines[13]. Aliphatic alcohols competitively inhibit the enzyme, and the $K_i$ values for methanol, ethanol, propanol and butanol are 1.2, 0.8, 0.5 and 0.3 M, respectively[12]. SCHAEFFER AND VOGEL[13] found that the inhibitory effectiveness of the 9-alkyladenines increases with the increasing of the alkyl chain. They also found that the adenine derivatives bearing a short hydroxyalkyl chain (2 or 3 carbon atoms) at the 9 position are more powerful inhibitors than the corresponding 9-alkyl derivatives, while the compounds with a longer hydroxyalkyl chain (4 or 5 carbon atoms) are weaker inhibitors than the corresponding 9-alkyl derivatives. From their results it appears that an apolar region exists near the active site of the enzyme, and a hydrophilic region is located between this hydrophobic region and the region of the active site occupied by the N-9 atom of the purine nucleus. The *N*-alkyl-substituted ureas probably bind at this apolar region with hydrophobic bonds. However, some evidence indicates that the urea moiety of the inhibitors is also involved in the binding. In fact the $K_i$ values for the aliphatic alcohols are higher than the $K_i$ values for the *N*-alkyl-substituted ureas. If we compare the $K_i$ of ethylurea (0.08 M) with those of ethanol (0.8 M), and ethylamine (0.8 M, 0.7 M, 0.8 M at pH 6, 7 and 8, respectively), we can see that ethylurea is the best inhibitor. Phenylalanine is a competitive inhibitor with a $K_i$ (0.1 M) higher than phenylurea.

The presence of hydrophobic regions in or near the active site of adenosine deaminase was proposed to explain the low reactivity with the alkylating reagents of an –SH group which is essential for the activity[14]. The specificity of adenosine deaminase towards the 9-substituted adenines can also be explained by assuming the presence of such a hydrophobic region. The ribose-binding site has a bulk tolerance concerning the presence and the stereochemistry of hydroxyl groups of the pentose moiety of the substrate. In fact 2′- or 3′-deoxyadenosine, 9-(β-D-xylofuranosyl)-, 9-(5-deoxy-β-D-xylofuranosyl)- and 9-(β-D-arabinofuranosyl)adenine are substrates for the enzyme[15,16]. However 9-(β-D-fructofuranosyl)- and 9-(β-D-psicofuranosyl)-adenine and 2′-, 3′- or 5′-AMP are neither substrates nor inhibitors[15]. It is possible that the increased size of the substituent in the 9 position excludes these compounds from the active site. However the adenine derivatives in which a relatively large apolar substituent is present in the 9 position as 9-(*m*- or *p*-phenoxycarbonylaminobenzyl)adenine and 9-(*m*- or *p*-bromoacetamidobenzyl)adenine, are inhibitors[17].

The different behaviors of the compounds reported above can be explained by assuming that the hydrophilic binding site for the ribose moiety of the substrate is

located in a hydrophobic region. This region which delimits the ribose-binding site would orient the ribosyl radical, freely rotating around $N^9$–$C^1$ bond, toward its binding site. It is also likely that a hydrophobic environment, by excluding the aqueous solvent, will strengthen the electrostatic bonds (hydrogen bonds, dipole–dipole interaction, *etc.*) formed between the substrate and the enzyme[18]. The adenine derivatives with hydrophilic substituents in the 9 position, which owing to their structure cannot be oriented towards the ribose binding site, are excluded from the binding.

The microscopic heterogeneity of the protein surface (region of low dielectric constant adjacent to or delimited by regions of high dielectric constant) which has been taken into consideration in explaining the enzyme catalysis[18], can also be important, at least in some cases, in determining the enzyme specificity.

The denaturation mechanism of protein by reagents of urea and amidine classes probably involves both the specific binding between protein and denaturant and the breakdown of solvent structure[19]. From the data of GORDON AND JENCKS[2], who have studied the effectiveness of many protein denaturants on bovine serum albumin and ovalbumin, it appears that more than one kind of binding site on the proteins must be postulated. In fact no correlation can be demonstrated between the hydrogen-bonding ability (as either hydrogen donor or acceptor) of the denaturants and their effectiveness; bifunctional hydrogen bonding may explain this effectiveness, but it is necessary to postulate more than a single type of such bifunctional hydrogen bonding to explain the action of all the active compounds. In the case of ovalbumin there is a significant contribution of the hydrophobic character of the denaturants to their denaturing capacity, which is not observed for bovine serum albumin.

The enzymes competitively inhibited by reagents of urea and amidine classes are useful models for the study of the specific sites of binding for these denaturants. In fact the inhibition by urea and amidine derivatives is observed at concentrations of denaturants lower than those which produce alteration of the local water structure around macromolecules[19]; furthermore few molecules (1 or 2) are involved in the inhibitory process.

In the case of adenosine deaminase the kinetic study of the inhibition by urea and amidine derivatives has shown that two different kinds of binding sites for these compounds are present on the protein: one site has the highest affinity for the most basic compounds and the other one for the most hydrophobic denaturants.

Further studies are in progress to elucidate the factors underlying the specificity of binding and the related chemical mechanisms.

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