Kraut, J., Robertus, J. D., Birktoft, J. J., Alden, R. A., Wilcox, P. E., and Powers, J. C. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 117–123.
Krieger, M., Kay, L. M., and Stroud, R. M. (1974), *J. Mol. Biol. 83,* 209–230.
LéJohn, H. B. (1971), *J. Biol. Chem. 246,* 2116–2126.
Levy, H. R., and Vennesland, B. (1957), *J. Biol. Chem. 228,* 85–96.
Long, G. L., and Kaplan, N. O. (1973), *Arch. Biochem. Biophys. 154,* 711–725.
Moras, D., Olsen, K. W., Sabesan, M. N., Buehner, M., Ford, G. C., and Rossmann, M. G. (1975), *J. Biol. Chem. 250,* 9137–9162.
Ohlsson, I., Nordström, B., and Brändén, C. I. (1974), *J. Mol. Biol. 89,* 339–354.
Polgár, L. (1977), *Int. J. Biochem. 8,* 171–176.
Popják, G. (1970), *Enzymes, 3rd. Ed. 2,* 115–215.
Rao, S. T., and Rossmann, M. G. (1973), *J. Mol. Biol. 76,* 241–256.
Robertus, J. D., Alden, R. A., Birktoft, J. J., Kraut, J., Powers, J. C., and Wilcox, P. E. (1972), *Biochemistry 11,* 2439–2449.
Rossmann, M. G., Adams, M. J., Buehner, M., Ford, G. C., Hackert, M. L., Lentz, P. J., Jr., McPherson, A., Jr., Schevitz, R. W., and Smiley, I. E. (1971), *Cold Spring Harbor Symp. Quant. Biol. 36,* 179–191.
Rossmann, M. G., and Argos, P. (1975), *J. Biol. Chem. 250,* 7525–7532.
Rossmann, M. G., Liljas, A., Brändén, C. I., and Banaszak, L. J. (1975), *Enzymes, 3rd Ed. 11,* 61–102.
Rossmann, M. G., Moras, D., and Olsen, K. W. (1974), *Nature (London) 250,* 194–199.
Rühlmann, A., Kukla, D., Schwager, P., Bartels, K., and Huber, R. (1973), *J. Mol. Biol. 77,* 417–436.
Segal, D. M., Powers, J. C., Cohen, G. H., Davies, D. R., and Wilcox, P. E. (1971), *Biochemistry 10,* 3728–3738.
Tarmy, E. M., and Kaplan, N. O. (1968), *J. Biol. Chem. 243,* 2579–2586.
White, J. L., Hackert, M. L., Buehner, M., Adams, M. J., Ford, G. C., Lentz, P. J., Jr., Smiley, I. E., Steindel, S. J., and Rossmann, M. G. (1976), *J. Mol. Biol. 102,* 759–779.

Influence of Substituent Ribose on Transition State Affinity in Reactions Catalyzed by Adenosine Deaminase†

Richard Wolfenden,* David F. Wentworth, and Gordon N. Mitchell

ABSTRACT: Adenosine deaminase from calf intestine hydrolyzes adenine at a limiting rate four orders of magnitude lower than that for adenosine, while K_m values for these substrates are about the same (Wolfenden, R., et al. (1969), *Biochemistry 8,* 2412–2415). Reactivity of 6-substituents, toward nucleophilic displacement, is found to be affected only slightly by removal of ribose as a 9-substituent, in model reactions. Substituent ribose thus appears to stabilize, selectively, the transition state for enzymatic deamination. In contrast with the small influence of substituent ribose on the apparent binding affinity of substrates, removal of substituent ribose from a potential transition state analogue, 1,6-dihydro-6-hydroxymethylpurine ribonucleoside, results in a lowering of its affinity for the enzyme by several orders of magnitude. The synthesis of the analogue and related compounds is described, and their properties compared with those of other photoadducts and of the naturally occurring inhibitors covidarabine and coformycin. Binding of these inhibitors is found to result in the appearance of ultraviolet-absorbing bands in the neighborhood of 323 nm.

Hydrolytic deaminases, that act on adenosine and related compounds, also catalyze the hydrolytic removal of a variety of leaving groups other than the normal product, ammonia (Cory and Suhadolnik, 1965). That such disparate leaving groups as ammonia and chloride are hydrolyzed from purine ribonucleosides at similar limiting rates, despite vast differences in the stability of their bonds to carbon, provided an early indication that the rate of reaction was at least partly determined by some step other than cleavage of the scissile bond in substrates (Wolfenden, 1966; Bär and Drummond, 1966). It was at first believed that this step might be the hydrolysis of a common intermediate, formed as a result of displacement of the leaving group by enzyme, but it was later found that the rate of this reaction was hardly affected by substituting deuterium oxide for substrate water (Wolfenden, 1969). This suggested that the transition state might be reached very early during the reaction, and a mechanism involving direct water attack on the substrate (Figure 1) was considered as a possibility. A route to a very approximate analogue of a tetrahedral intermediate, formed by water attack on the substrate adenosine, was provided by photoaddition of methanol to purine ribonucleoside, and the adduct proved to be a strong inhibitor of deaminase activity, as described in a preliminary communication (Evans and Wolfenden, 1970).

Pteridine, earlier shown to be covalently hydrated in aqueous solution (Perrin, 1962; Albert et al., 1966), had been found to be a good inhibitor of adenosine deaminase, consistent with the activity of the enzyme on 4-aminopteridine as a substrate (Wolfenden et al., 1969). When an attempt was made to compare pteridine and its hydrate as inhibitors, the enzyme proved unexpectedly to be a *catalyst* of the reversible hydration of pteridine (Evans and Wolfenden, 1972). The hydratase activity of the enzyme provided support for a mechanism in-

† From the Department of Biochemistry, University of North Carolina, Chapel Hill, North Carolina 27514. *Received June 20, 1977.* Supported by Grant Number GM-18325 from the National Institutes of Health, United States Public Health Service.

TABLE I: Effect of Ribose Substituents on Nonenzymatic Reactivity of Purine Derivatives.

Nucleophile	k (M^{-1} min^{-1})		
	9-Ribofuranosyl-6-chloropurine	6-Chloropurine	6-Chloro-9-methylpurine
Glutathione[a]	2.5 ($\Delta\epsilon_M = 1.7 \times 10^4$)	0.51[b] ($\Delta\epsilon_M = 1.5 \times 10^4$)	0.64 ($\Delta\epsilon_M = 1.7 \times 10^4$)
Hydroxylamine[c]	0.025 ($\Delta\epsilon_M = 3.9 \times 10^3$)	0.0096 ($\Delta\epsilon_M = 3.5 \times 10^4$)	

[a] At 290 nm, 25 °C, ionic strength 0.5, pH 8.46, for 0.04 M glutathione buffer at 25 °C, ionic strength 0.5. [b] Calculated from the rate constant (0.087 M^{-1} min^{-1}), obtained at 0.30 M glutathione, by correcting for the fraction of 6-chloropurine in the anionic form (presumably unreactive) using the pK_a of 7.8 observed at 25 °C and ionic strength 0.5. [c] At 290 nm, 45 °C, ionic strength 4.0, pH 6.10, for 0.5 M hydroxylamine buffer at 25 °C.

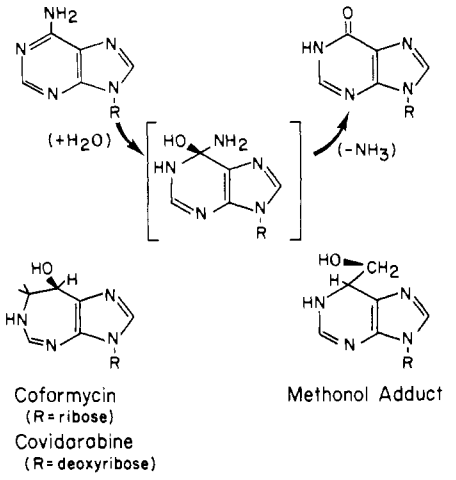

FIGURE 1: Proposed mechanism of action of adenosine deaminase, and structures of the inhibitory methanol photoadduct and of the antibiotics coformycin and covidarabine.

volving direct water attack on substrates for hydrolysis, especially when it was found that the rates of pteridine hydration and of 4-aminopteridine hydrolysis were comparable, when catalyzed by the enzyme. The hydration reaction was somewhat the more rapid of the two reactions, consistent with the hypothesis that it might be an analogue of a partial reaction in the normal hydrolytic process. Not unexpectedly, the hydration reaction was found to be stereospecific, the hydrate being a more potent inhibitor than pteridine itself (Evans and Wolfenden, 1973). Further support for this mechanism was provided by the discovery of the structures of two antibiotics, covidarabine (Woo et al., 1974) and coformycin (Ohno et al., 1974; Nakamura et al., 1974), that inhibit the enzyme very strongly indeed (Cha et al., 1975) and bear an evident structural resemblance to the hypothetical adduct (Figure 1).

The action of adenosine deaminases is anomalous in being insensitive to fundamental changes in the structures of substrates at the sites where reaction occurs, but profoundly sensitive to the presence or absence of substituent ribose at the 9-position. The kinetic parameter affected is V_{max}, whereas K_m changes very little (Wolfenden et al., 1969). Structural changes in a substrate that alter the catalytic effectiveness of an enzyme are expected to produce corresponding effects on the binding of inhibitors that resemble activated intermediates in catalysis (Wolfenden, 1970). Adenosine deaminase seemed to provide an opportunity for exploring this relationship, by comparing the effect of substituent ribose on enzymatic and nonenzymatic reactions, and on the relative binding affinity of substrates and potential transition state analogues. It also seemed desirable to examine the effects of varying structure on the effectiveness of synthetic inhibitors, and to compare the stoichiometry of binding of synthetic and naturally occurring inhibitors.

Results

Nonenzymatic Model Reactions. It was previously suggested that the difference in enzymatic deamination rates between adenosine and adenine might result, at least in part, from the electron-withdrawing character of ribose (Wolfenden et al., 1969). To test this possibility, 6-chloropurine and the corresponding ribonucleoside were compared with respect to their susceptibility to nucleophilic attack by a sulfur nucleophile (glutathione) and a nitrogen nucleophile (hydroxylamine). Observed second-order rate constants for glutathione attack on these two substrates, under identical conditions, differed considerably (Table I). The observed pK_a of 6-chloropurine (7.8 under these conditions of temperature and ionic strength) is such that this compound is largely present as a monanion at the pH at which glutathione attack was investigated. The corresponding ribonucleoside remains uncharged at normal pH values. Correcting for the proportion of 6-chloropurine present as the neutral species (presumably more reactive than the monanion), the calculated rate constant for glutathione attack on this species was fivefold lower than that for attack on the ribonucleoside, and 1.2-2-fold lower than that for attack on 6-chloro-9-methylpurine (Table I). This correction was unnecessary in reactions with hydroxylamine, where attack could be studied at a pH value (6.1) at which 6-chloropurine was present almost entirely as the uncharged species. In this case nucleophilic attack on 6-chloropurine was found to be 2.5-fold slower than attack on the corresponding ribonucleoside (Table I). These results show that the effect of substituent ribose on the nonenzymatic reactivity of 6-chloropurine is real but modest, and comparable with the 4-fold effect of ribose in lowering the K_a value of the adenine cation (Jordan, 1955). It seems reasonable to conclude that adenine and adenosine are unlikely to differ much in their susceptibility to nucleophilic substitution at the 6-position.

In another experiment it was found that adenosine fails to undergo reaction with glutathione at detectable rates at 25 °C and concentrations of glutathione buffer (pH 8.77) as high as 1.0 M. Reaction would have been detectable if it had occurred with a rate constant 5000 times lower than that for the reaction of 9-ribofuranosyl-6-chloropurine with glutathione.

Inhibitor Binding. The large V_{max} observed for the action of adenosine deaminase on adenosine (as compared with adenine) (Wolfenden et al., 1969), and the slight difference between these substrates in their expected rates of nonenzymatic reaction, suggest that adenosine is very much more tightly bound than adenine in the transition state for enzymatic deamination. It might be expected that this difference would

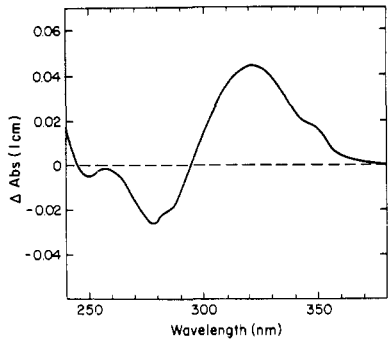

FIGURE 2: Difference spectrum obtained by mixing adenosine deaminase (final concentration 2.6 mg/mL) and 1,6-dihydro-6-hydroxymethylpurine ribonucleoside (final concentration 1.5×10^{-4} M). The reference cuvette contained the unmixed components in separate compartments.

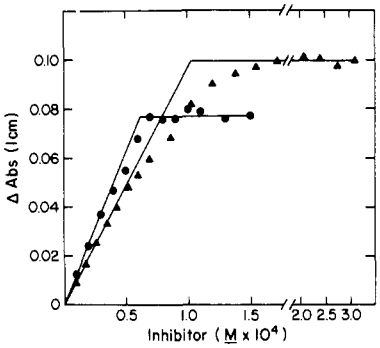

FIGURE 3: Spectrophotometric titration, at 320 nm, obtained by adding 1,6-dihydro-6-hydroxymethylpurine ribonucleoside (▲) or covidarabine (●) to a solution containing adenosine deaminase (5.6 mg/mL) in the presence of potassium phosphate buffer (0.05 M, pH 7.0).

be reflected in the binding of stable analogues of the altered substrates in the transition state. To test this hypothesis, the inhibitory properties of 1,6-dihydro-6-hydroxymethylpurine, prepared by the method of Linschitz and Connolly (1968), were compared with those for the 9-ribonucleoside, a potent inhibitor of adenosine deaminase (Evans and Wolfenden, 1970). Using 4-aminopteridine as the substrate, initial velocities were conveniently obtained by following the decrease in absorbance at 350 nm, where the absorbance of high concentrations of inhibitor was of no practical consequence. Inhibition by II was found to be competitive and the K_i value observed for 1,6-dihydro-6-hydroxymethylpurine (1.3×10^{-3} M) was ~1800-fold higher than the K_i (7.6×10^{-7} M) observed for the corresponding ribonucleoside (Evans and Wolfenden, 1970).

Covidarabine, coformycin, and 1,6-dihydro-6-hydroxymethylpurine ribonucleoside were each found to produce difference spectra when mixed with calf intestinal adenosine deaminase. Figure 2 shows the difference spectrum observed with the synthetic inhibitor, comparing a mixed sample of enzyme and inhibitor with the unmixed components present in the individual compartment of a tandem cuvette. There was no appreciable time lag in the appearance of the difference spectra, which were qualitatively similar for each of the inhibitors and showed a pronounced shoulder in the neighborhood of 320–330 nm. Spectrophotometric titration of enzyme showed a behavior consistent with moderately tight binding of the synthetic inhibitor (with enzyme present at a concentration approximately two orders of magnitude above K_i), and very tight binding of covidarabine (Figure 3). Virtually identical titration curves were observed for covidarabine and for coformycin (not shown), and the end point corresponded to

TABLE II: Inhibition of Adenosine Deaminase by Photoadducts of Purine Ribonucleoside.

Inhibitor[a]	Obsd rate (% of control)[b]
None	100
Purine ribonucleoside (I)	80
CH_3OH adduct (II)	24
CH_3OH di-adduct (II′)	84
6-Hydroxymethylpurine ribonucleoside (III)	81
C_2H_5OH adduct	98
$CH_3CHOHCH_3$ adduct	98
CH_3NH_2 adduct	98
CH_3ONH_2 adduct	85
CH_3NHOH adduct	67
CH_3SH adduct	98
HCHO adduct	98
HCOOH adduct	67
$HCONH_2$ adduct	83

[a] Adducts prepared by photolysis of purine ribonucleoside with the appropriate addend as the neat compound or in aqueous solution, as for methanol (see text). In each case addition was allowed to proceed essentially to completion, as judged by monitoring the absorbance of starting material at 264 nm. [b] Measured with adenosine (10^{-4} M); inhibitor concentration = 10^{-5} M.

approximately 2.5 mol of enzyme per mol of inhibitor, based on a molecular weight of 35 000 (cf. Zielke and Suelter, 1971) and an extinction coefficient of 0.916 mg^{-1} mL^{-1} at 280 nm (see Experimental Section). It was previously demonstrated that adenosine deaminase does not appear to contain impurities detectable by sedimentation or gel electrophoresis (Wolfenden et al., 1968), and the homogeneity of the present preparation was also established by electrophoresis in sodium dodecyl sulfate (see Experimental Section). The results suggested that inactive protein was present in deaminase preparations, but remained undetected by other analytic procedures. In view of the finding (see below) that the synthetic inhibitor consists of a mixture of diasteromers, it was of interest to compare the stoichiometry of apparent binding of 1,6-dihydro-6-hydroxymethylpurine ribonucleoside with values obtained for covidarabine and coformycin, antibiotics of natural origin that exist in a single stereochemical configuration (Woo et al., 1974; Nakamura et al., 1974). As shown in Figure 3, an approximate end point was reached with the synthetic inhibitor at a concentration about 60% greater than that with covidarabine.

In other experiments, products obtained by photoreaction of purine ribonucleoside with compounds other than methanol were examined for possible inhibitory effects on adenosine deaminase. As shown in Table II, these compounds tended to be less effective than the starting material.

Products of Photoaddition. The methanol photoadduct of purine ribonucleoside is a strong inhibitor of adenosine deaminase (Evans and Wolfenden, 1970), and the corresponding triacetate is an intermediate in the synthesis of the antibiotic coformycin (Ohno et al., 1974), also a powerful inhibitor. Photolysis in the presence of air resulted in three products separable by thin-layer chromatography (Evans and Wolfenden, 1970). The order of elution was I (purine ribonucleoside), III, II, and II′, and II and II′ were considered to be diastereomers (Evans and Wolfenden, 1970) of which II was very much the more potent inhibitor. Recently Ohno et al. (1974) reported that their photolysis of the triacetate of I resulted in a product that was "considered to be free of isomeric impurities", and concluded that photoaddition of methanol was stereospecific, based on the stereochemical purity of the

product obtained by photolysis in methanol under argon. This prompted our reinvestigation of the photolysis of I in the presence and absence of air.

Thin-layer chromatographic analysis at timed intervals, of the photolysis of I in methanol in the presence of air, showed that II appears first, followed by III and II′. When photoaddition was carried out under nitrogen, only one product was detected by thin-layer chromatography. This corresponded to the II reported earlier, and by comparison of nuclear magnetic resonance and ultraviolet spectra with those reported by Linschitz and Connolly (1968) was undoubtedly 9-β-D-ribofuranosyl-6-hydroxymethyl-1,6-dihydropurine.

It appeared that these observations might be explained by the sequence of events outlined in Figure 4. According to this scheme, photoaddition of methanol to III would form 9-β-D-ribofuranosyl-6,6-di(hydroxymethyl)-1,6-dihydropurine, and the structure originally proposed for II′ (Evans and Wolfenden, 1970) was incorrect. Linschitz and Connolly (1968) had reported that 6-methylpurine undergoes photoaddition alcohol, presumably to yield 6-hydroxyalkyl-6-methyl-1,6-dihydropurine. The formulation of II′ as a 6-disubstituted compound also appeared consistent with the very weak inhibition observed for this compound in comparison with II, which might be understood in terms of its greater steric requirements.

To confirm this hypothesis, II was prepared under nitrogen, and excess methanol was removed by evaporation. Photolysis of II in water in the presence of air gave the expected III in about 80% yield (as indicated by nuclear magnetic resonance analysis), and also small quantities of I and formic acid. When III was subjected to photolysis in methanol under nitrogen, the major product was II′ as demonstrated by thin-layer chromatography, and the NMR[1] spectrum of his product confirmed the structure as indicated in Figure 4. One apparent discrepancy in this identification is the mass spectrum originally reported, with a parent peak at $m/e = 284$. However, the parent peak of a primary or secondary alcohol is often found to be small, and intramolecular hydrogen bonding of the methylene hydroxyls to ring nitrogens as reported by Linschitz and Connolly (1968) might be expected to facilitate transfer of hydride to the molecular ion and elimination of CH_2O to give a fragment with $m/e = 284$. Analogous reversal of the radical alkylation would explain the peroxide-catalyzed conversion of II or II′ to III as reported earlier (Evans and Wolfenden, 1970). Similar effects have been observed in a series of hydroxymethylene aminosteroids (Longevialle et al., 1971).

In order to determine whether formation of II was stereospecific, as had been suggested for the triacetate by Ohno et al. (1974), II was examined by ^{13}C NMR. The longer relaxation times of the purine carbons resulted in their saturation, so that essentially the only carbon atoms observed were those of the ribose and the 6-hydroxymethylene group. Each carbon of the ribose moiety showed two very close peaks of nearly equal intensity, with shift differences of 0.068–0.122 ppm. The 6-hydroxymethylene carbon showed two peaks of equal intensity at 79.90 and 78.05 ppm. This demonstrates that photoaddition of methanol produces nearly equal amounts of both possible diastereomers. Although it was not possible to separate them, presumably one diastereomer is more effective than the other as an inhibitor, since the enzyme is stereoselective in its catalysis of the hydration of pteridine (Evans and Wolfenden, 1973). Presumably photoaddition of methanol to the triacetate of I is also not stereospecific. Ohno et al. (1974) reported that their product corresponded to the natural isomer of coformycin, but the yield was only 35%. This allows for the possibility of fractional separation of the unnatural isomer at some point in the synthetic sequence.

Discussion

The action of adenosine deaminases is anomalous in being insensitive to fundamental changes in the structure of substrates at the sites at which reaction occurs. The rate of reaction is little affected by replacement of the leaving group ammonia by such different leaving groups as chloride or methoxide, nor is the rate appreciably altered by substitution of deuterium oxide for substrate water (Wolfenden, 1969). The rate of reaction is profoundly sensitive, on the other hand, to the presence or absence of substituent ribose at the 9-position. The kinetic parameter affected is V_{max}, whereas K_m changes very little (Wolfenden et al., 1969).

Analogues of intermediates in catalysis, to the extent that they share the special binding characteristics of the altered substrates in the transition state, are expected to be bound by enzymes in a way that reflects the structural requirements of substrates for efficient catalysis. It is therefore of special interest that the affinity of adenosine deaminase for 1,6-dihydro-6-hydroxymethylpurine ribonucleoside is reduced by a factor of 1800 when hydrogen replaces ribose as a 9-substituent.

The actual nature of the rate-determining step or steps in enzymatic deamination has proven to be elusive. Many substrates are hydrolyzed at a rate that approaches the upper limit observed with adenosine, but a few compounds fall far short of this limit. Thus *lin*-benzoadenosine and *lin*-benzoadenine are hydrolyzed at rates closely similar to that of adenosine despite substantial differences in structure and probable intrinsic reactivity (Leonard et al., 1976). In another series, it is found that the deamination of 4-aminopteridine, the hydration of pteridine and the dehydration of pteridine hydrate are all catalyzed by calf intestinal adenosine deaminase at limiting rates that differ by a factor of less than 4, although these rates are substantially lower than those observed in the first set (Evans and Wolfenden, 1973).

It seems likely that these reactions may be limited by an early transition state, that precedes substantial chemical alteration of the substrate.[2] The competitive inhibition observed with inosine, against the substrate adenosine, tends to rule out the possibility that isomerization of the free enzyme may be rate determining. If rate-determining isomerization occurred in the enzyme–substrate complex, before the substrate underwent much chemical transformation, it would not be surprising if the rate showed some "muffled" sensitivity to changing substrate structure, and indeed minor differences in reaction rates are observed when the leaving group is varied (Wolfenden, 1969).

[1] Abbreviation used: NMR, nuclear magnetic resonance.

[2] If an enzyme reaction is limited in rate by some step such as isomerization of free enzyme, isomerization of an enzyme–substrate complex without chemical transformation of the substrate, or product release, then the reaction rate would not be expected to bear a simple relationship to stereoelectronic events occurring during the catalytic process. The rate-determining step has no counterpart in the nonenzymatic reaction, and semantic difficulties arise in considering the possible nature of a "transition state analogue". However, it appears that in such cases the activated complex in substrate transformation must be stabilized to an extent even greater than the rate comparison suggests, and thus analogues of highly activated intermediates in substrate transformation should be even more strongly inhibitory than is indicated by the usual scheme for expressing transition state affinity (Wolfenden, 1972, 1976).

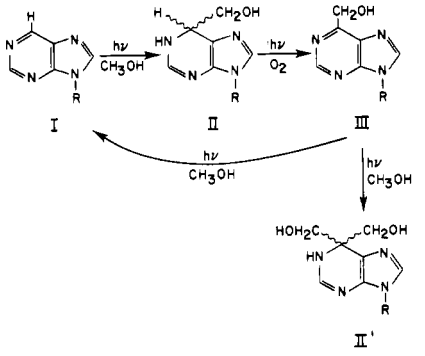

FIGURE 4: Reactions occurring during photolysis of purine ribonucleoside (I). In the absence of air, product II accumulates, whereas further reactions occur in the presence of air.

The weak activity of the enzyme on adenine is consistent with a specific substituent requirement at the 9-position in the transition state. Other substituents have been shown to replace ribose with varying success: 9-(2-hydroxymethoxymethyl)-adenine, for example, appears to be bound about 4-fold less tightly than adenosine, and is deaminated at a limiting rate 1.4% that of adenosine (Schaeffer et al., 1971). The hydroxyl group of this substrate can be rotated in such a way as to occupy the same position as might the 5′-hydroxyl group of substituent ribose, and its replacement by hydrogen results in a total loss of substrate activity with little change in apparent affinity for the enzyme (Schaeffer et al., 1971).[3] The ribose effect might result from ribose-dependent distortion of either the substrate or the enzyme from their native configurations, after formation of the encounter complex, in such a way that their structures more closely approach the structures present in the transition state. However, such explanations, involving the possible intervention of "strain" of "induced fit" in the enzyme–substrate complex, leave unexplained the absence of any ribose effect in the enzymatic deamination of *lin*-benzoadenine and *lin*-benzoadenosine, and this paradox deserves further investigation. Both the enzymatic and the nonenzymatic reactivities of adenine are enhanced by substitution of oxygen or sulfur for the –CH group at the 8-position, to such an extent that the sulfur analogue of adenine actually approaches adenosine in V_{max} (Wolfenden, 1969). Insofar as catalysis by adenosine deaminase is concerned, factors that enhance the intrinsic reactivity of adenine may go far to correct the deficiencies introduced by removal of ribose. Such changes presumably reduce the activation barrier to spontaneous reaction, and hence the need for catalysis. If the bound substrate were sufficiently reactive, effects of substituent ribose on the relative stability of intermediates in substrate transformation might not be apparent if some "noncovalent" process (such as isomerization of an enzyme–substrate complex or release of a product) were already the slow and rate-determining step.

In summary, the influence of ribose on enzymatic deamination of adenosine appears to be associated almost entirely with the catalytic process. Substituent ribose exerts only a slight effect on nonenzymatic reactivity and on the apparent affinity of adenosine deaminase for substrates, but enhances V_{max} and the enzyme's affinity for a potential transition state analogue by several orders of magnitude.

[3] Another analogue, adenine with an erythro(2-hydroxy-3-nonyl) substituent at the 9-position, is a very effective inhibitor (Schaeffer and Schwender, 1974); this compound is not a substrate and presumably binds in a nonproductive mode. Preliminary experiments, in collaboration with Dr. Howard Schaeffer, show that this hydrophobic substituent, combined with 1,6-dihydro-6-hydroxymethylpurine, results in an inhibitor that is not as effective as either of the parent compounds. This is understandable if the steric requirements of catalytic and nonproductive binding are in conflict.

Experimental Section

Materials. Of the compounds used in this study, 1,6-dihydro-6-hydroxymethylpurine was prepared by photolysis of purine as described by Connolly and Linschitz (1968); the corresponding ribonucleoside was prepared similarly, by photolysis or purine ribonucleoside. In contrast to a synthetic procedure (Evans and Wolfenden, 1970) involving photolysis that was carried out in the presence of air and found in the present study to result in formation of a diadduct of methanol in addition to the compound desired, these and other photolyses were carried out under nitrogen. 1-Methyladenosine was prepared from the hydriodide by the method of Jones and Robins (1963). 1-Methyl-6-hydroadenosine was prepared by borohydride reduction of 1-methyladenosine as described by Macon and Wolfenden (1968). 4-Aminopteridine and 9-ribofuranosyl-6-chloropurine were obtained from Cyclo Chemical Co.; 6-*N*-hydroxyaminopurine was obtained from Nutritional Biochemicals Corp.; and 6-chloro-9-methylpurine was purchased from Vega-Fox Biochemicals Co. Glutathione, purine, and 6-chloropurine were obtained from Sigma Chemical Co. The apparent pK_a of 6-chloropurine was determined as 7.77 at 25 °C, by comparison of spectra of the compound (10^{-4} M) in potassium phosphate buffers (0.05 M) with ionic strength maintained at 0.5 by appropriate addition of KCl, in reasonable agreement with the results of Albert and Brown (1954).

Kinetics of Nonenzymatic Reactions with Nucleophiles. The rate of reaction of 9-ribofuranosyl-6-chloropurine and of 9-methyl-6-chloropurine with glutathione was measured by 25 °C by following the increase in absorbance of these compounds (5×10^{-5} M) in glutathione buffers of various concentrations (0.01–0.08 M), with ionic strength adjusted to 0.5 with KCl, at 290 nm, as described for the ribonucleoside by Walsh and Wolfenden (1967). Rates of reaction with hydroxylamine were similarly determined from the rate of increase in extinction (Table I), but reaction with this nucleophile was so slow that higher concentrations of buffer nucleophile (1.0–4.0 M, with ionic strength maintained at 4.0) and a higher temperature, 40 °C, were required. Products of reaction with hydroxylamine exhibited ultraviolet spectra identical with those reported for 6-hydroxylaminopurine (Giner-Sorolla and Bendich, 1958) and 6-hydroxylaminopurine ribonucleoside (Giner-Sorolla et al., 1966).

Enzyme Experiments. Adenosine deaminase from calf intestine was obtained from Boehringer Mannheim Co. as a suspension in 3.2 M ammonium sulfate and dialyzed against potassium phosphate buffer (0.1 M, pH 7.0) before use, in experiments involving kinetic measurements or difference spectra and spectrophotometric titration of the enzyme with inhibitors. To determine the extinction coefficient of the enzyme, it was first established by polyacrylamide gel electrophoresis in sodium dodecyl sulfate (Pett et al., 1963) that the enzyme was at least 95% homogeneous, in accord with earlier findings (Wolfenden et al., 1968). Samples of the enzyme, dialyzed against four changes of deionized water, each containing about 2 mg of protein in a volume of about 0.2, were dried over P_2O_5 under vacuum at room temperature until they reached a constant weight, as determined with a Cahn electrobalance. Comparison of dry weight with extinctions of the dialyzed protein yielded an extinction coefficient of 0.916 per mg of protein at 280 nm, with a cell of 1-cm path length.

Spectrophotometric titrations of the enzyme with inhibitors were performed by first recording the spectrum of the enzyme in potassium phosphate buffer (0.05 M, pH 7.0), using a cuvette containing buffer alone as a reference. To the cuvettes, each containing 1.0 mL of solution at the outset, were added aliquots (1 μL) of inhibitor, and spectra were recorded successively with a Zeiss PMQII or Cary 14 spectrophotometer.

Photolysis of 9-β-D-Ribofuranosylpurine (I) in Methanol with Air. Photolysis of 250 mg of I in 50 mL of methanol was carried out under standard conditions in the presence of air. The reaction was monitored at regular intervals by thin-layer chromatography (Eastman Silica Gel sheets, eluting with 30% methanol in chloroform). The first product detected had a R_f 0.52 (II) and increased until this was the major product. Products of R_f 0.30 (II′) and R_f 0.60 (III) were detected concurrently. The amount of III, under these conditions, was not large, while the amount of II′ increased steadily. During this time, the UV band at 262 decreased steadily while the absorbance at 293 increased. The impressions obtained from the thin-layer chromatography were not quantitative and involved subjective judgements.

Photolysis of 9-β-D-Ribofuranosylpurine (I) in Methanol under Nitrogen. Photolysis of 250 mg of I in 50 mL of methanol, monitored by ultraviolet spectra, was carried out until no further increase was observed in the absorbance at 293 nm (usually about 100 min). In this manner, a sample of II was obtained which showed one spot by thin-layer chromatography. The methanol was removed by rotary evaporation. The residue was dissolved in D_2O and lyophilized prior to recording the NMR. In some cases of incomplete photolysis, I was also observed, as well as other trace impurities: ^{1}H NMR δ 3.80 (m, 4, 6-CH_2OH and 5′-CH_2OH), 4.32 (m, 1, 2′-C*H*OH), 4.68 (m, 1, 3′-C*H*OH), 4.82 (s, HOD), 5.04 (t, 1, 4′-C*H*), 5.77 (d, 1, 1′-C*H*), 7.23 (s, 1, 2 H), 7.66 (s, 1, 8 H). ^{13}C NMR shift (rel intensity, assignment[4]) δ_c 75.0746 (51, 5′-CHOH), 78.0512 and 79.9040 (37 and 37, 6-CH_2OH); 84.1988 and 84.2707 (41 and 36, 3′-CHOH); 87.1715 and 87.2779 (36 and 25, 2′-CHOH); 99.1080 and 99.2298 (35 and 35, 4-CHOC–); 101.3410 and 101.4092 (34 and 40, 1′-OCHNO), 162.1509 (31, ?).

Photooxidation of 9-β-D-Ribofuranosyl-6-hydroxymethyl-1,6-dihydropurine (II) to 9-β-D-Ribofuranosyl-6-hydroxymethylpurine (III). II was prepared as above and the methanol removed by rotary evaporation under vacuum with gentle heating. The residue II was dissolved in 50 mL of water and photolyzed (monitored by ultraviolet spectra and thin-layer chromatography) for about 50 min. Thin-layer chromatography indicated the presence of I as well as III, which was confirmed by NMR to contain approximately 15% (I): ^{1}H NMR of III; δ 3.94 (m, 2, 5′-CH_2OH), 4.34 (q, 1, 2′-C*H*OH), 4.51 (t, 1, 3′-CHOH), 4.85 (HOD singlet obscuring multiplet, of 4′-C*H*OC), 5.14 (s, 2, 6-CH_2OH), 6.21 (d, 1, J = 6 Hz, 1′-OC*H*N), 8.70 (s, 0.5, apparent deuterium exchange, 8 H), 8.88 (s, 1, 2 H). In addition to the impurity I peaks in the sample, another singlet at 8.5 ppm was assigned to formic acid. An attempt was made to separate I from III by column chromatography over E. Merck silica 60 eluting with increasing percentages of methanol in chloroform, but this was not completely successful.

Photolysis of 9-β-D-Ribofuranosyl-6-hydroxymethylpurine (III) in Methanol under Nitrogen. Thirty milligrams of chromatographed III from above containing 15% I was dissolved in 20 mL of methanol and photolyzed for about 30 min (until the reaction was complete by ultraviolet spectra). Methanol was removed under vacuum and the sample lyophilized from D_2O. ^{1}H NMR of II′ δ 3.67 (s, MeOH), 3.48–3.88 (m, 6 H, 5′-CH_2OH and 6,6-di(-CH_2OH)), 4.24 (q, 1, 2′-C*H*OH), 4.35 (d-d, 1, 3′-CHOH), 4.64 (m, 1, 4′-CHOH), 4.82 (s, HOD), 5.77 (d, 1, J = 6 Hz, 1′-OCHN), 7.32 (s, 1, 2 H), 7.68 (s, 1, 8 H). Chemical ionization mass spectrum of II′ (with the exception of the $M^+ + H$ peak at *m/e* 315, only peaks with relative intensity greater than 10% are reported): *m/e* (relative intensity) 315 (8), 298 (13), 297 (43), 284 (27), 283 (87), 282 (26), 268 (20), 267 (62), 253 (17), 184 (18), 183 (88), 167 (13), 166 (12), 165 (36), 152 (22), 151 (78), 150 (98), 149 (20), 138 (16), 136 (23), 135 (100), 134 (13), 132 (27), 121 (39).

Acknowledgments

We are grateful to Professor H. Umezawa for providing a sample of coformycin and to Dr. H. W. Dion for a gift of the covidarabine used in these studies. Chemical ionization mass spectra were kindly obtained by Dr. Joan Bursey at the Research Triangle Center for Mass Spectrometry.

References

Albert, A., Batterhan, T. J., and McCormack, J. J. (1966), *J. Chem. Soc. B,* 1105–1113.

Albert, A., and Brown, D. S. (1954), *J. Chem. Soc.,* 2060–2068.

Bär, H., and Drummond, G. I. (1966), *Biochem. Biophys. Res. Commun. 24,* 584–589.

Cha, S., Agarwal, R. P., and Parks, R. E., Jr. (1975), *Biochem. Pharmacol. 24,* 2187–2197.

Connolly, J. S., and Linschitz, H. (1968), *Photochem. Photobiol. 7,* 791–806.

Cory, J. G., and Suhadolnik, R. J. (1965), *Biochemistry 4,* 1733–1735.

Evans, B. E., and Wolfenden, R. (1970), *J. Am. Chem. Soc. 92,* 4751–4752.

Evans, B. E., and Wolfenden, R. (1972), *J. Am. Chem. Soc. 94,* 5902–5903.

Evans, B. E., and Wolfenden, R. (1973), *Biochemistry 12,* 392–398.

Giner-Sorolla, A., and Bendich, A. (1958), *J. Am. Chem. Soc. 80,* 3932–3937.

Giner-Sorolla, A., Medrek, L., and Bendich, A. (1966), *J. Med. Chem. 9,* 143–144.

Johnson, L. F., and Jankowski, W. C. (1972), Carbon-13 NMR Spectra, New York, N.Y., Wiley.

Jones, J. W., and Robins, R. K. (1963), *J. Am. Chem. Soc. 85,* 193–199.

Jordan, D. O. (1955), in The Nucleic Acids, I, Chargaff, E., and Davidson, J. N., Ed., New York, N.Y., Academic Press, p 447.

Leonard, N. J., Sprecker, M. A., and Morrice, A. G. (1976), *J. Am. Chem. Soc. 98,* 3987–3994.

Linschitz, H., and Connolly, J. S. (1968), *J. Am. Chem. Soc. 90,* 2979–2980.

Longevialle, P., Einhorn, J., Alazard, J. P., Diatta, L., Milliet, P., Monneret, C., Khoung-Huu, Q., and Lusinchi, X. (1971), *Org. Mass Spectrom. 5,* 171–186.

Macon, J. B., and Wolfenden, R. (1968), *Biochemistry 7,* 3453–3458.

Nakamura, H., Koyama G., Iitaka, Y., Ohno, M., Yagisawa, N., Kondo, S., Maeda, K., and Umezawa, H. (1974), *J. Am.*

[4] The assignments are based primarily on those reported for adenosine (Johnson and Jankowski, 1972).

Chem. Soc. 96, 4327-4328.
Ohno, M., Yagisawa, N., Shibahara, S., Kondo, S., Maeda, K., and Umezawa, H. (1974), *J. Am. Chem. Soc. 96,* 4326-4327.
Perrin, D. D. (1962), *J. Chem. Soc.,* 645-660.
Pett, D. M., Vanaman, T. C., and Joklik, W. K. (1973), *Virology 52,* 174-186.
Schaeffer, H. J., Gurwara, S., Vince, R., and Bittner, S. (1971), *J. Med. Chem. 14,* 367-369.
Schaeffer, H. J., and Schwender, C. F., (1974), *J. Med. Chem. 17,* 6-11.
Walsh, B. T., and Wolfenden, R. (1967), *J. Am. Chem. Soc. 89,* 6221-6225.
Wolfenden, R. (1966), *J. Am. Chem. Soc. 88,* 3157-3158.
Wolfenden, R. (1969), *Biochemistry 8,* 2409-2412.
Wolfenden, R. (1970), *Biochemistry 9,* 3404-3407.
Wolfenden, R. (1972), *Acc. Chem. Res. 5,* 10-18.
Wolfenden, R. (1976), *Annu. Rev. Biochem. Bioeng. 5,* 271-306.
Wolfenden, R., Kaufman, J., and Macon, J. B. (1969), *Biochemistry 8,* 2412-2415.
Wolfenden, R. Tomozawa, Y., and Bamman, B. (1968), *Biochemistry 7,* 3965-3970.
Woo, P. W. K., Dion, H. W., Lange, S. M., Dahl, L. F., and Durham, L. J. (1974), *J. Heterocycl. Chem. 11,* 641-643.
Zielke, C. L., and Suelter, C. H. (1971); *Enzymes, 3rd Ed. 4,* 47-78.

Allosteric Regulation of Aspartate Transcarbamoylase. Changes in the Sedimentation Coefficient Promoted by the Bisubstrate Analogue *N*-(Phosphonacetyl)-L-aspartate†

G. J. Howlett‡ and H. K. Schachman*

ABSTRACT: Although it is known that aspartate transcarbamoylase from *Escherichia coli* undergoes a 3.6% *decrease* in sedimentation coefficient upon the addition of the substrate, carbamoyl phosphate, and succinate, an analogue of the second substrate, aspartate, it has been difficult to establish an exact relationship between the conformational change and the extent of ligand binding because of the low affinity of the enzyme for succinate. Hence studies were performed on the enzyme with the bisubstrate analogue, *N*-(phosphonacetyl)-L-aspartate (PALA) since its binding is determined readily. The maximal change in sedimentation coefficient, −3.1%, was attained when only four of the six active sites in the enzyme were saturated. Carbamoyl phosphate and ATP facilitated the conformational transition promoted by PALA while CTP retarded the transition. Moreover the effects of carbamoyl phosphate and the nucleotide effectors were additive; with both carbamoyl phosphate and ATP present only 2.5 mol of PALA per mol of enzyme was required to produce a maximal decrease in sedimentation coefficient. ATP or CTP alone caused a reduction in sedimentation coefficient of about −0.5%. In contrast, when carbamoyl phosphate was also present, the change promoted by ATP was −1.1% whereas that caused by CTP was −0.5%. The conformational changes in aspartate transcarbamoylase appear to be concerted and the results are consistent with the two-state model of allosteric proteins (Monod, J., Wyman, J., and Changeux, J.-P. (1965), *J. Mol. Biol. 12,* 88). Analogous studies with the purified catalytic subunit, which exhibits noncooperative enzyme kinetics, showed that the *increase* in the sedimentation coefficient, +1.4%, promoted by PALA was closely linked with binding.

All molecular models proposed to account for the cooperativity exhibited by allosteric enzymes implicate ligand-promoted conformational changes of the oligomeric protein from a constrained or low-affinity state to a relaxed form with catalytic sites having a high affinity for substrates (Monod et al., 1965; Koshland et al., 1966). In this series of papers on the regulatory enzyme, aspartate transcarbamoylase (ATCase)[1] (EC 2.1.3.2; carbamoylphosphate:L-aspartate carbamoyltransferase) from *Escherichia coli,* we present studies of the conformational changes of the enzyme as a function of its extent of saturation by ligands in order to test whether the experimental results can be explained by the simple hypothesis that the allosteric transition from one state to the other is concerted.

As shown by Gerhart and Schachman (1968) the addition of the substrate, carbamoyl phosphate, and the substrate analogue, succinate, to ATCase led to a 3.6% decrease in the sedimentation coefficient and to a six-fold increase in the reactivity of the sulfhydryl groups of the regulatory subunits of the enzyme. These changes appeared to attain their maximal values prior to the saturation of the enzyme by succinate (Changeux et al., 1968). However, the earlier binding data for succinate are not readily rationalized with the present structural evidence that ATCase contains six catalytic polypeptide chains distributed within two catalytic trimers (Weber, 1968; Wiley and Lipscomb, 1968; Meighen et al., 1970; Rosenbusch and Weber, 1971; Cohlberg et al., 1972). Hence it seemed

† From the Department of Molecular Biology and the Virus Laboratory, Wendell M. Stanley Hall, University of California, Berkeley, California 94720. *Received March 1, 1977; revised manuscript received August 2, 1977.* This work was supported by National Institutes of Health Research Grant GM 12159 from the National Institute of General Medical Sciences, by National Institutes of Health Training Grant CA 05028 from the National Cancer Institute, and by Grant GB 32812X from the National Science Foundation.

‡ Present address: Department of Biochemistry, La Trobe University, Bundoora, Victoria, Australia 3083.

[1] Abbreviations used are: ATCase, aspartate transcarbamoylase; PALA, *N*-(phosphonacetyl)-L-aspartate; DEAE, diethylaminoethyl.