Rates of Spontaneous Disintegration of DNA and the Rate Enhancements Produced by DNA Glycosylases and Deaminases†

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ABSTRACT: To estimate the relative importance of alternate routes of spontaneous degradation of DNA and the rate enhancements produced by enzymes catalyzing these reactions, rate constants and thermodynamic activation parameters for the degradation of 2′-deoxynucleosides at 25 °C were determined by extrapolation of rates observed in the temperature range between 90 and 200 °C in neutral phosphate buffer. Rates of deamination of 2′-deoxycytidine, 1-methylcytosine, and cytidine were found to be identical within experimental error ($t_{1/2} \approx 20$ years, 37 °C). Rate constants for deamination of 2′-deoxyadenosine and 2′-deoxyguanosine, which could not be determined directly because of rapid glycoside cleavage, were estimated by assuming that methyl replacement should generate reasonable model substrates. The rates of deamination of 9-methyladenine and 9-methylguanine were found to be similar to each other ($t_{1/2} \approx 6000$ years, 37 °C) and $\sim 10^2$-fold slower than the rates of glycoside cleavage in 2′-deoxyadenosine and 2′-deoxyguanosine. The deamination of 2′-deoxyadenosine, 2′-deoxyguanosine, and 2′-deoxycytidine led to accelerated rates of glycoside cleavage. In the exceptional case of 2′-deoxycytidine, deamination and glycoside hydrolysis proceed at very similar rates at all temperatures. Glycoside cleavage proceeds with half-times ranging from 4 years for 2′-deoxyinosine to 40 years for 2′-deoxycytidine (37 °C). The rate enhancements produced by DNA glycosylases, estimated by comparison with the rates of these uncatalyzed reactions, are found to be substantially smaller than those produced by deaminases and staphylococcal nuclease.

With a typical half-life of ∼140000 years for hydrolysis in neutral solution at 25 °C (*1*, *2*),[1] the phosphodiester bonds of DNA are well-suited to conserving the integrity of genetic information, but other chemical linkages in nucleic acids are less robust. The exocyclic amino groups of cytosine, adenine, and guanine undergo hydrolytic deamination; the nucleotides are susceptible to hydrolytic cleavage at the *N*-glycoside bond, and the ring systems of purines and pyrimidines also disintegrate slowly in water. Each of these processes represents a threat to genome stability that becomes more acute in organisms growing at elevated temperatures (*3*, *4*).

It would be of interest to have quantitative information about the relative rates of each of these processes in neutral solution for several reasons. That information would be useful for assessing the rates of decomposition of DNA in a clinical or forensic setting and might furnish a basis for judging the integrity of ancient samples of DNA extracted from archaeological or paleontological sources. The relative rates of disintegration of nucleic acids, by the various routes available at ordinary and elevated temperatures, are also of interest in considering the selective pressures that presumably led to the evolution of different kinds of DNA repair enzymes and their levels of expression. This information is also expected to be useful in considering the design of transition state analogue inhibitors. In an ideal case, an enzyme should bind such an inhibitor more tightly than the substrates, to an extent that matches the rate enhancement that the enzyme produces. Enzymes have been found to act upon their substrates at rates that fall within a relatively narrow range that renders them useful to cells with limited division times, but the rates of the corresponding spontaneous reactions, proceeding in the absence of a catalyst, vary greatly (*5*). For example, rates of water-consuming reactions span a range of ∼17 orders of magnitude in the absence of enzymes. The identification of enzyme reactions that proceed exceptionally slowly in the absence of an enzyme is expected to be useful in identifying enzymes that should furnish especially sensitive targets for inhibition.

Current information about the rates of deamination, glycoside cleavage, and ring opening, proceeding spontaneously in neutral solution, is extensive but fragmentary (for reviews, see refs *6* and *7*). Most earlier efforts to estimate the rates of hydrolytic deamination and glycoside cleavage involved experiments conducted at extreme pH values where they are subject to acid and base catalysis, and the acid- and base-catalyzed terms in the rate equation were then extrapolated to neutrality (*6*−*10*). As noted by Shapiro (*7*), the validity of those extrapolations rests on the assumption that the rate equation contains no significant term for "uncatalyzed" (or water-catalyzed) hydrolysis that might contribute to the rate of reaction near neutrality. In many cases, effects

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[1] This half-life refers to the rate of C−O bond cleavage of dimethyl phosphate (*1*). The rate of P−O bond cleavage, the reaction catalyzed by phosphodiesterases, is much slower with an estimated half-life of ∼30 million years at 25 °C (*2*).

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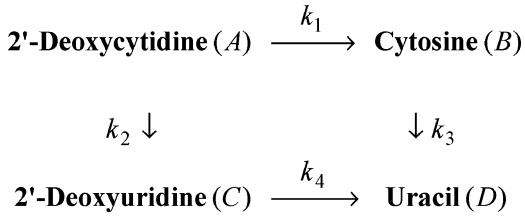
Scheme 1: Degradation Pathways for 2′-Deoxycytidine



of temperature on reaction rates were not determined, or the range of observations was limited.

Here, we report a re-examination of the rates and mechanisms of decomposition of nucleosides and related molecules in neutral solution, in sealed quartz tubes at elevated temperatures. Using proton NMR to analyze reaction mixtures after various time intervals, reaction rate constants and thermodynamic activation parameters were determined with sufficient precision to allow extrapolation to ordinary temperatures. The results furnish a basis for comparing the catalytic proficiencies of enzymes that catalyze the corresponding reactions in nucleoside salvage and DNA repair and provide some indication of the relative importance of the various modes of decomposition of DNA at the temperatures faced by mesophiles and thermophiles.

MATERIALS AND METHODS

9-Methylhypoxanthine was purchased from Vega Biochemicals (Tucson, AZ); 9-methyladenine was purchased from Cyclo Chemical Corp. (Los Angeles, CA), and other compounds were obtained from Sigma-Aldrich Co.

Reaction mixtures contained nucleosides or nucleoside models (10^{-3} M) in potassium phosphate buffer (0.1 M, pH 6.8). Samples were sealed in quartz tubes under vacuum, and the tubes were placed in convection ovens (Barnstead/Thermolyne Corp., model 47900) maintained at constant temperatures ranging from 90 to 200 °C (temperature variation of ±1.5 °C as determined with an ASTM thermometer) for varying periods of time. The contents were then diluted with D_2O containing pyrazine (10^{-3} M, added as an integration standard) and analyzed by proton NMR (Varian Inova 500 or 600 MHz spectrometer). Data were acquired for a minimum of four transients using a water suppression pulse sequence and processed using SpinWorks (*11*).

The rates of deamination of 2′-deoxyadenosine and 2′-deoxyguanosine could not be determined directly at neutral pH because glycoside cleavage occurs too rapidly (see below). To circumvent that difficulty, rates of deamination were estimated using the stable models 9-methyladenine and 9-methylguanine. Because the pK_a values (N1) of the 9-methylated derivatives are slightly higher than those of the original purine nucleosides ($\Delta pK_a \sim 0.4$; see the Discussion), one might expect their susceptibility to nucleophilic attack (at C6 of adenine or C2 of guanine) to be somewhat reduced, but those effects are expected to be small.

2′-Deoxycytidine differs from 2′-deoxyadenosine and 2′-deoxyguanosine in that glycoside cleavage (k_1) and deamination (k_2) occur at similar rates. In principle, rate constants for these alternative routes of breakdown could be obtained from the product ratio, but the initial products of 2′-deoxycytidine deamination (2′-deoxyuridine) and glycoside cleavage (cytosine) were found to undergo further decomposition, and at different rates [k_3 and k_4 (Scheme 1)]. The temperature dependence of k_3 and k_4 was therefore determined, in separate experiments, and a correction was made for the decomposition of these initial products (cytosine and 2′-deoxyuridine), at each temperature.

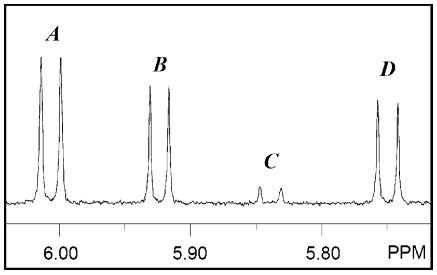


FIGURE 1: Proton NMR spectrum (500 MHz) of 2′-deoxycytidine after incubation at 130 °C for 17 h (pH 6.8). C5 proton resonances are as follows, in accordance with Scheme 1: A, 2′-deoxycytidine; B, cytosine; C, 2′-deoxyuridine; and D, uracil.

The expected kinetic relationships are shown in the following equations:

$$[A] = [A]_0 e^{-k_T t} \quad (1)$$

$$[B] = \frac{k_1[A]_0}{k_3 - k_T}(e^{-k_T t} - e^{-k_3 t}) \quad (2)$$

$$[C] = \frac{k_2[A]_0}{k_4 - k_T}(e^{-k_T t} - e^{-k_4 t}) \quad (3)$$

where [A] is the 2′-deoxycytidine concentration, $[A]_0$ is the initial 2′-deoxycytidine concentration, [B] is the cytosine concentration, [C] is the 2′-deoxyuridine concentration, t is time, $k_T = (k_1 + k_2)$, and $k_1 - k_4$ are defined as in Scheme 1. Equations 2 and 3 describe product concentrations as a function of time, where the initial concentration of each product is 0 at $t = 0$. The C5 proton resonances of molecules A−D (Scheme 1), which were well-resolved (Figure 1), were used to monitor the concentrations of all four compounds as a function of time. At time t, the only unknown variable is the desired rate constant (k_1 or k_2). As reported earlier in the deamination of cytidine (*12*), ring opening made no significant contribution to the rate of decomposition of 2′-deoxycytidine.

RESULTS

Rate Constants and Thermodynamics of Activation for Glycoside Cleavage. Apparent first-order rate constants for glycoside bond cleavage (pH 6.8) were determined at various temperatures for 2′-deoxyadenosine, 2′-deoxyguanosine, 2′-deoxycytidine, and 2′-deoxyinosine. Earlier we reported the rates of cleavage of thymidine and thymidylyl(3′−5′)-thymidine (TpT)[2] under the same conditions (*2*), but the actual data are shown here for the first time. In each case, the free base was the only detectable product of hydrolysis, consistent with earlier observations that direct cleavage of the glycoside bond predominates over ring opening at low alkalinity (*7*, *8*, *13*). Under the conditions of these experiments, deoxyribose decomposes virtually instantaneously, presumably by reverse aldol condensation. The apparent first-

[2] Abbreviations: dA, 2′-deoxyadenosine; dG, 2′-deoxyguanosine; dC, 2′-deoxycytidine; dU, 2′-deoxyuridine; T, thymidine; dI, 2′-deoxyinosine; dX, 2′-deoxyxanthosine; TpT, thymidylyl(3′−5′)thymidine; DMSO-d_6, deuterated dimethyl sulfoxide.

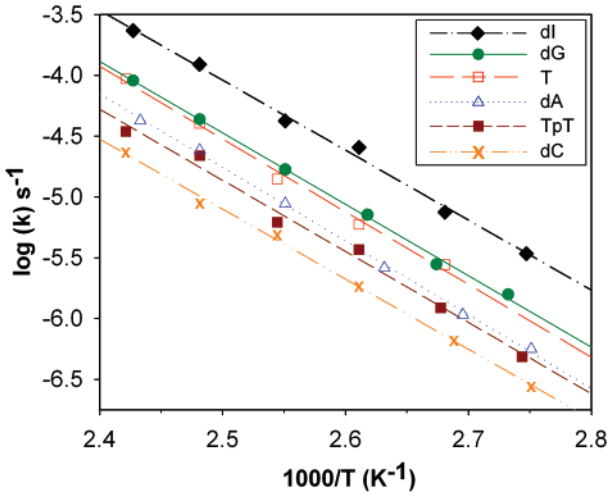


FIGURE 2: Arrhenius plot of the rate constants for the glycoside cleavage (at pH 6.8) of 2′-deoxynucleosides.

Table 1: Rate Constants and Thermodynamics of Activation[a] for Glycoside Cleavage of 2′-Deoxynucleosides at pH 6.8 and 25 °C (rate constants and $t_{1/2}$ values at 37 °C also included)

	$\Delta H^{\ddagger}$	$\Delta G^{\ddagger}$	$T\Delta S^{\ddagger}$	$k^{25\,°C}$ (s^{-1})	$k^{37\,°C}$ (s^{-1})	$t_{1/2}$ (37 °C)
dC[b]	25.8	31.0	−5.2	9.4×10^{-11}	5.1×10^{-10}	43 years
dA	27.1	30.9	−3.8	1.2×10^{-10}	6.8×10^{-10}	32 years
TpT	26.3	30.8	−4.5	1.4×10^{-10}	7.6×10^{-10}	29 years
T	26.9	30.5	−3.6	2.3×10^{-10}	1.4×10^{-9}	16 years
dU[c]	28.6	30.5	−1.9	2.5×10^{-10}	1.7×10^{-9}	13 years
dG	26.3	30.3	−4.0	3.2×10^{-10}	1.8×10^{-9}	12 years
dI[d]	25.7	29.6	−3.8	1.1×10^{-9}	5.6×10^{-9}	4 years

[a] Values reported in kilocalories per mole, with an estimated error of ±0.5 kcal/mol. [b] Calculated as described in the text. [c] 2′-Deoxyuridine (see Figure 1 of the Supporting Information). [d] 2′-Deoxyinosine.

order rate constants are shown as an Arrhenius plot in Figure 2, and the thermodynamics of activation at 25 °C are summarized in Table 1. Half-times for glycoside cleavage ranged from ~4 years for 2′-deoxyinosine to ~40 years for 2′-deoxycytidine (37 °C).[3] Table 1 includes an additional entry for 2′-deoxyuridine, the properties of which were examined under the same conditions (for the Arrhenius plot, see Figure 1 of the Supporting Information).

Rate Constants and Thermodynamics of Activation for Ring Opening and Deamination. Apparent first-order rate constants for the thermal degradation (pH 6.8) of 9-methylhypoxanthine (▲) and 9-methylxanthine (○) are shown as an Arrhenius plot in Figure 3 (for the thermodynamics of activation, see the first two entries of Table 2). The proton NMR spectrum of a product mixture obtained from 9-methylhypoxanthine, shown in Figure 2 of the Supporting Information, showed resonances consistent with the formation of two ring-opened products (for an interpretation, see the Discussion). Similar results were obtained for 9-methylxanthine.

Apparent first-order rate constants for deamination (pH 6.8) were determined at various temperatures for 2′-deoxycytidine, 1-methylcytosine, cytosine, 9-methyladenine, and 9-methylguanine. The temperature dependence of the deamination rate constants for 2′-deoxycytidine (■) and 1-methylcytosine (□) were identical within experimental error

[3] Interestingly, similar rates have been observed for the uncatalyzed hydrolysis of *O*-methyl-2-deoxyribofuranosides (*14*).

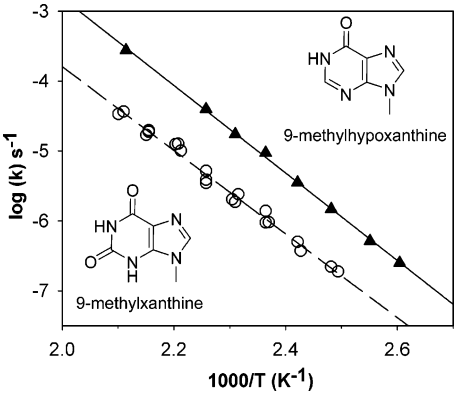


FIGURE 3: Arrhenius plot of the rate constants for the thermal degradation (at pH 6.8) of 9-methylhypoxanthine (▲) and 9-methylxanthine (○).

Table 2: Rate Constants and Thermodynamics of Activation[a] for Purine Ring[b] Degradation and 2′-Deoxynucleoside[b] Deamination at pH 6.8 and 25 °C

	$\Delta H^{\ddagger}$	$\Delta G^{\ddagger}$	$T\Delta S^{\ddagger}$	$k^{25\,°C}$ (s^{-1})	$k^{37\,°C}$ (s^{-1})	$t_{1/2}$ (37 °C)
Ring Opening						
dI[b,c]	28.1	32.8	−4.7	5.0×10^{-12}	3.3×10^{-11}	670 years
dX[b,d]	26.8	33.6	−6.8	1.3×10^{-12}	7.4×10^{-12}	3000 years
Deamination						
dA[b]	27.4	33.6	−6.2	1.2×10^{-12}	7.6×10^{-12}	3000 years
dG[b]	29.1	34.3	−5.3	3.6×10^{-13}	2.4×10^{-12}	9000 years
dC[e]	24.8	30.7	−5.9	1.8×10^{-10}	9.3×10^{-10}	24 years
1-MeC[f]	24.6	30.6	−6.0	2.0×10^{-10}	1.0×10^{-9}	22 years
cytidine[g]	22.1	30.4	−8.3	2.7×10^{-10}	1.2×10^{-9}	19 years
cytosine[h]	22.9	30.6	−7.7	2.1×10^{-10}	1.1×10^{-9}	20 years

[a] Values reported in kilocalories per mole, with an estimated error of ±0.5 kcal/mol. [b] 9-Methyl derivatives were used as models for purine 2′-deoxynucleosides (see the text). [c] 2′-Deoxyinosine, modeled by 9-methylhypoxanthine. [d] 2′-Deoxyxanthosine, modeled by 9-methylxanthine. [e] Calculated as described in the text. [f] 1-Methylcytosine. [g] From ref *12*. [h] Figure 3 of the Supporting Information.

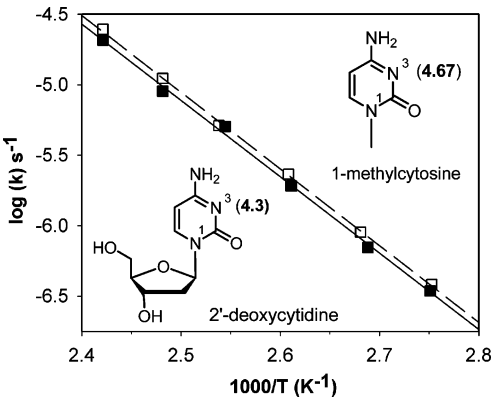


FIGURE 4: Arrhenius plot of the rate constants for the deamination (at pH 6.8) of 2′-deoxycytidine (■) and 1-methylcytosine (□). The pK_a values for the N3 protons are indicated (in parentheses) on the appropriate structures.

(Figure 4). The thermodynamics of activation (Table 2) were similar to those determined for cytosine (Figure 3 of the Supporting Information) and to those reported earlier for cytidine (*12*). Apparent first-order rate constants for the deamination of 9-methyladenine and 9-methylguanine are shown as an Arrhenius plot in Figure 5A, and the corresponding thermodynamics of activation are listed in Table 2. Rate constants for the deamination of these model compounds were almost identical over the experimental range

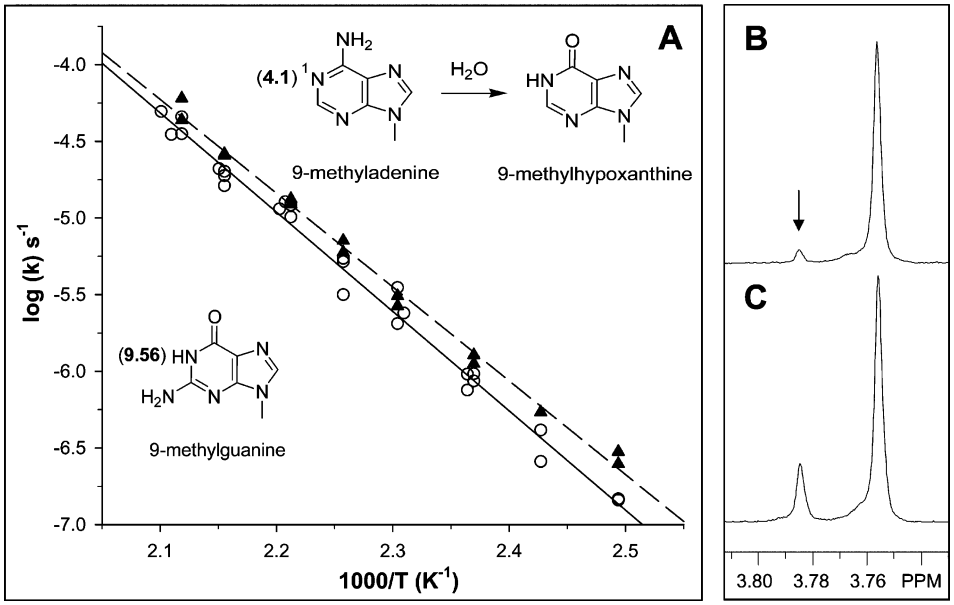


FIGURE 5: Deamination of 2′-deoxyadenosine and 2′-deoxyguanosine models. The pK_a values for the N1 protons are indicated (in parentheses) on the appropriate structures. (A) Arrhenius plot of the rate constants for the deamination (at pH 6.8) of 9-methyladenine (▲) and 9-methylguanine (○). (B) Proton NMR spectrum (600 MHz) of 9-methyladenine (rightmost resonance) after incubation at 140 °C for 3 days. The arrow indicates the newly formed product. (C) Spectrum of the same sample as in panel B, but with the addition of authentic 9-methylhypoxanthine.

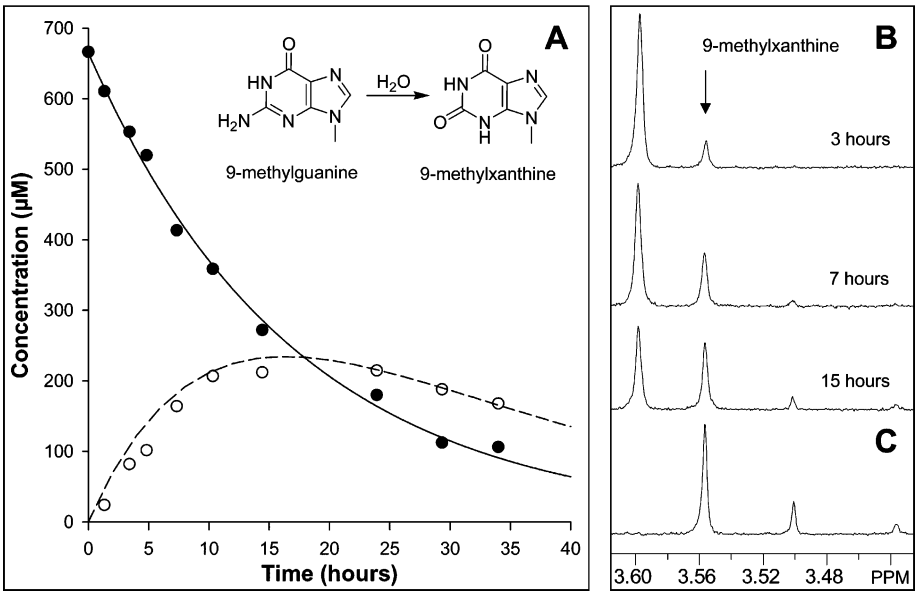


FIGURE 6: Time course for the deamination of the 2′-deoxyguanosine model. (A) Plot of 9-methylguanine (●) and 9-methylxanthine (○) concentrations as a function of time at 191 °C (pH 6.8). The solid line is a fit of the data to an exponential decay curve ($R^2 > 0.99$), and the dashed line represents a simulated time course for 9-methylxanthine based on its own rate of breakdown at 191 °C (see the text). (B) Proton NMR spectra (600 MHz) for three of the time points shown in panel A (leftmost resonance is 9-methylguanine). (C) NMR spectrum of 9-methylxanthine alone, under the same conditions.

from 130 to 200 °C, although the guanine derivative appears to be the slightly more stable of the two at all temperatures (Figure 5A). Figure 5B shows the proton NMR spectrum of 9-methyladenine (rightmost resonance) after incubation at 140 °C (3 days). The arrow indicates a newly formed peak, which was identified as the expected product of deamination, 9-methylhypoxanthine, by addition of the authentic material (Figure 5C). It was possible to account for the decomposition of 9-methyladenine entirely by deamination (see Discussion), with no detectable ring opening.

Figure 6A shows a plot of 9-methylguanine (●) and 9-methylxanthine (○) concentrations as a function of time at 191 °C (pH 6.8). The first-order rate constant for decay (k_5) and the known rate of 9-methylxanthine degradation at 191 °C (k_6, calculated from Figure 3) were used to simulate the expected concentration of 9-methylxanthine as a function of time. The dashed line in Figure 6A is based on a modified form of eq 2 (where $k_1 = k_T = k_5$, $k_3 = k_6$, $[A]_0$ is the initial 9-methylguanine concentration, and [B] is the 9-methylxanthine concentration), assuming two consecutive first-order reactions. That simulation, based on the assumption that the guanine ring is stable under these conditions, fit the data satisfactorily (estimated $R^2 \approx 0.9$). Figure 6B shows proton NMR spectra obtained (leftmost resonance is 9-methylgua-

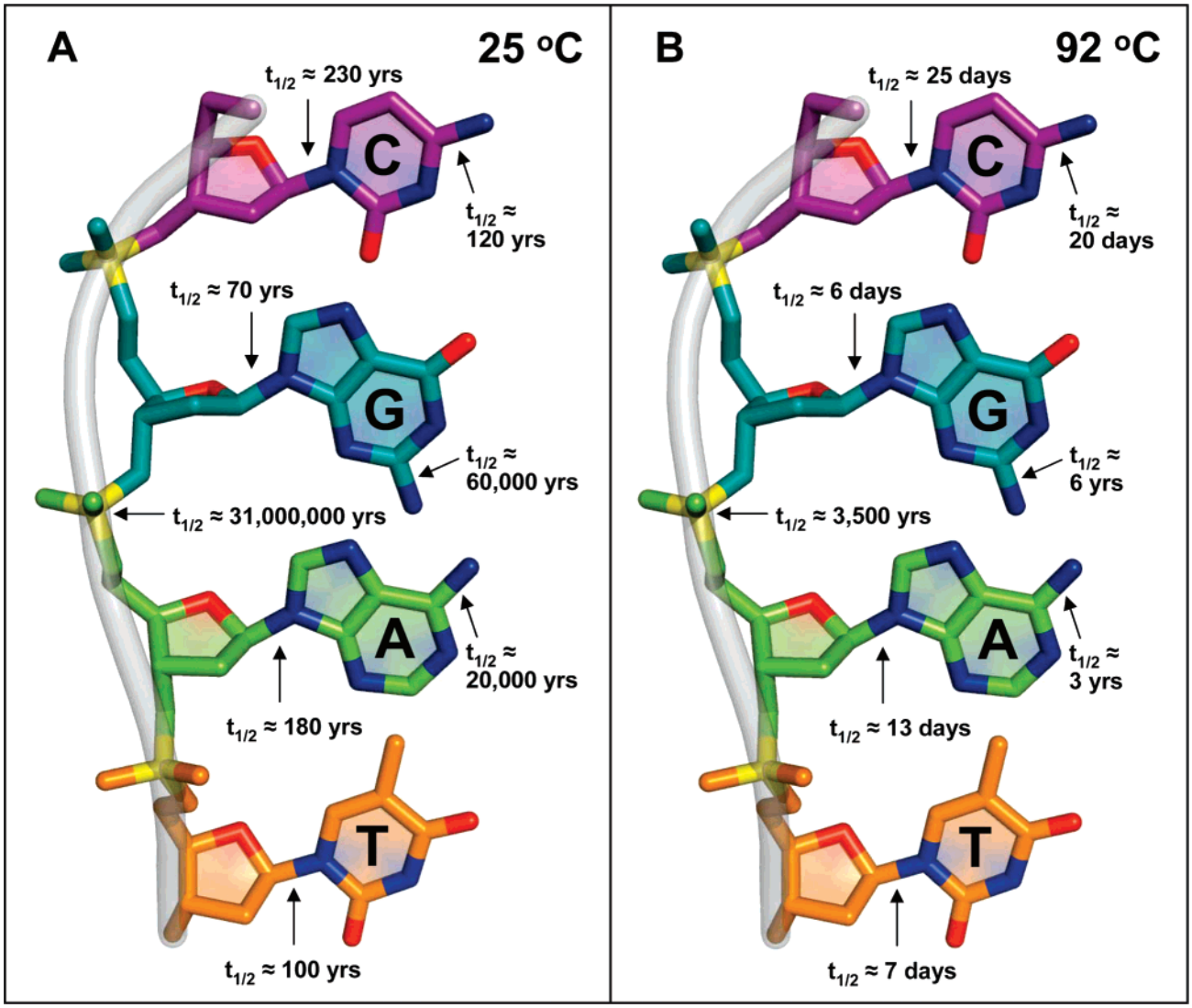


FIGURE 7: Summary of the stabilities (half-times) determined for the bonds of DNA at pH 7, based on work with individual 2′-deoxynucleosides and representative model compounds. (A) Values extrapolated to 25 °C. (B) Values extrapolated to 92 °C. Atoms are colored as follows: yellow for phosphorus, blue for nitrogen, and red for oxygen. The oxygen atoms of the backbone were left uncolored for the sake of simplicity. DNA nucleotides are colored as follows: purple for 2′-deoxycytidine (C), teal for 2′-deoxyguanosine (G), green for 2′-deoxyadenosine (A), and orange for thymidine (T). The DNA strand was adapted from PDB entry 1JO2 in PyMol (DeLano Scientific, Palo Alto, CA).

nine) at three different time points. The NMR spectrum of 9-methylxanthine alone (at 191 °C), also included in Figure 6C, shows that all additional peaks resulting from the breakdown of 9-methylguanine correspond to derivatives of the deamination product, 9-methylxanthine. These results indicate that deamination occurs directly and does not depend on prior ring opening of the starting material.

DISCUSSION

Figure 7A shows various possible routes for the decomposition of DNA at pH 7 at 25 °C. The specific hydrolytic reactions addressed in this work are glycoside cleavage (vertical arrows, Figure 7A), deamination (slanted arrows, Figure 7A), purine ring opening (not shown in Figure 7A), and phosphodiester cleavage (horizontal arrow, Figure 7A).

Hydrolysis of the Glycoside Bonds of 2′-Deoxynucleosides in Neutral Solution. The use of individual 2′-deoxynucleosides as models for DNA (particularly under acidic conditions) is well-documented (*6*−*10*), but detailed kinetic information about the glycoside cleavage of 2′-deoxynucleosides in the pH range near neutrality is limited. Only thymidine and 2′-deoxyuridine appear to have been studied extensively, with results consistent with a significant contribution from an uncatalyzed reaction in neutral solution (*13*). The model nucleosides 2′-deoxyadenosine, 2′-deoxyguanosine, and 2′-deoxyinosine all exhibit acid-catalyzed glycoside bond cleavage (*10*, *15*). In each of those three cases, the extrapolated rate constant is smaller than the rate constant determined directly at pH 6.8 in these experiments.[4]

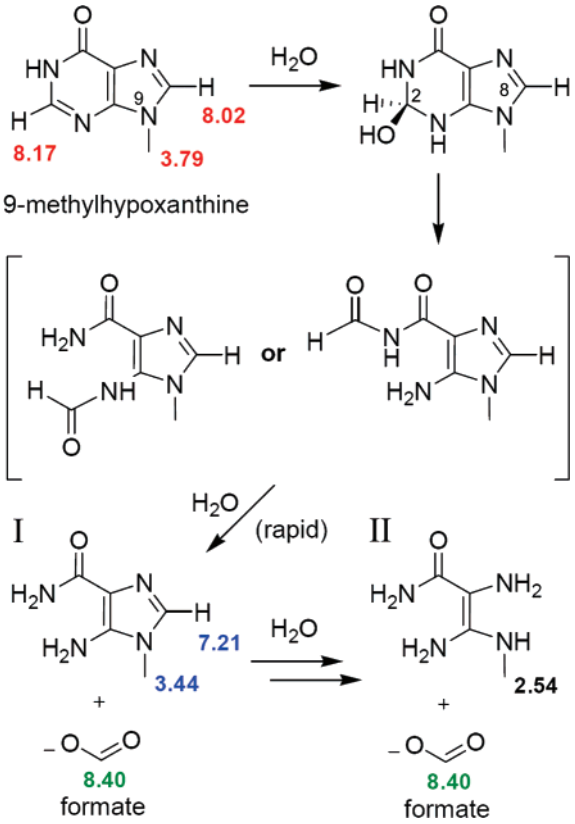
In the case of 2′-deoxycytidine, glycoside bond cleavage was shown earlier to be acid-catalyzed (*8*); extrapolation of those results to pH 6.8 (*6*) yields a rate constant ~10-fold lower than the rate constant determined in the experiments presented here. That result is consistent with a major role for an uncatalyzed reaction at pH 7. It should be noted that 2′-deoxycytidine differs from other nucleosides in that glycoside hydrolysis and deamination occur at similar rates.

Our experiments account for both of these competing reactions.

We have recently shown that the rates of glycoside hydrolysis of thymidine and TpT differ by a factor of less than 2 in neutral solution, implying that the presence of a 3′- or 5′-phosphoryl group has little effect on the rate of glycoside cleavage (*2*). It seems reasonable to infer that the behavior of thymidine, and by extension the other deoxynucleosides, furnishes a reasonable model for hydrolysis of the glycoside bonds of deoxynucleotide residues in single-stranded DNA.

Deamination of 9-Methylpurine Derivatives in Neutral Solution. Rate constants and thermodynamics of activation for the hydrolytic deamination of purine 2′-deoxynucleosides could not be determined directly because glycoside hydrolysis occurs too rapidly. Accordingly, rates of deamination were determined for 9-methylated analogues in which a methyl group replaces deoxyribose, eliminating the possibility of glycoside cleavage. Because the pK_a values of the methylated derivatives are slightly higher than those of the native deoxynucleosides, one might anticipate a minor reduction in susceptibility to nucleophilic attack. In the case of the pyrimidines (Figure 4), the rate constants observed for deamination of 2′-deoxycytidine [pK_a = 4.3 (*16*)], 1-methylcytosine [pK_a = 4.67 (*17*)], and cytidine [pK_a = 4.2 (*18*)] are similar (Table 2), indicating that the effect of small differences in the pK_a of the pyrimidine ring nitrogen is slight. In the case of the purine nucleosides, the small differences among the pK_a values (N1) for 9-methyladenine [4.1 (*19*)], 2′-deoxyadenosine [3.5 (*20*)], and adenosine [3.6 (*19*)] and among the values for 9-methylguanine [9.56 (*19*)], 2′-deoxyguanosine [9.3 (*7*)], and guanosine [9.22 (*19*)] show that the nature of the N9 substituent exerts only a modest influence on the electronic properties of the purine ring. Moreover, rate constants for the deamination of 2′-deoxycytidine (5.2×10^{-7} s^{-1}, 95 °C) and 9-methyladenine (4.4×10^{-8} s^{-1}, 110 °C) differ by a factor of less than 3 from those reported for the direct deamination of these residues in single-stranded DNA at neutrality [2×10^{-7} s^{-1} (*21*) and 4×10^{-8} s^{-1} (*22*), respectively]. Thus, the structure of single-stranded DNA seems likely to exert only a minor effect on the rate of deamination. In contrast, double-stranded DNA is known to be relatively resistant to deamination (*23*).[5] Base pairing presumably increases electron density at the carbon atom to which the exocyclic amino group is attached and may also render that carbon atom less accessible to the approach of nucleophiles.

Scheme 2: Proposed Mechanism for 9-Methylhypoxanthine Breakdown, with Proton Resonances Indicated (in parts per million) for Each Compound Observed by NMR



Ring Opening of Deaminated 9-Methylpurine Derivatives in Neutral Solution. The stability of the purine ring was tested using the model compounds 9-methylhypoxanthine and 9-methylxanthine. Scheme 2 shows the proposed route of decomposition of 9-methylhypoxanthine, with proton NMR resonances (parts per million) indicated for each compound. After 5.5 h at 170 °C, 9-methylhypoxanthine, compound **I**, compound **II**, and formate were present in a ratio of 1:0.8:0.2:1.2 (for the complete NMR spectrum in D_2O, see Figure 2 of the Supporting Information). These observations suggest that the predominant mode of inosine ring opening at neutral pH involves water attack at C2, followed by rapid loss of formate. The proton resonances observed for compound **I** match those reported (*24*) for 5-amino-1-methylimidazole-4-carboxamide in DMSO-d_6 (Figure 6 of the Supporting Information), whose ribonucleotide is formed as an intermediate during the biosynthesis of inosine 5′-phosphate (*25*). In accord with Scheme 2, the amount of formate produced initially was equivalent to the combined concentrations of compounds **I** and **II**. At longer times, the major product shifted to compound **II** with the concomitant disappearance[6] of compound **I**. In the case of 9-methylxanthine, the resonance of the major product (at all time points) was similar to that of compound **II**, suggesting that direct hydrolytic cleavage at C8 is the primary mode of ring opening for this compound.

Whereas the ring structure of 9-methylxanthine is more stable than that of 9-methylhypoxanthine at neutral pH

[4] Thus, for example, the reported rate of glycoside cleavage of 2′-deoxyinosine extrapolated to neutral pH (from acid) is 4.4×10^{-8} s^{-1} at 60 °C (*15*), and the rate of glycoside cleavage of 2′-deoxyinosine determined directly at pH 6.8 (extrapolated to 60 °C) is 1.1×10^{-7} s^{-1}. These comparisons suggest that uncatalyzed hydrolysis and acid-catalyzed hydrolysis make contributions of comparable magnitude to the rate of glycoside bond cleavage of 2′-deoxyadenosine, 2′-deoxyguanosine, and 2′-deoxyinosine at neutral pH. Figures 4 and 5 of the Supporting Information show that the enthalpy of activation for 2′-deoxyadenosine glycoside hydrolysis is lower (24 kcal/mol) at pH 1 than the enthalpy of activation at pH 7 [27 kcal/mol (Table 1)]. Because of the ~3 kcal/mol higher enthalpy of activation for the spontaneous reaction, the switch in mechanism will move to a progressively lower pH with an increase in temperature.

[5] The cytosine residues of native double-stranded DNA are deaminated at <1% of the rate observed with poly-2′-deoxycytidine (*23*).

[6] As proposed in the mechanism presented here, attack at C8 has been reported for cleavage of the adenine ring of 2′-deoxyadenosine in alkali (*9*).

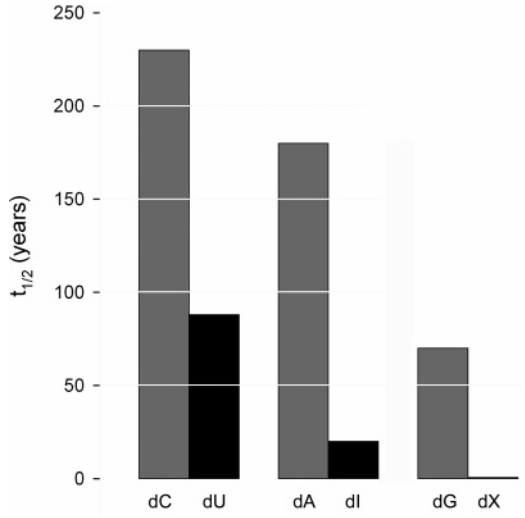


FIGURE 8: Comparison of the half-times for glycoside bond cleavage (25 °C, pH 7) of 2′-deoxycytidine (dC), 2′-deoxyadenosine (dA), 2′-deoxyguanosine (dG), and their deaminated products: 2′-deoxyuridine (dU), 2′-deoxyinosine (dI), and 2′-deoxyxanthosine (dX). The $t_{1/2}$ for dX was based on its ~100-fold faster depurination rate from single-stranded DNA vs that of dG (*26*).

[which has also been observed for the free bases (*25*)], the glycoside bond of 2′-deoxyxanthosine is less stable than that of other deoxyribonucleosides.[7] Figure 8 compares the half-times (pH 7, 25 °C) for glycoside cleavage of 2′-deoxycytidine, 2′-deoxyadenosine, and 2′-deoxyguanosine with those of the products of their deamination. In every case, the stability of the glycoside bond was reduced by ring deamination. With respect to the stability of the purine rings before deamination, ring-opened degradation products of 9-methyladenine and 9-methylguanine were not observed in these experiments. It is also evident that, under neutral conditions, the glycoside bonds of 2′-deoxyinosine and 2′-deoxyxanthosine (*26*) are significantly less stable than the bonds that join the purine ring themselves (Tables 1 and 2). In summary, deamination renders purine nucleosides more susceptible to glycoside bond cleavage as well as ring opening.

DNA Decomposition in Mesophiles and Thermophiles. Figure 7 summarizes the half-times at 25 and 92 °C for decomposition of the bonds of DNA at pH 7, based on the properties of the individual 2′-deoxynucleosides and representative model compounds. It is clear that hydrolytic damage represents a significant threat to the genomic integrity of hyperthermophiles such as *Ignisphaera aggregans*, which grows optimally at 92 °C (*27*). The double-stranded nature of DNA affords some protection against glycoside cleavage (*28*, *29*) and deamination (*23*, *30*), but single-stranded DNA is formed transiently during transcription, replication, and recombination. During these processes, cytosine residues are particularly susceptible to deamination, especially at high temperatures. For that reason, it seems understandable that many organisms contain redundant copies (in some cases as many as three) of uracil DNA glycosylase (*4*, *31*) and that some thermophiles express unique uracil DNA glycosylases that appear to be absent from mesophiles (*32*−*34*). As noted by Lindahl and Wood (*4*), such a multiplicity of repair enzymes for a single type of lesion is not observed for other types of DNA damage.

Rate Enhancements Produced by Glycosylases Involved in DNA Salvage and Repair. Of the numerous enzymes involved in DNA repair, many catalyze the cleavage of glycoside bonds of damaged DNA components (*35*, *36*), or normal bases opposite those that have been damaged (as in the removal of adenine from oxidized guanine·adenine pairs). Table 3 summarizes the kinetic properties of some repair enzymes, and the corresponding rate enhancements that can be estimated from these findings. The calculated rate enhancements for glycoside bond cleavage should be regarded only as lower limits because, as demonstrated by Stivers (*37*), some of the enzymatic rates (k_{cat}) may be limited by product release rather than glycoside cleavage. Moreover, as noted above, the natural single-stranded/double-stranded DNA substrates may be somewhat more stable than predicted by our models. [For example, glycoside bond cleavage is retarded by incorporation of the deoxyribonucleoside into single-stranded DNA, but those contributions are generally less than ~10-fold for depurination (*7*, *28*) and ~50-fold for depyrimidination (*29*) and may vary with the nature of the neighboring bases (*26*).] Despite those reservations, it seems clear that these enzymes exhibit very low k_{cat} values (many less than 1 s^{-1}) and relatively modest rate enhancements (10^7−10^{11}-fold) compared with those of more conventional enzymes such as cytidine deaminase, guanosine deaminase, and adenosine deaminase [10^{12}−10^{14}-fold (Table 3)]. Moreover, the rate enhancements produced by these enzymes are many orders of magnitude lower than those produced by typical *O*-glycoside hydrolases [~10^{17}-fold (*5*)]. It seems reasonable to suppose that the demands placed on enzymes involved in DNA repair are different from those placed on enzymes in high-flux metabolic pathways. Within the repair group, uracil DNA glycosylase produces the largest rate enhancement. Of the enzymes in Table 3, thymine DNA glycosylase produces the smallest rate enhancement. It has been noted that the low activity of this enzyme toward thymine probably contributes to the particularly mutagenic aspects of 5-methylcytosine (*3*, *51*).

Rate Enhancements Produced by Deaminases. In contrast to the DNA glycosylases for which product release is often rate-determining, C−N bond breaking is known to be rate-limiting (*52*, *53*) for adenosine and cytidine deaminase (Table 3). Thus, k_{cat}/k_{non} can be considered to represent the true rate enhancement produced by these enzymes. Table 3 includes a recently identified *S*-adenosylhomocysteine deaminase from *Thermotoga maritima* (*47*), for which the rate-determining step remains to be established. The rate enhancements produced by these deaminases are comparable with those observed for other C−N bond-cleaving enzymes, including proteases and jack bean urease (*5*), but substantially larger than those produced by the DNA glycosylases in Table 3.

The rate constants and thermodynamics of activation presented in Tables 2 and 3 for the deamination of 9-methyladenine show that earlier efforts in this laboratory aimed at monitoring the spontaneous deamination of adenosine by HPLC led to a serious overestimate of that rate constant at 37 °C [1.8×10^{-10} (*49*)]. It is now clear that the meaning of those preliminary experiments was clouded by the multiple routes of decomposition available to adenosine, which were unrecognized at the time.

[7] This difference in stability is indicated by the finding that the rate of depurination of 2′-deoxyxanthosine from single-stranded DNA is ~100-fold greater than that of 2′-deoxyguanosine (*26*).

Table 3: Kinetic Constants (k_{cat} and K_m) and Rate Enhancements (k_{cat}/k_{non}) of Enzymes Involved in DNA Editing and DNA Processing, Obtained by Comparison with Rate Constants for the Corresponding Uncatalyzed Reactions (k_{non}) [reactions shown in order of increasing rate enhancement (k_{cat}/k_{non})]

model substrate, T (°C)	k_{non} (s^{-1})	substrate	enzyme (source)	k_{cat} (s^{-1})	K_m (μM)	k_{cat}/k_{non}	ref
Glycosylases							
T, 37	1.4×10^{-9}	dsDNA G·T pair	thymine DNA glycosylase (*Homo sapiens*)[a]	0.02[b]	–	1.4×10^{7}	*38*
dG, 25	3.2×10^{-10}	dG	deoxyribonucleosidase (*Leishmania donovani*)	0.1	50	9.1×10^{7}	*39*
dA, 37	7.3×10^{-10}	dsDNA oxoG·A pair[c]	adenine mismatch specific glycosylase, MutY (*Escherichia coli*)[a]	0.2[b]	–	2.7×10^{8}	*40*
dI,[d] 25	1.1×10^{-9}	dI[d]	deoxyribonucleosidase (*L. donovani*)	0.5	41	4.5×10^{8}	*39*
dA, 30	2.6×10^{-10}	dsDNA oxoG·A pair[c]	MutY homologue, SpMYH (*Schizosaccharomyces pombe*)[a]	0.5	4.1	1.9×10^{9}	*41*
dU,[e] 37	1.7×10^{-9}	5′···AUG···3′	uracil DNA glycosylase (*H. sapiens*)[f,g]	9.1	0.4	5.3×10^{9}	*42*
dI,[d] 25	1.1×10^{-9}	dI[d]	2′-deoxyinosine hydrolase (*Trypanosoma cruzi*)	28.5	27	2.6×10^{10}	*43*
dU,[e] 25	2.5×10^{-10}	AUAA	uracil DNA glycosylase (*E. coli*)[h]	120[b,i]	4.7	4.8×10^{11}	*44*
Deaminases							
dC, 25	1.8×10^{-10}	dC	cytidine deaminase (*E. coli*)	704	60	3.9×10^{12}	*46*
9-MeA,[j] 30	2.6×10^{-12}	AdoHcy[k]	Tm0936 (*Thermotoga maritima*)[l]	12	210	4.6×10^{12}	*47*
9-MeG,[m] 37	2.5×10^{-12}	guanosine	guanosine deaminase (*Polyrhachis convexa* No. 149)	12	36	4.8×10^{12}	*48*
9-MeA,[j] 25	1.2×10^{-12}	adenosine	adenosine deaminase (*Rattus norvegicus*)	375	26.8	3.1×10^{14}	*49*
Phosphodiesterase							
Np_2P^-,[n] 25	7×10^{-16}	3′,5′-pdTp[o]	staphylococcal nuclease (*Staphylococcal aureus*)	95	9.5	1.4×10^{17}	*50*

[a] The enzyme acts on double-stranded DNA (dsDNA); the substrate used to determine kinetic parameters had a minimum of 24 bp. [b] Enzymatic rates were determined using single-turnover experiments so that rate-limiting product release is not expected to affect rate comparisons. [c] 8-Oxoguanine·adenosine pair. [d] 2′-Deoxyinosine. [e] 2′-Deoxyuridine. [f] The enzyme acts on single-stranded DNA. [g] A 25-base oligonucleotide was used to determine the kinetic parameters. [h] The maximal turnover rate (115 s^{-1}) and the Michaelis constant ($K_m = 0.03$ μM) were also determined for a double-stranded substrate (*45*). [i] The value for k_{cat} (13.5 s^{-1}) was also determined for AUAA. [j] 9-Methyladenine. [k] *S*-Adenosylhomocysteine. [l] *S*-Adenosylhomocysteine deaminase. [m] 9-Methylguanine. [n] Dineopentyl phosphate (anion). [o] Thymidine 3′,5′-diphosphate.

Susceptibility to Transition State Analogue Inhibitors. These findings have some bearing on the design of enzyme inhibitors. After the initial binding of the substrate in the ground state, an enzyme's affinity for the altered substrate in the transition state increases by a factor that matches the rate enhancement produced by the enzyme (k_{cat}/k_{non}). An enzyme is therefore expected to be especially sensitive to inhibition by an ideal transition state analogue inhibitor, i.e., a stable compound that mimics perfectly the structure of the altered substrate in the transition state (for a review, see ref *54*). In view of the magnitudes of the rate enhancements produced by nucleoside deaminases (Table 3), it seems understandable that adenosine deaminase is inhibited by 1,6-dihydroinosine with a K_i value of 3×10^{-13} M (a value $\sim 10^8$-fold lower than the K_m value of substrate adenosine) and that cytidine deaminase is inhibited by 3,4-dihydrouridine with a K_i value of 1×10^{-12} M (a value $\sim 10^7$-fold lower than the K_m value of substrate cytidine) (*55*). While no stable molecule can be expected to capture the binding affinity of an ideal transition state analogue inhibitor ($\sim 10^{-17}$ and $\sim 10^{-16}$ M for adenosine and cytidine deaminase, respectively), these molecules capture nearly 70% of the free energy of binding expected in the ideal case.[8]

Rate Enhancements Produced by Deoxyribonucleases. The final entry in Table 3 describes the hydrolysis (by P−O cleavage) of the phosphodiester bonds of the DNA backbone. The rate of that uncatalyzed reaction was estimated earlier [7×10^{-16} s^{-1} (*2*)] using the model compound dineopentylphosphate (Np_2P^-), for which hydrolysis proceeds exclusively by attack at phosphorus (the reaction catalyzed by staphylococcal nuclease). Unlike the repair enzymes described above, deoxyribonucleases such as staphylococcal nuclease are exceedingly proficient enzymes, producing a rate enhancement (10^{17}-fold) exceeded only by certain phosphomonoesterases (*5*). It should be possible, at least in principle, to design deoxyribonuclease inhibitors that are even more powerful than the deaminase inhibitors mentioned above.

[8] Uracil DNA glycosylase is inhibited by a 1-aza sugar oxocarbenium ion analogue with a dissociation constant of 10^{-10} M (*44*). This inhibitor captures approximately 50% of the free energy of binding expected of an ideal transition state analogue [$k_{non}/(k_{cat}/K_m) = (2.5 \times 10^{-10})/(2 \times 10^8) \approx 10^{-18}$ M (*45*)].

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SUPPORTING INFORMATION AVAILABLE

Additional Arrhenius plots for the hydrolysis of 2′-deoxyuridine, cytosine, and 2′-deoxyadenosine (pH 1), a time course for 2′-deoxyadenosine decomposition at 35 °C and pH 1, and the 500 MHz proton NMR spectrum of a heated sample of 9-methylhypoxanthine in both D_2O and DMSO-d_6. This material is available free of charge via the Internet at http://pubs.acs.org.

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