Glycosyl conformational and inductive effects on the acid catalysed hydrolysis of purine nucleosides

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Received 25 January 1977

ABSTRACT

The log kobs vs. pH profiles were determined in the intermediate acidity region for the glycosyl hydrolysis of guanosine and its 8-amino, 8-monomethylamino, 8-dimethylamino and 8-bromo derivatives. The decreased rate of the 8-amino and enhanced rate of the 8-bromo compound compared to guanosine support an A type mechanism: base protonation followed by glycosyl bond cleavage. All three 8-amino guanosines exhibited log kobs - pH profiles clearly showing that both mono and di-base protonated nucleosides undergo hydrolysis. The 700 fold rate acceleration of 8-N(CH3)guanosine compared to 8-NHCH3guanosine and the 110 fold rate acceleration of 8-N(CH3)2adenosine compared to 8-NHCH3adenosine could be unequivocally attributed to the fixed syn glycosyl conformation of both 8-dimethylamino compounds and relief of steric compression upon hydrolysis in these molecules. The lack of anomerization of all substrates during the course of the reaction supports an A rather than a Schiff-base mechanism.

INTRODUCTION

A detailed knowledge of the mechanism of C-N glycosyl hydrolysis in nucleosides and nucleotides is of importance for several reasons. Hydrolysis in strong acid is a standard degradation technique for determining nucleic base content in polynucleotides. The class of enzymes known as nucleosidases (phosphorylase and hydrolase) perform this function. Such hydrolysis also leads to depurination of DNA and may lead to "local loss of genetic information".

In model systems both acidic and basic hydrolytic pathways have been observed. The acid catalyzed pathways have been more fully explored for both purine and pyrimidine nucleosides. Under acid conditions two principal pathways have been suggested. (B is purine or pyrimidine, X is H or OH):
Parenthetically, in an A-2 scheme (S_N2 like), water would attack synchronously with departure of the nucleic base.

Scheme 2 (Schiff-base)

While earlier reports favored the Schiff-base mechanism, more recently evidence favoring the A-1 mechanism has been presented for purine nucleosides based on the work of Zoltewicz et al. and of others. Zoltewicz et al. showed that the rate of hydrolysis at low pH increased with increasing hydronium ion concentration and leveled off in very strong acid, supposedly where diprotonation of the nucleic base was completed. For no purine nucleoside had there been provided direct evidence for the existence of two reactive substrates - mono and diprotonated as suggested by Zoltewicz et al. (Scheme 1). Such evidence does exist for pyrimidine nucleosides.

The C8 position in purines is the closest one to the glycosyl C-N bond capable of substitution. We have chosen the -Br, -H, -NH2, -NHCH3 and -N(CH3)2 substituents on the guanosine C8 position to test the electronic and glycosyl conformational factors influencing the acid catalyzed hydrolysis mechanism.

We here report that steric effects play a very important role in the acid catalyzed hydrolytic pathway. In addition, the observed Inductive effects support the A-1 mechanism. We present the first literature evidence for two distinct ionization states of a purine substrate as reactive intermediates as well as chemical evidence favoring an A-1, rather than a Schiff-base type mechanism.

**EXPERIMENTAL**

**Reagents.** G was purchased from Sigma; A, 8-Br-A, from Aldrich; 95% aqueous hydrazine from Fisher; monomethylamine and dimethylamine from Matheson Gas Products. All inorganic reagents employed were high purity reagent grade chem-
Nucleosides used without further purification.

$8$-Br-G was synthesized from G$^9$, $8$-NH$_2$-G from $8$-Br-G$^{10}$, $8$-NHCH$_3$-G from $8$-Br-G$^{11}$, $8$-N(CH$_3$)$_2$-G from $8$-Br-G$^{12}$. $8$-NHCH$_3$-A and $8$-N(CH$_3$)$_2$-A were synthesized from $8$-Br-A.$^{13}$ All nucleosides synthesized in this laboratory exhibited spectral properties (uv and pmr) and gave elemental analyses in satisfactory accord with literature values.

**Determination of rate constants.** Most rates were studied at $100.6^\circ C$ (a temperature also employed by Zoltewicz et al.$^{4a}$) since most rate constants were conveniently measured at this temperature. Typically, $5 - 6$ ml aliquots of buffered nucleoside solution (0.005M) were sealed in a pyrex tube. On the average 15 such samples per run were placed in an oil bath (temperature maintained at $100.6\pm0.1^\circ C$ with a Honeywell proportional temperature controller). Periodically a tube was removed and the reaction quenched in an ice bath. Reaction time was measured from the time the tube was immersed in the oil bath until the time of quenching in the ice bath. At least 10 of the 15 tubes were removed within the first half-life of the reaction. The infinity time sample was kept in the oil bath for nearly 10 half lives. After the tubes were opened (including the zero time unreacted one), the solutions were diluted 100 fold with 0.1N NaOH. This was done since the uv absorption spectra of nucleoside and the corresponding nucleic base exhibit greatest differences under these conditions. The rates of hydrolysis of $8$-Br-G and $8$-N(CH$_3$)$_2$-G were determined by uv measurements of the quenched solution at pH7.

For each nucleoside the absorbance was recorded at two wavelengths: $\lambda_1$ - the wavelength at which the maximum difference in absorbance between the nucleoside and corresponding base is observed, and $\lambda_2$ - the wavelength corresponding to the isosbestic point apparent in the hydrolysis mixture. The $\lambda_1$ and $\lambda_2$ values selected for each nucleoside are given below. The data were analyzed assuming pseudo first order conditions: $\ln(C/Co) = kt$ where $Co$ is the initial concentration of nucleoside $C$ is the concentration at time $t$. Using the absorbance ratios at two wavelengths reduces any experimental errors due to dilution.

Reactions with very long half-lives sometimes gave absorbance ratios indicating that further reaction may have taken place. In such cases an iterative procedure was employed which incremented the absorbance ratios corresponding to infinity time until the best (signified by the highest linear least squares correlation coefficient) straight line was obtained. Even in these cases the final iterated absorbance ratios did not vary from the experimental-
ly found ones by more than 11%. The following \( \lambda_1 \) and \( \lambda_2 \) values were employed (all in nm's): G 260, 275; 8-Br-G 230, 248; 8-NH\(_2\)-G 260, 280; 8-NHCH\(_3\)-G 260, 280; 8-N(CH\(_3\))\(_2\)-G 230, 246.6; 8-NHCH\(_3\)-A 280, 300; 8-N(CH\(_3\))\(_2\)-A 280, 300.

The above method is only applicable if the tube is immersed in the oil bath for at least 10 minutes. Otherwise, significant nonlinearity of the pseudo-first-order plots was observed.

Hydrolysis at low pH's was performed in the thermostatted cell compartment of the spectrophotometer. 3.0 ml of buffer solution was equilibrated inside the spectrophotometer for 10 minutes at the desired temperature. 30 \( \mu \)l of nucleoside solution (dissolved in the same buffer) was then added via a Hamilton syringe to give a final concentration of nucleoside of ca. 5 \( \times \) 10\(^{-5}\) M. The reaction was followed at that single wavelength giving rise to the maximum difference in absorbance between nucleoside and base. The absorbance change was followed as a function of time via a recorder as well as by regular recording of the digital readout reading. The time of injection of nucleoside was taken as zero time and the absorbance corresponding to this initial time was obtained by back-extrapolation of the absorbance vs. time curve.

All pseudo-first-order data exhibited strict linearity for at least three half-lives and a linear least squares correlation coefficient of at least 0.990.

UV measurements were performed on a Cary 14 or Beckman Acta III spectrophotometer.

Analysis of Products. Thin layer chromatographic analyses were performed on the hydrolysates. The plates were coated with SilicAR-7GF (Mallinkrodt) and Ethyl Acetate-n-Propanol-H\(_2\)O (4:1:2 v/v) was the solvent.

pK Determinations were performed both spectrophotometrically and potentio-metrically.

The buffers in the Ho range were made up as wt% H\(_2\)SO\(_4\)-H\(_2\)O according to Johnson et al.\(^{13}\). Since 8-Br-G hydrolyzes rapidly in strong acid, absorbances for this substrate had to be extrapolated to zero time of mixing.

RESULTS

Test for buffer effects. As log \( k_{obs} \) vs. pH profiles were to be constructed for all substrates, it was necessary to show that the rates at the same pH were independent of the buffer employed. Our data indicate that the change in buffer system introduces less than 3% change in \( k_{obs} \). Hence, there appears to be no interaction of substrate with the buffers employed.

Zoltewicz et al.\(^{48}\) found that the hydrolysis rate of G at 100.6\(^{\circ}\)C depended on the concentration of formate buffer at constant pH. A tenfold increase
In buffer concentration resulted in a nearly 70% increase in observed rate constant. According to our results general acid-base catalysis, if present, within experimental error is insignificant.

Since most hydrolysis kinetics were performed at 100.6°C and the buffers were prepared near ambient temperatures, the effect of temperature change on pH had to be estimated. For a buffer composed of a weak acid and its salt, the \( \Delta \text{pH}/\Delta T \) could be estimated according to literature formula giving for HCl, HCOOH and CH\(_3\)COOH \( \Delta \text{pH} (25^\circ \text{C} \to 100.6^\circ \text{C}) \) of 0.036, 0.08 and 0.015, respectively. While uncertainty exists concerning this approach, for four acids, (tartrate, phthalate, phosphate, tetroxalate) whose \( \Delta \text{pH} (25^\circ \text{C} \to 95^\circ \text{C}) \) is known for this \( \Delta T \), \( \Delta \text{pH} \) is less than 0.13. The true pH can be estimated to within ± 0.1 units at the reaction temperatures here employed.

**pK\(_a\)'s of C8-substituted Purine Nucleosides.** In order to interpret the kinetic data a knowledge of relevant ionization constants is essential. The first protonation of all guanosine derivatives employed in this study is most likely at N7\(^{1,5,16}\). In addition, based on a combination of pmr and uv spectral results under aqueous and nonaqueous conditions we have found that all guanosine compounds here studied exist in the tautomeric form,

\[
\text{N}\begin{array}{c}
| \text{O} \\
| \text{H}_2N
\end{array}\text{H}_{2N}\begin{array}{c}
| \text{N} \\
| \text{X}
\end{array}\]

i.e. the exocyclic 8-amino group does not enter tautomeric equilibrium (within experimental error) with the purine ring.

**Table I. First Base Protonation pK\(_a\)'s of C8 Substituted Purine Nucleosides at 25°C**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Spectrophotometric(^a)</th>
<th>Potentiometric (conc, M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Guanosine</td>
<td>2.1 ± 0.1</td>
<td>1.8 ± 0.1 (4.46 x 10(^{-3}))</td>
</tr>
<tr>
<td>8-Br-Guanosine</td>
<td>0.2 ± 0.1</td>
<td></td>
</tr>
<tr>
<td>8-NH(_2)-Guanosine</td>
<td>4.66 ± 0.07</td>
<td>4.66 ± 0.005 (7.63 x 10(^{-3}))</td>
</tr>
<tr>
<td>8-NHCH(_3)-Guanosine</td>
<td>4.68 ± 0.08</td>
<td>4.68 ± 0.005 (7.22 x 10(^{-3}))</td>
</tr>
<tr>
<td>8-N(CH(_3))(_2)-Guanosine</td>
<td>3.34 ± 0.08</td>
<td>3.10 ± 0.005 (8.37 x 10(^{-3}))</td>
</tr>
<tr>
<td>8-NHCH(_3)-Adenosine</td>
<td>3.82 ± 0.008</td>
<td>3.82 ± 0.008 (9.94 x 10(^{-3}))</td>
</tr>
<tr>
<td>8-N(CH(_3))(_2)-Adenosine</td>
<td>3.84 ± 0.007</td>
<td>3.84 ± 0.007 (1.04 x 10(^{-2}))</td>
</tr>
<tr>
<td>Adenosine</td>
<td>3.63</td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Concentration 5 x 10\(^{-5}\) M throughout; Ionic strength is 0.1 maintained with KCl in all determinations.
Table 1 presents all relevant spectrophotometric and potentiometric pK results at 25°C. Those substrates exhibiting similar spectrophotometric and potentiometric pK values are subject to intermolecular interactions that do not change over the entire concentration range employed. The most prominent feature is the difference in pK between 8-NH$_2$-G and 8-NHCH$_3$-G on the one hand and 8-N(CH$_3$)$_2$-G on the other. 8-N(CH$_3$)$_2$-G has the "normal" value of pK, and the other two compounds form unusually stable triply hydrogen bonded hemiprotonated base pairs thus increasing the pK values.

Protonation in adenosines is predominantly at N1$^1$ and the remote 8-NHCH$_3^-$ and 8-N(CH$_3$)$_2^-$ groups appear to have only a minor electron donating function.

Changes in pK with temperature can be estimated. Usually, the dissociated form is more favored at higher temperature (decreased pK with increased temperature). Perrin estimated dpK/dT of 0.008/°C for purine and 0.009/°C for adenosine$^1$. Raising the temperature from 25 to 100°C results in a decrease of pK by 0.60 and 0.68 units, respectively for purine and adenosine$^1$. For some G derivatives the kinetic results (vide infra) allow direct determination of the high temperature pK's (subject to the small uncertainty discussed before due to changes in pH with temperature).

The second protonation pK's (pK$_{a2}$), on the other hand increase with temperature. For example, pK$_{a2}$ of G at 25°C is -2.43$^{4b}$ and would be shifted to -1.82 at 100.6°C.

Rate - pH Profiles. The following rate expression is a minimal one to account for all the observed results. \[
\text{rate} = k_{\text{obs}} S_T = k_1 (\text{SH}^+) + k_2 (\text{SH}^2+) \quad (1)
\]
where $k_{\text{obs}}$ is the pseudo first order rate constant, $S_T$ is the total nucleoside concentration, (SH$^+$) and (SH$^2+$) are the concentrations of mono and di-base protonated nucleoside, respectively.

\[
k_{\text{obs}} = \frac{k_1}{K_{a1}(\text{H}^+) + (\text{H}^+)/K_{a2} + 1} + \frac{k_2}{(K_{a2}/(\text{H}^+))(K_{a1}/(\text{H}^+) + 1) + 1} \quad (2)
\]
where $k_1$ and $k_2$ are the specific rate constants, and $K_{a1}$ and $K_{a2}$ the conjugate acid (protonated base) dissociation constants for the mono and di-protonated substrates, respectively.

Guanosine. The linear increase of log $k_{\text{obs}}$ with decreasing pH as reported by Zoltewicz et al.$^4$ and substantiated by Hevesi et al.$^6$ was again confirmed in this study in a very limited pH range at 100.6°C. We obtained a $k_2 = 2.0 \times 10^{-2}$M$^{-1}$sec$^{-1}$, compared to $1.78 \times 10^{-2}$M$^{-1}$sec$^{-1}$ reported by Zoltewicz et al.$^4$.

8-NH$_2$-Guanosine and 8-NHCH$_3$-Guanosine. These two substrates exhibit two portions of linear log $k_{\text{obs}}$ vs. pH behavior separated by a pH independent region (Figures...
I and 2, respectively). Superimposed on the experimental points the theoretical curve is drawn assuming the relationship outlined in Eq 2.

8-N(CH₃)₂-Guanosine. Since at 100.6°C this substrate reacted too fast to be studied in a wide pH range, it was studied at both 100.6°C and 79.5°C. At the lower temperature a pH independent region was found for this substrate as well (Figure 3).

8-Br-Guanosine. To check the kinetic behavior of this substrate in a wide pH range, data were collected at 100.6°C and 61.0°C. Throughout the pH (and H⁺) range studied there is a linear increase in log kobs with decreasing pH (Figure 4).

The rate pH profiles of all 8-amino derivatives were analyzed by computer fitting. An inspection of Eq 2 reveals that a plateau (pH independent kobs) could be observed under conditions such that kₐ₂ > (H⁺) > kₐ₁ and k₂(H⁺)/kₐ₂ < k₁. All three amino derivatives exhibit a plateau region from which a k₁ value can be estimated. Fitting of the high pH data allows estimation of k₁ under the reaction conditions. If the rate leveled off in strong acid one could also determine k₂ and kₐ₂ with precision. With our data we are only in a position to ascertain the ratio k₂/kₐ₂ for 8-NH₂-G and 8-NHCH₃-G.
Table II presents the results of computer fitting on the amino compounds. The reason for the plateau in the log $k_{obs}$ vs. pH profiles becomes obvious: it is due to $k_1/K_{a1} > k_2/K_{a2}$. Zoltewicz et al. reasoned that the lack of a plateau in $G$ was due to $k_1/K_{a1} \approx k_2/K_{a2}$ in this compound. Presumably, the same reasoning applies to the linear log $k_{obs}$ - pH profile in 8-Br-G.

Relative Rates. Since we could not measure total hydrolytic profiles of all three amino compounds at the same temperature, an estimate of the kinetic constant $k_1$ for the 8-N(CH$_3$)$_2$-G at 100°C is needed. One can do this two ways. Inspection of the 79.5 and 100.6°C results for this compound indicates (near pH 5, away from the plateau), that the temperature difference should bring about a eight to ninefold rate acceleration. Alternatively, employing the activation parameters of $G$, one can estimate a rate acceleration of about 7.3 fold going to 100.6°C.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>$k_1/K_{a1}$</th>
<th>$k_1$ (hr$^{-1}$)</th>
<th>$pK_{a1}$</th>
<th>$K_{a1}$</th>
<th>$k_2/K_{a2}$</th>
<th>$T$, °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>8-NH$_2$-G</td>
<td>2.40 x 10$^3$</td>
<td>0.240 ± 0.015</td>
<td>4.0 ± 0.1</td>
<td>15 ± 2</td>
<td></td>
<td>100.6</td>
</tr>
<tr>
<td>8-NHCH$_3$-G</td>
<td>2.08 x 10$^3$</td>
<td>0.125 ± 0.010</td>
<td>4.25 ± 0.10</td>
<td>25 ± 2</td>
<td></td>
<td>100.6</td>
</tr>
<tr>
<td>8-N(CH$_3$)$_2$-G</td>
<td>1.50 x 10$^4$</td>
<td>12 ± 1</td>
<td>3.07 ± 0.1</td>
<td>--</td>
<td></td>
<td>79.5</td>
</tr>
</tbody>
</table>

$^a$Derived from log $k_{obs}$ -pH profiles according to Eq. 2.

$^b$Extrapolated from the 79.5° and 100.6° data shown in Figure 3.
Table II presents the values of $k_1$ for the three 8-amino derivatives. 8-NH$_2$-G is somewhat faster than 8-NHCH$_3$-G, but 8-N(CH$_3$)$_2$-G is enormously faster than either of the former two (several hundred times).

Comparison of the relative rates of hydrolysis of all five substrates at the same temperature is more difficult yet. Both G and 8-Br-G have kinetic profiles which do not allow for the dissection of the kinetic parameters. What we do have, however, are the room temperature pK$_{al}$ values of all five compounds as well as the knowledge that G and 8-Br-G obey the approximate relationship: $k_1/K_{al} \approx k_2/K_{a2}$. As an approximation one can compare the $k_{obs}$ values at pH's corresponding to the room temperature pK$_{al}$'s of the substrates (assuming all five substrates experience similar pK$_{al}$ shifts with temperature) and all at 100.6°C (this also necessitates extrapolation of the results for 8-Br-G and 8-N(CH$_3$)$_2$-G to this temperature). Such estimates are presented in Table III. The differences are large enough to allow delineation of trends notwithstanding the approximation in extrapolation to higher temperatures and the uncertainties in pK and pH changes with increased temperatures.

The rates of hydrolysis of 8-NHCH$_3$-A and 8-N(CH$_3$)$_2$-A were determined at a single pH and temperature only. The 25°C pK$_{al}$'s of these two substrates are so close to each other (3.82 and 3.84 respectively) that relative rates can be determined very simply (Table III).

**DISCUSSION**

Zoltewicz et al. presented substantial evidence in favor of an A-1 mechanism in their studies on 7-methylguanosine. However, none of the unsubstituted purine nucleosides reported in the literature gave evidence of both

<table>
<thead>
<tr>
<th>Nucleoside</th>
<th>at pH</th>
<th>$k_{obs}$ (hr$^{-1}$)</th>
<th>$k_{rel}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>G</td>
<td>2.1</td>
<td>0.65</td>
<td>1</td>
</tr>
<tr>
<td>8-NH$_2$-G</td>
<td>4.66</td>
<td>0.043</td>
<td>0.066</td>
</tr>
<tr>
<td>8-NHCH$_3$-G</td>
<td>4.67</td>
<td>0.034</td>
<td>0.052</td>
</tr>
<tr>
<td>8-N(CH$_3$)$_2$-G</td>
<td>3.34</td>
<td>27$^a$</td>
<td>41</td>
</tr>
<tr>
<td>8-Br-G</td>
<td>0.20</td>
<td>300$^b$</td>
<td>461</td>
</tr>
<tr>
<td>8-NHCH$_3$-A$^c$</td>
<td>3.36</td>
<td>0.038</td>
<td>1</td>
</tr>
<tr>
<td>8-N(CH$_c$)$_2$-A$^c$</td>
<td>3.36</td>
<td>4.27</td>
<td>110</td>
</tr>
</tbody>
</table>

$^a$Estimated from data in Figure 3. $^b$Estimated from data in Figure 4. $^c$pK$_a$'s are near 3.82 for both; rates determined at 84°C.
mono- and di-base protonated substrates hydrolyzing. All 8-aminoguanidines exhibit a plateau region in the log $k_{obs}$-pH profiles. The plots for 8-NH$_2$-G and 8-NHCH$_3$-G provide clear indication of two forms of the substrate undergoing hydrolysis. Our kinetic plots provide base protonation $pK_a$ values for the three amino compounds clearly showing that the high pH behavior corresponds to hydrolysis of a base monoprotonated substrate according to an A type mechanism. We do not have enough kinetic data to disprove the possibility of the Schiff-base mechanism intervening in strong acid. We do offer chemical evidence below favoring an A type mechanism under all our reaction conditions for all our substrates.

Recently Cadet and Teoule presented a technique for chemically distinguishing between the A-1 (or A-2) and Schiff-base schemes. They found that in 2M HClO$_4$ at 90°C thymidine and 2'-deoxyuridine (but not 2'-deoxycytidine or 5-bromo-2'-deoxyuridine) underwent anomerization ($\beta \leftrightarrow \alpha$) and isomerization (furanoside $\rightarrow$ pyranoside) prior to glycosyl hydrolysis. Their results proved that for some substrates, at least, Schiff-base formation (from initial ribose ring oxygen protonation followed by ribose ring opening) according to Scheme 2, was a reversible process. Whether hydrolysis in these substrates resulted from the Schiff-base or the A-1 pathway (pursuant to reversible ribose ring opening) had not been established. We undertook experiments to check for anomerization of our substrates prior to hydrolysis. At each specified reaction condition three samples were compared: partially hydrolyzed (reacted for less than 2/3 half lives), completely hydrolyzed (reacted for ten half lives) and unhydrolyzed. The thin layer chromatographic system employed is documented to be capable of separating $\beta$-D-guanosine from $\alpha$-D-guanosine ($R_{\alpha}/R_{\beta} = 1.20$). Under all conditions employed (G at $H_0 = 2.0$, 30°C and pH's 2.0 and 3.5, 100.6°C; 8-NH$_2$-G at pH's 1.2 and 3.5, 100.6°C; 8-NHCH$_3$-G at pH's 1.2 and 3.5, 100.6°C; 8-N(CH$_3$)$_2$-G at pH = 3.5, 80°C; 8-Br-G at $H_0 = -2.00$, 30°C and at pH = 0.20, 60°C) only two spots were detected corresponding to the unhydrolyzed $\beta$-nucleoside and the completely hydrolyzed C8-substituted base. Hence no anomerization was apparent for any of our guanosine substrates. The Schiff-base mechanism could be ruled out for all conditions here employed unless the $\beta$-anomer is at least 20 times more stable than the $\alpha$-anomer (i.e. 5% detection limit) for all our substrates, not a likely possibility.

The substituent effects (Table III) support an A type mechanism. C8-NH$_2$-G and C8-NHCH$_3$-G hydrolyze slower and C8-Br-G faster than G, i.e. electron donation retards, withdrawal accelerates heterolytic C-N fission.
This is in accord with an A-1 or A-2 scheme but not with rate limiting Schiff-base formation which should have exactly the opposite electron demands to form immonium ion at N9. Similar inductive effects to those here found were reported in the acid hydrolysis of 8-Br-A and 8-OCH$_3$-A (admittedly only at a single pH) and for the 5-Bromo substituent in the 2'-deoxycytidine series$^{8,20}$. Theoretical calculations from this laboratory$^{21}$ showed that C8-NH$_2$ increases the electron density at N9, C8-CI decreases it compared to C8-H in guanines, and protonation at N7 invariably decreases the electron density at N9. These calculated trends give qualitative support to both pK$_a$ and rate results, especially for an A-type mechanism.

The most striking and unanticipated result is seen from a comparison of k$_1$ values among the three C8-aminoguanosines (Table II). 8-N(CH$_3$)$_2$-G has a specific rate constant hundreds of times larger than do 8-NH$_2$-G and 8-NHCH$_3$-G. The rate-pH profiles and product studies both suggest that at least in the weak acid region (pH $>$ 2) similar mechanisms operate for all three substrates. We have also shown that the same tautomeric structures predominate for all three (see pK$_a$ results above).

We have shown elsewhere$^{22}$ that in the proton magnetic resonance spectra of these three compounds the ribose C1'-H's have very similar chemical shifts (inductive effects mediated through the glycosyl bond are minimal). However, the C2'-H chemical shifts are very different at all pH's (neutral and strong acid) and in the entire temperature range employed in the present kinetic study. In fact, the behavior of the C2'-H chemical shift in 8-N(CH$_3$)$_2$-G clearly identifies this molecule to be locked in its syn glycosyl conformation$^{23,24}$, as expected for a bulky C8-substituent on purines$^{25}$. The other amino compounds are flexible syn-anti mixtures. The greater hydrolytic instability of 8-N(CH$_3$)$_2$-G suggests intrinsic instability of this compared to the other amino compounds. Since 8-NHCH$_3$-G and 8-NH$_2$-G can participate in very strong triply hydrogen bonded hemi-protonated base pairs$^{16}$ it was necessary to prove that the hydrolytic rate differences observed were not due to the hydrogen bonding scheme differences. 8-NHCH$_3$-A and 8-N(CH$_3$)$_2$-A were synthesized and their pK$_a$'s were determined to be virtually identical. Proton magnetic resonance studies indicated 8-N(CH$_3$)$_2$-A to be syn again, 8-NHCH$_3$-A to be a flexible syn-anti mixture$^{22}$. The 110-fold rate acceleration for 8-N(CH$_3$)$_2$-A compared to 8-NHCH$_3$-A (Table III) observed in this pair can only be attributed to differences in glycosyl conformation and intrinsic relative stabilities.

Panzica et al.$^5$ reported kinetic data (in 1.02 M HCl) for the hydrolysis of a number of 7'-D- and 9'-D- ribofuranosyl purines. The faster rates
found for 6-amino-7-D- than 6-amino-9-D-ribofuranosyl purines were attributed to steric effects. They suggested that as steric crowding near the glycosyl C-N bond accelerated the rate (in 7-D-substrates) an A-1 rather than an A-2 mechanism was operative. This claim was based on the premise that S_N^1 ionization reactions are accelerated by steric crowding near the reaction site, S_N^2 types are usually slowed down by such crowding^26.

Very recently it was shown that demethylation of N-methylpyridinium ions by triphenylphosphine (undoubtedly an S_N^2 reaction) was accelerated by steric bulk of substituent on the C2^*pyridinium position^27 i.e.

\[
\text{In addition, electron withdrawing R groups accelerated the reaction. Insofar that N7 protonated guanosines bear a vague resemblance to this substrate, the inductive and steric effects we find cannot clearly distinguish between an A-1 and A-2 (:OH_2 acting as a nucleophile) mechanisms. The best evidence in favor of an A-1 mechanism under acidic conditions is from Garrett and Mehta who showed that in acid 2^*deoxyadenosine hydrolyzed very much faster than adenosine^7 but in base (OH^- nucleophile in an A-2 pathway) the two hydrolyzed at nearly equal rates^28.}

Relief of steric compression in the ground state of 8-N(CH_3)_2-G and 8-N(CH_3)_2-A (rigid syn conformers) appears to be the source of the large rate accelerations found. Solvation may play an additional role: if the syn conformers interact less favorably with H_2O than the anti ones, solvent would further stabilize the anti ground state compared to the syn one.

Based on these findings substrate distortion would be a likely function for a nucleosidase enzyme, were it to function according to an A-1 mechanism. Acknowledgments. We are grateful to the Rutgers University Research Council and the Biomedical Research Support (NIH) for partial support of this work. Abstracted in part from the Ph.D. dissertation of H.N. submitted to the Graduate Faculty, Rutgers University, 1976.

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