Contribution to the Mechanism of the Acid-Catalyzed Hydrolysis of Purine Nucleosides[1]

L. Hevesi, E. Wolfson-Davidson, J. B. Nagy, O. B. Nagy,* and A. Bruylants

Contribution from the Université Catholique de Louvain, Laboratoire de Chimie Générale et Organique, B-3000 Louvain, Belgium. *Received July 12, 1971*

Abstract: The kinetics of the acid-catalyzed hydrolysis of the naturally occurring purine nucleosides (adenosine, guanosine, deoxyadenosine, and deoxyguanosine) is reinvestigated. The analysis of the relatively complex pH profile and of the other kinetic data bears out fully the mechanism proposed by Zoltewitz and coworkers. The possibility of influencing the reaction rate by external charge-transfer complexing agents is also explored.

In connection with research on charge-transfer complexes a systematic study is being carried out in our laboratory in order to explore the possibility of influencing reaction rates by complexation of the reagents.[2] Many investigators have shown that catalytic or inhibitory effects, brought about by complexing agents, may be of importance not only in fundamental research, but also in pharmacology and biological chemistry.[3-19] For this reason, we undertook the study of the acid-catalyzed hydrolysis of some naturally occurring purine nucleosides. Our choice was guided by the great importance of these molecules and also by the fact that the purine nucleus is known to form at least weak molecular associations.[5] No pertinent interpretation of the observed kinetic effects can be made until the detailed mechanism of the reaction is known. Despite the fact that acidic hydrolysis of nucleic acids is common in biochemistry,[20-24] only relatively little kinetic work has been done in order to determine the mechanism of the C–N bond cleavage.

Some authors have carried out kinetic measurements without proposing any mechanism,[25-27] while others have discussed the mechanistic aspects of the problem in a more qualitative manner.[28] It has been proposed that the N_9 protonated species is transformed into a charged Schiff base through sugar ring opening, which then generates hydrolytic products by water addition. The most fundamental studies on purine nucleosides are due to Garrett[32] and to Zoltewitz and coworkers.[33] Garrett studied the hydrolysis of psicofuranine (6-amino-9-D-psicofuranosylpurine) in a large pH range at different temperatures. He has shown that the reaction is not subject to general acid–base catalysis. He has also determined activation enthalpies from simplified kinetic analysis. Despite the fact that he takes into account several reactive species (unprotonated and monoprotonated substrates) to explain the kinetic behavior, he did not propose any mechanism. An interesting feature of this work is the appearance of a curvature on the pH profile in the vicinity of the pK_a.

Zoltewitz and coworkers examined several nucleosides. For the naturally occurring ones they have found a linear pH profile, determined some activation parameters as well as kinetic solvent isotope effects, and proposed a detailed reaction mechanism. This implies the breakdown of mono- and diprotonated species on the purine ring leading to purine and sugar by the monomolecular cleavage of the glycosidic linkage.

Although the mechanism proposed by Zoltewitz and coworkers can be considered as definitely established, the difference in the form of the pH profile as given by Garrett on one hand and by Zoltewitz and coworkers on the other prompted us to reinvestigate the problem.

Results and Discussion

Kinetics of the Acid-Catalyzed Hydrolysis. Four naturally occurring nucleosides, having a purine deriva-

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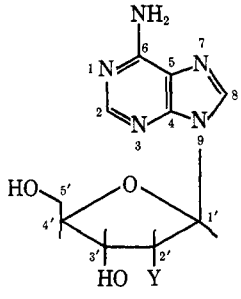
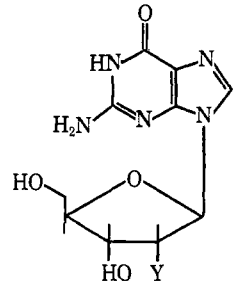
Table I. Temperature Dependence of the Pseudo-First-Order Rate Constants

	$k_{obsd} \times 10^3$ min^{-1} —Temp, °C—										
Compd	23.0	37.2	43.2	48.6	52.0	53.4	57.6	58.4	63.6	75.3	*N* HCl
A			3.25	4.56		9.23	14.2				1
dA	6.58	37.4	104		256				573		0.1
G			5.35	7.57	8.1			17.1	35.4	113	1
dG	6.92	39.1	91.9		228				486		0.1

tive such as aglycone, namely adenosine (A), guanosine (G), 2′-deoxyadenosine (dA), and 2′-deoxyguanosine (dG), have been studied.



Y = OH; A
Y = H; dA



Y = OH; G
Y = H; dG

For practical reasons only the last compound could be investigated in a sufficiently large pH range. In the presence of an excess of acid all the kinetics exhibited first-order behavior. The observed pseudo-first-order rate constants, k_{obsd}, are given in Tables I and II. The

Table II. Variation of the Pseudo-First-Order Rate Constants of dG, k_{obsd}, with Acidity[d]

pH	a_{H^+}	k_{obsd}, min^{-1} [b]	n[c]
4.1	7.94×10^{-5}	2.44×10^{-4}	5
3.2	6.34×10^{-4}	1.44×10^{-3}	5
2.8	1.585×10^{-3}	3.39×10^{-3}	5
2.5	3.158×10^{-3}	6.94×10^{-3}	5
2.42	3.8×10^{-3}	8.66×10^{-3}	5
2.255	5.56×10^{-3}	10.94×10^{-3}	5
2.205	6.237×10^{-3}	14.52×10^{-3}	5
2.0	1.0×10^{-2}	3.485×10^{-2}	5
1.5	3.158×10^{-2}	7.56×10^{-2}	5
H_0	h_0[a]		
0.98	0.105	1.574×10^{-1}	5
0.65	0.224	3.378×10^{-1}	6
0.44	0.363	5.467×10^{-1}	7
0.32	0.479	7.698×10^{-1}	6
0.10	0.795	1.38	7
−0.07	1.175	1.857	6
−0.20	1.585	2.577	7

[a] Acidity function h_0 defined by $h_0 = a_{H^+}(\gamma_B/\gamma_{BH^+})$ and calculated from $H_0 = -\log h_0$. B designates the indicator. [b] In the pH range 1–4.1 the ionic force was kept constant at the value of 0.31. At higher acidities the ionic force was not controlled. [c] Number of measurements. [d] $T = 48.4°$; λ 263.1 nm; $[dG]_0 = 1 \times 10^{-4}$ mole l.$^{-1}$.

pH profile for the reaction of dG, as obtained from the data of Table II, is represented in Figure 1. At pH < 1, H_0 was used instead of pH.

It should be noted that this pH profile is not a straight line as was found by Zoltewitz and coworkers, but reveals curvatures and in this respect bears out, at least partially, the findings of Garrett.

More information can be obtained from the nonlogarithmic plot of k_{obsd} *vs.* a_{H^+} (Figure 2), where (at $[H^+] > 10^{-2}$) activities were replaced by h_0.[34] The activation parameters are collected in Table III.

Table III. Activation Parameters of the Acidic Hydrolysis of Purine Nucleosides

Compound	$\Delta H^{\ddagger}$, kcal mole^{-1}	$\Delta S^{\ddagger}$, eu	$\Delta G^{\ddagger}$, kcal mole^{-1}
A	21.3	−10.8	24.8
G	20.9	−11.3	24.6
dA	23.7 (23.3 ± 0.5)[a]	+8.4 (+8.4 ± 1.3)[a]	21.1
dG	22.5 (25.0 ± 1.0)[a]	+4.3 (+12.7 ± 2.5)[a]	21.2

[a] Values taken from ref 33. It can be seen that the values for dA agree very well with those given by Zoltewitz and coworkers while a large difference exists for dG.

We have also measured the kinetic solvent isotope effect in the case of the deoxy derivatives. At 37° and at 0.093 *N* DCl or HCl concentration the results are $k_{D_2O}/k_{H_2O} = 2.14$ for dG and $k_{D_2O}/k_{H_2O} = 2.64$ for dA (in comparison with 2.4 in ref 33).

Figure 1 shows that at high acid concentration the logarithm of the rate constant varies linearly with H_0, whereas at lower concentrations this dependence is more complex. As a matter of fact, we can distinguish two curvatures: the first, more pronounced, is situated between pH 1 and pH 2.2; the second, less apparent in this figure, is between pH 2.2 and 4.1. This is even more visible in Figure 2, which suggests that the curvature at pH 2.2–4.1 may be due to saturation effects with increasing proton concentration, while the other, at pH 1–2.2, should correspond to the intervention of a higher order term in the rate expression.

Since the saturation effect occurs in the pH range involving the pK_a (2.31)[33] of the first protonation of the substrate, the first reactive species must be the monoprotonated dG formed in a rapid preequilibrium step as was previously indicated.[33] The observed kinetic solvent isotope effect also supports this hypothesis.

Therefore, the reaction is subject to specific acid catalysis.

In the same way we propose that the rapidly increasing slope of the curve following the levelling off is due to the reaction of the doubly protonated species. The intervention of this species has also been proposed,[33] but in a much more intuitive manner.

Accordingly, the kinetic scheme may be written as in Scheme I, where S = substrate, SH^+ = monoprotonated substrate, SH_2^{2+} = diprotonated substrate, P = products; K_1 and K_2 are the acidity constants of the mono- and diprotonated substrates, respectively.

(34) This fact does not bring about any essential difference, because the linearity of the corresponding part shows that the ratio γ_B/γ_{BH^+} is constant in our case.

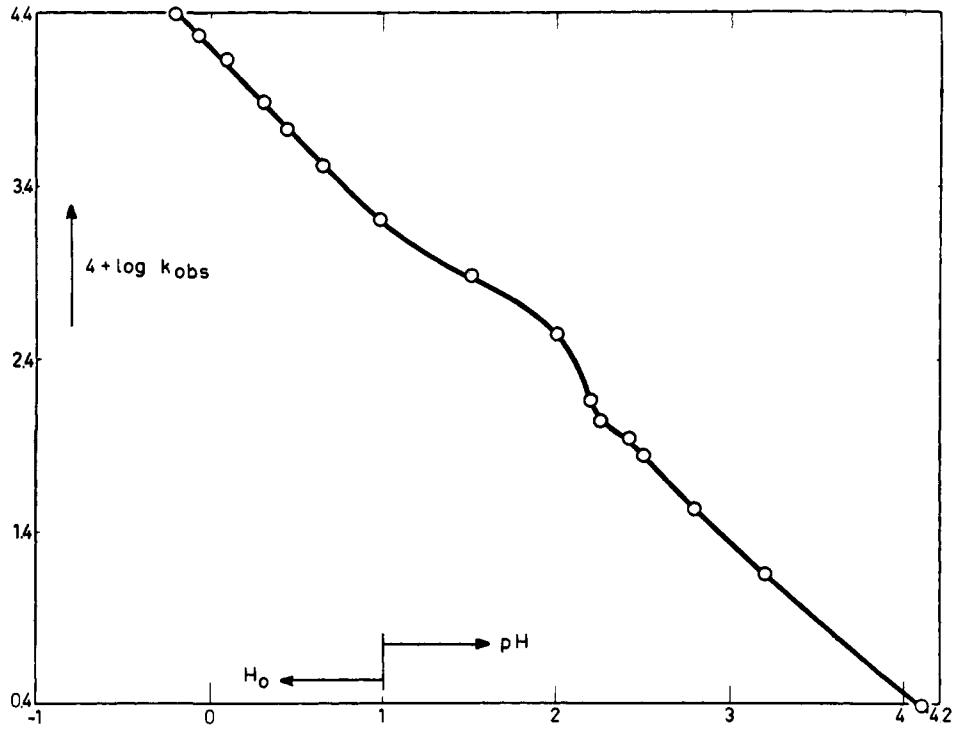


Figure 1. pH profile for the acidic hydrolysis of dG.

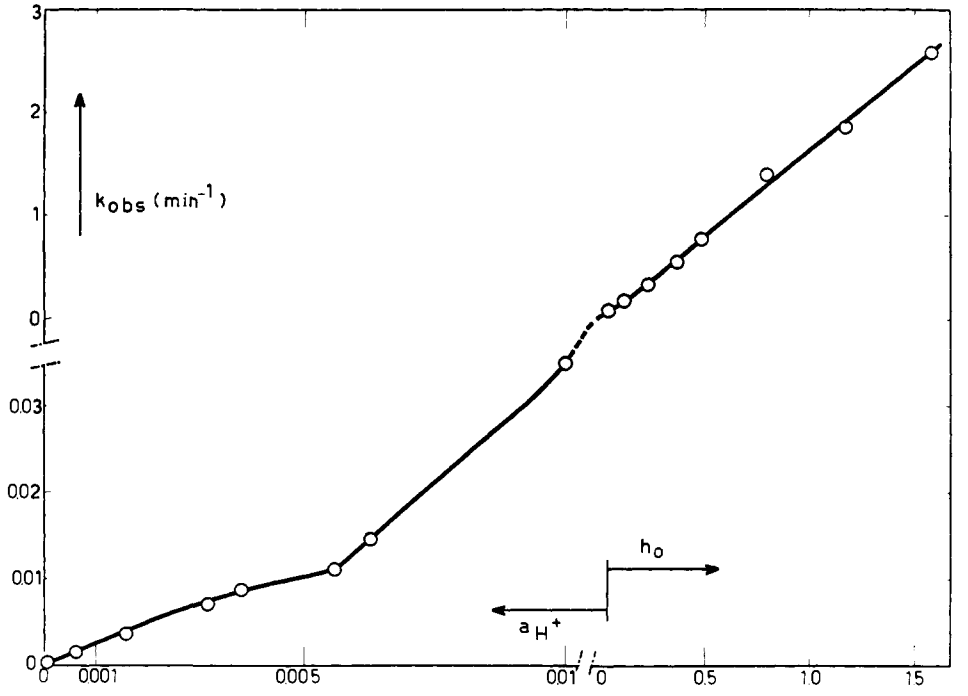


Figure 2. Dependence of the dG hydrolysis rate constant on the acidity of the medium.

Scheme I

$$\begin{array}{ccccc} S + H^+ & \underset{K_1}{\rightleftharpoons} & SH^+ & \xrightarrow{k_1} & P \\ & & H^+ \downarrow \uparrow K_2 & & \\ & & SH_2^{2+} & \xrightarrow{k_2} & P \end{array}$$

Scheme I led Zoltewitz and coworkers to the general expression of the observed rate constant

$$k_{obsd} = \frac{k_1 K_2 a_{H^+} + k_2 (a_{H^+})^2}{K_1 K_2 + K_2 a_{H^+} + (a_{H^+})^2} \qquad (1)$$

which accounts well for all our results. Under the actual experimental conditions no uncatalyzed reaction (k_{H_2O}) could be detected. Indeed, Table II and Figure 2 suggest that if such a reaction occurs, its contribution to the overall rate should be negligibly small. As this observation is valid for one of the most reactive substrates, *i.e.*, deoxyguanosine, it should certainly hold for the less reactive ones.

At very low acid concentration only the monoprotonated substrate plays a significant role. Furthermore, $[K_2 a_{H^+} + (a_{H^+})^2]$ can be neglected with respect to

K_1K_2 and k_{obsd} becomes

$$k_{obsd} = \frac{k_1}{K_1}a_{H^+} \quad (2)$$

This equation satisfies the very beginning of the curve of Figure 2. As the acidity increases, the term $(a_{H^+})^2$ can still be neglected, but $K_2a_{H^+}$ becomes more and more important. The new expression (eq 3) would

$$k_{obsd} = \frac{k_1a_{H^+}}{K_1 + a_{H^+}} \quad (3)$$

lead to a levelling off by saturation. This can also be seen on Figure 2. However, before attaining complete saturation, the second-order term, *i.e.*, the reaction of the doubly protonated substrate, comes into play and dominates the kinetics at higher acidities; the reaction is accelerated and the full expression (eq 1) applies (with $(a_{H^+})^2$ in the denominator still small). If the acidity further increases (from about 10^{-2} mole $l.^{-1}$) the term $K_2a_{H^+}$ at first causes a slight downward curvature, then completely controls the denominator: $[K_1K_2 + (a_{H^+})^2] \ll K_2a_{H^+}$.

Here the expression of k_{obsd} is

$$k_{obsd} = k_1 + \frac{k_2}{K_2}a_{H^+} \quad (4)$$

which explains well the last linear portion.

Equations 2 and 4 have also been used by Zoltewitz and coworkers to explain their linear pH profile.

This analysis is based on the hypothesis that $K_2 \gg K_1$; more precisely, K_2 should be at least of the order of magnitude of 10^2, which is quite reasonable from general properties of polybasic acids.

Unfortunately, exact values for the kinetic parameters k_1 and k_2 can not be extracted from the treatment of the data, because the reaction of SH_2^{2+} interferes very early with that of SH^+ and no linearization is possible. However, taking the tangent at the origin of the curve of Figure 2 as an approximation for eq 2, we obtain $k_1/K_1 = 2.5$. With $K_1 = 10^{-2.31}$, this gives $k_1 = 1.2 \times 10^{-2}$ min^{-1} $mole^{-1}$ l. This value is in good agreement with that obtained by the extrapolation to zero acidity of the linear part of the curve (eq 4): $0 < k_1 \leqslant 10^{-2}$.

In principle, from the slope of this linear part, $k_2/K_2 = 1.58$, k_2 could be obtained. However, two difficulties arise: first, since h_0 was used in place of a_{H^+}, the slope involves the ratio γ_B/γ_{BH^+} which can not be determined; second, the exact value of K_2 is unknown. If we suppose that γ_B/γ_{BH^+} remains reasonably constant in the whole h_0 range and that its value is approximately unity, using $K_2 = 10^2$ moles $l.^{-1}$ yields $k_2 = 1.58 \times 10^2$ min^{-1} $mole^{-2}$ $l.^2$.

When these values for k_2 and K_2 are introduced in eq 1, the experimental curve is not reproduced. Therefore, a full iterative numerical analysis was carried out with k_1 and k_2 taken as parameters. After stabilization of k_1 at the value of 1.4×10^{-2} the analysis was pursued by varying k_2. The best fit was obtained with $k_2 \geqslant 10^4$ and $K_2 \geqslant 6.33 \times 10^3$. It turned out that the results were insensitive to further increase of k_2: values like 10^5 or 10^6 would be equally acceptable. Accordingly, the exact value of k_2 can not be determined. It is noteworthy that the value of k_1 obtained by this procedure agrees very well with that obtained previously, so it should be considered as a final result. The high value of k_2 causes K_2 to be much greater than previously expected.

The activation parameters have been determined in the high acidity range between 23° and 75.3°. The excellent Arrhenius plots obtained with eq 4 in each case suggest that k_1 is negligible compared to k_2/K_2 supporting our previous conclusions. Although the activation parameters are composite quantities, it can be seen from Table III that $\Delta G^{\ddagger}$ is smaller for the deoxy derivatives, in accordance with the reactivity sequence generally established.[35] Furthermore, the $\Delta H^{\ddagger}$ values are higher for the deoxy compounds; their $\Delta S^{\ddagger}$ is positive, while for the oxy compounds it is negative.

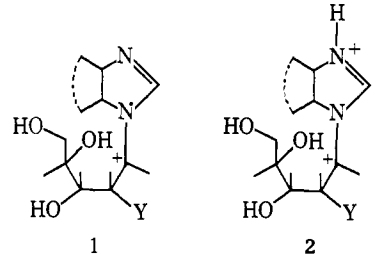
It is clear that our data lead also to the mechanism proposed by Zoltewitz and coworkers: mono- and diprotonated substrates undergo a rate-determining C–N cleavage. The absence of water in the rate-determining step is further supported by the good linearity of the plot k_{obsd} *vs.* H_0 (slope = −1.03, Zucker–Hammett hypothesis).

The following thermodynamic considerations are also in favor of the proposed mechanism. Realizing that, on account of the equilibria between transition states and the corresponding reagents on one hand, and between the mono- and diprotonated reagents on the other, a virtual equilibrium exists between the two transition states $M_1^{\ddagger}$ and $M_2^{\ddagger}$,[36] the corresponding equilibrium constant $K_{\ddagger}$ can be shown to be

$$K_{\ddagger} = K_2\frac{k_1}{k_2} \quad (5)$$

As the transition state of a monomolecular bond cleavage lies very far along the reaction path, the structures of $M_1^{\ddagger}$ and $M_2^{\ddagger}$ should be similar to those of the corresponding products. Accordingly, $K_{\ddagger}$ should also be similar to the dissociation constant of the monoprotonated guanine. It turns out that this is indeed the case: $K_{\ddagger} = 8.86 \times 10^{-3}$ mole $l.^{-1}$ and $K_{guanine} = 1.2 \times 10^{-3}$ mole $l.^{-1}$.

This argument rules out reactions through species protonated on the ring oxygen of the sugar moiety. In this latter case the monomolecular cleavage of the C–O bond would lead to intermediates **1** and **2** whose

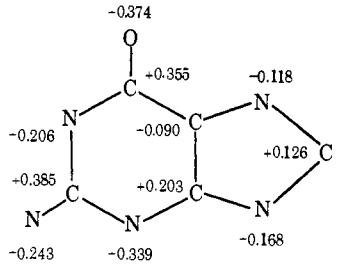


1 2

acidity should be considerably different from that of protonated guanine. The delocalization of the positive charge of the carbonium ion alters seriously the acid–base properties of the guanine ring. The SCF–MO CNDO calculations carried out for a model of **1**

(35) See the review by J. N. Be Miller, *Advan. Carbohyd. Chem.*, **22**, 25 (1967).
(36) J. L. Kurz, *J. Amer. Chem. Soc.*, **85**, 987 (1963).

in the approximation of Pople and Segal[37] give the following charge distribution



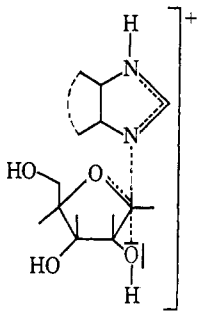
guanine

It can be seen that, as a result of the presence of the positive charge, the electron densities of all the possible protonation sites are considerably reduced. Consequently, the acid–base properties of **1** and **2** will be different from those of the free base. In addition, intermediate **2** is highly unstable and its formation is certainly not favorable.

It should be kept in mind that these arguments only establish that the N-protonated species are mainly responsible for the reaction, but they can not exclude completely the contribution of oxygen-protonated species.

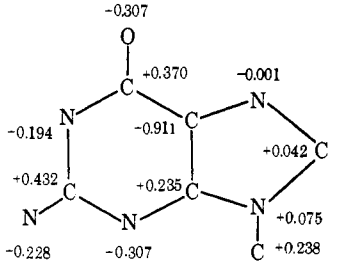
These latter may well be present in the reaction mixture, but they would give rise to negligible slower reactions.

The proposed mechanism[33] makes it possible to interpret the activation parameters given in Table III. Despite the $-I$ effect of the 2′-hydroxyl group which is expected to hinder the reaction, it can be seen that the $\Delta H^{\ddagger}$ values are smaller for the oxy compounds than for the deoxy derivatives. It seems that the presence of the hydroxyl group helps the departure of the protonated purine base by neighboring group participation.



Following the Evans–Polanyi–Bell–Ingold–Leffler–Hammond[38–42] postulate this means that the transition state of these compounds lies closer to the reagents on the reaction path than that of the deoxy derivatives. Therefore, the C–N bond is less broken in the former than in the latter case.

The entropy of activation is *a priori* expected to be positive for both kinds of compounds. The neighboring group participation and the lower degree of C–N bond breaking lead to a more ordered transition state structure. This results in a negative entropy of activation for the hydroxy derivatives. Moreover, solvation



model of **1**

differences may also contribute, but probably in a minor extent, to the observed activation parameters.

In spite of the relative magnitudes of the $\Delta H^{\ddagger}$ values, the reactivity sequence is reversed by the large differences in the $\Delta S^{\ddagger}$ values.[43]

Effect of External Complexing Agents. Purine and pyrimidine bases of nucleic acids are known to form molecular associations not only by hydrogen bonds but also through charge-transfer interaction.[44] The resulting complexes are relatively weak.

We examined the complex-forming ability of adenosine and the kinetic role of the complexes of adenosine, deoxyadenosine, and deoxyguanosine.

Nmr technique was used for the determination of the stability constant of the adenosine–α-methylnaphthalene complex by means of eq 6[45] where δ_A, δ_e, and δ_{AD} are

$$\frac{\delta_A - \delta_e}{[D]_0} = (\delta_A - \delta_{AD})K - (\delta_A - \delta_e)K \qquad (6)$$

the chemical shifts of the free, partially complexed, and fully complexed adenosine, respectively; $[D]_0$ is the molality of α-methylnaphthalene and K is the stability constant in kilogram per mole units.

Equation 6 is only valid if the donor D is used in large excess. The results are summarized in Table IV.

Table IV. Determination of the Stability Constants of the Complex between Adenosine and α-Methylnaphthalene in Dimethyl Sulfoxide at 22°[a,b]

$[D]_0$ (moles kg^{-1})	0.879	2.199	3.918	6.571
δ_{e_1} (Hz)	497.7	505.8	511.4	515.7
δ_{e_2} (Hz)	368.7	379.2	386.6	392.7

[a] $[A]_0 \simeq 0.1$ mole kg^{-1}, $\delta_{A_1} = 490.5$ Hz and $\delta_{A_2} = 357.9$ Hz. Indices 1 and 2 refer to H_2 of the adenine ring and to $H_{1'}$ of the sugar part, respectively. [b] $\delta_{A_1D} = 528.5$ Hz. $K_1 = 0.31$ kg mole^{-1} $\delta_{A_2D} = 409.9$ Hz. $K_2 = 0.32$ kg mole^{-1}.

It can be seen that the variation of the chemical shifts with increasing donor concentration shows saturation. This means that a genuine association equilibrium is present.

Furthermore, the presence of the donor causes a downfield shift of the acceptor protons, showing that

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(43) As was mentioned earlier, the data of Table III are composite values. However, this explanation remains valid, since the contribution of the protonation equilibrium must be similar for the two kinds of compounds.
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Table V. Effect of Charge-Transfer Complexing on the Rate of Hydrolysis at 23°

Substrate[a]	Complexing agent and concn, mole l.$^{-1}$	Reaction medium	$10^3 \times k_{obsd}$, min^{-1} Without complexing agent	$10^3 \times k_{obsd}$, min^{-1} With complexing agent
A	Pyridine, 1×10^{-2}	HCl, 1.0 *N*	16.6	16.1
dA	Hexamethylbenzene, 1.25×10^{-2}	CH_3CN + 20% HCl, 0.5 *N*	25.9	26.3
dG	Durene, 1.26×10^{-1}	Abs alcohol + 5% HCl, 5 *N*	24	24
	Caffeine, 2.5×10^{-3}	HCl, 0.1 *N*	7.31	6.60
	Cytosine, 2.5×10^{-3}	HCl, 0.1 *N*	7.31	6.60
	Pyridine, 5×10^{-3}	HCl, 0.1 *N*	7.31	7.93
	Acenaphthene, 1×10^{-3}	CH_3CN + 20% HCl, 0.5 *N*	22.8	23.3
	Hexamethylbenzene, 1.25×10^{-2}	CH_3CN + 20% HCl, 0.5 *N*	22.8	24.3

[a] Concentration, 1×10^{-4} mole l.$^{-1}$.

the donor molecule is located parallel to the adenine ring in such a way that the examined protons fall outside of its diamagnetic anisotropy cone. Moreover, the stability constant determined from the adenine ring protons (K_1) and that determined from the sugar proton (K_2) are in excellent agreement. Their magnitudes correspond to the weak complex-forming ability of purine bases.[44,46]

According to these results the question arose whether or not such a complexation would have any effect on the rate of hydrolysis. In order to answer this important question the reaction rates of A, dA, and dG have been measured in the presence of a number of complexing agents. The results are collected in Table V. Inspection reveals that under these experimental conditions the effects are at most small. Indeed, the largest observed effect amounts about to 10% in the case of dG in the presence of caffeine or cytosine. Moreover, the latter two have an inhibitory effect, while the other complexing agents cause the rate to increase. With durene there is no effect at all. Hexamethylbenzene accelerates slightly the reaction of dG and has almost no effect on that of dA.

If the mechanism of Zoltewitz and coworkers is essentially correct, these effects might be interpreted as being due to an increase of substrate basicity through complexation by donor molecules such as pyridine, acenaphthene, and hexamethylbenzene. The increased basicity favors protonation; consequently the rate is also increased. Since caffeine and cytosine decrease the rate of reaction, they should be considered as electron acceptors with respect to dG. Solvent reorganization might also contribute to the observed effects.

Nevertheless, this explanation should be accepted cautiously because the smallness of the effects and the paucity of the data prevent one from drawing a definite conclusion. In order to settle this problem, further experiments are being carried out using model compounds that allow more favorable experimental conditions.

Experimental Section

Materials. All the nucleosides are commercially available products. Adenosine, guanosine, and deoxyguanosine are of biochemical grade from Merck, whereas deoxyadenosine is a BDH product. Paper chromatography did not reveal any impurity and the products were used without further purification.

The complexing agents used are all commercially available products and were purified by classical methods.

Kinetic Measurements. The kinetics were followed spectrophotometrically at 259 nm for A and dA and at 263.1 nm for G and dG. The temperature was kept constant within ±0.1°.

The order of the reaction was degenerated with respect to the acid catalyst. The pseudo-first-order rate constants were calculated by the usual method, except when the infinite time absorbancy value could not be obtained. In this case Guggenheim's method was used. Every measurement was repeated several times (5–7) and the results were submitted to statistical analysis. This allowed us to attain high accuracy reaching even 1% in some cases.

In the range of pH 2–3 glycine–HCl or sodium citrate–HCl buffers were employed, while at pH 3–4.1 NaH_2PO_4–citric acid buffer was used.[47] In each case the final pH was checked potentiometrically.

Determination of the Stability Constants. The stability constants of the complex of adenosine with α-methylnaphthalene were determined by nmr spectroscopy. The experiments were carried out in dimethyl sulfoxide at 22° using TMS as internal reference. For practical reasons the concentrations are expressed in molality.

Equipment. The spectrophotometer used was a Cary 16; nmr spectrometer, Jeol C-60 working at 60 MHz; pH meter, Metrohm E388.

Computation. All the computations were carried out at the Computation Center of the University. For the kinetic simulation a Fortran IV program was used. The SCF-MO CNDO calculations were made using the program of Pople and Segal as modified by Andre, Degand, and Delhalle.

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