Kinetics and Mechanism of the Acid-Catalyzed Hydrolysis of Some Purine Nucleosides[1,2]

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Abstract: Rates of acid-catalyzed hydrolysis were obtained by spectrophotometric methods for the natural nucleosides guanosine (Guo), 2′-deoxyguanosine (dGuo), and 2′-deoxyadenosine (dAdo) and for betaines, 7-methylguanosine (7MeGuo) and 7-methyl-2′-deoxyguanosine (7MedGuo), as well as the salt 1,7-dimethylguanosinium iodide (1,7diMeGuoI). In solvolyzing, to a purine and ribose or deoxyribose all show specific hydronium ion catalysis over acidity regions where mono- and dicationic forms of substrate are present. Rate profiles for Guo, dGuo, and dAdo are linear even when pH $\simeq$ pK_a. 7MeGuo and 1,7diMeGuoI undergo pH-independent hydrolysis in weakly acidic solution. 7MedGuo shows two regions linearly dependent on acidity and a pH-independent region. Kinetic and equilibrium pK_a values for 7MedGuo are identical. Guo, 7MeGuo, and 1,7diMeGuoI hydrolyze at essentially identical rates at low pH. While dGuo is 520 times more reactive than Guo, dAdo and dGuo have similar reactivity. It is suggested that these nucleosides hydrolyze by a mechanism involving pre-equilibrium protonation of the purine followed by rate-limiting purine–glycosyl bond cleavage.

Nucleosides,[4] fragments of nucleic acids (RNA or DNA) which contain a purine or pyrimidine heterocyclic base and a ribose or deoxyribose group, may hydrolyze to their components by enzyme[5] or acid-catalyzed reactions. Little is known about the hydrolysis mechanism of either process.

Kinetic studies of the acid-catalyzed hydrolysis of nucleosides are of wide, potential interest. They can provide useful insight into the degradation of nucleic acids, the synthesis of natural and new nucleic acid fragments, and the mechanism of action of nucleoside enzymes.

Much is known qualitatively about the acid-catalyzed hydrolysis of nucleosides[6] but little quantitative information is available. Deoxyribonucleosides are more labile than ribonucleosides; purine nucleosides are more reactive than pyrimidine substrates.[6] The position of attachment of the sugar group to the purine[7-9] as well as the configuration of the sugar at the site of attachment influence hydrolytic reactivity.[7] Structural changes on the purine also influence reactivity.[10,11] Most kinetic studies have involved pyrimidine nucleosides[12,13] with a few exceptions.[13,14] Unfortunately, evidence for a mechanism of hydrolysis has not been forthcoming.

There has been considerable speculation, largely based upon qualitative observations of reactivity, regarding possible mechanisms of acid-catalyzed hydrolysis. A recent statement of a popular view is, "for hydrolysis to occur at an appreciable rate the proton must become attached to the ring oxygen of the glycosyl moiety."[15]

We have investigated the rates of acid-catalyzed hydrolysis of the natural purine nucleosides guanosine (Guo), 2′-deoxyguanosine (dGuo), and 2′-deoxyadenosine (dAdo). Synthetic substrates include betaines 7-methylguanosine[16-18] (7MeGuo) and 7-methyl-2′-deoxyguanosine[16-18] (7MedGuo), and the salt 1,7-dimethylguanosinium iodide[19] (1,7diMeGuoI).

The thermodynamically favored site of protonation of Guo and dGuo is N-7.[20] Both 7MeGuo and 7MedGuo have a methyl group at this position and this group introduces positive charge into the purine as does a proton. 7MeGuo and 7MedGuo are more basic and more hydrolytically labile than their natural counterparts.[16-18] 1,7-diMeGuoI has positive charge unambiguously built into the purine ring and therefore is a substrate designed to test the possibility that hydrolysis proceeds by simple fragmentation of the purine–ribose bond.

(1) The authors acknowledge gratefully the support of this work through a research grant (11798) from the National Institutes of Health, Division of General Medical Sciences.

(2) Taken in part from the Ph.D. Dissertation of D. F. C. and T. W. S., University of Florida, 1968.

(3) National Institutes of Health Predoctoral Fellow, 1965–1968.

(4) For reviews dealing with the chemistry of nucleosides, structure proofs of starting materials, and hydrolysis products see, for example, (a) "The Nucleic Acids," Vol. 1, E. Chargaff and J. N. Davidson, Ed., Academic Press, New York, N. Y., 1955; (b) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Academic Press, New York, N. Y., 1963.

(5) M. Friedkin and H. Kalckar in "The Enzymes," 2nd ed, P. D. Boyer, H. Lardy, and K. Mrybuch, Ed., Vol. 5, Academic Press, New York, N. Y., 1961, Chapter 15.

(6) Reference 4b, pp 26–27.

(7) J. A. Montgomery and H. J. Thomas, *J. Amer. Chem. Soc.*, **87**, 5442 (1965).

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(9) J. Baddiley, J. G. Buchanan, and G. O. Osborne, *J. Chem. Soc.*, 3606 (1958).

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(13) H. Venner, *Z. Phys. Chem.*, **339**, 14 (1964). Our results agree only partially with the data presented herein. Reactions were followed with the aid of paper chromatography.

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(15) Reference 4b, p 26.

(16) J. W. Jones and R. K. Robins, *J. Amer. Chem. Soc.*, **85**, 193 (1963).

(17) J. A. Haines, C. B. Reese, and Lord Todd, *J. Chem. Soc.*, 5281 (1962).

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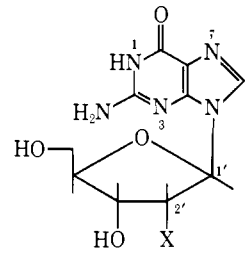
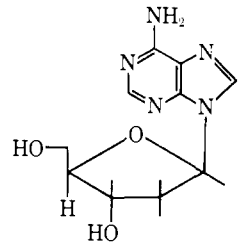
(20) (a) W. Pfleiderer, *Ann.*, **647**, 167 (1961); (b) M. Tsuboi, Y. Kyogoku, and T. Schimawauchi, *Biochim. Biophys. Acta*, **55**, 1 (1962); (c) J. M. Broomhead, *Acta Cryst.*, **4**, 92 (1951).

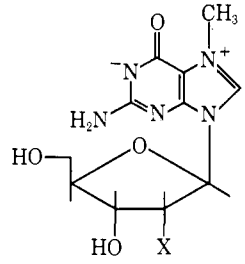
Contrary to earlier suggestions,[6,21-23] we have concluded that the mechanism of acid-catalyzed hydrolysis of these purine nucleosides involves preequilibrium protonation of the purine followed by rate-limiting cleavage of the glycosyl–purine bond. This is the simplest mechanism consistent with our data.



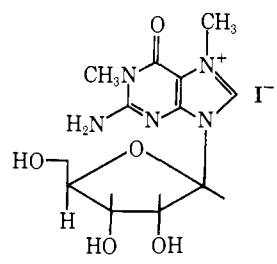
X = OH, Guo
X = H, dGuo



dAdo



X = OH, 7MeGuo
X = H, 7MedGuo



1, 7diMeGuoI

Experimental Section

Materials. 2′-Deoxyadenosine (dAdo), guanosine (Guo), 2′-deoxyguanosine (dGuo), adenine, guanine, and ribose were obtained from Calbiochem. Chromatography, paper and thin layer, indicated they were free of impurities. Alkaline solutions were standardized with potassium acid phthalate and acidic solutions with Fisher primary "THAM." Salts, buffers, and acids were reagent grade. $DClO_4$ was made by mixing D_2O (99.8%) and 70% $HClO_4$.

The known mono- and dimethylnucleosides prepared as indicated below all had ultraviolet absorption spectra in acidic, neutral, and basic solutions in agreement with those reported in the literatures. R_f values in several solvents were in agreement with reported values. Details may be obtained from the Ph.D. dissertation of D. F. C.

7-Methylguanosine (7MeGuo) and 7-methyl-2′-deoxyguanosine (7MedGuo) were prepared by methylation.[16] The yields were 30 and 10%, respectively. There was some discrepancy between the reported and observed "melting point" for 7MeGuo. Our samples discolored at 159–160° and then decomposed about 200° [lit.[16] mp 159–160° for the dihydrate; mp 165° for the hemihydrate[18]]. Our material analyzes as 7MeGuo dihydrate. *Anal.* Calcd for $C_{11}H_{15}N_5O_2 \cdot 2H_2O$: C, 39.6; H, 5.7. Found: C, 39.49; H, 5.51.

Equipment. Absorbance changes were followed with a Zeiss PMQ II or a Beckman DU spectrophotometer. The wavelength generally used in each kinetic run was selected as that which gave the maximum difference in absorbance between starting material and product. Once this wavelength was selected, the setting was never varied throughout a run. For reactions carried out inside the cell compartment of the PMQ II, the spectrophotometer was fitted with a hollow brass cuvette holder thermostated at the reaction temperature by a Lauda constant temperature circulating bath. The temperature of reaction mixtures followed in this direct way was measured at the end of a kinetic run by means of a copper-constantan thermocouple attached to a Rubicon potentiometer.

All pH measurements were made with a Beckman Research pH meter using Radiometer or Metrohm glass electrodes and Radiometer calomel electrodes or a Sargent combination electrode. For kinetic experiments involving ribosides at 100°, pH values were measured at 30.0°. All other measurements were taken at the reaction temperature, determined by a National Bureau of Standards certified thermometer. Electrodes were thermostated by a Haake EDe constant temperature circulator and standardized by the method of Bates.[24]

The thin layer chromatography plates used for identification of products were prepared with a 0.25-mm layer of cellulose or a 0.50-mm layer of either silica gel GF_{254} or aluminum oxide GK_{254}, Brinkman Instruments. The tlc plates used in qualitative kinetic studies had a 0.75-mm layer of the alumina. For all paper chromatography work, spots were made on Whatman No. 1 paper. A Fischer uv mineralight was used to detect all spots.

Ultraviolet absorption spectra were scanned using a Cary 15 spectrophotometer. A Varian A-60A was employed for nmr work. Titrations were carried out using a Radiometer TTT-1c titrator.

Buffer Solutions. The buffers used in the kinetic studies were prepared so that the total ionic strength generally was 0.10 and the concentration of undissociated buffer acid was present in more than 100-fold excess over that of nucleoside. Solutions (pH range) employed include: perchloric acid (1–2.5); formic acid–formate ion (2.5–4); acetic acid–acetate ion (3.5–5); dihydrogen phosphate ion–hydrogen phosphate ion (5–7.5); hydrochloric acid–borax (8–9).

1,7-Dimethylguanosinium Iodide. When the method of Jones and Robins[19] for the synthesis of the iodide salt of 1,7-dimethylguanosine was followed, a mixture of 1-methylguanosine and 1,7-dimethylguanosinium iodide was usually isolated. Therefore, the following preparation was developed: a suspension was made by mixing 3.0 g of guanosine and 30 ml of dimethyl sulfoxide containing 1.8 g of powdered anhydrous potassium carbonate. To this was added 1.0 ml of methyl iodide, and the suspension was stirred in a stoppered flask for 6 hr. An additional 0.5 g of potassium carbonate and 0.3 ml of methyl iodide were added, and stirring was continued for another 2 hr. The suspension was filtered through Celite and the filtrate was added slowly to 250 ml of methylene chloride. The precipitate was recrystallized from methanol giving 2.5 g (50%) of product: uv max (pH 1) 259 mμ (ε 11,300) and (pH 13), 271 mμ (ε 14,000). It was judged to be pure by chromatographic analysis using reported solvents.

Characterization of the Purine Product Resulting from the Acid Hydrolysis of Some Nucleosides. Guanosine, deoxyadenosine, and deoxyguanosine in ~0.1 *M* $HClO_4$ and 7-methylguanosinium iodide[16] in water were hydrolyzed. Samples of the reaction mixtures were spotted on thin layer plates or on paper along with authentic adenine and guanine. Development of the chromatograms indicated that only the expected purine hydrolysis product was formed. Ribose spots were visualized using *p*-anisidine. (Adenine and guanine are only very slowly degraded in acidic solution.[25,26])

Isolation of 7-Methylguanine from the Hydrolysis of 7-Methyl-2′-deoxyguanosine or 7-Methylguanosine. When heated in water or 0.1 *N* hydrochloric acid for 1 hr over a steam bath, both 7-methylguanosine[16] and 7-methyl-2′-deoxyguanosine[16] gave 7-methylguanine[20a,27] in 90% yield. Adjusting the pH to 7.5 and allowing the solution to cool for several hours at 5–10° resulted in the recovery of product: uv max (pH 1) 251 mμ (ε 10,000); (H_2O) 248 (ε 8000) and 243 mμ (ε 6000); (pH 11) 280 mμ (ε 8000); nmr (NaOD) δ 4.1 (s, 3) and 7.9 (s, 1). Sugar hydrolysis products have been characterized.[16]

Isolation of 1,7-Dimethylguanine from the Hydrolysis of 1,7-Dimethylguanosinium Iodide. The iodide salt of 1,7-dimethylguanosine[19] was heated over a steam bath in 0.1 *N* perchloric acid for 30 min. The reaction mixture was cooled to room temperature and the pH adjusted to 7 with concentrated ammonia. The mixture was refrigerated for 1 hr and then filtered. The product (95% yield) was found to have the properties reported for 1,7-dimethylguanine: mp 327–330° [lit.[27] 330–331°]; uv max (pH 1) 252 mμ (ε 10,000); (pH 12) 283 mμ (ε 6000); nmr (D_3O^+) δ 3.3 (s, 3), 4.0 (s, 3), and 8.7 (s, 1). The ribose component had been characterized earlier.[19]

(21) G. W. Kenner in "Ciba Foundation Symposium on the Chemistry and Biology of Purines," Little, Brown and Co., Boston, Mass., 1957, p 312.
(22) C. A. Dekker, *Ann. Rev. Biochem.*, **29**, 453 (1960).
(23) F. Micheel and A. Heesing, *Chem. Ber.*, **94**, 1814 (1961).
(24) R. Bates, "Determination of pH. Theory and Practice," John Wiley & Sons, Inc., New York, N. Y., 1964.
(25) R. Abrams, *Arch. Biochem. Biophys.*, **30**, 44 (1951).
(26) A. Marshak and J. H. Vogel, *J. Biol. Chem.*, **189**, 597 (1951).
(27) E. Fischer, *Chem. Ber.*, 31, 542 (1898); W. Traube and H. W. Dudley, *ibid.*, **46**, 3839 (1913).

Stability of Nucleosides and Their Hydrolysis Products toward Alkali. The absorption of solutions of 7-methylguanosine and 7-methylguanine in 1 *M* sodium hydroxide at room temperature was measured periodically from 270 to 295 mμ over a 2-week interval. No change was observed. Under these conditions 7MeGuo, 7MedGuo, and 1,7diMeGuoI undergo rapid imidazole ring opening to give stable products.[16-19]

Guanosine, 2′-deoxyguanosine, guanine, and adenine were reported to be stable toward 1 *N* alkali at 100° for 1 hr. Under these conditions, 2′-deoxyadenosine degraded somewhat.[28]

Kinetics. A. Alkaline Quench Method. This method was especially useful for reaction mixtures giving rise to small absorption charges in acidic solution. Most of our data were obtained in this way.

Solutions of guanosine, 2′-deoxyguanosine, 2′-deoxyadenosine, 7-methylguanosine, or 1,7-dimethylguanosinium iodide in buffers were prepared. For reactions at 100°, 5–6-ml aliquots were sealed in Pyrex tubes. About 15 such samples were placed in an oil bath. Tubes were withdrawn periodically and quenched in ice water; reaction times were measured to the time of contact of the tube with the ice bath. Sample tubes were opened and diluted five or tenfold with NaOH.

For reactions at lower temperatures a volumetric flask containing the reaction mixture was immersed in a bath. Periodically 5-ml aliquots were removed and the reaction was quenched by diluting them to 25 or 50 ml with NaOH. Time was measured to the first contact of the aliquot with the quenching solution.

In both cases samples were refrigerated until all could be analyzed. At least 10 samples were taken over the first three half-lives. Infinity samples were taken after at least 10 half-lives. The infinity samples for slow runs involving 2′-deoxyadenosine were taken from solutions incubated at elevated temperatures. Water or a sample of substrate stock solution added to NaOH generally was employed as an optical blank in making absorbance measurements.

Plots of $\log (A - A_\infty)$ or $\log (A_\infty - A)$ *vs.* time were constructed. Wavelengths at which absorbance (A) changes were followed are given in Table I. Plots generally were linear over 2–4 half-lives. Rate constants are in Tables III–IV.

Table I. Wavelengths Employed in Kinetic Studies

Substrate[a]	Quench method, mμ	Direct method, mμ
Guo	265[b]	
dGuo	255[c]	254, 256
dAdo	253.5[c]	
7MeGuo	285, 295[b]	
7MedGuo		260,[d] 310[e]
1,7diMeGuoI	295[b]	

[a] $\sim 10^{-3}$ *M* before quenching; $\sim 10^{-4}$ *M* for direct method. [b] ~ 1 *M* NaOH. [c] ~ 0.4 *M* NaOH quenching solution. [d] pH < 8. [e] pH > 8.

Rates of hydrolysis of guanosine at 100.6° were found to be slightly dependent on formate buffer concentration at constant pH. A tenfold increase in buffer results in about a 70% increase in the observed rate constant. While this has the appearance of general acid–base catalysis, it is likely that substrate is formylated and this derivative undergoes hydrolysis. Other substrates in formate buffers at lower temperatures did not show this effect. Of the two points at highest pH given for the hydrolysis of Guo in Figure 1, one comes from formate buffer runs extrapolated to zero buffer concentration and the other from dilute $HClO_4$.

B. Direct Method. Solutions of 2′-deoxyguanosine or 7-methyl-2′-deoxyguanosine in buffers of constant ionic strength (KCl) were added to a tightly stoppered cuvette inside a thermostated brass block. After the sample came to thermal equilibrium about twenty absorbance measurements were made covering a period of about four half-lives. Where possible several infinity points were taken, starting after ten half-lives. In some instances it was impossible to obtain infinity values directly because of degradation of deoxyribose; an extrapolation method (see "Iterative Method") was employed. Degradation of deoxyribose was noticeable at 52° below pH 2 but was insignificant at 30° at pH > 0.2. In many instances the quench method (above), was employed to check on rate constants obtained in runs complicated by the formation of the new chromophore. Both methods gave results in satisfactory agreement; for example, 75% of the runs for dGuo at 52.6° were obtained by the direct method, Figure 1.

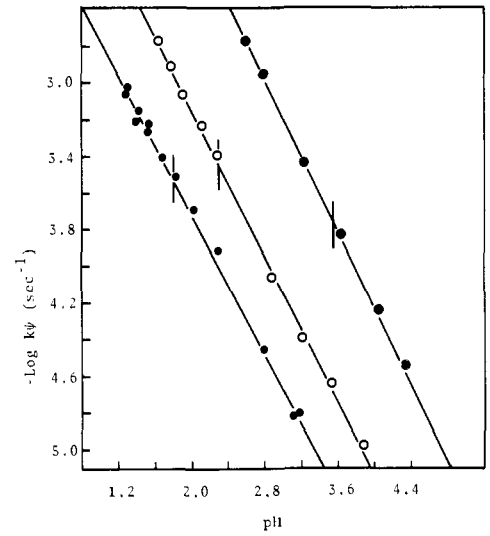


Figure 1. pH–rate profiles for the hydrolysis of guanosine (●) at 100.6°, 2′-deoxyguanosine (○) at 52.6°, and of 2′-deoxyadenosine (◉) at 70.0°. Vertical cross lines indicate pK_a values under the conditions of the kinetic runs.

The formation of a new chromophore at longer reaction times apparently results from the specific acid-catalyzed degradation of 2-deoxyribose by a sequential series of reactions to give 5-methyl-3(2H)-furanone.[29] The rate of appearance of this chromophore (λ_{max} 261 mμ) is not strictly first order. At <50° and at pH <2 this chromophore does not seem to form but one at 225 mμ does. The 261-mμ chromophore shifts to 293 mμ in alkali and disappears by a first-order process. Ribose does not degrade under the acidic conditions giving rise to degraded deoxyribose.[29,30]

Iterative Method to Determine Infinity Values in Kinetic Studies. Whenever the end point was not stable, the following method of estimating the infinity value was employed: a plot of absorbance *vs.* time was constructed. Three points with a time span covering at least one half-life but not more than three and an estimated infinity value then were selected from the plot. The equation $\log (A - A_\infty) = kt +$ constant was applied to the two absorbance values associated with the shorter times. The unknowns, k and the constant (which is an approximation of $\log (A_0 - A_\infty)$), are evaluated. Using the third absorbance value, $\log (A - A_\infty)$ was calculated and compared with the value for $(-kt +$ constant$)$. The entire process of estimating and calculating was repeated until self-consistent values were obtained. In this way it was generally possible to obtain linear plots through four half-lives. Agreement among rate constants obtained by this and the alkaline quench method giving stable infinity readings was generally within experimental error.

pK_a Determinations. Potentiometric Method.[31] A typical method is as follows: a 1.43×10^{-3} *M* solution of 7-methyl-2′-deoxyguanosine containing 0.1 *M* KCl was prepared using glass-distilled water which had been boiled previously to free it from carbon dioxide. To a jacketed titration cell, sealed with a rubber stopper and maintained at 52.4 ± 0.1°, was added 30 ml of the stock solution. Increments of 1.03×10^{-2} *M* HCl were added. After each addition, 1 min was allowed for mixing and thermal equilibration prior to measuring the pH. (Two minutes of equilibration was employed for 2′-deoxyadenosine at 70°.) At 52°

(28) A. S. Jones, A. M. Mian, and R. T. Walker, *J. Chem. Soc.* (*C*), 692 (1966).

(29) J. K. Seydel and E. R. Garrett, *Anal. Chem.*, 37, 271 (1965); J. K. Seydel, E. R. Garrett, W. Diller, and K. J. Schaper, *J. Pharm. Sci.*, 56, 858 (1967).

(30) Ribose does not interfere with the spectrophotometric analysis of nucleosides in acidic solution. See for example, H. S. Loring, J. L. Fairley, H. W. Bortner, and H. L. Seagran, *J. Biol. Chem.*, 197, 809 (1952).

(31) A. Albert and E. D. Serjeant, "Ionization Constants of Acids and Bases," John Wiley & Sons, Inc., New York, N. Y., 1962.

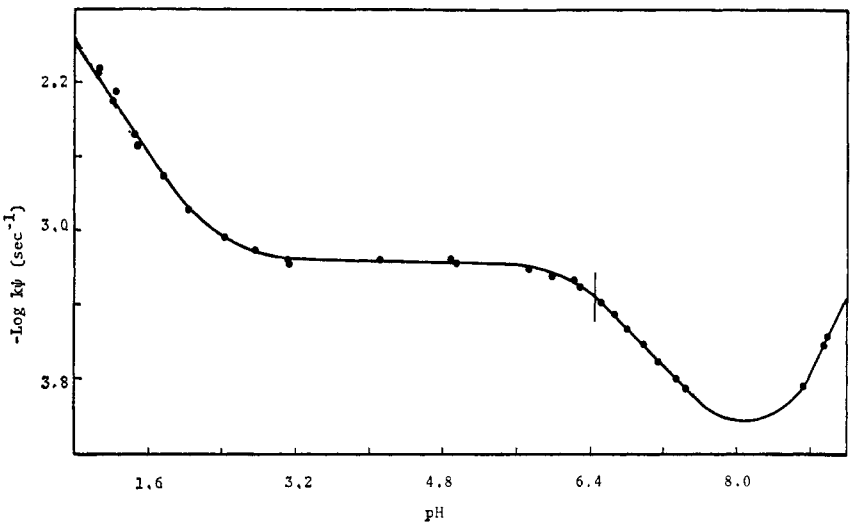


Figure 2. pH–rate profile for the hydrolysis of 7-methyl-2′-deoxyguanosine at 52.4°. Vertical cross line indicates the pK_a value under the conditions of the kinetic runs. The curve was calculated using eq 4 and the constants of Table IV.

hydrolysis of protonated substrate in the more acidic solutions took place; only four values at higher pH were employed in the calculation of pK_a. The pK_a (average of the antilogs) so determined is 6.44 ± 0.04. Similarly the pK_a of 2′-deoxyadenosine is 3.56 ± 0.05 (70.0°) and 3.79 ± 0.04 (29.9°).

Spectrophotometric Method. A. Dilute Acid.[31] Five-milliliter aliquots of 6.06×10^{-4} *M* solution of 2′-deoxyguanosine in water were diluted to 50 ml with buffer solution or a solution of known perchloric acid concentration. Samples of the resulting solutions, 0.10 in ionic strength (KCl), were placed into a cuvette thermostated at 52.5°. A timer was actuated and the absorbance at 248.5 mμ was measured 5 times at 1-min intervals. At low pH it was necessary to extrapolate the absorbance measurements to zero time. Change in absorbance due to hydrolysis was minimized by measuring at a wavelength close to the isosbestic point for the hydrolysis. The pH of the solutions was measured in a manner similar to that employed for the kinetic measurements. The value 2.31 ± 0.06 for the pK_a was obtained from an average of the antilogarithms of 10 determinations.

B. Molar Acid. Preliminary determinations of the pK_a were made so that the bulk of the measurements were in the vicinity of the pK_a value and the acidity necessary for complete conversion to each species was established. All solutions were about 23°.

A series of acids of known molarity was prepared before the actual determination. To these stock acids was added an aliquot of approximately 10^{-3}*M* nucleoside or purine dissolved in $HClO_4$ (usually about 1 *M*) so that a tenfold dilution resulted. Using these premixed acids the effects of heat of mixing were minimized. The stock solution of nucleoside was prepared no more than 1 hr before measurements were made. In the more concentrated acids there was evidence of nucleoside hydrolysis. At the wavelengths used in the determinations the absorbance change with time was so small that extrapolation of absorbance back to the time of mixing of stock nucleoside and concentrated acid was unnecessary. Absorbances were measured at two wavelengths corresponding to increase and decrease in absorbance.

Guanine, 7-methylguanosine, and guanosine each gave spectra with an isosbestic point as acidity of the absorbing solution was changed. Analytical wavelengths were selected to maximize the absorbance differences between pure cation and dication.

Two methods were used to obtain pK_a values.[32] In the first, the difference in absorbance at two wavelengths is plotted against H_0 according to the method of Davis and Geissman.[33] The method of Hammett was used in the second.[34] Values of H_0 were those determined for $HClO_4$ by Yates and Wai.[35] Results are given in Table II. It is to be noted that the nucleosides are fortuitously close to being true Hammett indicators in their response to protonation. Slopes in Hammett plots are close to the ideal value of 1.00.

Table II. Spectrophotometrically Determined pK_a Values in Moderately Concentrated $HClO_4$[a]

Compound	λ, mμ	pK_a[b]	pK_a[c]
Guanine	235, 280	−1.33	−1.26[d]
Guanosine	245, 280	−2.43	−2.42[e]
7-Methylguanosine	238, 280	−2.63	−2.61[f]

[a] 23°. [b] Davis–Geissman method. [c] Hammett method. [d] Slope, 0.85. [e] Slope, 0.95. [f] Slope, 1.02.

Results

Natural Nucleosides. Rates of hydrolysis of Guo, dGuo, and dAdo to guanine or adenine and ribose or deoxyribose were measured as a function of pH. The rates of these hydronium ion-catalyzed reactions are given by eq 1.[36] Plots of log $k\psi$ *vs.* pH are shown in

$$\text{rate} = k\psi[S] = k_2 a_H[S] \quad (1)$$

$$k\psi = a_H k_2 \quad (2)$$

Figure 1. pH values for runs at 100° were measured at room temperature but all others are at the temperature of the kinetic runs. Slopes of lines range from −0.95 to −1.03 as compared to the theoretical −1.00. Second-order constants are presented in Table III along with pK_a values. Changes in slope in the vicinity of the pK_a for each nucleoside could not be detected.

The influence of the concentration of potassium chloride on the rate of hydrolysis of Guo at pH 2.30 was determined. A change in the ionic strength from 0.10 to 1.00 resulted in only a 32% increase in the rate constant.

From the data in Table III it may be concluded that dGuo is 520 times more reactive than Guo at 100° and that dAdo is 2.2 times more reactive than dGuo at 30°

(32) For a recent discussion of the methods employed in the molar acid region to obtain pK_a values see: D. S. Noyce and M. J. Jorgenson, *J. Amer. Chem. Soc.*, **84**, 4312 (1962).

(33) C. T. Davis and T. A. Geissman, *ibid.*, **76**, 3507 (1954).

(34) L. A. Flexser, L. P. Hammett, and A. Dingwall, *ibid.*, **57**, 2103 (1935).

(35) K. Yates and H. Wai, *ibid.*, **86**, 5408 (1964).

(36) Although purine nucleosides are known to self-associate in aqueous solution to give vertically stacked purine rings, only a small fraction should be present in this form at the 10^{-3}–10^{-4} *M* concentration level employed in our rate studies: S. J. Gill, M. Downing, and G. F. Sheats, *Biochemistry*, **6**, 272 (1967); S. I. Chan and J. H. Nelson, *J. Amer. Chem. Soc.*, **91**, 168 (1969), and references cited therein.

Table III. Rate Constants for the Hydrolysis of Some Natural Purine Nucleosides and Their Dissociation Constants

Compound	T, °C	k_2, M^{-1} sec^{-1}	pK_a [a]
Guo[b]	100.6	1.78×10^{-2} [c]	1.8[d]
dGuo[b]	52.6	6.98×10^{-2}	2.31
	50.0	4.04×10^{-2}	
	30.0	3.16×10^{-3}	
dAdo[e]	70.0	7.02×10^{-1}	3.56[f]
	29.9	6.93×10^{-3}	3.79
	29.9[g]	1.66×10^{-2}	

[a] Temperature and ionic strength the same as in kinetic runs. [b] 0.10 ionic strength. [c] pH measured at room temperature. [d] Extrapolated value using the data given by L. G. Bunville and S. J. Schwalbe, *Biochemistry*, **5**, 3521 (1966). ΔH° = 2.15 kcal/mol; ΔS° = −2.5 eu. [e] In 1.0 ionic strength. [f] ΔH° = 3.33 kcal/mol; ΔS° = −6.5 eu. [g] In D_2O; k_2^D/k_2^H = 2.4.

(no correction was applied for the ionic strength difference at 30°).

Hydrolyses in deuterium oxide were treated in the same manner as those in proteo water. Since fewer runs were carried out in the former solvent, rate constants are less certain. For dAdo k_2^D/k_2^H = 2.4. Activational parameters for the hydrolysis of dAdo and dGuo are: ΔH^* = 23.3 ± 0.5 and 25.0 ± 1.0 kcal/mol and ΔS^* = 8.4 ± 1.3 and 12.7 ± 2.5 eu, respectively.

N-Methyl Nucleosides. The known compounds 7MeGuo,[16-18] 7MedGuo,[16-18] and 1,7diMeGuoI[19] were prepared by methylating the natural nucleosides. Although there was much confusion regarding the position of alkylation of nucleosides, the structures of these methylated guanine nucleosides have been established unambiguously.[16,19] From the hydrolysis of each on a preparative scale a minimum of 90% of 7-methylguanine or 1,7-dimethylguanine was recovered.

Plots of log $k\psi$ *vs.* pH are shown in Figures 2 and 3.[37] Unlike similar plots for the natural nucleosides, plots for the methylated substrates show several linear rate regions. 7MedGuo was studied the most extensively. Its hydrolysis rate is linearly dependent on proton concentration in the pH range 1–2, is insensitive to pH in the region 3–5.5, and then in the region pH 6.5–7.5, which goes beyond its pK_a (6.44), the rate again is linearly dependent on proton concentration. At high pH it is known that 7MedGuo undergoes imidazole ring fission and not purine–deoxyribose cleavage.[17,18] This ring fission was studied to provide rate data enabling corrections to be made to those regions where both types of hydrolyses occurred.

Since it is known that 7MeGuo[16-18,38] and 1,7-diMeGuoI[19] also undergo ring fission at high pH, rate profiles were obtained only at low pH where this reaction is not competing. Profiles for the latter two are similar to that for 7MedGuo in the same acidity region; purine–sugar cleavage takes place at a rate independent of pH between 3 and 5 and then by an acidity sensitive process at lower pH.

The pH dependence of the hydrolysis of 7MedGuo is readily explained if (excluding ring opening): (1) in addition to the second-order, hydronium ion catalyzed hydrolysis of monoprotonated substrate, terms for

(37) Qualitatively, our conclusions regarding the reactivity of 7MeGuo and 7MedGuo agree with those given in ref 18. Quantitative comparison of our rate constants with those in this more limited study are not possible, owing to temperature differences.

(38) L. B. Townsend and R. K. Robins, *J. Amer. Chem. Soc.*, **85**, 242 (1963).

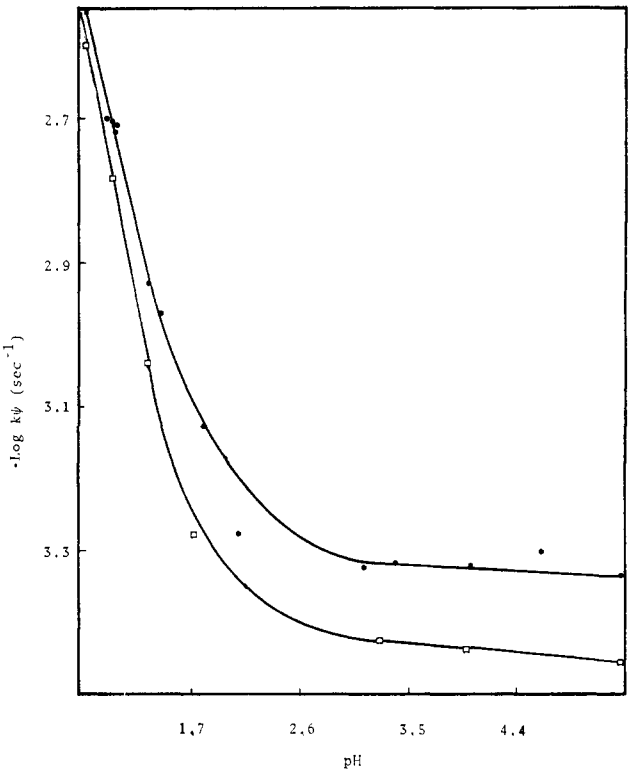


Figure 3. pH–rate profiles for the hydrolysis of 7-methylguanosine (top curve) and of 1,7-dimethylguanosinium iodide at 100.3°.

first-order decomposition of the monocation and the betaine itself are included in the kinetic expression; and (2) allowance is made for the betaine form as the pH is raised. Equation 3 is the rate expression for this case and eq 4 is the derived equation for $k\psi$ in terms of

$$\text{rate} = k\psi[S]_t = k_1[SH] + k_2 a_H [SH] + k_{H_2O}[S] \quad (3)$$

$$k\psi = \frac{a_H}{a_H + K_a}\left[k_1 + k_2 a_H + \frac{k_{H_2O} K a}{a_H}\right] \quad (4)$$

measured quantities and the first- and second-order rate constants k_1 and k_2. $[S]_t$ = concentration of nucleoside in all forms; $[SH]$ = concentration of monoprotonated nucleoside; $[S] = [S]_t - [SH]$ = concentration of betaine; and $K_a = a_H[S]/[SH]$ = ionization constant. It is to be understood that the "water" term k_{H_2O} may apply to the fragmentation reaction and/or to the ring opening reaction.

The data for hydrolysis of 7MedGuo were treated as follows in order to obtain values for the constants in eq 4. The second-order rate constant for imidazole ring opening was obtained from a plot of $k\psi$ *vs.* K_w/a_H for the region pH > 8.5; the value of the rate constant for ring opening, k_{RO}, so obtained is 4.27 M^{-1} sec^{-1}. A plot of $k\psi - k_{RO}K_w/a_H$ *vs.* a_H for the pH region 6.5–7.5 has slope $k_1/K_a = 1.72 \times 10^3$ M^{-1} sec^{-1} and intercept $k_{H_2O} = 5.0 \times 10^{-5}$ sec^{-1}. At low pH ring opening is unimportant and a plot of $k\psi$ *vs.* a_H for the region pH 1.0–2.0 has slope $k_2 = 6.03 \times 10^{-2}$ M^{-1} sec^{-1} and intercept $(k_1 + k_{H_2O}) = 7.0 \times 10^{-4}$ sec^{-1}. The "kinetic" pK_a was obtained from a log–pH plot where the term $[k\psi - (k_{RO}K_w/a_H + k_{H_2O})]$ was employed. The pK_a value obtained at the point of intersection of the two linear regions in this plot is 6.44. The pK_a determined potentiometrically under the temperature and salt con-

Table IV. Rate Constants for the Hydrolysis of Some Methylated Purine Nucleosides and Their Equilibrium Constants at 0.10 Ionic Strength

Compound	T, °C	k_1, sec^{-1}	k_2 M^{-1} sec^{-1}	pK_a
7MedGuo[a]	52.4	6.50×10^{-4}	6.03×10^{-2}	6.44
7MeGuo[b]	100.3	4.70×10^{-4}	1.73×10^{-2}	6.94[c,d]
1,7diMeGuoI[b]	100.3	3.40×10^{-4}	1.72×10^{-2}	

[a] $k_{H_2O} = 5.0 \times 10^{-5}$ sec^{-1}; $k_{RO} = 4.27$ M^{-1} sec^{-1} for imidazole ring opening. [b] pH measured at room temperature. [c] At 23°. [d] 6.88 (0.15 M NaCl) reported by A. M. Michelson and F. Pochon, *Biochem. Biophys. Acta*, **114**, 469 (1966).

ditions of the kinetic runs is 6.44. Figure 2 shows the agreement between observed values and the curve calculated using eq 4 and the constants of Table IV.

The hydrolysis of 7MedGuo had an apparent solvent isotope effect $k\psi^D/k\psi^H = 2.0$ at pD = pH 7.72 and 7.10. The value on the rate plateau was 1.0.

Observed rate constants for the hydrolysis of 7MeGuo and 1,7diMeGuoI are described by eq 5; values are given in Table IV.

$$k\psi = k_1 + k_2 a_H \quad (5)$$

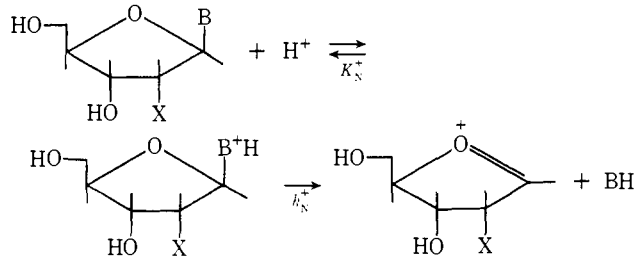
Rate constants for the hydrolysis of Guo, 7MeGuo, and 1,7diMeGuoI (100°) and of dGuo and 7MedGuo (52°) at low pH are essentially identical. Rates of hydrolysis of 7MeGuo are 40% faster than those of 1,7-diMeGuoI in the pH-independent region. By contrast, the second-order rate constant for the solvolysis of 7MedGuo at high pH is 25,800 times greater than that for dGuo. Under the same conditions 7MedGuo is 13,500 times more basic than dGuo. In contrast to the natural nucleosides which show a constant, linear dependence of rate on pH even in the vicinity of their pK_a's, neutral 7MedGuo hydrolyzes with a rate constant which is 30,000 times larger than that for protonated 7MedGuo.

That purine nucleosides may undergo diprotonation was revealed by pK_a studies (23°) in the molar acid region. Guo and 7MeGuo have very similar pK_a values, −2.43 and −2.62, respectively, for dissociation of their diprotonated forms. The acid strengthening effect of the ribose substituent is indicated by comparison with guanine, pK_a −1.30. This similarity between the nucleosides contrasts with the very large difference in their pK_a's in the dilute acid. Guo has pK_a 2.17 (25°) but 7MeGuo is very much more basic with pK_a 6.94 (23°).

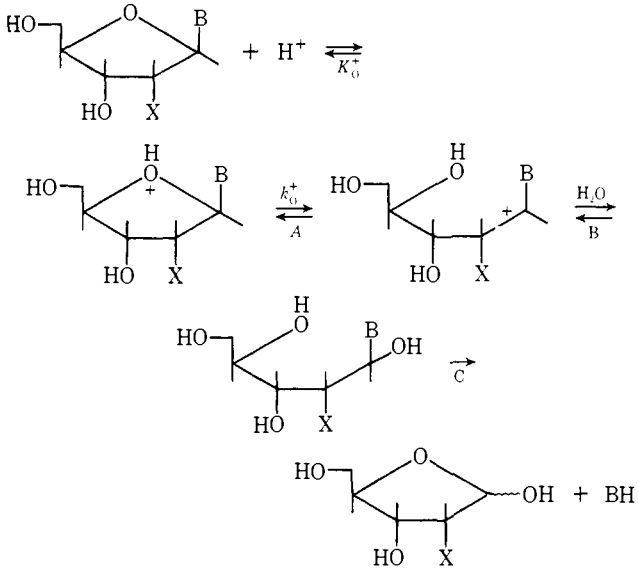
Discussion[39]

Three mechanisms have been suggested over the years as possible pathways for nucleoside hydrolysis.[21,22] Two are given schematically (B = purine base; X = OH or H). In mechanism 1 protonation on nitrogen is followed in the slow step by cleavage of the CN bond between the purine and sugar rings. The resultant cyclic carbonium–oxonium ion form of the sugar then reacts with solvent. In mechanism 2 ring opening of protonated sugar gives a charged Schiff base which fragments in subsequent reactions. Possible rate-limiting steps in mechanism 2 include (A) ring opening, (B) addition of water to the Schiff base to form a carbinolamine, and (C) decomposition of the carbinolamine.[40,41] Mechanism 3 is a special case of 2 and involves ring opening[42–44] coupled with intramolecular proton transfer from N-3 to the annular oxygen of the sugar.[22]

Mechanism I



Mechanism II



Mechanism 2 involves the formation of a cationic Schiff base and its further hydrolysis. We suggest that step B involving addition of water to a charged Schiff base to give a carbinolamine and step C involving decomposition of a carbinolamine are unlikely to be rate-limiting steps in the hydrolysis of the purine nucleosides considered here. This conclusion is suggested by a consideration of data on Schiff base hydrolysis.[45]

Substituted benzylidene-*t*-butylamines containing electron-withdrawing groups[46] and N-arylglucosylamines[47–49] both form cationic Schiff bases during hy-

(39) Symbols used in mechanisms: K is an acid dissociation constant. Superscripts + and 2+ indicate mono- and diprotonated acids; subscripts N and O indicate dissociation from nitrogen and oxygen centers, respectively. Rate constant k^+ and k^{2+} apply to the reactions of mono- and diprotonated substrates involving CN or CO bond cleavage (subscripts N and O).

(40) Elimination of purine may occur before or during ring closure to form furanoside.

(41) Another mechanism involving hydrolysis by way of an intermediate Schiff base has been proposed. It was suggested that a water molecule adds to the imidazole ring of a purine nucleoside prior to hydrolysis by mechanism 2.[42] This mechanism cannot operate generally. Purines generally do not undergo covalent hydration.[43] 8-Bromopurine ribosides cleave to an 8-bromopurine on acid-catalyzed hydrolysis.[44] Were the proposed mechanism operating, an 8-hydroxypurine would result instead.

(42) R. Shapiro, *Progr. Nucl. Acid Res., Mol. Biol.*, **8**, 73 (1969).

(43) A. Albert, *Angew. Chem. Intern. Ed. Engl.*, **6**, 919 (1967).

(44) R. E. Holmes and R. K. Robins, *J. Amer. Chem. Soc.*, **86**, 1242 (1964).

(45) For a review see, W. P. Jencks, *Progr. Phys. Org. Chem.*, **2**, 63 (1964).

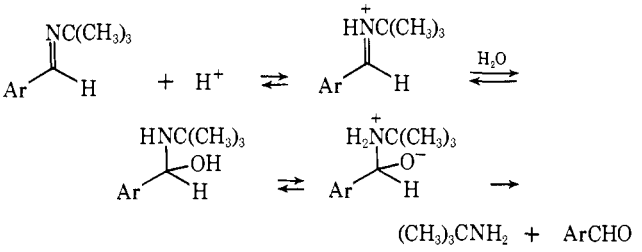
(46) E. H. Cordes and W. P. Jencks, *J. Amer. Chem. Soc.*, **85**, 2843 (1963).

(47) B. Capon and B. E. Connett, *J. Chem. Soc.*, 4497 (1965).

(48) H. Simon and P. Palm, *Chem. Ber.*, **98**, 433 (1965).

drolysis and both show a common type of pH–rate profile. With increasing acidity, rates increase to a maximum and then decrease. Consider the high pH region: here the rate-limiting step is addition of water to the cationic Schiff base formed in a preequilibrium step (Scheme I). With the substituted benzylidine-*t*-butyl-

Scheme I



amines the effect of substituents on the hydrolysis rate is small. For the rate-limiting step an extreme structural change such as replacing a *p*-nitro by a *p*-methoxyl group results in a rate decrease of only a factor of 550 at 25°. Reactivity differences are even less for the overall reaction. It is important to note that substituent effects are small despite the possibility of inductive and resonance effects. Consider now the low-pH region: here the rate-limiting step is decomposition of the carbinolamine. Rates show an inverse dependence on acidity.

Mechanism 2C involving decomposition of a carbinolamine as the rate-limiting step can be eliminated from consideration of nucleoside hydrolysis since no inverse dependence of rate on acidity has been observed.

Mechanism 2B involving rate-limiting addition of water to a charged Schiff base is unlikely. The rate decrease resulting from substitution of a 2′-hydrogen atom of the sugar by a hydroxyl group is too large to be accounted for by this mechanism.

The rate retardation associated with the substitution is likely to be due primarily to an inductive, electron-withdrawing effect.[50] In 2B the effects of this substitution on the preequilibrium formation of charged Schiff base and on rate-limiting addition of the water nucleophile are in opposite senses. The 2′-hydroxyl group ought to destabilize the charged Schiff base and so suppress its formation but this cation ought to be more reactive. The effect of a 2′-substituent on the initial protonation of the annular oxygen atom should be small, owing to large separation. However, Guo is 520 times less reactive than dGuo at 100°. This large rate decrease associated with an inductive effect contrasts with the kinetic changes found among benzylidine-*t*-butylamines where both inductive and resonance effects of substituents influence reactivity.

The large difference in reactivity between the ribosides and the deoxyribosides strongly suggests a reaction pathway involving preequilibrium protonation followed by rate-limiting bond breaking to give a C-1′ carbonium ion. Reactivity changes are similar in magnitude with those found for the hydrolysis of other kinds of substrates giving rise to carbonium ions by such a sequence. Thus, acetals[52] and glycosides[53] which undergo specific acid-catalyzed hydrolysis show a similar substituent effect. With simple acetals the inductive effect of a 2-hydroxyl group (carbonyl component) decreases the rate by a factor of 300 (25°).[54] One of the methyl glycopyranosides hydrolyzes about 2000 times faster than its 2-hydroxyl counterpart.[55] The glycosidic ring remains intact during the reaction.

A C-1′ carbonium ion could be formed by cleavage of either a CN bond, mechanism 1, or a CO bond, mechanism 2A. Consideration of the effects of the heterocycle on reactivity provides a way to distinguish between these two possibilities.

It should be mentioned at this point that it is futile to attempt to establish mechanism 1 on the grounds that purine protonation is overwhelmingly favored over sugar protonation. Both sugar and purine protonated forms are present in acidic solution and both are present in a constant ratio which is given by the ratio of their dissociation constants.[56] Moreover, the maximum degree of conversion to purine and sugar-protonated forms occurs at the same pH. This same reasoning also applies to diprotonation.

The hydrolysis reaction of Guo, dGuo, and dAdo each show a simple, continuous, first-order dependence on hydrogen ion activity, even in those regions where pH $\simeq$ pK_a, Figure 1. This provides good evidence against rate-limiting sugar ring cleavage and strong evidence for rate-limiting purine–sugar cleavage. Our equilibrium studies clearly indicate that neutral purine nucleosides can accept one or two protons. At pH > pK_a monoprotonated substrate solvolyzes but at pH < pK_a diprotonated material reacts. In order for the linear rate–acidity dependence to hold the apparent second-order rate constants for these two reactions must be the same. This is not reasonable for the pathway involving CO cleavage.

Scheme II is a representation of this CO bond cleavage mechanism. Because intermediate I $\leftrightarrow$ II resulting from the monoprotonation sequence is expected to be more stable and hence more readily formed than intermediate III $\leftrightarrow$ IV resulting from the diprotonation pathway, k_0^+ must be greater than k_0^{2+}; K_0^{2+} is expected to be greater than K_0^+. Thus k_0^+/K_0^+, the second-order rate constant for reaction of substrate at high pH, cannot equal k_0^{2+}/K_0^{2+}, the second-order constant for low pH reaction. This inequality eliminates any further need to consider slow CO bond cleavage.

Essentially this same reasoning can be applied to mechanism 3 to eliminate it.

Scheme III presents the CN bond cleavage mechanism for reaction of mono- and diprotonated forms of Guo and dGuo.[58] Rate equations relating the constants in this scheme to observed rate constants are given in the Appendix. Since Guo, dGuo, and also

(49) N-arylglucosylamines which are said to hydrolyze by a Schiff base mechanism[47,48] show buffer catalysis, unlike the hydrolysis of our purine nucleosides.

(50) Conformational changes associated with the substitution are likely to produce smaller rate effects.[51]

(51) E. L. Eliel, N. L. Allinger, S. J. Angyal, and G. A. Morrison, "Conformational Analysis," John Wiley & Sons, Inc., New York, N. Y., 1965, Chapter 6.

(52) For a review see: E. H. Cordes, *Progr. Phys. Org. Chem.*, **4**, 1 (1967).

(53) For a review see: J. N. BeMiller, *Advan. Carbohydrate Chem.*, 22, 25 (1967).

(54) M. M. Kreevoy and R. W. Taft, Jr., *J. Amer. Chem. Soc.*, 77, 5590 (1955).

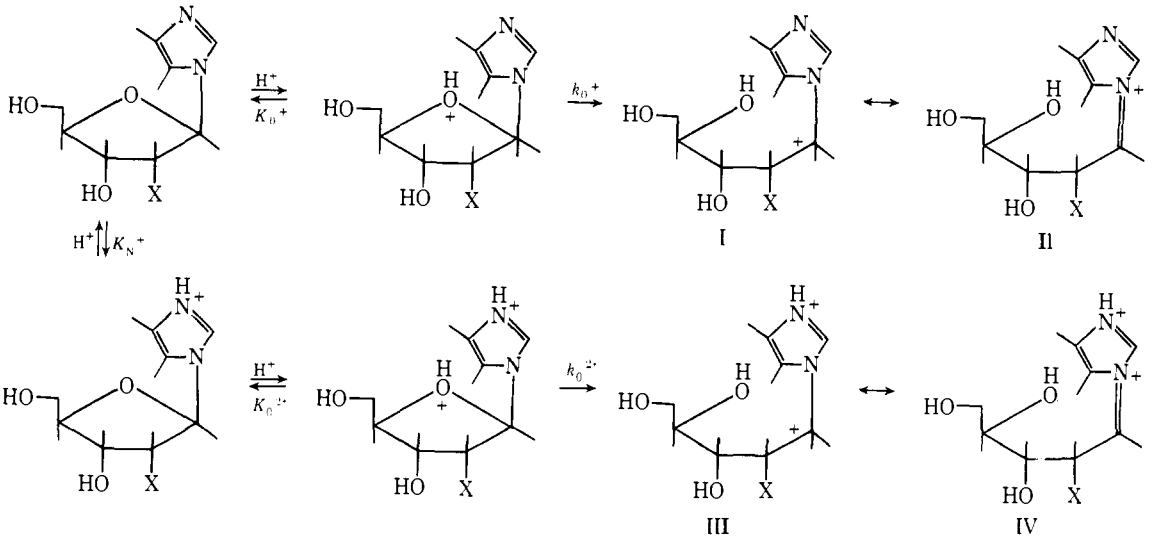
(55) C. Armour, C. A. Bunton, S. Patai, L. H. Selman, and C. A. Vernon, *J. Chem. Soc.*, 412 (1961).

(56) The pK_a of the sugar, roughly estimated from the value for tetrahydrofuran, is < -2.1.[57]

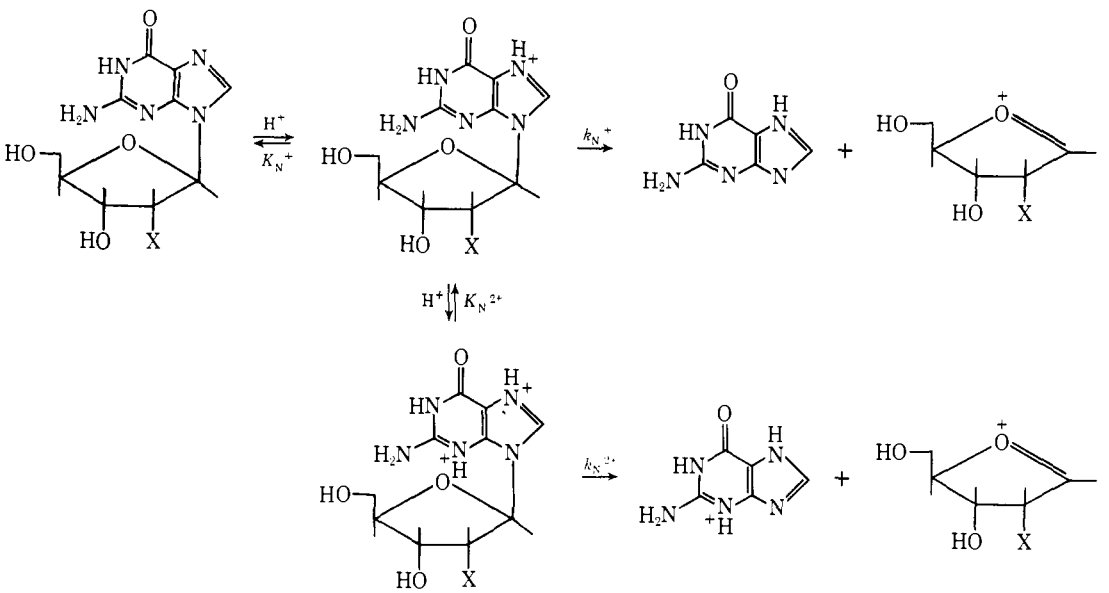
(57) E. M. Arnett, *Progr. Phys. Org. Chem.*, 1, 223 (1963).

(58) The second proton arbitrarily has been added to N-3.

Scheme II



Scheme III



dAdo exhibit a linear dependence of rate on acidity, then for this mechanism to hold, we must assume that the apparent second-order rate constants for reaction by both protonated forms are the same, *i.e.*, $k_N^+/K_N^+ = k_N^{2+}/K_N^{2+}$. This is possible. Diprotonated purine is a stronger acid than is monoprotonated purine, $K_N^{2+} > K_N^+$ and diprotonated purine is expected to be a better leaving group than monoprotonated purine, $k_N^{2+} < k_N^+$. In other words, while purine dication is formed less readily than purine monocation, it is more reactive. Hence k_N^+/K_N^+ may equal k_N^{2+}/K_N^{2+} because changes in the rate constants could be cancelled by changes in the equilibrium constants.

It is to be noted that compensating or partially cancelling effects on the rates of multistep reactions are not uncommon. Low sensitivity of the reaction rate to structural changes gives rise to low Hammett ρ values. Examples include the acid-catalyzed hydrolysis of thiazolines[59] and aryl α- and β-glucopyranosides,[60] among others. The ρ value for the α-glucosides is only -0.006. While these examples illustrate compensating substituent effects, our substrates exhibit compensating protonation effects.

The hydrolysis data for 1,7diMeGuoI, Figure 3, provide evidence against mechanisms 2 and 3 and a critical test of mechanism 1. If 1,7diMeGuoI were to react according to mechanism 2 or 3, protonation at the annular oxygen atom of the sugar or at N-3 would have to take place. Rates would have to depend on p*H* in the dilute acid region. But rates of hydrolysis are found to be independent of pH. Mechanism 3 is further eliminated since 1,7diMeGuoI is considerably more reactive than Guo and there are no protons on the dimethylguanine moiety available for intramolecular proton transfer to the annular oxygen atom of the sugar.[61]

According to mechanism 1, rupture of the sugar–purine bond is facilitated by charge on the purine; positively charged purine is expected to be a better leaving group than is neutral purine. Since positive charge is unambiguously built into the purine moiety of 1,7diMeGuoI, it need not be protonated to react

(59) G. L. Schmir, *J. Amer. Chem. Soc.*, 87, 2743 (1965).

(60) R. L. Nath and H. N. Rydon, *Biochem. J.*, 57, 1 (1965); A. N. Hall, S. Hollingshead, and H. N. Rydon, *J. Chem. Soc.*, 4290 (1961).

(61) It is possible to rule out a mechanism of hydrolysis of 1,7diMeGuoI involving both specific acid and specific base catalysis in a ring-opening pathway. The rate constant for hydroxide ion attack on the conjugate acid of 1,7diMeGuoI would exceed 10^{10} M^{-1} sec^{-1}.

according to mechanism 1. In fact this substrate does undergo hydrolysis in dilute acid by a pH independent process. At low pH another reaction commences, Figure 3. Substrate first protonates to give purine dication which then undergoes CN bond cleavage.

It is likely that the monomethylated nucleosides as well as the natural and dimethylated nucleosides react according to mechanism 1. Supporting this are the rate-retarding effect of a 2′-hydroxyl group, the similar reactivity at low pH of the natural and methylated guanine ribosides or deoxyribosides, and the similar reactivity at high pH of the mono- and dimethylated guanine ribosides.

The very similar reactivity of the natural and methylated guanine nucleosides at low pH is easily understood. As our equilibrium studies show, at low pH where dication is formed the basicity difference between natural and methylated substrate almost vanishes. It makes little difference whether a proton or a methyl group is bonded to nitrogen-7. Moreover, it is expected that the presence of either a proton or a methyl group on nitrogen should have little influence on the leaving group characteristics of the guanine moiety. Rates which reflect protonation and loss of a guanine group should therefore be similar for natural and methylated guanine nucleosides.

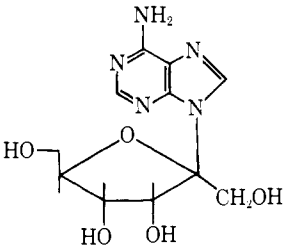
The very similar reactivity of 1,7diMeGuoI and 7MeGuo at high pH also is readily apparent in terms of mechanism 1. In the pH range where 7MeGuo is monoprotonated the leaving group characteristics of the two substituted guanine groups are expected to be nearly the same. Rates are similar since they reflect CN bond cleavage alone.

By contrast, at high pH monomethylated substrates are very much more reactive than their natural counterparts. 7MedGuo is 25,800 times more reactive than dGuo. This large difference does not signal a change in mechanism or the operation of a special effect. Rather, the explanation is straightforward. 7MedGuo is 13,500 times more basic than dGuo. The reactivity may be attributed almost entirely to the basicity difference.

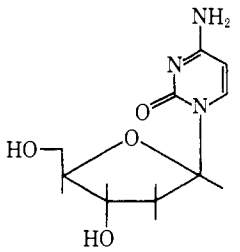
It is to be noted that the accidental equality of the apparent second-order rate constants for dGuo reacting by mono- and diprotonated forms is not found for 7MedGuo, Figure 2. This is so essentially because the greater basicity of 7MedGuo facilitates monocation formation. In other terms, $k_N^+/K_N^+ = 30{,}000\, k_N^{2+}/K_N^{2+}$.

Values for the solvent isotope effect and ΔS^* also are consistent with a hydrolysis pathway involving preequilibrium protonation followed by slow CN bond cleavage. At pD values where both steps operate, $k^D/k^H \sim 2$ (dAdo and 7MedGuo) but when the rates reflect only the latter step, there is essentially no isotope effect (7MedGuo). Positive ΔS^* values are commonly found in two-step, acid-catalyzed hydrolysis reactions.[62] For dAdo and dGuo the values are +8.4 and +12.7 eu, respectively.

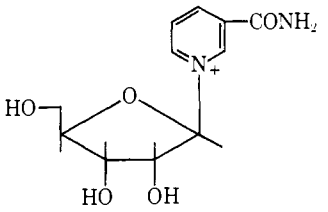
Still other nucleosides probably hydrolyze by mechanism 1. It is premature to suggest how extensive this pathway is, owing to the lack of detailed kinetic studies. However, some examples of nucleosides and related substances having diverse heterocyclic groups which probably hydrolyze according to mechanism 1 include psicofuranine,[14] 2′-deoxycytidine (dCyd),[63] and the pyridine–ribosyl bond of nicotinamide–adenine dinucleotide (NAD).[64,65]



psicofuranine



dCyd



NAD

Finally, it is worthwhile to review the origin of the point of view that nucleosides do not hydrolyze by simple CN bond cleavage, mechanism 1, but instead react by a Schiff base pathway. Notably, kinetic data on the hydrolysis of nucleosides themselves had not been obtained and employed in an attempt to establish a mechanism. Rather, reasoning by analogy was employed. Since the quaternary salts of aliphatic glycosylamines are "stable" toward hydrolysis, it was suggested that nucleosides having positive charge on the heterocyclic group would be "inert" as well. It was suggested, therefore, that positive charge must reside on the annular oxygen atom of the sugar in order for nucleoside hydrolysis to take place. This viewpoint, advanced repeatedly, fails to take into account the possibility that mechanism 1 involving cleavage of the glycosyl–nitrogen bond becomes likely as the quality of the nitrogen leaving group is improved.[66]

Appendix

For a substrate reacting according to mechanism 1 by the sequence given in Scheme III rate $= k\psi(S)_t = k_N^+(SH) + k_N^{2+}(SH_2)$ and

$$k\psi = \frac{a_H K_N^{2+}}{K_N^+ K_N^{2+} + a_H K_N^{2+} + a_H^2} X\left[k_N^+ + a_H \frac{k_N^{2+}}{K_N^{2+}}\right]$$

Under the conditions employed $a_H < K_N^{2+}$

$$k\psi = \frac{a_H}{K_N^+ + a_H} X\left[k_N^+ + a_H \frac{k_N^{2+}}{K_N^{2+}}\right]$$

This has the form of eq 4 which defines the hydrolysis of 7MedGuo. When $a_H/(K_N^+ + a_H) = 1$ the form of eq 5 results.

If $k_N^+/K_N^+ = k_N^{2+}/K_N^{2+} = k/K_N$, then $k\psi =$

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$a_H(k/K_N)$. This is similar to eq 2 which describes the rates of hydrolysis of Guo, dGuo, and dAdo. (A spontaneous or "water"-catalyzed reaction is not considered in these derivations.)

Acknowledgment. We are grateful to Professor Joseph F. Bunnett for allowing this project to commence in his laboratory (Brown University) and for initial, valuable discussions.

Relaxation Spectra of Ribonuclease. VII. The Interaction of Ribonuclease with Uridine 2′, 3′-Cyclic Phosphate[1]

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Abstract: Stopped-flow temperature-jump studies were made of the interaction of ribonuclease A with uridine 2′,3′-cyclic phosphate in 0.2 *M* NaCl. A bimolecular reaction of enzyme and substrate was observed at pH 6.5, 15°, with a second-order rate constant of approximately $1 \times 10^7\ M^{-1}\ \text{sec}^{-1}$ and a dissociation rate constant of $2 \times 10^4\ \text{sec}^{-1}$. A second relaxation process was observed at pH 6.0 and 6.5, 15°, which could be associated with an isomerization of the enzyme–substrate complex and the corresponding rate constants were determined. The isomerization of "free" ribonuclease was studied as a function of uridine 2′,3′-cyclic phosphate concentration at 25° in the pH range 5.5–7.5. The data suggest a mechanism where both isomeric forms of the enzyme bind the substrate, and an analogous isomerization can also occur with the enzyme–substrate complexes.

Earlier papers in this series dealt with the interaction of ribonuclease A with cytidine 3′-phosphate,[3,4] cytidine 2′,3′-cyclic phosphate,[5] cytidilyl-3′,5′-cytidine,[6] and uridine 3′-phosphate.[7] These studies have indicated that the mechanism of interaction of ribonuclease with all of the mentioned substrates involves an initial association between enzyme and substrate followed by an isomerization or conformational change of the enzyme–substrate complex. Three ionizable groups on the enzyme with approximate p*K* values of 5.4, 6, and 6.5 have been implicated in the binding mechanism and presumably the catalytic reaction. Moreover, the enzyme itself was found to undergo an isomerization at neutral pH values, with an ionizable group on the enzyme with a p*K* of approximately 6 being involved in the conformational change.[8] The results of these kinetic investigations have been correlated with the known three-dimensional structure of ribonuclease,[9,10] and a mechanism for the action of the enzyme can be postulated in molecular terms.[7]

The work reported here is concerned with stopped-flow temperature-jump studies of the interaction of ribonuclease A with uridine 2′,3′-cyclic phosphate. A relaxation effect was observed at pH 6.5, 15°, which is related to the initial association of enzyme and substrate; a second relaxation process is seen at pH 6 and 6.5, 15°, which corresponds to an isomerization of the enzyme–substrate complex. The isomerization of the "free" enzyme[8] was studied as a function of substrate concentration at pH 5.5–7.5, and the pH dependence of the enzyme isomerization at high substrate concentrations indicates that both isomeric forms of the enzyme bind the substrate. Moreover, the enzyme–substrate complexes themselves undergo an isomerization similar to that of the free enzyme.

Experimental Section

Bovine pancreatic ribonuclease A (phosphate free, lyophilized) was purchased from Worthington Biochemical Corp. and was used without further purification. Its concentration was determined spectrophotometrically as described previously.[11] Uridine 2′,3′-cyclic phosphate was prepared as previously described[11] and all other reagents were of analytical grade.

A combined stopped-flow temperature-jump apparatus[12,13] was used. All solutions used in the temperature-jump experiments were 0.2 *M* in NaCl, and were prepared from freshly boiled deionized distilled water. The pH changes accompanying the relaxation processes were observed using 2×10^{-5} *M* pH indicators. Methyl red (pH range 4.4–6.2), chlorophenol red (pH range 5.2–6.6) and phenol red (pH range 6.6–8) were used and the absorbance changes were observed at 520, 573, and 558 mμ, respectively. The pH of the solutions was adjusted with NaOH and HCl using a Radiometer Model 26 pH meter.

Steady-state experiments were done in 0.1 *M* Tris acetate–0.1 *N* NaCl buffers at 15.0 ± 0.1° using a Cary 14 spectrophotometer. The experimental procedure was as previously described[11] except that the time allowed for temperature equilibration was 10 min, mixing was by inversion, and the absorbance change was recorded using a 0–0.1 absorbancy unit slidewire.

Results and Treatment of Data

The steady-state kinetic parameters for the ribonuclease-catalyzed hydrolysis of uridine 2′,3′-cyclic phosphate to uridine 3′-phosphate at 15°, pH 6.0 and 6.5,

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(2) Predoctoral Fellow under the University of the Philippines–Cornell University graduate education program.

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