Secondary Deuterium Isotope Effects for Acid-Catalyzed Hydrolysis of Inosine and Adenosine[1]

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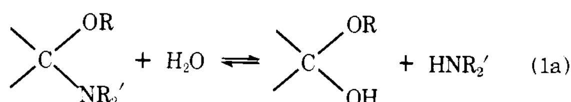
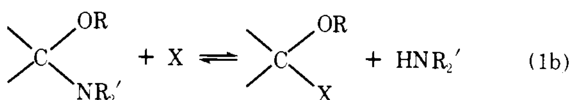
Abstract: Kinetic α deuterium isotope effects have been measured for acid-catalyzed hydrolysis of inosine and adenosine. For inosine hydrolysis, values of k_H/k_D follow: in 1.0 M HCl, 1.21 and 1.20 at 25 and 50 °C, respectively; in 0.1 M HCl, 1.19 and 1.18 at 25 and 50 °C, respectively. For adenosine hydrolysis, k_H/k_D is 1.23 in 0.1 M HCl at 25 °C. The values require that the transition states for hydrolysis of both the monocation and dication of inosine and the dication of adenosine have marked oxocarbonium ion character. Detailed mechanisms which accord with this and other experimental observations include (1) a classical A1 mechanism in which the C–N bond is largely cleaved in the transition state; (2) a mechanism involving some form of nucleophilic participation by solvent in which bond cleavage is advanced relative to bond formation in the transition state; or (3) complete C–N bond cleavage with rate-determining diffusion apart of oxocarbonium ion and purine base.

The decomposition of compounds bearing both oxygen and nitrogen substituents on one carbon atom with cleavage of the carbon–nitrogen bond constitutes an important class of reactions in chemistry and enzymology. At the carbonyl level of oxidation, these reactions include the reverse of carbinolamine formation, the hydrolysis of simple glycosylamines (1a),





the hydrolysis of nucleosides (1a), and ribosyl transfer reactions of nucleosides (1b). The last two reactions are subject to enzymatic catalysis. Enzymes in class EC 3.2.2 catalyze the hydrolysis of purine and pyrimidine nucleosides as well as that of nicotinamide coenzymes. Enzymes of class EC 2.4.2 catalyze the transfer of the ribosyl moiety of nucleosides to phosphate, pyrophosphate, purines, and pyrimidines. The extent to which the mechanism is understood for these related reactions varies markedly. The mechanism of formation of carbinolamines is understood in considerable detail.[2,3] Hydrolysis of simple glycosylamines has been investigated in less detail, although the principal features of the reaction pathway, involving formation of a Schiff base as an intermediate, seem reasonably secure.[4] The mechanism of hydrolysis of nucleosides is less well established,[4] although several recent studies have added substantially to understanding of purine nucleoside[5-10] and pyrimidine nucleoside hydrolysis.[11-13] The early view that nucleosides hydrolyze via a Schiff base intermediate,[14-16] as do simpler glycosylamines, has been strongly criticized.[4,5,7-9,11,12,17] The bulk of the evidence suggests that the rate-determining step for acid-catalyzed nucleoside hydrolysis involves a unimolecular dissociation of the mono- and dications of the substrates with rupture of the carbon–nitrogen bond and formation of a carboxonium ion or similar reaction pathway.

Enzymatic catalysis of cleavage of the carbon–nitrogen bond of nucleosides and nicotinamide coenzymes is not well understood. However, a number of enzymes in the classes EC 2.4.2 and EC 3.2.2 have been extensively purified and careful kinetic studies have been carried out in some cases.[18-27] This work provides the basis for design and execution of studies intended to answer basic questions concerning the enzyme-catalyzed reactions. For example, does C–N bond cleavage occur by a unimolecular decomposition reaction or with participation of solvent or groups on the enzymes as nucleophilic reagents? If the unimolecular pathway obtains, what is the extent of C–N bond cleavage in the transition state?

Useful information concerning these and related questions has been provided for related reactions, including carbonyl addition reactions[28,29] and hydrolysis of acetals,[30] through determination of kinetic α secondary deuterium isotope effects.[31] In the present study, we have initiated our investigations of mechanism and catalysis for nucleoside hydrolysis by determining kinetic α deuterium isotope effects for acid-catalyzed hydrolysis of inosine and adenosine. These studies have been extended to the enzymatic cleavage of the same substrates, work to be reported in a subsequent manuscript.

Experimental Section

Materials. Ribose-*1*-*d* was prepared as described previously.[32]

Adenosine-*1'*-*d* was synthesized through coupling 6-benzamido-α-(chloromercuri)purine to 1-chloro-2,3,5-tri-*O*-benzoyl-D-ribose-*1*-*d*. The procedure of Thomas et al.[33] for preparation of 2-aminoadenosine was followed without modification. 6-Benzamidopurine,[34] 1-*O*-acetyl-2,3,5-tri-*O*-benzoyl-β-D-ribose-*1*-*d*,[35] and anhydrous sodium methoxide[36] were prepared by published procedures. All reagents and solvents were dried or purified, usually by procedures collected by Perrin et al.[37] Ribose-*1*-*d* (10.0 g, 0.067 mol) was converted to adenosine-(β)-*1'*-*d* (6.44 g, 0.024 mol) in 36% yield. Purity was ascertained by ^{1}H NMR spectroscopy (HR-220). No resonance corresponding to a proton on C-1' was observed, despite repeated scanning of the appropriate region of the spectrum.

Inosine-*1'*-*d* was obtained by deamination of adenosine-*1'*-*d* with adenosine deaminase (Boehringer-Mannheim, calf intestine, specific activity 200 units/mg) at 20 °C. The reaction solution consisted of 200 mL of 0.1 M ammonium formate buffer, containing 650 mg of adenosine-*1'*-*d* and 20 units of adenosine deaminase. The course of the reaction was followed by measuring the decrease in absorbance at 260 nm; the velocity and end point were in agreement with results obtained with unlabeled adenosine. Inosine-*1'*-*d* was recovered as the residue following lyophilization of the reaction mixture and was subsequently recrystallized from ethanol, yield 482 mg (74%).

Inosine-*8*-^{14}C-*1'*-*d* was prepared by exchange of hypoxanthine-*8*-^{14}C into inosine-*1'*-*d*, catalyzed by calf spleen nucleoside phosphorylase in the presence of inorganic phosphate. The reaction solution contained 55.3 μmol of inosine-*1'*-*d*, 5.36 μmol of phosphate, 2 μmol of hypoxanthine-*8*-^{14}C (100 μCi, specific activity 50 μCi/μmol), and 0.5 units of crystalline nucleoside phosphorylase in 33.3 mL of 0.015 M tris(hydroxymethyl)aminomethane buffer, pH 7.45. The reaction mixture was kept at 4 °C for 23 days. Enzyme was denatured by heating at 75 °C for 7 min. Inosine was separated from hypoxanthine by two successive ion exchange chromatography runs; negligible ^{14}C activity was present in the hypoxanthine fractions obtained in the second pass through the column. Specific activity of the inosine-*1'*-*d* was 2 μCi/μmol, consistent with complete equilibration between inosine and hypoxanthine.

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Inosine-*2*-^3H was prepared by treatment of adenosine-*2*-^3H (Amersham-Searle, Inc.) with adenosine deaminase. The reaction solution contained 0.01 M tris(hydroxymethyl)aminomethane buffer, pH 7.50, 250 μCi adenosine-*2*-^3H (0.0156 mol, specific activity 16 000 μCi/μmol), and 2 units of adenosine deaminase in 12.7 mL. The reaction mixture was kept at 4 °C for 6 days, heated to 75 °C for 7 min to denature the enzyme, and purified by the ion exchange chromatographic technique described above for separation of inosine and hypoxanthine.

Hypoxanthine-*2*-^3H was obtained from adenine-*2*-^3H (Amersham-Searle, Inc.) by a variation of the procedure of Levene and Tipson.[38] Adenine-*2*-^3H (0.185 μmol, 5 μCi) was incubated in 1.00 mL of an aqueous solution 0.170 M in KNO_2 containing 30 μL of glacial acetic acid for 6 days in the dark, following which it was combined with 1.5 μCi of hypoxanthine-*8*-^{14}C and 7 μmol of hypoxanthine, adjusted to pH 9, and chromatographed as described above.

Adenosine-*8*-^{14}C-*1'*-*d* was prepared through the base exchange of inosine-*1'*-*d* with adenine-*8*-^{14}C (New England Nuclear) catalyzed by *E. coli* purine nucleoside phosphorylase. A 2.2×10^{-5} M KH_2PO_4 solution (100 mL) and 0.015 M Tris-HCl, pH 7.5, was added to 5.4 mg of inosine-*1'*-*d*, 2.7 mg of adenine, and 0.50 mL of an adenine-*8*-^{14}C solution (53.5 μCi/μmol). To this solution 2 units of *E. coli* PNPase was added and the reaction mixture maintained at 25.0 °C for 20 h. The enzyme was heat denatured, the solution centrifuged, and the supernatant collected and lyophilized. The lyophilate was dissolved in 3 mL of distilled water and chromatographed on a Sephadex G-10 column. Adenosine-*8*-^{14}C-*1'*-*d* was produced in 48% yield and had a specific activity of 2.3 μCi/μmol. Proof of structure was based on two criteria: (1) coincident chromatography with adenosine-*2*-^3H on a Sephadex G-10 column and (2) ^1H NMR (HR-220), which showed a minimal 95% incorporation of deuterium label at position C-1'.

Calf spleen purine nucleoside phosphorylase was a crystalline preparation from Boehringer-Mannheim Biochemicals, specific activity 25 units/mg protein.

E. coli purine nucleoside phosphorylase was partially purified according to a modification of the procedure of Jensen and Nygaard.[39]

Determinations of kinetic α deuterium isotope effects were carried out utilizing purified mixtures of the appropriately labeled substrates. Thus adenosine-*8*-^{14}C-*1'*-*d* and adenosine-*2*-^3H or inosine-*8*-^{14}C-*1'*-*d* and inosine-*2*-^3H were mixed in an appropriate ratio, lyophilized to dryness, dissolved in a 0.5 mL of distilled water, and chromatographed on Sephadex G-10. Coincident peaks of ^{14}C and ^3H were obtained in both cases. These mixtures were then stored as stock solutions until use in isotope effect determinations.

Kinetic Procedure. Even though it is possible to use a polarimetric method to follow the progress of hydrolysis of inosine in acidic media,[40] a chromatographic method was employed for several reasons. First, a knowledge of the fate of all reactants and products of hydrolysis or side reactions, if any, is required to apply the double-labeling technique, the method chosen to measure the kinetic α deuterium isotope effects. A balance of material through the entire course of the reaction is desirable. Second, the availability of a clean and powerful method of gel chromatography on Sephadex G-10 for separating unreacted inosine from hypoxanthine and ribose, the other products of hydrolysis, and any salt used is attractive. This chromatographic method has the added advantage of using distilled water as the eluent, a feature that simplifies and makes the application of the double-labeling technique more straightforward.

A weighed amount of inosine (0.0231 g), the hydrochloric acid solution, and a clean all-glass reaction vessel with a stopcock valve were brought to the temperature of reaction in a thermostated oil bath. Reaction was initiated by adding enough hydrochloric acid solution (50.0 mL) to reach a concentration of inosine of 1.72×10^{-3} M. After an appropriate time, the reaction was stopped by neutralization with a solution of sodium hydroxide of approximately the same molarity as the hydrochloric acid solution, containing 1.72×10^{-3} M citric acid, giving a final volume of approximately 4 mL. All aliquots were kept frozen until chromatography was performed.

These aliquots were applied to a Sephadex G-10 chromatographic column, and fractions were collected with an ISCO fraction collector Model 1200 Pup (ISCO 4700 Superior, Lincoln, Neb.), at a flow rate of 0.75 ± 0.10 mL/min. The absorbance of each fraction at 254 nm was determined with a Zeiss PMQ II spectrophotometer in order to detect the peaks corresponding to inosine and hypoxanthine or other chromophores. The inosine peak appeared first and the hypoxanthine peak eluted subsequently. Three or more fractions separated the two peaks. All the fractions of each peak were pooled and the total volume was brought up to 50 mL. The absorbance of each solution was recorded, and the extent of reaction was calculated using the equation

$$\text{extent} = \frac{(\text{OD} \times e)_{\text{in}} V_{\text{in}}}{(\text{OD} \times e)_{\text{in}} V_{\text{in}} + (\text{OD} \times e)_{\text{hx}} V_{\text{hx}}}$$

Values of 9.22×10^{-3} and 9.38×10^{-3} M^{-1} L^{-1} cm^{-1} were used as molar absorptivity (e) for inosine and hypoxanthine, respectively. V is the volume of the pooled peaks, usually 50 mL for both compounds. OD_{in} and OD_{hx} are the absorbances of these solutions.

Chromatographic Methods. Usually, a Sephadex G-10 column (inside diameter 0.8 cm, length 120 cm) eluted with 5.0×10^{-4} M acetic acid was used for the separation of adenosine and adenine. Typically, the adenosine peak appears after 130 mL of elution and the adenine peak after 220 mL of elution. A similar column was also employed to separate inosine and hypoxanthine employing distilled water as eluent. In both cases, excellent separations were obtained.

For one set of experiments, inosine and hypoxanthine were separated by ion exchange chromatography on a Bio-Rad AG 1-XB, 200-400 mesh, anion exchange resin in the chloride form, using as eluent a 0.04 M NaCl, 0.01 M Tris, 0.0025 M potassium tetraborate buffer adjusted to pH 9.00 with HCl. Column dimensions were 27 cm × 0.875 cm^2, and the flow rate was 170 mL/h. Samples contained 7 μmol of hypoxanthine and 3-50 μmol of inosine, and were applied as aqueous solutions in 0.01 M Tris buffer adjusted to pH 9 which contained as much as 0.5 M sodium chloride or 0.03 M phosphate. Hypoxanthine eluted as a nearly symmetrical peak with a maximum at 760 mL of eluent and a width at half-height between 120 and 220 mL, depending on salt content of the sample. Inosine began to elute at 1200-1300 mL as an extremely wide band; consequently, after 1200 mL of eluate had been collected the first eluent was replaced with a second consisting of 0.01 M Tris adjusted to pH 7.00, to give a sharp peak that eluted shortly after the change in buffers. Fractions were monitored by their absorbance at 250 nm.

The hypoxanthine peak eluted by ion-exchange chromatography as described above was inhomogeneous with respect to the $^{14}C/^3H$ ratio, which progressively increased from 0.434 to 0.558 to 0.662 for the leading, middle, and trailing fractions, respectively, in one typical run. This inhomogeneity is apparently the consequence of a small secondary tritium isotope effect on the extent of ionization of hypoxanthine; the site of tritiation (the 2 position) is close to the site of ionization (N-1) and chromatography was carried out at a pH, 9.0, close to the pK for anion formation, 8.9. The tritiated species, being slightly less ionized at 9.0, would travel slightly faster than the protio species, labeled with ^{14}C, and would account for relative enrichment of fractions with ^{14}C as chromatography proceeded. Chromatography of an authentic mixture of hypoxanthine-*2*-^3H and hypoxanthine-*8*-^{14}C led to qualitatively the same change in $^{14}C/^3H$ ratio across the peak, confirming this assumption. Consequently, in determination of the isotope effects the entire hypoxanthine peak was pooled prior to taking aliquots for counting.

The ^3H label at C-2 of hypoxanthine exchanges slowly with solvent in a base-catalyzed reaction. The rate of exchange at pH 9.00 is ca. 0.1% per hour, as determined from the ^3H background in the eluate. To obviate systematic errors in isotope effects due to exchange during chromatographic separation, hypoxanthine $^{14}C/^3H$ ratios were compared with the $^{14}C/^3H$ ratios of the reisolated inosine, rather than with the $^{14}C/^3H$ ratio of the starting inosine. The inosine reisolated from the reaction and product hypoxanthine suffer identical exposure to buffers of high pH. Identical exposure times to pH 9 were achieved by neutralizing the fractions comprising the hypoxanthine peak at the same time that the unreacted inosine was eluted with pH 7 buffer. As a consequence of these experimental difficulties, chromatography on Sephadex G-10 as described above was routinely employed in preference to ion exchange chromatography.

Measurement of Isotope Effects. An aliquot of the stock solution of the mixture of isotopically labeled inosines or adenosines was removed and added to an aqueous solution containing an appropriate concentration of hydrochloric acid. The reaction mixture was maintained in a constant-temperature bath for a predetermined time, usually chosen so as to permit 5% or less of the substrate to hydrolyze. Subsequently, the entire reaction mixture or some aliquot thereof was

Table I. First-Order Rate Constants for Hydrolysis of Inosine at 50 °C as a Function of Acid Concentration

acid concn, M	$k_{obsd} \times 10^5$, s^{-1}	acid concn, M	$k_{obsd} \times 10^5$, s^{-1}
1.49	4.28	0.103	0.97
0.100	3.14	0.103	0.94
0.498	1.88	0.078	0.78
0.377	1.84	0.052	0.58
0.252	1.51	0.026	0.37
0.174	1.25		

removed and the reaction quenched by adjusting the pH to neutrality employing 1 M Tris buffer. When necessary, these solutions were reduced in volume to about 1.0 mL through lyophilization prior to chromatography on Sephadex G-10 as described above. Elution of unreacted nucleoside and product base was monitored by ultraviolet spectrophotometry. In those cases in which ion-exchange chromatography was employed following partial nucleoside hydrolysis, the reaction was quenched by adjusting the pH to 6 with ammonium hydroxide-citrate buffer and the preparations were maintained refrigerated until chromatography. Prior to counting, all chromatographic fractions containing a single product were pooled. The unreacted nucleoside fraction was diluted with distilled water to a known volume in a volumetric flask and three aliquots were withdrawn and added to counting vials. These were taken to dryness by lyophilization, the residue was dissolved in 1.00 mL of distilled water added with a microburet, and 10.0 mL of Bray's solution was added with a volumetric pipet. The purine base fraction was divided equally among three counting vials and these were treated as just described. Counting of samples and calculation of isotope effects were carried out as described previously.[32]

Results

Validation of Methodology. Kinetic α secondary deuterium isotope effects are inherently small. Moreover, mechanistic interpretations may depend on modest differences observed as a function of a number of variables. Consequently, we have gone to some lengths to develop and validate the experimental procedures employed. In order to obtain reliable and reproducible results, it is essential to observe the procedures outlined in the Experimental Section and in ref 32. A very detailed set of procedures has been published.[41] Basically, the procedures employed in this work are based on those originally developed by Raftery and co-workers, who introduced the double-label technique in the first place.[42,43]

Two essential control experiments were carried out in order to establish the reliability of our methodology, including our ability to measure intrinsic counting efficiency accurately, dependence of measurements on the nature of the scintillation cocktail employed, and acceptability of chromatographic procedures. These two controls test the same variables; nonetheless, it seemed desirable to carry out both to validate the methodology as thoroughly as possible. The first control involved incorporation of a mixture of hypoxanthine-*8-¹⁴C* and hypoxanthine-*2-³H* into *unlabeled* inosine in the presence of catalytic amounts of phosphate and calf spleen purine nucleoside phosphorylase. Assuming that our methodology is sound, this experiment should yield an observed "isotope effect" of unity, except for the possible influence of very small effects resulting from the isotopic labels in the purine base.

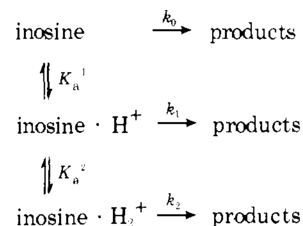
A mixture of inosine-*8-¹⁴C-1′-d* and inosine-*2-³H* (3.4 μmol) was completely hydrolyzed (1.0 M HCl, 50 °C, 34.6 h). The reaction was stopped by neutralization in the cold (pH 4.0), and the mixture was frozen and lyophilized. The dry mixture was dissolved in 2 mL of distilled water and chromatographed as described above. The hypoxanthine-containing fractions were pooled, frozen, and lyophilized. The incorporation reaction of this mixture of hypoxanthines was initiated by adding 10 mL of a solution that was 4.00×10^{-4} M in unlabeled inosine, 1.5×10^{-2} M in Tris, 3.32×10^{-5} M in phosphate at pH 7.5, and which contained 1.51×10^{-2} units of enzyme/mL. The reaction mixture was kept at 4 °C for 3 days. The reaction was stopped by denaturing the enzyme at 60 °C for 12 min. The reaction mixture was frozen, lyophilized, redissolved in 2 mL of distilled water, and chromatographed as described. The inosine- and hypoxanthine-containing fractions were pooled separately and 100-, 200-, and 500-μL aliquots were withdrawn and placed in counting vials, frozen, and lyophilized. Ten milliliters of the different counting solutions was subsequently added. Three counting solutions were used: Aquasol (New England Nuclear, Boston, Mass.) with the sample previously dissolved in 1 mL of distilled water, Bray's solution added directly without water, and Bray's solution added to the sample previously dissolved in 1 mL of water. The observed "isotope effect", measured as $(^3H/^{14}C)_{product}/(^3H/^{14}C)_{reactant}$, did not vary significantly as a function of counting solution employed. The observed value derived from a number of such experiments is 1.017 ± 0.008, a result quite close to unity. The small deviation from the ideal value may reflect a slightly preferential loss of tritium from inosine compared to that from hypoxanthine (see below) or possibly a subtle effect of isotopic substitution in the ring as noted above.

The second control experiment involved making a mock determination of the secondary isotope effect for hydrolysis of the mixture of inosine-*8-¹⁴C* and inosine-*2-³H* prepared in the control experiment just described. Again, since no deuterium substitution is present at C-1′, one expects a measured "isotope effect" of unity. Hydrolysis was initiated by dissolving a dry mixture of the inosines in 1.0 M HCl and was carried out at 50 °C. For a reaction mixture incubated 2 h and which proceeded 22% to completion, the observed effect was 1.024 $\pm$ 0.011; a similar reaction incubated for 6 h and which went 54% to completion yielded an effect of 1.046 ± 0.009. The fact that the observed effect increases with increasing extent of reaction requires that a preferential loss of tritium from inosine compared to hypoxanthine occur; extrapolation of the data to zero extent reaction yields an "isotope effect" within 1% of unity, that is, within experimental error of the ideal value.

We consider that these results establish the reliability of our measurements.

pH-Rate Profile for Inosine Hydrolysis. The kinetics of hydrolysis of inosine were determined in aqueous solution at 50 °C at several concentrations of HCl by the chromatographic procedure described. In each case, excellent first-order kinetic behavior was observed. First-order rate constants measured are collected in Table I; the logarithms of these rate constants are plotted as a function of pH in Figure 1. The rate constants increase with increasing concentration of acid; however, no sharp break of the profile is observed at the pK_a of the inosine monocation, ca. 0.5.

The kinetic data were fitted to the scheme



The rate constants are $k_0 = 0.17 \times 10^{-5}\ s^{-1}$; $k_1/K_a^1 = 7.7 \times 10^{-5}\ M^{-1}\ s^{-1}$; $k_2/K_a^2 = 1.7 \times 10^{-5}\ M^{-1}\ s^{-1}$. p$K_a^1$ has been reported to be 0.9 and 1.2 at 25 °C.[44]

Assuming that the pK_a of the inosine monocation varies with temperature in the same way as does that of the guanosine monocation,[45] we estimate that the pK_a at 50 °C is 0.5. This yields a value for k_1 of $2.45 \times 10^{-5}\ s^{-1}$. The solid line in Figure

Table II. Kinetic α Deuterium Isotope Effects for Acid-Catalyzed Hydrolysis of Inosine and Adenosine

substrate	temp, °C	[HCl], M	k_H/k_D[a]
inosine	50	1.0	1.175 ± 0.009
inosine	25	1.0	1.190 ± 0.008
inosine	25	1.0	1.23 ± 0.02[b]
inosine	50	0.1	1.196 ± 0.008
inosine	25	0.1	1.211 ± 0.010
adenosine	25	0.1	1.229 ± 0.012

[a] Error limits indicated are standard deviations from the mean; each isotope effect was measured three to six times. [b] Value determined employing the ion-exchange methodology for product separation (see text).

1 is a theoretical line based on these rate constants and the rate law

$$k_{obsd} = \frac{1}{1 + \frac{(H^+)}{K_a^1} + \frac{(H^+)^2}{K_a^1 K_a^2}} \times \left[k_0 + \frac{k_1}{K_a^1}(H^+) + \frac{k_2}{K_a^1 K_a^2}(H^+)^2\right]$$

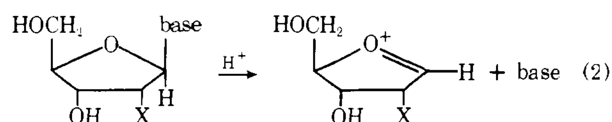
The kinetics of inosine hydrolysis in aqueous solution have been extensively probed by Suzuki.[46] He has established clear evidence for the pH-independent reaction detected here, although comparison of rate constants is not possible owing to the differences in temperature of measurement. He has reported a value of 1.8×10^{-4} M^{-1} s^{-1} for the second-order rate constant for hydrolysis of the inosine monocation, a value about twice that observed in this work.

α Secondary Deuterium Isotope Effects for Hydrolysis of Inosine and Adenosine. Isotope effects for the acid-catalyzed hydrolysis of inosine were measured at 0.1 M HCl and 1.0 M HCl at both 25 and 50 °C. Results are collected in Table II. In the presence of 0.1 M HCl, the hydrolysis of inosine occurs about 17% through the pH-independent pathway, 77% through the monocation, and 6% through the dication, as judged from the pH-rate data presented above. Consequently, the isotope effect measured under these conditions largely reflects that for decomposition of the monocation. In the presence of 1.0 M HCl, the neutral pathway contributes about 1%, the monocation pathway about 58%, and the dication pathway about 41% to the overall rate of hydrolysis of inosine. The fact that the isotope effects measured at the two acidities are about equal strongly suggests that the intrinsic isotope effects for hydrolysis via the mono- and dications are about the same.

The kinetic α deuterium isotope effect for acid-catalyzed hydrolysis of adenosine was measured at 25 °C in the presence of 0.1 M HCl; the value obtained is included in Table II. On the basis of the pH-rate profile for hydrolysis of this compound previously determined by Garrett,[8] adenosine hydrolyzes 99% via the dication under these conditions.

Discussion

There is considerable evidence to suggest that hydrolysis of purine nucleosides occurs via unimolecular decomposition of the protonated and diprotonated forms of the substrate to form an oxocarbonium ion (eq 2). The crucial observations include



the following. First, values of entropy of activation for these reactions are usually near zero or positive,[6-9] suggesting a carbonium ion pathway rather than a reaction involving

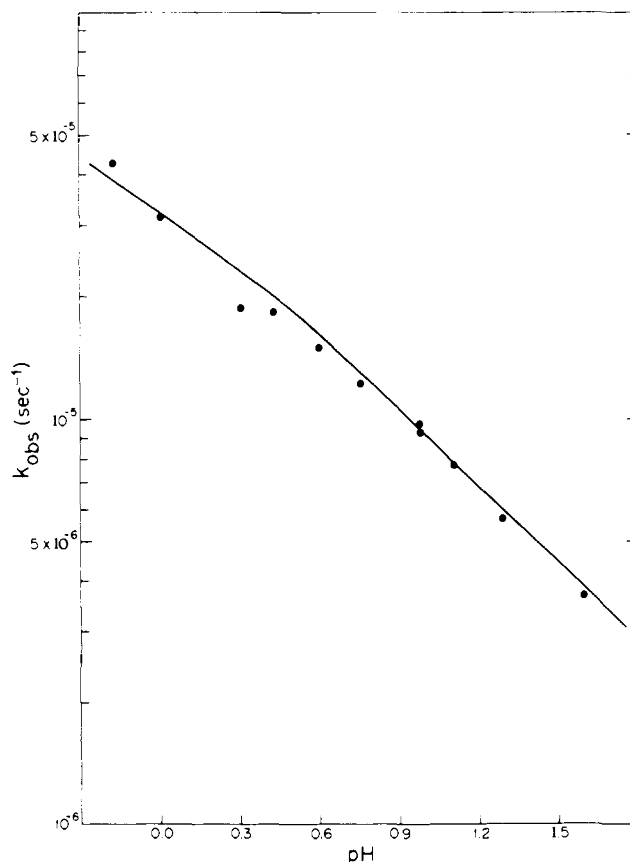


Figure 1. Logarithms of first-order rate constants for hydrolysis of inosine in aqueous solution at 50 °C plotted as a function of pH. The solid circles are experimental values; the solid line is theoretical, calculated as described in the text.

nucleophilic attack by solvent in the transition state.[45] Second, 2-deoxy nucleosides are much more reactive than the ribonucleosides,[4,5,8,9] consistent with the mechanism shown in eq 2. Third, the hydrolysis of 1,7-dimethylguanosine shows a marked pH-independent reaction.[5] This is consistent with rate-determining C–N bond cleavage but difficult to explain on the basis of C–O bond cleavage, a reaction for which no driving force would exist. Fourth, there is no evidence for anomerization concomitant with hydrolysis of purine nucleosides[4] as would be expected were C–O bond cleavage more rapid than C–N bond cleavage. Finally, hydrolysis rates are decreased by electron-releasing substituents at C6.[7,8] This, too, suggests C–N bond cleavage in the transition state.

Kinetic α deuterium isotope effects measured in this work for hydrolysis of the inosine and adenosine dications and the inosine monocation are all large and near 1.20 (Table II). This value requires that these reactions occur via transition states having considerable carbonium ion character. The simplest interpretation is that the reactions are classical Al, involving unimolecular decomposition of the protonated substrate to yield the free oxocarbonium ion as an intermediate.

The secondary deuterium isotope effects measured in this work will not distinguish between C–N and C–O bond cleavage in the transition state. Assuming that the former alternative obtains, as discussed above, careful theoretical calculations, employing the complete Bigeleisen–Mayer equation and the computer program developed by Burton et al.,[47] were made for the *limiting* α deuterium isotope effect for nucleoside hydrolysis, that is, the equilibrium isotope effect for interconversion of the nucleoside and the oxocarbonium ion. The value obtained is 1.21–1.25.[48] This value together with the experimental ones strongly suggests that the C–N bond is largely or completely cleaved in the transition state. Thus, the transition

state must closely resemble the oxocarbonium ion, regardless of the precise details of the mechanism. One possibility that cannot be excluded is that the C–N bond is completely cleaved in the transition state and that the rate-determining step is diffusion apart of the oxocarbonium ion and the purine base (or protonated purine base). These conclusions hold for both hydrolysis of the purine nucleoside mono- and dications.

Interpretation of the acid-catalyzed hydrolysis of nucleosides in terms of a simple unimolecular carbonium ion pathway is clouded by recent evidence of Young and Jencks which suggests that free oxocarbonium ions derived from *O*- and *N*-glycosides, which includes the nucleosides, are expected to have lifetimes so short as to call into question whether they can be reaction intermediates.[49] Thus the possibility of some form of oxocarbonium ion stabilization, perhaps by nucleophilic participation of solvent, cannot be excluded. However, the isotope effects measured in this study are large and, as noted above, nearly equal to those calculated for complete carbonium ion formation. This would appear to argue against a significant role for solvent as nucleophilic reagent in the transition state. Moreover, Jones et al.[50] have argued that glycosyl carbonium ions of the type of interest in the work reported here are about as stable as the diphenylmethyl cation, considered to have a lifetime of about 10^{-9} s in solution.[51] This is consistent with and support for the intermediacy of glycosyl carbonium ions in nucleoside hydrolysis. In any case, the measured isotope effects require that bond cleavage be far advanced relative to any bond formation process that may occur in the transition state.

The results just discussed contrast with those obtained for the pH-independent hydrolysis of nicotinamide nucleosides, reactions which bear a strong formal resemblance to hydrolysis of purine nucleosides via the monocations. In the former case, kinetic α deuterium isotope effects are near 1.11,[32,52] a value consistent with formation of an oxocarbonium-ion-like transition state but well below the limiting effect. Consequently, bond breakage in the transition state for the hydrolysis of the nicotinamide nucleosides is less advanced than is the case for purine nucleosides.

The hydrolysis of β-galactopyranosylpyridinium ion exhibits an α secondary deuterium isotope effect of 1.16.[50] It is not clear precisely how the transition state structure, as indicated by the secondary deuterium isotope effects, is related to substrate structure and reactivity. Nicotinamide is less basic than pyridine by about two orders of magnitude and ought, therefore, to be a better leaving group. Considerations based on the Hammond postulate[53] suggest that the transition state for nicotinamide nucleoside hydrolysis should be reached earlier than that for the pyridine glycosides, consistent with the measured isotope effects. However, purine cations, the leaving group for decomposition of the nucleoside dications, are probably better leaving groups than either of the other two, yet the isotope effects for both neutral and cationic purine departure are larger than for the other cases and are about equal to each other. Clearly, considerations other than the basicity of the leaving groups are important in determining the extent of C–N bond cleavage in the transition state.

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