Uncatalyzed Hydrolysis of Deoxyuridine, Thymidine, and 5-Bromodeoxyuridine*

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ABSTRACT: The *N*-glycosyl bonds of deoxyuridine, thymidine, and 5-bromodeoxyuridine undergo slow hydrolysis in aqueous solution, pH 3–7. The reaction rates are independent of the pH in this range, and of the nature and concentration of the buffer. The reactions have positive entropies of activation. A linear correlation exists between the logarithms of the rate constants for the three nucleosides and the ionization constants for the 1-protons of the corresponding bases. It is proposed that the reaction involves a simple SN1 ionization mechanism. The existing data on the known acid-catalyzed hydrolyses of these nucleosides is reexamined. It has been considered that the acid-catalyzed hydrolyses of nucleosides proceeds *via* sugar ring opening to an intermediate immonium ion. It is suggested that an A-1 mechanism, analogous to the hydrolysis in neutral solution, might better fit the data for deoxyuridine derivatives.

The integrity of the *N*-glycosyl bonds that bind the bases of DNA to deoxyribose is important to the proper functioning of DNA. The cleavage of one of these bonds can lead to mutations (Freese, 1963), or by causing subsequent chain breakage, to inactivation of the DNA (Strauss and Wahl, 1964). For these reasons it is important that the conditions causing hydrolysis of these bonds, and the chemical principles involved, be defined precisely.

In this paper we will discuss the hydrolysis of thymidine, a constituent of DNA, its important analog, 5-bromodeoxyuridine, and the parent compound, deoxyuridine. It is well known that these compounds are cleaved under acidic conditions (see the Discussion section). We wish to report now that they are also slowly hydrolyzed at physiological pH by a pH-independent process, and to suggest a mechanism for this process. The possible mechanisms involved in the acid hydrolysis will also be discussed.

Experimental Section

Methods and Materials. Nucleosides were obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y., and Mann Research Laboratories, New York, N. Y. The methods of preparation of buffers, instruments for determining pH and ultraviolet spectra, and procedures for thin-layer chromatography were analogous to those described in a previous paper (Shapiro and Klein, 1966). Reactions were run in volumetric flasks in a thermostat maintained at the indicated temperature ±0.1°. The initial concentration of nucleoside was 0.05 M. Aliquots were withdrawn from time to time and analyzed by the procedure described below.

Kinetic Determinations. A direct spectrophotometric method was used. The general details of this method have been described (Loring, 1955). As a precaution, each aliquot was added to excess 0.1 N NaOH solution and allowed to stand for 16 hr at 25°. It had been reported (Garrett *et al.*, 1966; Seydel *et al.*, 1967) that hydrolysis of deoxyuridine derivatives in HCl led to the formation of a chromophore which interfered with the spectrophotometric determinations. This chromophore was destroyed by alkaline treatment. However, in our hydrolyses at less acidic pH, this substance was not observed by thin-layer chromatography. Only the deoxyuridine derivative, the corresponding base, and, in the alkaline runs, the brown materials described below were detected. The relative concentrations of nucleoside and base were determined in the following way.

HYDROLYSIS OF DEOXYURIDINE. The absorptions of the solution in 0.1 N NaOH at 290 and 260 mμ were determined. From the ratio of A_{290}/A_{260} (R), and from the extinction coefficients of deoxyuridine and uracil at 290 and 260 mμ in 0.1 N NaOH, the fraction X of deoxyuridine hydrolyzed was calculated. This calculation was performed using the equation, $X = (7350R - 40)/(3270R + 4960)$.

In the hydrolyses run at pH 10 and 10.7, a considerable amount of brown color was formed in the reaction mixtures. Each aliquot was neutralized and worked up by thin-layer chromatography in 2-propanol–HCl–H_2O (65:18.4:16.6, v/v). The brown materials (presumably derived from free deoxyribose) ran at the solvent front, while deoxyuridine and uracil ran together. The combined bands deoxyuridine and uracil were eluted together into 0.1 N NaOH and analyzed by the above procedure.

HYDROLYSIS OF THYMIDINE. The ratio, R, of absorbances at 300–260 mμ was determined, in 0.1 N NaOH. From this, and from the extinction coefficients of thy-

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TABLE I: Rate Constants for Hydrolysis.

pH	Buffer	Temp (°C)	10^6K (sec^{-1})
	Deoxyuridine		
1.9	0.5 M citrate	95	4.54
3.1	0.5 M citrate	95	3.27
3.9	0.5 M citrate	95	3.05
4.0	0.25 M citrate	95	2.85
4.0	1.0 M citrate	95	2.96
4.3	0.5 M citrate	95	3.18
4.8	0.5 M citrate	95	2.98
5.3	0.5 M citrate	95	3.10
6.0	0.5 M citrate	95	3.2
6.5	H_2O	95	2.80
7.0	0.1 M phosphate	95	3.05
9.0	0.1 M borate	95	2.16
10.0	0.1 M borate	95	1.09
10.7	0.5 M phosphate	95	0.814
6.5	H_2O	85.5	0.707
6.5	H_2O	75	0.210
	Ethanol	75	0.0252
	Thymidine		
4.0	0.25 M citrate	95	1.05
4.0	0.5 M citrate	95	1.26
5.5	0.5 M citrate	95	1.16
6.5	H_2O	95	1.14
7.0	0.1 M phosphate	95	1.19
6.5	H_2O	85.5	0.308
6.5	H_2O	75	0.0806
	5-Bromodeoxyuridine		
4.0	0.5 M citrate	95	52.8
5.5	0.5 M citrate	95	56.6
7.0	0.1 M phosphate	95	49.6
6.5	H_2O	85.5	15.7
6.5	H_2O	75	3.90

midine and thymine at 300 and 260 mμ in 0.1 N NaOH, the fraction, X, of thymidine hydrolyzed was calculated, using the equation, $X = 6700R/(3000R + 3950)$.

HYDROLYSIS OF 5-BROMODEOXYURIDINE. Ultraviolet measurements were made upon solutions which had finally been brought to pH 11 by addition of Na_2HPO_4. The ratio, A_{300}/A_{275}, was determined, as were the extinction coefficients of 5-bromouracil and 5-bromodeoxyuridine at pH 11. The fraction, X, of 5-bromodeoxyuridine remaining was calculated, using the equation, $X = (6500R - 880)/(2460R + 5720)$.

Results

The kinetics of the hydrolyses of the *N*-glycosyl bonds of deoxyuridine, thymidine, and 5-bromodeoxyuridine were followed by a direct spectrometric method. The data from the runs, when plotted, gave curves indicating a first-order reaction in nucleoside. The rate constants were determined by a least-squares program on a CDC 6600 electronic computer. The standard deviations were generally in the range of 2–8.6%, with the slower hydrolyses having the less accurate rate constants. The rate constants are complied in Table I.

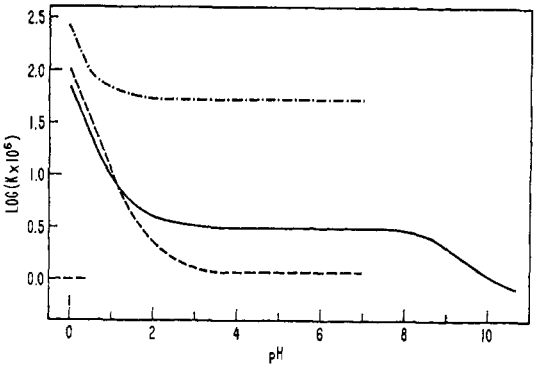


FIGURE 1: Plot of the logarithms of the rate constants, at 95°, *vs.* pH for the hydrolysis of deoxyuridine (———), thymidine (– – –), and 5-bromodeoxyurine (· – · –).

The hydrolysis of deoxyuridine was studied the most extensively. As indicated in Table I, the rate of this hydrolysis was (within experimental error) independent of pH and the nature and concentration of the buffer, in the pH range 3–7. The rate constant for hydrolysis declined with increasing pH in the pH range 9–11. Hydrolysis at pH 1.9 went more rapidly than in the pH range 3–7. A solvolysis run in ethanol at 75° revealed the rate to be 12% of the rate in H_2O at that temperature. One hydrolysis was run in D_2O, and the uracil produced was isolated and examined by nuclear magnetic resonance spectroscopy. No exchange of the 5-proton for deuterium was observed. This made unlikely the possibility of a hydrated intermediate, of the type implicated in the hydrolytic deamination of cytidine to uridine (Shapiro and Klein, 1967).

Less work was done with thymidine and 5-bromodeoxyuridine. However, the rates of hydrolysis of these nucleosides also appeared to be independent of the pH and of the nature and concentration of the buffer, in the pH range 4–7. A similar hydrolysis of 5-iododeoxyuridine in acetate buffers has been observed by other workers (Garrett *et al.*, 1964). The variation of the rates of hydrolysis of deoxyuridine, thymidine, and 5-bromodeoxyuridine with pH, at 95°, is summarized in Figure 1. This figure was constructed by assuming that the rates of hydrolysis are equal to the sums of the rates of the uncatalyzed and acid-catalyzed reactions. The acid-catalyzed rates were extrapolated to 95° from data by Garrett *et al.* (1966).

From the rates of hydrolysis at 75, 85.5, and 95°, the enthalpies and entropies of activation for the uncatalyzed reactions were calculated, using the absolute rate equation, $K = (kT/h)e^{-H^{\ddagger}/RT}e\Delta S^{\ddagger}/R$. The values are, for deoxyuridine, $\Delta H^{\ddagger} = 32.1$ kcal/mol, $\Delta S^{\ddagger} = 8.7$ eu; for thymidine, $\Delta H^{\ddagger} = 34.5$ kcal/mol, $\Delta S^{\ddagger} = 3.5$ eu; and for 5-bromodeoxyuridine, $\Delta H^{\ddagger} = 32.4$ kcal/mol, $\Delta S^{\ddagger} = 10.3$ eu (entropies of activation evaluated at 75°.

The relative order of reactivity of the three nucleosides in the uncatalyzed hydrolysis was 5-bromodeoxy-

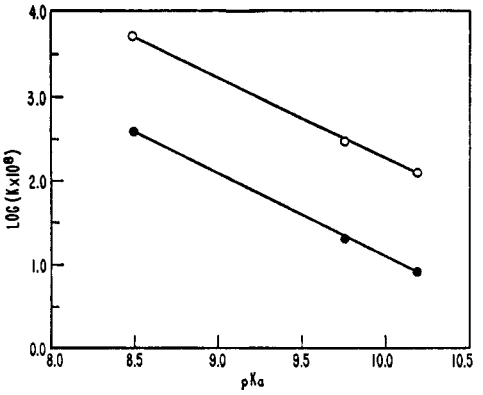
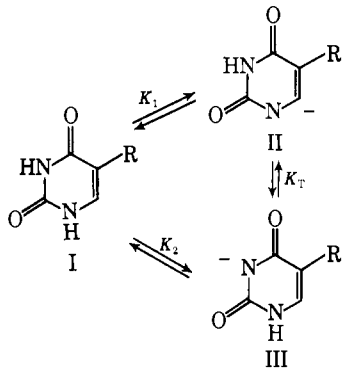


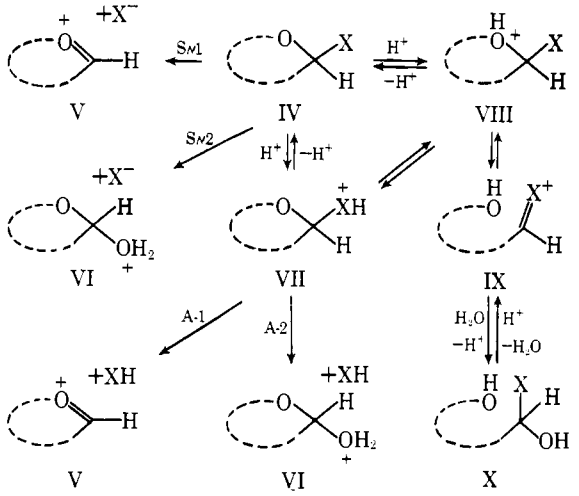
FIGURE 2: Plot of the logarithms of the rate constants for hydrolysis of deoxyuridine, thymidine, and 5-bromodeoxyuridine at 95° in 0.5 M citrate buffer (○) and 75° in H_2O (●) *vs.* the pK_a values for dissociation of the 1-hydrogens of the corresponding bases.

uridine > deoxyuridine > thymidine. It was desired to make a quantitative comparison between the hydrolysis rates of the deoxynucleosides and the dissociation constants for the 1-hydrogens of the corresponding bases. Dissociation constants, K_A, of 9.45 for uracil (Levine *et al.*, 1926), 9.87 for thymine (Wittenburg, 1966), and 8.05 for 5-bromouracil (Berens and Shugar, 1963) have been reported. These dissociations, however, are composites of the ionizations of both the 1- and 3-hydrogens (Nakanishi, *et al.*, 1961; Wempen and Fox, 1964; Wittenburg, 1966). The equilibria between the "1-anions" (II, and other contributing resonance structures) and the "3-anions" (III and other contributing resonance structures) have been determined. The



"1-anion" is present to the extent of 49% in uracil monoanion (Nakanishi *et al.*, 1961), 48% in thymine monoanion (Wittenburg, 1966), and 36% in 5-bromouracil monoanion (Wempen and Fox, 1964). Using this data and the relationships, $K_A = K_1 + K_2$, and K_T (tautomerism constant) = K_1/K_2, K_1 was calculated to be 9.76 for uracil, 10.19 for thymine, and 8.49 for 5-bromouracil. A plot of these values against the hydrolysis rates of the corresponding deoxyribosides in 0.5 M citrate buffer at 95°, pH 4.0, yielded a straight line (Figure 2). The objection may be raised that the dissociation constants for the bases were determined in aqueous solution at room temperature, and that they would be altered in a citrate buffer at 95°. However, the bases are

SCHEME I



structurally similar, and it seems likely that the dissociation constants of each would be altered to a similar extent by the change in conditions, leaving the relationship valid. To test this, the hydrolysis rates at 75° in H_2O were also plotted against the calculated dissociation constants for the 1-hydrogens (Figure 2). A linear relationship was again obtained.

Discussion

A number of the mechanistic paths available for the hydrolysis of glycosyl derivatives of sugars have been indicated in Scheme I. Examples of the operation of each of these mechanisms have been reported. The *O*-methyl and *O*-acylglycosyl halides (IV, X = halide) usually solvolyze *via* an Sn1 ionization, although in the latter case the situation may be complicated by neighboring group participation. If a strong nucleophile is present, in a poorly ionizing solvent, the displacement of halide can proceed by the alternative Sn2 path, to intermediate VI (Capon *et al.*, 1964; Vernon, 1967). The acid-catalyzed hydrolysis of the methyl and phenyl α- and β-D-glucopyranosides, and the corresponding thiopyranosides, involves the protonated species, VII (Bunton *et al.*, 1955; Banks *et al.*, 1961; Overend *et al.*, 1962; Bamford *et al.*, 1962). This dissociates *via* an A-1 process to afford the free alcohol or thiol and the same type of resonance-stabilized oxonium ion, V, that is formed in the Sn1 mechanism. The hydrolysis of several aldofuranosides, on the other hand, appears to involve an A-2 substitution on VII (Overend *et al.*, 1962; Capon and Thacker, 1967). Yet another pathway is followed by simple alkyl- and arylglycosylamines. Their hydrolysis in acid involves protonation of the sugar ring oxygen to give cation VIII, which then undergoes ring opening to give the immonium ion, IX. This, in turn, is attacked by water to form intermediate X, which subsequently expels the amine (Isbell and Frush, 1958; Capon and Connett, 1964; Simon and Palm, 1965).

The prevailing theory with respect to the mechanism of hydrolysis of purine and pyrimidine nucleosides ap-

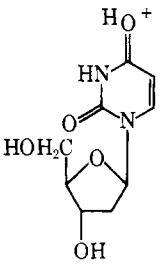
pears to be that of Kenner (1957) as modified by Dekker (1960). The nucleosides are considered to be analogous to the simpler glycosylamines, and the pathway involving intermediates VIII, IX, and X is preferred. In the event that initial protonation occurs upon the base to give VII, the ability of the base to transfer the proton directly to the sugar ring oxygen, to form VIII, is considered to be an important factor.

Our data indicate that deoxyuridine, thymidine, and 5-bromodeoxyuridine slowly hydrolyze to the free bases in the pH range 3–7, in an uncatalyzed reaction. The solvolysis proceeds much more rapidly in water than in ethanol and an ionic process, either SN1 or SN2, appears to be involved. Further evidence for this is provided by a study of the relative rates of hydrolysis of the three nucleosides. The ionization constants of the 1-hydrogens of the corresponding bases (I) were calculated from data available in the literature. The "1-anions" (II) produced by these dissociations are the same species liberated by the uncatalyzed hydrolyses of the nucleosides. A plot of the pK_A for the dissociations *vs.* the log of the rate constants for the hydrolyses is linear (Figure 2). This suggests that a Hammett-type free-energy relationship (Gould, 1959) exists for the two processes. The lability of 5-bromodeoxyuridine relative to thymidine in phosphate buffer (pH 6.8) had been noted earlier by Greer and Zamenhof (1962) and is accounted for by this relationship.

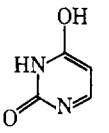
Only a few studies of the hydrolysis of deoxyuridine in alkaline solution were run. The extensive formation of dark brown decomposition products (presumably derived from the decomposition of free deoxyribose) made a more tedious procedure necessary. The data available indicate that the rate of hydrolysis falls off in the pH range 9–11. This is not surprising, as deoxyuridine ionizes its N-3 proton with a pK_A of 9.3. Thus the concentration of the neutral form of the nucleoside corresponding to IV would be decreased in alkaline solution.

The above data do not distinguish between the SN1 and SN2 possibilities for the reaction. Some information on this can be obtained by considering the entropies of activation for the reactions, which range from +3 to +10 eu for the three nucleosides. In studies of A-1 and A-2 hydrolyses of alkyl and aryl glycosides and SN1 and SN2 reactions of related systems, it was found that the unimolecular processes were usually associated with positive entropies of activation. Bimolecular reactions, on the other hand, had $\Delta S^{\ddagger}$ values about 20 eu lower, and these were usually negative in sign (Schaleger and Long, 1963; Overend *et al.*, 1962; Long *et al.*, 1957). On this basis it seems more likely that the uncatalyzed hydrolysis of deoxyuridine derivatives involves an SN1 rather than an SN2 mechanism.

Uracil derivatives accept a proton upon the 4-carbonyl group in strongly acidic solution (Beaven *et al.*, 1955; Sobell and Tomita, 1964). It is reasonable to assume that the deoxyribosides involved in this study will do so as well. In acidic solution, they will be converted in part into structure XI. An accelerated rate of hydrolysis in acid would therefore be expected, due to the greater ability of the neutral structure XII to act as a



XI



XII

leaving group, as compared with the learning ability of the monoanion II. This mechanism would correspond to the A-1 path, VII → V, in Scheme I. We indeed found that the hydrolysis of deoxyuridine at pH 1.9 was faster than could be accounted for by the uncatalyzed SN1 process. More extensive data about this acid-catalyzed hydrolysis of deoxyuridine derivatives have been compiled by other workers (Shapiro and Chargaff, 1957; Wacker and Trager, 1963; Pfitzner and Moffatt, 1964; Garrett *et al.*, 1966; Venner, 1966). The reaction rate has been found to be directly proportional to hydrogen ion concentration (Garrett *et al.*, 1966). These workers have preferred to discuss the acid-catalyzed hydrolysis in terms of the Kenner–Dekker proposals (VII → IX → X), rather than in terms of the A-1 path. The data at hand are not sufficient to unequivocally distinguish the two alternative paths, and it may be that both take place simultaneously. However, we feel that the evidence is more in accord with the A-1 route than with the one involving the immonium ion, IX.

The following order of reactivity has been shown for the acid-catalyzed hydrolysis: 5-bromodeoxyuridine > thymidine > deoxyuridine (Garrett *et al.*, 1966). This is readily understood in terms of an A-1 mechanism. It has been pointed out in connection with the A-1 hydrolyses of the phenyl α- and β-D-glucopyranosides that the substitution of an electron-attracting or -withdrawing group in the phenyl ring results in a composite effect, both enhancing and retarding the hydrolysis (Overend *et al.*, 1962). An electron-attracting group, for example, tends to lower the concentration of the protonated species, VII, in the solution, but enhances the rate of its decomposition to V. These effects cancel to some extent, so that no clear order of reactivity is apparent. It is more difficult to reconcile the observed reactivity order of deoxyuridine derivatives with an immonium ion mechanism. If the rate-determining step were the formation of the immonium ion, IX, one would expect that the substitution of an electron-withdrawing group on the base would retard the rate greatly, rather than enhance it, as is observed with 5-bromodeoxyuridine. Both the formation of the protonated species VIII and its rate of ring opening to IX would be retarded. If the rate-determining step were IX → X, or later, as is the case with the glucosides of *p*-nitroaniline and *p*-toluidine, the situation would become more complex, kinetically. However, the glucoside of *p*-toluidine still hydrolyzes more quickly than that of *p*-nitroaniline in all solutions of pH > 0 (Simon and Palm, 1965). In 0.5 M $HClO_4$, a Hammett ρ constant of −2.0 has been calculated for this reaction (Capon and Connett, 1964). Thus, the

kinetic details of the hydrolysis of deoxyuridine derivatives do not seem to resemble those of the hydrolysis of glucosylamines, where an immonium ion mechanism is followed. Finally, it should be mentioned that the glucosylamines show other reactions such as anomerization, rearrangement, and transglycosidation, which are compatible with the ready formation of an immonium ion (Isbell and Frush, 1958; Ellis and Honeyman, 1955). These reactions have never been observed, to our knowledge, in the hydrolysis of the *N*-glycosyl bond of uracil nucleosides.

The A-1 mechanism readily explains the relative ease of hydrolysis of uracil nucleosides in which the sugar residue varies. Thus, deoxyuridine is far more readily hydrolyzed in acid than uridine (Pfitzner and Moffatt, 1964). The retarding effect of a neighboring hydroxyl group upon a solvolysis in neutral or acidic solution is well known (Streitweiser, 1962). As would be expected, the 3′-hydroxyl group plays a lesser role in retarding the hydrolysis (Pfitzner and Moffatt, 1964; Kowollik and Langen, 1968).

The observed linear dependence of the hydrolysis rate in acid with the H^+ concentration is also compatible with an A-1 mechanism. The rate would depend upon the concentration of protonated nucleoside VII (=XI), which would be proportional to the term, $[H^+]/(K_A + [H^+])$, where K_A is the dissociation constant of the protonated nucleoside. If K_A were large relative to $[H^+]$, then the second term in the denominator could be neglected. Thus the A-1 mechanism appears to be compatible with all of the existing data on the acid-catalyzed hydrolysis of deoxyuridine derivatives. In order to establish this mechanism firmly, however, further kinetic studies will be needed, particularly in strongly acidic solution.

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