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Nucleic Acids. IX. The Structure and Chemistry of Uridine Photohydrate*

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ABSTRACT: The kinetics of the "photohydration" of uridine in H_2O, HDO, and D_2O have been determined as well as the extent of "heat reversibility" of the photoproducts.

The structure of "uridine hydrate" has been determined by proton magnetic resonance spectroscopy. Two isomers of 6-hydroxydihydrouridine and four isomers of 5-deuterio-6-hydroxydihydrouridine were observed when the reaction was carried out in water and deuterium oxide, respectively. Under the conditions of dehydration (*i.e.*, "heat reversal") C-5 protons or deuterium were exchanged more rapidly than the elimination of water or deuterium oxide. The mechanistic consequences of these observations are discussed.

The structure of uridine photohydrate and the chemistry of its formation and dehydration depend upon indirect evidence (Moore and Thompson, 1957; Wang, 1962; Wierzchowski and Shugar, 1961). We have examined samples of the photohydrate prepared in water and in deuterium oxide and have established the structure of the photohydrate (**1a,b**) and determined the course of the acid–base-catalyzed dehydration reaction by proton magnetic resonance spectroscopy. From these studies we are able to make conclusions as to the mechanism of the photoaddition to and elimination of water from substituted pyrimidines. These mechanistic pathways may also serve as models for the photochemistry of α,β-unsaturated carbonyl compounds.

Materials and Methods

Uridine (grade A, Calbiochem) was dissolved at 0.005 M in either glass-distilled H_2O or in D_2O (99.7%, New England Nuclear). A 100-ml sample was placed in a plastic pan (8.3 × 32.5 cm), the bottom of which was *ca.* 1.3 cm from the filters (Corning No. 9863) covering a Chromato-Vue Lamp (Ultraviolet Products, Inc.) containing two 25-W General Electric germicidal lamps. The use of this apparatus for the bulk preparation of photoproducts has been previously described (Smith and Aplin, 1966).

To ensure that the photohydrate was not destroyed by heat from the lamps over these long irradiation times, the plastic pan was maintained in an ice bath. The photolysis was monitored by following the loss in absorbance at 260 nm. When the absorbance had decreased to less than 10% of that at the start of the experiment, the sample was taken to dryness at room temperature in a rotary evaporator. The sample was then taken up in a minimum volume of D_2O for proton magnetic resonance spectroscopy.

The elimination reactions were carried out by heating solutions of the photoproducts in H_2O or D_2O in a boiling-water bath.

Results and Discussion

From earlier work on the acid–base-catalyzed exchange of H5 of uridine in water (Wechter and Kelly, 1968; Wechter, 1969), it was evident particularly with *ara*-uridine (and *ara*-cytidine) that hydroxyl groups of the sugar portion of the nucleoside participate to form cyclo-Michael addition products as intermediates A. Further, in the case of uridine itself species B as well as C is an intermediate in the exchange reaction especially in strong base (Wechter and Kelly, 1967; Wechter, 1969). Thus we hypothesized that uridine photohydrate, heretofore (Moore and Thompson, 1957; Wang, 1962; Wierzchowski and Shugar, 1961) assigned structure C might be the anhydronucleoside B containing a mole of water not covalently bound. It also appeared important to determine whether the hydrate was a C-5- rather than a C-6-oxygenated species. Analysis of the proton magnetic resonance spectra of the photoproduct of uridine prepared in water (Figure 1) led to the unequivocal assignment of the pair of epimers **1a** and **1b** as the principal photoproducts. Attempts to separate the 60:40 (approximate) mixture of diastereomers by crystallization have been unsuccessful to date. Neither the cyclonucleoside B nor the 5-hydroxylated species could be detected in the crude photoproduct by proton magnetic resonance spectroscopy. The limit of detection is about 5%. The gross structure of cytidine hydrate was recently confirmed by Miller and Cerutti (1968) by chemical degradation. These authors could not, however, distinguish the diastereomers.

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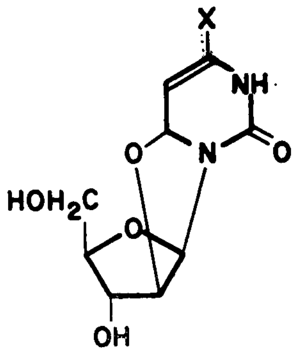
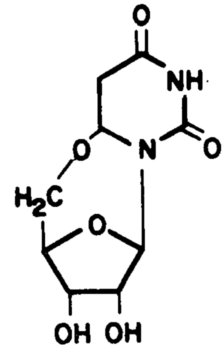
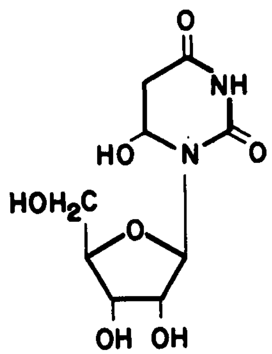
[1] D. Santi, private communication.



A, X = OH, NH_2

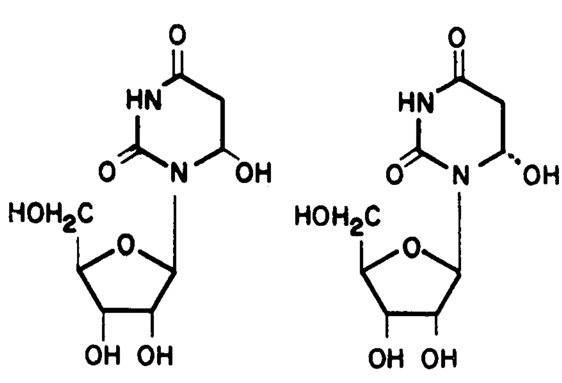


B



C

The assignment of chemical shifts of the H5, H6, H1′, and 5′ protons of **1a** and **1b** was based on dihydrouridine (**2**) as a model and all proton magnetic resonance data are summarized in Table I. The H5a, H5b, and H6 protons exhibited a typical ABX pattern. The presence ot two diastereomers was indicated by a doubling of the ABX (H5a, H5b, and H6) and H1′ patterns owing to small differences in chemical shifts of the two epimers. The magnetic nonequivalence of these protons results from molecular assymmetry (Martin and Martin, 1966). Thus the set of H1′ doublets at δ 5.73 and 5.69 and the sets of AB (H5a and H5b) resonances (see Figure 1) result from assymmetry at C-6. On the other hand, the overlapping quartets at *ca.* δ 5.5 appear as a quintet (*ca.* 1, 2, 2, 2, and 1) due to assymmetry at H1′. That the chemical shifts of the H5, H5′, and H1′ protons and coupling constants of the latter two are essentially the same as those protons in dihydrouridine (**2**) is in complete agreement with structures **1a** and **1b** and inconsistent with a 5-hydroxylated species. The assignment of the H5 protons was confirmed by their ready exchange in hot water or deuterium oxide (see below). We



Ia, b

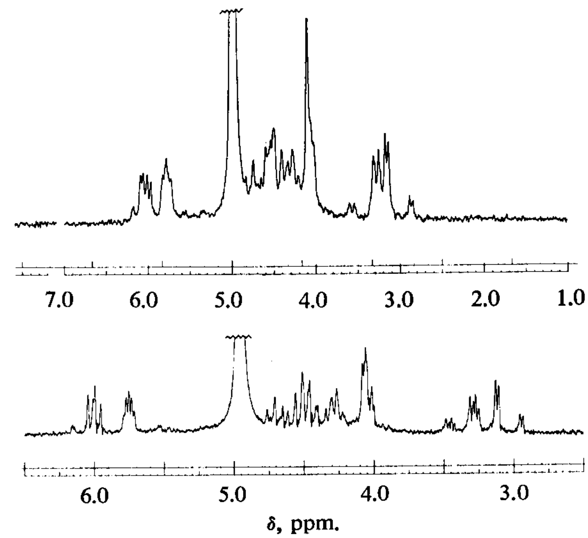


FIGURE 1: Proton magnetic resonance spectra of 6-hydroxydihydrouridine. The upper spectrum was measured at 60 MHz and the lower curve at 100 MHz on Varian spectrometers. Hexamethyldisilazane was used as an external standard but the data in Table I are corrected to external dimethylsilapentanesulfonate (conversion factor 0.25 ppm).

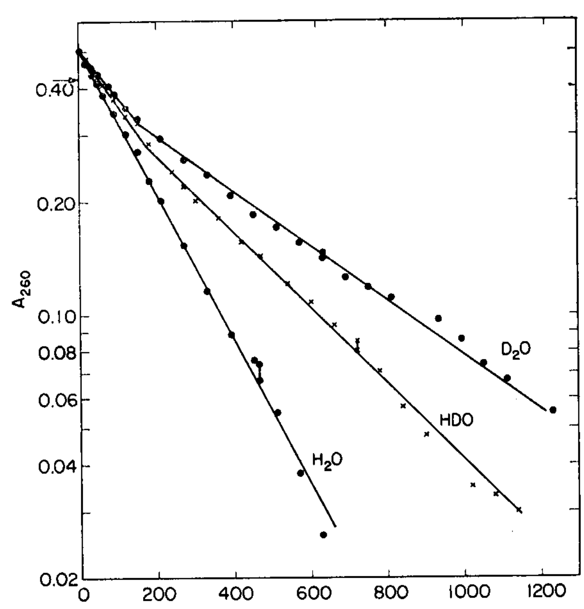
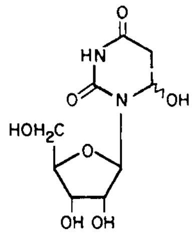
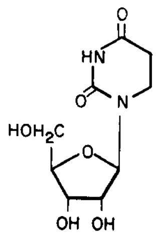
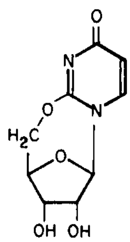
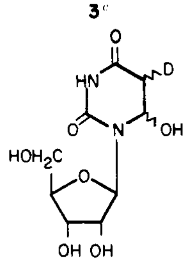


FIGURE 2: The loss of absorbance of ultraviolet-irradiated solutions of uridine. Uridine at 0.005 M in H_2O, HDO, or D_2O was irradiated (2537 Å) and the change in absorbance at 260 nm was determined in a Beckman DU spectrophotometer.

TABLE I: Proton Magnetic Resonance Data for Uridine Analogs.[a]

	H6	H5a	H5b	H1′	H5′	Isomer Rel %
 **1**	(a) $\sim$5.5	$\sim$3.1	$\sim$2.8	5.73 (d, 5)	$\sim$3.8 (m)	60
	(b) $\sim$5.5	$\sim$3.0	$\sim$2.8	5.69 (d, 5)	$\sim$3.8	40
 **2**[b]	3.53 (t, 6–7)	2.75 (t, 6–7)		5.78 (d, $\sim$5)	3.7 (m)	
 **3**[c]	8.08	6.20		5.72 (d, <1)	4.25 (m)	
 **4**	(a, b) $\sim$5.5 (m)	3.1 (m)	D	5.73 (d, 5)	3.8 (m)	60
	(c, d) $\sim$5.5 (m)	D	2.75 (m)	5.69 (d, 5)	3.8 (m)	40

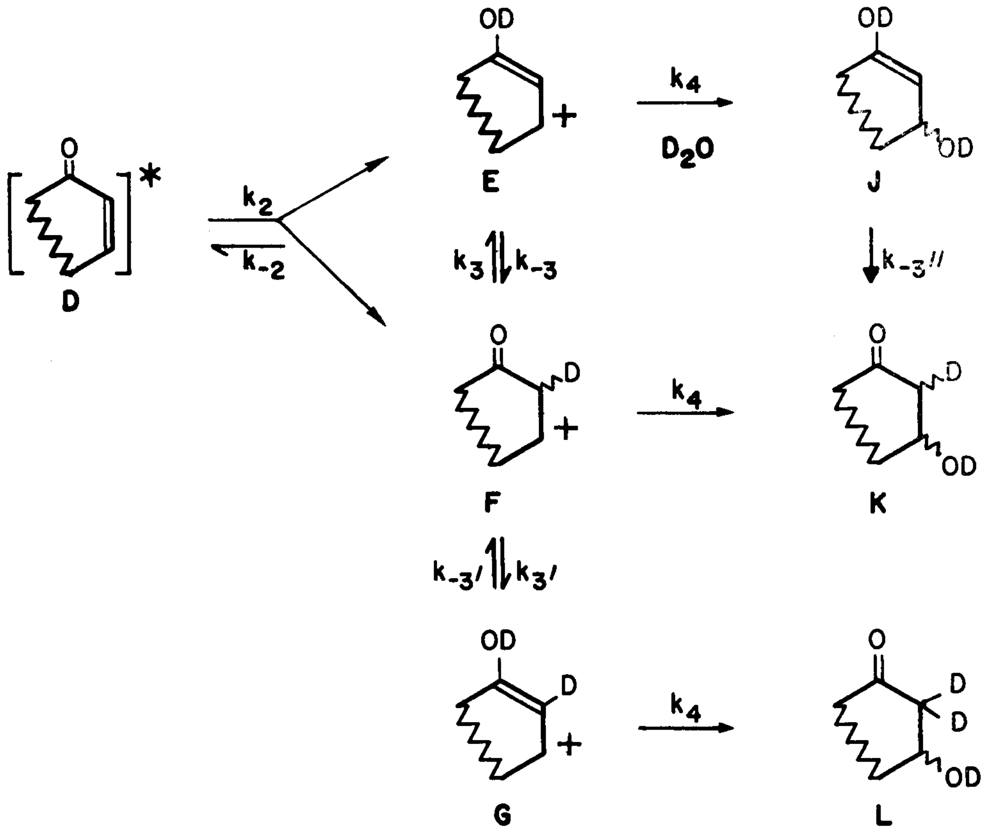
[a] Proton magnetic resonance spectra were measured in D_2O (except where noted) on Varian spectrophotometers at both 60 and 100 MHz and chemical shifts (δ) were recorded in parts per million downfield from external dimethylsilapentanesulfonate (line multiplicities and coupling constants, in cycles per second, appear in parentheses). [b] Data of Hanze (1967). [c] Spectrum run in dimethylformamide-d_7–D_2O. This compound was a gift of Dr. A. R. Hanze of The Upjohn Co.

were also able to exclude the cyclonucleoside B on the basis of the proton magnetic resonance spectrum of model compound **3** (Table I). This compound exhibited both the expected downfield shift of the C-5 methylene protons (−0.5 proton magnetic resonance) and a reduction of the $J_{1,2}$ coupling to <1 cps owing to the stereochemical reorganization of the fused-ring system. While this model is not ideal, we concluded from our study of molecular models that it would predict the $J_{1,2}$ coupling.

When uridine was irradiated in deuterium oxide (99.7%), stable photohydrates were again formed. The rate of the photoaddition was much slower in deuterium oxide (Figure 2) as noted earlier (Wierzchowski and Shugar, 1961), leading to a greater proportion of other photochemical products of the nucleoside. Thus, on heating, only $\sim$70% of the absorbance at 260 nm could be regenerated as opposed to $\sim$90% in the case of the "water hydrate," **1a,b**.

We also found that the rate of photolysis of uridine in HDO was intermediate between that of water and deuterium oxide (Figure 2). In both experiments involving isotopic water a break in the curve can be noted at about 20% loss of absorbance. This break is not evident in the normal water experiment. We are unable to account for the break in the rate curves but presently assume that it results from a change in pD of the unbuffered solution

SCHEME I



resulting from basic photolysis products of the nucleoside. We have attempted to characterize by proton magnetic resonance spectroscopy the product(s) present after 20% photolysis but its insolubility after removal of the solvent has thwarted our efforts to date.

The proton magnetic resonance spectrum of the product of photolysis in D_2O revealed that the principal photoproduct was a 60:40 (approximately) mixture of two sets of diastereomers (four isomers in all) of 5-d_1-6-hydroxydihydrouridine (**4a,b** and **4c,d**).

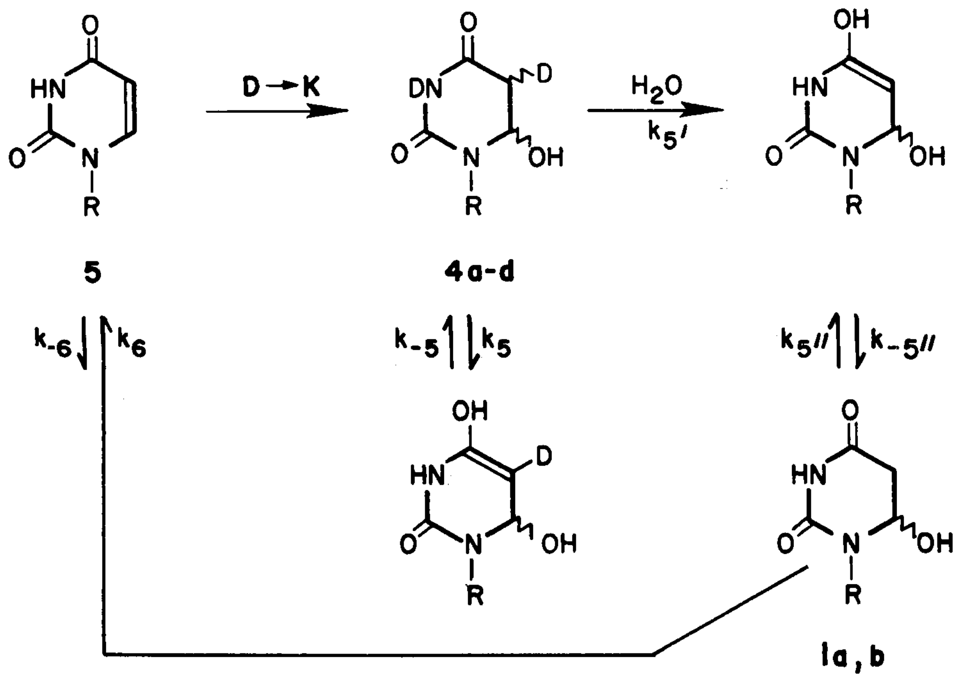
The addition of D_2O was not stereospecific (*i.e.*, either *cis* or *trans*), yet involved the addition of a single proton (deuterium). The product (**4a–d**) was stable under the reaction conditions (*i.e.*, compounds **4a–d** were stable toward further photolysis in H_2O). The proton magnetic resonance spectrum of the photohydrate produced in D_2O was simpler than that of **1a,b** owing to the replacement of one AB proton (H5a or H5b) by deuterium. The chemical shifts of all protons were identical with those of **1a,b** except for the fact that each new AX-deuterium system lacked one resonance (H5a or H5b) in each diastereomeric pair, **4a,b** and **4c,d**, and like **1a** and **1b** were present in *ca.* 60:40 ratio. The pairs **4a,b** and **4c,d** obviously each represent a single configuration at C-6 (*i.e.*, each set contains one *erythro* and one *threo isomer*) and a 1:1 mixture of epimers at C-5 since the approximate 60:40 ratio was maintained both in the H5 and H1′ protons. When the photolysis was carried out in HDO, we not only realized an intermediate rate of hydrate formation, but also that the product was largely undeuterated at C-5. This is consistent with the difference in autoprotolysis of H_2O and of D_2O (Wiberg, 1955).

When the acid–base-catalyzed elimination reaction (Wierzchowski and Shugar, 1961) was carried out by heating the mixed isomers **4a–d**, a rapid exchange, compared with the elimination reaction, of the C-5 deuterium was seen by monitoring the reaction course by proton magnetic resonance spectroscopy. Thus **4a–d** on heating in water yielded a proton magnetic resonance spectrum identical with that of **1a,b** (confirming the 60:40 ratio of isomers about C-6). Thus, the rapid exchange reaction predicted from earlier solvent-exchange studies (Wechter and Kelly, 1967; Wechter, 1969) in which **1a,b** is the intermediate for H5 exchange in uridine was realized. As noted above, this exchange also confirms the assignment of the chemical shift of the H5 protons. The exchange reaction was followed by a slower elimination reaction to give almost exclusively unlabeled uridine (**5**).

Mechanisms

From the work of Burr *et al.* (1968) the first chemical step in the mechanism of uracil photohydration appears to be protonation of the excited singlet state. Furthermore, the work of Marshall and Wurth (1967) indicates that the photochemical addition of alcohols to olefin triplet excited states also involves protonation followed by solvolysis of the carbonium ion thus produced. Reasoning from the conclusion of Burr *et al.* (1968), the close analogy of the work of Marshall and Wurth (1967) and the observed nonstereospecific addi-

SCHEME II



tion of a single deuterium, we propose the following sequence, consistent with our findings for such α,β-unsaturated carbonyl systems. Protonation of the singlet D may take place either on oxygen to give E or on C-5 to give F. If protonation is on oxygen then k_3 must be a very slow reaction compared with k_{-2} ($k_3 > k_{-2}$ and k_3/k_{-3} very small) as only one and not both C-5 hydrogens are exchanged in the deuterated media (*i.e.*, species L is not formed in detectable amounts; Scheme I).

Since in the excited-state singlet D carbon is probably more electronegative than oxygen, we favor the pathway E → F → K with the alternative D → E → J → K being significant if k_{-3} is similar to k_4. This pathway is quite different from that proposed by Wierzchowski and Shugar in 1961 yet is consistent with the isotope effect $k_{H_2O}/k_{D_2O} = 2.2$ found by these workers and confirmed by us (Figure 2). This isotope effect is consistent with k_{-2} being rate determining since neither k_4 nor k_{-3} should be affected by the higher activation energy of ionization of D_2O (Wiberg, 1955) (*i.e.*, lower autoprotolysis constant). Further, the finding of Burr *et al.* (1968) that the rate was some function of pH correlates well with this conclusion.

It is possible that in the photolysis of nucleotides and oligonucleotides that the very nucleophilic phosphate anion may substitute for water in the solvolysis of the carbonium ion intermediate, E or F, to yield photohydrates in which the substitutent at C-6 is phosphate, yielding phosphodi- or triesters, respectively. Such a reaction might help to explain some of the intermediates observed by Helleiner *et al.* (1963) and Brown *et al.* (1966) in the photolysis of uridylyl-(3′-5′)-uridine.

The elimination of water would appear to follow the following path consistent with what has been concluded about this system. For the case of the deuterated products **4a–d** there is a rapid exchange of D-5 ($k_{5'}$). This reaction was observed to be much faster than the elimination reaction, k_6, which is probably an E-2 elimination. This point has been reported (Chambers, 1968) subsequent to the completion of our work. That the exchange must be fast compared with the elimination follows from our earlier work on solvent exchange (Wechter and Kelly, 1967; Wechter, 1969). In this work k_{-6} was evaluated and found to be negligible under these conditions. For reactions, see Scheme II.

The earlier workers (Wierzchowski and Shugar, 1961) studying this reaction were not able to observe the exchange reaction employing only ultraviolet spectroscopy as an analytical tool. They did, however, note deuterium isotope effects in the elimination reaction. They observed (at pH or pD 1.6 and 70°) that the hydrate prepared in water to which we now assign structures **1a,b** eliminated about twice as fast in deuterium oxide as it did in water. This is consistent with our mechanism involving first rapid exchange to give **1a,b** in which both C-5 substituents were either hydrogen or deuterium. On the other hand, Wierzchowski and Shugar (1961) reported that the hydrate prepared in heavy water, to which we now assign structures **4a–d**, was slower (by a factor of 2) than **1a,b** when their elimination rates were compared in heavy water. This fact is not consistent with either our work or that of Chambers (1968) since in heavy water both **1a,b** and **4a–d** should exchange to give largely the 5,5-dideuteriohydrate before elimination. The suggestion by Chambers that the rapid exchange of uridine hydrate might be applied as an analytical technique for tRNA is not compatible with known proton-exchange reactions for guanosine, adenosine, and cytidine. These nucleosides would be expected to exchange (Wechter and Kelly, 1967; Wechter, 1969) under mild conditions. One would thus have to degrade the macromolecule and look at the uridine fraction individually.

Acknowledgment

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Added in Proof

We recently became aware of similar work on tritium labeling of the 5′-CH_2 by Nossen and Froholan (1967).

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