Photochemistry of Cytosine Derivatives. 2. Photohydration of Cytosine Derivatives. Proton Magnetic Resonance Study on the Chemical Structure and Property of Photohydrates†

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ABSTRACT: Photohydrates of cytidine and cytidylic acids have been definitively characterized to be isomeric 6-hydroxy-5,6-dihydrocytosine derivatives. It has also been demonstrated by nuclear magnetic resonance spectroscopy that (1) the stereochemistry of photohydration is random, (2) the C_5-H trans to the C_6-OH undergoes a rapid selective exchange in the presence of proton acids, and (3) the dehydration of photohydrates is a trans-elimination. The mechanism of these processes is discussed.

The photohydration of pyrimidines plays an important role in the photochemistry and photobiology of nucleic acids (Fisher & Johns, 1976; Patrick & Rahn, 1976). Photohydration of uridine has been correlated with ultraviolet inactivation of RNA (Remsen et al., 1970). The formation of photohydrate of cytidine units in polynucleotides and DNA has been demonstrated (Vandehoek & Cerutti, 1973; Grossman & Rogers, 1968) and related to the mutagenic effect of UV irradiation (Grossman, 1968). It has been suggested that cytosine photohydrate formed in the UV-irradiated poly(rC) may be recognized in a RNA polymerase system as uracil or thymine (Ono et al., 1965; however, see Singer & Fraenkel-Conrat, 1970). Chemically, the photohydration of pyrimidines is thermally reversible; i.e., photohydrates of pyrimidines undergo dehydration and revert back to pyrimidines under relatively mild experimental conditions.

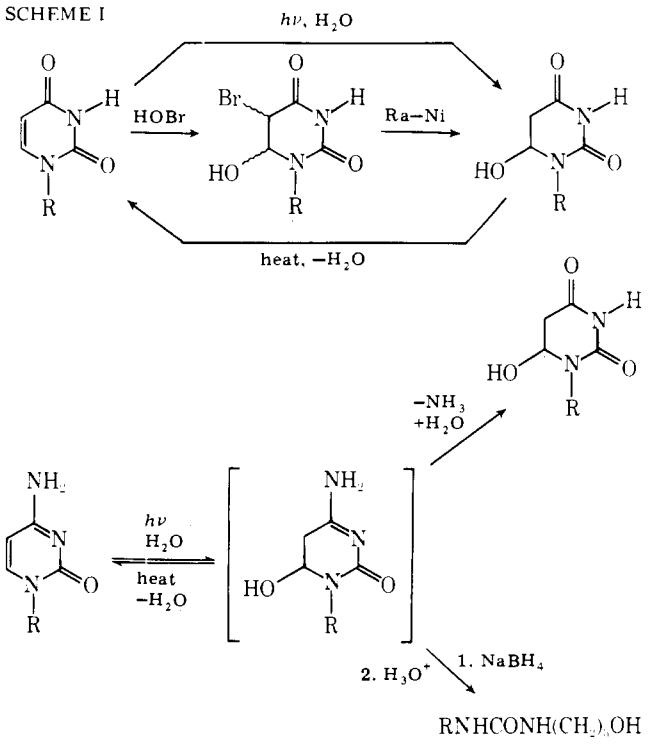
The structures of photohydrates of uracil and its derivatives have been established unambiguously to be 6-hydroxy-5,6-dihydrouracils by chemical synthesis (Wang et al., 1956; Moore & Thomson, 1957; Moore, 1958; Gattner & Fahr, 1963; Ducolomb et al., 1976), by their reactions with sodium borohydride (Miller & Cerutti, 1968), and by NMR spectroscopy (Wechter & Smith, 1968; Ducolomb et al., 1976). However, photohydrates of cytosine and its derivatives are considerably less stable than their uracil analogues. Their half-lives are estimated to be only minutes under ordinary experimental conditions (DeBoer et al., 1970). Fahr and his co-workers had attempted to synthesize photohydrates of cytosine and its derivatives, but the products obtained were either cytosines or mixtures containing cytosines. They were able to demonstrate, however, that one of the products in their attempted synthesis of 6-hydroxy-5,6-dihydrocytidylic acid has the same R_f value as the photoproduct formed from the irradiation of cytidylic acid (Kleber et al., 1965; Fahr et al., 1966). Although the structures of photohydrates of cytosine and its derivatives have been inferred indirectly to be 6-hydroxy-5,6-dihydrocytosines by their borohydride reduction in situ (Miller & Cerutti, 1968) and by their deamination to uracil hydrate derivatives (Johns et al., 1965), there has been no direct characterization of photohydrates of their structures (Scheme I). We wish to report in this communication the characterization of photohydrates of cytosine derivatives by high resolution NMR spectroscopy (270 MHz) and some of their chemical reactions.

Experimental Section

Materials. Analytically pure nucleosides and nucleotides were purchased from commercial sources. Uridine 5′-phosphate (sodium), uridine 2′-phosphate (lithium), 2′-deoxyuridine 5′-phosphate (sodium), cytidine 3′-phosphate (lithium), and cytidine-5′-phosphate (sodium) were from P-L Biochemicals. Cytidine was from Nutritional Biochemicals, and uridine was from Aldrich. They were used directly without purification.

Nuclear Magnetic Resonance. NMR spectra (CW and FT

SCHEME I



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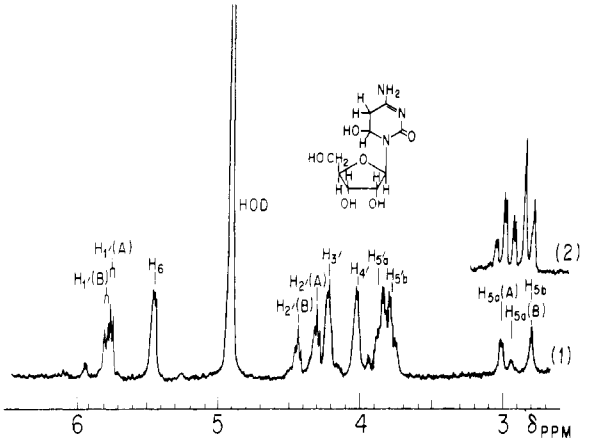


FIGURE 1: NMR spectrum (270 MHz) of 6-hydroxy-5,6-dihydrocytidine in D_2O. (1) FT-NMR spectrum of a sample irradiated in D_2O. (2) Spectrum taken in the CW mode for a sample irradiated in H_2O.

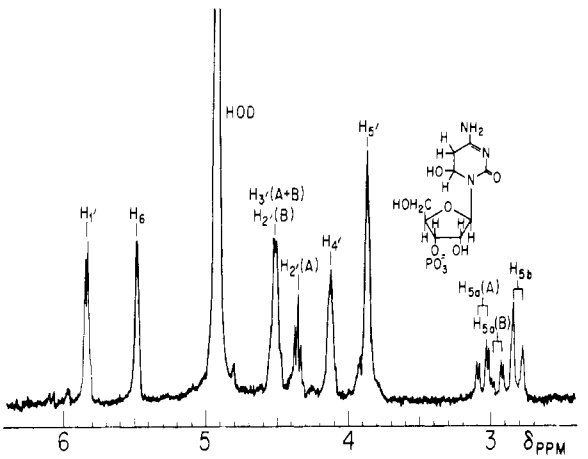


FIGURE 2: FT-NMR spectrum (270 MHz) of 6-hydroxy-5,6-dihydrocytidine 3'-phosphate in D_2O.

TABLE I: Irradiation Condition in the Preparation of Photohydrates.

compounds	concn (M)	pH	irradiation time (h)
cytidine (**1a**)	0.02	6.7	1½
cytidine 3'-phosphate (**1b**, lithium)	0.02	8.5	1⅔
cytidine 5'-phosphate (**1c**, sodium)	0.02	7.7	2
uridine (**3a**)	0.01	5.7	1
uridine 3'-phosphate (**3b**, lithium)	0.005	7.6	⅔
uridine 5'-phosphate (**3c**, sodium)	0.005	8.1	⅔

mode) were recorded on a Bruker HS-270 spectrophotometer (270 MHz) as described in the preceding paper (Liu & Yang, 1978). For spectra taken in CW mode, TSP was used as the lock and reference signals. For FT-NMR spectra, deuterium resonance of D_2O was used as the lock signal. The pulse duration time was 24 μs and the delay time was 1 s.

Preparative Irradiation. Solutions of nucleosides or nucleotides in 30 mL of unbuffered distilled water were irradiated. The irradiation apparatus consists of a 450-W Hanovia medium pressure mercury lamp in a water-cooled quartz immersion well fitted with a Vycor filter sleeve and a Pyrex outer-jacket to contain the irradiation solution (optical path 2 mm). The whole assembly was immersed in a low temperature bath at 0 °C. The solutions were deaerated and agitated by ebullition with nitrogen. The irradiations were monitored by the loss of absorbance of the starting material at 260–265 nm and were discontinued when most of the starting material was consumed. The concentrations and pHs of irradiation solutions and the irradiation time are listed in Table I. The irradiated solutions were lyophilized (for cytidine and cytidylic acids, 5 mL of aliquots was lyophilized). Samples were then dissolved in 0.5 mL of D_2O for NMR studies.

Photohydrates of Cytidine and Cytidine 5'-Phosphate for FT-NMR Studies. Cytidine (**1a**) or cytidine 5'-phosphate (**1c**, sodium salt) was dissolved in D_2O (Aldrich, 99.8 atom %) and lyophilized. This process was repeated once in order to exchange all the exchangeable protons to deuterium. The residue was then dissolved in D_2O (Aldrich, 100 atom %) to a final concentration of 0.01 M. The solution (0.4 mL) was irradiated in a quartz UV cell (1 mm light path) attached to the above-mentioned immersion well by rubber bands. After 1.33 h (or 3 h for cytidine 5'-phosphate), only a shoulder remained at 270 nm in the UV spectrum. The irradiated solution was immediately transferred to an NMR tube (5 mm) and the NMR spectrum was taken in the FT mode by the accumulation of 200 scans.

Photohydrate of Cytidine 3'-Phosphate for FT-NMR Studies. A solution of cytidine 3'-phosphate (**1b**, lithium salt) in 30 mL of nonbuffered distilled water (0.01 M) was irradiated in the same manner as the preparative irradiations for 1.5 h. Two aliquots (0.6 mL each) of irradiated solution were lyophilized, 0.5 mL of D_2O was added, and the solution was again lyophilized. The residues were combined and dissolved in 0.4 mL of D_2O and ^1H FT-NMR spectrum was obtained by the accumulation of 50 scans.

Quantum Yield Determination. The determination of quantum yield of photohydration of cytidine 3'-photophate was performed on a conventional "merry-go-round" apparatus. A low-pressure mercury lamp was used as the light source to provide the 253.7-nm light, and the ferrioxalate actinometry was used to determine the intensity of the incident light, which was found to be 3.17×10^{-7} einstein/(min sample). The irradiation tubes were made from a single length of quartz tubing of uniform o.d. and 7.5 mm i.d. Each tube was sealed through a graded seal to an Ace Teflon right-angle valve. The volume of samples used was 1.5 mL.

The samples to be irradiated were 0.01 M phosphate buffer solutions (pH 7.1) containing 7×10^{-4} M in cytidine 3'-phosphate. All samples were purged with argon for 1 h before irradiation. The progress of the reaction was monitored by UV absorbance at 270 nm (1-mm quartz cell).

Results and Discussion

After solutions of cytidine (**1a**) and cytidine 5'-phosphate (**1c**, sodium salt) in D_2O (0.01 M) were irradiated at 0 °C, more than 95% of the starting materials was consumed in each case. NMR spectra of irradiated solution were taken in the FT mode at 270 MHz. Spectra corresponding to cytidine "photohydrate" (**2a**, Figure 1) and cytidine 5'-phosphate "photohydrate" (**2c**, sodium salt, Figure 2) of higher than 90% purity were obtained. Proton FT-NMR spectrum (Figure 3) of cytidine 3'-phosphate "photohydrate" (**2b**, lithium salt) of similar purity was obtained from a sample originally irradiated in H_2O followed by exchange with D_2O. NMR spectra (CW mode) were also obtained from photohydrates of 60–80% purities prepared from the preparative irradiations as described in the Experimental Section. Since pertinent peaks are well resolved, these spectra are good enough for the study of isotope exchange at C_5 and spin-decoupling experiments as discussed below.

TABLE II: Chemical Shifts (ppm from Internal Standard TSP) and Coupling Constant (Hz) of Dihydropyrimidine Protons of Photohydrates **2** and **4** (Unbuffered D_2O, 20 ± 1 °C).

photohydrate of		H_6	H_{5a}	H_{5b}
uridine (**4a**)	A	5.53 (br s)[a]	3.13 (dd 4.1, 17.3)	2.81 (br d, 17.3)
A:B = 3:2	B		3.10 (dd 4.1, 17.3)	
uridine 3′-phosphate (**4b**)	A	5.56 (br s)	3.16 (dd 4.1, 17.4)	2.82 (td 2.2, 17.4)
A:B = 2:1	B		3.10 (dd 4.1, 17.4)	
uridine 5′-phosphate (**4c**)	A	5.70 (br s)[b]	3.14 (dd 4.1, 17.3)	2.86 (br d, 17.3)
A:B = 7:6	B	5.80 (br s)[b]	3.09 (dd 4.1, 17.3)	
cytidine (**2a**)	A	5.47 (m)[c]	3.02 (dd 3.9, 18.0)	2.81 (br d, 18.0)
A:B = 3:2	B		2.95 (dd 3.9, 18.0)	
cytidine 3′-phosphate (**2b**)	A	5.49 (br s)	3.05 (dd 4.2, 17.4)	2.82 (d, 17.4)
A:B = 7:5	B		2.95 (dd 4.2, 17.4)	
cytidine 5′-phosphate (**2c**)	A	5.63 (br s)[d]	3.00 (dd 4.5, 17.1)	2.85 (d, 17.1)
A:B = 3:2	B	5.74 (br s)[d]	2.94 (dd 4.5, 17.1)	

[a] Two singlets after both H_5's exchanged to D. [b] Well-resolved doublet of doublets in 0.1 M DCl (J_{H_{5a},H_6} = 4.1 Hz, J_{H_{5b},H_6} = 1.9 Hz). [c] Two doublets after H_{5a} exchanged completely to D (J_{H_{5b},H_6} = 1.9 Hz). [d] A doublet after H_{5a} exchanged completely to D ($J_{H_6,H_{5b}}$ = 1.9 Hz).

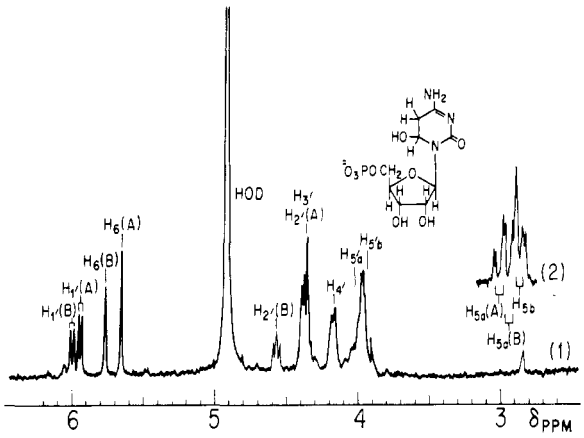


FIGURE 3: NMR spectrum (270 MHz) of 6-hydroxy-5,6-dihydrocytidine 5′-phosphate in D_2O. (1) FT-NMR spectrum of a sample irradiated in D_2O. (2) Spectrum taken in the CW mode for a sample irradiated in H_2O.

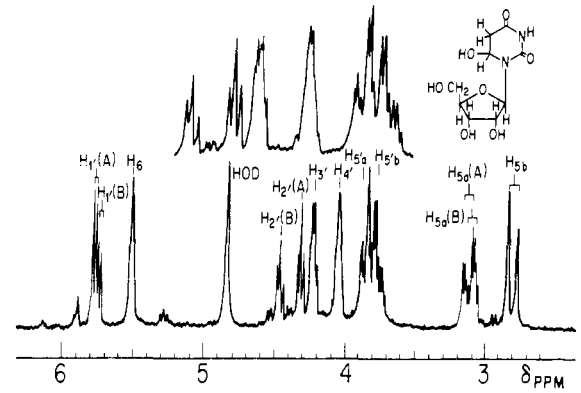


FIGURE 4: NMR spectrum (270 MHz) of 6-hydroxy-5,6-dihydrouridine in D_2O. The upper spectrum is the expansion of the region δ 3.5-4.5 ppm.

Since photohydrates of uracil derivatives are more stable, the 270-MHz NMR spectra of photohydrates of uridine (**4a**, Figure 4), uridine 3′-phosphate (**4b**, lithium salt), and uridine 5′-phosphate (**4c**, sodium salt) of 95% purities were obtained from preparative irradiation for comparison purposes.

NMR Spectra of Photohydrates. The proton assignments were based on the knowledge of chemical shifts of reference compounds and hyperfine splitting patterns and were verified by spin decoupling experiments as exemplified by the following discussion on the uridine photohydrate (**4a**).

NMR spectrum (270 MHz) of uridine photohydrate (**4a**, Figure 4) exhibits a broad singlet at δ 5.53. It also exhibits a broad doublet at δ 2.81 (J = 17.3 Hz) which is coupled to two overlapping doublet of doublets at δ 3.13 and 3.10 (J = 17.3 and 4.1 Hz). Irradiation at δ 5.53 sharpens the former and collapses the latter to two doublets. Therefore, the presence of two sets of ABX protons is evident indicating the presence of two isomers. The signal at lower field (X) is assigned to the H_6 proton and the signals at higher field (A and B) are assigned to the H_5 protons. The H_5 proton at lower field (H_{5a}) is subsequently assigned to the proton trans to C_6-OH and the one at higher field (H_{5b}) cis to C_6-OH (vide infra).

Spectrum of **4a** also exhibits two doublets at δ 5.79 and 5.76 with an integration of 0.6 proton and 0.4 proton, respectively, which are assigned to the $H_{1'}$ proton of two isomers in a ratio of 6:4. Irradiation at δ 5.79 and δ 5.76 collapses the triplets at δ 4.32 and δ 4.48, respectively, to doublets. The latter signals are therefore assigned to the $H_{2'}$ proton. Since the chemical shift of the $H_{2'}$ proton of dihydrouridine is at δ 4.30 (Deslauriers et al., 1971), the $H_{2'}$ proton of one isomer is deshielded by 0.16 ppm downfield to that of the other isomer. There are also a triplet of AB quartets centered at δ 3.82, which is assigned to the $H_{5'}$ protons, and two multiplets at δ 4.05 and 4.23, which are assigned to the $H_{4'}$ and $H_{3'}$ proton, respectively.

Recently, the syntheses of both diastereomers of (+)- and (−)-6-hydroxy-5,6-dihydrouridine have been achieved (Ducolomb et al., 1976) by the reduction of trans-(+)- and -(−)-

TABLE III: Chemical Shifts (ppm from Internal Standard TSP) and Coupling Constants (Hz) of Ribose Ring Protons of **2** and **4** (Unbuffered D_2O, 20 ± 1 °C).

photohydrate of		$H_{1'}$	$H_{2'}$	$H_{3'}$	$H_{4'}$	$H_{5'}$
uridine (**4a**)	A	5.79 (d, 6)	4.32 (t, 5.7)	4.23 (m)	4.05 (m)	3.82 (m)
	B	5.76 (d, 6)	4.48 (t, 5.7)			
uridine 3′-phosphate (**4b**)	A	5.83 (d, 5.4)	4.38 (t, 5.4)	4.54 (m)	4.18 (m)	3.87 (m)
	B	5.78 (d, 4.8)	4.54[a]			
uridine 5′-phosphate (**4c**)	A	5.90 (d, 7.0)	4.43 (t, 6.8)	4.37 (m)	4.19 (m)	3.97 (m)
	B	5.91 (d, 6.9)	4.59 (t, 6.8)			
cytidine[b] (**2a**)	A	5.78 (d, 5.8)	4.32 (t, 6.0)	4.23 (m)	4.03 (m)	3.82 (m)
	B	5.81 (d, 6.0)	4.44 (t, 6.0)			
cytidine 3′-phosphate[b] (**2b**)	A	5.84 (d, 5.4)[c]	4.37 (t, 5.7)	4.52 (m)	4.14 (m)	3.87 (m)
	B		4.52[a]			
cytidine 5′-phosphate (**2c**)	A	5.94 (d, 6.7)	4.36[a]	4.36 (m)	4.17 (m)	3.97 (m)
	B	5.99 (d, 6.9)	4.57 (t, 6.9)			

[a] Overlaps with $H_{3'}$ signal. [b] FT-NMR. [c] Two doublets in 0.1 M DCl.

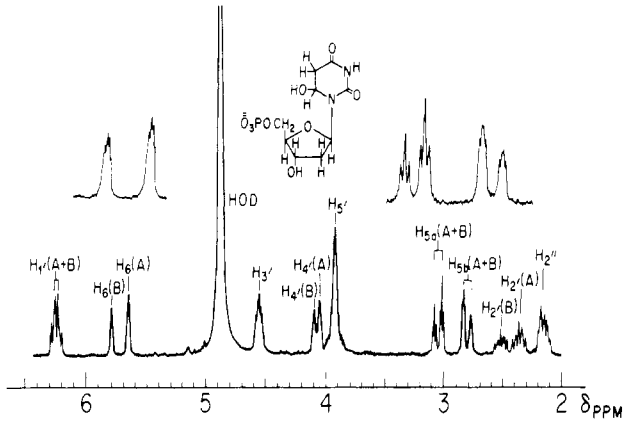


FIGURE 5: NMR spectrum (270 MHz) of 6-hydroxy-5,6-dihydro-2′-deoxyuridine 5′-phosphate in D_2O. The upper spectrum is the expansion of signals for the H_5 and H_6 protons.

iodohydrins, respectively. Our spectra of uridine photohydrates and the sum of the NMR spectra (250 MHz) of two diastereomers are in complete agreement, indicating that two isomers A and B are two diastereomers with a difference in the stereochemistry at C_6.

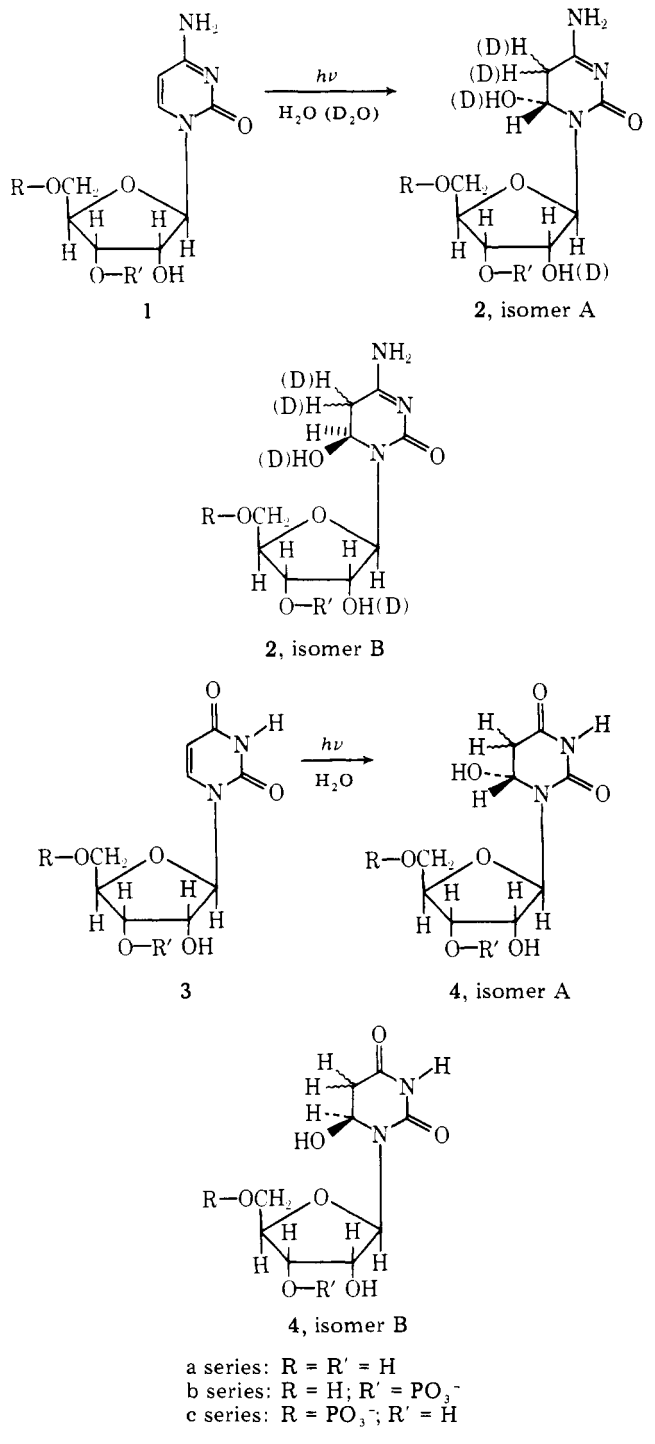
The NMR spectrum of cytidine photohydrate (Figure 1) closely resembles that of uridine photohydrate in every aspect, and this indicates the structural similarity between these two compounds. Such spectral resemblance also exists among the corresponding nucleotides; therefore, all spectra were analyzed in the same manner. The chemical shifts and coupling constants are summarized in Table II for the pyrimidine ring protons and Table III for the ribose protons. All spectra exhibit the following characteristic peaks: (1) Two sets of ABX protons, a single broad peak or two peaks at δ 5.5–5.8 (X) for the H_6 protons; two overlapping doublets of doublets at δ 2.94–3.16 (A) and a broad doublet at δ 2.81–2.86 (B) for the H_5 protons. J_{AB}'s are in the range of 17.1–18.0 Hz, a typical geminal coupling constant. J_{AX}'s are in the range of 3.9–4.5 Hz and J_{BX}'s are smaller. In some spectra fine structures of the X proton or the B proton are evident, as indicated in Table II. J_{BX}'s are resolved for those spectra and are in the range of 1.9–2.2 Hz. (2) Two doublets at δ 5.76–5.99 for the $H_{1'}$ proton. (3) Two triplets at δ 4.32–4.43 and at δ 4.44–4.59 for the two $H_{2'}$ protons. For photohydrates of cytidine 3′-phosphate and uridine 3′-phosphate the $H_{3'}$ signal overlaps with the $H_{2'}$ signal at the lower field. However, the presence of the $H_{2'}$ proton signals may be demonstrated by the spin decoupling experiment.

We conclude from the NMR spectra that all photohydrates exist as two isomers of similar structure. The ratio of two isomers can be obtained from the integration of two $H_{1'}$ (or H_6) peaks and is included in Table II. The more abundant isomers are designated as the isomer A and the less abundant ones are designated as the isomer B. It is noteworthy that the isomer B has the $H_{2'}$ proton at lower field than the isomer A for all photohydrates.

Stereochemistry of Photohydration. In the FT-NMR spectrum for **2a** obtained from irradiation in D_2O, one of the H_5's becomes deuterated due to the addition of D_2O instead of H_2O into the pyrimidine ring (Scheme II). The proton signals for H_5 at higher field change to three broad singlets at δ 3.02 (H_{5a}, isomer A), 2.95 (H_{5a}, isomer B) and 2.81 ppm (H_{5b}). The spectrum of **2a** irradiated in H_2O (Figure 1) shows the same patterns as for uridine photohydrate in this region. The presence of both H_{5a} (trans to C_6-OH) and H_{5b} (cis to C_6-OH) in **1a** obtained in D_2O indicates that photohydration of cytidine is stereochemically random as in the case of the photohydration of uracil derivatives (Wechter & Smith, 1968; Summer et al., 1973); i.e., both cis addition and trans addition of water to cytidine occur in approximately equal proportions.

Structures of Photohydrates. Although the chemical structure of uridine photohydrate has been determined in various ways to be 6-hydroxy-5,6-dihydrouridine (**4a**) (Fisher & Johns, 1976), there is no unambiguous structural proof for cytidine photohydrate and its derivatives. We have now, by NMR studies, a definitive evidence that the photohydrates of cytidine and cytidylic acids are mixtures of isomeric 6-hy-

SCHEME II



1

2, isomer A

2, isomer B

3

4, isomer A

4, isomer B

a series: R = R′ = H
b series: R = H; R′ = PO_3^-
c series: R = PO_3^-; R′ = H

droxy-5,6-dihydrocytidines and their nucleotides (**2a–c**).

Fahr (1969) suggested that two isomeric uridine photohydrates may be either two diastereomers A and B or two isomers differing in their H bonding involving the $C_{2'}$-OH group of the riboside. NMR spectra (270 MHz) of photohydrates of 2′-deoxyuridine and 2′-deoxyuridine 5′-phosphate (Figure 5), which contain no hydroxyl group in the $C_{2'}$ position, are still very similar to that of uridine photohydrate except that the $H_{2'}$ protons are now at high field (δ 2.1–2.5). The existence of two distinct sets of signals for H_{5a}, H_{5b}, H_6, and $H_{1'}$ are still evident, which indicates the presence of two isomers. One of the $H_{2'}$'s of one isomer is also shifted downfield relative to the other isomer. Our results indicate the hydrogen bonding of $C_{2'}$-OH is not important in these compounds. The structures are adequately represented by A and B as suggested by Wechter & Smith (1968).

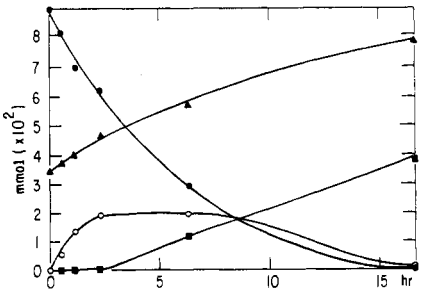


FIGURE 6: Growth and decay of monodeuterio-**2c** (Scheme V, ●), dideuterio-**2c** (○), **1c** (▲), and 5-deuterio-**1c** (■) after one of the H_5 protons of 6-hydroxy-5,6-dihydrocytidine 5′-phosphate (**2c**) has exchanged completely to D in D_2O.

Conformation of Photohydrate. NMR spectra of photohydrates also give valuable information for the conformation of these compounds. Two aspects of the NMR spectra will be analyzed: (1) the conformation of the dihydropyrimidine ring and (2) the conformation of the riboside ring. The case of uridine photohydrate (Figure 4) is discussed as the representative compound. Due to the close similarity in spectra of all photohydrates, the results may also be applied to other photohydrates.

(1) Nofre and co-workers (Chabre et al., 1966; Rouillier et al., 1966a,b) investigated the conformation of dihydropyrimidines by NMR spectroscopy. From the chemical shift and coupling constants (J_{N_1H,H_6}, J_{H_5,H_6}, etc.) of a number of 5- and 6-substituted dihydropyrimidines, they were able to obtain a general picture of the conformation of these compounds. The dihydropyrimidine ring is in the half-chair conformation, and substituents at the C_5 or C_6 position may assume either the pseudo-axial or pseudo-equatorial orientation. It has been shown by X-ray diffraction analysis that dihydropyrimidines are in a half-chair conformation in the solid state (Fuberg & Jensen, 1968; Rohrer & Sundaralingam, 1970). Nofre and co-workers classified all 5- and 6-monosubstituted dihydropyrimidine compounds they had studied into two groups according to the size of the coupling constants J_{ax} and J_{bx} (H_x is the proton α to the substituent and H_a and H_b are β-methylene protons). In one group, compounds have J_{ax} in the range of 9.52–10.3 Hz and J_{bx} in the range of 4.06–7.2 Hz. These coupling constants can best be rationalized by a conformation in which the substituent has a preference for the equatorial orientation. In the other group, compounds have J_{ax} in the range of 3.6–4.3 Hz and J_{bx} in the range of 1.7–3.0 Hz. These couplings can best be rationalized by a conformation in which the substituent has a preference for the axial orientation. In the latter group, the H_a proton, which is trans to the substituent (OH or Br) and in the axial orientation, is at lower field than H_b, which is cis to the substituent and in the equatorial orientation. Also, J_{ax} (J_{cis} or J_{ae}) is larger than J_{bx}(J_{trans} or J_{ee}).

For all the photohydrates we have studied, $J_{5a,6}$ is in the range of 3.9–4.5 Hz and $J_{5b,6}$ is 1.9–2.2 Hz. This is most consistent with a half-chair conformation with the C_6-OH group being predominantly in the pseudo-axial orientation and H_{5a} being trans to C_6-OH and H_{5b} being cis to C_6-OH. It is to be noted that *H_{5a} is the proton at lower field and H_{5b} is the proton at higher field.*

The coupling constants $J_{5a,6}$ and $J_{5b,6}$ for the photohydrates of uracil (Hollis, 1976) and 1-ethyluracil (Summer et al., 1973) are all in the same respective range indicating that the con-

TABLE IV: Coupling Constants of Ribose Protons of Uridine Photohydrate and Dihydrouridine (Hz).

	dihydrouridine[a]	uridine photohydrate A	uridine photohydrate B
$J_{1'2'}$	6.3	6.00	6.15
$J_{2'3'}$	6.0	5.40	5.40
$J_{3'4'}$	3.6	4.50	4.50

[a] Deslauriers et al. (1971).

formation of dihydropyrimidine ring does not change from the base to the corresponding nucleoside and nucleotide.

(2) Coupling constants $J_{1'2'}$, $J_{2'3'}$, and $J_{3'4'}$ of uridine photohydrate are compared with those of dihydrouridine (Deslauriers et al., 1971) in Table IV. The data in the table demonstrate that the conformation of riboside in uridine photohydrate is similar to that in dihydrouridine which may be interconverting rapidly between conformers but with the equilibrium shifted toward $C_{2'}$ endo and $C_{3'}$ exo, or has mainly the *S* conformation, based on the pseudo-rotational analysis (Altona & Sundaralingam, 1973).

Exchanges of Protons at C_5. The exchange of protons at C_5 position is common in many 5,6-dihydropyrimidines. Hydrogen isotope exchange at C_5 with D_2O or T_2O has been observed in dihydrocytidine (Skaric et al., 1974), 1-alkyldihydrocytosine (Brown & Hewlins, 1968), 5,6-dihydrouracil-6-sulfonate (Hayatsu et al., 1970; Shapiro et al., 1970) as well as photohydrates of uridine (Wechter & Smith, 1968), uridylic acid (Chambers, 1968), cytidine and cytidylic acid (Grossman & Rogers, 1968; DeBoer & Johns, 1970).

We have also observed the H–D exchange at C_5 position of all photohydrates in D_2O in our NMR studies. Some qualitative pictures were obtained by following the H–D exchange and dehydration of photohydrates using NMR spectroscopy. The major finding was that photohydrates of uracil derivatives and cytosine derivatives behaved very differently in the exchange at C_5. The half-life of exchange and dehydration of uridine photohydrate (**4a**) in D_2O and the percentage of D incorporation in the uridine recovered after dehydration are summarized in Table V.

From this result, it may be concluded that exchange at C_5 of uridine photohydrate is catalyzed by bases, while dehydration is catalyzed by both acids and bases. Photohydrates of uridine 3′-phosphate and uridine 5′-phosphate behave similarly.

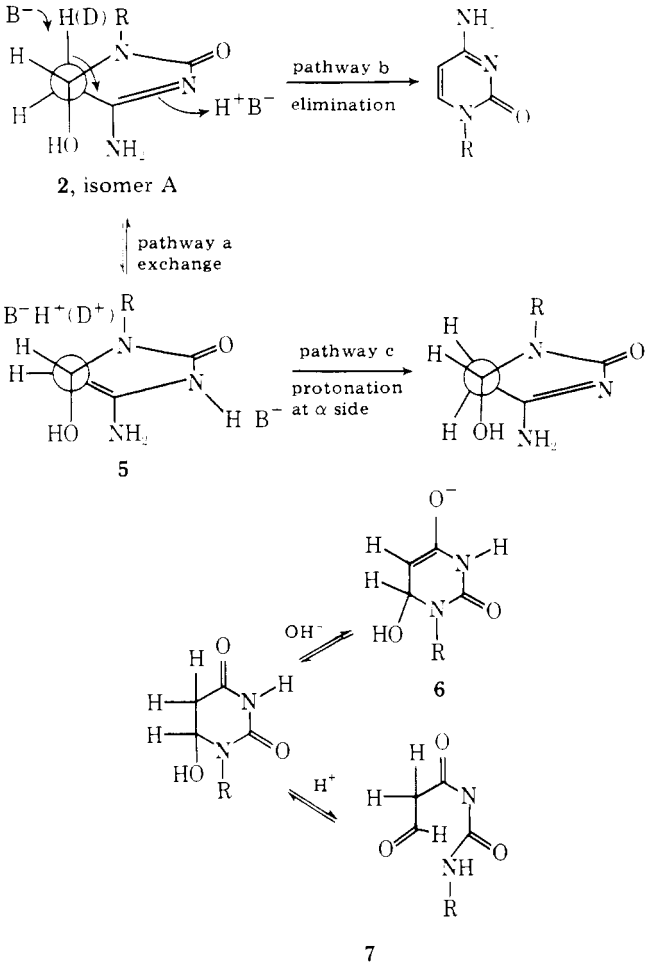
On the other hand, cytidine photohydrate (**2a**), cytidine 3′-phosphate (**2b**, Li salt) and cytidine 5′-phosphate (**2c**, Na salt) slowly underwent H–D exchange at the C_5 position in unbuffered D_2O (0.2 M solution, pD 9.4–9.6) with no selectivity while they dehydrated quickly. However, in phosphate buffers of similar pH ranges, the initial exchange of these photohydrates was very fast and highly stereoselective. When **2a**, **2b**, or **2c** was dissolved in 0.1 M phosphate buffer in D_2O (pD's of these solutions were 9.0, 9.4, and 9.4, respectively), one of the H_5 protons, H_{5a}, which is trans to the hydroxyl group at C_6 in both isomers A and B, exchanged much faster than the other proton. For example, a lyophilized sample of **2c** (sodium salt, 80% purity) prepared from the preparative irradiation (see Experimental Section) was dissolved in 0.1 M phosphate buffer in D_2O to give a 0.2 M solution (pD 9.4). The solution was kept at 20 ± 1 °C, and the exchange was followed by the integration of the H_5 and H_6 signals of **2c** in the NMR spectrum. Signals for H_{5a} (δ 3.00 and 2.94) disappeared almost completely in 30 min ($\tau_{1/2}$ < 5 min), while signals for H_{5b} changed to a singlet (δ 2.84) and showed little decrease in integration. This initial

TABLE V: Exchange and Dehydration of Uridine Photohydrate in D_2O.[a]

	$\tau_{1/2}$ exchange	$\tau_{1/2}$ dehydration	D% incorp
unbuffered D_2O (pD 6.34)	30 h	20 days	80%
0.1 M phosphate buffer (pD 8.4)	2 h	3 days	80%
0.1 M DCl (pD 1.4)	negligible exchange	15 h	<5%

[a] In 0.4 M solution at 25 °C.

SCHEME III



rapid and stereoselective exchange was followed by a slower exchange at H_{5b} to give the 5,5-dideuterated photohydrate ($\tau_{1/2}$ was estimated to be 3 h) which occurred simultaneously with the dehydration of **2c**. Such rapid stereoselective exchange also occurred when **2a**, **2b** (Li salt), or **2c** (Na salt) was dissolved in 0.1 M DCl (pD's of these solutions were 1.0, 6.5, and 6.7, respectively). This phenomenom was not observed in uracil derivatives under similar conditions.

The selectivity in the hydrogen isotope exchange at the C_5 position of photohydrates of cytosine derivatives may be accounted for by a simple mechanism formulated in the Scheme III. Since the favorable conformation of these photohydrates is a pseudo chair with the 6-OH in the pseudo-axial position (vide supra), the removal of 5-H trans to the 6-OH may result in two chemical consequences, the isomerization of the photohydrate to a 3,6-dihydropyrimidine (**5**), and the dehydration of photohydrates. The isomerization process is analogous to

the rearrangement of an acylamide to a vinyl amide. In this instance, this process may occur via the removal of the pseudo-axial 5-H and the concerted protonation of the 3-N from the opposite side (pathway a), while the dehydration will involve the trans-antiparallel elimination of a molecule of water (pathway b).

The 3,6-dihydropyrimidine **5** formed in the pathway a does not contain a conjugated system and may revert back to the more stable photohydrate **2.** Although the protonation of the 4,5-double bond may take place from either side of the molecule, the reverse reaction with the proton (or deutron) added to the original site is favored, because the photohydrate thus formed will be in the original pseudo-chair conformation, while the protonation on the opposite side will lead to the formation of **2** in the less favorable pseudo-boat conformation (pathway c). In view of the high basicity of 3-N in dihydropyrimidines, this reversible isomerization, which will lead to the stereoselective H–D exchange at the C_5 position, may occur with greater facility than the dehydration.

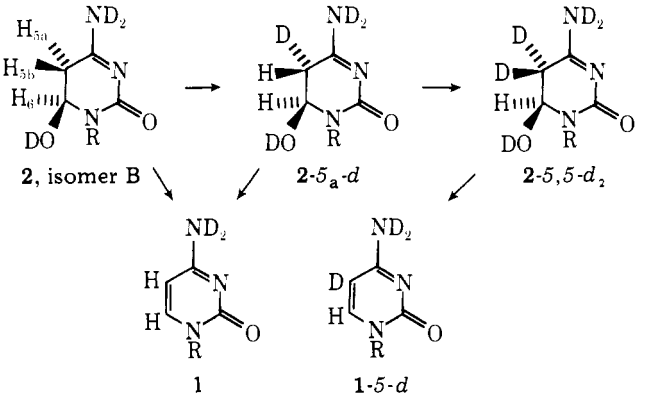
The observed pH and buffer dependence of the stereoselective exchange is in accordance with the proposed mechanism. In nonbuffered D_2O at alkaline pH (pD 9), there was no selective exchange at C_5 in **2a–c.** This may be due to the lack of a proton donor in the solution since the phosphate group of the nucleotide is completely dissociated above pH 9. In the phosphate buffer, the HPO_4^{2-} ion in solution may serve as a proton donor to the 3-N. Although the pH values of these solutions are about the same as those of the nonbuffered solutions, the isomerization may occur via the concerted mechanism. The selective exchange was also observed at acidic pH because there are both general base and specific acid present under such conditions. All these exchanges may occur with both isomers of **2** (A and B) of the photohydrates. However, the stereoselective exchange of the H_5 proton did occur at low concentrations (0.01 M) of **2c** (Na salt) in nonbuffered D_2O (Figure 2) which may be attributed to the lower pH of the medium.

The formulated mechanism will also account for the observation that this selective exchange process will occur for cytosine photohydrates only, since uracil photohydrates (**4**) do not contain a basic 3-N. The exchange at C_5 in **4** may involve only the keto–enol equilibrium (**4** ⇌ **6**), which does not exhibit any selectivity. The formation of 5,5-dideuterated photohydrates of cytosines from the monodeuterated compounds may involve a similar equilibrium.

Two isomers of uridine photohydrate are known to undergo equilibration in acidic media, although there is neither detectable exchange at the C_5 position nor appreciable dehydration under this experimental condition. The observation suggests that the isomerization of uracil photohydrates may involve a simple ring–chain tautomerism (**4** ⇌ **7**) (Ducolomb et al., 1976).

Stereochemistry of Dehydration of Cytidine 5′-Phosphate Photohydrate **2c.** Because of the stereoselective exchange at the C_5 position of cytidine in cytidine photohydrates, cytidine photohydrates with stereoselectively labeled deuterium at the C_5 position may be thus prepared which enable us to study the stereochemistry of their dehydration. A sample of **2c** (Na salt) labeled at H_{5a} with deuterium was prepared by the preparative irradiation followed by the rapid exchange with D_2O in 0.1 M phosphate buffer (pD 9.4; see the Experimental Section). The sample thus prepared was about 80% pure and the only contaminant was cytidine 5-phosphate (**1c**). The decay of 5_a-monodeuterio-**2c** to **1c,** 5,5-dideuterio-**2c,** and 5-deuterio-**1c** (Scheme IV) at 20 ± 1 °C was monitored by NMR spectroscopy at 270 MHz. The results are shown in Figure 6. The graph

SCHEME IV

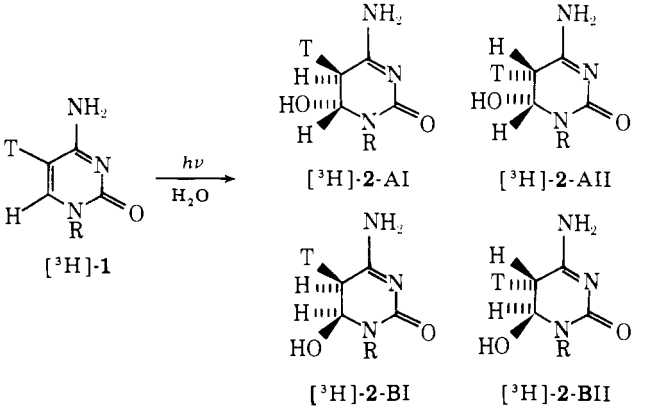


shows that concomitant with the decrease in the concentration of 5_a-monodeuterio-**2c** are the formations of 5,5-dideuterio-**2c** and of **1c.** 5-Deuterio-**1c** is not formed in the initial period and starts to appear only after the concentration of dideuterio-**2c** has been built up. From this result, it may be concluded that the dehydration of **2c** is also highly stereoselective. More specifically, the proton 5_a trans to the C_6-hydroxyl group which exchanges much faster than the proton 5_b is eliminated during the dehydration; i.e., the dehydration is a trans elimination.

The stereoselective hydrogen exchange at the C_5 position and the stereospecific dehydration of **2** may account for the observation by DeBoer & Johns (1970) that, in contrast to the observation by Grossman & Rogers (1968), the release of tritium from [5-^{3}H]cytosine derivatives via photohydrates was not quantitative. Since photohydration of cytosine derivatives is a stereochemically random process, four stereoisomers ([^{3}H]-**2**-AI, AII, BI, and BII) may be formed in comparable proportions when [5-^{3}H]cytidine or -cytidylic acid ([5-^{3}H]-**1**) is photohydrated. Compounds AI and BII, which contain a tritium atom trans to the C_6-OH, can release the tritium atom very fast by exchanging with the hydrogen ion in the phosphate buffer medium. However, compounds AII and BI, which contain the tritium atom cis to the C_6-OH, will release the tritium atom only slowly. The tritium atom is not released from AII and BI during their dehydration either, since the dehydration of **2** is a highly stereoselective trans elimination. This is in accordance with the observation of DeBoer & Johns (1970) that very little loss of tritium arose from the elimination of ^{3}HOH during the dehydration (Scheme V).

Quantum Yield of Photohydration. The quantum yields for photoreactions of cytosine derivatives in dilute aqueous solutions have been measured in several laboratories and a range of values have been reported (for a review, see Fisher & Johns, 1976). The measurement was complicated by the thermal re-

SCHEME V



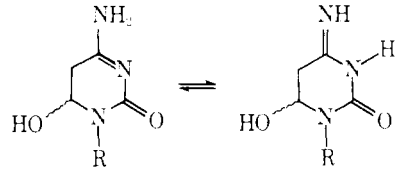
version of the photoreaction and by the fact that the photoproduct has an appreciable UV absorption in the range of the absorption of the starting material. By running the reaction at lower temperature (7 °C) in order to reduce the thermal reversion, Wiezchowski & Shugar (1961) obtained quantum yields for the photochemical transformation of several cytosine derivatives at 254 nm and at several different pH values after the background absorption of photoproducts had been corrected. The values at neutral pH are 0.0013 for cytosine, 0.01 for cytidine, 0.0052 for cytidine 5′-phosphate, and 0.014 for cytidine 2′-phosphate. The value reported by Sinsheimer (1957) for cytidine 3′-phosphate is 0.017. Grossman & Rodgers (1968) reported a quantum yield of 0.0162 for cytidine 5′-phosphate by an assay method based on the hydrogen–tritium exchange at the C_5 position of cytosine photohydrates formed. Since the release of tritium from the labeled photohydrate may not be quantitative as discussed above, the quantum yield may be in fact higher than the value reported. Therefore, the quantum yield determination for the photohydration of cytidine 3′-phosphate was undertaken. When a dilute solution of cytidine 3-phosphate (7×10^{-4} M) in 0.01 M phosphate buffer (pH 7.1) was irradiated at 254 nm, the UV absorbance of the solution at 270 nm decreased by 5.6% of its original value after 20 min of irradiation in our apparatus. Subsequent changes in absorbance were not found to follow simple zero-order kinetics which was attributed to the build-up of photoproducts and their thermal reversal. Therefore, the quantum yield was calculated on the basis of the absorbance changes during the first 20 min of irradiation after the absorbance values had been corrected for the product formation. A value of 0.011 ± 0.001 was obtained which is in good agreement with that reported by Wiezchowski & Shugar (1961).

The quantum yield of photohydration of cytidylic acid units in DNA (0.0016) is even lower than those in single strand DNA (0.0051) or poly(C) (0.0068) (Grossman & Rodgers, 1968), and the result has been attributed to the hindrance on the accessibility of water molecules by the base stacking. The quantum yield of photohydration of poly(C) apparently increases as the reaction proceeds, and the increase may be due to the breakdown of the secondary structure of the polynucleotide (Lomart & Fresco, 1972). If this is the case, cytosine photohydrate may be formed in higher quantum yield in locally denatured portion of DNA caused by other photochemical processes such as pyrimidine photodimerizations.

Conversion of Cytosine Derivatives to Uracil Derivatives via Photohydrates and Its Implication to Photobiology. The major portion of UV-induced mutation in bacteriophages corresponds to the C to T base transition (Drake, 1963, 1966a,b; Howard & Tessman, 1964). In the accompanying communication (Liu & Yang, 1978), we have discussed the possibility of the conversion of the C bases in DNA to the U bases via pyrimidine photodimerization which may be miscoded as T during replication. The sequential events of the photohydration of C bases in DNA, the deamination of the C photohydrates to the U photohydrates, the dehydration of the U photohydrates to U bases, and the miscoding of the U bases as T may provide another molecular basis for the mutagenic action of ultraviolet light (see Scheme I).

Johns & co-workers (1965) have made a thorough study of the thermal reversion and the deamination of photohydrates of cytidylic acid. They observed that 2.5 to 14% of the photohydrates of cytidylic acid was converted to those of uridylic acid depending upon the pH of the medium, while the balance of the photohydrates underwent thermal reversion. The deamination reached a maximum at pH 9. We have observed a similar amount of conversion of cytidine and cytidylic acids to uridine and uridylic acids, respectively, in our work. In a typical experiment, a solution of 0.01 M cytidine 3′-phosphate (**1b,** Li salt) in unbuffered water (pH 8.5) was converted to the photohydrate **2b.** The irradiated solution was allowed to stand at 21 ± 1 °C until there was no further change in the product composition. Uridine 3′-phosphate was formed in 10.0 ± 1.0% yeild together with the recovered **1b** as determined by TLC and NMR spectroscopy.

Brown & Hewlins (1968) suggested an alternative mechanism for the miscoding of photohydrates of cytosine as T. 5,6-Dihydrocytosines including the photohydrates may exist partially as the imino tautomer (**8**). The imino form of cytosine photohydrates in the UV-light transformed DNA may code as T during replication. The relative significance of these two mechanisms for the mutagenic action of ultraviolet light is not known at this moment.



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Conclusion

The structures of photohydrates of cytidine and cytidylic acids have been established to be the respective isomeric 6-hydroxy-5,6-dihydrocytosine derivatives by 270 MHz spectroscopy. By the application of deuterium isotope labeling, we have shown that the photohydration of cytidine and its derivatives is a stereochemically random process, the photohydrates undergo a novel rapid stereoselective exchange at the C_5-H trans to the C_6-OH group, and the dehydration of the photohydrates is a trans elimination. Finally, photohydration of C bases in DNA may initiate a sequential event which may account for the mutagenic action of ultraviolet light.

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Effect of Inhibition of DNA Synthesis on Histone Synthesis and Deposition†

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ABSTRACT: We have reinvestigated the degree of coupling between DNA and histone synthesis in mammalian cells. In at least one cell line (HTC cells), the coupling is not nearly as tight as had previously been inferred from experiments with HeLa cells. The site of deposition of such histones which continue to be made in the presence of sufficient hydroxyurea to depress DNA synthesis almost totally has been studied. Deposition seems to be on material which absorbs at 260 nm. This material is not a part of the bulk chromatin and binds histone in a relatively tight manner. The possible role of such a material in histone synthesis and deposition is discussed.

There is a notion that histone synthesis is very tightly coupled to DNA synthesis (Spalding et al., 1966). This idea has been most exhaustively demonstrated in HeLa cells (Robbins and Borun, 1967; Gallwitz and Mueller, 1969). The origin of this coupling appears to lie in the availability of cytoplasmic histone mRNA, since it seems that this RNA is made throughout the cell cycle (Jacobs-Lorena, et al., 1972; Thompson et al., 1976; Stein et al., 1977; Melli et al. 1977) but appears in polyribosomes primarily during the S phase. Furthermore, the addition of inhibitors of DNA synthesis to S-phase cells causes a loss of cytoplasmic histone mRNA even though its nuclear synthesis continues.

However, the tight coupling of DNA and histone synthesis is not always observed. For example, in developing frog oocytes, Adamson and Woodland (1974) have noted a vast excess of histone synthesis which appears to function as a reservoir for histones during the very rapid phase of DNA synthesis following fertilization. Furthermore, a lack of coupling has also been reported in mammalian systems during SV 40 (Kay and Singer, 1977) and HTC replication (Balhorn et al. 1973). Thus, it seems that the tight coupling observed in HeLa cells might prove to be an extreme case. Since we have previously (Balhorn et al., 1973) observed only partial coupling between

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