STRUCTURE OF THE PHOTOHYDRATION PRODUCTS OF CYTIDINE AND URIDINE*

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Attempts to obtain unambiguous, chemical proof for the structures of the major photoproducts of cytidine and uridine and their derivatives in aqueous solution have failed because of the instability of these products toward acid, base and elevated temperature. From indirect evidence the structures of 6-hydroxy-5,6-dihydrocytidine (I, Fig. 1) and 6-hydroxy-5,6-dihydrouridine (IV) have tentatively been assigned to these products.¹⁻⁴ We have now obtained a stable derivative of the major photoproduct of cytidine, which unambiguously proves the correctness of this assignment. New evidence, which strongly supports structure IV as the major photoproduct of uridine, is also presented.

Materials and Methods.—Sodium borodeuteride was obtained from Merck & Dohm, Canada. A 250-w mercury high-pressure lamp (Hanovia type S654A-36) filtered by a vycor liner was used as the light source in the photochemical experiments. Special quartz cells with a semicircular cross section and a wall-to-wall distance of 1 cm were used as reaction vessels and were arranged at a distance of 5 cm from the burner. The entire photochemical apparatus was immersed in a bath which was kept at 10°. The reaction solution was freed from oxygen and thoroughly mixed during the irradiation with a constant stream of nitrogen.

Ultraviolet absorption spectra were measured on a Cary model 15 spectrometer; nuclear magnetic resonance spectra on a Varian A60A spectrometer in D₂O with sodium-3-(trimethylsilyl)-1-propane-sulfonate (in D₂O) as an external standard; infrared spectra (KBr) on a Perkin-Elmer Infracord. Solvent systems used for thin-layer chromatography were: *n*-butanol-water, 86:14 (system A); and *n*-propanol-conc. ammonia, 8:1 (system B). The mobility of the compounds is given relative to D-ribose (*R_r*).

(1) *Reduction of the major photoproduct of cytidine (I) to N₁-(α,β -D-ribofuranosyl)-N₃-(γ -hydroxypropyl)urea (III):* A solution of cytidine (400 mg, 1.6 mmole) in 400 ml of water was irradiated under the conditions described above until the absorption at 270 m μ had virtually disappeared. Sodium borohydride (4 gm, 108 mmoles) was then added and the reaction allowed to proceed for 18 hr. The reaction mixture was treated with Dowex 50W-X8(H⁺) to destroy excess sodium borohydride and the pH was adjusted to 5. The acidified solution and the resin were placed on a column (Dowex 50W-X8(H⁺), 2 $\times$ 15 cm) to ensure complete removal of sodium and unreacted cytidine. The products were eluted with about 500 ml of water. Unreacted cytidine (220 mg) was recovered by elution with 300 ml of 2 *N* ammonia. The aqueous eluate was evaporated to dryness *in vacuo*. Boric acid was removed as methylborate by repeated evaporation with methanol. The residue was purified on a silica gel column (Merck 0.02 to 0.5 mm; 2 $\times$ 17.5 cm; *n*-butanol-water, 86:14). Fractions of 5 ml were collected and analyzed on thin-layer plates using system A. The spots were developed with the anisaldehyde reagent.⁵ Fractions 31-118 contained the major product (*R_r* = 0.55) and were pooled and evaporated to dryness. The same product in a similar yield was obtained if the reduction of I was carried out in the dark. $\alpha_D^{24} + 1.8^\circ$ (*c* = 5.6, H₂O). *Analysis:* Calculated for C₉H₁₈N₂O₆·1/2 H₂O: C, 41.70; H, 7.39; N, 10.81. Found: C, 41.91; H, 7.38; N, 11.02.

IR (cm⁻¹): 1668 (ureido); NMR (ppm): 1.76 (five-line multiplet, *J* = 6.5 cps) (—ND—CO—ND—CH₂—CH₂—CH₂—OD); 3.28 (triplet, *J* = 6.5 cps) (—ND—CO—

FIG. 1.—Reduction of the photohydration products of cytidine and uridine with sodium borohydride.

ND—CH₂—CH₂—CH₂—OD); 3.59, 3.69, 3.79 (—ND—CO—ND—CH₂—CH₂—CH₂—OD); 4.98 (singlet) and 5.09 (triplet, $J = 2.0$ cps) anomeric proton.

Compound III (28 mg) was treated with a solution of 19 mg of potassium nitrite in 1 ml of 0.1 *N* hydrochloric acid for 4 hr at 4°. Potassium was removed with Dowex 50W-X8(H^+). The sample was evaporated to dryness and the products were analyzed by thin-layer chromatography in systems A and B. The spots were made visible with the anisaldehyde reagent and with *p*-dimethylaminobenzaldehyde/HCl. D-ribose and 3-ureidopropane-1-ol were detected as the major cleavage products.

Reduction of I with sodium borodeuteride using identical conditions for the reaction and the isolation gave N_1 -(α,β -D-ribosepyranosyl)- N_3 -(γ -hydroxypropyl- d_α , d_β , d_γ) urea as the major product.

NMR (ppm): 1.76 (doublet, $J = 6.5$ cps) (—ND—CO—ND—CD₂—CH₂—CHD—OD); 4.98 (singlet) and 5.09 (triplet, $J = 2.0$ cps) anomeric proton.

(2) *Synthesis of N_1 -(α,β -D-ribosepyranosyl)- N_3 -(γ -hydroxypropyl)urea (III) by the condensation of D-ribose with 3-ureidopropane-1-ol:* D-ribose (225 mg, 1.5 mmole) and 3-ureidopropane-1-ol (240 mg, 2 mmole) were dissolved in 4 ml of 1% hydrochloric acid and allowed to stand for 60 hr at 55°. The solvents were removed *in vacuo* and the residue was purified on a silica gel column (Merck 0.02 to 0.5 mm; 2×22 cm; *n*-butanol–water, 86:14). Unreacted 3-ureidopropane-1-ol (130 mg) and D-ribose (70 mg) were first eluted followed by the major product. After evaporation of the solvents, 198 mg (55%) of a colorless glass remained. On thin-layer chromatograms using system A, a R_f value of 0.55 was obtained. $\alpha_D^{24} = -1.9^\circ$ ($c = 2.6$, H_2O).

IR (cm^{-1}): 1668 (ureido); NMR (ppm): 1.76 (five-line multiplet, $J = 6.5$ cps) (—ND—CO—ND—CH₂—CH₂—CH₂—OD); 3.27 (triplet, $J = 6.5$ cps) (—ND—CO—ND—CH₂—CH₂—CH₂—OD); 3.58, 3.69, 3.78 (—ND—CO—ND—CH₂—CH₂—CH₂—OD); 4.97 (singlet) and 5.10 (triplet, $J = 2.0$ cps) anomeric proton.

(3) *Reduction of the major photoproduct of uridine (IV) to α,β -D-ribosepyranosylurea (VII):* Uridine (800 mg, 3.3 mmole) in 400 ml of water was irradiated as described above until the absorption at 260 $m\mu$ had disappeared. Sodium borohydride (6 gm, 162 mmole) was added and the reaction allowed to proceed for 18 hr. Excess sodium borohydride was

removed as described in section (1). No uridine was recovered. The major product was isolated on a silica gel column (Merck 0.02 to 0.5 mm; 2×18.5 cm; *n*-butanol-water, 86:14; 5-ml fractions). Fractions 60–220 containing the major product were pooled and evaporated to a hygroscopic syrup (294 mg, 46% yield). An R_f value of 0.44 was found on thin-layer chromatograms (system A, development of the spots with the anisaldehyde reagent). The same major product was obtained in the reduction of IV with sodium borohydride in the dark. $\alpha_D^{24} - 1.1^\circ$ ($c = 4.7$, H_2O).

IR (cm^{-1}): 1682 (ureido); NMR (ppm): 4.92 (singlet) and 5.04 (multiplet) anomeric proton.

When VII was treated with nitrous acid under the conditions described in section (1), urea and D-ribose were found as cleavage products by thin-layer chromatography (systems A and B; anisaldehyde reagent and *p*-dimethylaminobenzaldehyde/HCl for the development of the spots).

(4) *Preparation of α,β -D-ribofuranosylurea (VII) from 2,3,5-tri-O-benzoyl- β -D-ribofuranosylurea*³: A mixture of 2,3,5-tri-O-benzoyl- β -D-ribofuranosylurea (1.5 gm, 3 mmole) in 200 ml of 50% ethanol containing 9 ml of *N* sodium hydroxide was stirred at room temperature until solution occurred. Sodium was removed by treatment with Dowex 50W-X8(H^+). The resin was removed by filtration and the filtrate evaporated *in vacuo*. The residue was dissolved in 50 ml of water and benzoic acid extracted with chloroform. The aqueous layer was evaporated to dryness and the residue applied to a silica gel column (Merck 0.02 to 0.5 mm; 2×32 cm; *n*-butanol-water, 86:14). The major product was characterized as described in section (3). $R_f = 0.44$; $\alpha_D^{24} - 19^\circ$ ($c = 1.9$, H_2O).

IR (cm^{-1}): 1682 (ureido); NMR (ppm): 4.89 (singlet) and 5.03 (multiplet) anomeric proton.

Results and Discussion.—Cytidine and uridine in 4.1×10^{-3} *M* aqueous solution were irradiated at 10° until the absorption in the ultraviolet at 270 $m\mu$ and 260 $m\mu$ had virtually disappeared. A 65 *M* excess of sodium borohydride was then added and the reaction allowed to proceed for 18 hours in the presence or absence of light.⁶ Excess sodium borohydride was destroyed with Dowex 50W-X8(H^+) and boric acid removed as methylborate. Considerable amounts of cytidine, but not uridine, were recovered which had formed by reversion of the photoproduct during the reduction. The major products were isolated by column chromatography on silica gel (*n*-butanol-water, 86:14).

The major photoproduct of cytidine (I) is deaminated and reduced with sodium borohydride to N_1 -(α,β -D-ribofuranosyl)- N_3 -(γ -hydroxypropyl)urea (III; 63% yield). The structure of III follows from spectroscopic evidence (NMR, IR) and analytical data. The data of the oxidation with sodium periodate and the orcinol assay for ribose are summarized in Table 1. In the NMR (D_2O) a five-line multiplet at 1.76 ppm, a triplet at 3.28 ppm, and signals at 3.59 and 3.69 ppm were assigned to the methylene protons at C_β , C_α , C_γ , respectively. In the spectrum of the deuterated compound, which was obtained when sodium borodeuteride was used as the reducing agent, the absorption of the C_α methylene group had disappeared, the signal of the C_β -protons was simplified to a doublet (at 1.75 ppm), and the signals attributed to the protons at C_γ had disappeared or were lowered in intensity. A singlet at 4.98 ppm and a multiplet at 5.09 ppm were attributed to the anomeric proton of the ribopyranose portion of the molecule. Further proof for the structure of III was obtained from the cleavage with nitrous acid.⁷ D-ribose and 3-ureidopropane-1-ol were identified as major components of the reaction mixture by thin-layer chromatography (silica

TABLE 1. *Analytical data.*

	Orcinol assay (moles ribose/ mole)	Periodate Oxidation		Oxidation	
		Moles IO ₄ ⁻ Consumed at	Mole at	Moles HCOOH Liberated at	Mole at
N ₁ -(α,β -D-ribofuranosyl)-N ₃ -(γ -hydroxy-propyl)urea (III)	1.10	1.99	2.08	0.90	1.29
N ₁ -(α,β -D-ribofuranosyl)-N ₃ -(γ -hydroxy-propyl)urea (obtained by the condensation of 3-ureidopropane-1-ol with D-ribose)	0.94	1.70	1.75	0.69	0.73
α,β -D-ribofuranosylurea (VII)	1.03	1.90	2.05	0.75	1.05
α,β -D-ribofuranosylurea (obtained by debenzoylation of 2,3,5-tribenzoyl- β -D-ribofuranosylurea)	1.36	1.79	2.20	0.77	1.15
Ethyl- β -D-ribofuranoside	1.02	1.95	1.91	1.20	1.14

gel G, system A and B). Compound III was also obtained by the condensation of 3-ureidopropane-1-ol and D-ribose in dilute hydrochloric acid. The major product of this reaction was identical with the compound obtained from the reduction of I with sodium borohydride.

Reduction of the photoproduct of uridine yielded α,β -D-ribofuranosylurea (VII, 46% yield) as the major product. Compound VII, in contrast to III, gave a yellow color with *p*-dimethylaminobenzaldehyde/HCl on chromatograms, indicating the presence of a monosubstituted urea derivative. Almost identical absorption was found in the NMR for the sugar protons of III and VII. No signals at higher field were observed in the spectrum of VII. Urea, D-ribose, and a number of unidentified by-products were obtained from the cleavage of VII with nitrous acid. Compound VII was also obtained as a major product after debenzoylation of 2,3,5-tri-O-benzoyl- β -D-ribofuranosylurea⁸ with mild ethanolic base.

The pyranose structure was assigned to the sugar portion of III and VII on the basis of the data obtained from the oxidation with sodium periodate (see Table 1). The large upfield shift of the signals of the anomeric protons in the NMR spectrum of III and VII further supports this assignment.⁹ The reduction products III and VII have to be considered as mixtures of both anomers and only very small specific rotations were measured (III, $\alpha_D^{24} +1.8^\circ$, $c = 5.6$, H₂O; VII, $\alpha_D^{24} -1.1^\circ$, $C = 4.7$, H₂O). It is likely that the open chain intermediates II and VI are first being formed. Subsequent ring closure leads to mixtures of the anomers of the pyranosides III and VII.¹⁰

The key step in the reaction of I and IV with sodium borohydride is best described as the reductive cleavage of a carbinolamide. A prerequisite for the cleavage of the 1-6 bond in the photoproducts of cytidine and uridine by this mechanism is the presence of a hydroxyl group at C₆. The formation of III and VII therefore unambiguously proves the position of the hydroxyl function in C₆ and excludes 5-hydroxy-5,6-dihydrocytidine or -uridine as the major photoproducts.

The observation that deoxyribosylurea is obtained from the photoreduction of thymidine with sodium borohydride¹¹ may indicate the formation of 6-hydroxy-

5,6-dihydrothymidine as a photoproduct of thymidine in aqueous solution. The fact that this product has so far not been identified as a photoproduct of thymidine could be due to its fast reversion to starting material,¹² which may lead to a very low steady-state concentration of the product under the conditions normally used for the photolysis. Only the trapping of the product in the form of a stable derivative would then allow its detection. The formation of a photohydration product of thymidine could be important for our understanding of the photochemistry and photobiology of DNA, especially in view of the fact that intra- and intermolecular interactions in the polymer may substantially alter the stability of such a product. This problem is now under investigation.

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⁶ Formation of the same products in similar yields was observed if the reductions were carried out in the dark.

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⁸ This compound was a gift of Dr. Max Hoffer, Hoffmann-La Roche, Inc., Nutley, New Jersey, and was synthesized by the condensation of urea with 2,3,5-tri-O-benzoyl-D-ribose in methanolic HCl. It is assumed that the rearrangement of the sugar portion to the pyranoside form has taken place after the debenzoylation.

⁹ Ethyl β -D-ribosepyranoside was prepared according to Jeanloz, R., H. G. Fletcher, and C. S. Hudson, *J. Am. Chem. Soc.*, **70**, 4055 (1948), for comparison. A doublet at 4.78 ppm was observed for the anomeric proton in the NMR. The presence of small amounts of the corresponding furanosides cannot be excluded.

¹⁰ Cf. Naito, T., and T. Kawakami, *Chem. Pharm. Bull. (Tokyo)*, **10**, 627 (1962). Condensation of D-ribose with urea under mild acidic conditions lead to a mixture of α and β -D-ribosepyranosylurea. It is possible that VI is also an intermediate in this reaction.

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