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218. Tyunosin Ukita, Harumi Okuyama, and Hikoya Hayatsu*1 : Modifications of Nucleosides and Nucleotides. I. Reaction of Ethylene Oxide with Uridine and Uridylic Acid.

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Previous works have shown that nucleic acids react with ethylene oxide under mild conditions.[1,2] The detailed mode of the reaction, however, has not yet been completely clarified.

Windmueller and Kaplan reported that the reaction of ethylene oxide with adenosine at pH 6.5 at room temperature yielded 1-(2-hydroxyethyl)adenosine[3] and that a similar reaction with deoxyguanosine at pH 7.0 gave 7-(2-hydroxyethyl)guanine and deoxyribose.[4]

The present communication deals with several new observations concerning the reaction of ethylene oxide with uridine and uridine 5′-phosphate.

In order to gain fundamental knowledge about the reaction of ethylene oxide with uridine-derivatives, 1-methyluracil (I)[5] was alkylated with the reagent in an aqueous solution of pH 10 at room temperature. Paper chromatography (PPC) of the reaction mixture revealed that the starting material (I) had completely disappeared after two days and an ultraviolet-absorbing product, II, having a larger Rf value than that of I was formed. The pH of the reaction mixture was unchanged during the period. The product was isolated from the mixture as colorless needles, m.p. 136.5~138.5°. Elemental analysis gave a molecular formula corresponding to a mono-hydroxyethylated derivative of I.

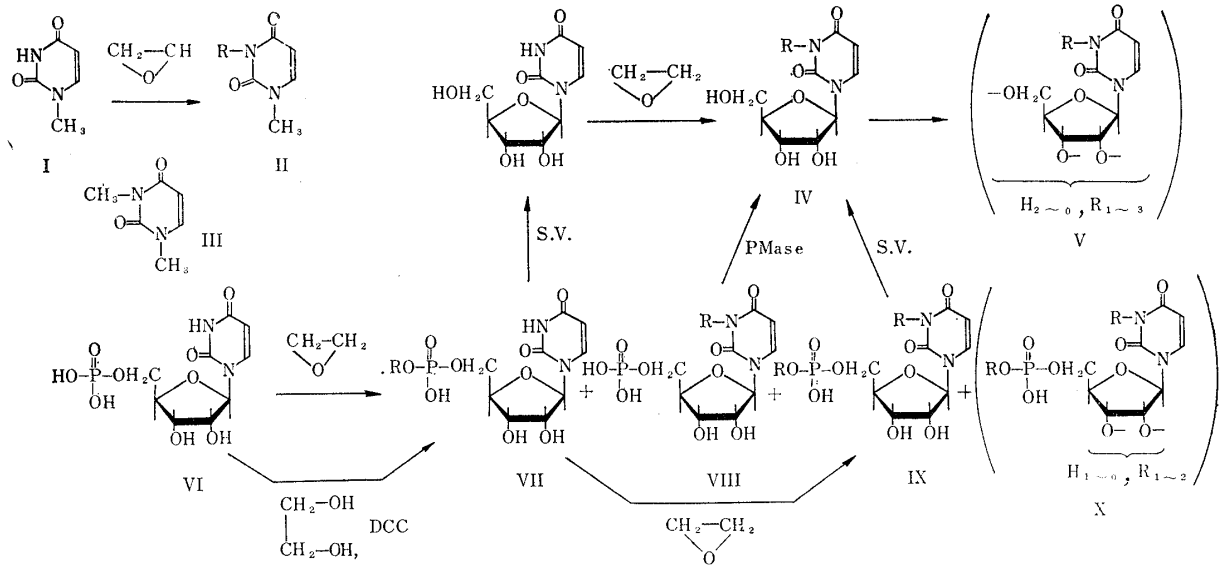

Chart. 1.

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1) K. A. Stacey, M. C. Cobb, S. F. Cousens, P. Alexander : Ann. N. Y. Acad. Sci., **68**, 682 (1958).
2) H. Fraenkel-Conrat : Biochim. Biophys. Acta, **49**, 169 (1961).
3) H. G. Windmueller, N. O. Kaplan : J. Biol. Chem., **236**, 2716 (1961).
4) *Idem* : Biochim. Biophys. Acta, **61**, 307 (1962).
5) P. A. Levene, L. W. Bass, H. S. Simms : J. Biol. Chem., **70**, 229 (1926).

The ultraviolet absorption spectrum of II (λ_{max} 267 mμ) was similar to that of 1,3-dimethyluracil (III).[6] Thus, the absorption spectra in alkaline solution of these compounds did not show any difference from those in neutral solution, whereas a considerable spectral change was observable between the spectra of compound (I) taken in alkaline and neutral media owing to its dissociable proton (pK_a 9.75).[6] In paper electrophoresis (PEP) at pH 11.5, II behaved as a neutral compound, while the starting compound (I) migrated to anode as a mono-valent anion. Furthermore, in the infrared spectrum, II gave absorptions characteristic of primary hydroxyl groups at 1049 cm^{-1} (C-O stretching vibration of a primary alcohol) and 3500 cm^{-1} (O-H stretching). These properties, together with its stability in both acid and alkaline media, indicated that the product (II) is 1-methyl-3-(2-hydroxyethyl)uracil.

A similar reaction was performed with uridine. A solution of uridine in ca. 10% aqueous ethylene oxide at pH 9 was kept at room temperature. Paper chromatography of the reaction mixture gave, in the early step of the reaction, two spots, that of uridine (Rf_1 0.37) and that of a reaction product (IV) (Rf_1 0.64). This product showed an ultraviolet absorption maximum at 262 mμ and gave no spectral change in either neutral or alkaline solution.

After a prolonged reaction period, disappearance of the spot of uridine was observed, followed by an appearance of an elongated spot of another product (V) having Rf_1 of 0.72~0.85. At the end of the reaction (about 10 days at room temperature) the only product found was V which gave an ultraviolet absorption similar to that of IV. The spot of V on a paper chromatogram did not consume periodate in contrast to compound (IV). These observations strongly suggest that a N^3-alkylation of uridine initially took place to give IV and might be followed by alkylation of the hydroxyl group(s) of the sugar moiety to give V which, consequently, lacked the vicinal hydroxyl groups.*2

At a stage when the reaction mixture contained mainly compound (IV), the reaction was stopped and the intermediate product (IV) was isolated as colorless needles. The elemental analysis of IV coincided well with those calculated for 3-(2-hydroxyethyl)-uridine. In PEP at pH 11.5, IV behaved as a netural compound. According to the procedure of Yu and Zamecnik,[7] IV was oxidized with periodate to a dialdehyde-compound which was subsequently degraded by cyclohexylamine to furnish a pyrimidine base. This pyrimidine base gave a similar ultraviolet absorption spectrum to that of 3-methyluracil and was assumed to be 3-(2-hydroxyethyl)uracil.

Every attempt to crystallize product (V) was unsuccessful, presumably because V is a mixture of derivatives of 3-(2-hydroxyethyl)uridines differently alkylated in the ribofuranose moiety,

The above reaction of ethylene oxide with uridine was then performed in aqueous solutions of various pH's. In ca. 10% aqueous ethylene oxide solutions both the reaction of uridine to IV and IV to V occurred more rapidly at a higher pH. At pH 2, no reaction took place; at pH 4, ca. 50% of uridine used was converted into IV by keeping the mixture for 30 days; at pH 5~6, IV began to appear after 6~7 days and all of the starting material was changed into IV after an additional 24 days; at pH 7.0, the appearance of IV in the mixture after two days was followed by the conversion of a small amount of IV to V after 30 days' storage; at pH 8~9, all of uridine used was converted into IV within the first two days and after 4 days the mixture contained both IV and V. Above pH 10, uridine disappeared from the mixture within the first several hours to

*2 The alkylations of starch and cellulose (W. Ziese : Z. Physiol. Chem., **229**, 213 (1934); **235**, 235 (1935)) and of sucrose (J. W. LeMaistre, R. B. Seymour : J. Org. Chem., **13**, 782 (1948)) in alkaline medium have been reported.

6) D. Shugar, J. J. Fox : Biochim. Biophys. Acta, **9**, 199 (1952).

7) C. Yu, P. C. Zamecnik : *Ibid.* **45**, 148 (1960).

give IV and V. From this pH dependence of the reaction rate, the alkylation of uridine seems to occur in its dissociated form.

In the alkylation of uridine 5′-phosphate with ethylene oxide at various pH's, a rise of the pH of the reaction mixture was observed. The elevation of the pH might be caused by the esterification of VI at its phosphoryl group as reported in the case of ethylene oxide with adenosine triphosphate in which the hydroxyethylation occurred both at the adenine moiety and at the terminal phosphate position.[3] On separation of the products by PPC and/or PEP, three phosphodiester-type compounds (VII, IX, and X) and one phosphomonoester-type compound (VIII) were detected.

Compound (VII) showed an uridine-type absorption spectrum in the ultraviolet and was identified by PPC in two solvent systems as uridine 5′-phosphate-2-hydroxyethyl ester by a comparison with an authentic sample synthesized by condensation of ethylene glycol with uridine 5′-phosphate in the presence of dicyclohexyl carbodiimide. Uridine was derived from VII by treatment with *N* barium hydroxide at 70~80° for 2 hours,[*3] or by digestion with crude snake venom which contains both phosphodiesterase and 5′-nucleotidase.

Compound (VIII) gave an ultraviolet absorption similar to that of IV, a M_{UMP} value of 1.0 in PEP run at pH 9.2 and, upon dephosphorylation with intestinal alkaline phosphatase of beef, 3-(2-hydroxyethyl)uridine (IV). Consequently, VIII must be 3-(2-hydroxyethyl)uridine 5′-phosphate.

Product (IX) was shown to be 3-(2-hydroxyethyl)uridine 5′-phosphate-2-hydroxyethyl ester from the following observations; IX had a ultraviolet absorption similar to that of IV, gave a M_{UMP} value of 0.8 in PEP and was converted into 3-(2-hydroxyethyl)-uridine by digestion with crude snake venom. Compound (IX) was also derived from VII by further treatment of VII with ethylene oxide.

Compound (X) behaved in PEP as a phosphodiester and gave V upon digestion with the crude snake venom. Therefore, the phosphodiester (X) was assumed to be derived from IX by a further alkylation of hydroxyl group(s) in the sugar portion.

In these alkylations of uridine 5′-phosphate to the above products, the rate of hydroxyethylation of uracil and ribofuranose moiety was dependent on the pH of the reaction mixture as was observed for uridine. The esterification of the phosphoryl group, however, seemed independent of pH; thus, between pH 2 and 11 the reaction occurred at least within the first 24 hours.

Experimental[*4]

Methods——In order to prevent loss of the volatile ethylene oxide, its reaction with uridine or uridine 5′-phosphate was carried out in a flask shown in Fig. 1 equipped with a glass tube. An aliquot

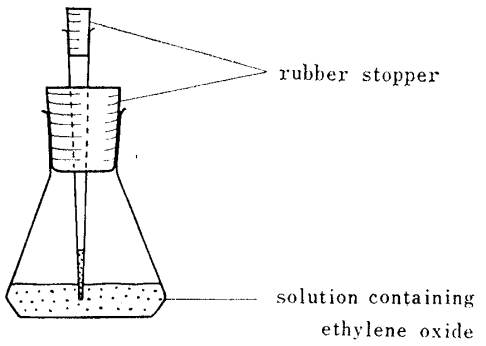

Fig. 1.

[*3] Alkaline hydrolysis of alkyl(2-hydroxyethyl)phosphate to give alcohol and ethylene glycol-phosphate has been reported (T. Ukita, A. Hamada : This Bulletin **9**, 369 (1961)).

[*4] All melting points are uncorrected.

was taken up through the glass tube by use of a capillary. The pH of the reaction mixture was adjusted by NaOH or $HClO_4$.

Paper chromatography was performed ascendingly on Toyo Roshi No. 53. The following solvent systems were employed: (1) iso-PrOH–conc. NH_4OH–H_2O (7:1:2); (2) BuOH–EtOH–H_2O (4:1:5); (3) BuOH–AcOH–H_2O (4:1:5); (4) BuOH–H_2O (86:14); (5) iso-PrOH–AcOH–H_2O (6:1:3). The Rf values found in these solvent systems are represented by Rf_1, Rf_2, Rf_3, Rf_4 and Rf_5 respectively and are summarized in Table I.

Table I.

	Rf				
	1	2	3	4	5
1-Methyluracil (I)	0.59	0.53	0.56	—	—
1-Methyl-3-(2-hydroxyethyl)uracil (II)	0.78	0.65	0.69	0.57	0.77
Uridine	0.37	0.36	0.35	—	0.35
3-(2-Hydroxyethyl)uridine (IV)	0.64	0.41	0.42	0.28	0.48
3-(2-Hydroxyethyl)uracil	0.68	—	—	—	0.64
Uridine 5′-phosphate (VI)	0.06	—	0.05	—	—
Uridine 5′-phosphate 2-hydroxyethyl ester (VII)	0.20	—	—	—	—
3-(2-Hydroxyethyl)uridine 5′-phosphate (VIII)	0.10	—	0.10	—	—
3-(2-Hydroxyethyl)uridine 5′-phosphate 2-hydroxyethyl ester (IX)	0.48	0.13	—	—	—
V	0.72～0.85	—	0.46～0.64	—	—
X	0.60	0.24	0.23	—	—

Paper electrophoresis was performed on Toyo Roshi No. 53 at 10～30 v./cm. for 50～90 min. using buffer solutions of pH 9.2 (BuOH–Pyridine–AcOH–H_2O, 20:10:2:977 adding ammonia to the pH)and pH 11.5 (Na_2CO_3–HCl), and mobilities of the compounds tested are represented by M_{UMP} (taking that for uridine 5′-phosphate as standard, $M_{UMP}=1.0$) and M_U (taking that for uridine as standard, $M_U=1.0$) respectively.

The spots on the paper were located by an ultraviolet lamp and by spraying the chromatograms with molybdate-perchloric acid[8] and subsequent irradiation with ultraviolet light.[9] Compounds which contained vicinal hydroxyl groups were detected on chromatograms with periodate-benzidine spray.[10]

The measurement of the UV spectra was carried out with a Cary Model 11 recording spectrophotometer.

1-Methyl-3-(2-hydroxyethyl)uracil (II)——To a solution of 1-methyluracil (I)[5] (500 mg.) in H_2O (35 ml.), pH 10.2, was added 5 ml. of 50% (v./v.) aqueous ethylene oxide solution and the mixture well stoppered and left at room temperature for 2 days. The pH of the mixture was found unchanged during this period. After confirmation by PPC of the complete conversion of I to II, the reaction mixture was treated with a small amount of Dowex 50 (H^+) resin to remove Na ions. The resin was removed by filtration and washed with aqueous EtOH. The filtrate and washings were combined and evaporated *in vacuo* leaving a white crystalline mass. Recrystallization from EtOH afforded 458 mg. (68%) of II as colorless needles, m.p. 136.5～138.5°. UV: $\lambda_{max}^{pH\ 5\ or\ 13}$ 267 mμ (ε 8260). cf. 1,3-dimethyluracil: $\lambda_{max}^{pH\ 1\sim14}$ 266 mμ (ε 8900). IR: (C–O of primary alcohol) 1049 cm^{-1}(S) (KBr), (O–H) 3500 cm^{-1}(S) (KBr). *Anal.* Calcd. for $C_7H_{10}O_3N_2$: C, 49.41; H, 5.92; N, 16.46. Found: C, 49.86; H, 6.26; N, 16.16. $M_U=0.7$ at pH 11.5.

The stability of II upon treatment with 0.1*N* HCl or 0.1*N* NaOH at 100° for 20 min., was confirmed by PPC.

3-(2-Hydroxyethyl)uridine (IV)——Uridine (560 mg.) was dissolved in 20 ml. of H_2O at pH 5.4 and to the solution was added 5 ml. of 50% aqueous ethylene oxide solution and the reaction mixture was kept at room temperature (ca. 10°). After 24 days the reaction was found incomplete, so a drop of 2*N* NaOH was added to promote the reaction and the mixture was kept around 20° for an additional 10 days. PPC indicated that the conversion of uridine to IV was just complete.*5 Na ions were removed by treatment with Dowex 50 (H^+) resin, and, after removal of the resin by filtration, the filtrate was evaporated under reduced pressure leaving a yellow viscous oil. The oil was dissolved in a small amount of EtOH and then Et_2O was added to cloudiness. After standing overnight, the needle clusters

*5 If the reaction is allowed to proceed further and a partial alkylation of the hydroxyl group(s) in sugar moiety takes place, the isolation of IV becomes difficult and the yield of crystalline (IV) isolated drops considerably.

8) C. S. Hanes, F. A. Isherwood: Nature, **164**, 1107 (1949).
9) R. S. Bandurski, B. Axelrod: J. Biol. Chem., **193**, 405 (1951).
10) J. A. Cifonelli, F. Smith: Anal. Chem., **26**, 1132 (1954).

that appeared were collected by filtration and recrystallized from EtOH-Et_2O to yield colorless needles melting at 142~142.5°. Yield : 322 mg. (49%). UV mμ (ε) : $\lambda_{max}^{pH\ 5\ or\ 13}$ 262 (9010), $\lambda_{min}^{pH\ 5\ or\ 13}$ 233. *Anal.* Calcd. for $C_{11}H_{16}O_7N_2$: C, 45.83; H, 5.59; N, 9.72. Found : C, 46.09; H, 5.81; N, 9.76. M_U=0.7 at pH 11.5.

On oxidation with periodate at pH 5.5, IV consumed 1.01 moles of the oxidant within 30 min. and further consumption was not observed. Uridine, used as a control, analogously consumed 1.01 moles of the oxidant within 30 min.

Cleavage of Glycosidic Linkage of 3-(2-Hydroxyethyl)uridine——To a solution containing ca. 10 mg. of IV in 0.5 ml. H_2O was added ca. 10 mg. of potassium periodate and the mixture was warmed at 35° for 2 hr. Excess potassium periodate was removed by centrifugation and to the supernatant-dialdehyde solution was added 2 drops of cyclohexylamine and the mixture was kept at 35° for 2 hr. PPC with solvent system No. 5 revealed a single UV absorbing spot at Rf_5 0.64. UV absorption of aqueous extract of the spot was quite similar to that of 3-methyl uracil,[6] the former being $\lambda_{max}^{pH\ 5}$ 259 mμ, λ_{min}^{pH5} 233 mμ, $\lambda_{max}^{pH\ 13}$ 283 mμ, $\lambda_{min}^{pH\ 13}$ 243 mμ and the latter $\lambda_{max}^{pH\ 7.2}$ 259 mμ, $\lambda_{min}^{pH\ 7.2}$ 230 mμ, $\lambda_{max}^{pH\ 12.0}$ 283 mμ, $\lambda_{min}^{pH\ 12.0}$ 244 mμ.

When the above procedure was applied to uridine, uracil was detected as a single UV-absorbing product on paper chromatogram.

Reaction of Ethylene Oxide with Uridine 5′-Phosphate——A typical experiment is as follows.

To an aqueous solution (2 ml.) of disodium uridine 5′-phosphate (ca. 20 mg.), pH 5, was added 50% aqueous ethylene oxide solution (0.5 ml.) and the mixture was left at room temperature. PPC (solvent system No. 1) was performed after 28 days giving a chromatogram shown in Fig. 2. The number attached to each spot corresponds to that attached to compounds mentioned on chart 1.

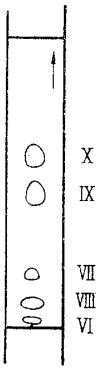

Fig. 2.

Spot (VI) is a very faint one and corresponds to the starting material, uridine 5′-phosphate.

Compound (VII) is shown to be uridine 5′-phosphate 2-hydroxyethyl ester by comparison with the specimen prepared by condensation of uridine 5′-phosphate with ethylene glycol in the presence of dicyclohexyl carbodiimide. Comparison of these compounds was performed by PPC (solvent system No. 1 and 3), PEP (two dimentional; PPC by solvent system No. 1 and then PEP at pH 9.2) and by UV absorption spectrum.

UV absorption spectrum of VIII does not change from neutral to alkaline pH; λ_{max} 262 mμ. In addition to this, the result of two dimensional PEP, M_{UMP}=1.0, indicates that VIII is 3-(2-hydroxyethyl)uridine 5′-phosphate. This indication was further confirmed by the following evidence.

Preparative PPC followed by elution of the zone of VIII with H_2O and subsequent lyophilization of the elute gave an amorphous powder of VIII. The powder was treated with beef intestinal alkaline phosphatase (Sigma Chem. Co,) in 0.1*M* Tris-buffer (pH 8.0) at 37° for 3 hr. PPC of the reaction mixture revealed that VIII was cleaved to IV and inorganic phosphate (solvent system No. 1 and 2). Control solution lacking enzyme from above mixture gave a single spot of VIII.

Spot (IX), which is the thickest one among these spots, showed λ_{max} 262 mμ in its UV absorption spectrum at both neutral and alkaline media. M_{UMP}=0.8 at pH 9.2.

An amorphous powder of IX was prepared employing a method mentioned above in the case of VIII. The powder was treated with crude snake venom for 5 hr. at 37° in 0.1*M* Tris-buffer at pH 8.0 in the presence of a trace amount of Mg^{++}. PPC of the enzyme-treated mixture gave a single UV-absorbing spot corresponding to that of IV (solvent system No. 1, 2 and 3). Control solution which lacks enzyme from above mixture showed a single spot of IX. From these evidences, IX is believed to be 3-(2-hydroxyethyl)uridine 5′-phosphate 2-hydroxyethyl ester.

Similar treatment of X (M_{UMP}=0.8) with crude snake venom yielded V (PPC with solvent system No. 1), so the compound (X) is assumed to be a substance produced on further alkylation of IX at the hydroxyl group(s) in its sugar portion.

Uridine 5′-Phosphate-2-hydroxyethylester (VII), from Uridine 5′-Phosphate and Ethylene Glycol by Treatment with Dicyclohexyl Carbodiimide——A mixture consisted of pyridinium uridine 5′-phosphate (1.1 mmoles), ethylene glycol (30 ml.), dicyclohexyl carbodiimide (13 g., 5.5 mmoles) and Me_2CO (20 ml.)*6 was placed in a well stoppered flask and kept at room temperature for 2 days. PPC (solvent No. 1) gave two UV-absorbing spots of the desired phosphodiester (VII) and uridine.*7 Dicyclohexyl urea which precipitated from the reaction mixture was removed by filtration. To the filtrate was added 13 ml. of MeOH saturated with $BaBr_2$ and the mixture was left standing at room temperature for 1 day. Crude barium salt of VII that separated out was collected by centrifugation and washed several times with

*6 Dry acetone was added to make the mixture more homogeneous.

*7 Uridine was probably derived from the diester (VII). Precise investigation at this point is now under way.

warm Me_2CO to remove uridine. The barium salt was then dissolved in 2 ml. of H_2O, equal volume of EtOH was added to the solution and a small amount of barium uridine 5′-phosphate precipitated was removed by centrifugation. Supernatant solution of VII was concentrated to dryness under diminished pressure, the residue repeatedly treated as above to remove uridine 5′-phosphate completely, and finally washed with warm Me_2CO giving colorless powder of barium uridine 5′-phosphate 2-hydroxyethyl ester. Dried material over $CaCl_2$ weighed 95 mg. *Anal.* Calcd. for $C_{11}H_{16}O_{10}N_2PBa_{1/2}\cdot 5H_2O$: C, 25.02; H, 4.97; N, 5.31. Found : C, 25.18; H, 4.48; N, 5.62. $M_{UMP}=0.8$ at pH 9.2.

The barium salt of VII (50 mg.) was dissolved in H_2O (1 ml.) and treated with 50% ethylene oxide (0.7 ml.) at room temperature. After 3 days a single spot of IX was detected on paper chromatogram. On standing further, alkylation of hydroxyl group(s) in sugar moiety was observed.

Upon treatment of VII with *N* $Ba(OH)_2$ for 2 hr. at 70~80°, uridine was detected as a sole UV-absorbing spot on paper chromatogram.

Incubation of VII in 0.1*M* Tris-buffer, pH 8.0, with crude snake venom at 37° for 5 hr. in the presence of Mg^{++} produced uridine as a single UV-absorbing product.

The authors are indebted to Takeda Chemical Industries for the gift of the starting materials. Thanks are due to Mr. K. Tamaki for carring out the measurement of ultraviolet absorption spectra. They also express deep thanks to the members of the Central Analysis Room of the Faculty of Pharmaceutical Sciences, University of Tokyo for performing the micro analyses and carring out the measurement of IR spectra.

Summary

The reaction of ethylene oxide with uridine derivatives in an aqueous solution at room temperature was investigated. 1-Methyluracil gave 1-methyl-3-(2-hydroxyethyl)-uracil (II) quantitatively upon reaction with ethylene oxide at pH 10. The reaction of uridine with ethylene oxide at neutral pH results in the formation of 3-(2-hydroxyethyl)-uridine (IV) quantitatively. These products, II and IV, were isolated as crystals and the structure well defined. The rate of N^3-alkylation of uridine increases with the increase of pH value. This indicated that the reaction proceeds through the dissociated form of uridine. At a high-pH region, alkylation of the hydroxyl group(s) of ribose moiety took place concomitantly with the N^3-alkylation and gave multiply 2-hydroxyethylated derivatives of uridine. In the reaction of uridine 5′-phosphate with ethylene oxide, besides the N^3-hydroxyethylation, phosphodiester formation was also observed. In a high-pH solution, the hydroxyethylation of sugar-hydroxyl group(s) took place as in the case of uridine. Synthesis of uridine 5′-phosphate 2-hydroxyethyl ester by the reaction of uridine 5′-phosphate with ethylene glycol in the presence of dicyclohexyl carbodiimide was described.

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