[Chem. Pharm. Bull. 12(10)1240~1246(1964)] UDC 612.398.145

173. Hatsuhiko Mizuno, Harumi Okuyama, Hikoya Hayatsu, and Tyunosin Ukita : Modifications of Nucleosides and Nucleotides. II.*1 Reaction of Ethylene Oxide with 1-Methylcytosine.

(*Faculty of Pharmaceutical Sciences, University of Tokyo**2)

Ethylene oxide, as an alkylating agent, has been reported to induce a gene mutation of *Drosophila*,[1] which could have been ascribed to some modifications by this reagent of nucleic acids in the organism.

It seems to be important to have a fundamental knowledge on the mode of reactions of this agent with constituents of nucleic acids, in order to understand the structural modifications occurring in the nucleic acids.

The mode of reaction of ethylene oxide under mild conditions with adenosine,[2] deoxyguanosine,[3] uridine,[4] and uridylic acid[4] has recently been reported. As for the reaction of this reagent with cytidine or its derivatives, however, no available data have been encountered.

The present communication deals with the mode of reaction of ethylene oxide with 1-methylcytosine to obtain a fundamental knowledge about the alkylation of cytidine with this reagent.

The results obtainable from the reaction of 1-methyl counterparts of nucleosides with a given reagent have often played important roles in the elucidation of the structure of chemically modified nucleic acids.[4~6]

When 1-methylcytosine was treated with aqueous ethylene oxide at room temperature, the pH of the reaction mixture was elevated gradually and formation of three new products was observed by paper chromatography and paper electrophoresis. Two of these products, Ⅱ and Ⅲ, have cationic charges under neutral conditions, and, therefore, were assumed to have quaternary-base structures. The third product, Ⅳ, was identical with an authentic specimen of 1-methyl-3-(2-hydroxyethyl)uracil prepared by alkylation of 1-methyluracil with ethylene oxide.[4]

The first step of the reaction was the formation of Ⅱ, then compound (Ⅲ) came to appear, followed by Ⅳ. After standing for a long period, the sole ultraviolet-absorbing material in the reaction mixture was Ⅳ.

In order to isolate the intermediates, Ⅱ and Ⅲ, the reaction was carried out in an anhydrous medium, expecting the prevention of the hydrolysis reactions, presumably occurring in the conversion of Ⅱ or Ⅲ to Ⅳ.

In anhydrous methanol, compound (Ⅰ) reacted a little more slowly with ethylene oxide than in aqueous medium, and from the mixture, by fractional extraction using ether and acetone, Ⅱ and Ⅲ were successfully isolated as crystals.

The 1-methyl-3-(2-hydroxyethyl)cytosine structure was assigned to the compound (Ⅱ). This assignment was based on the following observations : (1) the results of the elemental

*1 Part I. T. Ukita, H. Okuyama, H. Hayatsu : This Bulletin, **11**, 1399 (1963).
*2 Hongo, Tokyo (水野初彦, 奥山治美, 早津彦哉, 浮田忠之進).
1) I. A. Rapoport : Doklady Akad. Nauk, S. S. S. R., **60**, 469 (1948).
2) H. G. Windmueller, N. O. Kaplan : J. Biol. Chem., **236**, 2716 (1961).
3) *Idem* : Biochim. et Biophys. Acta, **61**, 307 (1962).
4) T. Ukita, H. Okuyama, H. Hayatsu : This Bulletin, **11**, 1399 (1963).
5) D. M. Brown, P. Schell : J. Mol. Biol., **3**, 709 (1961).
6) H. Hayatsu, T. Ukita : Biochem. Biophys. Res. Commun., **14**, 198 (1964).

analysis support the structure; (2) II has a strong basicity (pKa 9.6), characteristic of N-alkylated iminopyrimidines;[7] (3) ultraviolet spectrum of II closely resembles that of 1,3-dimethylcytosine;[8] (4) II is the first intermediate appeared throughout the reaction steps, and (5) II is convertible into the N^3-alkylated compound, IV, in an aqueous solution. The elevation of pH in the reaction mixture of I with aqueous ethylene oxide can be attributed to the strong basic property of the reaction product, II.

The second intermediate, III, was found to be produced not only from II but also from 1-methyl-N-(2-hydroxyethyl)cytosine (V)*3 by treatment of these compounds with aqueous ethylene oxide. Consequently, it was concluded that compound (III) should be assigned the 1-methyl-N,3-bis(2-hydroxyethyl)cytosine structure. This structural assignment was confirmed by elemental analysis.

The product, III, also has strong basicity (pKa 9.4), and showed ultraviolet absorption maximum at somewhat longer wave length than that of II (Table I). In an aqueous solution, III was hydrolysed to IV, but, different from the case of II, with simultaneous liberation of ethanolamine. The over-all reaction is summarized in Chart 1.

Table I. The pKa and Spectral Data

Compounds	pKa[a]	λ_{max} mμ (ε)	
		Neutral form	Protonated form
1-Methylcytosine (I)	4.55[9]	274 (8, 200)	283 (12, 300)
1-Methyl-3-(2-hydroxyethyl)cytosine (II)	9.6	276 (8, 000)	283 (10, 900)
1-Methyl-N,3-bis(2-hydroxyethyl)cytosine (III)	9.4	285 (7, 750)	291 (12, 700)
1-Methyl-N-(2-hydroxyethyl)cytosine (V)	4.0	275 (10, 100)	287 (13, 800)
		275[9]	288[9]
1,3-Dimethylcytosine[8]	9.4	272	280.5

a) Determined spectroscopically.

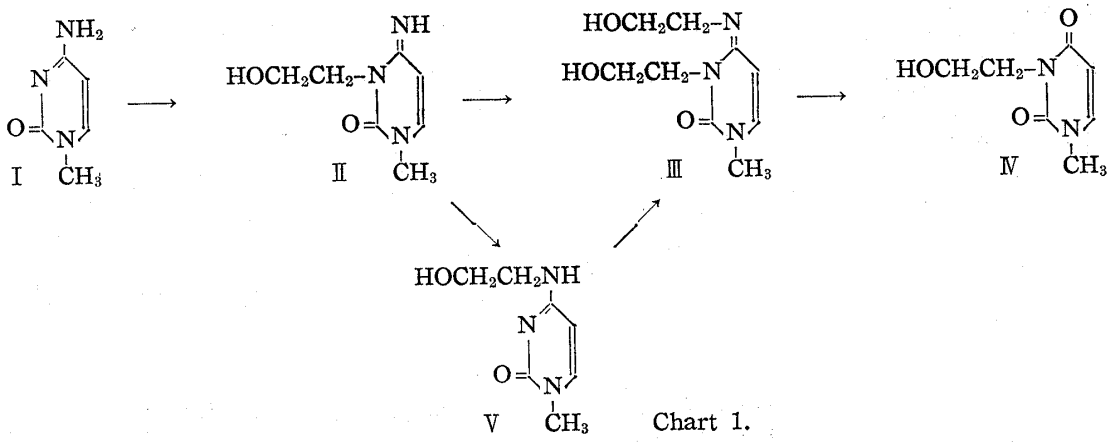


Chart 1.

By use of paper chromatography and paper electrophoresis, the reaction rate of each step in the whole reaction course as well as the pH-dependence of the reaction rates were estimated.

1-Methylcytosine was incubated in 9% aqueous ethylene oxide at 25° and at pH's of 3, 5, 7 and 10. The pH's of the mixtures were adjusted and maintained by appropriate

*3 See following descriptions.

7) D. J. Brown, J. S. Harper : J. Chem. Soc., **1963**, 1276.
8) P. Brookes, P. D. Lawley : *Ibid.*, **1962**, 1348.
9) T. Ueda, J. J. Fox : J. Am. Chem. Soc., 85, 4024 (1963).

Table II. The Rate of Reaction of 1-Methylcytosine (I) with Ethylene Oxide at Various pH's[a]

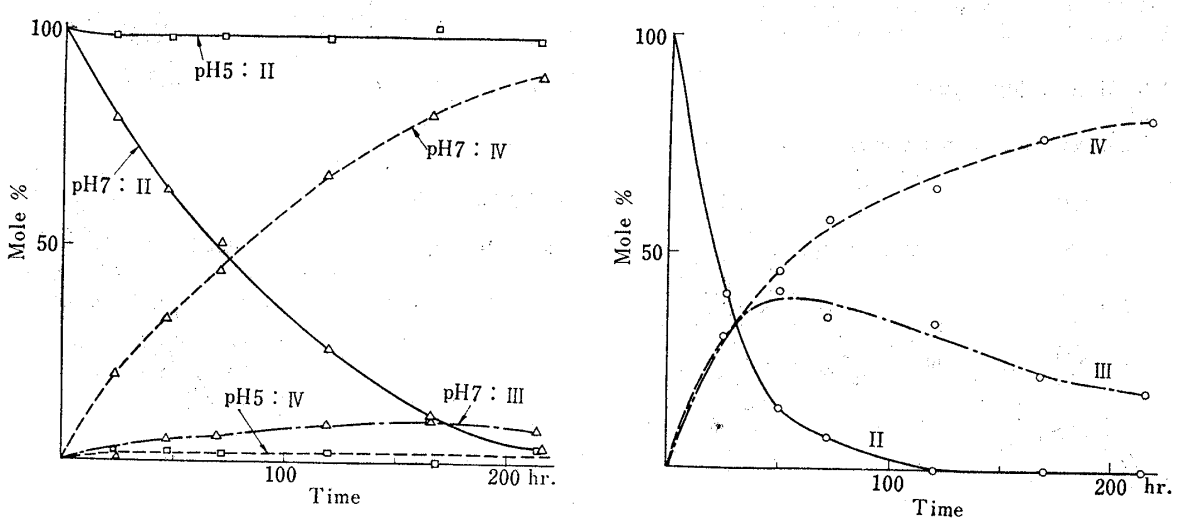
pH	Molar ratio of products (%)	
	1-Methylcytosine (I)	1-Methyl-3-(2-hydroxyethyl)cytosine (II)
3[b]	100	—
5	82	18
7	82	18
10	82.5	17.5

a) Reactions were performed at 25° for 9 hr. As for the method in detail, see Experimental.
b) In this case, the reaction mixture was allowed to stand at room temperature for three days.

addition of perchloric acid or sodium hydroxide.*4 The results are given in Table II. It can be seen that no reaction occurred at pH 3, and at pH's above 5, there existed practically no difference in the yield of II in each reaction mixture after 9 hours' incubation.

These results clearly indicate that a protonated form of I (pKa 4.55) can not attack ethylene oxide, and that at pH above 5, the N^3 ring-nitrogen of neutral form of I is participating in nucleophilic substitution reaction at the carbon atom of ethylene oxide, affording II as the product.

The rates of conversions of II into the products, III and IV, were determined and the results are given in Fig. 1. The steps involved in the total reaction are, the alkylation



a) pH 5 and pH 7 b) pH 10

Fig. 1. Reaction of 1-Methyl-3-(2-hydroxyethyl)cytosine (II) with Ethylene Oxide at Various pH's[a]

a) Reactions were carried out at 25°. Other experimental conditions are described in Experimental.

of II to III, hydrolysis of III to IV, and the direct hydrolysis of II to IV. As can be seen in Fig. 1, the rate of reactions strongly depends on the pH. Thus, at pH 5, practically no reaction occurred between II and the reagent up to 200 hours (Fig. 1a); at pH 7, a remarkable decrease of II paralleled with the formation of IV was observed, but at this

*4 We could not find any buffer solution available to fix the pH of this reaction mixture. Even though an acetate buffer (pH 5) has been used as a solvent in the reaction of ethylene oxide with TMV-RNA (H. Fraenkel-Conrat : Biochim. et Biophys. Acta, 49, 169 (1961)), we found out that the acetate buffer itself had reacted with ethylene oxide to raise the pH during the reaction (see Experimental).

pH only a small amount of Ⅲ accumulated during the reaction (Fig. 1a); at pH 10, the disappearance of Ⅱ was more rapid than that at pH 7, and the accumulation of a fairly large amount of Ⅲ, which reached the maximum at the first 50 hours of the reaction time, was observed (Fig. 1b).

Hydrolysis of Ⅱ and Ⅲ at pH's 5, 7 and 10 was performed, and the amounts of the product, Ⅳ, were plotted against reaction time (Figs. 2 and 3).

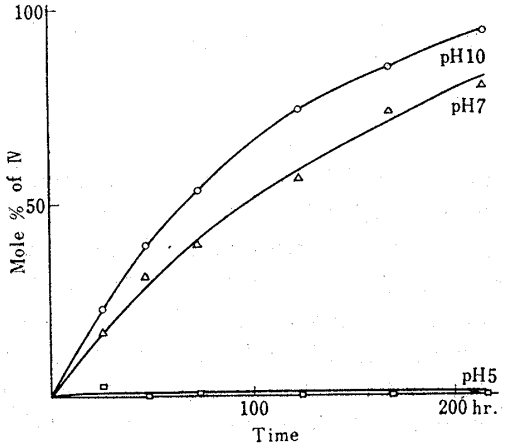


Fig. 2. Hydrolysis of 1-Methyl-3-(2-hydroxyethyl)cytosine (Ⅱ) to 1-Methyl-3-(2-hydroxyethyl)uracil (Ⅳ) at Various pH's and at 25°

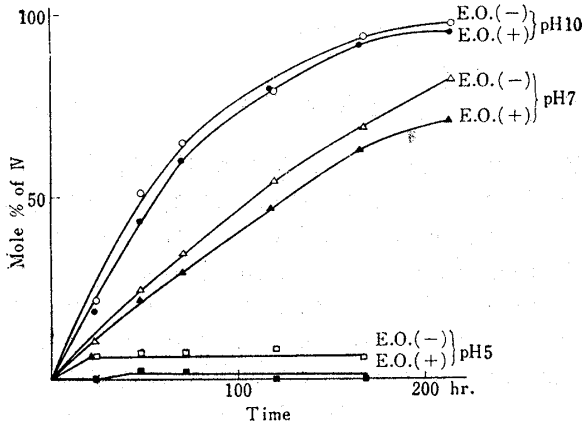


Fig. 3. Hydrolysis of 1-Methyl-N,3-bis(2-hydroxyethyl)cytosine (Ⅲ) at Various pH's in the Presence or Absence of Ethylene Oxide[a)]

a) Reaction conditions are given in Experimental. E.O. (+) and (−) represent the presence and absence of ethylene oxide, respectively.

As is given in Fig. 2, at pH 5 no hydrolysis of Ⅱ could be observed, whereas a high rate of hydrolysis was found at pH's 7 and 10, and the rate at pH 10 was somewhat higher than that at pH 7.

The pH-profile of hydrolysis of Ⅲ (Fig. 3) is almost similar to that of Ⅱ. Thus, the curves in Fig. 3 indicating the production of Ⅳ at three pH's are almost superimposable with those in Fig. 2. The presence of ethylene oxide in the reaction mixtures gave little, if any, effect on the rate of formation of Ⅳ from Ⅲ at each pH tested.

It is interesting that in the rate of formation of Ⅲ from Ⅱ (Fig. 1) there existed a large difference between the reactions at pH 7 and 10, while in the production of Ⅳ from Ⅱ or Ⅲ by hydrolysis there existed only a small difference between the reactions at these pH's (Figs. 2 and 3). Furthermore, the actual difference between the formation of Ⅲ from Ⅱ at pH 7 and that at 10 should still be larger than that can be seen in Fig. 1, because the rate of production of Ⅳ from Ⅲ at pH 10, as is indicated in Fig. 3, is higher than that at pH 7. These characteristics in the pH-profile of the reactions described above could be interpreted in terms of the reaction mechanisms proposed in the followings.

In the reaction of Ⅱ to Ⅲ, the N^4 imino-nitrogen of a neutral form of Ⅱ (pKa 9.6) would make a nucleophilic attack at a carbon atom of ethylene oxide molecule. Thus, the reaction rate at pH 7 is remarkably lower than that at pH 10, *i.e.*, at pH 7 the neutral form occupies only a very small portion of the whole molecules, whereas at pH 10 the neutral form prevails over the protonated form.

In the hydrolysis of Ⅱ and Ⅲ, the main reacting species should be both the protonated forms of Ⅱ or Ⅲ and the hydroxide ions present in the reaction mixture. Concentrations of both these species would determine the reaction rate. At pH 7, the amount of the protonated forms of Ⅱ or Ⅲ (pKa 9.4) should be considerably larger than that at

pH 10, whereas the hydroxide-ion concentration should be much lower than that at pH 10. Thus, the rate of production of Ⅳ from Ⅱ or Ⅲ at pH 7 was slightly smaller than that at pH 10.

II : R=−H
III : R=−CH_2CH_2OH

Chart 2.

By treatment with 1*N* sodium hydroxide, Ⅱ underwent Dimroth rearrangement[7] to give a known compound, 1-methyl-N-(2-hydroxyethyl)cytosine.[9] In this reaction mixture, no hydrolysis product, Ⅳ, was detected by paper chromatography. This observation is in agreement with the proposed mechanism of hydrolysis of Ⅱ. Thus, the attack of an excess of hydroxide ions to the neutral forms of Ⅱ cleaved the C^2-N^3 linkage in pyrimidine ring and the subsequent reclosure between the C^2 and the imino nitrogen resulted in the formation of N-alkylated compound, Ⅴ. In this reaction condition, hydrolysis of Ⅱ to Ⅳ could not take place since the protonated form of Ⅱ was practically absent at pH 14.

Experimental*5

Methods——To prevent a loss of volatile ethylene oxide, the reaction of this agent was carried out in an equipment described in the previous paper.[4] Aliquots were taken up from the reaction mixture under ice-cooling.

Paper chromatography (PPC) was performed ascendingly on Toyo Roshi No. 53. Unless otherwise mentioned, 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) EtOH-conc. NH_4OH-H_2O (80:2:8). Rf values are summarized in Table Ⅲ.

Table Ⅲ. The Rf's in Paper Chromatography and the Mobilities in Paper Electrophoresis

Compounds	Rf's in solvent systems			Moving distances to cathode (cm.)
	1	2	3	
1-Methylcytosine (Ⅰ)	0.58	0.33	0.62	5.7
1-Methyl-3-(2-hydroxyethyl)cytosine (Ⅱ)	0.77	0.20	0.77	9.7
1-Methyl-N,3-bis(2-hydroxyethyl)cytosine (Ⅲ)	0.82	0.22	0.85	8.0
1-Methyl-3-(2-hydroxyethyl)uracil (Ⅳ)	0.78	0.65	0.82	5.7
1-Methyl-N-(2-hydroxyethyl)cytosine (Ⅴ)	0.64	0.42	0.72	5.7

Paper electrophoresis (PEP) was performed at 20 v./cm. for 60 min. using, unless otherwise noted, 0.02*M* borax solution (pH 9.4). The moving distances in PEP of the compounds tested are summarized in Table Ⅲ.

The spots on paper were detected by scanning over ultraviolet lamp. The measurement of UV spectra was carried out with a Cary Model 11 recording spectrophotometer.

1-Methyl-3-(2-hydroxyethyl)cytosine (II)——To an ice-cooled solution of 1.0 g. (8 mmoles) of 1-methylcytosine[10] in 54 ml. of abs. MeOH was added 6 ml. of ethylene oxide. The mixture was well stoppered and kept at 36~38° for 48 hr. The solvent was evaporated and from the residue 1-methy-N,3-bis(2-hydroxyethyl)cytosine (Ⅲ) was extracted with Et_2O using Soxhlet extraction apparatus. After complete removal of Ⅲ, which could be detected by PEP, the residue was then extracted with acetone. Acetone was evaporated from the latter extract, and the residue was recrystallized from EtOH to give 431 mg.

*5 All melting points are uncorrected.

10) E. H. Flynn, J. W. Hinman, E. L. Caron, D. O. Woolf, Jr. : J. Am. Chem. Soc., **75**, 5867 (1953).

(2.55 mmoles; 32%) of II as colorless needles which melted at 191~193°. This product gave a single spot on PEP and the melting point was raised to 194~196° by repeated recrystallization from EtOH. *Anal.* Calcd. for $C_7H_{11}O_2N_3$: C, 49.69; H, 6.55; N, 24.84. Found : C, 50.01; H, 6.73; N, 24.73.

1-Methyl-N,3-bis(2-hydroxyethyl)cytosine (III)——The Et_2O extract, obtained from the reaction product of 1-methylcytosine with methanolic ethylene oxide as stated above, was concentrated. After complete removal of Et_2O, the residue was recrystallized from benzene yielding 309 mg. (1.45 mmoles; 18%) of III as colorless needles, m.p. 100.5~102.5°. Since this material contained a trace amount of II detectable in PEP, recrystallization was repeated twice from benzene to give an electrophoretically pure substance, m.p. 103~104.5°. *Anal.* Calcd. for $C_9H_{15}O_3N_3$: C, 50.69; H, 7.09; N, 19.71. Found : C, 50.59; H, 6.96; N, 19.77.

From the residue of the successive extraction with Et_2O and acetone in the above experiments, 68 mg. (0.55 mmoles) of 1-methylcytosine was recovered by recrystallization from MeOH.

Reaction of 1-Methylcytosine with Ethylene Oxide in Aqueous Solution. a) Identification of the Reaction Products——Three products formed in the reaction mixture of I with aqueous ethylene oxide were identical with II, III and IV, the former two compounds being prepared by reaction of I in methanolic ethylene oxide and the last compound, IV, by reaction of 1-methyluracil with aqueous ethylene oxide.[4] The identification was performed by PPC (solvent 1~3) and PEP and by comparison of UV spectra.

b) Preliminary Experiments——To a 2 ml. aqueous solution of ca. 10 mg. of 1-methylcytosine (pH 7.5) was added 0.3 ml. of ethylene oxide. The reaction tube was well stoppered and set aside at room temperature. After 5 days, the pH of the reacton mixture was elevated to 10.8, and four compounds, I, II, III and IV, were detected by PPC (solvent 1) followed by two dimensional PEP using acetate buffer of pH 5.8. After keeping for 39 days, compound (IV) was the sole UV absorbing material in the reaction mixture.

c) Effect of pH on the Reaction——The pH's of 2 ml. aqueous solutions, each containing 4 mg. of 1-methylcytosine were separately adjusted to 5, 7 and 10 with 0.1*N* $HClO_4$ or 0.1*N* NaOH. To each solution was added 0.2 ml. of ethylene oxide and the mixtures were kept at 25°. After every 3 hr's interval, the pH's of the solutions were adjusted under ice-cooling. By this treatment, the pH's could be controlled within 1 pH-unit of the original pH values. Reaction was continued for 9 hr. Aliquots were withdrawn from each mixture and applied to PEP using buffer of pH 5.8. The paper after electrophoresis was submitted to two-dimensional ascending PPC using solvent system 3. The spots of I and II were extracted with 5 ml's of 0.05*M* Tris-buffer (pH 7.0) and the extracts were submitted to the spectrophotometrical estimation by determining the absorption at λ_{max} of each compound. The results are given in Table I. In the case of the solution of pH 7 and 10, very faint spots of III and IV were observed, but their quantitative estimation was not performed. In the solution of pH 3, no reaction was observed within 3 days at room temperature.

Reaction of 1-Methyl-3-(2-hydroxyethyl)cytosine (II) with Ethylene Oxide——Each 6 mg. of compound (II) was dissolved in a small amount of 0.02*N* $HClO_4$ and the pH of solution was adjusted to 5 and 7 with additional amount of the $HClO_4$ solution. Water was added to each mixture to make final volume of 2.7 ml. Another 6 mg. of II was dissolved in H_2O to make 2.7 ml. solution which showed pH of 10.

To each solution thus prepared was added 0.3 ml. of ethylene oxide and the solution kept at 25°. Aliquots were taken up at intervals and applied to PEP and the spots of II, III and IV separated were eluted with 0.05*M* Tris-buffer (pH 7.0) and submitted to spectrophotometrical estimation. During these reactions, the pH's of reaction mixtures were practically constant. The results are given in Fig. 1.

Hydrolysis of 1-Methyl-3-(2-hydroxyethyl)cytosine (II)——Aqueous solutions of II having pH's of 5, 7 and 10 were similarly prepared as stated above excepting the addition of ethylene oxide and kept at 25°. Aliquots were withdrawn at intervals and the formation of IV was estimated by PPC (solvent 2) and quantitatively determined by subsequent spectrophotometrical analysis. The results are given in Fig. 2.

Hydrolysis of 1-Methyl-N,3-bis(2-hydroxyethyl)cytosine (III)——Two series of three 3.0 ml. solutions of pH 5, 7 and 10 each containing 6 mg. of III were prepared similarly to the above experiment, one series of which containing ethylene oxide. The formation of IV was followed by PPC (solvent 2) and the rate was determined by spectrophotometry. The results are given in Fig. 3.

Detection of Ethanolamine formed in the Hydrolysis of III to 1-Methyl-3-(2-hydroxyethyl)uracil (IV)——An aqueous solution of III (pH 10) was kept at room temperature for seven days. The mixture was applied to PPC run in three solvent systems and the spot which colored reddish brown with Ninhydrin was found identical with that of ethanolamine. The Rf's of ethanolamine found are 0.43 (solvent 1), 0.31 (BuOH–AcOH–H_2O; 4:1:5) and 0.08 (BuOH–H_2O; 86:14).

1-Methyl-N-(2-hydroxyethyl)cytosine (V). Dimroth Rearrangement of 1-Methyl-3-(2-hydroxyethyl)-cytosine (II)——A mixture of 200 mg. of II and 2 ml. of *N* NaOH was heated on a water bath at 90~95° for 15 min. PPC of the reaction mixture revealed that V was the sole UV absorbing material in the mixture. The ice-cooled mixture was applied to a Dowex 50 (NH_4^+-form, 50~100 mesh) column (0.9×7 cm.) and eluted with H_2O. The eluate was evaporated and the oily residue was extracted with EtOH. On removal of the solvent from the ethanolic extract, and trituration of the residue with acetone, 29 mg.

of needles were obtained. This was recrystallized from aceton to colorless, somewhat hygroscopic needles, mp. 156~158° (reported 161~163°[9]). *Anal.* Calcd. for $C_7H_{11}O_2N_3 \cdot \frac{3}{4}H_2O$: C, 46.02; H, 6.90; N, 23.00. Found : C, 45.81; H, 7.01; N, 22.50.

The UV absorption maxima of this compound, as given in Table I, are in good accord with those of reported ones.[9] The yield of V estimated spectrophotometrically was 62%.

Reaction of 1-Methyl-N-(2-hydroxyethyl)cytosine (V) with Ethylene Oxide——To a solution of 3 mg. of V in 1.8 ml. of H_2O was added 0.2 ml. of ethylene oxide. The mixture was kept at 25° for 24 hr. Either PEP or PPC (solvent 3) gave a spot of III which had the same mobility or Rf as that of III prepared *via* alkylation of II with ethylene oxide. UV absorption spectra of the extract of the spot also showed the identity of this product with III. During this reaction, pH of the solution was elevated as in the case of that of 1-methylcytosine with ethylene oxide.

Influences of Ethylene Oxide on pH Value of Buffer Solutions——A mixture of 0.2 ml. of ethylene oxide with 1.8 ml. of 0.1*M* acetate buffer, pH 5.0, was kept at 25°. The pH of the mixture as estimated after 1, 4 and 8 days were 6.0, 6.8 and 7.0, respectively. A mixture of ethylene oxide with 0.2*M* borate buffer, pH 7.0, showed pH of 8.4 and 8.8 after keeping for 1 and 2 days. These observations indicated that ethylene oxide reacted with the constituents of the buffers.

Summary

The reaction of aqueous ethylene oxide, a biologically important alkylating agent, with 1-methylcytosine (I) was investigated. At pH above 5, a hydroxyethylation was found to take place at N^3-position of I to yield 1-methyl-3-(2-hydroxyethyl)cytosine (II). When the pH of the reaction mixture was higher than 7, the reaction was followed by a further alkylation at the N^4-position of II to give 1-methyl-N,3-bis(2-hydroxyethyl)-cytosine (III) and was also followed by hydrolysis of these products, II and III, to 1-methyl-3-(2-hydroxyethyl) uracil (IV). Mechanisms of these reactions are discussed in the light of reaction-rate differences at various pH's. At pH 14, II underwent Dimroth rearrangement to give 1-methyl-N-(2-hydroxyethyl)cytosine (V).

(Received July 13, 1964)