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Selective Chemical Alkylation of Nucleotides with Triethyloxonium Fluoroborate*

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Triethyloxonium fluoroborate (TEOF), which is one of the ethylating agents, was substantially inactive to adenosine 5′-monophosphate (AMP) at various pHs. TEOF reacted on cytidine 2′(3′)-monophosphate (CMP), uridine 2′(3′)-monophosphate (UMP) and guanosine 5′-monophosphate (GMP) in pH 7—11 buffer solutions having a moderate ionic strength ($\mu \simeq 0.6$) to give the corresponding ethylated nucleotides. The extent of ethylation was influenced by the pH and especially the ionic strength of the buffer employed. At lower ionic strength ($\mu < 0.2$) TEOF was much less reactive toward CMP and UMP.

When ribonucleic acid (RNA) was treated with TEOF in a buffer solution at pH 8.9 and $\mu = 0.1$ and hydrolyzed with hydrochloric acid, the resulting hydrolysate contained approximately 50% of the ethylated guanine residue by base analysis. Deoxyribonucleic acid (DNA) was ethylated to a similar extent under the same conditions. In both cases, the chromatographic analysis revealed that uracil (thymine), cytosine, and adenine residues were left practically intact. The ethylated DNA contained a small amount of dialyzable material which was identical with 7-ethylguanine. The physical properties showed that DNA on ethylation underwent partial loss of tertiary structure although the molecule retained considerable double strandedness.

Alkylation of the biomacromolecular substances has aroused much interest[2] in connection with mutagenic, carcinogenic and antitumor effects in recent years. Chemical alkylation[3] with a number of alkylating reagents has been extensively employed for the modification of polynucleotides and nucleic acids providing the physico-chemical and biological information, but the chemical process was not so simple because of polyfunctionality of the polynucleotides. The present paper describes a selective method for ethylation of the guanine residues in deoxyribonucleic acid (DNA) and ribonucleic acid (RNA) upon treatment with triethyloxonium fluoroborate (Meerwein reagent) (TEOF) under particular reaction conditions.

Experimental[4]

Substrates and Reagent——GMP disodium salt was obtained from the Seikagaku Kogyo Co. Ltd. AMP, UMP sodium salt and GMP were purchased from the Kohjin Chemical Co. Yeast RNA was kindly supplied by Dr. A. Kuninaka, Laboratory of the Yamasa Shoyu Ind., and salmon DNA was purchased from the Sigma Chemical Co. TEOF was prepared according to the literature.[5]

Buffers——Buffers used in this study were phosphate for the range of pH 6 to 8 and carbonate for the range of pH 9 to 11 having a range of ionic strengths (μ) between 0.1 to 1.0.

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* Dedicated to the memory of Prof. Eiji Ochiai.

1) Location: *Aobayama, Sendai.*

2) P.D. Lawley, "Progr. Nucl. Acid Res. Mol. Biol.," Vol. 5, ed. by J.N. Davidson and W.E. Cohn, Academic Press Inc., New York, 1966, p. 89; J.W. Drake, "The Molecular Basis of Mutation," Holden-Day, San Francisco, 1970; L. Fishbein, W.G. Flamm, and H.L. Falk, "Chemical Mutagens," Academic Press Inc., New York, 1970.

3) W.G. Verly, *Biochem. Pharmac.*, 23, 3 (1974).

4) Abbreviation used: GMP, guanosine 5′-monophosphate; AMP, adenosine 5′-monophosphate; UMP, uridine 2′(3′)-monophosphate; CMP, cytidine 2′(3′)-monophosphate; UR, uridine; Et-UMP, 3-ethyluridine 2′(3′)-monophosphate; Et-CMP, 3-ethylcytidine 2′(3′)-monophosphate; Et-GMP, 7-ethylguanosine 5′-monophosphate.

5) H. Meerwein, "Organic Synthesis," Vol. 46, ad. by W.G. Dauben, John Wiley and Sons, Inc., New York, 1966, p. 113.

Ethylation Procedure——An aqueous solution of mononucleotides (25 μmol) in the buffer and 10 molar equivalents of TEOF were shaken together at 20° for 60 min. In case of nucleic acids, a solution of substrates and 5 equivalents of TEOF in carbonate buffer, pH 8.9 ($\mu=0.1$), was incubated at 20° for 60 min. The reaction mixture was dialyzed overnight against 10^{-3} M sodium chloride solution or passed through a column of Sephadex G10, and then lyophilized.

Paper Chromatography——Toyo Roshi No. 51A and Whatmann No. 3MM papers were used for descending paper chromatography with the following solvent systems: A, MeOH–conc. HCl–H_2O (7: 2: 1); B, phenol–*tert*–BuOH–HCOOH–H_2O (52: 3: 5: 50); C, isobutyric acid–0.5 N NH_4OH (10: 6); D, 95% EtOH–1 M $AcONH_4$ (15: 6); E, EtOH–H_2O–conc. NH_4OH (80: 18: 2); F, MeOH–EtOH–conc. HCl–H_2O (50: 25: 6: 19).

3-Ethyluridine——To a solution of uridine (1 g) in 83 ml of carbonate buffer (pH 9.4, $\mu=0.6$) was added 10 molar equivalents of TEOF portionwise with vigorous stirring. After stirring for 60 min at room temperature, the reaction mixture was evaporated *in vacuo* and the residue was chromatographed on a column of silica gel ($CHCl_3$–MeOH, 95: 5). Recrystallization from acetone afforded colorless needles, mp 111—112.5°. Yield, 145 mg.

7-Ethylguanine——GMP (190 mg) was ethylated with TEOF in a carbonate buffer solution (pH 8.9, $\mu=0.6$) as described above. Ethylated GMP was heated with 1N HCl under reflux for 10 min. The hydrolyzate was applied to a 20×250 mm column of Dowex 50×2 (H^+ form). Elution of the column with 0.1 N HCl (0.25 ml/min) gave 22 mg of guanine while continued elution gave 57 mg of 7-ethylguanine. Recrystallization from water gave colorless needles (25 mg), mp>250°, which is identical with an authentic specimen of 7-ethylguanine.[6]

Analysis of Ethylated Mononucleotides——Aliquots of the ethylating pyrimidine nucleotides amounting to 20 μl were chromatographed in the system B, C, or D (UMP), and C, E, or F (CMP). The nucleotides on the strip paper were eluted with 0.1 N HCl and the concentrations were determined by measuring the optical density at the λ_{max}.[7] The results are given in Fig. 1, Fig. 2, and Fig. 3.

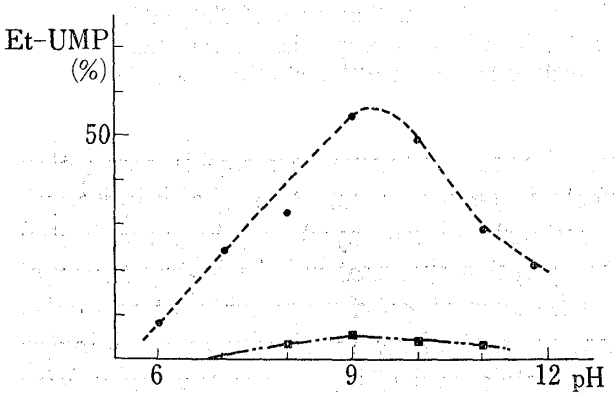

Fig. 1. Extent of Ethylation of UMP in Various Buffers

To a solution of 25 μmol of UMP sodium salt in 0.5 ml of various buffers was added 250 μmol of TEOF. The reaction mixture was shaken at 20° for 60 min. Aliquots of 20 μl were spotted on a Toyo Roshi No. 51A paper and the bases were separated by descending technique in the solvent system B, C, or D. Each spot containing UMP or Et-UMP was eluted from paper with 0.1N HCl and the concentration was determined by measuring the absorbance at 260 nm.

●——●: $\mu=0.6$, ■–·–·–■: $\mu=0.1$

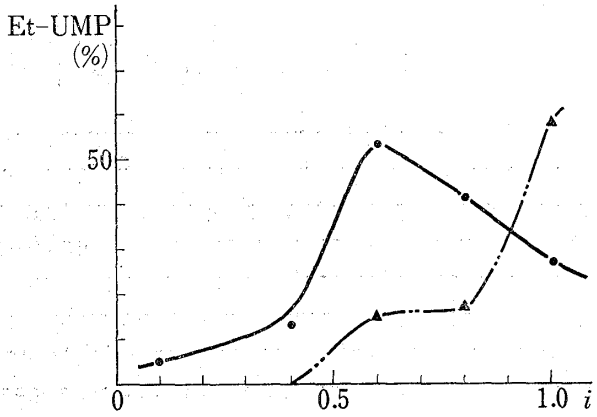

Fig. 2. Dependence of Ethylation on the Ionic Strength of Buffer Solutions

Ethylation of UMP was performed in a pH 8.9 carbonate buffer solution having various ionic strengths ($\mu=0.2$—1.0) (●—●, percent of Et-UMP). Aliquots were determined by chromatography as described in Fig. 1. Raising the ionic strength of buffer solutions the tendency of the hydrolysis of the phospholic bond increased (▲–·–·–▲, percent of UR).

●——●: pH 8.9, ▲–·–·–▲: UR

Aliquots of the ethylating purine nucleotides amounting to 0.1 ml were lyophilized. The residue was incubated with 0.1 ml of 1N HCl for 60 min at 100° in a sealed vessel. 20 μl of the hydrolysate was chromatographed in the system A. Quantitative analyses of the ethylated and unethylated purine bases were carried out by elution of the spots with 0.1 N HCl and determination of the optical density at the λ_{max}.[7] The results are given in Fig. 4. Structural assignments were in most instances made from comparison of *Rf* values and ultraviolet (UV) spectra with those of authentic compounds.

Methods for the Separation and Characterization of Ethylated Nucleic Acids——For base analysis, ethylated RNA (4.0 mg) was hydrolyzed with 0.2 ml of 1N HCl at 100° for 60 min in a sealed Pyrex tube. 20 μl of the hydrolysate was submitted to two dimensional paper chromatography (Toyo Roshi No. 51A) and mapped with the solvent system A and subsequently with the solvent system F. The concentrations of the four bases

6) B.C. Pal, *Biochem.*, **1**, 558 (1962).
7) J.D. Smith and R. Markham, *Biochem. J.*, **46**, 509 (1950).

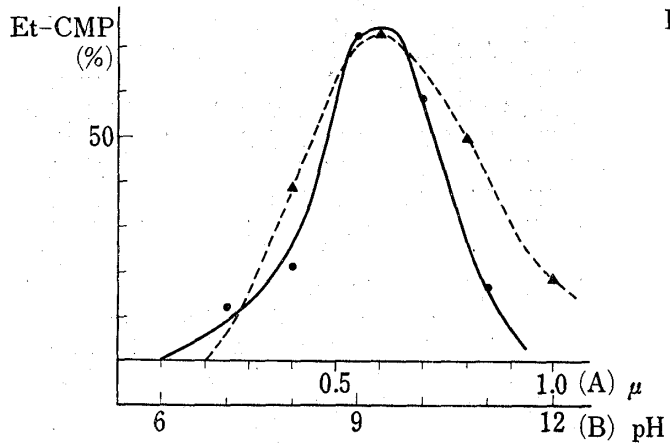

Fig. 3. Extent of Ethylation of CMP in Various Buffers

To a solution of 25 μmol of CMP in 0.5 ml of various buffers was added 250 μmol of TEOF. The reaction mixture was shaken at 20° for 60 min. Aliquots of 10 μl were separated by descending paper chromatography in the solvent system C or F. Each spot containing CMP or Et-CMP was eluted with 0.1N HCl and the concentration was determined by measuring the absorbance at 280 nm. (A) The dotted curve shows the percents of Et-CMP which were performed ethylation in buffers at pH 8.9 having various ionic strengths (scale A). (B) The solid curve shows the percents of Et-CMP which were performed ethylation in the pH range of 6—12 at the ionic strength of 0.6 (scale B).
----▲----: (A) pH 8.9, ——●——: (B) μ=0.6

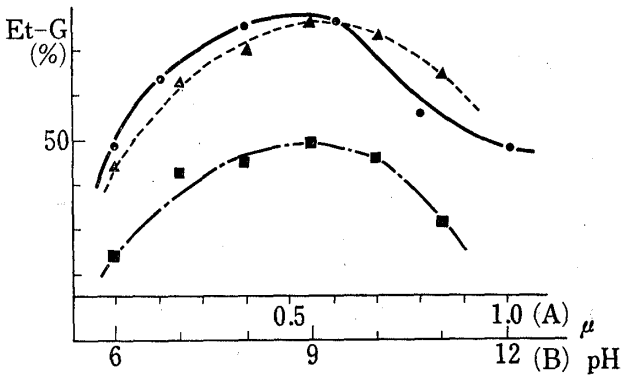

Fig. 4. Extent of Ethylation of GMP in Various Buffers

To a solution of 25 μmol of GMP disodium salt in 0.5 ml of various buffers was added 250 μmol of TEOF. The reaction mixture was shaken at 20° for 60 min and then Aliquots of 0.1 ml were lyophilized. The residue was incubated with 0.1 ml 1 N HCl at 100° for 60 min in the sealed vessel. 20 μl of hydrolysate was separated by descending paper chromatography in the solvent system A. Each spot of guanine or ethylguanine (Et-G) was eluted with 0.1N HCl and the concentration was determined by measuring the absobrance at 249 nm. (A) The solid curve shows the percents of Et-G which were performed ethylation in buffer solutions at pH 8.9 having various ionic strengths (scale A). (B) The dotted and dashed curves showed the percents of Et-G which were performed ethylation in the pH range of 6—12 at the ionic strengths of 0.6 (▲----▲) and 0.1 (■-·-·-■) (scale B).
——●——: (A) pH=8.9, ----▲----: (B) μ=0.6,
---■---: (B) μ=0.1

and the ethylated base were estimated by the spectrophotometric method.[7] Ethylated DNA (4.2 mg) was hydrolyzed with 0.1 ml of 70% $HClO_4$ at 100° for 60 min in a sealed Pyrex tube. Aliquot of the hydrolysate amounting to 10 μl was separated by two dimensional paper chromatography (Whatmann No. 3 MM) described above. The concentrations of the bases were estimated by the same manner above mentioned. The results are given Table I and Table II.

TABLE I. The Base Composition of Yeast RNA before and after Treatment with TEOF

mole % of	U_p	C_p	A_p	G_p	Et-G_p
Native yeast RNA	26.5	21.0	25.9	26.6	—
Ethylated RNA	26.1	21.3	25.8	13.5	13.3

RNA was incubated with 5 equivalents of TEOF in carbonate buffer (pH 8.9, μ=0.1) at 20° for 60 min. The reaction mixture was dialyzed overnight against 10^{-3}M NaCl aq. solution, and then lyophilized. Ethylated RNA was hydrolyzed with 1N HCl and the hydrolysate was mapped by two dimensional paper chromatography (Toyo Roshi No. 51A). The concentrations of each base were estimated by the spectrophotometric method.

TABLE II. The Base Composition of Salmon DNA before and after Treatment with TEOF

mole % of	dT_p	dC_p	dA_p	dG_p	Et-dG_p
Native salmon DNA	27.1	20.3	27.1	25.5	—
Ethylated DNA	26.9	20.1	27.4	15.0	10.6

Ethylation of DNA was performed as the described manner in RNA. The reaction mixture was passed through a column of Sephadex G10 and lyophilized. After hydrolysis with 70% $HClO_4$, the hydrolysate was mapped by two dimensional paper chromatography (Whatmann No. 3MM). The base composition of ethylated DNA was determined by the spectrophotometric method.

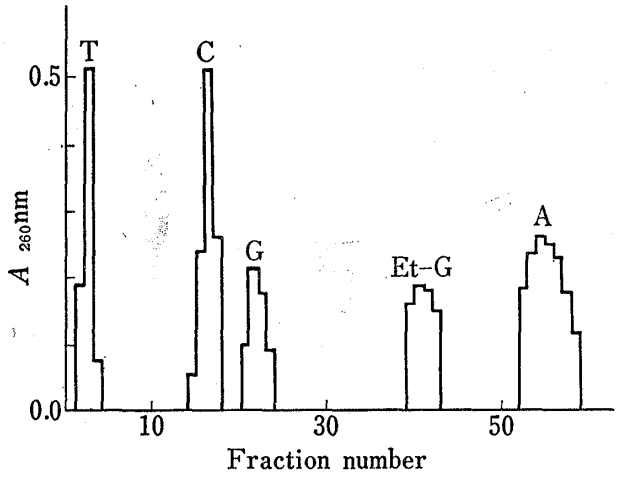

Fig. 5. Elution Profile of Ethylated DNA upon Dowex 50×2 Chromatography

The hydrolysate obtained from 1 mg of ethylated DNA was chromatographed over a column of Dowex 50×2 (4 ml) (H^+ form). The column was eluted with 0.25N HCl. Rate of flow, 0.25 ml/min, fraction size, 4 ml. Absorbances were measured at 260 nm.

Alternative separation was accomplished by column chromatography on ion-exchange resin. The hydrolysate obtained from 1 mg of ethylated DNA was put on a column of Dowex 50×2 (H^+ form, 4 ml). The column was eluted with 0.25 N HCl (rate of flow, 0.25 ml/min) and the optical densities of eluates at 260 nm were plotted using a Hitachi 124 spectrophotometer. The elution profile of the chromatogram is shown in Fig. 5.

Physicochemical Measurements——Ultraviolet absorption spectra were measured on a Hitachi 124 spectrophotometer. Optical melting curves were taken at 260 nm with a Hitachi EPS-3T spectrophotometer fitted with a thermostatted cell holder and a linear temperature programmer. The temperature of the sample was measured with a thermistor inserted into the reference liquid. Circular dichroism (CD) spectra were determined on a Jasco J-20A spectropolarimeter. The gradients were centrifuged in a RPS 65T rotor at 40000 rev./min at 4° for 6 hr. DNAs (750—870 μg) were layered on 5 ml linear 1—15% (w/v) sucrose gradient in carbonate buffer (pH 8.9, μ=0.21) or 0.5 N NaOH containing 0.1 M sodium chloride. Fractions were collected and diluted with buffer for the determination of the absorbance at 260 nm.

Results

The extent of ethylation of the nucleotides by TEOF depended not only on the pH value, but on the ionic strength of the applied buffer solutions. To determine the optimum conditions ethylation of the mononucleotides was carried out in various buffer solutions. Reaction of UMP with TEOF in low ionic strength buffer (μ=0.1) scarcely proceeded over the range of pH 7—11 and unreacted UMP was recovered over 95%. Ethylation in moderate ionic strength buffer (μ=0.6) took place smoothly, in the range between pH 8.9 to 10.2, producing about 50% Et-UMP besides UR both of which were identified by comparison of the chromatographic behavior with an authentic specimen of Et-UMP or UR. The results are given in Fig. 1.

The tendency of the hydrolysis to occur was proportional to the ionic strength of the applied buffers (Fig. 2). As the ionic strength increases TEOF would tend to be more reactive on the phosphate residue than the N-3 position in UMP. The resulting uridine ethyl phosphate is readily hydrolyzed to UR during the incubation

Fig. 3 shows the extent of ethylation of CMP as a function of pHs and ionic strengths. CMP was unreactive to TEOF in lower ionic strength (μ<0.2) buffer solutions. At the optimum conditions in the pH range of about 8.5—9.5 and in the ionic strength around 0.6, CMP gave mainly Et-CMP (up to 72%).

On the other hand, the purine nucleotides showed a characteristic profile for ethylation. AMP was much less reactive in ethylation by TEOF in the pH range of 5 to 11 and in the ionic strength range of 0.1 to 1.0. In contrast with AMP, GMP was easily alkylated with TEOF in various buffer solutions. Treatment in the buffer (pH 8.9, μ=0.1) gave 48.8% ethylation and 51.2% recovery of guanine, whereas treatment in the buffer (pH 8.9, μ=0.6) gave 76.2% ethylation and 23.8% recovery. Dependences on the ionic strength and the pH value of applied buffer on ethylation of GMP are shown in Fig. 4. Under the conditions determined as optimum, pH 8.9, μ=0.6, Et-GMP was formed as the predominant product in about 76% yield.

From the above experiments at the mononucleotide level, it would appear that TEOF is a selective ethylating agent for the guanine residue under controlled conditions. Ethylation of UMP and CMP in the buffer with lower ionic strength ($\mu \simeq 2$) gave practically no Et-UMP and Et-CMP, respectively.

When native RNA was incubated with five equivalents of TEOF at pH 8.9, $\mu=0.1$, a small amount of dialyzable material was detected in the ethylated RNA, which contained 49.6% of the ethylated guanine residue by base analysis (Table I). 7-Ethylguanine and adenine in the hydrolysate of ethylated RNA whose *Rf* values were close to each other on the paper chromatography were quite well separated on a Dowex 50×2 column by elution with 0.1 N HCl or on a two dimensional paper chromatography. Native DNA was ethylated to a similar extent under the same conditions and ethylated DNA contained 41.4% of the ethylated guanine residue (Table II). In the both cases, the paper and column chromatographic analyses revealed that uracil (thymine), cytosine and adenine residues were left unreacted.

Ethylated DNA gradually decomposed to give DNA with apurinic sites. After 500 hr in buffer (pH 8.9) the depurination went essentially to completion. The dialyzable fragments were separated on chromatography and confirmed by spectrophotometry as 7-ethylguanine (Fig. 6) which was released according to the same depurination process as after methylation of DNA.[8] Other ethylated bases and small oligonucleotide fragments were not detected. The resulting apurinic DNA included no 7-ethylguanine residue by base analysis. The optical melting curves (T_m) and the CD spectra of the

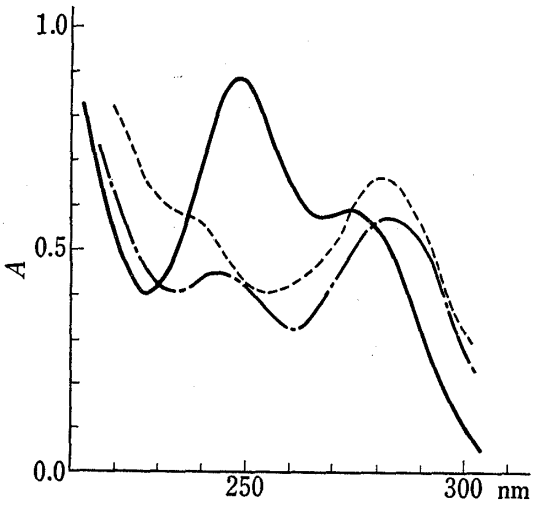

Fig. 6. Ultraviolet Absorption Spectra of 7-Ethylguanine

Ethylated DNA (30 mg) was incubated in 10 ml of a pH 8.9 carbonate buffer solution ($\mu=0.21$) at 37° for 500 hr. The solution was dialyzed against 10^{-3}M NaCl aq. and the dialyzate was evapolated *in vacuo*. For the spectra, 8.28×10^{-5}M solution was used.
----: in pH 11, ·—·: in pH 7.4, ——: in pH 0.5

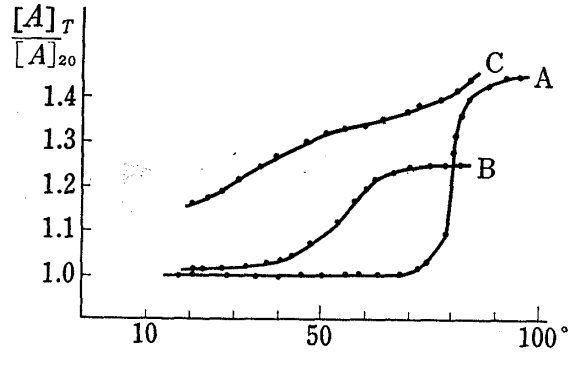

Fig. 7. Optical Melting Curves (T_m) of Native DNA, Ethylated DNA, and Heat-denatured DNA

T_ms were determined in a carbonate buffer (pH 8.9, $\mu=$ 0.11). A, native DNA; B, ethylated DNA; C, heat-denatured DNA. Vertical scale represented the quotients dividing the absorbance of each DNA at several temperature by the absorbance of native DNA at 20°.

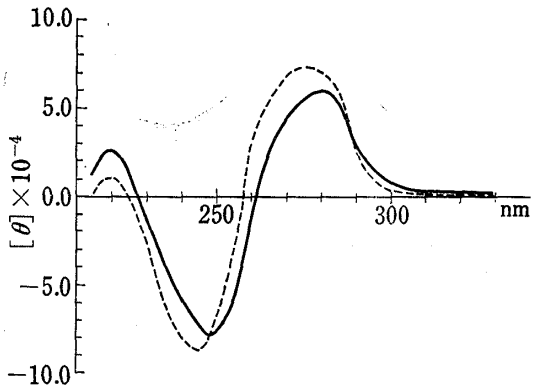

Fig. 8. Circular Dichroism Spectra of Native DNA and Ethylated DNA

Spectra in carbonate buffer solutions(pH 8.9, $\mu=0.21$) were recorded using 1 cm cuvettes. ----: native DNA, ——: ethylated DNA

8) B.S. Strauss and T. Hill, *Biochim. Biophys. Acta*, 213, 14 (1970).

DNAs are shown in Fig. 7 and Fig. 8. In contrast with Handler's observation,[9] the hyperchromicity of ethylated DNA at 20° increased 0.8% compared with the original absorbance. The CD spectrum of ethylated DNA showed a slight shift of the maxima to longer wavelengths and a decrease in the molecular ellipticity.

Discussion

Although extensive studies on alkylation of nucleotides as well as nucleic acids by the monofunctional alkylating agents have been reported,[3,10–19] comprehensive understanding of the effect was mainly confined to physico-chemical studies. It might be attributed to the fact that the complexity of chemical alkylation made a simple interpretation of effects on cellular processes difficult.

There are a few reports[11,14,15] on the ethylation of polynucleotides (nucleic acids), in which some different biological effects to methylation aroused interest. Ethyl methanesulfonate and ethyl ethanesulfonate showed analogous mutagenity as dimethyl sulfate toward TMV-RNA, whereas diethyl sulfate showed to be ineffective.[16]

It has been previously reported that the hydrolysate of native DNA which was treated with dimethyl sulfate, methyl methanesulfonate and ethyl methanesulfonate included 7-ethylguanine[14,17,18] as the predominant product with small amounts of 3-methyladenine.[17,18] On the contrary, methylation of RNA or denatured DNA produced 1-methyladenine in addition to 3-methyladenine.[19] Other typical alkylating agents, diazomethane or diazoethane gave more complex results.

An appropriate alkylating agent, TEOF which has been previously used as a selective alkylating agent for uridine[20] was highly reactive toward the guanine residue in the nucleic acids.

In the experiments on the mononucleotide level, TEOF reacted on UMP, CMP, GMP but not AMP, in buffer solutions. The extents of ethylation of the mononucleotides were profoundly influenced by the pH and the ionic strength of buffer employed. In general, ethylation with TEOF compared with other alkylating agents gave simple results. For instance, ethylation of guanosine with TEOF gave 7-ethylguanosine as the predominant product, whereas ethylation with ethyl iodide and ethyl methanesulfonate produced various N- and O-ethyl substituted guanosines.[21] The behavior of TEOF against mononucleotides in the buffer at lower ionic strength was especially characteristic and was shown to give ethylation only on the guanine residue.

When native RNA was incubated with TEOF in a buffer solution at the pH 8.9 and the ionic strength around 0.1 approximately 50% of the guanine residues were converted to 7-ethylguanine and other bases were left quite intact. This suggested that TEOF is applicable

9) S. Hendler, E. Fürer, and P.R. Srinivasan, *Biochem.*, **9**, 4141 (1970).
10) B.E. Griffin, "Methods in Enzymology," Vol. 12A, ed. by S.P. Colowick and N.O. Kaplan, Academic Press Inc., New York, 1968, p. 141.
11) R.H. Hall, "The Modified Nucleosides in Nucleic Acids," Columbia University Press, New York, 1971.
12) P.D. Lawley, D.J. Orr, S.A. Shah, P.B. Farmer, and M. Jarman, *Biochem. J.*, **135**, 139 (1973).
13) N.P. Trifunac and A.I. Krasna, *Biochem.*, **13**, 2403 (1974).
14) H. Rhasese and E. Freese, *Biochim. Biophys. Acta*, **190**, 418 (1969).
15) A. Loveless, *Nature*, **223**, 206 (1969).
16) B. Singer and H. Fraenkel-Conrat, *Virology*, **39**, 395 (1969).
17) E.L. Uhlenhopp and A.I. Krasna, *Biochem.*, **10**, 3290 (1971) and the references cited herein.
18) S. Zamenhof and S. Arikawa, *Mol. Pharmac.*, **2**, 570 (1966).
19) P. Brookes and P.D. Lauley, *Biochem. J.*, **80**, 496 (1961).
20) Y. Kanaoka, E. Sato, M. Aiura, O. Yonemitsu, and Y. Mizuno, *Tetrahedron Letters*, **1969**, 3361.
21) B. Singer, *Biochem.*, **11**, 3939 (1972).

to ethylation of cellular nucleic acids without the toxic action which is connected with adenine alkylation as opposed to guanine alkylation.[22]

Native DNA was ethylated to a similar extent under the same conditions. The ethylated DNA contained a small amount of dialyzable material which was identified as 7-ethylguanine. It seemed that the ethylated DNA could be prevented from concomitant depurination[6] under appropriate conditions. The optical melting curve of the ethylated DNA is indicative of the partial loss of tertiary structure although the molecules remain double stranded (Fig. 7). This fact is in agreement with a decrease in the molecular ellipticity (Fig. 8). The zone sedimentation profiles of the ethylated DNA, shown in Fig. 9A and Fig. 9B demonstrated that DNA on ethylation caused chain scission to large segments, which presumably resulted at sites of depurination.

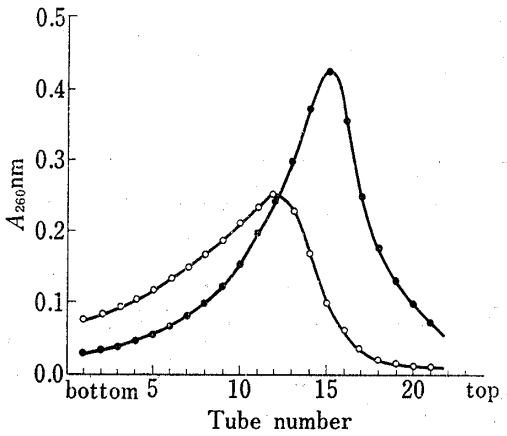

Fig. 9A. Zone Sedimentation Profiles of Native DNA and Ethylated DNA in Sucrose Density Gradient

Conditions of analyses were described in Experimental. —○—: native DNA, —●—: ethylated DNA

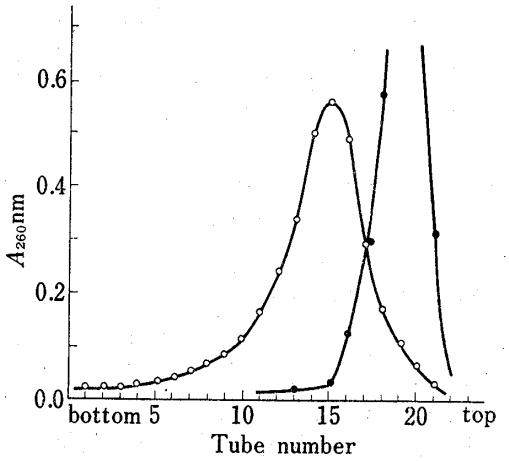

Fig. 9B. Zone Sedimentation Profiles of Native DNA and Ethylated DNA in Alkaline Sucrose Density Gradient

Conditions of analyses were described in Experimental. —○—: native DNA, —●—: ethylated DNA

Acknowledgement The authors are grateful to Dr. B. Witkop, National Institutes of Health, U.S.A. for his interest and encouragement.

22) B. Papirmeister, J.K. Dorsey, C.L. Davison, and C.L. Gross, *Fed. Proc.*, **29**, 2 (1970).