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# Alkylation of Ribose in RNA Reacted with Ethylnitrosourea at Neutrality†

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ABSTRACT: Ribose oxygens in TMV-RNA are ethylated by the carcinogen ethylnitrosourea in neutral aqueous solution (pH 6.1–7.3). 2′-*O*-Ethyladenosine, 2′-*O*-ethylguanosine, 2′-*O*-ethyluridine, and 2′-*O*-ethylcytidine have been identified as reaction products. The four 2′-*O*-ethyl nucleosides are found in approximately equal amounts and the total extent of ribose alkylation is about 15% of the total ethylation. This finding, in conjunction with earlier results showing that all ring and phosphate oxygens can be ethylated, signifies that every oxygen in RNA or polyribonucleotides can react with ethylnitrosourea. The possible biological significance of ribose alkylation, resulting from chemical rather than enzymatic reaction, is discussed. The preparation of the new derivative 2′(3′)-*O*-ethylguanosine is described.

*N*-Nitroso compounds, particularly ethylating nitroso compounds, have been shown to alkylate preferentially the oxygens of nucleosides rather than the nitrogens. We have reported that, in RNA and DNA treated with ethylnitrosourea in vitro and in vivo, the phosphodiesters are the major site of ethylation and, of the nucleophilic sites of the bases, the $O^6$ of guanine is modified to a high extent (Singer and Fraenkel-Conrat, 1975; Sun and Singer, 1975).

Cytidine in neutral aqueous solution is alkylated on the $O^2$, with the ethylating agents being more effective than the analogous methylating ones (Singer, 1976). All nucleophilic centers of the uracil ring in poly(U)[1] ($O^2$, N-3, and $O^4$) are found reactive when treated with carcinogenic alkylating agents at neutrality; the ratio of O/N alkylation is about 300 when ethylnitrosourea is used (Kuśmierek and Singer, 1976b). In addition to the ring alkylation, phosphodiesters and ribose are alkylated in poly(U) (Kuśmierek and Singer, 1976b).

The finding that the ribose-OH group is also susceptible to

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[1] Abbreviations used are: Et, ethyl; Ado, Guo, Cyd, and Urd are the four ribonucleosides; O′ indicates ribose substituted on the 2′(3′) position; poly(U), poly(uridylic acid); uv, ultraviolet; EDTA, ethylenediaminetetraacetic acid.

alkylation under these conditions and the identification of 2′-*O*-alkyluridine as a major product of alkylation of a polyribonucleotide (Kuśmierek and Singer, 1976b) led to the present work in which we report that all four 2′-*O*-alkylnucleosides are formed in TMV-RNA when reacted at pH 6.1–7.3 with the carcinogen ethylnitrosourea. The possible biological consequences of ribose alkylation are discussed.

## Experimental Section

*Materials.* [$^{14}$C]Ethylnitrosourea (4.1 Ci/mol) was obtained from Farbwerke Hoechst and was used for reactions with, as well as without, dilution with the nonradioactive reagent.

Wheat germ phosphatase, bacterial alkaline phosphatase, ribonuclease A, and snake venom phosphodiesterase were obtained from Worthington. $T_1$ ribonuclease was from Sankyo. Ribonuclease $U_{4B}$ was a gift from Dr. Ann Blank (Blank and Dekker, 1972).

*Chromatographic Systems.* Four chromatographic systems and electrophoresis were used to separate and characterize *O*′-alkyl nucleosides. No attempt was made to resolve 2′-*O*-alkyl and 3′-*O*-alkyl nucleosides. Solvent I is 1-butanol–ethanol–water (80:10:25) on Whatman 3 MM, descending 16–24 h. Solvent II is 1-butanol–concentrated ammonium hydroxide–0.8 M boric acid (200:0.8:27) on Whatman 3 MM, saturated with 0.1 M ammonium borate and dried, descending 22 h (Al-Arif and Sporn, 1972). Solvent III is 1-butanol–concentrated ammonium hydroxide–$H_2O$ (85:2:12) on Whatman 3 MM, descending 18 h. Solvent IV is acetone–benzene (2:1) on activated silica gel sheets, ascending 2–3 h (Kuśmierek and Singer, 1976a). (The separations and $R_f$'s reported for this system are greatly dependent on the batch of silica gel used.) Electrophoresis on Whatman 3 MM was in 0.1 M, pH 9.2, sodium borate (1000 V, 3 h, 30 cm).

*Preparation of 2′(3′)-O-Alkyl Nucleosides.* 2′(3′)-*O*-Ethylcytidine and 2′(3′)-*O*-ethyluridine were obtained by enzymatic dephosphorylation of the corresponding 5′-nucleotides (Kuśmierek and Shugar, 1973) and then purified by chromatography in solvent I.

2′(3′)-*O*-Ethyladenosine was synthesized following the procedure used by Robins and Naik (1971) for 2′- and 3′-*O*-methyladenosine. Diethyl ether was used as a carrier for diazoethane (Robins et al., 1974) rather than 1,2-dimethoxyethane in order to avoid mixed alkylation (Pike et al., 1974; Kuśmierek and Singer, 1976a). The reaction mixture, which contained 0.5 mmol of reacted adenosine, was evaporated, redissolved in 20 ml of water, and then passed through a Dowex 1-X2 (200–400 mesh) column (1.5 × 10 cm) which was in the borate form (the chloride form of Dowex 1-X2 was washed with 0.1 M sodium borate until no $Cl^-$ was detected. The excess of borate was removed by washing with water until the pH was neutral). The column was washed with 100 ml of water which eluted all uv-absorbing material. Final purification was by chromatography in solvent I. The overall yield after this step was about 70%. 2′(3′)-*O*-Ethyladenosine has the same uv spectral characteristics as adenosine but the chromatographic behavior is different (Table I).

2′(3′)-*O*-Ethylguanosine was prepared using the following procedure. Guanosine (300 mg) and $SnCl_2 \cdot 2H_2O$ (50 mg) were dissolved in 100 ml of dimethylformamide. Ethereal diazoethane was added in small aliquots (1–2 ml) to the stirred reaction mixture at 30 °C. The disappearance of guanosine was monitored by thin-layer chromatography in solvent I (guanosine has a lower $R_f$ than derivatives; Table I; Singer, 1972) and the addition of diazoethane was stopped when 80–90% of the guanosine had reacted (about 25 ml of diazoethane or about 10 mmol over a 5-h period). The reaction mixture was evaporated to dryness, redissolved in 10 ml of $H_2O$, and again evaporated. This last procedure was repeated three times. The dry residue was suspended in 10 ml of $H_2O$ and centrifuged to remove the precipitate which was insoluble in both water and 0.1 M formic acid and which was probably an ethylated Sn derivative. The supernatant was loaded on a Dowex 1-X2 (200–400, borate form) column (1.5 × 20 cm). The column was washed with about 500 ml of $H_2O$ or until all uv-absorbing material was eluted. The eluate contained 6800 $A_{260}$ units of 2′(3′)-*O*-ethyl nucleosides which were, after evaporation to a smaller volume, chromatographed in solvent I. Ninety-five percent of the material was separated into $O^6$,2′(3′)-*O*-diethylguanosine and 1,2′(3′)-*O*-diethylguanosine, the ratio being 2:1. The $O^6$,2′(3′)-*O*-diethylguanosine fraction was hydrolyzed with alkali (1 N KOH, 100 °C, 75 min) to dealkylate the ring and, after chromatography in solvent I, 2′(3′)-*O*-ethylguanosine was obtained with 70% yield. 2′(3′)-*O*-Ethylguanosine has the same uv spectral characteristics as guanosine but the $R_f$ value in solvent I is very different (Table I). The overall yield of 2′(3′)-*O*-ethylguanosine, based on the original guanosine, is about 20%.

TABLE I: $R_f$ Values of Ribose-Ethylated Nucleosides.[a]

| Compound | Solvent I $R_f$ | Solvent II $R_{2'(3')\text{-}O\text{-EtUrd}}$ | Solvent III $R_f$ |
|---|---|---|---|
| Adenosine | 0.30 | | |
| 2′(3′)-*O*-EtAdo | 0.55 | 0.89 | 0.65 |
| Guanosine | 0.10 | | |
| $O^6$-EtGuo | 0.65 | | |
| 1-EtGuo | 0.33 | | |
| $O^6$-Et,2′(3′)-*O*-EtGuo | 0.79 | | |
| 1-Et,2′(3′)-*O*-EtGuo | 0.60 | | |
| 2′(3′)-*O*-EtGuo | 0.33 | 0.45 | 0.31 |
| Uridine | 0.30 | | 0.13 |
| 2′(3′)-*O*-EtUrd | 0.62 | 1.0 ($R_f$ 0.48) | 0.53 |
| Cytidine | 0.15 | | 0.06 |
| 2′(3′)-*O*-EtCyd | 0.45 | 0.71 | 0.52 |
| Adenine | 0.47 | | 0.30 |
| Guanine | 0.12 | | 0.09 |
| 2′(3′)UMP | 0 | | 0 |
| 2′(3′)CMP | 0 | | 0 |

[a] Chromatographic methods and solvent systems are described in Materials and Methods.

*Identification of 2-O-Ethylribose and 3-O-Ethylribose.* Ten to twenty absorbancy units each of 2′(3′)-*O*-ethyladenosine and 2′(3′)-*O*-ethylguanosine were hydrolyzed with 0.3 N HCl at 100 °C for 60 min in stoppered tubes and then subjected to electrophoresis in 0.1 M sodium borate. Ribose or alkylribose was identified using a sugar color test. The paper was dipped in a solution of 2 g of aniline in 100 ml of ethyl acetate containing 2% trichloroacetic acid and then heated at 85 °C for 5 min (Block et al., 1958). 2-*O*- and 3-*O*-alkylribose appear as dark spots well separated from each other and from ribose. The mobilities, which are similar to those reported for methyl riboses by Brown et al. (1954), are relative to ribose: 0.37 for 2-*O*-ethylribose and 0.81 for 3-*O*-ethylribose (picric acid is 1.20 and 2-deoxyribose is 0.40; this latter compound should and does behave like 2-*O*-alkylribose in this system).

*Alkylation of TMV-RNA.* One milligram of TMV-RNA

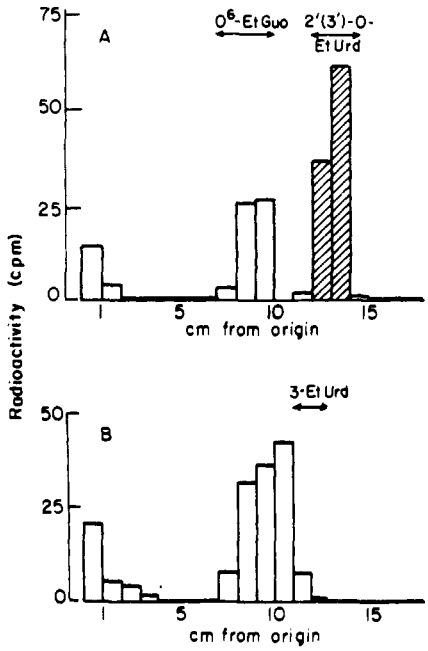


FIGURE 1: Radioactivity profiles of chromatograms of enzyme digests of [$^{14}$C]ethylnitrosourea-treated TMV-RNA. Samples were chromatographed in solvent I and the area cochromatographing with $O^6$-ethylguanosine was eluted and rechromatographed on silica gel sheets in solvent IV (Kuśmierek and Singer, 1976b). (A) Digested with snake venom phosphodiesterase and phosphatases according to method I. (B) Digested at pH 5 with RNase $U_{4B}$ and wheat germ phosphatase according to Singer and Fraenkel-Conrat (1975). Double arrows indicate the position of added uv absorbing markers. $O^6$-EtGuo, 3-EtUrd, and 2'(3')-$O$-EtUrd were also present as external markers and had the same $R_f$ values as the internal markers. The shaded strips in Figure 1A were eluted and rechromatographed in solvent II where the radioactivity was coincident with 2'(3')-$O$-ethylurdine ($R_f$ 0.48). Radioactivity was determined on 1-cm strips immersed in 5 ml of scintillation fluid (14.3 g of Omnifluor/3 kg of toluene) and counted in a Bekman liquid scintillation counter.

in 0.6 ml of 0.5 M (pH 6.1 or pH 7.3) cacodylate buffer was reacted with 15 mg of [$^{14}$C]ethylnitrosourea in 0.2 ml of ethanol at 37 °C until decolorized (2 h at pH 6.1 and 1 h at pH 7.3). Alkylated RNA was freed of reagent by repeated precipitation at 0 °C with 3 volumes of ethanol containing 0.1 M (pH 5) acetate. Constant specific activity was obtained after five or six precipitations. The specific activity varied from 50 to 250 cpm/$\mu$g depending on the specific activity of the reagent and the extent of alkylation (20–50 ethyl groups/6400 nucleotides).

*Methods for Hydrolysis of Alkylated RNA.* Two methods of enzyme digestion and one of acid hydrolysis were used, although not all were on the same sample in every case.

Method I was digestion with snake venom phosphodiesterase and phosphatases. RNA (200 $\mu$g) in 0.25 ml of 0.02 M (pH 7.2) Tris buffer, containing 0.01 M $MgCl_2$, was incubated with 50 $\mu$g of snake venom phosphodiesterase, 50 $\mu$g of bacterial alkaline phosphatase, and 50 $\mu$g of wheat germ phosphatase for 18–24 h at 37 °C. Snake venom phosphodiesterase has been shown to hydrolyze the phosphodiester bond adjacent to the 2'-$O$-alkyl group in polynucleotides (Gray and Lane, 1967). The neutral pH permits recovery of labile $O$-alkylnucleosides and other alkylated nucleosides such as 7-alkylguanosine and 1-alkyladenosine which are ring opened and rearranged, respectively, at the usual pH used for venom phosphodiesterase digestion. The high enzyme/substrate ratio is necessary for maximum digestion.

Method II was digestion with ribonucleases and phosphatase. RNA (200 $\mu$g) in 0.25 ml of 0.2 M acetate buffer (pH 5) containing $10^{-3}$ M EDTA (pH 7) was incubated with 100 units of RNase $U_{4B}$ (Blank and Dekker, 1972), 50 units of RNase $T_1$, 1000 units of RNase A, and 50 $\mu$g of wheat germ phosphatase at 37 °C for 20 h. This method, which is a variation of that previously used by Singer and Fraenkel-Conrat (1975), should not hydrolyze 2'-$O$-alkyl nucleotides since ribonucleases require a free 2'-OH for hydrolysis. However, if the enzymes contain traces of diesterase, then some ribose alkylated products could be released. We find that, with the large amounts of enzymes used, low levels of $O'$-alkyl nucleosides can be detected in digests.

Method III was hydrolysis in a sealed tube with 1 N HCl at 100 °C for 1 h. One milliliter of ethanol was added and the alcohol distilled off. One milliliter of alcohol was again added and again distilled. The second distillate contained about 15% of the total released radioactivity. Repeated 1 N HCl hydrolysis did not release additional alkyl groups. The remaining material was evaporated to dryness, 50 $\mu$l of 70% $HClO_4$ added, and the sample hydrolyzed in a sealed tube at 100 °C for 1 h. One milliliter of alcohol was added and distilled. The addition of alcohol and distillation were repeated to obtain quantitative data.

Radioactivity in the HCl distillate represents alkyl groups bound to the oxygens of the bases, and to the phosphodiesters. Radioactivity then released by $HClO_4$ hydrolysis represents alkyl groups bound to ribose (Baskin and Dekker, 1967; Abbate and Rottman, 1972). In an earlier paper, Singer and Fraenkel-Conrat (1975) discuss the fact that 1 N HCl does not volatilize all alkyl groups on ribose phosphates. However, the present technique of repeated 1 N HCl hydrolysis and alcohol distillation is more effective and nonribose $O$-alkyl groups are completely hydrolyzed.

## Results and Discussion

The first indication that ribose alkylation occurs when RNA is alkylated in neutral aqueous solution came from experiments in which we compared the products in snake venom phosphodiesterase plus phosphatase digests of ethylnitrosourea-treated RNA with those from ribonuclease $U_{4B}$ plus phosphatase digests using a one-dimensional chromatographic system (solvent I). The area of the chromatogram of the snake venom digest cochromatographing with $O^6$-ethylguanosine was found to contain radioactivity which was not volatilized when hydrolyzed with 1 N HCl (100 °C, 60 min), in contrast to $O^6$-ethylguanosine which is deethylated. When this material, prior to hydrolysis, was chromatographed in solvent IV, part of the radioactivity moved ahead of all ethylated nucleosides and coincided with 2'(3')-$O$-ethyluridine (Kuśmierek and Singer, 1976a) (Figure 1A). The 2'(3')-$O$-ethyluridine area from solvent IV was rechromatographed in solvent II and all radioactivity again moved with the authentic ribose alkyl nucleoside. This product, which represented about 3% of all ethylation, was completely absent in the RNase $U_{4B}$ digest (Figure 1B), further proving that, in accord with its lack of a free 2'-OH, 2'-*O*-*ethyluridine in a polynucleotide is not appreciably released by RNase.*

In order to analyze for other ribose-ethylated nucleosides it was first necessary to prepare authentic derivatives. 2'-$O$-Ethyluridine and 2'-$O$-ethylcytidine had been prepared by Ransford et al. (1974) but we chose to use a different method for their preparation (Kuśmierek and Shugar, 1973). 2'-$O$-Ethyladenosine 5'-phosphate was described by Tazawa et al. (1972) but it proved more desirable to obtain the nucleoside by modifying the procedure used by Robins and Naik (1971)

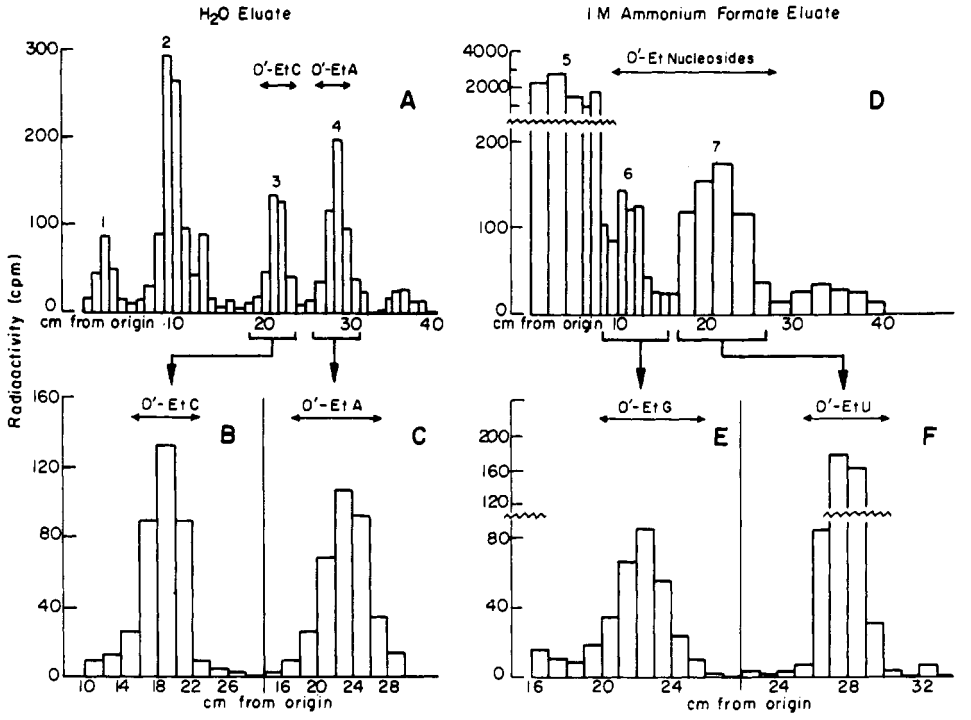


FIGURE 2: Radioacitvity profiles of chromatograms of [$^{14}$C]ethylnitrosourea treated TMV-RNA digested according to method I. The digest containing 50–200 $\mu$g was first fractionated on a 0.125 × 14 cm Dowex 1-X2 200–400 mesh column which was in the borate form (see Materials and Methods). The water eluate (10 ml) (left panels) and the 1 M ammonium formate eluate (5 ml) (right panels) were treated separately. (A) Chromatography in solvent III of the water eluate. Peaks 3 and 4 which cochromatograph with 2′(3′)-*O*-EtCyd and 2′(3′)-*O*-EtAdo, respectively, were eluted and rechromatographed in solvent II as shown in B and C. (D) Chromatography in solvent II of the ammonium formate eluate. The areas of the chromatogram designated as 6 and 7 were each rechromatographed with 2′(3′)-*O*-EtGuo and 2′(3′)-*O*-EtGuo and 2′(3′)-*O*-EtUrd, respectively, as shown in E and F. In the case of B, C, E, and F only those portions of the chromatograms containing significant radioactivity are shown. Radioactivity was determined on paper strips of various widths as indicated by the bars.

for 2′-*O*-methyladenosine. 2′(3′)-*O*-Ethylguanosine had not previously been prepared and it was found that a rather indirect route could be utilized successfully. This method relied on the fact that diazoethane in nonaqueous solution preferentially ethylates oxygens (Kuśmierek and Singer, 1976a), and a major derivative of guanosine reaction is $O^6$-2′(3′)-*O*-diethylguanosine which, after separation from other products, can be converted to 2′(3′)-*O*-ethylguanosine by deethylating the $O^6$ in alkali.

Complete separation of all four ribose ethyl nucleosides from each other, and also from all other ethyl derivatives, was not possible in any single chromatographic system. Since the 2′(3′)-alkyl ribonucleosides lack the ability to form borate complexes, in contrast to ribonucleosides with unsubstituted 2′,3′-*cis*-hydroxyl groups, their separation and tentative identification were based on this property. Two borate systems were used: the anion-exchanger column in borate form and a borate-containing paper chromatographic system (solvent II).

The anion exchanger in borate form retains base alkylated and unmodified ribonucleosides as well as anionic derivatives such as alkyl esters of nucleotides which are among the digestion products of ethylnitrosourea-treated RNA. Due to their anionic character, 2′(3′)-*O*-alkyluridine and 2′(3′)-*O*-alkylguanosine are also retained by the borate form of Dowex 1-X2. We thus find that, when a digest of ethylnitrosourea-treated RNA (method I) is applied to this column, the fraction eluted by water contains 2′(3′)-*O*-alkyladenosine, 2′(3′)-*O*-alkylcytidine, and an unidentified compound (Figure 2A, peak 2). The remaining radioactivity is eluted from the column with 1 M ammonium formate and contains 2′(3′)-*O*-alkylguanosine and 2′(3′)-*O*-alkyluridine as well as all other alkyl derivatives (Figure 2D, peak 5). The identity of ribose-alkylated nucleosides from the two eluates was shown by chromatography in solvents I, II, and III (Figure 2). Solvent II contains borate and serves to further identify a radioactive alkyl group as being on the ribose. Solvent II clearly separates the ribose derivatives of guanosine from those of uridine (Table I, Figure 2E,F) but does not resolve 2′(3′)-*O*-ethyladenosine from 2′(3′)-*O*-ethylcytidine as well as solvent III (Table I, Figure 2B,C). By using combinations of the borate column and the three chromatographic systems, all four 2′-*O*-ethyl nucleosides were identified as products of both pH 6.1 and pH 7.3 ethylnitrosourea treatment of TMV-RNA (Figure 2). In addition [$^{14}$C]2-*O*-ethylribose was a product of acid hydrolysis of [$^{14}$C]-2′-*O*-ethylguanosine. After isolation of the ribose-alkylated guanosine (Figure 2E), the acid digest was subjected to electrophoresis in borate and the radioactivity comigrated with authentic 2-*O*-ethylribose and not 3-*O*-ethylribose (data not shown; methods and mobilities given in Experimental Section).

Perchloric acid or formic acid hydrolysis has been used to "selectively" volatilize the 2′-*O*-alkyl group in nucleic acids since in general the alkyl groups on the base nitrogens are stable. Some examples of this usage are Munns et al. (1974) who studied the methylated constituents of tRNA from cells or rats given labeled methionine and Pegg (1972) who did a similar study with labeled ethionine. In neither case, or others in which RNA is hydrolyzed with strong acid, is cognizance taken of the even greater acid lability of other oxygen-linked alkyl groups such as alkyl ribophosphotriesters, *O*-alkylpyrimidines, and $O^6$-alkylguanosine. All these derivatives are dealkylated in 1 N HCl, 100 °C, 1 h. This difference in acid stability makes it possible to determine the amount of ribose

alkylation as well as the total of other O-alkylation. The amount of ribose ethylation as determined by acid hydrolysis is in good agreement with the total ribose ethylation using enzyme digestion.

In quantitative terms ribose alkylation represents about 15% of the total alkyl groups bound and each of the four ethyl ribose nucleosides is present in approximately equal amounts (Figure 2). There was no significant difference in the extent of ribose ethylation when the reaction was performed at pH 6.1 and 7.3.

Since the 2′-*O*-alkyl group inhibits digestion with ribonucleases, we expected that 2′-*O*-alkyl nucleosides would not be released using digestion method II. However, while there was a great difference between the patterns of *O*-alkyl nucleosides from venom diesterase and ribonuclease digestion, there was a detectable amount of each of the four *O*′-ethyl nucleosides when the latter digest was chromatographed in a borate-containing solvent (data not shown). The radioactivity from the RNase digest cochromatographing with 2′-*O*-ethyl nucleosides amounted to about one-third of that from snake venom digests. In the case of the experiment in Figure 1B, only a single ribonuclease (RNase $U_{4B}$) was used and the enzyme/substrate ratio was much lower which probably accounts for the lack of detectable 2′-*O*-ethyluridine in the RNase $U_{4B}$ digest.

The number of systems used to isolate ribose alkylated nucleosides appears sufficient to give us assurance that ethylnitrosourea alkylates the 2′-O at neutrality. However, we did not investigate the identity of a number of products which during separation of products did not behave as expected for the known alkyl derivatives; for example, in Figure 2A, peak 2 does not correspond in properties to either ribose-, base-, or phosphate-alkylated nucleosides. One possibility for this to be eluted with water from the borate column is that peak 2 is an ethylated base but, since the enzyme digestion was at neutrality (37 °C, 18 h), it is very unlikely that even N-3 and N-7 ethylpurines would be liberated.

*Biological Effects of Ribose Alkylation.* Chemical ethylation, in general, occurs in a different pattern than methylation. This is also true for enzymatic ethylation of nucleic acids in cells which results when ethionine is injected into rats (Rosen, 1968; Swann et al., 1971; Pegg, 1972). Ethionione is an hepatic carcinogen and the mechanism of its biological action is unknown but is under investigation. Both Pegg (1972) and Rosen (1968) report that ethionine administered to rats causes, among other ethylations, a high proportion of ribose ethylation (approximately 50%). Since methylation of ribose in tRNA by *S*-adenosylmethionine is a normal process, the harmful effects of ethionine or *S*-adenosylethionine may be due to modification of the ribose of nucleosides that are normally not methylated. Alternatively the ethyl group as such may not be biologically acceptable. However, although we do not present data in this paper, methylnitrosourea, which like ethylnitrosourea is a carcinogen, methylates the 2′-O of ribose (Kuśmierek and Singer, 1976b). This would indicate that substitution of the larger ethyl group for the normal methyl group is not, per se, the "carcinogenic" event. We may, however, speculate that the carcinogenic nitroso compounds exert their effect in cells by alkylation of sites, such as ribose, which affect conformation, enzyme resistance, or ribosome binding.

One important consequence of ribose alkylation in an RNA is that enzyme susceptibility is greatly changed. Certainly at the relatively low enzyme levels present in cells, ribonucleases would not digest *O*′-alkyl sequences and other nucleolytic enzymes would also probably be ineffective since it takes 100 times more 5′-nucleotidase to attack 2′-*O*-alkyl-5′-CMP than 5′-CMP (Kuśmierek and Shugar, 1973). In the many recent papers which report that mRNAs contain an unusual 5′ terminus, a 2′-*O*-methyl nucleoside is frequently part of the "capped" sequence in animal cells and viruses (e.g., Shatkin, 1974; Keith and Fraenkel-Conrat, 1975; Wei and Moss, 1975; Perry et al., 1975). It is likely that the ribose methylation is there as a simple means of preserving the terminus from enzymatic attack. However, if ribose methylation occurs in a random fashion, then normal processing of a nucleic acid may be prevented.

Rottman and co-workers (Rottman and Heinlein, 1968; Rottman and Johnson, 1969; Dunlop et al., 1971; Khan and Rottman, 1972; Rottman et al., 1974) and Shugar and coworkers (Janion et al., 1970; Zmudzka and Shugar, 1970; Kuśmierek and Shugar, 1973; Kuśmierek et al., 1973) have studied the incorporation of 2′-*O*-alkyl nucleoside diphosphates into hetero- and homopolymers using polynucleotide phosphorylase and, in general, methylation or ethylation of ribose does not prevent polymerization, although it is, in some cases, necessary to modify conditions for polymerization, probably due to steric hindrance (Tazawa et al., 1972; Khan and Rottman, 1972; Kuśmierek and Shugar, 1973). The effect of 2′-*O*-alkyl substituents on the ability of a polynucleotide to function as a messenger or template depends to a great extent on the specific polynucleotide and on the experimental system. It does appear that introduction of low levels of 2′-*O*-methyl nucleosides into ribopolynucleotides does not eliminate template activity or cause misreading; yet such modified polymers do exhibit differences compared with the unmodified polymer when tested for amino acid incorporation in a cell-free system (Dunlap et al., 1971). Perhaps the most marked effect of ribose alkylation is on polymer structure where the 2′-O-alkylated polymer has a more ordered structure than the analogous ribopolymer (Zmudzka and Shugar, 1970; Khan and Rottman, 1972; Kuśmierek et al., 1973; Rottman et al., 1974). The 2′-*O*-ethyl group has an even greater effect on $T_m$ than does the 2′-*O*-methyl group, and it is possible that even a few 2′-*O*-ethyl substituents could affect the secondary structure and, thus, the biological function of a nucleic acid.

Acknowledgment

The authors are grateful to Dr. H. Fraenkel-Conrat for his continued interest and advice.

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# Studies on the Noncooperative Binding of the *Escherichia coli* DNA Unwinding Protein to Single-Stranded Nucleic Acids†

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ABSTRACT: The noncooperative binding of the *Escherichia coli* DNA unwinding protein to single-stranded DNA oligomers has been studied by means of equilibrium dialysis. Dialyses were performed under a number of solution and temperature conditions using oligomers of varying length and base composition. The results of these studies, which include a Scatchard analysis of the binding, have allowed us to propose a model for the cooperative binding of the protein to single-stranded DNA. The results of experiments dealing with the interaction of the protein with single-stranded RNA are also presented.

During the past several years a number of proteins which bind preferentially and cooperatively to single-stranded DNA have been isolated from viral, prokaryotic, and eukaryotic sources utilizing the DNA-cellulose chromatography method of Alberts and Herrick (1971). T4 gene 32 product, the first such protein isolated (Alberts and Frey, 1970), was shown through genetic analysis (Tomizawa et al., 1966; Kozinski and Felgenhauer, 1967; Alberts et al., 1968; Sinha and Snustad, 1971) to be required for both replication and recombination. Subsequent studies (Huberman et al., 1971; Delius et al., 1972) determined that gene 32 product (a) holds single-stranded DNA in an extended conformation, (b) specifically stimulates the activity of T4 DNA polymerase, and (c) forms a weak complex with T4 DNA polymerase in the absence of DNA.

Of the other DNA-binding proteins isolated, the one which has shown properties similar to nearly all of those catalogued for T4 gene 32 product is the *E. coli* DNA unwinding protein (Sigal et al., 1972). The protein holds single-stranded DNA in an extended conformation and stimulates the activity of *E. coli* DNA polymerases II and III* but not *E. coli* DNA polymerase I or T4 DNA polymerase (Sigal et al., 1972; Molineux and Gefter, 1974; Weiner et al., 1975). It has also been shown to form a weak complex with *E. coli* DNA polymerase II (Molineux et al., 1974). While no mutations in the gene coding for the protein have been found, the stimulation of the two *E. coli* DNA polymerases indicates that it is involved in both replication and repair.

The native form of the protein in solution is a tetramer composed of four identical 19 000–20 000-dalton monomers (Molineux et al., 1974; Weiner et al., 1975). The initial studies of Sigal et al. (1972) indicated that the protein bound to single-stranded DNA in a ratio of one protein monomer per eight bases. Electron microscopy of DNA–protein complexes by the

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