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Light-induced free radical alkylation of polynucleotides and their enzymatic digestion

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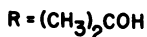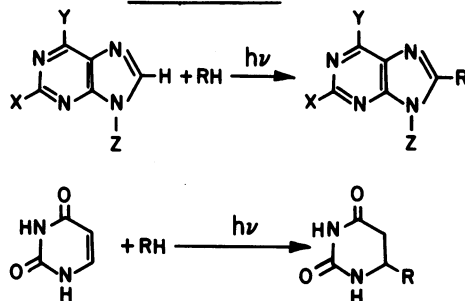
ABSTRACT

Ultraviolet light-induced free radical alkylation with 2-propanol or D-ribose, initiated with di-tert-butyl peroxide, of poly(G), poly($U_{20}G$), and poly(A) led to the substitution of the appropriate group for the H-8 atom of the purines and addition across the 5,6-double bond of the pyrimidines. The alkylated polynucleotides were subjected to nucleolytic digestion with several nucleases. T_1-RNase digestion of poly(G) irradiated with 2-propanol gave a mixture of the modified and non-modified mononucleotides. Similarly, pancreatic RNase digestion of the irradiated poly($U_{20}G$) resulted in a mixture of the appropriate mononucleotides. A T_2-RNase treatment of poly(A) irradiated with 2-propanol gave the modified Ado-2':3'-P, while T_2-RNase digestion of poly(A) irradiated with D-ribose led to the cyclic modified mononucleotides, in addition to the modified mononucleotides.

INTRODUCTION

Purines and purine nucleotides have been shown to react photochemically with a variety of organic compounds, like alcohols (1), amines (2), ethers and acetals (3). These reactions, which involve free radical intermediates, lead to the substitution of the appropriate group for the H-8 atom of the purine system. The application of these reactions to synthetic polynucleotides and natural biopolymers, i.e. DNA (4,5) and RNA, might give a clue as for the role that purines play in photochemical and free radical transformations, and in the subsequent biological effects. It is noteworthy that uracil and its derivatives also react photochemically with some organic compounds, e.g. alcohols, to yield the corresponding 6-substituted-5,6-dihydro-uracils (6-8). These reactions can further be utilized to extend the use of photochemistry as a probe of polynucleotide structure and interaction. The photoproducts can thus serve as suitable substrates for the study of structure-function relationship, e.g. in their behavior towards enzyme action.

Initially, we were concerned whether the phosphodiester bonds of the modified nucleotides are split by the appropriate enzyme in comparable rates to those of the non-modified moieties (9-12). The present publication includes

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Scheme I

R = (CH$_3$)$_2$COH

X = H; Y = NH$_2$; Z = ribose (adenosine)

X = NH$_2$; Y = OH; Z = ribose (guanosine)

a description of the light-induced free radical alkylation with 2-propanol or D-ribose, initiated with di-tert-butyl peroxide [(Bu^{t}O)$_2$] of poly(G), poly(U$_{20}$G), and poly(A), and the subsequent digestion of the irradiated polynucleotides with T$_1$-, pancreatic-, and T$_2$-RNase, respectively.

MATERIALS AND METHODS

Materials. Poly(G) was obtained from Miles Laboratories. Poly(A) was purchased from Sigma, while poly(U$_{20}$G) was kindly provided by Dr. H. Sorek (The Weizmann Institute). T$_1$-RNase was obtained from Worthington, T$_2$-RNase and pancreatic RNase were purchased from Sigma. 1,3-^{14}C-2-propanol (9.1 mCi/mmole) and 5-^{3}H-D-ribose (2.9 Ci/mmole) were purchased from The Radiochemical Centre, Amersham. (Bu^{t}O)$_2$ was obtained from Schuchardt, Munich; while tert-butanol and 2-propanol were obtained from Frutarom, Haifa. D-Ribose was purchased from Fluka, and AMP from Merck.

Irradiation Procedure. Irradiations were carried out with a Wild Universal unit equipped with an Osram 200W high pressure mercury vapor lamp. The irradiated mixture, which was placed in a 1 cm path-length Pyrex cell (at a distance of 15-20 cm), consisted of a solution of the polynucleotide and the radioactive material in a mixture of buffer or water and tert-butanol (3:1). Samples were irradiated for 6-12 hr, and the photoinitiator, (Bu^{t}O)$_2$, was added portionwise in 2 hr intervals.

Irradiation of AMP with D-ribose was carried out with Hanovia 450 W high pressure mercury lamp with internal water cooled jacket.

Enzymatic Digestion. Incubations were carried out at 37°C. The RNase

digestion mixtures (0.5 ml) consisted of the polynucleotide (10-15 O.D. units),
Tris-HCl pH 7.5 (0.05M), EDTA (0.002M), and T_1-RNase (1000 units). Incubation
time was 90 min. The pancreatic ribonuclease digestion mixture (0.5 ml)
contained the polynucleotide (40 O.D. units), Tris-HCl pH 7.5 (0.05 M), EDTA
(0.002 M), and pancreatic RNase (0.02 mg). Incubation time was 2 hr. For
T_2-RNase (9), the mixture (0.5 ml) contained the polynucleotide (12-16 O.D.
units), acetate buffer pH 4.5 (10 mM) and the enzyme (10-20 units). Incubation
was conducted for 3 hr.

Analysis of Digests. The nuclease digests were made 7M in urea, adjusted
to pH 7.4 with Tris-HCl, and chromatographed on a glass column (0.5x70 cm) of
DEAE-cellulose (Whatman DE-52). The resin was suspended in 0.01 M Tris-HCl
pH 7.4 containing 0.5 M NaCl and 7M urea (10). The column was packed and then
equilibrated with starting buffer Tris-HCl (0.01 M), NaCl (0.01 M)and urea(7 M).
Nucleotides were eluted with a linear gradient of 250 ml each of 0.01 M and
0.25 M NaCl in 7M urea and 0.01 Tris-HCl pH-7.4, at flow rate of ca 20 ml/hr.
Fractions of 2 ml were collected, and UV absorption was determined by a Zeiss
PMO II Spectrophotometer. Peaks were pooled and desalted on a column (2x100cm)
of polyacrylamide gel (Biogel P-2) at neutral pH at room temperature (11). The
recovery of nucleotidic material was nearly quantitative in all cases.

Nucleotides thus obtained were chromatographed on Whatman No. 3MM paper.
The following solvent systems were used: Solvent A, 2-propanol: concentrated
ammonium hydroxide:water (55:10:35); solvent B, isobutyric acid:concentrated
ammonium hydroxide:water (58:7:35); solvent C, isobutyric acid:concentrated
ammonium hydroxide:water (66:1:33); solvent system D, ethanol: 1M ammonium
acetate pH-7.5 (7:3). Elution of the nucleotidic material from the paper
chromatograms was carried out with ammonium hydroxide (0.1 M). Recovery of the
ultraviolet-absorbing material and of the radioactive materials was usually in
the range of 90%. R_f values of the products are given in Table I.

Photoalkylation of AMP with D-ribose. A mixture of D-ribose (20 g), AMP
(Ado-5'-P) (1.5 g), water (150 ml), and $(Bu^tO)_2$ (6 portions of 5 ml each were
introduced at 24 hrintervals) was irradiated (Pyrex filter) at room temperature
under nitrogen for 168 hr. Progress of the reaction was followed qualitatively
by tlc on cellulose plates using solvent system C. Following irradiation, the
solvent was removed under reduced pressure and the residue was chromatographed
on Dowex-2 ion exchanger column (30x1.4 cm). Nucleotides were eluted with a
linear gradient of 500 ml each of 0.01 M and 1 M NH_4HCO_3. Sugars and uncharged
irradiation products were eluted with the front and the nucleotides with 0.6 M
NH_4HCO_3. The nucleotidic solution was lyophilized and the residue was

TABLE I. R_f Values of Purine and Pyrimidine Photoproducts

Photoproduct	Solvent System			
	A	B	C	D
8-α-hydroxyisopropyl-Guo-3'-P		0.47		0.09
6-α-hydroxyisopropyl-5,6-dihydro-Urd-3'-P	0.55			0.023
6-α-hydroxyisopropyl-Ado-3'-P			0.55	0.3
8-α-hydroxyisopropyl-Ado-2':3'-P			0.65	0.35
8-ribofuranosyl-Ado-5'-P			0.3	0.1
8-ribofuranosyl-Ado-3'-P			0.3	0.1
8-ribofuranosyl-Ado-2':3'-P			0.38	0.12

chromatographed on Whatmann No. 17 paper using a mixture of isobutyric acid-water (67:33). Fluorescent strips were cut and the nucleotidic material (150 mg; crude) was eluted with distilled water. R_f values of the photoproduct in two solvent systems are given in Table I.

	pH-2	pH-7	pH-13
λ_{max}	265	265	263
λ_{min}	238	238	243

NMR (D_2O): τ 1.2 (s, 1H, C-2-H); 4.1 (apparent d, 1H, H-1'); 5.6-6.5 (m, 10H, H-2', H-3', H-4', 2H-5').

RESULTS AND DISCUSSION
Reactions of Polynucleotides with 2-Propanol.

I. T_1-RNase Digestion of Irradiated Poly(G). Poly(G) was irradiated with light of λ>290 nm in the presence of ^{14}C-labeled-2-propanol and $(Bu^tO)_2$, the latter serving as a photoinitiator. The reaction mixture was then dialized, freeze-dried and the residue was treated with T_1-RNase. The nucleotidic material was fractionated on DEAE-cellulose column. Figure 1a shows that the T_1-RNase treatment of the irradiated poly(G), which contained 4% (calculated by the monitored radioactivity) of modified residues, led to a single ultraviolet absorbing peak of mononucleotidic material (13.7 O.D. units), which coincided with the radioactive peak. After desalting on Biogel P-2, the mononucleotide mixture was analyzed by paper chromatography in solvent systems B and D. It was shown to consist of Guo-3'(2')-P and 8-α-hydroxyisopropyl-Guo-2'(3')-P by comparison with authentic samples.
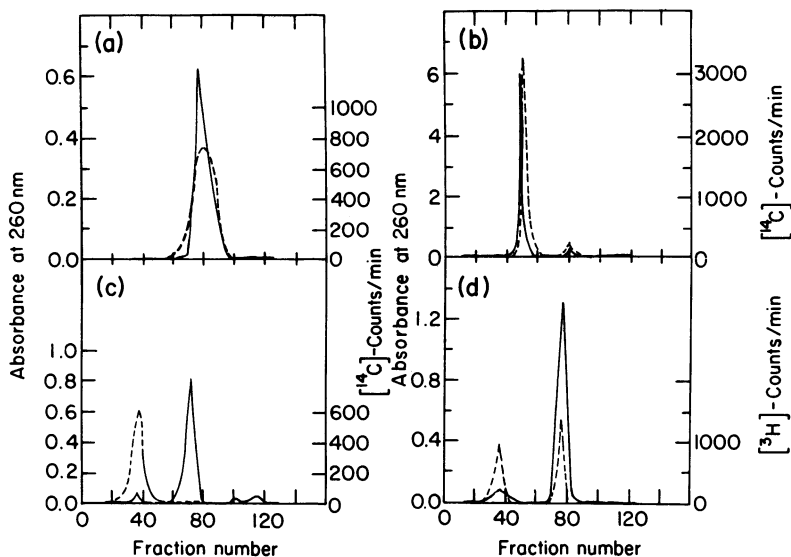
LEGEND TO FIGURE 1

Figure 1: Ion exchange chromatography of enzymatic digests of irradiated polynucleotides on DEAE-cellulose (0.5x70 cm) eluted with a linear gradient (2x250 ml) of 0.02-0.25 M NaCl in 7M urea-Tris HCl pH 7.4. Fractions of 2 ml were collected at flow rate of 20 ml/hr. UV absorption at 260 nm (———) . Radioactivity (----).

a T_1-RNase digest of irradiated poly(G) with 2-propanol. A mixture of poly(G) (15 O.D. units), in buffer solution (1 mM KH_2PO_4, 0.1 M KCl, 5 mM $MgCl_2$)(1.5 ml), 1,3-^{14}C-2-propanol (100μl from a stock solution of 1.20^8 cpm/ml in buffer solution: tert-butanol 1:1), tert-butanol (750 μl), $(Bu^tO)_2$ (five 4 μl portions added in two-hour intervals), and 2-propanol (7.5 μl) was irradiated for 12 hours and worked up as described under methods.

b Pancreatic RNase digest of irradiated poly(U_{20}G) with 2-propanol. A mixture of poly(U_{20}G) (40 O.D. units) in buffer solution (0.75 ml), 1,3-^{14}C-2-propanol (100 μl) of stock solution, tert-butanol (250 μl), $(Bu^tO)_2$ (three 5 μl postions added in two-hour intervals) was irradiated for 6 hr and worked up as described under methods.

c T_2-RNase digest of irradiated poly(A) with 2-propanol. A mixture of poly(A) (12 O.D. units), in water (1.2 ml), 1,3-^{14}C-2-propanol (100 μl) of stock solution, 2-propanol (7.5 μl), and $(Bu^tO)_2$ (three 10 μl portions added in two-hour intervals) was irradiated for 8 hours and worked up in the usual way.

d T_2-RNase digest of irradated poly(A) with D-ribose. A mixture of poly(A) (16 O.D. units) in water (900 μl), 5-^{3}H-D-ribose (1 mCi/ml, 100 μl), D-ribose (0.15 mmole), tert-butanol (200 μl), and $(Bu^tO)_2$ (five 5 μl portions added in two-hour intervals) was irradiated for 12 hours and worked up in the usual way.

II. Pancreatic RNase Digestion of Irradiated Poly(U_{20}G). The irradiated poly(U_{20}G) was dialized, freeze-dried, the residue was treated with pancreatic RNase, and chromatographed on DEAE-cellulose as described above. The elution pattern is given in Figure 1b. The digestion pattern consists of two ultraviolet absorbing peaks: a mononucleotidic one (33.4 O.D. units) and a dinucleotidic one (3.2 O.D. units), which virtually overlap with the respective radioactive

peaks. The first peak contains 3.2% modified material, while the second contains 0.4% modification. The ratio between the peaks is ca 10:1, which is consistent with the calculated value of Urd-2'(3')-P:GpUp obtained by pancreatic digestion. After desalting, the mononucleotide peak was shown to consist of Urd-2'(3')-P and 6-α-hydroxy-isopropyl-5,6-dihydro Urd-3'(2')-P by comparison with their migration on Whatman No. 3 MM paper in solvents A and D and were detected according to Hanes and Isherwood (13). The smaller peak consists of GpUp and a mixture of modified GpUp.

III. T_2-RNase Digestion of Irradiated Poly(A). The irradiated polynucleotide (containing 3% modified moieties) was treated as described above and digested with T_2-RNase. The elution profile of the digest from the DEAE-cellulose column is given in Figure 1c. The first peak which was ultraviolet absorbing (ca 0.5 O.D. units) and possessed radioactivity, was eluted from the column with the same elution volume as Ado-2':3'-P. The pooled fractions were desalted on Biogel P-2 column at neutral pH and then treated with 1N HCl to hydrolyze the cyclic phosphate. Chromatography in solvents C and D revealed a single product as those of 8-α-hydroxyisopropyl-Ado-3'(2')-P. This indicates that the product of the enzymatic digestion was 8-α-hydroxyisopropyl-Ado-2':3'-P. The second peak (9.4 O.D. units) was ultraviolet light absorbing only and possessed no radioactivity. It has been shown to consist of Ado-2'(3')-P by comparison of its migration in solvents C and D with an authentic sample.

T_2-RNase Digestion of Irradiated Poly(A). The irradiated mixture was dialized against water for 24 hr and freeze-dried. The residue was treated with T_2-RNase and chromatographed on DEAE-cellulose column. The elution pattern is given in Figure 1d and consists of two ultraviolet absorbing peaks of mononucleotidic mixtures which coincide with the radioactive peaks of 1.0×10^3 cpm and 1.3×10^3 cpm. The peaks were passed through a Biogel P-3 column. The smaller one was treated with 1N HCl and chromatographed on Whatman No. 3 MM in solvents C and D. The radioactive material (0.4% modified residues) had the same R_f values as those of 8-ribofuranosyl-AMP. This product results from the hydrolysis of 8-ribofuranosyl-Ado-2':3'-P, which was the primary product of the T_2-RNase digestion of the modified polynucleotide.

The other peak was chromatographed in solvents C and D and the radioactive material (0.6% modified residues) had R_f values similar to those of authentic 8-ribofuranosyl-AMP. It also included unreacted Ado-3'(2')-P.

The proposed structure for the AMP (Ado-5'-P)-D-ribose adduct, i.e. 8-ribofuranosyl-AMP is based on the nmr spectrum of the photoproduct, in which the absorption of the H-8 proton of AMP is absent. Additional proof for the

absence of H-8 in the product is given by the fact that heating with D_2O at 105° (a method for the exchange of H-8 protons) (14) does not lead to any changes in the spectrum. There are no changes in the absorption of the H-2', H-3', H-4', and H-5' protons of the sugar moieties linked either to N-9 or C-8, while there is a shift to a higher field in the absorption of the anomeric proton (H-1') of the ribose moiety linked to N-9. A similar shift has been observed previously in the absorption of the anomeric proton of 8-α-hydroxy-isopropyladenosine as compared to that of adenosine (1).

Our results indicate that the photoalkylation reactions can be applied for the modification of polynucleotides, and that the modified polynucleotides are sensitive to RNase. The 2-propanol-modified moieties consist either of uracil residues which underwent addition of the alcohol across the 5,6-double bond (6-8) or adenine or guanine residues, which were alkylated at the C-8 position. With irradiated poly(G) and 2-propanol, T_1-RNase hydrolyzed the polynucleotide, under standard conditions, in the normal pattern, and the digestion resulted in a mixture of Guo-2'(3')-P and 8-α-hydroxyisopropyl-Guo-2'(3')-P. These results indicate that alkylation at the C-8 position of guanine residue with the α-hydroxyisopropyl group does not lead to the inability of T_1-RNase to rupture the phosphodiester bonds of the alkylated guanine moiety. It is noteworthy, that 8-bromo-Guo-2':3'-P was digested by T_1-RNase with the same rate as that of Guo-2':3'-P (15).

In T_1-RNase digestion of Poly(G), the 8-position of the purine moiety in the nucleotide is not in intimate contact with the binding site of the enzyme (15). It is, therefore, feasible that 8-modified purine moieties are still as good a substrate as the non-modified moieties for the nucleases; differing only, if at all, in the rates of the spliting of the cyclic phosphate inter-mediates.

The 2-propanol modified poly($U_{20}G$) showed the normal pattern of nucleotides upon digestion with pancreatic RNase. The digestion mixture consisted of a mononucleotide fraction, which included Urd-3'(2')-P and modified Urd-2'(3')-P, as well as dinucleotidic fraction consisting of UpGp and a mixture of modified UpGp. This indicates that pancreatic RNase ruptures the phosphodiester bond of the 6-α-hydroxyisopropyl-5,6-dihydro Urd-2'(3')-P moieties despite the presence of a sizable group at C-6 of the pyrimidine system and, therefore, digests the modified poly($U_{20}G$) (16-18). It is noteworthy, that 6-hydroxy-5,6-dihydro-Urd-2':3'-P was cleaved by the RNase, while the corresponding uridine cyclobutane dimer was not (12).

The T_2-RNase digestion of the irradiated poly(A) and 2-propanol resulted in

Ado-2'(3')-P and 8-α-hydroxyisopropyl Ado-2':3'-P. These results indicate that splitting of the phosphodiester bond of 2-propanol-modified Ado-2'(3')-P moieties stops at the cyclization step under the conditions described.

The T_2-RNase digestion of D-ribose-modified poly(A) resulted, however, in a mixture of modified Ado-2':3'-P and the open form of Ado-2'(3')-P.

To summarize, the enzymes which digest the polynucleotides split the phosphodiester bonds of photochemically alkylated moieties of Guo, Ado and Urd; however, in some cases the cyclic modified mononucleotide results either exclusively or together with the open form of the mononucleotide.

We thank Professor A. Zamir and Dr. J. Sperling for most useful comments and remarks.

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