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Preparation and template activities of polynucleotides containing O^2- and O^4-alkyluridine

[DNA-dependent RNA polymerase (nucleotidyltransferase)/mispairing bases/mutation/*N*-nitroso alkylating agents]

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ABSTRACT **O^2-Ethyl-UDP and O^4-methyl-UDP have been prepared and copolymerized in various proportions with UDP or CDP, using polynucleotide phosphorylase. The copolymers were used as templates for DNA-dependent RNA polymerase in the presence of Mn^{2+}.**

Both of the *O*-alkylated uridines caused similar misincorporations. When copolymerized with U they led to incorporation of CMP and GMP into the poly(A). No AMP or UMP incorporation seemed to be caused by the introduction of *O*-alkyluridines into either poly(U) or poly(C).

The mispairing of O^2- and O^4-alkyluridine to behave like C or G represents mutagenic events. O^2 alkylation of U or T is, in contrast to O^4 alkylation, a relatively frequent result of treatment of double-stranded nucleic acids with *N*-nitroso alkylating agents. In single-stranded nucleic acids both O^2 and O^4 alkylations of U and T occur to similar extents. Thus, the observed mutagenic effects of O^2 and O^4 alkylation of U may be involved in the high carcinogenicity of these alkylating agents.

Mutation and malignant transformation resulting from chemical modification of nucleic acids by alkylating agents have been ascribed to miscoding of certain alkyl bases. The first modified base to be studied in these terms was 3-alkyl-C, the formation of which correlated with an increased mutation frequency (1, 2). When 3-alkyl-CDP was copolymerized with CDP and used as template for DNA-dependent RNA polymerase, A, U, or C was incorporated into the complementary polynucleotide, thus confirming biological evidence that this reaction was under certain circumstances mutagenic (3–6). In the same way, the formation of O^6-alkyl-G was found to correlate with increased carcinogenicity, and in polymers O^6-alkyl-G directed the misincorporation of A and U (7).

More recently, we have found that the highly carcinogenic *N*-nitroso alkylating agents not only favor the alkylation of the O^6 of G, but also react to a high extent with all three pyrimidine oxygens (O^2 and O^4 of U or T, O^2 of C) as well as the $—O^-$ of the phosphodiesters and, in RNA, the $O^{2'}$ of ribose (8–12).

O-Alkylpyrimidines are labile to various extents in acid and alkali (13), and one portion of this paper describes methods for preparing the corresponding nucleoside diphosphates without loss of the alkyl group or sugar, and for their incorporation in polynucleotides. The various copolymers containing O^2-ethyl-U or O^4-methyl-U were then studied in terms of their base-pairing.

EXPERIMENTAL

Preparation of *O*-Alkyluridines. O^4-Methyluridine was synthesized following the procedure of Robins and Naik (14). O^2-Ethyluridine was obtained by ethylation of uridine with diazoethane according to Kuśmierek and Singer (15). ^{14}C-Labeled O^2-ethyluridine was prepared by the same procedure with ethylnitrosourea containing ^{14}C in the ethyl group. O^2-Ethyluridine was recrystallized from ethanol to give colorless crystals with mp 170.5°–172°, UV (H_2O) λ_{max} 227, 252 nm (ϵ = 10,400, 10,500 M^{-1} cm^{-1}), λ_{min} 211, 238 nm (ϵ = 6900, 8900 M^{-1} cm^{-1}). The use of O^2-ethyluridine rather than O^2-methyluridine was due to difficulty in obtaining large enough amounts of the methyl derivative (15).

Preparation of 5′-Phosphates of *O*-Alkyluridines. 5′-Phosphates of *O*-alkyluridines were obtained by enzymatic phosphorylation of modified nucleosides using crude wheat shoot extract as a source of phosphotransferase (16). A typical reaction mixture containing 0.3 mmol of *O*-alkyluridine, 3 ml of 1 M pH 4.5 sodium acetate buffer, 6 ml of wheat shoot extract, and 1.2 g of *p*-nitrophenylphosphate was incubated 16–20 hr at 37°. The isolation of nucleotides was by chromatography on an anion exchange column [Dowex 1 X 2 (HCO_3^- form) using a gradient of 0.1–0.5 M triethylammonium bicarbonate]. The yield of the nucleotides varied between 30% and 60%, depending on the wheat shoot extract preparation. During the enzymatic reaction up to 5% of the alkyl groups is lost. However, the dealkylation products (uridine and 5′-UMP) are easily separated by the column chromatographic system used.

Contaminating inorganic phosphate is deleterious to the next phosphorylation step and must be separated from the nucleotide. Inorganic phosphate was determined on aliquots of each fraction from the column, using the method of Chen *et al.* (17). In the case of O^2-ethyl-UMP, 60–70% of the nucleotide peak did not contain inorganic phosphate and could be used for the next reaction after evaporation to dryness. Additional material was obtained by rechromatography of the phosphate-containing part of the nucleotide peak. In the case of O^4-methyl-UMP, for which the inorganic phosphate peak almost symmetrically covered the nucleotide peak, the separation of nucleotide from inorganic phosphate was as follows (the same procedure is applicable and was used for O^2-ethyl-UMP): 0.1 mmol of nucleotide was dissolved in 5 ml of water in a centrifuge tube. Approximately 3 ml of 0.5 M barium acetate was added in 1-ml portions until a white precipitate of barium phosphate formed. The precipitate was centrifuged off, washed with 2 ml of water, and centrifuged again. The combined supernatants (containing the nucleotide and an excess of barium acetate) were passed through the bed of 10 ml of Dowex 50 W X 1 in the pyridinium form, then the bed was washed with 50 ml of water. From the combined effluents containing the nucleotide, the residual pyridinium acetate was removed by repeated evaporation from an aqueous solution.

Preparation of 5′-Diphosphates of *O*-Alkyluridines. The synthesis of 5′-diphosphates of *O*-alkyluridines follows the procedure of Hoard and Ott (18) for the preparation of 5′-tri-

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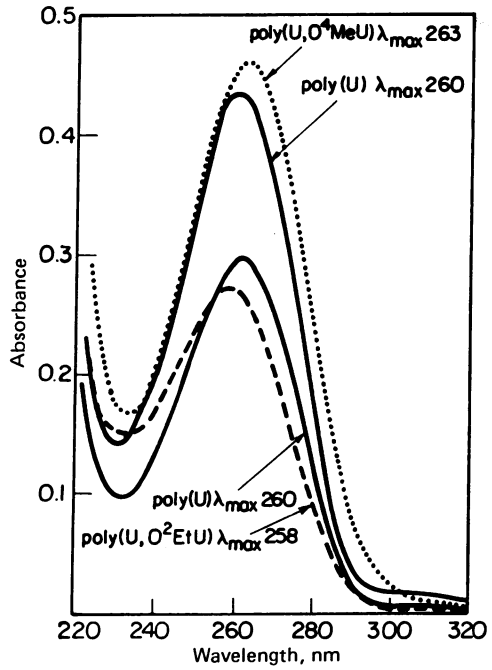

FIG. 1. UV absorbance spectra in water of poly(U) (—), poly(U,O^4MeU) 7:1 (····), poly(U,O^2EtU) 3:1 (- - -). The λ_{max} of each is shown in the figure. The λ_{max} (H_2O) of O^4-methyl-U is 272 nm and of O^2-ethyl-U, 227 nm and 252 nm.

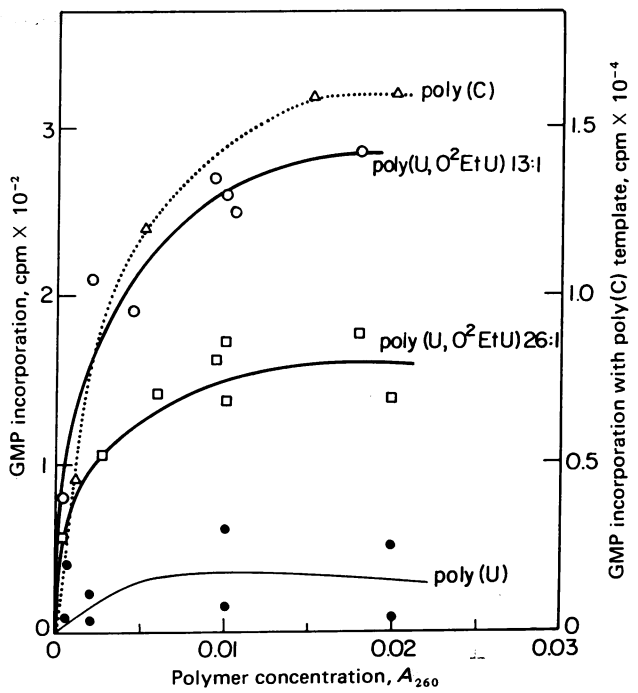

FIG. 2. Nucleotide incorporation as a function of template concentration in the presence of [^{3}H]GTP and unlabeled ATP, CTP, and UTP. The normal incorporation of GMP with a poly(C) template (△) is shown, with the level of incorporation on the right abscissa, and misincorporation of GMP with poly(U) (●), poly(U,O^2EtU) 26:1 (□), and poly(U,O^2EtU) 13:1 (○) on the left abscissa. The points for each curve are a composite of several experiments.

phosphates of 2′-deoxynucleosides, except that tributylammonium phosphate was used as a phosphorylating agent instead of tributylammonium pyrophosphate. The separation of 5′-diphosphates was by chromatography on an anion exchange column. The column was 2.5 × 30 cm Dowex 1 X 2, 200–400 mesh, in the HCO_3^- form. Fractions were eluted with a linear gradient of 0–0.95 M triethylammonium bicarbonate. The nucleotides eluted with 0.5 M solvent and the nucleoside diphosphates with 0.85 M solvent. After column separation the yield of 5′-diphosphate was 50–70%.

The 5′-diphosphate-containing eluate (0.05 mmol) was evaporated to dryness, and from the residue added ethanol was repeatedly evaporated to remove triethylammonium bicarbonate. The 5′-diphosphates were converted to the sodium salt by adding 1 ml of 1 M NaI in acetone to 1 ml of a methanolic solution of the triethylammonium salt. After vigorous mixing, 10 ml of acetone was added and the precipitate was centrifuged off, washed several times with acetone, then dissolved in water and reprecipitated with ethanol/acetone (1:1 vol/vol). The final yield of 5′-diphosphate was 30–55%.

The samples had the expected ratio of phosphorous to extinction coefficient and the UV spectra were identical to the parent nucleoside. Limited digestion with bacterial alkaline phosphatase gave a mixture of 5′-diphosphate, 5′-monophosphate, and nucleoside, while the product of prolonged digestion was a nucleoside.

Polymerization of 5′-Diphosphates of *O*-Alkyluridines. A typical incubation mixture (60 μl) contained a total of 1 mg of nucleoside 5′-diphosphate (or mixtures of two nucleoside diphosphates) and 20 μg of polynucleotide phosphorylase (from *Micrococcus luteus*, P-L Biochemicals 0302) in 0.1 M pH 8.2 Tris·HCl/0.5 mM EDTA/5 mM $MgCl_2$/bovine serum albumin at 0.04 mg/ml. The mixture was incubated at 37° for 16 hr. Polymers were separated from unreacted nucleoside diphosphate using thin-layer chromatography on cellulose (Eastman no. 6065). Details will be described elsewhere. When a mixture of unmodified and *O*-alkyl nucleoside diphosphate was polymerized, such chromatography also separated the unreacted two diphosphates from each other so that each could be eluted and quantitated.

Polymer yield and composition were determined in several ways. When O^2-[^{14}C]ethyl-UDP was present the specific radioactivity of the polymer was a measure of its incorporation. The UV absorbance (and radioactivity) of the unpolymerized diphosphates gave an independent value for the extent of polymerization. Polymers containing either O^2-ethyl-U or O^4-methyl-U showed UV spectral changes that were correlated with the amount of *O*-alkyl-U. The λ_{max} of poly(U), which is 260 nm, was shifted to the red by the presence of O^4-methyl-U (λ_{max} 272) and to the blue by O^2-ethyl-U (λ_{max} 252) (Fig. 1). In a series of polymers containing various input ratios of U and *O*-alkyl-U, superimposition of the spectra of the polymers showed spectral shifts which could be used to calculate the amount of *O*-alkyl-U in each polymer. Analysis of the spectral shifts in poly(C) containing *O*-alkyl-U was less feasible, and the amount of unlabeled *O*-alkyl-U incorporation was determined by difference after quantitating the unpolymerized diphosphates. Labeled *O*-alkyl-U incorporation in poly(C) was determined by the radioactivity in the polymer as compared to the unpolymerized nucleoside diphosphate.

RNA Polymerase Reactions. Incubations with RNA polymerase (*Escherichia coli* K-12, EC 2.7.7.6) were performed as previously described (19). Tubes containing enzyme, a ^{14}C-labeled nucleoside triphosphate and usually all four unlabeled nucleoside triphosphates, Mn^{2+}, 2-mercaptoethanol, and various amounts of template were incubated at 37° for 2 hr. The product was transferred to DEAE-paper disks and washed with Na_2HPO_4 followed by water and ethanol, and the bound radioactivity was determined. With poly(C) as template, GMP incorporation into polynucleotide was about 70% of the theo-

Table 1. Copolymerization of *O*-alkyl-UDP with UDP and CDP

Diphosphate	Input NDP,*	Polymerization,† %	NMP in polymer,‡ %
UDP	—	38§	
UDP	4% CDP	29	
UDP	6% O^2EtUDP	41	3.8
UDP	23% O^2EtUDP	23	7.5
UDP	27% O^2EtUDP	20	10
UDP	60% O^2EtUDP	11	23
UDP	4% O^4MeUDP	32	(3)
UDP	7% O^4MeUDP	20	(7)
UDP	13% O^4MeUDP	18	13
CDP	—	29§	
CDP	4% UDP	13	
CDP	4% ADP	20	
CDP	6% O^2EtUDP	27	8
CDP	13% O^2EtUDP	22	10
CDP	23% O^2EtUDP	17	10
CDP	7% O^4MeUDP	30	(5)
CDP	13% O^4MeUDP	20	(7)

* NDP stands for the added diphosphate, whether modified or unmodified.

† Not corrected for absorbance changes as a result of polymer hypochromicity.

‡ See *Experimental* for determination of % modified nucleotide in polymers. Numbers in parentheses are derived from data that are approximations.

§ Average of three experiments.

retical maximum after a 2-hr reaction. Complementary polymer formation increased with increasing amounts of template until the saturation level was reached (Fig. 2).

Nearest-Neighbor Analysis. RNA polymerase reactions were performed as above except that the scale of the reaction was increased 5-fold and all four triphosphates were present with the labeled nucleoside triphosphate in the α-^{32}P form. The procedure for isolation and hydrolysis was similar to that used by Ludlum (20). The four nucleotides were separated by electrophoresis on Whatman 3MM paper with 1 M pH 3.5 pyridinium acetate as solvent and each 3′-nucleotide UV-absorbing area was cut out and the ^{32}P radioactivity was determined.

RESULTS

Preparation of *O*-Alkyl-U-Containing Polynucleotides. We have recently shown that the alkyl group on either the O^2 or O^4 of uridine is unstable at both acid and alkaline pH (13). We have now developed methods to prepare the 5′-diphosphates of *O*-alkyluridines by enzymatic phosphorylation followed by gentle chemical phosphorylation (see *Experimental*), and have used these as substrates for polynucleotide phosphorylase. Both O^2-ethyl-UDP and O^4-methyl-UDP could be copolymerized with UDP or CDP. Data for the extent of polymerization and the composition of copolymers are shown in Table 1. Increasing the input of *O*-alkyl-U decreases the yield of polymer. This is partially due to a decreased rate of polymerization similar to that found by M. Kröger and B. Singer (unpublished) for the copolymerization of CDP and 2-thio-CDP or 2-(methyl)thio-CDP. The time of polymerization was standardized at 16 hr because maximal yields of poly(C) and poly(U) were obtained in that time.

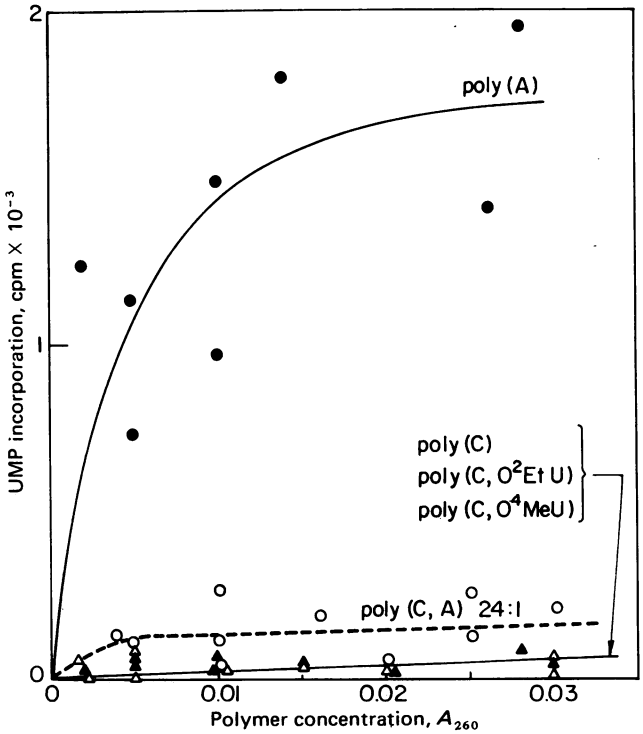

FIG. 3. Nucleotide incorporation as a function of template concentration in the presence of [^{3}H]UTP and unlabeled ATP, CTP, and GTP. Poly(C,O^2EtU) contained 10% O^2-ethyl-U and poly(C,O^4MeU) contained 7% O^4-methyl-U. Experiments with each of these polymers are shown with the symbol ▲ and poly(C), with the symbol △; normal incorporation of UMP with poly(A) (●) and with poly(C,A) 24:1 (○) are also shown. The points for each curve are a composite of several experiments.

Template Properties of *O*-Alkyl-U-Containing Polynucleotides. These polymers were then used as templates for RNA polymerase in the presence of Mn^{2+}. The effects of the presence of O^2- and O^4-alkyl-U were similar. Both directed the incorporation of G and C; neither paired with A or U.

Data for UMP incorporation directed by O^4-methyl-U or O^2-ethyl-U in poly(C) are shown in Fig. 3. Although poly(A) is not a very efficient template for poly(U) synthesis, a polymer containing C with 4% A incorporated a measurable amount of UMP, while the various poly(C,*O*-alkyl-U) samples gave no indication of UMP incorporation above the control poly(C) (Fig. 3).

Similar negative results were obtained when AMP incorporation was studied with poly(C,*O*-alkyl-U) as template while a control poly(C,U) containing 4% U incorporated the expected amount of AMP (data not shown). Furthermore, when O^4-methyl-U or O^2-Ethyl-U were incorporated in poly(U), AMP incorporation was progressively depressed as the amount of *O*-alkyl-U in the template increased, indicating that the alkylated bases are not recognized by RNA polymerase as uridine (Table 2).

The presence of O^2- or O^4-alkyl-U in poly(U) resulted in the incorporation of GMP and CMP into the product copolymer (Figs. 2, 4, and other data not shown). The efficiency of such misincorporation of GMP approached that of normally base-paired GMP, indicating that 30–70% of the modified bases in the template were transcribed. The efficiency of misincorporation of CMP was low, but no lower than its normal base-paired polymerization with poly(U,G) templates as observed by us and by Ludlum (20).

Table 2. Effect of O-alkyl-U on normal base pairing of poly(U) and poly(C)

Alkyl base	% in poly(U)†	AMP incorporation,‡ % of poly(U)	% in poly(C)†	GMP incorporation,§ % of poly(C)
O^2EtU	3.8	88 (92)		
	7.5	82 (90)		
	10	74 (84)		
	23	59 (82)		
			8	99 (107)
O^4MeU	4	81 (85)		
	7	77 (84)		
	13	56 (69)		
			7	30 (37)
			13	13 (26)

* Considerable UMP incorporation occurs with poly(U) as template in the presence of AMP, and considerably lesser CMP incorporation occurs with poly(C) as template in the presence of GTP; these effects are probably due to secondary transcription of the primary product. For this reason poly(U,*O*- alkyl-U) could not be used to test for UMP misincorporation, nor poly(C,*O*-alkyl-U) for CMP misincorporation.

† See Table 1.

‡ Average of two to four separate determinations. The numbers in parentheses are AMP incorporation corrected for the percent of U in the template.

§ Average of two separate determinations. The numbers in parentheses are GMP incorporation corrected for the percent of C in the template.

Over a wide range of modified base content, GMP and CMP incorporation was observed. The extent of misincorporation increased as the percent of modified base in the polymer increased (illustrated in Fig. 2). However, polymers containing more than 10% of either modified base were less efficient in misincorporation as well as in the normal incorporation of AMP basepairing to U [as compared to poly(U)] (Table 2). O^4-Methyl-U in poly(C) greatly decreased GMP incorporation, although the same alkyl base in poly(U) directed GMP incorporation. This is an unexplained oddity, because GMP incorporation was not noticeably affected by 8% O^2-ethyl-U in poly(C) (Table 2).

Nearest-neighbor analyses were performed after [α-^{32}P]GTP incorporation and clearly established that O^4-methyl-U and O^2-ethyl-U in poly(U) directed GMP incorporation into the complementary polymer in a random fashion as indicated by the ^{32}P transfer to Ap and not Gp. With poly(C), only poly(G) is synthesized, and thus the label remained in Gp. Data for a typical experiment are in Table 3. The small amount of G incorporation found with poly(U) as template was also internal and not terminal, because the ^{32}P label was found in Ap after KOH digestion. This type of experiment is extremely sensitive and it is not excluded that the UDP used to prepare poly(U) might contain 0.2% CDP [calculated from the data for poly(U_{24},C)], or that the triphosphates might not be absolutely pure.

Table 3. Nearest-neighbor analyses for products from templates containing *O*-alkyl-U

Template	[^{32}P]GMP incorporation, cpm in aliquot*	^{32}P cpm in Up	Gp	Ap	Cp
Poly(U)	147	225	160	890	160
Poly(U, 4% C)	940	295	240	3580	70
Poly(U, 7% O^4MeU)	853	165	240	3140	55
Poly(U, 8% O^2EtU)	617	255	270	2580	100
Poly(C)	24,800	300	101,000	360	95

* 20% of entire reaction mixture was placed on DEAE-paper and washed as described for RNA polymerase reactions.

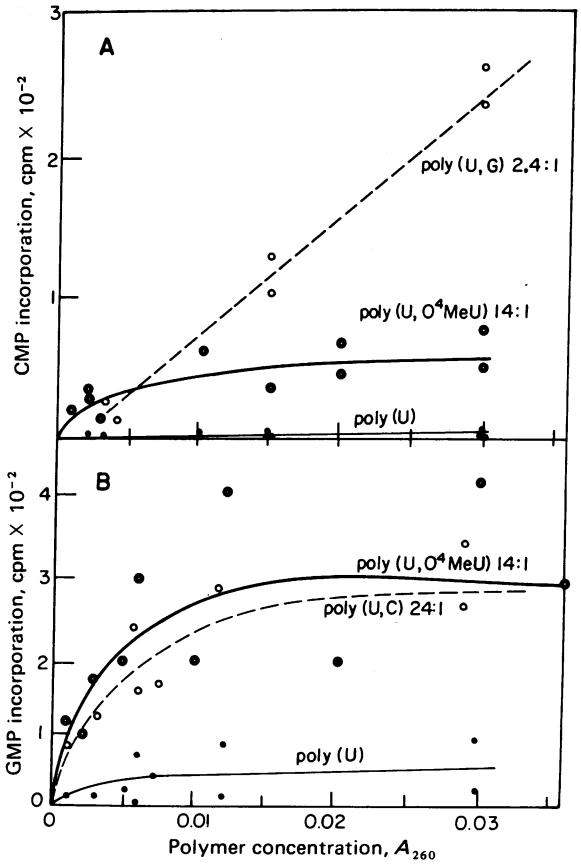

FIG. 4. Nucleotide incorporation as a function of template concentration. Points for each curve are a composite of several experiments. (*A*) [^{3}H]CMP incorporation in the presence of unlabeled ATP, GTP, and UTP. Poly(U) (●), poly(U,G) 2.4:1 (○), poly(U,O^4MeU) 14:1 (◐). (*B*) [^{3}H]GMP incorporation in the presence of unlabeled ATP, CTP, and UTP. Poly(U) (●), poly(U,C) 24:1 (○), poly(U, O^4MeU) 14:1 (◐).

DISCUSSION

Singer and coworkers have demonstrated in recent years that the highly carcinogenic alkyl nitroso compounds preferentially alkylate the oxygens when acting on nucleic acids (8–12). One objective of current research is to ascertain the biological consequences of specific modifications of this type. An *in vitro* technique to test for mispairing by modified bases has been successfully applied in this laboratory (5, 19) and that of Ludlum (3, 4, 6, 7, 20). Polynucleotides containing modified bases in homo- or heteropolymers with typical nucleotides are used as templates for RNA polymerase. We were able to validate this method by demonstrating good correlation between such *in vitro* misincorporation and mutagenesis observed *in vivo* (1, 2).

The present study concerned itself with the effect of alkylating either the O^2 or O^4 on its base-pairing capability when

copolymerized with either U or C, in ratios ranging from 3–23% of the alkylated residue.‡

Alkylation of either oxygen of U resembles O^6 alkylation of G in that it results in partially specific mutagenesis, but it causes apparent pairing with different based than reported for O^6-alkyl-G (7). Both alkylations of U are definitely mutagenic in that these residues no longer normally pair like U, nor would they be expected to, because they lack the necessary proton on the N-3 in addition to having the steric factor of an alkyl group next to this nitrogen. However, alkyl groups on the O^2 or O^4 do permit the incorporation of both G and C into the product, yet not A. To the extent that it could be tested this was true whether the alkylated Us were co-polymerized with U or C.

The efficiency of transcription of copolymers with modified bases is often lower than that of the homopolymers (Table 2), possibly due to *O*-alkyl-U residues affecting their secondary structure, which in turn may well affect transcription. This appears to be particularly the case when O^4-methyl-U is copolymerized with either C or U (Table 2).

The mechanism that permits insertion of any base opposite N-3 alkyl-C, but only A or U opposite O^6-alkyl-G, and only G or C opposite the O^2- and O^4-alkyl-U, is not understood. In some of these "pairings," hydrogen bonds can be postulated if one assumes that the unmodified base donates two protons. The more likely explanation for the observed misincorporations is that suggested by Ikehara and Hattori (21, 22), which attributes misincorporations largely to vertical stacking forces, although the role of changes in secondary structure resulting from a limited number of modified bases cannot yet be evaluated.

A prior study on the template properties of an *O*-alkylpyrimidine is that by Abbott and Saffhill (23). However, they attributed misincorporation of GMP solely to O^4-alkyl-T. The evidence that CMP was not incorporated lacks positive controls; in our work and that of Ludlum (20), CMP is always incorporated with low efficiency and thus at low levels its incorporation may be difficult to detect. O^2 alkylation of T, which Abbott and Saffhill did not report, occurs to a very much higher extent than O^4 alkylation (10). The alkylation of the O^2 of T has been recently corroborated by D. Jensen (personal communication), who reacted poly(dA·dT) with the same reagents used by Abbott and Saffhill and found that under all conditions of reaction of the double-stranded polymer with methylnitrosourea and ethylnitrosourea, O^2 alkylation was approximately 10 times that of O^4 alkylation.

As far as relating our observations to carcinogenesis is concerned, the predominant effect on nucleic acids of many poorly carcinogenic alkylating agents, the substitution of the N-7 of G, has been tested in *in vitro* systems and found to be lethal but nonmutagenic (24, 25). The *N*-nitroso carcinogens modify nucleic acids on several sites that can be demonstrated to have a mutagenic effect (N-3 of C, O^6 of G, O^2 of U or T, O^4 of U or T) and on other sites that have not yet been studied in these terms. Yet we do not regard these findings as evidence for the belief that there is a simple and direct relationship between mutation and carcinogenesis.

‡ The results of parallel studies of the translation capability of the same copolymers, using the wheat germ system, will be reported elsewhere. These experiments also showed varying depression in the amount of polypeptides made when alkylated uridines were present in the messenger, but mutation in terms of altered codon expression was not detected.

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