Proc. Natl. Acad. Sci. USA
Vol. 83, pp. 28–32, January 1986
Biochemistry

O^4-Methyl, -ethyl, or -isopropyl substituents on thymidine in poly(dA-dT) all lead to transitions upon replication

(*N*-nitroso carcinogens/DNA polymerase I/fidelity/mutagenesis)

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Contributed by H. Fraenkel-Conrat, August 26, 1985

ABSTRACT **In a previous paper, we reported that O^4-methyl dTTP can be incorporated into poly(dA-dT) in place of thymidine without distortion of the helical structure, but on replication it could behave as deoxycytidine and misincorporate dGTP. Only weak interactions are possible for any O^4-modified T·A pair. While O^4-alkyl T·G pairing should be favored, experiments to detect the ability of *Escherichia coli* DNA polymerase I (pol I) to utilize the triphosphate as dCTP were ambiguous. dTTPs with larger alkyl groups (ethyl, isopropyl) have now been synthesized and tested for their recognition as dTTP by pol I. Enhanced steric hindrance could be expected, particularly for O^4-isopropyl dTTP, which has a three-carbon branched chain. However, both compounds behaved qualitatively like O^4-methyl dTTP, being incorporated into poly(dA-dT) and then directing deoxyguanosine misincorporation by pol I. Quantitative comparisons of mutagenicity were not possible because of the finding that, unlike polymers made with O^4-methyl dTTP, those made with ethyl or isopropyl dTTP were resistant to hydrolysis by using a variety of nucleases. The frequent misincorporations of dGTP would be expected to produce transitions *in vivo*. O^4-ethyldeoxythymidine is very poorly repaired *in vivo*, which would also be expected for repair of O^4-isopropyldeoxythymidine. Therefore, under suitable conditions, these particular carcinogen products are likely to be initiators of carcinogenesis.**

O^4-alkyldeoxythymidine, formed both *in vitro* and *in vivo* by *N*-nitroso methylating and ethylating agents (1) has been implicated in initiation of carcinogenesis (2, 3). Neither O^4-methyldeoxythymidine (O^4-Me-dThd) nor O^4-ethyldeoxythymidine (O^4-Et-dThd) is rapidly repaired *in vivo* (2–4), and the mechanism for their repair is not, at present, identified in mammals. It does not appear to be due to the O^6-methyldeoxyguanosine methyltransferase, as is the case in bacteria (5). The analogous propylating agents are also carcinogenic (ref. 6 and refs. therein) and form both isopropyl and propyl bases (7, 8). However, O^4-isopropyl (or propyl) deoxythymidine (O^4-iPr-dThd) has not been reported as a product; on the basis that O^6-iPr-dGuo is formed *in vivo* (7) and that O^4-butyldeoxythymidine has been identified in *N*-butyl-*N*-nitrosourea-treated DNA (9), it can be assumed that O^4-iPr-dThd is also formed *in vivo*. Its repair would be expected to be even slower than repair of the methyl and ethyl analogues (2–7). Neither of the larger (ethyl, isopropyl) O^4-alkylthymidines has previously been investigated for potential mutagenicity and the ability to be utilized by polymerases, which would be indicative of carcinogenic potential.

Both O^4-Me-dThd and O^4-methyl- and ethyluridine appear to simulate cytidine in transcription, replication, or codon–anticodon binding (10–13). O^4-Methyl dTTP (O^4-Me-dTTP) could substitute for dTTP in primed poly(dA-dT) synthesis using *Escherichia coli* DNA polymerase I (pol I) (12). Upon copying such a polymer with the same enzyme, dGTP was incorporated, showing again that O^4-Me-dThd can act as deoxycytidine. Saffhill and Fox (14) were able to incorporate O^4-Me-dThd from the cell culture medium into V79 cell DNA, albeit at a low frequency. Deoxycytidine was a competitive inhibitor of incorporation, suggesting that the analogue was replacing deoxycytidine (15). In studies to be reported separately (B. D. Preston, L. A. Loeb, and B.S., and unpublished observations), each of the three O^4-alkyl dTTPs could be introduced in place of dTTP in ϕX174 DNA by polymerase-catalyzed extension of synthetic oligonucleotides.

In this paper, we report that both the two-carbon ethyl group or the three-carbon branched isopropyl group on the O^4 of T incorporated into poly(dA-dT) also lead to dGTP incorporation when the polymer is replicated. As with the polymers containing the analogous methyl derivative, no apparent distortion of the helical structure of poly(dA-dT) was observed.

MATERIALS AND METHODS

O^4-Me-dTTP was prepared as described (12). Deoxynucleoside triphosphates (dNTPs), double-stranded alternating poly(dA-dT), other polynucleotides, the Klenow fragment of pol I, and various nucleases were obtained from Pharmacia P-L Biochemicals. Radiolabeled dNTPs were obtained from New England Nuclear. Highly purified pol I was a gift from L. A. Loeb.

Preparation of O^4-Ethyl and O^4-Isopropyl dTTP. O^4-iPr-dThd, prepared as described (12), was phosphorylated to yield O^4-iPr-dTMP (10). The isopropyl group was displaced with sodium ethoxide in absolute ethanol previously dehydrated to contain <0.05% water. The dehydration of commercial absolute ethanol is necessary because the displacement reaction is extremely sensitive to water and, even with "dehydrated" ethanol, some hydrolysis of the alkyl group occurs. O^4-Et-dTMP was separated from unreacted O^4-iPr-dTMP and dTMP by sequential purification on a Dowex 1 × 4 (carbonate form) followed by a Sephadex A-25 (carbonate form). The conversion of O^4-Et-dTMP and O^4-iPr-dTMP to the triphosphates was carried out by the procedure of Hoard and Ott (16), and the products were isolated on a Sephadex A-25 (carbonate form) using a gradient of triethylammonium bicarbonate for elution. Inorganic pyrophosphate, which inhibits polymerization by pol I, cochromatographed with O^4-Et-dTTP. It was then removed by using an XAD-4 column. The alkyl dTTPs were converted to the sodium salt.

O^4-Et-dTTP was also obtained by direct ethylation of thymidine with diazoethane (17) followed by phosphoryl-

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Abbreviations: O^4-Me-dThd, O^4-Et-dThd, O^4-iPr-dThd, etc., O^4-methyl-, O^4-ethyl-, O^4-isopropyldeoxythymidine, etc.

ation. In subsequent experiments, there was no detectable difference in the two preparations. However, the yield was less than with the exchange reaction and separation of the three initial products and unreacted dTTP was more difficult.

The O^4-alkyl dTTPs were homogeneous on thin-layer chromatography and, on limited digestion with alkaline phosphatase, gave only the expected products. Neither O^4-Me-dThd, O^4-Et-dThd, nor O^4-iPr-dThd, the products of complete dephosphorylation, contained detectable dThd using HPLC chromatography (12) in which the retention times were as follows: dThd, 4.7 min; O^4-Me-dThd, 7.8 min; O^4-Et-dThd, 9.5 min; O^4-iPr-dThd, 10.8 min.

Preparation of Poly(dA-dT) Using dATP and Both dTTP and O^4-Alkyl dTTP. Synthesis of polymers was carried out as described (12) or using variations as indicated in the figure legends. Polymerization of dNTPs was monitored by the decrease in absorbancy at 260 nm using a Varian-Cary 219 spectrophotometer at 37°C. Occasional samples, not intended for fidelity measurement, were prepared with [^{3}H]dATP and aliquots were taken at numerous time points for determination of the rate of incorporation into acid-insoluble polymer. The final polymer products, judged by maximum hypochromicity (30–40%), were purified by separation from monomeric material using Biogel P-150 column chromatography. The absorbancy of the purified polymer, compared to that of the primer, was taken as the net synthesis.

Substitution of O^4-Me-dTTP for dCTP in Polymerization. Two polymers were used as templates for pol I (12). The polymers were poly(dG-dC), or poly(dA-dC)·poly(dT-dG), in which only synthesis of the poly(dT-dG) strand was monitored by using [^{3}H]dATP. In each case, dCTP, a normal complement, was omitted and O^4-Me-dTTP or dTTP was substituted.

Assay of Fidelity in Replication of Poly(dA-dT, O^4-Alkyl dThd). The methods are essentially those described (12, 13). Both pol I and the Klenow fragment of pol I were used. Reaction mixtures (50 μl) contained (per ml) 50 μmol of Tris·HCl (pH 7.8), 2 μmol of $MgCl_2$, 100 nmol of dTTP, 100 nmol of dATP, and 100 μmol of dGTP, 0.15 A_{260} poly(dA-dT), 8 units of pol I, or 4 units of the Klenow fragment, incubated 30 min at 37°C. In replicate experiments, either the dATP or dGTP was ^{3}H labeled. The specific activity of [^{3}H]dGTP was at least 10-fold that of dATP. Washing and counting procedures were as described (13). Blank values for samples lacking template were ≈10 cpm for [^{3}H]dATP and ≈200 cpm for [^{3}H]dGTP. Consistent values >50% above the dGTP blank were considered significant.

All fidelity experiments were performed several times in duplicate, usually on two different polymer preparations.

Thermal Denaturation. The methods and instrumentation were as reported (12).

RESULTS

Effect of O^4-alkyl dTTPs in Synthesis of Poly(dA-dT). All three O^4-alkyl dTTPs were substrates for pol I and its large fragment (Klenow). However, synthesis plateaued relatively early unless some dTTP was present. The previously published paper on this topic (12) dealt only with *O*-methyl dTTPs, which were not greatly inhibitory for poly(dA-dT) synthesis. This is not true for O^4-Et-dTTP or O^4-iPr-dTTP. Fig. 1 illustrates the initial decrease in absorbancy resulting from polymerization. After 2 hr, synthesis of poly(dA-dT) using equimolar amounts of dTTP and dATP reaches maximum hypochromicity (35%; data not shown). In contrast, when 90% of the dTTP is replaced by O^4-Me-dTTP, initial synthesis is rapid but slows down after 30–40 min. The same 10:1 ratio of O^4-Et-dTTP and dTTP does decrease the rate of synthesis noticeably. An even greater effect is found in the presence of O^4-iPr-dTTP, where after 30 min a maximum of ≈2.5% hypochromicity is reached.

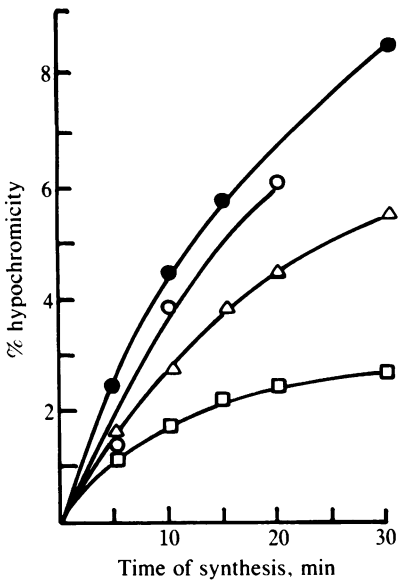

FIG. 1. Decrease in absorbancy of 100 μM dATP and 100 μM dTTP, or 100 μM dATP, 10 μM dTTP, and 100 μM O^4-alkyl dTTP with 0.03 A_{260} poly(dA-dT) primer and 20 units of pol I. ●, dATP and dTTP only; ○, added O^4-Me-dTTP; △, added O^4-Et-dTTP; □, added O^4-iPr-dTTP.

Nevertheless, appreciable polymer synthesis (30- to 50-fold increase over the primer) can be obtained by repeated additions of small amounts of dTTP as each plateau is reached (Fig. 2). This procedure, termed "forcing," does not abolish the difference in the inhibitory effect of the size of substituent, which is iPr > Et > Me.

The experiments described above were performed with 100 μM dATP, 10 μM dTTP, and 100 μM O^4-alkyl dTTP. The effect of varying the proportion of dTTP and O^4-Et-dTTP, with a high amount of poly(dA-dT) primer to extend the time during which synthesis is linear, is shown in Fig. 3. In Fig. 3*A*, 100 μM dATP and 25 μM dTTP are polymerized to a much higher extent than when 75 μM O^4-Et-dTTP were also added. As shown in Fig. 3*B*, reversal of the proportion of dTTP (75 μM) and O^4-Et-dTTP (25 μM) leads to a smaller difference in the rate of synthesis compared to using 100 μM dTTP only. In some reactions, incorporation of [^{3}H]dATP into an acid-insoluble product was monitored as an additional criterion of polymer synthesis. The rate and net polymer synthesis followed those in Fig. 3. Not shown in Fig. 3*B* is the parallel experiment using O^4-Me-dTTP, because there was no difference between synthesis using only dATP and dTTP and that with added 25 μM O^4-Me-dTTP substituting for the equivalent amount of dTTP.

Poly(dA-dT) containing O^4-Me-dThd was analyzed for composition after digestion using DNase I, snake venom phosphodiesterase, and acid and alkaline phosphatase (12). This procedure, or one using micrococcal nuclease and spleen phosphodiesterase for digestion, did not completely digest polymers made with O^4-Et-dTTP or O^4-iPr-dTTP. Both deoxyadenosine and deoxythymidine were released, although not quantitatively, because considerable oligomeric material was in the void volume of the cation exchange column (12). A parallel observation was made in attempting to quantitate these nucleosides in a ϕX174 primed with an oligonucleotide in which the O^4-alkyl dThd is adjacent to dA (B. D. Preston, L. A. Loeb, and B. S., unpublished observations). Since O^4-Et-dThd has been quantitated in enzyme digests of ethylated DNA (18), it seems likely that the problem resides in recognition of a specific sequence con-

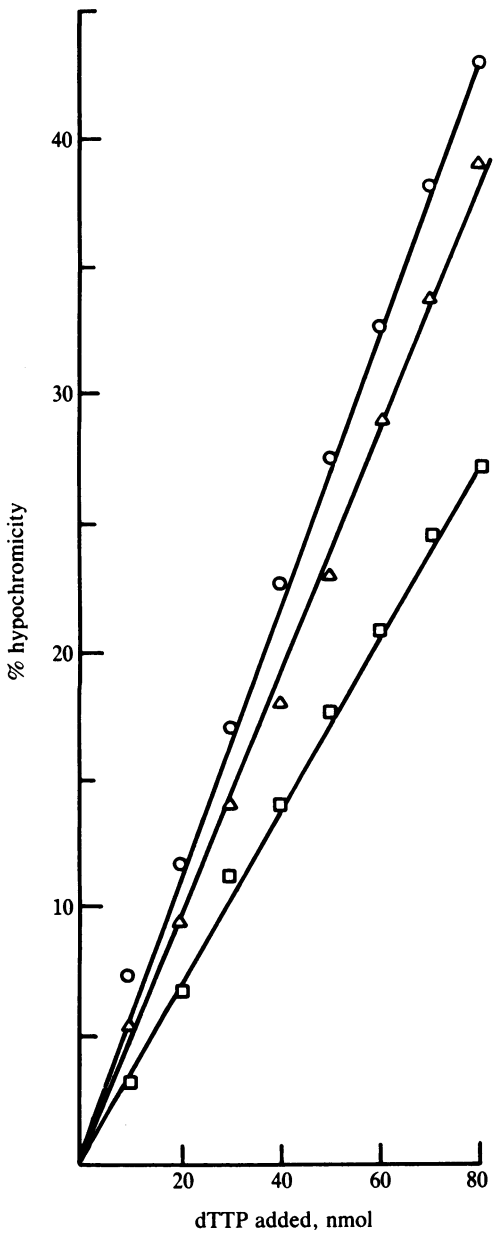

FIG. 2. Effect of repeated additions of 10 nmol of dTTP at 30-min intervals to 1-ml synthetic reaction mixtures shown in Fig. 1. Each point represents the cumulative hypochromicity after each 30-min polymerization. Net synthesis, relative to the poly(dA-dT) primer was 30- to 50-fold. ○, O^4-Me-dTTP present; △, O^4-Et-dTTP present; □, O^4-iPr-dTTP present. Complete enzymatic hydrolysis and analysis was only possible with the O^4-Me-dTTP polymer.

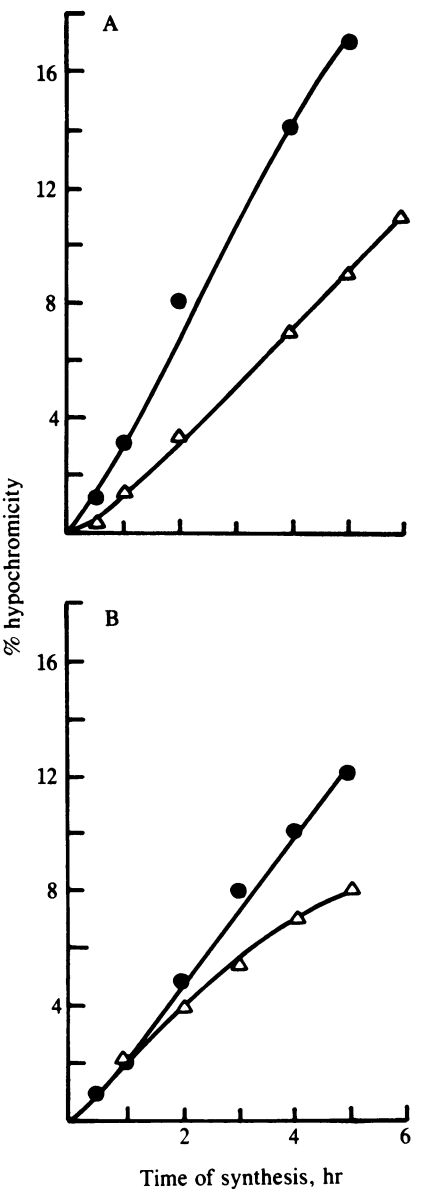

FIG. 3. Effect of varying the proportion of dTTP and O^4-Et-dTTP on rate and extent of polymer formation, measured by decreased absorbance in 1-ml reaction mixtures. (*A*) ●, 100 μM dATP, 25 μM dTTP, 0.5 A_{260} poly(dA-dT), 7 units of Klenow fragment; △, 75 μM O^4-Et-dTTP added to the reaction mixture. (*B*) ●, 100 μM dATP, 100 μM dTTP using the conditions described above; △, 100 μM dATP, 75 μM dTTP, 25 μM O^4-Et-dTTP, using the conditions described above. Not shown is the parallel experiment with O^4-Me-dThd in which the points are the same as for 100 μM dATP and 100 μM dTTP.

taining O^4-alkyl dThd, perhaps as a result of the orientation of the alkyl group and the phosphodiester. Another possibility is represented by the difficulty in digesting polymers with highly organized secondary structure—e.g., poly(dG)·poly(dC) (18).

It should be pointed out that the O^4-alkyl group is unstable in acid (19) so that hydrolysis can only be performed with enzymes.

Effect of O^4-Me-dTTP in Synthesis of Poly(dG-dC) and Poly(dC-dA)·Poly(dG-dT). Little polymer synthesis could be achieved by using alternating poly(dG-dC) as template/primer and the complementary dNTPs. Under our conditions, the use of O^4-Me-dTTP in place of dCTP did not lead to measurable incorporation. The less structured random double-stranded polymer was used in the presence of [^{3}H]dATP to measure synthesis of the dG-dT strand, which occurred at a much higher rate. When equimolar dATP and dTTP, without dCTP, were present, ≈6% synthesis compared to the normal level resulted, presumably as the result of a forced dG·dT wobble. The same experiment with O^4-Me-dTTP as the mismatched dNTP gave almost identical results to those with dTTP.

Thermal Stability of Synthesized Polymers. All of the polymers made with O^4-Me-dThd, O^4-Et-dThd, or O^4-iPr-dThd were indistinguishable from poly(dA-dT) in thermal stability, hyperchromicity, and breadth of transition (Table 1, Fig. 4).

Replication of Synthesized Polymers. Both pol I and the Klenow fragment of pol I were used for replication under conditions favoring high fidelity. The data were within experimental error for the two enzymes and thus have been pooled. Commercial poly(dA-dT) and polymer synthesized

Table 1. Thermal denaturation of poly(dA-dT)·poly(dA-dT) containing O^4-alkyl dThd

Substitution in poly(dA-dT)*	0.1 M Tris·HCl (pH 7.2)		
	t_m, °C	Δt, °C†	H, %‡
7% O^4-Me-dThd	61.3	1.8	45
2–5% O^4-Et-dThd	61.0	2.2	45
0.2–3% O^4-iPr-dThd	61.4	1.8	45
None	61.3	2.0	46

t_m, melting temperature.
*Percent substitution values calculated from the rate difference. See Fig. 1 and ref. 20.
†Temperature change betweeen 25% and 75% of total hyperchromicity.
‡Average hyperchromicity of three determinations.

as control to the present experiments gave the same error rate, which ranged from 1 dG per 25,000–80,000 bases synthesized. The higher fidelity represents the limit of detectability in our experimental system. These data are consistent with previously published error rates (12, 21).

The O^4-alkyl dThd-containing poly(dA-dT) were excellent templates and did not depress synthesis but did lead to much higher dG incorporation than the control (Table 2). The exact content of O^4-Et-dThd or O^4-iPr-dThd are not known, so that a calculation of the frequency of dG incorporation per modified dT cannot be made except for the O^4-Me-dThd polymer (12), in which 7% of the dT was replaced by the alkyl derivative. The calculated frequency of O^4-Me-dThd acting as dC is ≈1 dG per 25 O^4-Me-dThd. The previously published data (using less dGTP, which can increase fidelity due to pool bias) were ≈1 dG per 140 O^4-Me-dThd (12). In primed single-stranded polymers containing O^4-Me-dThd the frequency of misincorporation was ≈1 dG per 12 O^4-Me-dThd (13).

The relative incorporations of O^4-alkyl dThds, as determined by the ability of pol I to elongate a 15-mer ϕX174 primer, with O^4-alkyl dTTP substituted for dTTP are as follows: dTTP, 100; O^4-Me-dTTP, 70; O^4-Et-dTTP, 20; O^4-iPr-dTTP, 2 (20). Using these values as an estimate, together with our data on the initial rate of synthesis and extent of incorporation of O^4-Me-dThd as reference points (Fig. 1), we calculate that 2–5% O^4-Et-dThd and 0.2–3% O^4-iPr-dThd are

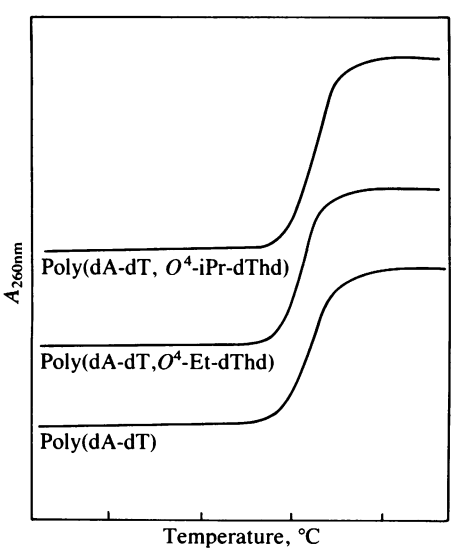

FIG. 4. Thermal melting profiles in 0.1 M Tris·HCl (pH 7.2) of polymers isolated from the reaction shown in Fig. 2. See Table 1 for data.

Table 2. Fidelity of synthesis with poly(dA-dT) containing O^4-alkyl dThd

Polymer*	dGMP/dAMP, $\times 10^{-4}$†	Average error rate‡
Poly(dA-dT)	<0.6 (ND–1.2)	<1/33,000
Poly(dA-dT, O^4-Me-dThd)	32 (18–49)	1/630
Poly(dA-dT, O^4-Et-dThd)	4.5 (3.6–6.1)	1/4440
Poly(dA-dT, O^4-iPr-dThd)	3.8 (3–5.2)	1/5200

*Polymers were prepared using pol I, as described in Figs. 1 and 2 and *Materials and Methods*, or as described in Fig. 3. Poly(dA-dT) refers to both the commercial polymer and that synthesized in this laboratory.
†Experiments were carried out six times in duplicate or triplicate, using either pol I or the Klenow large fragment. The range of data obtained (shown in parentheses) was the same for both enzymes. ND, not detected.
‡dGMP incorporated per total bases polymerized. The error rates are a pool of data using both enzymes and two different preparations of the methyl or ethyl polymers. Only one isopropyl-containing polymer was used.

present in the respective polymers. The lower values are likely to be an underestimate since the extent of polymer synthesis was eventually 30- to 50-fold in the presence of some dTTP.

DISCUSSION

The alkylated thymidines in this study are produced *in vivo* by dialkylnitrosamines and alkylnitrosoureas, found by Druckrey *et al.* (22) to be potent carcinogens. The major sites of tumor formation are dependent on the animal species, mode of administration, and other factors. These *N*-nitroso compounds can alkylate all oxygens and nitrogens in nucleic acids to varying extents (1, 23, 24). In the case of the well-studied *N*-nitroso methylating and ethylating agents, it was hypothesized that the formation and persistence of O^6-alkylguanosine correlated with the site of tumorigenesis (25). As more data accumulated, it became apparent that this simple explanation could not account for all tumor specificity (24).

O^4-alkyl dThd was suggested by Singer *et al.* (2) as a potential initiating event in carcinogenesis. Later experiments in Swenberg's laboratory (3, 4) also indicated that the persistence of O^4-alkyl dThd was implicated in initiation of hepatocellular tumors.

The recent study on methylnitrosourea-induced mouse mammary tumors containing a Ha-*ras* oncogene with a G→A mutation in the 12th codon of the P21 protein (26) has been supportive of the role of O^6-Me-Guo in transformation. However, it is not clear whether this mutated gene alone can transform primary cells (27, 28). Until such time as further work clarifies this point, we are assuming that the mutagenic properties of O^4-alkyl Thd or other pyrimidine derivatives (29) can also contribute to malignant transformation.

The aims of this study were to compare the substrate properties and mutagenic potential of higher homologues to those of the previously studied O^4-Me-Thd. There were three aspects: (*i*) Would pol I recognize O^4-Et-dTTP or O^4-iPr-dTTP as dTTP analogues and polymerize them; (*ii*) would their presence in the polymer affect its structural stability; and (*iii*) would such polymers serve as templates for replication and reveal their presence by misincorporation?

Concerning points *i* and *ii*, the data show that polymers are made but extensive elongation requires the presence of some unmodified dTTP (Fig. 3). [Data on the kinetics of polymerization of the O^4-alkyl dTTPs and dATP alone will appear in a separate paper (F. Chavez, B.S., and S.J.S., unpublished

data).] This is attributed to the poor 3'-OH primer terminus resulting when several O^4-alkyl Ts are incorporated opposite A. Renewed synthesis can then be achieved by adding small amounts of dTTP (Fig. 2). Synthesis even under these conditions is diminished with increasing alkyl chain length (Fig. 2). The final products, which in amount are greatly increased over the primer, are apparently, and surprisingly, unchanged in helix stability (Table 1).

The earlier study of the substrate properties of O^4-Me-dTTP (12) did not include data on the failure of O^4-Me-dTTP to replace dCTP using poly(dG-dC) as template/primer. We have extended these studies to a more effective template, poly(dA-dC)·poly(dT-dG). By using [^{3}H]dATP as a measure of synthesis, we restricted the study to observing only the product formation directed by the dT-dG strand. When dTTP was present, 6% as much synthesis occurred as with dCTP. Thus, when forced, in the absence of dCTP, a considerable number of dG·dT wobble pairs (30) resulted. The same effect was noted when dCTP was replaced by O^4-Me-dTTP. Thus, these data cannot differentiate between an O^4-Me-dT·dG wobble or simulation of dCTP. Data reported on incorporation of O^4-Me-dTTP into poly(dG-dC) lacked the dTTP control, and the interpretation is subject to similar doubts (31). However, when present in DNA as a result of modification, O^4-alkyl dThd acts occasionally as dC.

The third point, that of the effect on fidelity, showed that, qualitatively, the larger substituents behave as O^4-Me-dThd (12) and direct dG incorporation when replicated with pol I. The enzyme resistance conferred by the presence of ethyl or isopropyl groups in these alternating polymers prevented a quantitative comparison of the efficiency of mutation as a function of size of substitutent (Table 2). Based on our rough estimates of the ability of pol I to insert O^4-alkyl dTTPs, it is conceivable that O^4-iPr-dThd may be even more mutagenic than O^4-Me- or O^4-Et-dThd.

In DNA modified *in vivo* by *N*-nitroso carcinogens, the presence of O^4-alkyl T could lead to transition mutants with high frequency since these derivatives are poorly repaired and can accumulate markedly under conditions of chronic exposure (3).

The authors gratefully acknowledge the expert technical assistance of F. Chavez and the suggestions and critical reading of the manuscript by Drs. L. A. Loeb and B. D. Preston. This work was supported by Grant CA 12316 from the National Cancer Institute, National Institutes of Health (B.S.) and the Polish Ministry of Science, Higher Education and Technology, Project MR. 1.5 (J.T.K.).

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