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O^4-Methyl-, O^4-Ethyl-, and O^4-Isopropylthymidine 5′-Triphosphates as Analogues of Thymidine 5′-Triphosphate: Kinetics of Incorporation by *Escherichia coli* DNA Polymerase I†

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ABSTRACT: O^4-Methyl-, O^4-ethyl-, and O^4-isopropylthymidine 5′-triphosphates, which can be formed by *N*-nitroso carcinogens, were tested for their ability to substitute for thymidine 5′-triphosphate (dTTP) in synthesis catalyzed by *Escherichia coli* DNA polymerase I (Pol I) by using activated DNA or synthetic polymers as templates. All could substitute for dTTP for short periods, the rate and extent decreasing with the size of the alkyl group. Because the structure of O^4-alkylthymidine does not permit normal hydrogen bond formation with deoxyadenosine, it was inferred that eventual formation of a poor or frayed primer end was responsible for termination of synthesis. Synthesis of polymers at temperatures ranging from 0 to 40 °C showed that the extent of incorporation using the O^4-alkyl-dTTPs was favored, relative to dTTP, when the terminal helical structure was stabilized by low temperatures. K_m^{app} values were determined for each O^4-alkyldeoxynucleoside 5′-triphosphate. These values were 0.7 μM for dTTP, 5 μM for methyl-dTTP, 11 μM for ethyl-dTTP, and 33 μM for isopropyl-dTTP. O^4-Alkyl-dTTPs were tested for their ability to inhibit or compete with dTTP incorporation and found to have a minimal effect, even when present at high concentration. These experiments indicated that Pol I can incorporate deoxynucleotides with O^4-alkyl substituents into an ordered DNA structure. A postulated base-pairing scheme with deoxyadenosine is described.

O-Alkylpyrimidines have recently become a focus of interest since they are potential initiators of carcinogenesis. Studies on the di- or triphosphates of O^4-alkyluridine and O^4-alkylthymidine indicate that, when incorporated into polymers, they can act as T(U) or C, thus leading to mutation when G is misincorporated (Singer et al., 1978, 1983, 1984a, 1986). A variety of *N*-nitroso alkylating agents are potent carcinogens and alkylate the O^4 of pyrimidines to varying extents [reviewed by Singer & Grunberger (1983)]. In vivo studies with these carcinogens implicate O^4-methylthymidine (m^4dT) and O^4-ethylthymidine (e^4dT) in initiation of tumors (Singer et al., 1981; Swenberg et al., 1984; Richardson et al., 1985). In a strain of *Salmonella typhimurium* able to detect mutations by a TA → CG transition, it has been shown that pyrimidine modification is mutagenic (Hu & Guttenplan, 1985).

Our laboratory has found that when thymidine 5′-triphosphate (dTTP) is replaced in part by m^4dTTP, e^4dTTP, or O^4-isopropyl- (ipr^4) dTTP, these act as analogues and can substitute for dTTP in *Escherichia coli* DNA polymerase I (Pol I) directed polymer synthesis, albeit with a lower efficiency (Singer et al., 1983, 1986). In parallel work, the same derivatives in the absence of dTTP were site-directed by primer extension opposite a single A in φX174 (Preston et al., 1985). These experiments were primarily designed to synthesize polymers and measure mutagenicity.

The present studies investigate the initial rates of incorporation of the three O^4-alkyl-dTTPs into DNA and poly[d(A-T)] in the absence of any other source of dTTP. A comparable purine analogue, O^6-methyldeoxyguanosine (m^6dG) triphosphate, has been shown to substitute for dATP (Snow et al., 1984a), and the kinetic data are similar to ours for m^4dTTP. Other previous kinetic studies on utilization of

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Table I: Temperature Effect on Rate of Incorporation by Pol I of [^{3}H]dATP with O^4-Alkyl-dTTPs or dTTP[a]

template and dNTP tested	pmol of dATP incorporated/min		
	37 °C	18 °C	0 °C
activated DNA[b]			
T	3.15	0.88	0.08
m^4T	1.45	0.55	0.052
e^4T	1.0	0.24	0.036
ipr^4T	0.55	0.20	0.03
poly[d(A-T)][c]			
T	0.88	0.23	0.02
m^4T	0.28	0.09	0.009
e^4T	0.18	0.04	
ipr^4T	0.09	0.05	

[a] Rate determined from the linear portion of synthesis. See Figure 2 for the times used. [b] dCTP and dGTP are also present. [c] Reaction conditions are as given in the legend to Figure 1, except only dATP and dTTP, or O^4-alkyl-dTTP, were present at 100 μM.

nucleoside triphosphates apparently include only those derivatives that can form at least two hydrogen bonds with another base, such as 2-aminopurine, 5-alkyldeoxyuridines, N^4-methoxydeoxycytidine, etc. (Engel & von Hippel, 1974; Sagi et al., 1977, 1980, 1981; Yoshida et al., 1979; Kowalzick et al., 1982; Watanabe & Goodman, 1982; Hall & Saffhill, 1983; Snow et al., 1984b; Singer et al., 1984b).

The effect of the size of a substituent in a base-pairing position on the nucleotide's ability to be stably incorporated into polymers has not been established. In this paper we compare the 1-carbon methyl, 2-carbon ethyl, and 3-carbon isopropyl branched chain on the O^4 of dT in terms of their effect on polymer elongation, K_m^{app}, and inhibition or competition with dTTP, utilizing a DNA polymerase of high fidelity [reviewed by Loeb & Kunkel (1982)].

Experimental Procedures

Materials. Unlabeled deoxynucleoside triphosphates, poly[d(A-T)], and the large fragment Pol I (Klenow) were from P-L Biochemicals. *E. coli* Pol I was the generous gift of Dr. L. A. Loeb (University of Washington, Seattle, WA). Labeled deoxynucleoside triphosphates, [^{32}P]dNTPs and [^{3}H]dNTPs, were obtained from New England Nuclear. The alkylthymidine triphosphates were synthesized as described previously and had no detectable dTTP (Singer et al., 1983).

Temperature Studies. The conditions were as described previously by Singer et al. (1983). In addition to poly[d(A-T)], activated DNA was also used as template-primer (see Table I).

Determination of K_m. Either [^{3}H]dNTPs or [^{32}P]dNTPs were used to measure synthesis. The standard assay (100 μL) contained 200 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.8, 2 mM $MgCl_2$, 100 μM dATP, 0.15 A_{260} unit of poly[d(A-T)], [^{32}P]dATP at 0.15 μCi/nmol, dTTP at various levels, and 2 units of Pol I [diluted in 50 mM Tris-HCl, pH 7.8, 20% (v/v) glycerol, 1 mM DTT, and 1 mg/mL bovine serum albumin (BSA) to 0.20 mg/μL]. When the Klenow fragment was used, 0.4 unit was added. Reactions were initiated by addition of enzyme to samples. Aliquots were removed after 30, 60, and 120 s, spotted on Whatman DE81 disks, and washed as described previously (Singer et al., 1986).

K_m values were determined by using the direct linear analysis of Eisenthal and Cornish-Bowden (1974), which demands weaker assumptions than using least squares.

Inhibition Studies. The assay mixture was the same as for the K_m determination except for the following: polymer was present at either 42 or 83 μg/mL while m^4dTTP was present at either 25 or 100 μM, except in the control, which lacked m^4dTTP. Sampling times were 1, 2, 5, and 15 min.

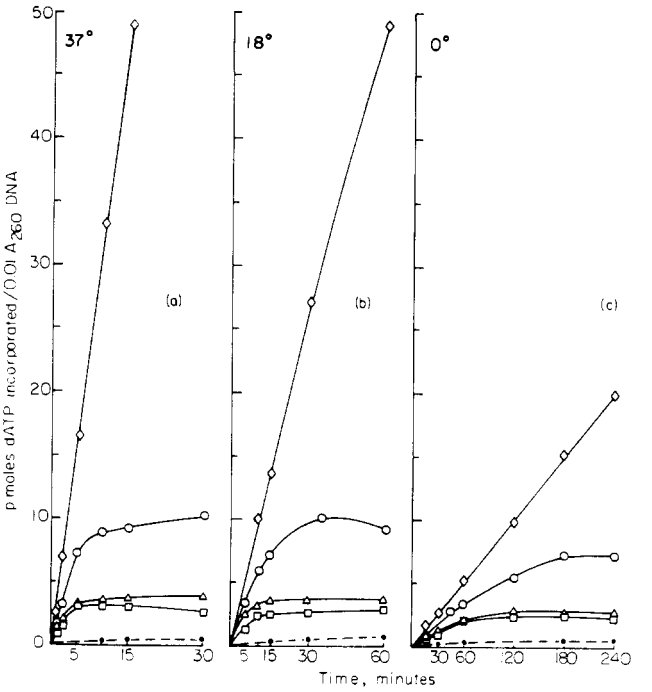

FIGURE 1: Effect of O^4-alkyl-dTTPs on extent of synthesis of DNA by Pol I at (a) 37, (b) 18, and (c) 0 °C. The assay mixture, as previously described (Singer et al., 1983), contained 200 mM Tris-HCl (pH 7.4), 2 mM $MgCl_2$, all four dNTPs at 100 μM each, 0.1 A_{260} unit of salmon sperm DNA, and 0.2 unit of Pol I. Further details are under Experimental Procedures. dTTP, ◇; m^4dTTP, ○; e^4dTTP, △; ipr^4dTTP, □; synthesis in the absence of any dTTP source, –·–.

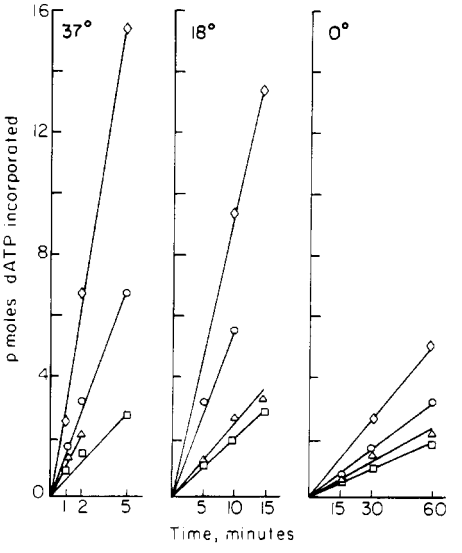

FIGURE 2: Rate of incorporation of O^4-alkyl-dTTPs during the linear portion of synthesis. Data from Figure 1 are expanded to show early time points. Symbols as in Figure 1. The level of incorporation in the absence of any dTTP has been subtracted from these points.

Results

Synthesis with O^4-Alkyl-dTTP as the Only dTTP Source. In the absence of added dTTP, both poly[d(A-T)] and activated DNA were templates for synthesis using Pol I, dATP (or dATP, dCTP, dGTP), and alkylated dTTP. On a molar basis, none of the O^4-alkyl-dTTPs were as effective in synthesis as dTTP. Over the time period in which dTTP gives a rapid and linear rate of synthesis, the synthesis with the analogues was depressed and the linear portion terminated much earlier

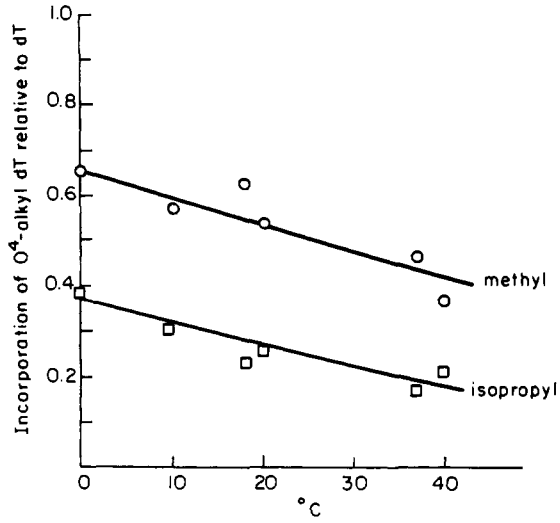

FIGURE 3: Relative incorporation of nucleotides by Pol I in the presence of m⁴dTTP and ipr⁴dTTP, compared to that with dTTP, as a function of incubation temperature. The level of dTTP incorporation is defined as 1.0 for each temperature. Data for e⁴dTTP overlap ipr⁴dTTP data and are not shown.

than with dTTP present (Figure 1). At 37 °C this decrease in rate and extent of synthesis was inversely related to the size of the alkyl group (methyl ≫ ethyl > isopropyl; see Figures 1 and 2). In the absence of any dTTP or its analogues, truncated synthesis was apparent, though not comparable to the level of synthesis in the presence of the least effective analogue ipr⁴dTTP (Figure 1a). Therefore, even without a labeled alkyl-dTTP, significant synthesis can be easily detected by using for quantification another labeled dNTP, usually [^{32}P]- or [^{3}H]dATP.

Effect of Incubation Temperature on Synthesis Using O⁴-Alkyl-dTTPs. The early termination of synthesis using any of the three O^4-alkyl-dTTPs may have been due to creation of a poor primer terminus. To explore this possibility, experiments were performed at temperatures ranging from 0 to 40 °C, always with a control mixture which contained dTTP. One experiment of this type using DNA as template is shown in Figures 1 and 2. Similar data were obtained with poly[d-(A-T)] or with other temperatures and either template. The pooled data from experiments with each template showed that the amount of elongation as a function of incubation temperature using m⁴dTTP or ipr⁴dTTP increased relative to the rate using dTTP (Figure 3). Data for e⁴dTTP are not shown since they are very similar to those for ipr⁴dTTP.

The rate of synthesis using unmodified dNTPs was changed by the expected factor of 2 for a 10-deg change in incubation temperature between 20 and 40 °C. For the alkyl triphosphates, there was significantly greater change as lower temperatures were used, which suggested that one or more steps in Pol I catalyzed synthesis were specifically enhanced for the alkyl-dTTPs at 0 or 10 °C. An Arrhenius plot for the rate of dT incorporation at all concentrations of dTTP yielded a straight line (not shown), as expected (McClure & Jovin, 1975).

Data for the rate of labeled dATP incorporation during linear synthesis are in Table II.

Kinetic Data. The apparent K_m values for each O^4-alkyl-dTTP and dTTP were determined under the standard conditions (see Experimental Procedures). On the basis of rapid onset of nonlinearity, early time points were used (30, 60, 120 s) in order to measure an initial rate. Concentrations were chosen to both give detectable levels of incorporation and avoid outlying points. The data from a series of experiments were

Table II: Temperature Effect on Extent of Incorporation by Pol I of [^{3}H]dATP with O^4-Alkyl-dTTPs or dTTP[a]

template and dNTP tested	total pmol of dATP incorporated: 37 °C, 5 min	18 °C, 15 min	0 °C, 60 min
activated DNA[b]			
T	16	13.4	5.0
m⁴T	6.7	6.9	3.1
e⁴T	2.7	3.3	1.8
ipr⁴T	2.6	2.1	1.8
poly[d(A-T)][c]			
T	4.4	3.5	1.2
m⁴T	1.4	1.4	0.6

[a] The time is chosen to be that of the period of linear synthesis using O^4-alkyl-dTTPs. [b] dCTP and dGTP are also present. The total average number of nucleotides incorporated is assumed to be 4 times these values for DNA; e.g., 16 pmol of dATP is equivalent to 64 pmol of total nucleotides. [c] It is presumed that the total average picomoles of dT or O^4-alkyl-dT is equivalent to the dATP incorporation.

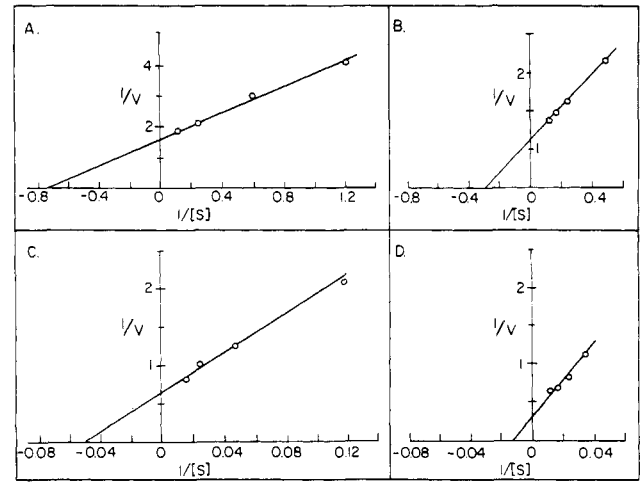

FIGURE 4: Representative plots for K_m^{app} of (A) dTTP, (B) m⁴dTTP, (C) e⁴dTTP, and (D) ipr⁴dTTP. The standard Lineweaver–Burk plot is shown. Within experimental sets the relative order of K_m values did not change although small changes in v could alter the K_m determined from a linear fit. The K_m values reported in the text were determined from these and other data by the method of Eisenthal and Cornish-Bowden (1974).

analyzed by the method of Eisenthal and Cornish-Bowden (1974). Data from a representative experiment are shown in Figure 4. The K_m^{app} for dTTP under our conditions with Pol I is 0.7 μM; for m⁴dTTP, 5 μM; for e⁴dTTP, 11 μM; and for ipr⁴dTTP, 33 μM. The same values using the Klenow fragment of Pol I were obtained for dTTP and m⁴dTTP. Scarcity of substrate limited these Klenow experiments to the nucleotide with the smallest alkyl group.

Inhibition and Competition of dTTP and O⁴-Alkyl-dTTP. The temperature studies and K_m values described above utilized only one source of dTTP or an analogue. In order to consider questions of inhibition and competition, three types of experiments were performed that used various radiolabels and ratios of the different necessary triphosphates.

In the studies on inhibition, a limiting amount of [^{3}H]dTTP (1% of dATP) was mixed up to a 300-fold molar ratio of m⁴dTTP (0.4–300-fold), and the extent of labeled dT incorporation in the presence of a nonlimiting amount of dATP was measured with poly[d(A-T)] as template. None of the 20 ratios of m⁴dTTP:dTTP tested in multiple experiments led to more than 10% inhibition of dT incorporation after a 15-min period of synthesis.

When instead the radiolabel was in dATP and the alkyl-dTTP was present at 100 times the limiting dTTP level (1%

Table III: Utilization of O^4-Alkyl-dTTP and dTTP in Pol I Catalyzed Poly[d(A-T)] Synthesis[a]

ratio to dATP[b]				pmol of dATP incorporated
dTTP	m^{4}dTTP	e^{4}dTTP	ipr^{4}dTTP	
0.01				32
	1.0			37
		1.0		18
			1.0	18
0.01	1.0			58
0.01		1.0		43
0.01			1.0	44

[a] Incubations were at 37 °C, 15 min. One picomole of dAMP incorporated was 270 cpm. [b] dATP was present at 100 μM.

of dATP), all three alkyl-dTTPs increased the incorporation of dATP, indicating their participation in synthesis. The amount of [^{32}P]dATP incorporated was approximately 15% less than would be expected from the two dTTPs individually (Table III). At lower ratios of the two T-like sources, e.g., 25:1:100 alkyl-dTTP:dTTP:dATP, the extent of incorporation was apparently additive. That is, both the alkyl-dTTP and dTTP appeared to be utilized independently, as measured by [^{32}P]dATP incorporation. Due to limited alkyl triphosphate availability, more conventional competition analysis could not be performed. However, the conclusions were drawn that O^4-alkyl-dTTPs were poor inhibitors of dTTP incorporation, but they could be shown, by these data and other (Singer et al., 1983; Preston et al., 1985), to be utilized by Pol I in the presence of dTTP.

Discussion

We had earlier demonstrated that m^{4}dTTP, in the presence of dTTP and dATP, could be incorporated into poly[d(A-T)] without significant change in the polymer structure. Although acting as dT in synthesis, in vitro replication of such polymers led to significant levels of dGTP incorporation. One might thus postulate that m^{4}dTTP should be able to replace dCTP. Hall and Saffhill (1983) did report such a replacement and proposed a probable structure of the putative base pair. Recent criticism of this structure based on NMR and crystallographic data (Birnbaum et al., 1986) has focused on placement of the alkyl group syn to the N-3 although pairing of O^{4}T and G can still be accommodated. High levels of T-G wobble observed in vitro precluded confirmation of this point. However, under conditions of high fidelity, replication of polymers containing O^4-alkyl-dT by Pol I led to dG incorporation (Singer et al., 1986). In addition, these studies also indicated that a dA·O^4-alkyl-dT structure could exist even with substituents as large as ethyl or isopropyl.

All the polymers synthesized in our previous studies utilized mixtures of the modified alkyl-dTTP and dTTP. The ability of the three O^4-alkyl-dTTPs to be utilized as the sole source of dTTP to elongate poly[d(A-T)] or a natural DNA was, not surprisingly, inversely related to the size of the substituent (Table I). However, the synthesis with either template-primer remained linear for only a short time (Figure 2) at the usual temperatures used for Pol I catalyzed synthesis and terminated after a relatively low level of synthesis (Figure 1a).

We reasoned that increasing numbers of dA·O^4-alkyl-dT pairs could eventually destabilize the primer terminus. This effect should be enhanced with a poly[d(A-T)] template compared to a DNA template which has additionally normal G·C base pairs (Tables I and II). In addition, lowering the temperature of synthesis should stabilize these primer ends (Smith & Gillam, 1981) and permit further synthesis. As predicted, at temperatures of 0–10 °C, extension of the primer

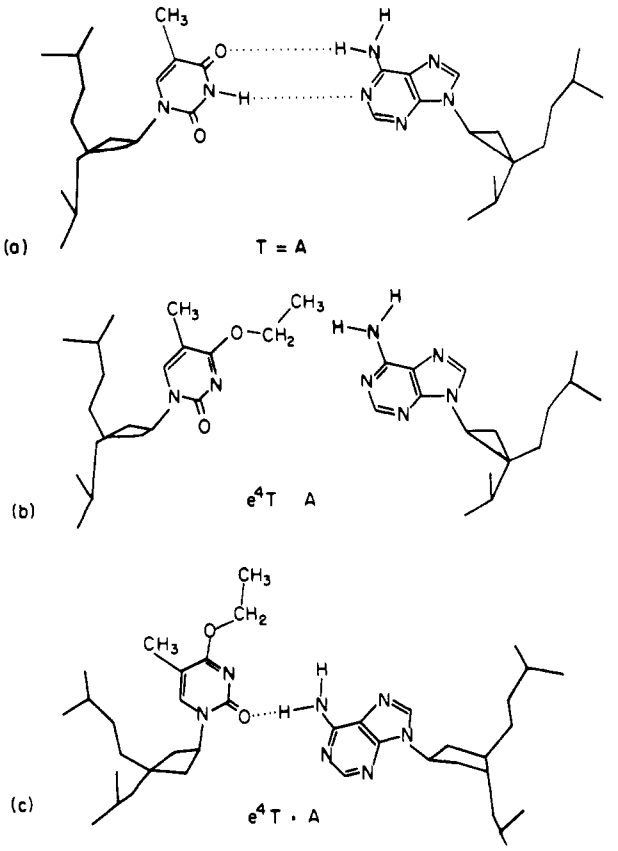

FIGURE 5: Base pairing of dT or O^4-ethyl-dT to dA: (a) Watson–Crick T-A base pair; (b) e^{4}T (syn) and A in Watson–Crick positions; (c) e^{4}T (syn) and A in an open-helix position in which one hydrogen bond can be formed. The position and size of the ethyl group are based on data of Birnbaum et al. (1986) and Allore et al. (1983).

ends utilizing the alkyl-dTTPs was relatively higher compared to that with dTTP (Figure 3).

An alternative explanation for termination of synthesis involves the proofreading function of the polymerase. Mismatches or poor matches are known to be excised at high frequency [e.g., Fersht & Knill-Jones (1981) and Snow et al. (1984a)]. This exonuclease activity produces a high turnover frequency but a low level of nucleotide incorporation. Data in Table II show that even in the linear period of synthesis significant elongation of the DNA has occurred. This, coupled with the temperature effect, strongly suggests that the helix and the polymerase (Kunkel et al., 1981; Rabkin & Strauss, 1984; Ollis et al., 1985) can accommodate even the bulky isopropyl adduct which is then probably stabilized by stacking.

Our evidence that the O^4-alkyl-dTTPs are both recognized and incorporated in place of dTTP by Pol I, coupled with new data on the orientation of the alkyl group (Birnbaum et al., 1986; Allore et al., 1983), has led us to propose a base-paired structure for O^4-alkyl-dT and dA that can readily be accommodated in a DNA helix (Figure 5). Other evidence for this structure is found in data on the rapid exchange of imino hydrogens in poly[d(A-T)] (Mirau & Kearns, 1985).

It has been proposed that alkylation of the dNTP pool can be a major source of modified nucleotides in DNA (Toorchen & Topal, 1983). While an attractive hypothesis, based on the high reactivity of monomers, our K_m values for dTTP and the three O^4-alkyl-dTTPs (Figure 4) indicate that in the case of these derivatives, as well as O^6-methyl-dGTP (Snow et al., 1984a), utilization of this type of precursor is statistically unlikely. The data from these experiments coupled with the analyses of DNA products resulting from in vivo alkylation

support a view that initiation of carcinogenesis reflects direct modification of DNA. Nevertheless, under cellular conditions, a polymerase could insert an occasional modified nucleotide, particularly in specific sequences that favor maintenance of base pairing or stacking.

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M1 RNA, the RNA Subunit of *Escherichia coli* Ribonuclease P, Can Undergo a pH-Sensitive Conformational Change†

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ABSTRACT: After purification from extracts of whole cells, M1 RNA, the catalytic subunit of ribonuclease P from *Escherichia coli*, apparently must undergo a change in conformation before it can function catalytically. The rate of this conformational change is dependent upon the duration of incubation at various temperatures and pH. $\Delta E^{\ddagger}$ of the transition at pH 7.5 is approximately 36 kcal/mol. The change in conformation is not sensitive to Mg^{2+} concentration between 10 and 100 mM. A decrease in A_{260} of M1 RNA in solution has been observed during the incubation period that potentiates the conformational change at 30 °C, but no direct correlation can yet be made to specific structural rearrangements.

Individual RNA molecules can assume different conformations in solution that depend on precise patterns of intramolecular or intermolecular hydrogen bonding. The ability to change their conformations with solution conditions in vitro, or with physiological states in vivo, can be important to the function of proteins and nucleic acids. RNA molecules that demonstrate this phenomenon include the leader sequences of trp operon mRNA in *Escherichia coli* (Lee & Yanofsky, 1977), tRNAs (Crothers & Cole, 1978), rRNAs (Chambliss et al., 1980; Kao & Crothers, 1980), col E1 primer RNA

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