Enzymatic Synthesis of Deoxyribonucleic Acid. XXXIV. Termination of Chain Growth by a 2′,3′-Dideoxyribonucleotide*

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ABSTRACT: 2′,3′-Dideoxyribonucleoside triphosphates are analogs of the natural 2′-deoxyribonucleotide substrates of deoxyribonucleic acid polymerase but lack the 3′-hydroxyl group required for deoxyribonucleic acid chain growth. Attachment of a dideoxynucleotide blocks deoxyribonucleic acid synthesis and inhibits related reactions (pyrophosphorolysis, pyrophosphate exchange, and hydrolysis) which occur at the primer site of deoxyribonucleic acid polymerase from *Escherichia coli*. Attachment of a chain-terminating dideoxythymidylate group to deoxyribonucleic acid and to oligo- and polydeoxynucleotide chains is approximately a thousand times slower than that of deoxyribothymidylate. Hydrolysis and pyrophosphate exchange are inhibited to a similar extent.

The requirement for a 3′-hydroxyl group for optimal rates of these reactions is discussed in terms of a model for deoxyribonucleic acid polymerase action. In the presence of an excess of polymerase, the extent of incorporation of dideoxythymidylate residues at the 3′ terminus of poly d(A-T) and of deoxyribonucleic acid chains is proportional to polynucleotide concentration, and thus permits a determination of available primer sites.

DNA polymerase from *Escherichia coli* catalyzes a nucleotidyl transfer that results in polymerization. This enzyme also hydrolyzes nucleotide residues from the 3′ terminus of polydeoxyribonucleotide chains (3′ → 5′ hydrolysis) and from the 5′ terminus at nicks in polynucleotide duplexes (5′ → 3′ hydrolysis). In the presence of suitable primer and template strands the polymerase also catalyzes pyrophosphate exchange into nucleoside triphosphates, and pyrophosphorolysis of residues at the 3′ terminus of the primer strand (for a recent review, see Kornberg, 1969). Analogs of the naturally occurring bases have been useful in establishing the importance of hydrogen-bonded base pairing in the nucleotidyl transfer reaction (Bessman *et al.*, 1958). Nucleotide analogs in which the sugar is modified might now be of considerable value in clarifying several aspects of the enzyme's multiple functions.

One of the simplest and most significant alterations in the sugar moiety is the replacement of the 3′-hydroxyl of the 2′-deoxyribonucleoside triphosphate by a group such as hydrogen. Would a nucleotide analog such as 2′,3′-dideoxythymidine 5′-triphosphate be a substrate for DNA polymerase? If so, it would block chain growth by preventing the attachment of additional nucleotide residues. Would chains bearing such a chain-growth terminator be susceptible to the hydrolytic and pyrophosphorolytic reactions (3′ → 5′) that take place at the 3′ terminus of the primer strand? Such modified chains, if insusceptible to 3′ → 5′ degradation, would then make certain studies of the 5′ → 3′ hydrolytic action of the enzyme more feasible and precise.

Another important use of a nucleotide analog bearing a 2′,3′-dideoxyribose moiety might be as a reagent to control and analyze DNA replication *in vivo*. In fact, the earliest application of this type of analog was the demonstration that 2′,3′-dideoxyadenosine irreversibly inhibits DNA synthesis and is lethal to *E. coli* cultures (Doering *et al.*, 1966).

Recent studies with pure *E. coli* DNA polymerase have shown that 2′,3′-dideoxynucleoside 5′-triphosphates bind to the triphosphate site (Englund *et al.*, 1969a) and are substrates for addition to a DNA chain (L. Toji and S. S. Cohen, 1969, submitted for publication; Deutscher and Kornberg, 1969; Atkinson *et al.*, 1969). Dideoxy-TTP also inhibits polymerization and related functions such as pyrophosphate exchange, hydrolysis, and pyrophosphorolysis at the 3′ terminus (Deutscher and Kornberg, 1969) but does not interrupt 5′ → 3′ hydrolysis (Cozzarelli *et al.*, 1969; Kelly *et al.*, 1969b).

The present work establishes the rate, stoichiometry, and linkage involved in attachment of 2′,3′-dideoxythymidine to synthetic polymers and DNA. The slow rate, 1000-fold less than that with natural substrates, was also reflected in a very slow rate of pyrophosphate exchange and degradation of chains terminated with the dideoxynucleotide. These observations along with comparable results on arabinosyl nucleotides as chain-growth terminators (M. R. Atkinson and A. Kornberg, unpublished results) provide new insights into the mechanism of DNA polymerase action and may also prove valuable in analyzing DNA replication *in vivo*.

Materials and Methods

2′,3′-Dideoxy-TTP. A solution of 20 mg of sodium 2′,3′-

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TABLE I: Positions of Attached Dideoxynucleotide Determined by Nuclease Hydrolysis and Nearest Neighbor Analysis.

Template-Primer	Nucleotide Hydrolysis Products[a]	Isolated Products[b] Nature	Amt (%)
Poly d(A-T)	5′	ddTMP	84
	3′	3′-dAMP	78
$d(pTpA)_{13}$	3′	3′-dAMP	>95
Poly dA + oligo dT	3′	3′-dTMP	>90[c,d]
DNA	5′	ddTMP	77
	3′	3′-dAMP	26
		3′-dCMP	16
		3′-dGMP	17
		3′-dTMP	21

[a] 5′-Nucleotides were produced by pancreatic DNase and venom diesterase digestion; 3′-nucleotides were produced by micrococcal and spleen nuclease digestion. [b] Values represent the ^{32}P released by enzymic hydrolysis which is associated with the products indicated; it is probable that much of the unidentified ^{32}P is in P_i. [c] The hydrolysis was incomplete, 54% of the ^{32}P remaining in dTp-dideoxy-T and dTpTp-dideoxy-T. The value shown represents the fraction of ^{32}P mononucleotides isolated as 3′-dTMP. [d] In a separate experiment, ddTMP was attached to single-stranded oligo d(T) with terminal transferase from calf thymus. Nearest-neighbor analysis indicated that 91% of the ^{32}P was associated with 3′-dTMP.

didehydro-2′,3′-dideoxy-TTP[1] (Russell and Moffatt, 1969) in 3 ml of water was hydrogenated at atmospheric pressure for 24 hr with 5% palladium on barium sulfate (50 mg). Chromatography of the nucleoside released by alkaline phosphatase (Russell and Moffatt, 1969) showed complete conversion to ddTTP. *Anal.* Thymidine:total P:acid-labile P, 1.00:2.95:1.98. The product was homogeneous on chromatography in isobutyric acid–1 M NH_4OH–0.1 M Na_4EDTA (100:60:1.6, v/v; R_F 0.38, M_{dTTP} 1.6) or 1-propanol–11 M NH_4OH (3:2, v/v; R_F 0.12) and on electrophoresis in 0.02 M citrate (Na^+, pH 3.2; M_{dTTP} 1.00). The triphosphate had λ_{max} 267 nm at pH 7 (ϵ 9600). [^{3}H]ddTTP[1] was prepared, in collaboration with Dr. Walter Hafferl, by reducing the didehydro derivative with tritium gas; details will be published separately.

[α-^{32}P]ddTTP was prepared from [^{32}P]ddTMP [(*cf.* Pfitzner and Moffatt, 1964); obtained in 60% yield at specific activities of 1–3 mCi/μmole by Symons' (1968) procedure] by three methods: (a) treatment with carbonyldiimidazole and pyrophosphate (Hoard and Ott, 1965; 41% yield); (b) phosphorylation with H_3PO_4 and dicyclohexylcarbodiimide (Smith and Khorana, 1958; 27% yield); (c) phosphorylation by ATP and phosphoenolpyruvate with T4 phage-induced nucleoside monophosphate kinase of *E. coli* and pyruvate kinase–nucleoside diphosphate kinase from rabbit muscle (Bello and Bessman, 1963; 41% yield). With the kinase system rates are less than 1% of the rate with dTMP.

DNA Polymerase, Oligonucleotides, and Polynucleotides. These materials were prepared as described previously (Deutscher and Kornberg, 1969; Cozzarelli *et al.*, 1969).

[^{3}H]Oligo dT with an average chain length of 170 nucleotides was prepared by the action of terminal transferase (Olivera and Lehman, 1967). The mean chain length was calculated from the extent of incorporation of [^{32}P]ddTMP residues on incubation with terminal transferase and [α-^{32}P]ddTTP. [^{3}H]Oligo dT with a [^{1}H,^{32}P]ddTMP residue at the 3′ terminus was isolated from the reaction mixture by passage through Sephadex G-100 in 0.25 mM EDTA–0.1 M potassium phosphate (pH 7.4), and was used in the hydrolysis experiments described below. Nearest-neighbor analysis showed ^{32}P esterified only to the 3′-hydroxyl of thymidylate residues (Table I).

Reaction Conditions and Assay Methods. Except where indicated in the text, reactions were carried out at 37° in 67 mM phosphate (K^+, pH 7.4)–6.7 mM $MgCl_2$–1 mM 2-mercaptoethanol with 33 μM nucleoside triphosphates as described by Deutscher and Kornberg (1969). Attachment of dideoxy nucleotides to oligo dT and to $d(pTpA)_{13}$ was monitored by chromatography on DEAE paper in 0.75 M NH_4HCO_3; the oligonucleotide products remained at the origin in this system. Oligonucleotides were eluted from the origin zones for nearest-neighbor analysis by extraction with 1 M triethylammonium bicarbonate (pH 8)–triethylamine (20:1, v/v) and were freed of salts by evaporation at 37° (0.1 mm) followed by evaporation with two portions of ethanol. With poly d(A-T) or poly d(A)·poly d(T), or DNA as primer, reactions (0.3 ml) were stopped by addition of 0.2 ml of 0.2 M tetrasodium pyrophosphate and 0.5 ml of 7% $HClO_4$. Precipitates were washed eight times with 10-ml portions of 0.1 M tetrasodium pyrophosphate–1 M HCl and four times with 10-ml portions of ethanol on Whatman glass fiber disks that had been soaked in 0.2 M tetrasodium pyrophosphate; nucleotide incorporation was measured by gas flow or scintillation counting. Polynucleotides were recovered from the disks for nearest-neighbor analysis by extraction with 1.5 M NH_4OH and evaporation of solvent.

Nearest-neighbor analyses were carried out by chromatography in the isobutyrate and propanol–NH_4OH systems and by electrophoresis in 0.05 M citrate (Na^+, pH 3.5) after hydrolysis with micrococcal nuclease and spleen diesterase (Josse *et al.*, 1961; Wu and Kaiser, 1968). The identity of incorporated dideoxy-TMP was confirmed by chromatography in the isobutyrate system after hydrolysis of polynucleotide to 5′-monophosphates with pancreatic DNase and snake venom phosphodiesterase (Lehman *et al.*, 1958).

Results

Attachment of ddTMP to Poly d(A-T). Rate of Attachment. With poly d(A-T), dATP, and [α-^{32}P]ddTTP as substrates, DNA polymerase catalyzed attachment of ddTMP to the polynucleotide (Figure 1). The only radioactive nucleotide detected after hydrolysis of the product to 5′-monophosphates was ddTMP (Table I), and exclusive attachment of [α-^{32}P]ddTMP

[1] Abbreviations used that are not listed in *Biochemistry 5*, 1445 (1966), are: ddTMP, dideoxy-TMP, 3′-deoxythymidine 5′-phosphate, (*i.e.*, 2′,3′-dideoxyribothymidine 5′-phosphate); ddTTP, dideoxy-TTP, 3′-deoxythymidine 5′-triphosphate; M_{dTTP}, chromatographic or electrophoretic mobility with respect to dTTP; ara-AMP and ara-CMP, arabinofuranosyl analogs of AMP and CMP.

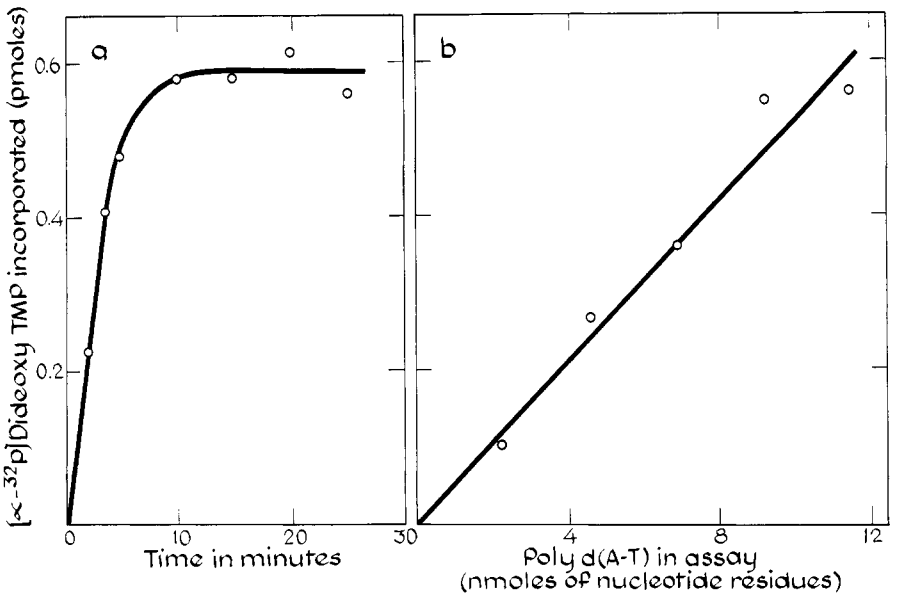

FIGURE 1: Rate (a) and extent (b) of incorporation of ddTMP into poly d(A-T). DNA polymerase (1.4 pmoles), dATP (10 nmoles), [α-^{32}P]ddTTP (7.5 nmoles), and the indicated amounts of poly d(A-T) in a final volume of 0.3 ml were incubated at 37° for the times shown and incorporation of ddTMP was measured. For all points in the time course (a) 10.7 nmoles of poly d(A-T) was used; the range of poly d(A-T) concentrations (b) was studied with a 30-min incubation.

residues to the 3′-hydroxyl of dAMP residues in the primer was shown by nearest-neighbor analysis (Table I). The initial rate of incorporation was 1.3×10^{-3} residue/sec per molecule of enzyme (Table II) and incorporation reached a limit within 20 min at the highest primer concentrations tested.

Stoichiometry. When incorporation of ddTMP proceeded to a limit (Figure 1), the extent of attachment of the analog was proportional to the concentration of poly d(A-T) in the system. One ddTMP residue was incorporated for each 19,000 nucleotide residues in the poly d(A-T) template–primer. Using a different sample of poly d(A-T), which was shown by the methods of Figure 1 to incorporate one [^{32}P]ddTMP residue/4000 nucleotide residues, net attachment of [^{3}H]ddTMP during 30 min was measured as a function of DNA polymerase concentration (Figure 2). At a low enzyme concentration approximately one residue of ddTMP was attached for each molecule of polymerase; at higher enzyme concentrations incorporation of the analog was limited by the number of available primer ends.

Even the highest extent of incorporation in Figure 1 was less than one ddTMP/molecule of polymerase. To test the possibility that a poly d(A-T) molecule terminated at the 3′ end with a ddTMP residue did not dissociate readily from DNA polymerase, the experiment of Figure 1 (in which maximal incorporation was 0.6 pmole/1.4 pmoles of polymerase) was repeated with 0.35 pmole of polymerase. Net incorporation reached a maximum of 0.30 pmole of ddTMP in 30 min and was 0.28 pmole at 60 min. A second addition of 0.35 pmole of polymerase at 30 min raised net incorporation to 0.42 pmole of ddTMP (constant from 45 to 60 min). Thus in no case was net incorporation greater than the number of polymerase molecules present.

TABLE II: Summary of Rates of Incorporation of ddTMP into Various Primers.[a]

Expt	Primer	Rate (Residues sec^{-1}) (Molecule of Enzyme)$^{-1}$	(Primer End)$^{-1}$
1	Poly d(A-T)	0.0013	0.0031
2	$d(pTpA)_{13}$	0.0106[b]	
3	Poly d(A)·poly d(T)	0.00013	0.002
4	Poly d(A)·oligo d(T)	0.0015	
5	DNA	0.0022	

[a] In the incubations, the primers were present in micromolar concentrations shown, based on total nucleotide residues; the nucleotides were present at or near 33 μM. Expt 1: see Figure 1; expt 2: see Figure 3; expt 3: poly dA, 14; poly dT, 12.5; enzyme, 5 nM; expt 4: see Figure 4; expt 5: see Figure 5. [b] V_{max} 0.0106 ± 0.0006; K_m 89 ± 8 μM ddTTP. With 33 μM dTTP as substrate, the rate was 1.8 molecules of dTMP incorporated sec^{-1}(molecule of polymerase)$^{-1}$. The other rates are observed values at the substrate concentrations indicated and are not calculated for saturating conditions.

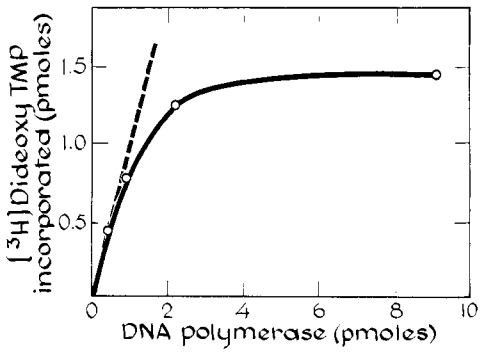

FIGURE 2: Influence of enzyme concentration on incorporation of ddTTP (10 nmoles) treated with dATP and poly d(A-T) (77 nmoles of nucleotide residues) as described in Figure 1b. The broken line is the relationship for incorporation of one ddTMP residue/molecule of polymerase.

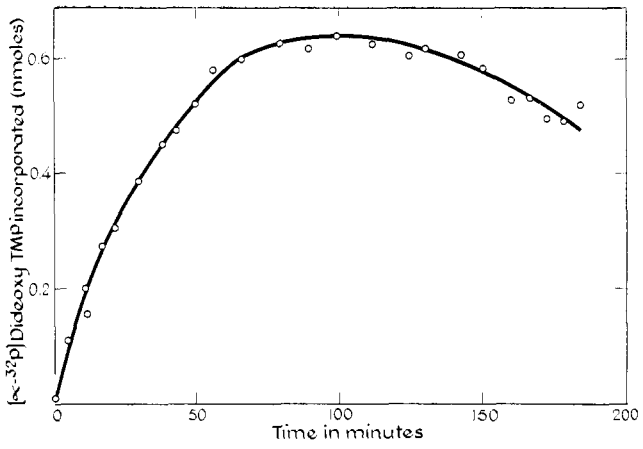

FIGURE 3: Incorporation of ddTMP into $d(pTpA)_{13}$. $d(pTpA)_{13}$ (21 nmoles), [α-^{32}P]ddTTP (37.5 nmoles), and DNA polymerase (70 pmoles) were incubated in the standard assay system (3 ml) at 37°. Incorporation was measured at the times indicated after chromatography of 0.1-ml samples on DEAE-cellulose in 0.75 M NH_4HCO_3.

Attachment of ddTMP to $d(pTpA)_{13}$, Poly dA·Poly dT, and Poly dA·Oligo dT. Table II summarizes the rates of attachment of ddTMP to a number of homo- and heteropolymer primers with DNA polymerase in the standard assay system. With $d(pTpA)_{13}$ as primer, the variation of incorporation rate with ddTTP concentration was studied, and the apparent Michaelis constant, calculated by Wilkinson's (1961) method, was 89 ± 8 μM ddTTP. The time course at one concentration of ddTTP is shown in Figure 3. With $d(pT)_{170}$ as primer in the presence of a limiting concentration of poly d(A) as template (Figure 4), net incorporation of the analog was 0.21 μM, a value nine times the enzyme concentration, indicating that in this system, as with $d(pTpA)_{13}$ (Table II and Figure 3), DNA polymerase dissociates from a primer terminus after attaching a ddTMP residue. Nearest-neighbor analyses (Table I) again confirmed that covalent attachment of ddTMP residues had taken place at primer 3′ termini that would have accepted dTMP residues from dTTP (*i.e.*, adjacent to dAMP residues with $d(pTpA)_{13}$ and to dTMP residues with oligo d(T)). Rates of attachment were in the range 10^{-3}–10^{-2} residue/sec per molecule of polymerase (or per primer end when there was an excess of polymerase molecules over primer ends). Rates of attachment of dTMP from dTTP in corresponding systems are in the range 1 to 10 sec^{-1} (*cf.* Deutscher and Kornberg (1969) and footnote to Table II).

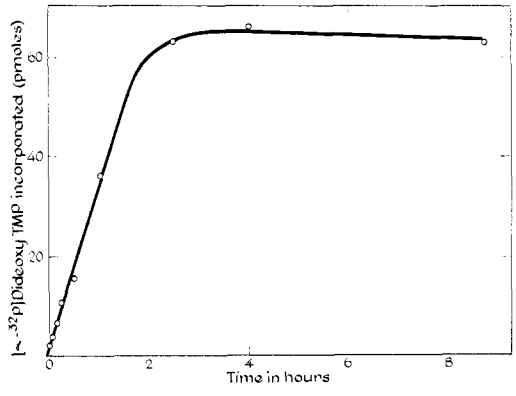

FIGURE 4: Incorporation of ddTMP into $d(pT)_{170}$·poly d(A)·$d(pT)_{170}$ (130 nmoles of residues), poly d(A) (16 nmoles), [α-^{32}P]ddTTP (19 nmoles), and DNA polymerase (6.8 pmoles) were incubated in the standard assay system (0.3 ml) at 37°. Incorporation was measured at the times indicated after chromatography of 25-μl samples as in Figure 3.

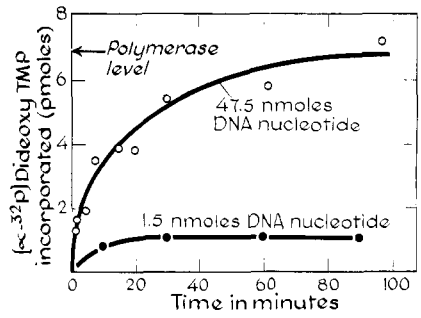

FIGURE 5: Time course of incorporation of ddTMP into thymus DNA. The incubation (0.3 ml) contained enzyme (6.9 pmoles), nicked calf thymus DNA (1.5 nmoles, or 47.5 nmoles, of nucleotide residues), [α-^{32}P]ddTTP (9.5 nmoles), and dATP, dCTP, and dGTP (10 nmoles each). The DNA was nicked by treatment with pancreatic DNase (5 μg/mg of DNA)for 25 min at 37° in the conditions described by Aposhian and Kornberg (1962).

Attachment of ddTMP to DNA. DNA polymerase catalyzes transfer of ddTMP residues from ddTTP to calf thymus DNA that had been treated with pancreatic DNase to increase the number of available primer ends. Figure 5 shows the time course of attachment and Figure 6 the proportionality of ddTMP attachment to DNA concentration. Net incorporation was 1 residue of ddTMP/1400 DNA nucleotide residues up to a total incorporation of 3.4 pmoles of ddTMP. In this system, which contained 6.9 pmoles of DNA polymerase, a tenfold increase in DNA concentration raised the total incorporation only to 7 pmoles during incubation for 90 min (Figure 5). In this instance, as with poly d(A-T), incorporation was not significantly greater than the enzyme concentration. The initial rate of incorporation at the higher DNA concentration (Figure 5) was approximately 2×10^{-3} molecule/sec per molecule of polymerase (Table II).

On enzymic hydrolysis to 5′-nucleotides, ddTMP was the only [^{32}P]nucleotide detected, while nearest-neighbor analysis showed the presence of 3′-phosphates of deoxyadenosine, deoxycytidine, deoxyguanosine, and thymidine (Table I), indicating covalent attachment adjacent to all four types of primer termini.

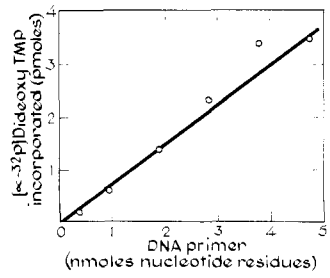

FIGURE 6: Proportionality between extent of incorporation of ddTMP and the amount of DNA added. Reaction conditions were as in Figure 5 with incubation at 37° for 30 min.

TABLE III: Pyrophosphorolysis and PP_i Exchange into ddTTP.[a]

Expt	Reactants	PP_i Incorporated (nmoles) dNTP[b]	dATP	dTTP	ddTTP
1	Poly d(A-T)	0.40 (0.34)[c]			
	Poly d(A-T), dTTP	1.59 (1.50)[c]			
	Poly d(A-T), ddTTP			0.18 (0.11)[c]	
2	Poly d(A-T)		0.16	0.18	–
	Poly d(A-T), dTTP		0.03	0.91	–
	Poly d(A-T), ddTTP		0.03	0.09	<0.01
3	$d(pTpA)_{13}$, dTTP		–	2.2	–
	$d(pTpA)_{13}$, ddTTP		–	–	0.0006
	$d(pTpA)_{13}$, ddTTP[d]		–	–	0.0016

[a] In expt 1, the reaction conditions and assay were as described by Deutscher and Kornberg (1969). Reaction mixtures (0.3 ml) contained 7.8 nmoles of poly d(A-T), 300 nmoles of [^{32}P]PP_i, and DNA polymerase (1 unit, 0.51 pmole). Where indicated, 10 nmoles of dTTP or ddTTP was added. ^{32}P incorporation into nucleotide was measured by adsorption on charcoal. Incubations were for 30 min at 37°. Experiment 2: Conditions as in expt 1 but with 35 pmoles of enzyme. Reactions were stopped with one-tenth volume of acetic acid and samples were chromatographed in two dimensions (propanol–NH_3 and isobutyrate systems) with dATP, dTTP, and ddTTP carriers. The separated nucleotides were adsorbed on charcoal and the radioactivity was measured. Experiment 3: The reaction mixture (0.03 ml) contained 0.14 nmole of $d(pTpA)_{13}$, 16 nmoles of [^{32}P]PP_i, 35 pmoles of enzyme, and 10 nmoles of dTTP or ddTTP. After 30 min at 37°, carrier triphosphates were added and the nucleotides were recovered by chromatography in the propanol–NH_4OH and isobutyrate systems. The identity of the [^{32}P]ddTTP was confirmed by elution from charcoal and chromatography with carrier in 0.4 M NH_4HCO_3 on DEAE paper. Controls with no enzyme or no primer gave blanks of less than 10% of the incorporation measured in the presence of primer. [b] dNTP represents a mixture of dATP and dTTP [c] Published values: Deutscher and Kornberg (1969). [d] This incubation was for 10 min and contained seven times as much ddTTP (2.4 mM).

TABLE IV: Inhibition of DNA Polymerase by Prior Incubation of Poly d(A-T) with Analogs of dTTP.[a]

Expt	Analog	Prior Incubn (min)	Analog Concn for 50% Inhibn (μM)
1	ddTTP	None	53
2	ddTTP	10	3
3	ddTTP	30	11
	3′-Iodo-ddTTP	30	275
	2′,3′-Didehydro-ddTTP	30	3

[a] In expt 1, DNA polymerase (0.14 pmole) was incubated with 40 nmoles of poly d(A-T), 10 nmoles of dATP, 10 nmoles of [^{3}H]dTTP, and seven levels of ddTTP (0–30 nmoles) in a final volume of 0.3 ml. Incorporation of [^{3}H]-dTMP into acid-insoluble material during 30 min was measured. In expt 2, conditions were as in 1 except that the [^{3}H]dTTP and half of the poly d(A-T) were added (in 0.02 ml) after a prior incubation of the mixture for 10 min. Incorporation during the following 30 min was measured. A sample preincubated in this way in the absence of ddTTP incorporated [^{3}H]dTMP at 96% of the rate in a control without prior incubation. In expt 3, conditions were as in 2, except that 20 nmoles of poly d(A-T) was present and the prior incubation was for 30 min. [^{3}H]dTTP was then added in 0.01 ml but no second addition of poly d(A-T) was made.

Does DNA Polymerase Catalyze PP_i Exchange into 2′,3′-ddTTP? Using adsorption on charcoal to detect incorporation of [^{32}P]PP_i into nucleotides, Deutscher and Kornberg (1969) found that ddTTP inhibited pyrophosphorolysis of poly d(A-T) in the presence of DNA polymerase. This method does not identify the radioactive nucleotides, so the formation of [β,γ-^{32}P]dATP, dTTP, and ddTTP was reinvestigated. As shown in Table III, pyrophosphate was exchanged into ddTTP in the presence of $d(pTpA)_{13}$. Nonenzymic incorporation was not detected in a control, and the identity of [^{32}P]ddTTP as the product was confirmed by the chromatographic methods indicated. The level of incorporation was close to the limit of detection by our methods, and it is not known if the level of net incorporation is a true measure of initial rate. The apparent rate of PP_i exchange (10^{-5}–10^{-4} molecule/sec per molecule of enzyme) is less than one-tenth the rate of attachment of ddTMP residues in this system. Formation of [^{32}P]dATP by pyrophosphorolysis is strongly inhibited by either dTTP or ddTTP.

Inhibition of Polymerization by Analogs of dTTP Correlated with the Inhibition of Pyrophosphorolysis and PP_i Exchange. As an inhibitor of pyrophosphorolysis and of PP_i exchange into dATP with poly d(A-T), 3′-iodo-ddTTP (Russell and Moffatt, 1969) proved to be less effective than ddTTP, but 2′,3′-didehydro-ddTTP was at least as effective (Deutscher and Kornberg, 1969). A similar order of effectiveness of the dTTP analogs was observed in their capacity to inhibit polymerization. As shown in Table IV, a prior incubation of DNA polymerase with poly d(A-T), dATP, and the dTTP analog inhibited subsequent incorporation of dTMP. Effectiveness of the inhibitors increased in the sequence 3′-iodo-ddTTP < ddTTP < didehydro-ddTTP. Concentrations for 50% inhibition were 275 μM, 11 μM, and 3 μM, respectively. With different preincubation conditions the concentration of ddTTP for 50% inhibition was 3 μM (Table IV).

Is a ddTMP-Terminated Chain Degraded by the 3′ → 5′ Exonuclease of DNA Polymerase? In the presence of ddTTP and a complementary poly dA strand, ddTTP blocked hydrolysis of oligo d(T) from the 3′ terminus by the 3′ → 5′ exonuclease activity of DNA polymerase (Kelly *et al.*, 1969b).

TABLE V: Hydrolysis of a ddTMP Residue from the Terminus of [^{3}H]d(pT)$_{170}$-[^{32}P]dd(pT).[a]

State of Poly dT	Initial Rate of Hydrolysis, sec^{-1}	
	^{32}P Release	^{3}H Release
Single strands (−poly d(A))	0.0005	0.06
Duplex strands (+poly d(A))	0.00011	0.50

[a] Reaction mixtures contained [^{3}H]d(pT)$_{170}$ with a [^{32}P]-ddTMP residue at the 3′ terminus (0.25 nmole of "ends," 42.5 nmoles of nucleotide residues) and polymerase (0.35 nmole) with or without poly d(A) (50 nmoles of nucleotide residues) in a final volume of 0.5 ml. Nucleotide release, measured by chromatography in the isobutyrate system (Cozzarelli *et al.*, 1969), was linear up to 50% hydrolysis. Rates are expressed as nucleotides released/sec per molecule of polymerase. We wish to thank Dr. R. B. Kelly for carrying out this analysis.

Because hydrolysis and pyrophosphorolysis at the 3′ terminus appear to be closely related reactions (Deutscher and Kornberg, 1969; Kornberg, 1969), the ability of DNA polymerase to hydrolyze a terminal [^{32}P]ddTMP from [^{3}H]oligo d(T) was examined. In the absence of ddTTP and poly d(A), there was a very slow release of the ddTMP residue at a rate of 5×10^{-4} residue/sec per molecule of polymerase (Table V); this was followed by a rapid release of [^{3}H]dTMP. In the presence of enough poly d(A) to form duplexes with the oligo d(T) the rate of release of [^{32}P]ddTMP was decreased at least fourfold. This probably reflects the preferential attack by polymerase from the 5′ terminus (5′ → 3′ nuclease), as indicated by the greatly increased rate of release of ^{3}H-labeled nucleotides (Kelly *et al.*, 1969b).

Binding of ddTMP-Terminated Chains to DNA Polymerase. Experiments described earlier in this paper suggested that a chain of poly d(A-T) or DNA to which a ddTMP residue was attached did not dissociate from polymerase. To obtain further evidence on such binding, the effect of a prior incubation of polymerase with poly d(A-T), dATP, and ddTTP on the rate of subsequent polymerization with extra poly d(A-T), dATP, and dTTP was studied. As shown in Table IV, such a prior incubation greatly increased the inhibitory effect of ddTTP, even when extra primer was added during the subsequent assay. With no prior incubation, 53 μM ddTTP was required for 50% inhibition, whereas with prior incubation 3 μM ddTTP gave 50% inhibition. A control preincubated for 10 min with poly d(A-T) and dATP was inhibited by only 4%. Since a large excess of poly d(A-T) was added with the [^{3}H]dTTP at the end of the preincubation period, it seems likely that the greater inhibition after preincubation resulted from binding of polymerase to poly d(A-T) molecules with a ddTMP residue at the 3′ terminus.

Discussion

One of the main aims of the current research on DNA polymerase is to find how the multiple catalytic functions of the enzyme are related, and to define the groups in the active center that participate in polymerization, nuclease reactions, and pyrophosphorolysis. With the recognition of the separate 3′ → 5′ and 5′ → 3′ exonuclease functions intrinsic to purified DNA polymerase from *E. coli* (Klett *et al.*, 1968; Deutscher and Kornberg, 1969; Kelly *et al.*, 1969b), a need has arisen for reagents that will selectively block degradation of polynucleotide primers from either the 5′ end or the 3′ end. In the presence of ddTTP, hydrolysis from the 3′ (primer) end of poly d(T) in duplexes is almost completely blocked (Kelly *et al.*, 1969b), and this reagent permits study of the specific cleavage of nucleotides from the 5′ end in duplexes by the 5′ → 3′ exonuclease function of DNA polymerase. Use of ddTTP to block 3′ ends has been of great value in characterizing excision of oligonucleotides containing thymine dimers and mismatched bases by the 5′ → 3′ exonuclease activity of DNA polymerase (Kelly *et al.*, 1969a). It is now shown that ddTTP protects the primer end from hydrolysis by attaching a ddTMP residue in the presence of DNA polymerase. Though the rate of attachment is slow (about 0.1% the rate of attachment of natural nucleotide residues), hydrolysis of ddTMP residues by DNA polymerase is even slower. Thus the terminal ddTTP residue, with no 3′-hydroxyl group available to accept a further nucleotide, is an effective covalent blocking group, stabilizing the primer end against hydrolysis, pyrophosphorolysis, or polymerization.

Nucleotide analogs that block DNA replication by covalent attachment are also of potential interest for control of growth and of DNA synthesis *in vivo*. In the absence of methods that permit delivery of exogenous nucleoside triphosphates to the active center of DNA polymerase in intact cells, inhibition of DNA synthesis by chain-terminating triphosphate analogs requires generation of these compounds by kinase action on nucleoside analogs that can penetrate target cells. 3′-Deoxythymidine is a very poor substrate for thymidine kinase from *E. coli* (M. R. Atkinson and A. Kornberg, unpublished observations), but 2′,3′-dideoxyadenosine does inhibit bacterial DNA synthesis irreversibly (Doering *et al.*, 1966), and its triphosphate attaches a dideoxy-AMP residue covalently to DNA in the presence of polymerase from *E. coli* (L. Toji and S. S. Cohen, submitted for publication). Though the ability of DNA polymerase to catalyze incorporation of nucleotide analogs modified in the purine or pyrimidine (*e.g.*, 5-bromo-dUMP, 2-amino-dAMP) is well known, there is little information on the specificity of the enzyme for nucleotides modified in the pentose residue. Ribonucleotides are incorporated by DNA polymerase, a reaction that is stimulated in the presence of Mn^{2+} (Berg *et al.*, 1963), and there have been reports that arabinosyl nucleotides are incorporated, though previous attempts to demonstrate this reaction with polymerase from *E. coli* have not been successful (for a review, see Cohen, 1966). It has now been shown that the arabinosyl analogs of dATP and dCTP are substrates of this enzyme (M. R. Atkinson and A. Kornberg, unpublished results) and with the demonstration that dideoxy-ATP (L. Toji and S. S. Cohen, 1969, submitted for publication) and dideoxy-TTP are also substrates, it is evident that limited changes in the pentose portion of nucleotide substrates of DNA polymerase are tolerated. This raises the possibility that other nucleoside analogs which are better substrates for kinases may form triphosphate analogs that will inhibit DNA syn-

thesis more effectively *in vivo* by covalent blocking of chain growth.

This paper shows that a dideoxy analog of thymidine nucleotide resembles the natural nucleotide in its capacity to be added to a DNA chain, to support pyrophosphate exchange, and to be removed by hydrolysis from the 3′ end of a polynucleotide chain. However, absence of the 3′-hydroxyl group in the dideoxy analog has a striking effect on the rates of these reactions, decreasing them all by a factor of about 1000 or more at equivalent concentrations. The marked inhibitory effect of this structural change provides information on the mechanism of action of DNA polymerase and the nature of its catalytic center. The enzyme from *E. coli* has in each molecule a single site which binds triphosphates, and since ddTTP is bound with about the same affinity as dTTP in the absence of primer (Englund *et al.*, 1969a), it is unlikely that discrimination against the analog arises through structural features of this site. It has been proposed that polymerization involves nucleophilic attack at the α-phosphorus of the nucleotide, generating an intermediate state which is identical with that generated by pyrophosphate attack at the primer end during pyrophosphorolysis or pyrophosphate exchange (*cf.* Kornberg, 1969). In a model where the intermediate state involves the pentacovalent α-phosphorus with its dsp^3 orbitals in a trigonal-bipyramidal configuration, the intermediate may be subject to pseudorotation (Westheimer, 1968). This will occur if the leaving groups (magnesium pyrophosphate in polymerization and the 3′-hydroxyl of the primer end in chain cleavage) are sterically hindered from occupying, during the nucleophilic attack, the apical position from which they must leave. Kornberg (1969) has discussed the evidence that 3′ → 5′ exonuclease action may involve an analogous intermediate in which OH rather than pyrophosphate is bound to the α-phosphorus in the bipyramidal structure generated during cleavage of the terminal residue from the primer end. The absence of a 3′-hydroxyl group in dideoxy analogs has a comparable effect on the rates of the three types of reactions catalyzed at the primer end. This suggests the possibility that groups in the catalytic center that facilitate displacements from the proposed intermediates have a strict requirement for a 3′-hydroxyl in the *ribo* configuration to permit rapid catalysis. This proposal is supported by our inability to demonstrate incorporation of the xylosyl analog of dAMP into poly d(A-T) or DNA (M. R. Atkinson and A. Kornberg, unpublished results).

There is further evidence from binding measurements suggesting that the 3′-hydroxyl of the primer end is recognized in that part of the catalytic center which holds the reaction intermediates. Equilibrium dialysis in the absence of primer shows that each molecule of polymerase has a single "monophosphate site" which is distinct from the triphosphate site and binds nucleoside 5′-monophosphates with a 3′-hydroxyl in the *ribo* configuration (J. A. Huberman and A. Kornberg, unpublished results; Atkinson *et al.*, 1969). Nucleoside 5′-monophosphates which lack the 3′-hydroxyl group (*e.g.*, dideoxy analogs) or have one in the opposite configuration (*e.g.*, xylosyl analogs) do not bind to the monophosphate site. Current attempts to correlate binding data with kinetic measurements of hydrolysis and polymerization may show if this site is responsible for binding the intermediates formed during reactions at the primer end of polynucleotides.

The studies of pyrophosphate exchange provide further evidence that this reaction is the reverse of polymerization. The rate of pyrophosphate exchange into ddTTP is even less than the low rate of attachment of ddTMP. The selective inhibitory effect (Table III) of ddTTP or dTTP on pyrophosphate incorporation into dATP during pyrophosphorolysis of poly d(A-T) is also consistent with the proposed mechanism of action of the polymerase (Kornberg, 1969); when a dAMP residue is at the primer end it is a substrate for attachment of ddTMP or dTMP residues and these reactions compete with pyrophosphorolysis, whereas a dTMP residue at the primer end of the alternating copolymer does not permit attachment of a further thymine nucleotide and is therefore available for pyrophosphorolysis. The relative rates of hydrolysis of dTMP and dAMP from the 3′ terminus of poly d(A-T) in the presence of [^{32}P]PP_i and ddTTP (Table III) were not measured. Differences in rates of hydrolysis might contribute to the different extents of pyrophosphorolysis.

The evidence obtained here for binding of DNA polymerase to poly d(A-T) or DNA molecules with ddTMP residues at their primer ends is indirect. We found that only one ddTMP residue was incorporated per molecule of polymerase even when an excess of primer ends was present. There was also an enhanced inhibitory effect of ddTTP when there was a prior incubation with polymerase and poly d(A-T), an expected result if the enzyme is bound to primer ends blocked with ddTMP residue and is unable to dissociate and interact subsequently with additional poly d(A-T). More direct physical measurements of the type described by Englund *et al.* (1969b) will be needed to measure the extent of such binding.

There has been no convenient method available for measurement of the number of available primer ends in a polynucleotide sample. Whereas polynucleotide kinase can be used to determine available 5′-hydroxyl groups, neither this method nor physical techniques such as centrifugation give an indication of the extent to which 3′ ends of polynucleotides are free. Dideoxyriboside triphosphates of known specific activity are potentially useful end group reagents for this purpose (Figures 1 and 6). The method is limited by the need for an excess of polymerase molecules over primer ends as discussed above, and measures only functional 3′ termini. It is not applicable to oligonucleotides because enzymic hydrolysis of the latter from the 5′ end leads rapidly to low molecular weight products. Recently we have used a labeled dideoxyriboside triphosphate to calibrate the number of primer sites in preparations of poly d(A-T) and thymus DNA for studies of the stoichiometry of ara-AMP and ara-CMP incorporation (M. R. Atkinson and A. Kornberg, unpublished results).

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Effect of Adenosine Monophosphate on the Reactivity of Thiol Groups of D-Fructose 1,6-Diphosphatase from Rabbit Liver*

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ABSTRACT: Adenosine monophosphate, an allosteric inhibitor of most fructose 1,6-diphosphatases, markedly reduces the reactivity of SH groups of rabbit liver fructose 1,6-diphosphatases toward 5,5′-dithiobis(2-nitrobenzoic acid) and 2,2′-dithiodipyridine at pH 7.5. Six out of the twenty SH groups react with 5,5′-dithiobis(2-nitrobenzoic acid) in the absence of added ligand, but only two groups react when 0.5 mM adenosine monophsophate is present. This effect is highly specific for adenosine monophosphate and deoxyadenosine monophosphate and is reversed by ethylenediaminetetraacetic acid, an activator of the enzyme. The activation by 2,2′-dithiobisethylamine and 2-hydroxyethyl disulfide is also diminished by adenosine monophosphate. The addition of the substrate D-fructose 1,6-diphosphate does not affect the reactivity of SH groups by itself, but it does exert a synergistic effect with adenosine monophosphate.

The presence of substrate, however, is not obligatory for the demonstration of altered SH reactivity in the presence of adenosine monophosphate. The disulfide reagents provide a sensitive indication that the enzyme may exist in an altered conformational state in the presence of its allosteric inhibitor.

Mammalian liver FDPase[1] catalyzes the conversion of fructose 1,6-diphosphate into fructose 6-phosphate in the presence of Mg^{2+} or Mn^{2+} and plays an essential role in the process of gluconeogenesis. Its catalytic activity is inhibited by AMP. This inhibition is reversible, noncompetitive with substrate, and maximal at neutral pH (Mendicino and Vasarhely, 1963; Gancedo *et al.*, 1965; Taketa and Pogell, 1965). Chemical modification of FDPases purified from rabbit liver and *Candida utilis* have indicated that the inhibition due to AMP occurs at sites on the enzyme which are distinct from those involved in catalysis (Horecker *et al.*, 1966; Pontremoli *et al.*, 1966; Rosen and Rosen, 1966). Furthermore, the FDPase isolated from *C. utilis* is dissociated into subunits by the addition of AMP at pH 9.5 (Rosen *et al.*, 1967). This drastic alteration in the quaternary structure at pH 9.5 may represent an exaggerated manifestation of subtle conformational changes exhibited by the enzyme in the presence of AMP at neutral pH. The FDPase isolated from rabbit liver, however, does not dissociate under these

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[1] Abbreviations used are: PMB, *p*-mercuribenzoate; DTNB, 5,5′-dithiobis(2-nitrobenzoic acid); FDPase, fructose 1,6-diphosphatase.