MOLECULAR PHARMACOLOGY, 17, 256–261

Inhibition of Calf Thymus DNA Polymerase-α by Deoxyribonucleoside Triphosphates

GERALD B. GRINDEY, MARJORIE WINKLER, MAUREEN OTTEN AND JAY A. STEINBERG

Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, Buffalo, New York 14263

Received July 23, 1979; Accepted October 4, 1979

SUMMARY

GRINDEY, G. B., M. WINKLER, M. OTTEN, AND J. A. STEINBERG. Inhibition of calf thymus DNA polymerase-α by deoxyribonucleoside triphosphates. *Mol. Pharmacol.* **17**: 256–261 (1980).

Kinetic evaluation of DNA polymerase-α (DNA nucleotidyltransferase, EC 2.7.7.7.) was conducted using the enzyme purified from calf thymus and obtained from a commercial source. At saturating levels of template, the reaction was first order with respect to dTTP and, at optimum dTTP, was first order with respect to homopolymer template. At subsaturating levels of template, however, the reaction yielded nonhyperbolic kinetics which were consistent with substrate inhibition by dTTP. This substrate inhibition was competitive with template and independent of Mg^{2+} concentration. The noncomplementary nucleotides, dCTP, dGTP, and dATP, all inhibited the reaction in a noncompetitive manner with respect to dTTP and in a competitive manner with respect to template. Noncomplementary homopolymer as well as pyrophosphate also inhibited the reaction competitive with template. This study suggests that the deoxyribonucleoside triphosphate precursors of DNA may regulate the replication of cellular DNA through substrate inhibition of DNA polymerase-α.

INTRODUCTION

The synthesis of the deoxyribonucleoside triphosphate precursors of DNA is tightly regulated by a complex network of feedback and activation loops controlling the activity of ribonucleotide reductase (1, 2) and deoxycytidylate deaminase (3). Evaluation of perturbations in the deoxyribonucleoside triphosphate pools in synchronized Chinese hamster ovary cells in culture by the addition of exogenous thymidine led Bjursell and Reichard (4) to the conclusion that the allosteric mechanisms shown with purified ribonucleotide reductase are functional in intact cells. Moreover, inhibition of DNA synthesis was correlated in time with the decrease in dCTP.

Using leukemic L1210 cells in culture, Lowe and Grindey (5) evaluated the effect of thymidine or deoxyguanosine on the deoxyribonucleoside triphosphate pools and correlated changes in these pools with decreased growth rate. With deoxyguanosine toxicity, both dTTP and dCTP decrease in parallel and in a linear manner while dATP remains unaffected and dGTP increases. In cells inhibited by thymidine, both dTTP and dGTP increase. Although both dCTP and dATP decrease, only their sum diminishes in proportion to growth inhibition. While these observations indicate that the synthesis of DNA may be related to the concentration of the deoxyribonucleoside triphosphates, the changes in these pools are not sufficiently great to explain the observed degree of growth rate inhibition by substrate limitation of DNA polymerase (5). Similar evaluation of CCRF-CEM cells in culture lends additional support to this conclusion (6). The addition of exogenous thymidine increases the pools of dTTP, dGTP and dATP. While a 42% decrease in dCTP occurs, a 73% inhibition of growth rate is observed. These studies indicate that increases rather than decreases in deoxyribonucleoside triphosphate pools appear directly responsible for the observed inhibition of DNA synthesis. Additional support for this hypothesis is derived from the studies of Lowe *et al.* (7) where deoxyadenosine toxicity was related primarily to modest increases in the intracellular concentration of dATP.

In the present study, the effect of deoxyribonucleoside triphosphates on the activity of a preparation of DNA polymerase-α obtained from calf thymus was evaluated. These results indicate that all four deoxyribonucleoside triphosphates can directly inhibit DNA polymerase-α in addition to serving as substrates for the enzyme.

MATERIALS AND METHODS

The following were obtained from commercial sources: crystalline BSA, dithiothreitol, and unlabeled deoxyribonucleoside triphosphates (Sigma Chemical Co.); om-

This investigation was supported by U. S. Public Health Service Research Grant CA-17156 from the National Cancer Institute.

0026-895X/80/020256-06$02.00/0

nifluor (New England Nuclear); [*methyl*-^{3}H]deoxythymidine 5′-triphosphate with a specific activity of 19 Ci/mmol (Schwarz/Mann). All DNA homopolymers were purchased from P-L Biochemicals. DNA polymerase-α, exonuclease free, was purchased from Worthington (Lot 95S200) and was isolated using a modification of the method of Bollum *et al.* (8) which included Sephadex G-100 and hydroxylapatite chromatography. Before use, the enzyme was diluted in 47 mM Tris–HCl, pH 7.8, 11.2% glycerol and 0.93 mM DTT such that 10 μl contained 1 unit of DNA polymerase activity. The assay conditions were: 20 mM K^+-phosphate buffer pH 7.2, 0.2 mg/ml BSA, 1 mM DTT with an additional 0.74 μM [^{3}H]dTTP to give a diluted specific activity of 0.167 Ci/mmol, 100 μM poly(dA), 20 μM oligo(dT)$_{(12-18)}$ and 10 μl of the diluted DNA polymerase-α in a total volume of 150 μl. All determinations were made in duplicate and each experiment contained zero time controls. The concentrations of [^{3}H]dTTP and poly(dA):oligo(dT) were varied as indicated in the legends to the figures.

All compounds, except enzyme, were added to tubes at 4°; after addition of enzyme and mixing, the reaction was initiated by incubating the rack of tubes for 15 min at 37°. The reaction was terminated by placing the tubes in ice water. This procedure gave the same results as boiling, or dilution of label by adding cold dTTP, and was more convenient. Cooled assay mixtures (100 μl) were spotted on Whatman 3MM filter paper disks (2.3 cm diameter), which were then immersed in ice-cold 5% TCA containing 1% $NaPP_i$, 20 ml/disk, for 1 hr. Washing procedures were similar to those of Lowe and Grindey (5), except that the disks were oven-dried at 80°F for 15 min. The disks were placed in 5 ml of a toluene–Omnifluor scintillation cocktail and radioactivity was determined in an Isocap Mark III (Amersham/Searle) instrument.

RESULTS

The use of homopolymers as templates for DNA polymerase allows the kinetic evaluation of each deoxyribonucleoside triphosphate as a specific substrate for the reaction (9, 10) and the potential interaction of the noncomplementary deoxyribonucleoside triphosphates. For example, the utilization of polydeoxyadenylate (500–600 nucleotides)–oligothymidylate (12–15 nucleotides) in a ratio of 5:1 was specific for the incorporation of dTTP with only marginal incorporation of the other three noncomplementary deoxyribonucleotides. Chang (10) has proposed that DNA polymerase-α operates in a distributive manner rather than with a processive mechanism in the replication of homopolymer templates *in vitro*, i.e., that the enzyme dissociates from the template after the incorporation of each deoxyribonucleoside triphosphate. Should this be the mechanism of DNA replication, then DNA polymerase must be considered as an eight-substrate rather than a four-substrate enzyme (assuming that the metal–nucleotide complex is the actual substrate). For the replication of homopolymer, this reduces to a simple two-substrate mechanism at optimal metal cofactor concentration.

Using the preparation of DNA polymerase-α obtained from Worthington, the incorporation of dTTP was evaluated using polydeoxyadenylate–oligothymidylate in a constant ratio of 5:1 and displayed first-order kinetics with an apparent K_m of 22 μM at saturating homopolymer concentrations (100 μM) (Fig. 1). These results were comparable with the characteristics of this enzyme previously reported by Bollum (9). Inhibition by the noncomplementary nucleotide, dCTP, was noncompetitive with respect to dTTP (Fig. 1). At nonsaturating concentrations of homopolymer (10 μM), the kinetics of dTTP incorporation were nonlinear using the Lineweaver–Burk plot (11) and were consistent with the characteristics of a substrate–inhibitor function (Fig. 2). In the presence of dCTP, both the degree of deviation from linearity and the inhibition were more pronounced (Fig. 2).

As shown in Fig. 3, the inhibition of dTTP incorporation by dCTP was competitive with the homopolymer template and the apparent K_i at this concentration of dTTP (75 μM) was 65 μM. In these experiments the apparent K_m for the homopolymer is meaningless since the degree of association between the oligothymidylate and polydeoxyadenylate is unknown. The apparent K_i for dCTP was calculated by assuming that this ratio was constant for the two conditions. The inhibition of dTTP incorporation by the other noncomplementary nucleotides, dATP and dGTP, was also competitive with template as shown in Fig. 4. The apparent K_i for dATP under these conditions was about 100 μM while that for dGTP was about 5 μM. Thus, the order of effectiveness of inhibition of the incorporation of dTTP into DNA by the noncomplementary nucleotides is dGTP > dCTP > dATP.

The kinetics of incorporation of dTTP were a function of template concentration (Figs. 1 and 2) and were an apparent substrate–inhibitor function at a low concentration of homopolymer. This inhibition by excess dTTP was also competitive with template concentration (Fig. 5), accounting for the apparent hyperbolic kinetics ob-

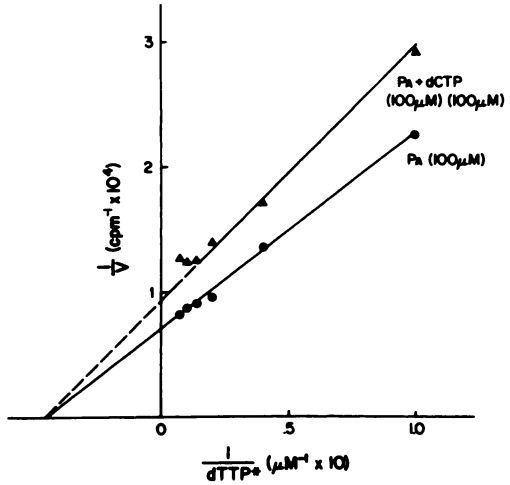

FIG. 1. *Kinetic analysis of the effect of dCTP on the incorporation of dTTP using DNA polymerase-α at saturating concentrations of template*

Reaction conditions are as described under Materials and Methods except that the concentration of dTTP was varied from 10 to 125 μM at constant homopolymer, P_A (100 μM). The asterisk indicates the tritium-labeled nucleotide utilized in the analysis. The symbols are: ●, P_A alone; ▲, P_A + 100 μM dCTP.

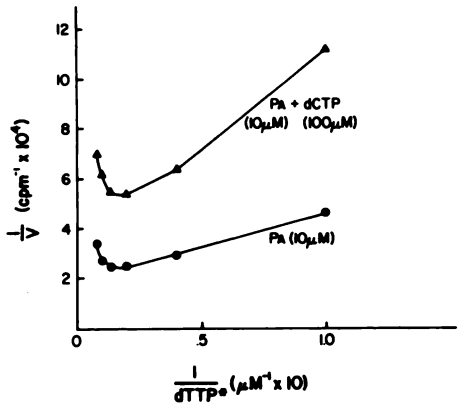

FIG. 2. *Kinetic analysis of the effect of dCTP on the incorporation of dTTP using DNA polymerase-α at nonsaturating concentrations of template*

Reactions conditions are as described under Materials and Methods except that the concentration of dTTP was varied from 10 to 125 μM at constant homopolymer, P_A (10 μM). The asterisk indicates the tritium-labeled nucleotide utilized in the analysis. The symbols are: ●, P_A alone; ▲, P_A + 100 μM dCTP.

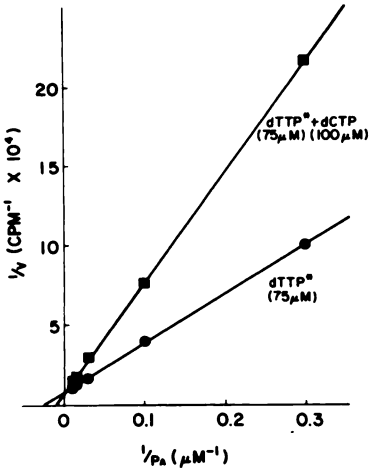

FIG. 3. *Competitive inhibition with respect to template of DNA polymerase-α by dCTP*

Reaction conditions are as described under Materials and Methods except that the concentration of homopolymer, P_A, was varied from 3.3 to 100 μM at constant dTTP (75 μM). The asterisk indicates the tritium-labeled nucleotide utilized in the analysis. The symbols are: ●, dTTP alone; ■, dTTP + 100 μM dCTP.

served at a saturating concentration of template (Fig. 1). While the above sets of data have been presented using Lineweaver–Burk plots, the same conclusions were drawn when the results were analyzed by Eadie plots (11).

Variation in the kinetics of template binding to enzyme can be achieved by two different methods. In the above experiments this was achieved by holding the ratio of polydeoxyadenylate to oligothymidylate constant and varying the total amount of polydeoxyadenylate–oligothymidylate added to the assay. An alternate procedure would be to keep the polydeoxyadenylate concentration constant and vary the amount of oligothymidylate added to the reaction mixture. As shown in Fig. 6, this procedure also yielded substrate inhibition by dTTP competitive with template concentration. Thus, variation in the amount of polydeoxyadenylate present in the reaction mixture was not responsible for the observed substrate inhibition by dTTP through indirect effects. The first procedure was utilized for the majority of the experiments since it was substantially less expensive.

The effect of varying concentrations of homopolymer on the ability of Mg^{2+} to serve as the metal cofactor for the incorporation of dTTP by DNA polymerase-α is shown in Fig. 7. At saturating concentrations of homopolymer (100 μM), the velocity-cofactor kinetics for Mg^{2+} were nonhyperbolic. There was an absolute requirement for Mg^{2+} with no incorporation of dTTP being detected in the absence of this cation. The optimum concentration of Mg^{2+} for the reaction was 6 mM and higher concentrations produced substantial inhibition. At lower concentrations of homopolymer, the optimum Mg^{2+} concentra-

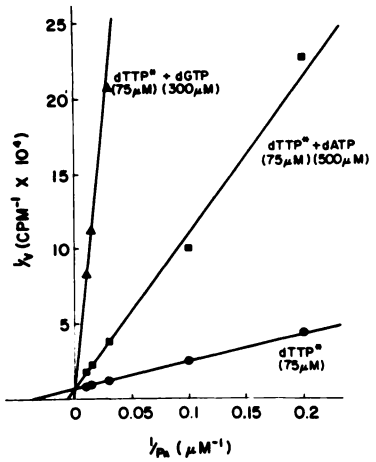

FIG. 4. *Competitive inhibition with respect to template of DNA polymerase-α by dATP and dGTP*

Reaction conditions are as described under Materials and Methods except that the concentration of homopolymer, P_A, was varied from 5 to 100 μM at constant dTTP (75 μM). The symbols are: ●, dTTP alone; ■, dTTP + 500 μM dATP; ▲, dTTP + 300 μM dGTP.

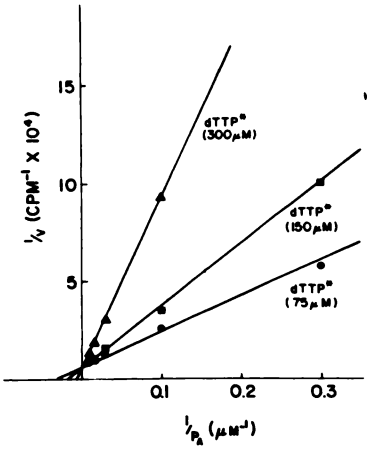

FIG. 5. *Kinetic analysis of the substrate inhibition of DNA polymerase-α by dTTP*

Reaction conditions are as described under Materials and Methods except that the concentration of homopolymer, P_A, was varied from 3.3 to 100 μM at three specific concentrations of dTTP. The symbols are: ●, 75 μM dTTP; ■, 150 μM dTTP; ▲, 300 μM dTTP.

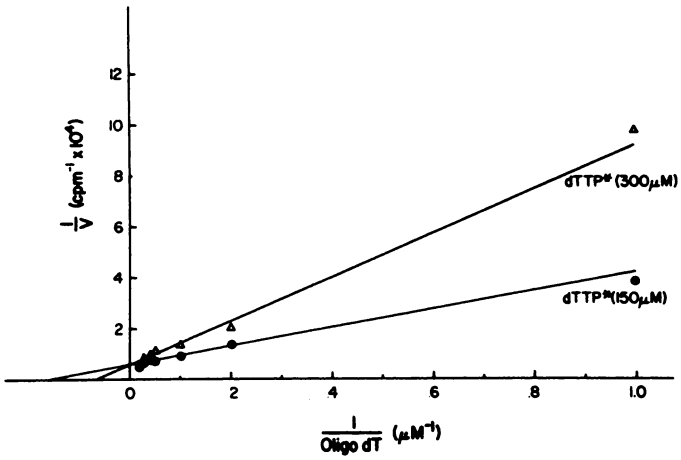

FIG. 6. *Kinetic analysis of the substrate inhibition of DNA polymerase-α by dTTP at a constant concentration of polydeoxyadenylate*

Reaction conditions are as described under Materials and Methods except that the concentration of oligothymidylate was varied from 1 to 200 μM at constant polydeoxyadenylate (100 μM) for two specific concentrations of dTTP. The symbols are: ●, 150 μM dTTP; Δ, 300 μM dTTP.

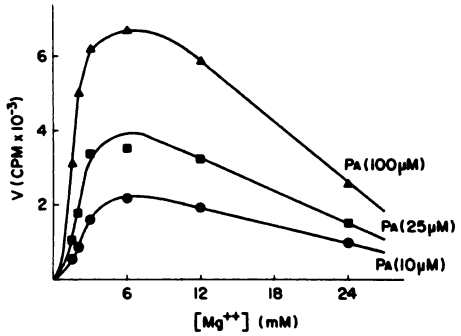

FIG. 7. *Evaluation of the Mg^{++} requirement for the incorporation of dTTP using DNA polymerase-α at various concentrations of homopolymer, P_A*

Reaction conditions are as described under Materials and Methods except that the concentration of Mg^{++} was varied from 1.5 to 24 mM at constant dTTP (75 μM) and at three concentrations of homopolymer. The symbols are: ●, 10 μM P_A; ■, 25 μM P_A; ▲, 100 μM P_A.

tion was still 6 mM and the sigmoidicity observed at the low concentrations of Mg^{2+} became more apparent. Increasing the concentration of homopolymer further did not completely abolish this sigmoidicity, but did broaden the peak Mg^{2+} concentration necessary for optimum activity (4–10 mM). The effect of dTTP is shown in Fig. 8. While increasing the concentration of dTTP above 75 μM inhibited the reaction, the degree of inhibition observed was similar at all concentrations of Mg^{2+}. Moreover, the optimum concentration of Mg^{2+} (6 mM) for the reaction was unaltered or affected only very slightly. That the inhibition by dTTP is not simply due to a depletion of metal cofactor is also indicated by the observation that ATP (data not shown) at the same concentration (300 μM) was not inhibitory.

As previously reported by Chang (10), the addition of noncomplementary polymer after the initiation of the reaction substantially inhibits dTTP polymerization. The addition of the noncomplementary polymer, polydeoxycytidylate–oligodeoxyguanylate in a ratio of 5:1, prior to the initiation of the reaction is also inhibitory, competitive with the concentration of the complementary homopolymer (data not shown). The end product of the polymerase reaction, PP_i, has been reported by Chang (12) to inhibit DNA polymerase-α in a mixed-noncompetitive manner with respect to dTTP. As shown in Fig. 9, this inhibition by PP_i was also competitive with the concentration of template.

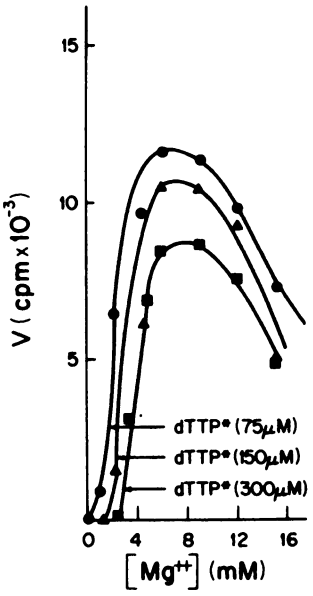

FIG. 8. *Evaluation of the Mg^{++} requirement for the incorporation of dTTP using DNA polymerase-α at various concentrations of dTTP and homopolymer, P_A*

Reaction conditions are as described under Materials and Methods except that the concentration of Mg^{2+} was varied from 1.5 to 15 mM at three specific concentrations of dTTP and P_A. The symbols are: ●, 75 μM dTTP + 50 μM P_A; ▲, 150 μM dTTP + 50 μM P_A; ■, 300 μM dTTP + 50 μM P_A.

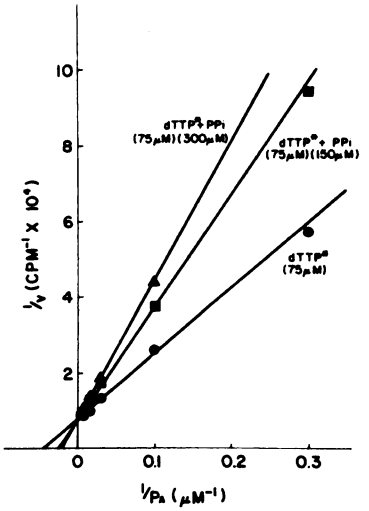

FIG. 9. *Competitive inhibition with respect to template of DNA polymerase-α by PP_i*

Reaction conditions are as described under Materials and Methods except that the concentration of homopolymer, P_A, was varied from 3.3 to 100 μM at constant dTTP (75 μM). The symbols are: ●, dTTP alone; ■, dTTP + 150 μM PP_i; ▲, dTTP + 300 μM PP_i.

DISCUSSION

The reactions of DNA polymerase I from *Escherichia coli* have been studied by Kornberg and co-workers (13) who describe two associated nuclease activities that complicate kinetic evaluation of this enzyme. However, two groups have recently evaluated the kinetics of this enzyme under conditions where the contribution of the two nuclease activities is minimized (14, 15). McClure and Jovin (14) utilized poly[d(A-T)] as the template–primer substrate and suggested an altered ordered Bi–Bi mechanism for the enzyme where template is bound prior to the deoxynucleotide. In agreement with Chang (10), they also indicated that the enzyme functions nonprocessively under certain conditions of low temperature. However, the results at higher temperature indicate that the enzyme dissociates from template after about 30 nucleotides are incorporated. The noncomplementary nucleotide, dGTP, is inhibitory when template is the variable substrate yielding a K_i of 1 mM.

Travaglini *et al.* (15), utilizing homopolymers for template, also observed that inhibition by the noncomplementary nucleotides is usually mixed-noncompetitive with respect to the complementary nucleotide. The K_m for the complementary nucleotides generally exceeds the respective K_i's by one to more than three orders of magnitude (15).

The mammalian DNA polymerases do not contain the associated 5′ → 3′ exonuclease activity (9, 12, 16) and thus are more suitable for kinetic evaluation (10). To date, only the low molecular weight enzyme, DNA polymerase-β, has been evaluated kinetically. Using homopolymers, Chang (17) concludes that this enzyme has a single binding site for deoxyribonucleoside triphosphates and one binding site for template. Moreover, this enzyme is strictly a two-substrate reaction in the replication of homopolymers, the initiated polynucleotide, and the complementary metal–nucleotide complex (17). In the presence of saturating polymer, the reaction is strictly first degree with respect to dTTP up to 100 μM. The fidelity of replication for this enzyme is quite good with a maximum of only one noncomplementary nucleotide incorporated per 10,000 complementary nucleotides.

Matsukage and co-workers (18) have isolated a high molecular weight membrane-bound DNA polymerase from mouse myeloma cells that has characteristics different from those of the α and β polymerases. This enzyme prefers polyriboadenylate–oligothymidylate for template and is also hyperbolic with respect to dTTP incorporation at saturating template.

The characteristics of the high molecular weight mammalian enzyme, DNA polymerase-α, have been reviewed (9, 16). The enzyme can catalyze pyrophosphate exchange and pyrophosphorolysis but has no detectable 5′ → 3′ exonuclease activity (12). For dTTP incorporation using homopolymer and commercial DNA polymerase, the optimum Mg^{2+} concentration of either dTTP or homopolymer does not affect this optimum. At saturating homopolymer concentrations, the reaction is apparent first order with respect to dTTP (Fig. 1) and, at optimum dTTP, is first order with respect to template (Fig. 3). The noncomplementary nucleotides are noncompetitive inhibitors with respect to dTTP (Fig. 1) and competitive inhibitors with respect to template (Figs. 3 and 4). The substrate inhibition by dTTP is also competitive with respect to template (Fig. 5) as is the inhibition by noncomplementary homopolymers and PP_i (Fig. 9).

In a classical sense, competitive inhibition is interpreted to mean that the two ligands compete for the same binding site on the enzyme and this usually occurs when the two ligands are structurally similar. However, in a kinetic sense, competitive inhibition simply implies that the two ligands bind to the same enzyme species (11). Thus, the inhibition by the deoxyribonucleoside triphosphates competitive with template observed in this study need not necessarily imply that the nucleotides are binding to the template site on the enzyme.

While the characteristics of DNA polymerase-α described herein were obtained using a commercial preparation of enzyme, subsequent studies (19, 20) show that many of the regulatory properties of DNA polymerase-α are conferred upon the enzyme by a separate regulatory protein; the procedure for isolation of DNA polymerase-α and this regulatory protein have recently been described (20). The characteristics of the commercial enzyme preparation indicate that deoxyribonucleoside triphosphates can serve as both substrates and inhibitors of DNA polymerase-α and that these nucleotides may play an important role in the regulation of DNA synthesis. These observations should stimulate further evaluation and identification of various proteins involved in this regulation.

REFERENCES

1. Brown, N. C., and P. Reichard. Role of effector binding in allosteric control of ribonucleoside diphosphate reductase. *J. Mol. Biol.* **46:** 39–55 (1969).
2. Moore, E. C., and R. B. Hurlbert. Regulation of mammalian deoxyribonucleotide biosynthesis by nucleotides as activators and inhibitors. *J. Biol. Chem.* **241:** 4802–4809 (1966).
3. Scarano, E., M. Rossi and G. Geraci. The regulation of the activity of deoxycytidylate aminohydrolase, in *The Regulation of Enzyme Activity and Allosteric Interactions* (E. Kramme and A. Pihl, eds.). Universitetsfordaget, Oslo, 145–165 (1968).
4. Bjursell, G., and P. Reichard. Effects of thymidine on deoxyribonucleoside triphosphate pools and deoxyribonucleic acid synthesis in Chinese hamster ovary cells. *J. Biol. Chem.* **248:** 3904–3909 (1973).
5. Lowe, J. K., and G. B. Grindey. Inhibition of growth rate and deoxynucleoside triphosphate concentrations in cultured leukemia L1210 cells. *Mol. Pharmacol.* **12:** 177–184 (1976).
6. Grindey, G. B., M. C. Wang and J. J. Kinahan. Thymidine induced perturbations in ribonucleoside triphosphate pools in human leukemic CCRF-CEM cells. *Mol. Pharmacol.* **16:** 601–606 (1979).
7. Lowe, J. K., B. Gowans and L. Brox. Deoxyadenosine metabolism and toxicity in cultured L5178Y cells. *Cancer Res.* **37:** 3013–3017 (1977).
8. Bollum, F. J., L. M. S. Chang, L. M. Tsiopalis and J. W. Dorson. Nucleotide polymerizing enzymes from calf thymus gland, in *Methods in Enzymology* (L. Grossman and K. Moldave, eds.). Academic Press, New York, 70–81 (1974).
9. Bollum, F. J. Mammalian DNA polymerases. *Progr. Nucleic Acids Res. Mol. Biol.* **15:** 109–144 (1975).
10. Chang, L. M. S. The distributive nature of enzymatic DNA synthesis. *J. Mol. Biol.* **93:** 219–235 (1975).
11. Wong, J. T. F. One-substrate mechanism, in *Kinetics of Enzyme Mechanisms.* Academic Press, New York, 1–3 (1975).
12. Chang, L. M. S., and F. J. Bollum. A comparison of associated enzyme activities in various deoxyribonucleic acid polymerases. *J. Biol. Chem.* **248:** 3398–3404 (1973).
13. Kornberg, A. Active center of DNA polymerase. *Science* **163:** 1410–1418 (1969).
14. McClure, W. R., and T. M. Jovin. The steady state kinetic parameters and non-processivity of Escherichia coli deoxyribonucleic acid polymerase I. *J. Biol. Chem.* **250:** 4073–4080 (1975).

15. Travaglini, E. C., A. S. Mildran and L. A. Loeb. Kinetic analysis of Escherichia coli deoxyribonucleic acid polymerase I. *J. Biol. Chem.* **250:** 8647–8656 (1975).
16. Weissbach, A. Vertebrate DNA polymerases. *Cell* **5:** 101–108 (1975).
17. Chang, L. M. S. Low molecular weight deoxyribonucleic acid polymerase from calf thymus chromatin. *J. Biol. Chem.* **248:** 6983–6992 (1973).
18. Matsukage, A., E. W. Bohn and S. H. Wilson. On the DNA polymerase III of mouse myeloma: Partial purification and characterization. *Biochemistry* **14:** 1006–1020 (1975).
19. Steinberg, J. A., and G. B. Grindey. Isolation of a regulatory protein associated with calf thymus DNA polymerase-α modulated by deoxyguanosine triphosphate. *Fed. Proc.* **38:** 484 (1979).
20. Steinberg, J. A., M. Otten and G. B. Grindey. Isolation of a DNA polymerase-α associated regulatory protein from calf thymus. *Cancer Res.* **39:** 4330–4335 (1979).

Send reprint requests to: Dr. Gerald B. Grindey, Department of Experimental Therapeutics, Grace Cancer Drug Center, Roswell Park Memorial Institute, 666 Elm St., Buffalo, N. Y. 14263.