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Nucleoside Diphosphokinase Activity Associated with DNA Polymerases

[*E. coli*/*Micrococcus luteus*/avian myeloblastosis virus/poly(dA–dT)·poly(dA–dT)]

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ABSTRACT **Nucleoside diphosphokinase activity is present in highly purified preparations of DNA polymerase from *Micrococcus luteus* and *Escherichia coli*, and in a partially purified DNA polymerase from avian myeloblastosis virus. The activity is also observed in the protein fragment of molecular weight 76,000 that is produced by subtilisin cleavage of DNA polymerase I from *E. coli*. The NDP kinase activity in DNA polymerase preparations from *M. luteus* uses various ribo- and deoxyribonucleoside di- and triphosphates as substrates. The presence of this activity in preparations of DNA polymerase results in the apparent use of deoxyribonucleoside diphosphates as substrates for DNA synthesis, provided that some triphosphate is present to serve as a phosphate donor.**

DNA polymerase from *Micrococcus luteus* (DNA:nucleotidyltransferase, EC 2.7.7.7) contains an exonucleolytic activity that is markedly stimulated by the presence of nucleoside triphosphates (1–3). Deoxynucleoside diphosphates and triphosphates are potent stimulators under certain conditions. Hence, it was realized that diphosphates could serve as substrates for DNA synthesis, if a triphosphate was also present. Investigation revealed that the diphosphates were rapidly converted to triphosphates, which then could serve as substrates for DNA polymerase.

MATERIALS AND METHODS

Materials

Radioactive and nonradioactive di- and triphosphates were purchased from Schwarz/Mann and Sigma and were carefully analyzed for radiochemical purity by paper chromatography in solvents A (isobutyric acid–concentrated ammonium hydroxide–water, 66:1:33) and B (60 g of ammonium sulfate–100 ml of 0.1 M sodium phosphate, (pH 6.8)–2 ml of propanol-1). The radioactive deoxyribonucleoside diphosphates contained no detectable deoxyribonucleoside triphosphates (less than 0.1%). Both dADP and dTDP were purified by column chromatography with DEAE-cellulose in the carbonate form (gift of R.W. Sweet). [^{3}H]dAMP was purchased from Schwarz/Mann and was further purified by preparative paper chromatography in solvent A. Poly(dA–dT)·poly(dA–dT) and DNA from *M. luteus* were prepared and characterized as described (4, 5).

Enzymes

The DNA polymerase from *M. luteus* was purified essentially as previously described (5) (sp. act., 1300). The enzyme was at least 70% pure, as analyzed by polyacrylamide gel electrophoresis under two conditions (manuscript in preparation).

Two preparations of the DNA polymerase from *Escherichia coli* were studied. Preparation *A* (gift of R.W. Sweet) was purified (6) through the phosphocellulose chromatography step; the specific activity was 5000 [poly(dA–dT)·poly(dA–dT) assay], and it gave a single band on polyacrylamide gel electrophoresis (7). Preparation *B* was purified by the entire published procedure (6), followed by an additional phosphocellulose chromatography step. This enzyme (gift of Dr. L. Loeb, Institute of Cancer Research, Philadelphia, Pa.) was homogeneous, as analyzed by isoelectric focusing, and had a specific activity of 20,000 (assay of activated DNA from calf thymus).

The fragment of molecular weight 76,000 (8, 9), produced by subtilisin cleavage of the highly purified DNA polymerase from *E. coli*, had a specific activity of 24,000 [poly(dA–dT)·poly(dA–dT) assay]. This fragment (gift of Dr. Hans Klenow, Biochemical Institute, Copenhagen, Denmark) was separated from the fragment of molecular weight 36,000 by chromatography on hydroxylapatite.

The DNA polymerase from avian myeloblastosis virus was purified 25-fold by sucrose gradient centrifugation and DEAE-Sephadex chromatography (Schendel and Wells, manuscript in preparation). At this stage, the enzyme is impure.

Conditions for DNA synthesis

Reaction mixtures contained in 0.10 ml total volume: 0.05 M Tris·HCl, (pH 7.8), 0.01 M $MgCl_2$, 5×10^{-4} M 2-mercaptoethanol, deoxyribonucleoside di- or triphosphates (see Legends to Figures), a DNA template [30 μM poly(dA–dT)·poly(dA–dT), or 50 μM DNA from *M. luteus*], and 4 units of DNA polymerase from *M. luteus*. Reaction mixtures were incubated at 37°C. At intervals, 15-μl samples were removed and assayed for acid-insoluble radioactivity (10).

Assay of nucleoside diphosphokinase (ATP: nucleoside diphosphate phosphotransferase, EC 2.7.4.6)

NDP kinase activity was measured directly by chromatographically monitoring the conversion of [^{3}H]dADP to [^{3}H]-dATP. Reaction mixtures, described in the Legends, were incubated at 37°C. At intervals, samples were diluted with an equal volume of nucleotide-marker solution (mixture of 5 μM dATP, 5 μM dADP, and 0.1 M EDTA). The mixture was analyzed by chromatography on Whatman No. 1 paper in sol-

Abbreviation: NDP kinase, nucleoside diphosphokinase.

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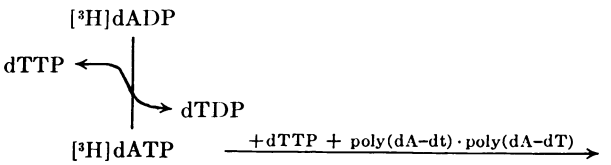
vent A for 18 hr. DNA was synthesized during some enzymic reactions and remained at the origin. The R_fs of dATP and dADP were 0.29 and 0.40, respectively. Ultraviolet-absorbing spots were visualized under a UV lamp and radioactivity was determined with a Packard radiochromatogram scanner equipped with a Disc integrator that indicated the relative proportion of products. The entire length of all chromatograms was monitored for radioactivity. Radioactivity was observed only in di- and triphosphate areas, unless noted otherwise. That the products of this reaction are triphosphates is also indicated by their capacity to serve as substrates for DNA polymerase (Figs. 1, 2, and 4).

RESULTS

Requirements for NDP kinase activity

The general requirements for nucleoside diphosphokinase activity of the DNA polymerase from *M. luteus* are demonstrated in Table 1*A*. NDP kinase has an absolute requirement for a divalent metal ion (Mg^{++}). Heating the enzyme for 10 min at 80°C destroys its activity. No reaction is observed in the absence of enzyme or dTTP. Thus, the conversion of dADP to dATP is not due to a myokinase type of reaction. The NDP kinase reaction occurs in the absence of DNA; hence, the concomitant polymerization of nucleotides is not necessary for expression of kinase activity. DNA from *M. luteus* has little effect on the rate of conversion of dADP to dATP. However, addition of poly(dA-dT)·poly(dA-dT) supports synthesis of DNA, since the dATP that is formed by the NDP kinase is a substrate for the DNA polymerase.



[³H]poly(dA-dT)·poly(dA-dT) + PPi

The rate of dATP formation by NDP kinase is not affected by DNA synthesis, but the final equilibrium of the reaction is shifted to minimize the concentration of dADP. In the absence of DNA synthesis, at equilibrium, dADP and dATP concentrations are about equal. In the presence of DNA synthesis, however, dATP is a substrate for polymer synthesis and, if sufficient substrate dTTP is provided, virtually all the isotope in the dADP appears in DNA.

Specificity of the reaction

Table 1*B* shows the substrate specificity of the nucleoside diphosphokinase activity. rATP or dCTP substitute equally well for dTTP as the phosphate donor. [³H]dAMP will not function as the phosphate acceptor. Neither [³H]dADP nor [³H]dATP are formed after incubation of [³H]dAMP and dTTP with the enzyme. Hence, the activity is not that of a general nucleotide kinase. All deoxynucleoside diphosphates can function as phosphate acceptors. Fig. 2 indirectly illustrates that [¹⁴C]dCDP can function as a phosphate acceptor.

Mechanism of the reaction

Table 1 demonstrates that dADP is converted to dATP by DNA polymerase prepared from *M. luteus* in the presence of a nucleoside triphosphate. The nucleoside triphosphate substrate is converted to the corresponding nucleoside diphosphate. When [α-³²P]dTTP is incubated with dADP, under

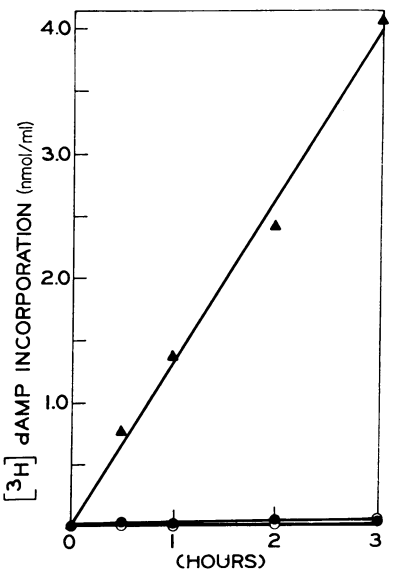


FIG. 1. Poly(dA-dT)·poly(dA-dT) synthesis by the use of [³H]dADP as a substrate. Reactions were performed as described in *Materials and Methods* (conditions for DNA synthesis). The template was poly(dA-dT)·poly(dA-dT). All reactions contained 100 μM [³H]dADP (spec. act. 6×10^4 cpm/nmol). [³H]dADP only (○), [³H]dADP plus 100 μM dTDP (●), and [³H]dADP plus 100 μM dTTP (▲).

conditions similar to those mentioned in Table 1, it is converted to [α-³²P]dTDP at a rate equivalent to the rate of appearance of [³H]dATP in an experiment analogous to that of Table 1 (data not shown). No other radioactive products are formed. Thus, the reaction appears to be of the general type

$$N'TP + N''DP \rightleftharpoons N'DP + N''TP$$

where the gamma-phosphate of N'TP is transferred onto the beta-phosphate of the N''DP.

Role of NDP kinase in DNA synthesis

The presence of NDP kinase activity was found originally by observation of the synthesis of DNA from a mixture of nucleoside di- and triphosphates as substrates for the DNA polymerase from *M. luteus*. Fig. 1 shows the kinetics of poly(dA-dT)·poly(dA-dT) synthesis when [³H]dADP and dTTP are provided as substrates for the DNA polymerase in the presence of a poly(dA-dT)·poly(dA-dT) template. As expected, DNA synthesis does not occur when only [³H]dADP is provided, since the template has a strictly alternating sequence. Also, no DNA synthesis is observed when [³H]dADP plus dTDP are provided as substrates. Discovery of the DNA-independent NDP kinase activity in the DNA polymerase from *M. luteus* gave an explanation, in that [³H]dADP is converted to [³H]dATP in the presence of dTTP. The [³H]dATP then serves as a substrate for DNA polymerase. dTDP cannot substitute for dTTP as a phosphate donor in the NDP kinase reaction.

In Fig. 2, one series of experiments shows the incorporation of isotope into DNA when [¹⁴C]dCDP, dTTP, dGTP, and dATP are the substrates and *M. luteus* DNA is the template. The reaction proceeds equally well when [¹⁴C]dCDP, dADP, dGTP, and dTTP are provided. Again, no synthesis is observed if only the appropriate deoxynucleoside diphosphates are used as substrates. The rate of DNA synthesis when all four deoxy-

nucleoside triphosphates are used is also illustrated in Fig. 2. A second series of reactions with [^{14}C]dGTP and various nucleoside di- and triphosphates was assayed for DNA synthesis with DNA from *M. luteus* as the template. DNA synthesis proceeds at the same rate when either all four deoxynucleoside triphosphates are provided as substrates or when dATP is replaced by dADP in the reaction. No detectable DNA synthesis occurs if one of the four nucleotides is omitted. Comparison of the rate of [^{14}C]dCDP and [^{14}C]dGTP incorporation in this system also shows that DNA synthesis is not retarded by replacement of one or two triphosphates by the corresponding diphosphates. Hence, the rate of conversion of diphosphates to triphosphates by the NDP kinase is sufficiently rapid for DNA synthesis using a native-DNA template from *M. luteus*.

Poly(dA-dT)·poly(dA-dT) is synthesized by the DNA polymerase from *M. luteus* much more rapidly than native *M. luteus* DNA. In the poly(dA-dT)·poly(dA-dT) system, the rate of synthesis observed with dATP and dTTP as substrates is 10-fold greater than the rate of synthesis with dADP and dTTP under conditions similar to those in Fig. 1. With dADP and dTTP supplied, the enzyme concentration is in excess for the polymerase reaction, but is limiting for the NDP kinase activity. This might imply that the NDP kinase is a trace contaminant of DNA polymerase. However, at this enzyme level the concentration of exonuclease, which is an integral part of the DNA polymerase (Miller and Wells, unpublished data), is also limiting. Furthermore, no attempt has been made to optimize the NDP kinase activity.

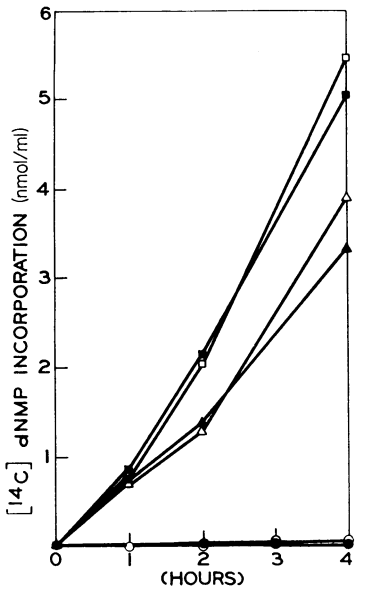


FIG. 2. Di- and triphosphates as substrates for DNA synthesis. Reactions were performed a described under conditions for DNA synthesis in *Materials and Methods*. The template was DNA from *M. luteus*. One series of reactions contained 100 μM [^{14}C]dGTP (spec. act. 8000 cpm/nmol) plus other nonradioactive di- or triphosphates (each 100 μM) as follows: [^{14}C]dGTP, dCTP, and dTTP (●), [^{14}C]dGTP, dCTP, dTTP, and dATP (△) and [^{14}C]dGTP, dCTP, dTTP, and dADP (▲). The other series of reactions contained 200 μM [^{14}C]dCDP (spec. act. 17,000 cpm/nmol) plus other nonradioactive di- or triphosphates (each 100 μM) as follows: [^{14}C]dCDP, dGDP, dTDP, and dADP (○), [^{14}C]dCDP, dGTP, dTTP, and dATP (□), and [^{14}C]dCDP, dGTP, dTTP, and dADP (■).

NDP kinase activity associated with other highly purified DNA polymerases

In addition to the DNA polymerase from *M. luteus*, several other highly purified DNA polymerase preparations were assayed for NDP kinase activity. All the polymerase preparations tested contained NDP kinase, even though they were isolated from widely different sources. Fig. 3 shows the kinetics of conversion of [^{3}H]dADP to [^{3}H]dATP, in the presence of dTTP, by DNA polymerase of avian myeloblastosis virus, DNA polymerase from *E. coli* (preparations A and B), and the *M. luteus* DNA polymerase.

The fragment of molecular weight 76,000 produced by subtilisin cleavage of the DNA polymerase of *E. coli* was also assayed for NDP kinase activity. Since this preparation was dilute, it was necessary to use a more sensitive assay to monitor [^{3}H]dADP conversion to [^{3}H]dATP than the chromatographic assay. NDP kinase activity was followed by the incorporation of [^{3}H]-labeled nucleotide into DNA using [^{3}H]dADP and dTTP as substrates and poly(dA-dT)·poly(dA-dT) as a template. Fig. 4 shows that the 76,000 molecular weight fragment of the DNA polymerase of *E. coli* has NDP kinase activity. The rate of [^{3}H] dADP to [^{3}H]dATP conversion per μg of protein is slightly faster than the rate observed for the two highly purified preparations of uncleaved DNA polymerase of *E. coli*. Due to an insufficiency of material, it was not possible to test the fragment of molecular weight 36,000 for NDP kinase activity.

These preparations were made in three different laboratories and were each assayed for polymerase activity under slightly different conditions. Hence, no meaningful ratio between NDP kinase and DNA polymerase activities has been

TABLE 1. *General requirements for nucleoside diphosphokinase activity*

Additions	[^{3}H]dATP formed in 1 hr (nmol/ml)
A.	
Complete system	23.6
− Mg^{++}	<0.3
− Enzyme	<0.3
− Enzyme, + heat-treated enzyme	<0.3
− dTTP	<0.3
+ *M. luteus* DNA (50 μM)	25.1
+ poly(dA–dT)·poly(dA–dT) (30 μM)	1.9*
B.	
− dTTP, + rATP (200 μM)	25.4
− dTTP, + dCTP (200 μM)	24.2
− [^{3}H]dADP, + [^{3}H]dAMP (57 μM)	<0.05

The reaction mixture (50 μl) contained 50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 5×10^{-4} M 2-mercaptoethanol, 55 μM [^{3}H]dADP (spec. act. 6.6×10^{5} cpm/nmol), 200 μM dTTP, and 4.0 units of DNA polymerase from *M. luteus*. The specific activity of [^{3}H]dAMP was 1.33×10^{6} cpm/nmol. For heat-treatment of the enzyme, a solution of the DNA polymerase (0.3 mg/ml) in storage buffer (50 mM potassium phosphate buffer, pH 6.9, 1 mM 2-mercaptoethanol, and 30% ethylene glycol) was kept at 80°C for 10 min. Samples (10 μl) of the reaction mixtures were removed at intervals over a 3-hr period and were assayed for NDP kinase activity (*Materials and Methods*).

* Synthesis of poly(dA–dT)·poly(dA–dT) resulted in the incorporation of 23.0 nmol/ml of the [^{3}H]dATP.

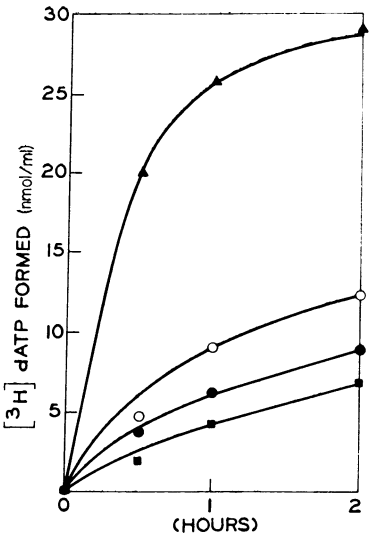


FIG. 3. Nucleoside diphosphokinase activity of DNA polymerase from *E. coli*, *M. luteus*, and avian myeloblastosis virus. The reaction mixtures (0.05 ml) contained 50 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 5×10^{-4} M 2-mercaptoethanol, 55 μM [^{3}H]-dADP (spec. act. 6.6×10^5 cpm/nmol), 200 μM dTTP, and the appropriate enzyme. NDP kinase activity was assayed as described in *Materials and Methods*. DNA polymerase from *M. luteus* (30 μg/ml) (●), DNA polymerase from *E. coli*, preparation A (60 μg/ml) (■), DNA polymerase from *E. coli*, preparation B (92 μg/ml) (○), and DNA polymerase from avian myeloblastosis virus (1 μg/ml) (▲).

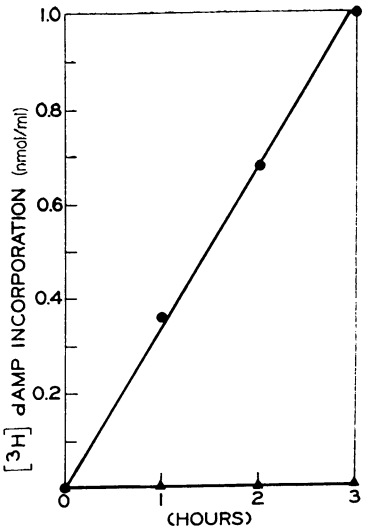


FIG. 4. [^{3}H]dADP as a substrate for the fragment (molecular weight 76,000) DNA polymerase of *E. coli*. The reaction mixture (50 μl) contained 50 mM phosphate buffer (pH 7.4), 5 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 60 μM poly(dA-dT)·poly-(dA-dT), 15 ng of the fragment (molecular weight 76,000) produced by subtilisin cleavage of the DNA polymerase from *E. coli*, 51.5 μM [^{3}H]dADP (specific activity 4.4×10^6 cpm/nmol), and 200 μM dTTP. The mixtures were incubated at 37°C, and at intervals 10-μl samples were removed and assayed for acid-insoluble radioactivity. [^{3}H]dADP + dTTP, (●); [^{3}H]dADP alone, (▲).

established for them. It is clear, however, that the enzymes from different sources have widely different polymerase to nuclease ratio (1, 6, 8, 9, Schendel and Wells, unpublished data).

Heat inactivation of activities

A highly purified preparation of the *M. luteus* DNA polymerase [0.3 mg/ml in storage buffer (5)] was kept at 41°C; samples were taken at intervals and assayed for both DNA polymerase and NDP kinase activities. Virtually all (94%) of the polymerase activity was destroyed in the first 10 min, whereas the kinase activity was lost at a linear rate of 10% per 10 min at 41°C. After 40 min of incubation at 41°C, 58% of the kinase activity remained, but no polymerase activity was detectable.

DISCUSSION

Nucleoside diphosphokinase activity was detected in highly purified preparations of DNA polymerases from *M. luteus* and *E. coli* and in a partially purified DNA polymerase from avian myeloblastosis virus. The kinase activity also was detected in the fragment of molecular weight 76,000 that is produced by subtilisin cleavage of the DNA polymerase from *E. coli* (8, 9). The fact that the activity is found in essentially pure preparations of DNA polymerases, obtained from a wide spectrum of organisms, suggests that the polymerase *per se* has kinase activity. However, thermal inactivation studies suggest that it may be a contaminant. In addition, RNA polymerase from *E. coli* (gift of Dr. Fred Blattner) also contains NDP kinase activity. Attempts to separate the NDP kinase and DNA polymerase activities have not been successful. It would be of interest to attempt to label the NDP-kinase active site by incubation of the enzyme in the presence of [gamma-^{32}P]-rATP, as has been done for the NDP kinases from yeast and from human erythrocyte (11, 12). The isolation of an enzyme–phosphate intermediate might be useful for studies of both purification and mechanism.

The partially purified DNA polymerase from avian myeloblastosis virus possesses NDP kinase activity. Also detergent-treated preparations of the Schmidt-Ruppin strain of Rous sarcoma virus possess nucleotide kinase activities (Mizutani and Temin, personal communication). Various enzymes that metabolize nucleic acids are associated with RNA tumor viruses (13, and previous papers cited therein); their possible role in the transformation of host cells is under intense investigation in many laboratories.

The specific activity of NDP kinases in preparations of DNA polymerase is low as compared to the specific activities of purified NDP kinases from other sources (14, 15). However, the rate of diphosphate to triphosphate conversion by the *M. luteus* DNA polymerase is adequate to support a rate of DNA synthesis, with bacterial DNA template, equal to the rate of polymerization with the use of all four triphosphates as substrates (Fig. 2).

Under physiological conditions, deoxynucleoside triphosphates are presumably synthesized from the deoxynucleoside diphosphates by NDP kinases using rATP as the donor. Because NDP kinases have high specific activities, even in crude extracts (16), the reaction is not considered to be a key step in regulating nucleotide synthesis (17, 18). The NDP kinase present in the preparations of DNA polymerase accounts for only a small fraction of the NDP kinase activity that is present in crude fractions. However, NDP kinases function in a number of different metabolic pathways, and multiple species of NDP kinase could be present in any organism.

Little is known about the size of *in vivo* pools of deoxynucleoside diphosphates and triphosphates. Recently, there have been attempts to determine the size of deoxynucleoside triphosphate pools (19, 20), but DNA polymerase was used as an analytical tool. It is now apparent that both deoxynucleoside diphosphate and triphosphate pools will be measured by such an assay system. In addition, recent studies (21) on the binding of diphosphates to the DNA polymerase from *E. coli* could be complicated by the presence of NDP kinase.

Other workers have recently found that deoxynucleoside diphosphates can serve as substrates for DNA synthesizing systems under certain conditions (22–24); some of these systems may also contain NDP kinase activity.

NOTE ADDED IN PROOF

Recent studies show that NDP kinase is present in a purified preparation of *E. coli* DNA polymerase II (gift of M. Gefter, Columbia University, New York). DNA synthesis was observed with [^{3}H]dADP, dTTP, dGTP, and dCTP as substrates for a reaction with exonuclease III-treated calf-thymus DNA as template. All four deoxynucleoside diphosphates did not serve as effective substrates for DNA synthesis unless a phosphate donor (ATP) was provided. The rate of synthesis, however, was only 5% of the rate observed with all four deoxynucleoside triphosphates as substrates for the reaction with exonuclease III-treated calf-thymus DNA as template.

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