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New substrates of DNA polymerases

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Abstract Bis-(2′-deoxynucleoside) 5′,5′-tetraphosphates and bis-(2′-deoxynucleoside) 5′,5′-triphosphates were shown to be a new type of substrate for several DNA polymerases of human, bacterial and viral origin. Their substrate properties depend both on their structure and on the nature of the enzyme. They are incorporated by both termini in correspondence with the template nucleotide program in the active center. The results obtained support the mechanism of their direct incorporation rather than prior hydrolysis to dNTP. The highest activity of these compounds was observed for HIV reverse transcriptase. The probable biological significance of the reaction is discussed.

Key words: Bis-(2′-deoxynucleoside) 5′5′-tetraphosphate; Bis-(2′-deoxynucleoside) 5′5′-triphosphate; DNA polymerase; Substrate

1. Introduction

It is well known that dNTPs are standard natural substrates of all DNA polymerases. In the process of DNA synthesis some unpredictable events can happen, which can delay or even stop the extension process. For example, DNA polymerases repair the 3′-termini of DNA chains synthesized if a mismatched or terminating nucleotide residue is incorporated into the 3′-terminus. Various DNA polymerases use different molecular mechanisms for correction [1]. Hydrolytic removal of the 3′-terminal nucleotide residue seems to be the most common, but it only is characteristic for DNA polymerases containing a 3′-5′ exonuclease active center located in the neighborhood of the polymerization active center.

However, some DNA polymerases (human and mammalian DNA polymerases α and β as well as viral reverse transcriptases among them) do not have the 3′-5′ exonuclease activity. Pyrophosphorolysis may be involved in another mechanism of repair, which results in the removal of the 3′-terminal nucleotide of the growing DNA chain and the formation of the corresponding dNTP. This reaction proceeds well in cell-free solutions, but it has not been observed directly in cells. There are two reasons against the last hypothesis. (i) Pyrophosphorolysis needs a high PPi concentration because its affinity to the DNA polymerase active center is 100–1000 times lower than that of dNTP. (ii) Inorganic pyrophosphatase presenting in the replicative fork rapidly hydrolyzes PPi to a phosphate thus favoring the elongation reaction. It is quite possible that PPi released in the elongation reaction remains bound to the active center of DNA polymerase until its translocation along the template occurs. However, these complexes have not been isolated so far.

It was recently shown that HIV RT catalyzes the formation of dideoxynucleoside 5′,5′-polyphosphates dNp_xdN, $x=4$ (**I**), and $x=3$ (**II**) by nucleotide-dependent removal of the primer chain-terminated residue [2]. A similar reaction proceeded in the presence of same DNA polymerases (*Escherichia coli* DNA polymerase I, Klenow fragment, human DNA polymerase α, and AMV RT), a non-terminated primer, and L-dNTP as substrates [3]. In both cases residues of dNTP, dNDP as well as of L-dNTP non-complementary to the template nucleotide served as PPi analogues.

We demonstrate in this work that dinucleoside polyphosphates **I** and **II** are effective substrates for some DNA polymerases in the process of DNA elongation.

2. Materials and methods

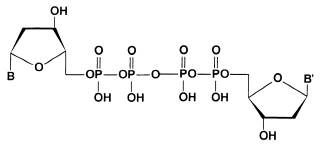
2.1. Compounds

Compounds **I–III** were synthesized according to the general method [4,5]. Their physicochemical data are given in Table 1.

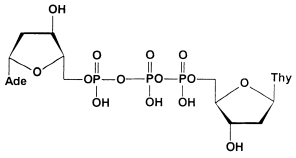
2.2. Enzymes and DNA

The following enzymes were used: *E. coli* DNA polymerase I Klenow fragment (Boehringer Mannheim), calf thymus TDT, HIV RT and T4 polynucleotide kinase (Amersham Corp.), *Taq* DNA polymerase (Pharmacia Biotech), human placenta DNA polymerases α and β were isolated as in [6,7]. Phage M13mp10 DNA was isolated according to [8]. The template-primer complexes (Scheme 1) were made as in [9].

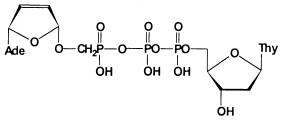
All experiments including measurements of K_m and V_{max} were carried out as in [9,10].



Ia, B=Ade, B′=Thy
Ib, B=B′=Ade
Ic, B=B′=Thy



II



III

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Abbreviations: dNTP and dNDP, 2′-deoxynucleoside 5′-triphosphates and 5′-diphosphates, respectively; PPi, inorganic pyrophosphate; ddNTP, 2′,3′-dideoxynucleoside 5′-triphosphates; HIV RT, reverse transcriptase of human immunodeficiency virus; *Taq, Thermus aquaticus* DNA polymerase

PII: S0014-5793(99)00615-8

The template-primer complex C1

3′- d(GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGA...)
5′-[^{32}P]- d(CCCAGTCACGACGT)

The template-primer complex C2

3′- d(GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGA....)
5′-[^{32}P]-d(GTAAAACGACGGCC)

Scheme 1.

3. Results

The substrate properties of compounds **I–III** were studied using the template-primer complexes C1 and C2. The structure of these complexes was selected in such a way that the next nucleotide residue incorporated in the primer was either deoxythymidylate (complex C1) or deoxyadenylate (complex C2) (Scheme 1).

In the first series of experiments the substrate properties of **I–III** were studied in the reaction catalyzed by HIV RT (Fig. 1). As is seen in Fig. 1a, **Ic** extended the primer in the complex C1 by a deoxythymidylate residue (series B, lanes 2–4) similarly to dTTP (series A; lanes 2–4). When dGTP is added into the incubation assay (series B, lane 5), the primer was extended by three deoxynucleotide residues (TGT) in correspondence with the template program. The same result was obtained in the presence of dTTP+dGTP (series A, lane 5). Compounds **Ia** (series C) and **II** (series D) also demonstrated substrate properties toward HIV RT, being incorporated into the DNA chain by both termini; after the addition of dGTP the primer is extended by seven nucleotide residues, TGTAAAA. A similar primer extension was observed in the presence of dTTP+dGTP+dATP (series A, lane 6). Substrate properties of **Ia**, **Ib**, **II** and dATP for complex C2 can be compared in Fig. 1b. It is obvious that all three compounds extended the primer chain both by a deoxyadenylic residue (series B, C and D, lanes 2–4) and by dATP (series A, lanes 2–3). When dGTP was added together with **Ib**, the primer was extended by two residues, AG (series B, lane 5), similarly to the control experiments with dATP+dGTP (series A, lane 4). At the same time, compounds **Ia** and **Ib** in the presence of dGTP extended the primer by four residues, AGTG (series C and D, lanes 5), being incorporated in the primer by both nucleotide termini of **Ia** and **Ib** molecules. Fig. 1c,d shows substrate activity of **III** (series D) with complexes C1 (Fig. 1c) and C2 (Fig. 1d). As can be seen, the activity of **III** was close to that of **II** (series B), and it also extended the primer by both nucleotide termini. When dGTP and **III** were added together, the primer in complex C1 was extended by only four nucleotide residues (Fig. 1c, series D, lane 5), rather than by seven, as was observed in the presence of **II** (series B, lane 5). This occurs because the modified isosteric dideoxydidehydroadenylic residue in **III** reacts as a terminating substrate.

For complex C1, the substrate activity of the compounds under study toward HIV RT was decreased in the order: dTTP = **Ic** > **Ia** = **III** > **II**; for complex C2, the compounds can be arranged in the order: dATP > **Ib** > **Ia** > **III** = **II**. The K_m and V_{max} for the most active substrates **Ib** and **Ic** are shown in Table 2.

Fig. 2 demonstrates the primer extension by compounds **I** and **II** for *E. coli* DNA polymerase I and *Taq* DNA polymerase. As seen in Fig. 2a, the primer in complex C1 was extended by **Id** (series B) to the same degree as dTTP (series A), whereas **Ia** (series C) was by one order of magnitude less active. Compound **II** (series D) did not manifest substrate activity to a concentration of 20 μM. The results for complex C2 (Fig. 2b) were partially different: **Ib** (series B) was 20 times, **Ia** (series C) 200 times, and **II** (series D) 2000 times weaker than dATP (series A).

It is worth mentioning that **Id** containing two thymidylate residues revealed activity close to that for dTTP when the reaction in the complex C1 was catalyzed by *Taq* DNA polymerase (Fig. 2c, series B and A, respectively). At the same time the activity of **Ia** (series C) was lower by three orders of magnitude, whereas **II** (series D) did not extend the primer up to a concentration of 0.5 mM.

Slightly different results were found for DNA polymerase α (Fig. 3). It can be seen in Fig. 3a that although compounds **Ic** and **Ia** extended primer C1, the activity of **Ia** (series B, lanes 2–5) toward the DNA synthesizing complex was 25–50-fold lower than that for dTTP (series A, lane *2*), whereas the activity of **Ic** (series D, lanes 2–5) was comparable with that for dTTP. Compound **II** (series C, lanes 2-5) was less active by more than two orders of magnitude than dTTP. As seen in Fig. 3b, compounds **Ia** (series C, lanes 2–5) and **Ib** (series B, lanes 2–5) extended the primer in complex C2 less effectively up to concentrations of 50 μM, whereas **II** (series D, lanes 2–5) was practically inactive. Thus, the activity toward DNA polymerase α was as follows: dTTP = **Ic** > **Ia** ≫ **II** (for complex C1) and dATP ≫ **Ia** = **Ib** (for complex C2).

A decrease in the activity of **I** and **II** was demonstrated for DNA polymerase β even to a higher degree (data not shown). The activity of **Ic** is five times lower in the presence of complex C1 compared with dTTP; for **Ia**, it was 2–3 orders of magnitude lower. The activity of **Ia** and **II** was approximately equal. In the presence of complex C2 the activity of com-

Table 1
Some physicochemical characteristics of dinucleoside polyphosphates[a]

Compound	R_f^b	λ_{max}, nm (pH 7)	^{31}P-NMR, Pα	^{31}P-NMR, Pβ
Ia	0.20	262.2	−11.36 br.s. (2P)	−22.84 br.s. (2P)
Ib	0.16	259.2	−10.76m (2P)	−22.85m (2P)
Ic	0.15	265.2	−11.51m (2P)	−23.14m (2P)
II	0.55	262.5	−11.25, −11.46 2d (2P, 16, 17 Hz)	−22.88 br.t. (1P, !6 Hz)
III	0.57	261.1	7.93m (1P, PCH_2) −11.28m (1P)	−22.67m (1P)

[a]^{1}H-NMR spectra support the **I** and **II** structures and not characteristic ones (data not shown) with the exception of spectra of **III**. **III**, ^{1}H-NMR spectra (D_2O, δ in ppm, *J* in Hz): 8.13 s (1H, H8 Ade); 7.87 s (1H, H2 Ade); 7.41 s (1H, H6 Thy), 6.82 s (1H, H1 Ade); 6.50 d (1H, H2′, Ade, $J_{H1',H2'}$ = 6.0); 6.46 d (1H, H3′, Ade, $J_{H3',H2'}$ = 6.0); 6.03 t H1′,Thy, *J* = 8); 5.99 s (1H, H4′, Ade); 4.65 m (1H, H3′, Thy); 3.99 m (3H, H4′,+H5′, Thy); 3.79 dd 1H, H_{PCH2}, Ade, *J* = 9.3, 13.2); 3.69 dd (1H, H_{PCH2}, Ade, *J* = 9.3, 13.2); 2.1 m (2H, H2′, Thy); 1.69 s (3H, 5Me, Thy). Mass: VISION 2000 (MALDI), calc. 694.36; found 691.7.
[b]Dioxane-acetonitrile-25% ammonium-water 4:2:1:3.

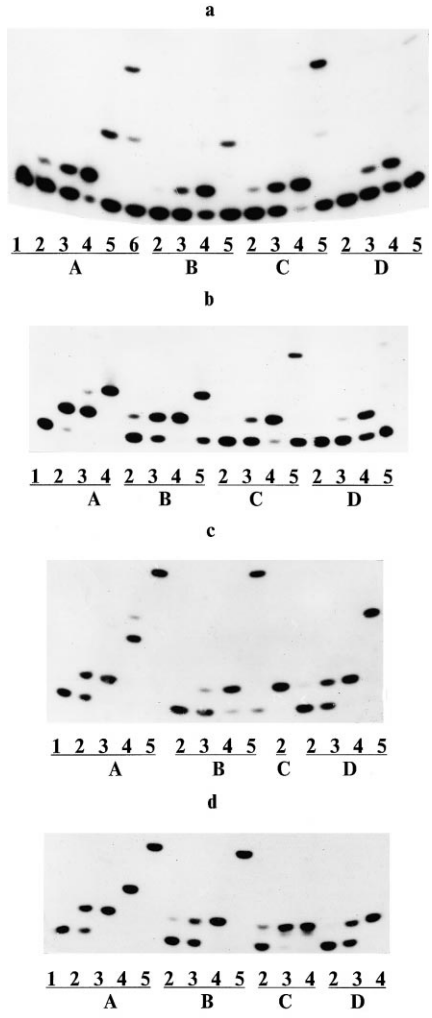

Fig. 1. Primer extension catalyzed by HIV RT. a: Complex C1. Series A: lane 1: primer+enzyme (control); 2–4: as in 1 +dTTP, 0.02 μM (2), 0.2 μM (3), and 2 μM (4); 5–6: as in 1 +0.2 μM dTTP+0.2 μM dGTP (5), +0.2 μM dTTP+0.2 μM dGTP+0.2 μM dATP (6). Series B: 2–4: as in 1 +0.02 μM (2), 0.2 μM (3), and 2 μM (4) **Ic**; 5: as in 1 +0.2 μM dGTP+0.2 μM **Ic**. Series C,D: 2–4: as in 1 +0.2 μM (2), 2 μM (3), and 20 μM (4) **Ia** and **II**, correspondingly; 5: as in 1 +0.2 μM dGTP+2 μM **Ia** and **II**, correspondingly. b: Complex C2. Series A: lane 1: primer+enzyme (control); 2–3: as in 1 +dATP, 0.02 μM (2), 0.2 μM (3); 4: as in 1 +0.2 μM dATP +0.2 μM dGTP. Series B–D: 2–4: as in 1 +0.02 μM (2), 0.2 μM (3), and 2 μM (4) **Ib**, **Ia** and **II**, correspondingly; 5: as in 1 +0.2 μM dGTP+0.2 μM **Ib**, **Ia** and **III**, correspondingly. c: Complex C1. Series A: lane 1: primer+enzyme (control); 2–3: as in 1 +dTTP, 0.2 μM (2), 2 μM (3); 4: as in 1 +2 μM dTTP+2 μM dGTP; 5: as in 1 +2 μM dTTP+2 μM dGTP+2 μM dATP. Series B and D: 2–4: as in 1 +0.2 μM (2), 2 μM (3), and 20 μM (4) **II** and **III**, correspondingly; 5: as in 1 +2 μM dGTP+20 μM **II** and **III**, correspondingly. Series C: 2: as in 1 +2 μM dTTP. d: Complex C2. Series A: lane 1: primer+enzyme (control); 2–3: as in 1 +dATP, 0.02 μM (2), 0.2 μM (3); 4: as in 1 +0.2 μM dATP+0.2 μM dGTP; 5: as in 1 +0.2 μM dATP+0.2 μM dGTP+0.2 μM dTTP. Series B and D: 2–4: as in 1 +0.2 μM (2), 2 μM (3), and 20 μM (4) **II** and **III**, correspondingly; 5: as in 1 +2 μM dGTP+20 μM **II** and **III**, correspondingly. Series C: as 1 +ddATP, 0.02 mM (2), 0.2 mM (3), and 2 mM (4).

pound **Ib** was also lower than that for dATP by two orders of magnitude. The activity for this enzyme is decreased in the order dTTP > **Ic** ≫ **Ia** = **II** (complex C1) and dATP ≫ **Ib** ≫ **II** (complex C2).

4. Discussion

As far as we know, only one group of natural substrates of DNA polymerases, dNTP, are known by now. Moreover, there is no question that dNTP are the only traditional substrates of polymerizing enzymes upon DNA replication, repair, and recombination as well as reverse transcription. One can assume, however, that in any unexpected situations (misincorporation, termination, and others) the problems that appear can be overcome by DNA polymerases via non-traditional pathways.

As mentioned above, it was recently found that, when the extension of newly synthesized DNA chains was stopped, some DNA polymerases can catalyze the pyrophosphorolysis reaction using instead of PPi natural or modified dNTP or dNDP as low molecular substrates. As a result, compounds of the **I** and **II** types and DNA chains shortened by one nucleotide residue were generated [2,3]. We shall not analyze the biological significance of this reaction, but regard it from the viewpoint of molecular mechanisms of reactions catalyzed by DNA polymerases. As follows from the main rule of enzymology, every enzymatic reaction is reversible. Therefore, one can imply that compounds of the **I** and **II** types can be substrates of the elongation reaction catalyzed by DNA polymerases. In this paper we report that dinucleoside polyphosphates are relevant substrates for DNA polymerases.

As it was found, HIV RT (as an example of viral reverse transcriptases), human DNA polymerase α (replicative DNA polymerase), human DNA polymerase β (repairing DNA polymerase), and bacterial DNA polymerases *Taq* and I from *E. coli* catalyze the reaction of DNA chain elongation when compounds **I** and **II** are used as substrates, the substrate activity of **I** and **II** being dependent both on their structure and on the properties of DNA polymerase. For HIV RT, the substrate activity of some **I** and **II** was close to that of the corresponding dNTP. In the case of other enzymes, this activity was either insignificantly or significantly lower. Compounds **I** were more active than **II**. The study of the dependence of the substrate activity versus the **I** and **II** structure needs additional experiments, which may provide interesting information on the structure and properties of DNA polymerase active centers.

Containing two nucleotide residues, compounds **I** and **II** must be able to extend DNA chains by any of them, which is defined by the template program. This was confirmed by our experiments: as seen in Figs. 1–3, both nucleotide residues can be incorporated into DNA chains, and this process is highly specific to the template nucleotide located in the active center.

One can propose two molecular mechanisms of the extension reaction. In accordance with one of them, compounds **I**

Table 2
The Michaelis constants (K_m) and reaction rates (V_{max}/V_{max} of dTTP or dATP) in one-step reaction catalyzed by HIV RT in the presence of complexes C2* or C1**

	K_m (μM)	V_{max} (%)	V_{max}/V_{max} for dATP	V_{max}/V_{max} for dTTP
Ib*	2.63 ± 0.13	4.54	0.06	
dATP*	0.052 ± 0.002	71.43	1	
Ic**	2.17 ± 0.22	4.00		0.96
dTTP**	1.25 ± 0.12	4.17		1

and **II** serve as donors of the nucleotide residues that are incorporated in the DNA chain (reaction 1). The second reaction mechanism implies the preliminary hydrolysis of **I** and **II** catalyzed by the active center of DNA polymerase, and dNTP formed serve as substrates (reaction 2).

Reaction 1: dNp_xpdN'+primer → primer-pdN+dN′TP (for **I**) or dN′DP (for **II**); or dNp_xpdN'+primer → primer-pdN′+dNTP (for **I**) or dNDP (for **II**).

Reaction 2: dNp_xpdN'+primer → dNTP+dN′MP (for **I**) or dN′ (for **II**), dNTP+primer → primer-pdN+PPi; or dNp_xpdN'+primer → dN′TP+dNMP (for **I**) or dN (for **II**), dN′TP+primer → primer-pdN′+PPi.

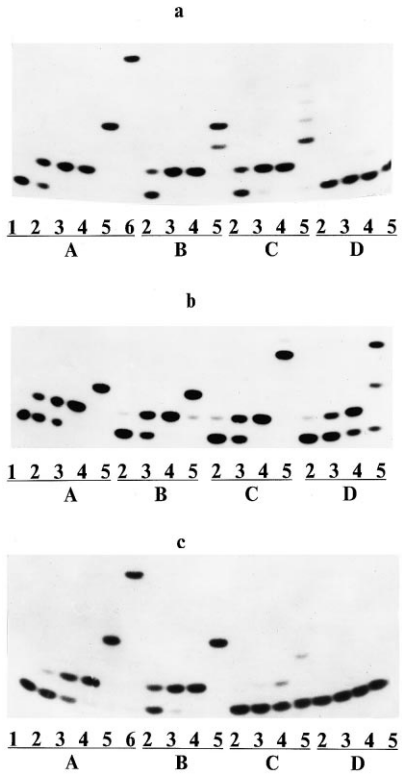

Fig. 2. a, b: Primer extension catalyzed by *E. coli* DNA polymerase I. a: Complex C1. Series A: lane 1: primer+enzyme (control); 2–4: as in 1 +dTTP, 0.02 μM (2), 0.2 μM (3), and 2 μM (4); 5–6: as in 1 +0.2 μM dTTP+0.2 μM dGTP (5) and +0.2 μM dTTP+0.2 μM dGTP+0.2 μM dATP (6). Series B: 2–4: as in 1 +0.02 μM, 0.2 μM, and 2 μM **Ic**, correspondingly; 5: as in 1 +0.2 μM **Ic**+0.2 mM dGTP. Series C and D: 2–4: as in 1 +0.2 μM, 2 μM, and 20 μM **Ia** and **II**, correspondingly; 5: as in 1 +0.2 μM dGTP+2 μM **Ia** and **II**, correspondingly. b: Complex C2. Series A: lane 1: primer+enzyme (control); 2–4: as in 1 +dATP, 0.005 μM (2), 0.01 μM (3), and 0.02 μM (4); 5: as in 1 +0.02 μM dATP+0.2 μM dGTP. Series B: 2–4: as in 1 +**Ib**, 0.02 μM (2), 0.2 μM (3), and 2 μM (4); 5: as in 1 +2 μM **Ib**+0.2 μM dGTP. Series C: 2–4: as in 1 +**Ia**, 0.2 μM (2), 2 μM (3), and 20 μM (4); 5: as in 1 +20 μM **Ia**+0.2 μM dGTP. Series D: 2–4: as in 1 + **II**, 20 μM (2), 100 μM (3) and 500 μM (4); 5: as in 1 +500 μM **II**+0.2 μM dGTP. c: Primer extension catalyzed by *Taq* DNA polymerase in the complex C1. Series A: lane 1: primer+enzyme (control); 2–4: as in 1 +dTTP, 0.2 μM (2), 2 μM (3), and 10 μM (4); 5–6: as in 1 +10 μM dTTP+2 μM dGTP (5), and +10 μM dTTP+0.2 μM dGTP+0.2 μM dATP (6), correspondingly. Series B and C: 2–4: as in 1 +2 μM (2), 20 μM (3), and 200 μM (4) **Ic** and **Ia**, correspondingly; 5: as in 1 +2 μM dGTP+200 μM **Ic** and **Ia**, correspondingly. Series D: 2–4: as in 1 +**II**, 20 μM (2), 200 μM (3), and 500 μM (4); 5: as in 1 +500 μM **II**+20 μM dGTP.

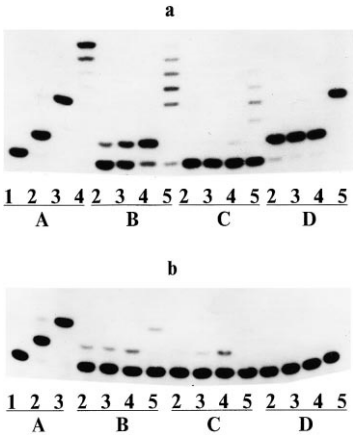

Fig. 3. Primer extension catalyzed by DNA polymerase α. a: Complex C1. Series A: lane 1: primer+enzyme (control); 2: as in 1 +2 μM dTTP; 3: as in 1 +2 μM dTTP+2 μM dGTP; 4: as in 1 +2 μM dTTP+2 μM dGTP+2 μM dATP. Series B–D: 2–4: as in 1 +2 μM (2), 10 μM (3), and 50 μM (4) **Ia**, **II**, and **Ic**, correspondingly; 5: as in 1 +2 μM dGTP+50 μM **Ia**, **II**, and **Ic**, correspondingly. b: Complex C2. Series A: lane 1: primer+enzyme (control); 2: as in 1 +2 μM dATP; 3: as in 1 +2 μM dATP+2 μM dGTP. Series B–D: 2–4: as in 1 +2 μM (2), 10 μM (3), and 50 μM (4) **Ib**, **Ia**, and **II**, correspondingly; 5: as in 1 +2 μM dGTP+50 μM **Ib**, **Ia**, and **II**, correspondingly.

To select one of the pathways, compound **III** was synthesized. Reaction 2 is impossible for this compound because of the stability to enzymatic hydrolysis of a P-C bond in the -P(O)(OH)-CH_2-O-CH_2- fragment. Since compound **III** extends DNA by both nucleotide residues (in accordance with the template program), we can conclude that the extension reaction proceeds by reaction 1.

We cannot yet explain the differences in the activity of various **I** and **II** toward DNA polymerases. It is probable that there are some steric hindrances upon binding in active center sites for some structural combinations of **I** and **II**, although other reasons cannot be excluded. Subsequent investigation is necessary, but the main conclusion can be drawn: the data obtained confirm the previous results that dNTP modified with bulky substituents at the γ-phosphate can form productive enzymatic complexes [9,11].

It is difficult to explain the contribution of the reaction under study to the biological functioning of DNA polymerases. One can propose that it may play an important role in replicative repair upon DNA synthesis, particularly for enzymes lacking the 3′-5′-exonuclease activity. The repairing pathway, which involves a cascade of reactions yielding compounds **I** followed by their participation in the DNA chain elongation, seems to be more productive than the reaction of repairing by PPi using pyrophosphorolysis. This hypothesis is supported by the rapid hydrolysis of PPi catalyzed by pyrophosphatase, whereas the stability of **I** in the replicative fork is significantly higher. Other reasons are also possible.

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