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Formation of phosphonester bonds catalyzed by DNA polymerase

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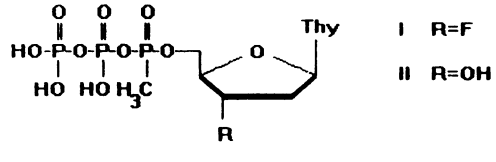
ABSTRACT

3′-Fluoro-2′,3′-dideoxythymidine 5′-(α-methylphosphonyl)-β,γ- diphosphate and 2′-deoxythymidine-5′-(α-methylphosphonyl)-β,γ- diphosphate have been synthesized. Both compounds are incorporated into DNA chains during catalysis by reverse transcriptases of human immunodeficiency (HIV) and avian myeloblastosis (AMV) viruses, DNA polymerase β from rat liver, terminal deoxynucleotidyl transferase from calf thymus and (at a very low rate) is by *E.coli* DNA polymerase I, Klenow fragment. The first compound is a termination substrate while the second is capable of multiple incorporation into the DNA chains. For instance, reverse transcriptase catalysis resulted in the appearance of 8 residues of second compound. DNA polymerases α and ε from human placenta incorporated none of the above compounds into DNA chains, although an inhibition of DNA synthesis by both compounds was observed with all enzymes mentioned. The 3′→5′-exonuclease activity of DNA polymerase I, Klenow fragment, hydrolyzed DNA fragments containing phosphonomethyl internucleoside groups, while such DNA fragments were resistant to the *E.coli* exonuclease III.

INTRODUCTION

Application of the DNA polymerase substrate analogues modified just at the chemical groups participating in the condensation reaction is a way of investigating of mechanisms of catalytic reactions during DNA synthesis involving DNA polymerases. Several DNA polymerases from different sources have been shown to catalyze the phosphorothioate bond formation [1–4], but do not catalyze the synthesis of phosphoroamide [5] and phosphorothioester bonds [6]. It has been shown recently that terminal deoxynucleotidyl transferase (TdT) from calf thymus catalyses also the formation of the methylphosphonoester bond, although with a low efficiency [7].

The study of molecular mechanism of nucleoside 5′-(α-methylphosphonyl)-β,γ-diphosphates action in the cell-free systems is reasonable because several 5′-phosphonates of sugar-modified nucleosides demonstrated their high inhibitory activity against HIV reproduction in cell cultures [8–11]. One of the possible mechanisms of action of 5′-phosphonates is the transformation of such nucleotides into the β,γ-diphosphate derivatives with following DNA chain elongation either by termination or competitive inhibition by the substrate, or both ways simultaneously. In this respect it was interesting to study properties of nucleoside 5′-(α-methylphosphonyl)- β,γ-diphosphates in reactions of DNA chain elongation catalyzed by different DNA polymerases. We have synthesized 3′-fluoro-2′,3′-dideoxythymidine 5′-(α-methylphosphonyl)-β,γ- diphosphate (I) and 2′-deoxythymidine 5′-(α-methylphosphonyl)- β,γ-diphosphate (II) and shown that the rat liver DNA polymerase β, calf thymus TdT, reverse transcriptases of human immunodeficiency virus 1 (HIV-1) and avian myeloblastosis virus (AMV) and to much less extent *E.coli* DNA polymerase I, Klenow Fragment (KFr) incorporate nucleoside 5′-methylphosphonate residues from I and II into the 3′-ends of the DNA chains. Moreover the appropriate residue from II is incorporated less readily than I into the DNA chains. At the same time DNA polymerases α and ε from human placenta did not incorporate nucleotide 5′-methylphosphonate residues into the DNA chains, although the synthesis is inhibited in the presence of I or II.



MATERIALS AND METHODS

3′-Fluoro-2′,3′-dideoxythymidine 5′-(α-methylphosphonyl)-β,γ- diphosphate (I) and 2′-deoxythymidine 5′-(α-methylphosphonyl)- β,γ-diphosphate (II)

Initial phosphonates have been synthesized according to the earlier described procedure [8,9]. 5′-methylphosphonate of 2′-deoxythymidine or 3′-fluoro-2′,3′-dideoxythymidine (0.2

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mmol) was dissolved in 5 ml of dry DMF and N,N'-carbonyldiimidazole (162 mg, 1 mmol) was added. The mixture was allowed to stay at the room temperature for 1 h followed by addition of 2 ml of 1 M bis-(tri-n-butylammonium) pyrophosphate (2 mmol) in DMF (2 ml). The mixture was stirred for 30 min and then diluted by water to the total volume 150 ml. The solution was loaded onto the column (22×3 cm) with Toyopearl DEAE (Toyosoda, Japan). The column was washed with water up to the absence of optical density in the eluate, and then the elution was carried out with the linear gradient of NH_4HCO_3, pH 7.5 (0→0.3 M, total volume 600 ml). Initial nucleoside 5'-methylphosphonates were eluted with 0.05 M buffer, while I or II were eluted with 0.1 M buffer. Fractions, containing I or II, were evaporated, reevaporated with water (5×10 ml) and repurified by the reversed phase chromatography of low pressure on the column (20×1 cm) with Lichroprep C-18 (Merck, Germany) in water and first peak was collected. I and II were obtained with yield about 30%. TLC, R_f0.51 (for I) and 0.48 (for II) in dioxan–water–25% NH_4OH 6:4:1 (v/v); HPLC, Nucleosil NH_2 column (Macherey Nagel, Germany) in a linear KH_2PO_4 gradient 0.05→1 M, 30 min, flow rate 1 ml/min, retention time for I is 19.5 min, for II—18.4 min. ^1H NMR spectra were recorded at 100 MHz using a Varian XL-100 spectrophotometer (USA) in D_2O and *tert.*—BuOH as internal standard. [^{31}P]NMR spectra were obtained with a Bruker AC 250(Germany) at the working frequency 101.26 MHz in D_2O (pH7.5) with triethylphosphate as an internal standard. The NMR data are given in Table 1.

dNTP (Sigma) were purified by HPLC on the column (150×4 mm) with Separon SCX C_{18} and then in a similar column with Tessek (CzSFR, Denmark) in acetonitrile linear gradient (0→15%) in 50 mM triethylammonium bicarbonate, pH 7.5, flow rate 1 ml/min.

Experiments with enzymes

M13mp10 phage DNA was isolated from the culture fluid of a recipient K12×L1 *E.coli* strain according to [12]. The primer tetradecadeoxynucleotide (see Scheme) was labelled at the 5'-position by T4 polynucleotide kinase (Amersham, England) as in [13] using [γ-^{32}P]ATP with a specific activity of 1500 Ci/mmol (Radioisotope, USSR). After phenol extraction and reprecipitation with ethanol the [^{32}P]-tetradecanucleotide was dissolved in water up to the concentration of 1 nmol/ml and used either directly in the reaction with TdT (Amersham) according to [14], or after primer-template complex purification on BioGel A-1.5M in reactions catalyzed by the KFr (Amersham), β from rat liver[15], α and ϵ from human placenta (paper in preparation), reverse transcriptases from AMV (Omutninsk Chemical Plant, USSR) and HIV-1 (the kind gift of Dr.S.Wilson), [α-^{32}P]dATP and [α-^{32}P]dCTP with the specific activity 2000 Ci/mmol (Radioisotope, USSR) have been used throughout this work.

The TdT catalyzed elongation of the [5'-^{32}P]oligonucleotide

A mixture containing in total volume 5 μl 100 mM Na-cacodylate, pH 7.2, 10 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM dithiotreitol, 50 μM dTTP or its analogues, 0.6 μM [5'-^{32}P]d(CCCAGTCACG-ACGT) and 3 activity units of the enzyme, were incubated for 40 min at 37°C. The reaction was stopped by addition of 2 μl of deionized formamide containing 0.1% of xylenecyanol and bromophenol blue and 20 mM EDTA, pH 8.0. The reaction products were analyzed by electrophoresis in denaturating 20% PAAG.

The template dependent elongation of [5'-^{32}P]-oligonucleotide catalyzed by DNA polymerases

The M13mp10 DNA (0.5 μM) was hybridized with 0.75 μM [5'-^{32}P]primer in the following buffers: 10 mM Tris-HCl, pH 7.9, 5 mM $MgCl_2$, 1 mM dithiotreitol for KFr; 10 mM Tris-HCl, pH 8.2, 5 mM $MgCl_2$, 40 mM KCl, 1 mM dithiotreitol (for reverse transcriptases); 10 mM Tris-HCl, pH 7.4, 6 mM $MgCl_2$, 0.4 mM dithiotreitol (for DNA polymerases α and ϵ) and the same as in the last case, but with pH 8.5 for DNA polymerase β. Incorporation of one template-complementary nucleotide residue to the [5'-^{32}P]primer's 3'-terminus was carried out in 5 μl of a mixture containing the appropriate buffer solution, enzyme (0.5 activity unit of KFr, 1 activity unit of DNA polymerases α or ϵ; 0.5 activity unit of DNA polymerase β; 4 activity units of each reverse transcriptase), 0.01 μM the primer-template complex, 50 μM dTTP or 100 μM II (for incorporation of three residues 4 μM dGTP was added to the reaction mixture). After incubation for 20 min at 37°C (10 min at 20°C for KFr) the reaction was stopped by adding of 2 μl of formamide containing dyes and EDTA and the reaction products were separated by electrophoresis in the denaturating 20% PAAG.

The [5'-^{32}P]-oligonucleotide hydrolysis by the KFr and *E.coli* exonuclease ExoIII

The pentadeca- and heptadecanucleotides obtained by incorporation of residues from the natural dNTP, I or II to 3'-end of the [5'-^{32}P]-primer and the starting primer were eluted from the preparative gel with 0.5 ml of water [12], desalted on Sephadex G-10, precipitated with ethanol and dissolved in 10 μl of water. Aliquots (5 μM) of oligonucleotide solutions were hybridized with the M13mp10 template and hybrids were hydrolyzed in the presence of 6 activity units of ExoIII (Amersham) in 50 mM Tris-HCl, pH 7.9, 10 mM $MgCl_2$, 50 mM KCl, 1 mM dithiotreitol during 1 hour at 37°C [16]. In both

Table 1. The NMR parameters for I and II

δ^1H, ppm	δ^{31}P,ppm.*			$^2J_{pp}$, Hz	
($^2J_{CH_3-P}$, Hz)	α-P	β-P	γ-P	$^2J_{\alpha-\beta}$	$^2J_{\beta-\gamma}$
I: 1.79d (18)	25.55d; 25.34d	−(22.76− −23.21)m	−7.57d; −7.61d	21.87; 22.03	20.66 20.85
II:1.69d (19)	25.18d; 25.08d	−(23.55− −23.99)m	−9.72d; −9.77d	21.31; 22.32	19.76; 19.25

* The chemical shift values were obtained by extrapolation relative to 85% H_3PO_4.

cases hydrolysis products were analyzed by electrophoresis in the denaturing 20% PAAG.

Inhibition of DNA synthesis catalyzed by DNA polymerases I and II

Reaction mixtures (6 μM) contained DNA polymerases in appropriate buffers, 0.02 μM primer-template complex, 20 μM dATP and dGTP, 10 μM dCTP (1−2 μCi [α-^{32}P]dCTP), 1 μM dTTP and I or II in different concentrations. The reactions proceeded for 15 min at 20°C (KFr, 0.5 activity unit); 15 min at 40°C (reverse transcriptases, 4 activity units, and DNA polymerase β, 0.5 activity unit) and 1 hour at 37°C (DNA polymerases α and ε, 1 activity unit) and were stopped by EDTA addition till 50 mM. The aliquots were placed on 0.5×1 cm strips of Whatman DE-81, the strips were washed with 0.2 M NaCl containing 0.5 mM EDTA, pH 8.0, fixed with ethanol, and the radiolabelled substrate incorporation into the DNA chain was measured by liquid scintillation counting.

The ability of I to terminate DNA synthesis catalyzed by reverse transcriptases was tested under standard conditions [12]. Mixtures (6 μl) containing 0.02 μM primer-template complex, 2−4 activity units of each enzyme, substrates and different amounts of the compound under investigation, were incubated for 20 min at 37°C, 1 μl of a solution containing 0.05 mM mixture of four dNTP was added and 10 min later the reaction was stopped by addition of 3 μl of formamide with dyes and EDTA. Controls contained 0.3 μM ddATP, 0.8 μM ddGTP, 0.8 μM ddCTP and 1.7 μM ddTTP, respectively. The termination pattern was analyzed after electrophoresis in 8% denaturing PAAG.

Incorporation of II into the (−)DNA chain, catalyzed by reverse transcriptases, was studied under similar conditions.

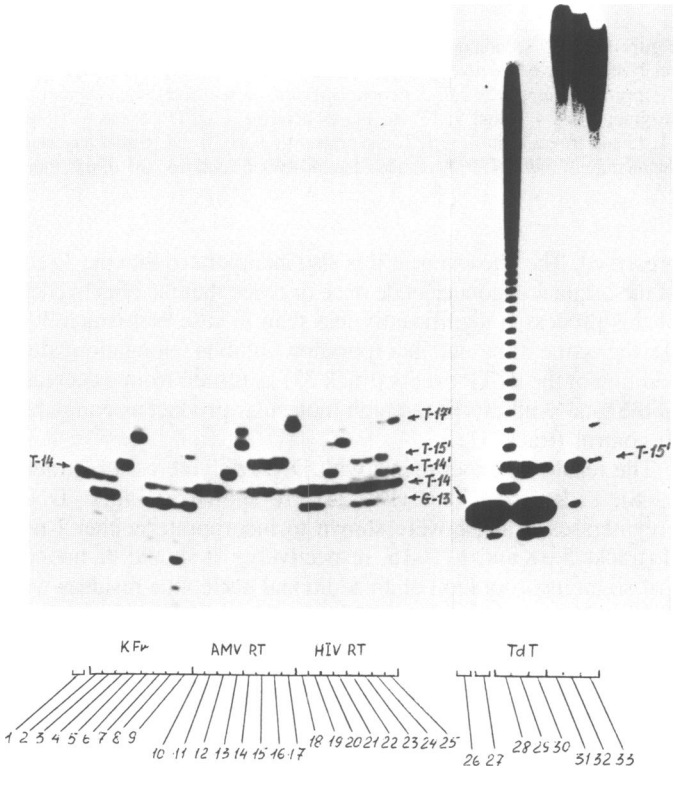


Figure 1. Radioautograph of the PAGE separation in 20% gel of products of [5′-^{32}P]primer elongation in the presence of dTTP or compounds I or II catalyzed by KFr (2−9), reverse transcriptases of AMV (10−17) and HIV (18−25) and TdT (26−33): 1-control (primer+template); 2,10,18−(primer + template) + an appropriate enzyme; 3,11,19−the same + dGTP; 4,12,20−(primer + template) + an enzyme + dTTP; 5,13,21−the same + dGTP; 6,14,22−(primer + template) + an enzyme + I; 7,15,23−the same + dGTP; 8,16,24 (primer + template) + an enzyme + II; 9,17,25−the same + dGTP; 26−control, [5′-^{32}P]tetradecanucleotide; 27−tetradecanucleotide + an enzyme; 28,31−the same as 27 + dTTP; 29,32−the same as 27 + I; 30,33−the same, as 27 + II; probes 26−33 were incubated for 40 min at 37°C then to reactions 31−33, 300 μM dTTP were added and incubation continued for 40 min more.

RESULTS

Synthesis of I and II was carried out by the method described earlier for II [7], with some modifications. I and II were characterised by a combination of methods including ^{1}H-NMR and ^{31}P-NMR (Table I). All signals of protons of I and II in the ^{1}H-NMR spectra were similar to those of starting nucleoside 5′-methylphosphonates. Only a shift of the CH_3-group signal at phosphorus by approximately 0.4 ppm down field compared with the starting nucleoside 5′-methylphosphonate was observed. I and II were isolated as a mixture of R_p and S_p isomers, as is seen from the ^{31}P-NMR spectra (two signals for each phosphorus) in equal ratio of isomers in the mixture.

Incorporation of I and II into the DNA chains by DNA polymerases was monitored by PAGE analysis of products. The results of KFr (tracks 2−9), AMV (10−17) and HIV (18−25) reverse transcriptases and TdT (26−33) are illustrated in Fig.1. The dTTP addition to the complex [enzyme + template + tetradecanucleotide primer] results in elongation of the primer by one nucleotide residue (tracks 4, 12 and 20) according to the template context (see Scheme). Inclusion of two substrates dTTP and dGTP leads to the primer elongation by three nucleotide residues resulting in the heptadecanucleotide formation (tracks 5, 13, 21). Additionally, there is an octadecanucleotide band,

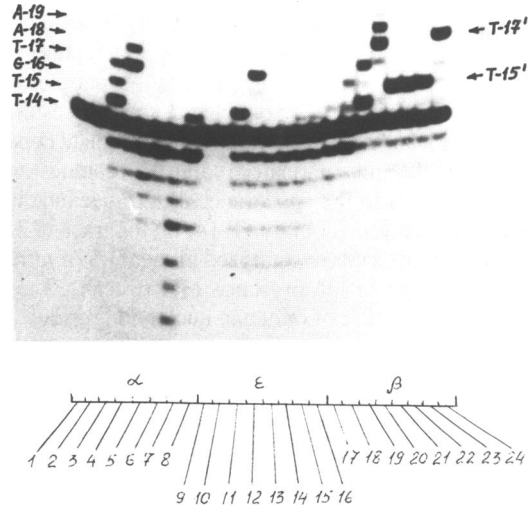
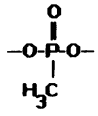


Figure 2. Radioautograph of the PAGE separation in 20% gel of products of [5′-^{32}P]primer elongation catalyzed by DNA polymerases α (1−8), ε (9−16) and β (17−24): 1,9,17−controls without dNTP ([primer + template] + an appropriate enzyme); 2, 10,18−the same + dGTP; 3,11,19−[primer + template] + an enzyme + dTTP; 4,12,20−the same + dGTP; 5,13,21−[primer + template) + an enzyme + I; 6,14,22−the same + dGTP; 7,15,23−[primer + template] + an enzyme + II; 8,16,24−the same + dGTP.

probably, due to incorporation of an additional dGMP residue with formation of an incorrect T:G pair. This has been noticed many times before in the systems with an reduced set of substrates, such as in [17, 18]. The AMV reverse transcriptase incorporates an additional dGMP nucleotide even more effectively than KFr (track 13). Participation of the HIV reverse transcriptase results in formation of several non-canonical pairs: in the presence of only dTTP (track 20) a heptadecanucleotide band is produced after incorporation of three dTMP residues to the primer with formation of two A:T pairs and the incorrect C:T pair. Addition of two substrates—dTTP and dGTP—leads to the primer elongation and octadeca- and nonadecanucleotides are formed (track 21). A decrease of dTTP and dGTP concentration reduces misincorporation (data not given). KFr incorporates phosphonates I and II into the DNA chain in a very low extent as can be seen from comparison of tracks 6–9 (Fig.1). In the presence of the phosphonate II (track 8) an increased 3′→5′ exonuclease hydrolysis is observed but the reasons are not clear. In track 9, this hydrolysis is limited by the tridecanucleotide formation evidently because dGTP presence in the reaction mixture stops hydrolysis on the dGMP residue located in that position. Pentadeca- and hexadecanucleotides are formed also. The AMV reverse transcriptase can effectively elongate the primer only by one nucleotide residue in the presence of I (track 14) and the addition of dGTP doesn't change the chain length (track 15). So, an uncharged methylphosphonate group is formed between tetradeca- and pentadecanucleoside residues:



Formation of an uncharged group results in a decreased electrophoretic mobility of the product (the pentadecanucleotide band on tracks 14 and 15 is located behind the corresponding band in track 12).

Phosphonate II has been also incorporated into the 3′-end of a chain but in contrast to I, phosphonate II does not terminate DNA synthesis and the nucleotide residues of dGTP and one more residue of II and again dGTP become incorporated successively into the chain (track 17). The HIV reverse transcriptase is slightly contaminated with the 3′→5′ exonuclease activity resulting in formation of significant tridecanucleotide. The template dependent incorporation of the phosphonate I and II residues to this tridecanucleotide leads to the appearance of tetradecanucleotides with lower electrophoretic mobility (T-14′ on the tracks 22–25). The HIV reverse transcriptase catalyzed elongation of tetradeca- and tridecanucleotides in the presence of I (tracks 22 and 23) is characterized by addition of only one nucleotide residue (bands T-14′ and T-15′, respectively) and further synthesis is stopped. The situation is similar for II (track 24, bands T-14′ and T-15′) but after addition of dGTP the chain is elongated by two more residues (track 25, band T-17′) indicating that incorporation of the nucleoside phosphonate residue of II into a chain doesn't prevent further elongation similar to the AMV reverse transcriptase.

Compound I also elongates a tetradecanucleotide by one residue (T-15′ in the track 29) and terminates further DNA synthesis during TdT catalysis (track 32) because after addition of excess dTTP tetradecanucleotide remaining in the reaction mixture undergoes transformation into a polymeric product, but pentadecanucleotide containing fluorinated sugar residue is

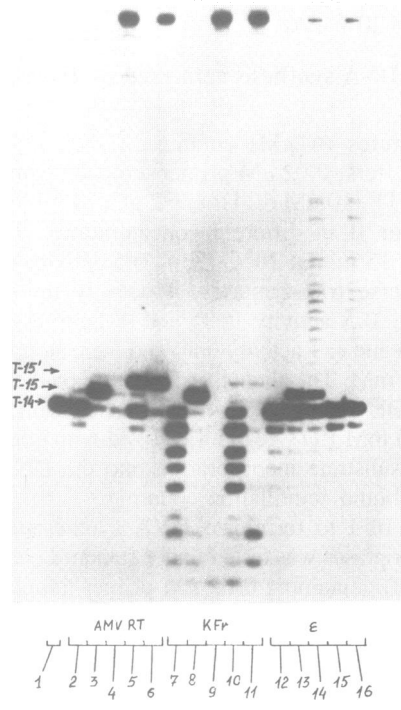


Figure 3. PAGE separation in 20% gel of products of [5′-^{32}P] primer elongation catalyzed by the AMV reverse transcriptase (2–6), KFr (7–11) and ε (12–16): 1–(primer + template); 2,7,12–controls (primer + template) + an appropriate enzyme; 3 and 4, 8 and 9, 13 and 14–as controls + dTTP; 5 and 6, 10 and 11, 15 and 16–as controls + I. To the probes 4,6,9,11,14 and 16 after incubation the mixture of four dNTPs was added and incubation continued for 10 min more.

preserved. The phosphonate II is also incorporated into the 3′-end of the original oligonucleotide once or twice, but the effectiveness of this process is significantly less than in case of I (track 30). At the same time its incorporation inhibits elongation after addition of the dTTP excess (track 33) as is seen from a decrease of the total synthesis of the high molecular product as compared to control (track 31).

The results of experiments with DNA polymerases α (tracks 1–8), ε (9–16) and β (17–24) are shown in Fig.2. DNA polymerases α and ε were shown to incorporate neither I nor II (tracks 5–8 and 13 –16, respectively). It should be noticed that slight incorporation of an additional nucleotide residues was observed in case of α enzyme (band G-16 of the track 3 and A-18 of track 4) while incorporation was completely absent in case of ε enzyme (tracks 11 and 12). This data corresponds to high specificity of these enzymes. In case of DNA polymerase β incorporation of additional residues was more pronounced (track 20, bands A-18 and A-19). DNA polymerase β elongated the primer in the presence of I and II (tracks 21–24) with the same efficiency as in case of the natural dTTP (track 19). After incorporation of the nucleoside phosphonate residue of II the T-15′ pentadecanucleotide undergoes effective further elongation (track 24, bands G-16 and T-17′).

The experiment presented in Fig.3 gives an additional confirmation of different ability of various enzymes to incorporate the phosphonate residue of I into the DNA chain. KFr (tracks 7–11), AMV reverse transcriptase (tracks 2–6) or DNA polymerase ε (tracks 12–16) were added to the template-primer complex. Addition of dTTP to the controls resulted in T-15

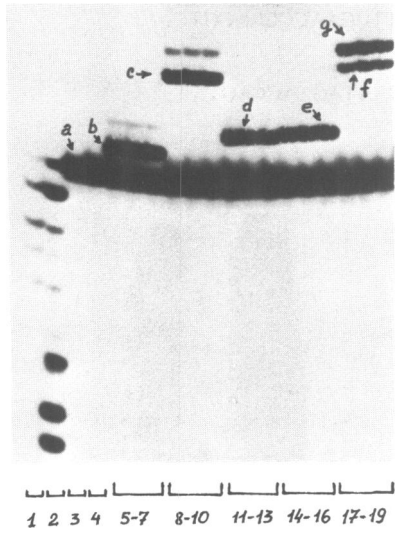


Figure 4. Preparative separation in 20% PAAG of [5′-^{32}P] oligonucleotides obtained after the [5′-^{32}P]primer elongation catalyzed by the AMV reverse transcriptase in the presence of 100μM dTTP (5–7), 100 μM dTTP + 40 μM dGTP (8–10), 1 mM I (11–13), 1 mM II (14–16), 1 mM II + 40 μM dGTP (17–19). 3–the enzyme free control; 4–the same with the enzyme; 1 and 2–as 3 + 0.5 and 0.2 units of KFr, respectively; a–tetradecanucleotide; b–pentadecanucleotide; c–octadecanucleotide; d and e–pentadecanucleotides with the residues from I and II at the 3′-end; f–heptadecanucleotide, containing 2 residues of II at the 1st and 3rd positions from the 3′-end; g–an octadecanucleotide formed by the dGMP residue biding to f.

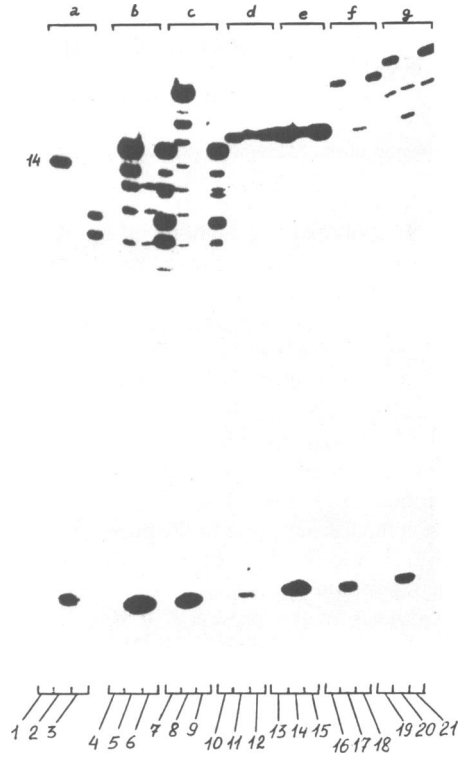


Figure 5. The radioautograph of PAGE separation (20% gel) by hydrolysis products of (5′-^{32}P)oligonucleotides marked in Fig.4 as follows: a–(tracks 1–3), b–(4–6), c–(7–9), d–(10–12), e -(13–15), f–(16–18), d–(19–21). Tracks 1,4,7, 10,13,16,19–control portions; tracks 2,5,8,11,14,17,20–samples after hydrolysis by KFr; tracks 3,6,9,12,15,18 and 21–samples hydrolyzed by ExoIII.

pentadecanucleotide formation (tracks 3, 8 and 13). When the reaction was over an excess of four dNTPs was added to the same probes and incubation continued. In this case the high molecular weight DNA was synthesized and the T-15 band disappeared (tracks 4 and 9) or became less intense (for ϵ, track 14). Experiments in which phosphonate I was added instead of dTTP were treated in a similar way. The AMV reverse transcriptase incorporated the nucleotide residue of I in the primer and led to the T-15′ pentadecanucleotide formation (track 5). This pentadecanucleotide is not elongated after addition of dNTP excess due to 3′-F presence at the chain terminal deoxyribose (T-15′, track 6). DNA polymerase ϵ did not form a pentadecanucleotide in the presence of I (there is no band T-15′ in tracks 15 and 16). KFr added a very small amount of I to the primer, and the terminated pentadecanucleotide T-15′, being formed in this case, still exists in the track 11 after addition of the dNTP excess (tracks 10 and 11).

Fig.4 shows preparative separation of products after reaction with I and II in the presence of the AMV reverse transcriptase. Oligonucleotides contained in the bands marked with letters were eluted from the gel and, after desalting, aliquots were hydrolyzed by 3′→5′ exonuclease activity of the KFr while others by the *E.coli* ExoIII (after repeated template hybridization).

Fig.5 shows the results of such hydrolysis. Pentadecanucleotide with the 3′-end residue of I (from band d, Fig.4) is shown to be only slightly hydrolyzed by KFr (track 11) and is not hydrolyzed by ExoIII under these conditions (track 12). Control tracks 2 and 3, 5 and 6, 8 and 9 (from bands a, b, c, respectively), synthesized with the participation of natural dTTP or a combination of dTTP and dGTP indicate that both enzymes hydrolyze 14-, 15- and 18-membered oligonucleotides. The situation is more complicated with oligonucleotides (from bands e, f and g, respectively) synthesized in the presence of II, in which the last or penultimate nucleotide from the 3′-end is joined to the chain by phosphonoester bond. In all cases KFr hydrolyzed such oligonucleotides but the rate limiting step is hydrolysis of a modified link. So, the 15-membered oligonucleotide bands in tracks 14 and 17 as well as 18-, 17-, 16- and 15-membered oligonucleotide bands in track 20 are visible and then there is a high rate hydrolysis to mononucleotides. ExoIII does not hydrolyze modified oligonucleotides at all, and it hydrolyzes at a very low rate the dGTP residue incorrectly incorporated into the octadecanucleotide chain after the nucleotide residue of II (track 21). A slight additional band is visible on this track at the level of a heptadecanucleotide, compared with the control track 19. The oligonucleotide with the 3′-end altered by the inclusion of nucleotide residue from II reacts slowly with the enzyme.

Table 2. The ratios of I and II concentrations to that of the dTTP substrate at which the DNA synthesis catalyzed by different DNA polymerases is inhibited by 50% (mol/mol)

DNA polymerases	[I]/TTP	[II]/TTP
KFr	>>1600	500
α	200	95
β	300	30
ϵ	130	40
HIV	40	10
AMV	100	10

Table 2 shows ratios of concentrations of I and II compounds to that of dTTP substrate, at which the DNA synthesis catalyzed

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                10          20          30          40          50
3'-GGGTCAGTGCTGCAACATTTTGCTGCCGGTCACGGTTCGAACCCGACGTC-
5'-CCCAGTCACGACGT
                60          70          80          90          100
-CAGCTGAGATCTCCTAGGGGCCCGCTCGAGCTTAAGCAATAGTACCAGTA
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Scheme. 3'-end region of the M13mp10 DNA with the 14-membered primer

by different DNA polymerases is inhibited by 50% (mol/mol). These ratios were obtained from the curves of inhibition of the (−)DNA chain synthesis catalyzed by different enzymes by these compounds. While HIV reverse transcriptase appeared to be most sensitive to I, both reverse transcriptases were equally inhibited by II; this inhibition was three times more effective than that of DNA polymerase β and 4−10 times more effective than inhibition of DNA polymerase α and ϵ.

The aim of the last series of experiments was to study the effect of I and II compounds on the DNA chain elongation catalyzed by the AMV reverse transcriptase in the presence of four dNTPs or dATP, dGTP and dCTP. Fig.6 shows the pattern of electrophoretic separation of reaction products obtained in several variants by synthesis in the presence of four dNTPs and the appropriate nucleoside phosphonates (for II—tracks 5 and 6, for I—tracks 13−17) and dATP, dGTP and dCTP with addition of II (tracks 2−4). Tracks 9−12 show DNA synthesis termination patterns in the presence of four ddNTPs. From tracks 13−17 it is seen that I is incorporated into DNA like ddTTP; beginning with a concentration of 250 μM (track 15), it gives distinctive termination bands. The band positions on tracks 15−17 are different from those on track 12 due to a charge absence at the phosphonodiester group. An increase of I concentration results in even stronger synthesis inhibition (tracks 15−16). But the efficiency of I is significantly lower than that of ddTTP (250 μM against 1.7 μM, compare tracks 15 and 12). I was shown to demonstrate its termination abilities at rather high concentrations.

A different situation is observed with the methylphosphonate II. Tracks 5−6 in Fig.6 show that addition of II to four dNTPs only increases the overall synthesis inhibition as the II concentration increases. But if three natural substrates only (dATP, dGTP and dCTP) were included in the reaction and II was substituted for dTTP (tracks 2−4), the primer elongation approximately by 50 nucleoside residues (tracks 2 and 3) was observed and the product length increased When the higher concentrations of II obtained. At the concentration of 100 uM II is incorporated into the DNA chain 7−8 times. Identification of the bands on the track 2 is rather difficult because incorporation of each nucleoside phosphonate residue from II into a chain changes the mobility of oligonucleotide formed in comparison with the control one.

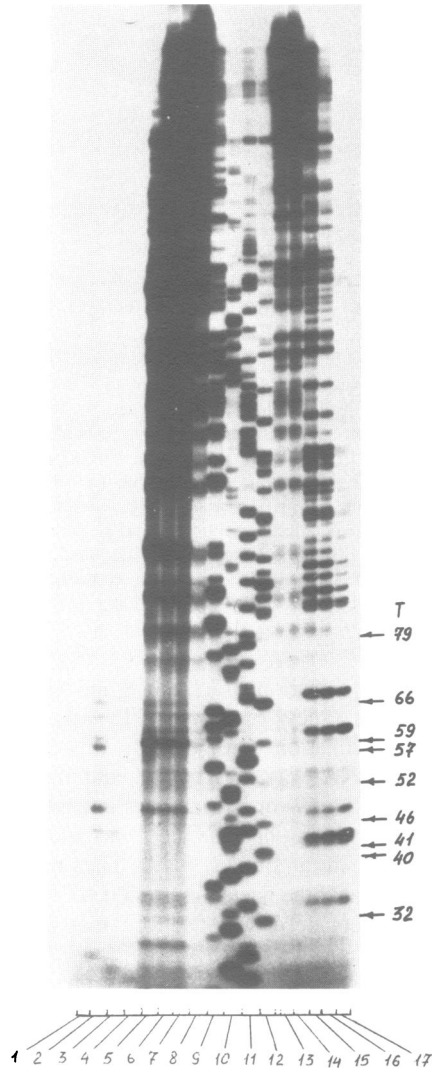

Figure 6. The radioautograph of PAGE separation of products of primer elongation by the HIV reverse transcriptase (2 units, 37°C, 20 min). Probes 1−7 without additional incubation with an excess of four dNTP; 8−17−with such incubation (10 min, 37°C). Track 1−control without one substrate (dTTP or II); tracks 2−4: synthesis in the presence of three dNTPs (20 μM of each dGTP, dCTP and 10 μM of [α-^{32}P]dATP) and compounds II, 500 μM (2), 100 μM (3) and 20 μM (4). Tracks 5 and 6−synthesis in the presence of four dNTPs (20 μM of each dGTP, dCTP, dTTP and 10 μM of [α-^{32}P]dATP) but with the addition of 100 and 20 μM of II, respectively. Tracks 7−the complete system with four dNTPs (control without additional incubation); 8−the same with additional incubation; 9−as 8 + 0.3 μM ddATP; 10−as 8 + 0.8 μM ddGTP; 11−as 8 + 0.8 μM ddCTP; 12−as 8 + 1.7 μM ddTTP; 13−17−as 8 but with addition of I: 20 μM (13), 100 μM (14), 250 μM (15), 500 μM (16) and 1000 μM (17).

DISCUSSION

Enzyme TdT from the calf thymus was reported previously to be able to incorporate one-two nucleotide residues with the methylphosphonate group [−OP(=O)(−CH_3)O−] into the deoxyoligonucleotide chain [7]. In the present paper following results have been obtained:

1. Several DNA polymerases are able to incorporate the residue of 3'-fluoro-2',3'-dideoxythymidine 5'-methylphosphonate from compound I into the 3'-end of the DNA chain. These are the HIV and AMV reverse transcriptases, DNA polymerase β, enzymes TdT and KFr. But DNA polymerases α and ϵ don't incorporate this residue.

2. The same specificity of DNA polymerases has been found relative to the compound II. Polymerase TdT incorporated it into the chain only one or two times, other DNA polymerases more than once. For instance, the AMV reverse transcriptase is able to incorporate this compound 7−8 times thus forming a DNA

fragment containing seven-eight internucleoside methylphosphonate groups bearing no negative charges. DNA polymerases α and ϵ do not incorporate the methylphosphonate nucleoside residue of II into the DNA chain.

3. The 3′→5′ exonuclease activity of DNA polymerase I KFr has hydrolyzed DNA fragments containing one or two internucleoside methylphosphonate groups while ExoIII did not hydrolyze such oligonucleotides. This correlates with the activities of these enzymes relative to phosphorothioate groups.

3′-Exonuclease from the snake venom is reported [7] not to hydrolyze an oligonucleotide having the 3′-methylphosphonate internucleoside bond.

4. Both dTTP analogues (I and II) turned out to be effective inhibitors of DNA synthesis catalyzed by the HIV and AMV reverse transcriptases as well as by other DNA polymerases (Table 2). Compound I appeared to be more effective inhibitor than II, evidently because of its higher affinity to enzymes. It should be noticed that in this case reverse transcriptases were less specific for substrate analogues than other DNA polymerases. This corresponds well to the earlier found regularity in the series of DNA polymerases [18−20].

5. The ability of reverse transcriptases to incorporate nucleoside 5′-triphosphate residues modified at the α-phosphate into the 3′-end of a DNA chain shed the light on possible mechanism of the modified nucleoside 5′-phosphonates action as inhibitors of HIV production in the cell cultures [8−11].

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