Volume 5 Number 4 April 1978 Nucleic Acids Research

The reactivity of phosphomono-and phosphodiester groups in oligonucleotides

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Received 19 December 1977

ABSTRACT

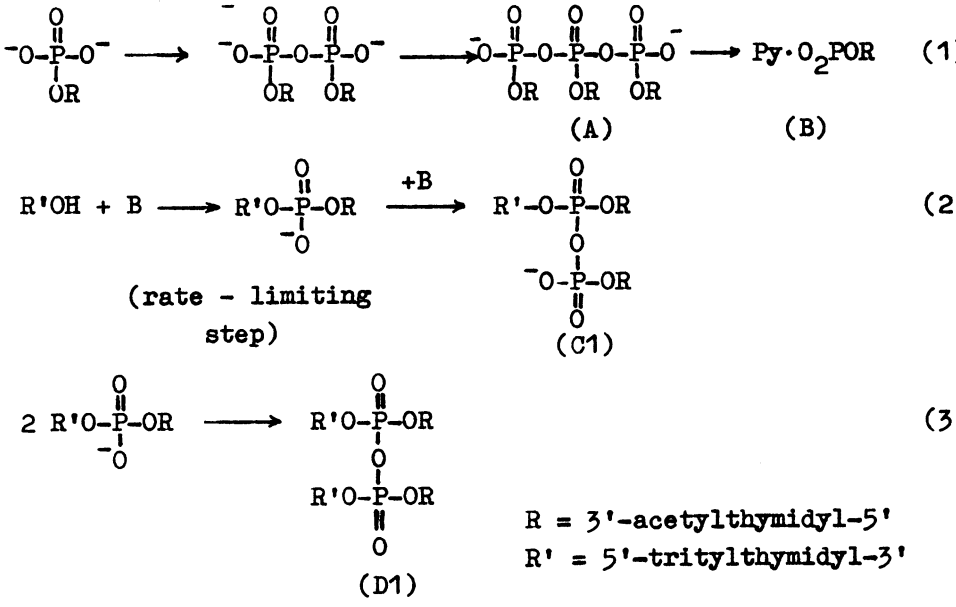
The rate constants were estimated by phosphorus NMR spectroscopy for the reactions of alcohols (Tr-dT, 2-cyanoethanol) in pyridine with the main types of the reactive phosphorylating intermediates formed by treatment of pdT-Ac, pdTpdT-Ac, Tr-dTpdT-Ac, Tr-dTpdTpdT-Ac with 2,4,6-triisopropylbenzenesulfonyl chloride (TPS): 1) B type derivatives with phosphomono ester (PME) group converted to a phosphoryl pyridinium residue; 2) C type derivatives with PME and phosphodiester (PDE) groups converted to trisubstituted pyrophosphate; 3) D type derivatives with PDE groups converted to tetrasubstituted pyrophosphate. The two latter types are partially present as cyclic intramolecular pyrophosphates Ci and Di. The reactivity of the intermediates decreases in the series $B \gg Ci \approx Di > C \approx D$. The Ci derivative of pdTpdT-Ac when obtained in dimethylformamide was found to be rather stable to hydrolysis and could be separated from the other dinucleotide derivatives by ion-exchange chromatography. The Arrhenius parameters of all steps of the conversion of PME group of pdT-Ac to B derivative and of the reaction of TPS with PDE group of dinucleoside phosphate Tr-dTpdT-Ac were measured.

INTRODUCTION

The reaction of phosphoester groups of mono- and oligonucleotides with nucleophiles in the presence of condensing reagents is the most essential step in the formation of new internucleotide bonds and in the protection of phosphate groups in the oligonucleotide synthesis.

In the last several years the use of the pulsed ^{31}P NMR spectroscopy permitted to follow the accumulation and subsequent transformations of the reactive phosphorylating derivatives of mono- and oligonucleotides forming by treatment with condensing reagents most commonly used in the oligonucleotide synthesis, namely triisopropylbenzenesulfonyl chloride (TPS) and dicyclohexylcarbodiimide (DCC) [1].

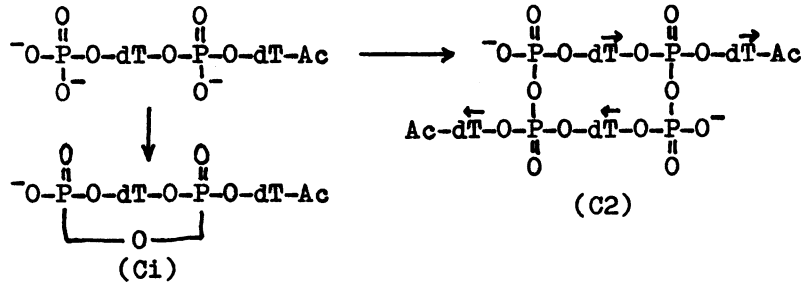
Three main types of intermediates were found: 1) B type derivatives with phosphomonoester group converted to highly reactive form with ^{31}P NMR and chemical properties expected either for nucleoside-5'-metaphosphate postulated by Todd [2], or phosphoryl pyridinium derivative postulated by Michelson [3]. These derivatives are formed only in pyridine solution thus indicating some participation of pyridine in stabilisation of the derivatives and , therefore, the latter were designed as $Py \cdot O_2POR$ (R - nucleoside) [1]; 2) C type derivatives with phosphomonoester and phosphodiester residues combined to trisubstituted pyrophosphate; 3) D type derivatives with two phosphodiester groups combined to tetrasubstituted pyrophosphate. The pathways established with the most simple representatives of mononucleotides and dinucleoside phosphates may be represented by the scheme [4-6].



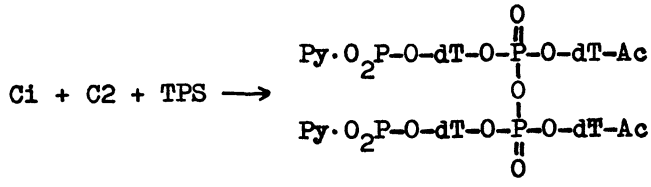
Scheme I

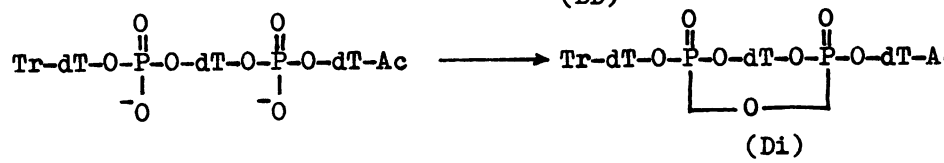
Similar types of derivatives were found to be formed in the reaction of dinucleotide pdTpdT-Ac [7,8] and trinucleoside diphosphate Tr-dTpdTpdT-Ac [9]. The difference is that the C type derivative of the former and the D type derivative of the latter oligonucleotide may be formed intramolecular thus lea-

ding to eight-member pyrophosphate cycles. These intermediates will be further designed as respectevily Ci and Di. In pyridine solution the C type derivatives of pdTpdT-Ac with TPS may be converted to highly reactive BD type derivative with phosphomonoester groups converted to the B form and phosphodiester groups combined to the D form.The main results may be represented by the scheme



(arrows in C2 indicate the orientation of the thymidine residues 5'→3')





(this compound is formed in parallel with intermolecular tetrasubstituted pyrophosphates)

Scheme II

It is obvious that the behaviour of oligonucleotides in the reactions with nucleophilic compounds in the presence of condensing reagents should depend both on the reactivity of phosphomonoester and phosphodiester groups towards condensing reagents and on the reactivity of the intermediate phosphorylating reagents towards nucleophiles. The main aim of the present work is to give quantitative estimates of these reactivities. The

former is characterised by the Arrhenius parameters of the reactions of TPS with the most simple representatives of mononucleotides and dinucleoside phosphates namely pdT-Ac and Tr-dTpdT-Ac. The reactivity of all main types of the intermediate phosphorylating derivatives is estimated using Tr-dT and 2-cyanoethanol as nucleophilic components.

MATERIALS AND METHODS

2,4,6-triisopropylbenzenesulfonyl chloride [10] , N,N-dicyclohexylcarbodiimide [11], prepared by commonly used methods, pdT-Ac produced by pilot plant of the Institute of Organic Chemistry (Novosibirsk, USSR) were used. Tr-dT, Tr-dTpdT-Ac, were prepared according to [12], O(pdT-Ac)$_2$ according to [13], Tr-dTpdTpdT-Ac according to [14], pdTpdT-Ac according to [15]. Pyridine with water content less than 0.05% stored on molecular sieves 4A was used as a solvent. Nucleotides and their derivatives were carefully dried by evaporation with absolute pyridine before use.

The paper chromatography was performed on FN-1 paper in 1M ammonium acetate (pH 7.5)-ethanol(2 : 5).

The ^{31}P NMR spectra were taken with a Bruker HX-90 pulse spectrometer operating at 36.43 MHz. Fourier transform was performed using a Bruker B-NC 12FFT computer after 100-500 accumulations. All spectra were recorded with heteronuclear spin-spin decoupling ^{31}P-{^{1}H}. The chemical shifts are given relative to 85% H_3PO_4 with the accuracy ± 0.1 ppm. D_2O was used as an external standard for the stabilization of the resonance conditions. Integral intensities were measured with accuracy ±10%. The kinetic measurements were performed using ^{31}P NMR spectroscopy in 10 mm tubes with 1.5-2.5 ml of the reaction mixture. The compounds were identified using values presented either in the table 1 or in the text. Concentrations of the components were determined as the total concentration of P atoms multiplied by the relative integral intensity of the signals of the components and divided by the number of P atoms in the molecule. The rate constants if not indicated otherwise were estimated by dividing the initial rates v_o of disappearance of the P atom containing component by the product of the initial concentra-

Table 1

^{31}P NMR chemical shift values δ for the compounds investigated in pyridine at 30°

Compound	δ (ppm)	Signal's structure	Ref
pdT-Ac	-1.2	singlet	[5]
pdT-Ac pdT-Ac	10.3	singlet	[5]
pdT-Ac pdT-Ac (A) pdT-Ac	11.5 21.6	doublet J_{POP}≈17 Hz triplet J_{POP}≈17 Hz	[5]
B derivative of pdT-Ac	5.1	singlet	[5]
Tr-dTpdT-Ac pdT-Ac (C1)	11-13	multiplet	[5]
pdTpdT-Ac (Ci)	13-17	multiplet	[7]
pdTpdT-Ac Ac-dTpdTp (C2)	11-13	multiplet	[7]
Tr-dTpdT-Ac Tr-dTpdT-Ac (D1)	13.75 13.9	two singlets*	[6]
Tr-dTpdTpdT-Ac (Di)	15-19	multiplet	[9]
BD derivative of pdTpdT-Ac	5.4 14.2	singlet splitting signal	[7]
Tr-dTp(OCH_2CH_2CN)dT-Ac	2.55 2.70	two singlets*	**
Tr-dTp_αdTp_βdT-Ac	1.0(P_α) 1.2(P_β)	singlet singlet	**
$NCCH_2CH_2O$p$_\alpha$dTp$_\beta$dT-Ac	0.85(P_α) 1.2 (P_β)	singlet singlet	**

* Due to asimmetric phosphorus atoms.

** The data of this paper.

The chemical shift values for the most compounds are taken in the reaction mixtures.

tions of the reagents taking into account the stoichiometry of the reaction. To measure v_0 the initial part of the kinetic curve c(t) was approximated by parabolic equation

$$c = c_0 + v_0 t + at^2$$

v_0 and a being calculated by the least square method.

Kinetic characteristics of the reactions of TPS in pyridine with pdT-Ac and its derivatives.

The rate constants of the reactions of TPS with pdT-Ac,

O(pdT-Ac)$_2$ and Tr-dTpdT-Ac (scheme I) were studied as described above. The conditions used are given in the legend to fig.1. The rate constants of the reaction of trisubstituted tripolyphosphate A with TPS were calculated using the kinetic curves of the disappearance of A and of the accumulation of B in the mixture of pdT-Ac (0.13-0.16M) and TPS (0.3-0.46M) by equation

$$k = \frac{1}{3}\left([B] - [B]_0\right) \Big/ \int_{t_0}^{t} [A][TPS]\, dt$$

taking $[TPS] = [TPS]_0 - 2[A] - [B]$; $[B]_0$ is the B concentration at time t_0 corresponding to maximal [A] value.

The reactivity of the B type derivatives.

B derivative of pdT-Ac was prepared by the treatment of 0.1-0.2M solutions of pdT-Ac with 3mole equivalents of TPS in pyridine during 2 hours. The BD derivative of pdTpdT-Ac was prepared by the treatment of 0.1-0.2M solutions of pdTpdT-Ac with 5 mole equivalents of TPS in the pyridine during 2-3 hours. Tr-dT was dissolved 1-2 hours before use in pyridine containing small amounts (0.2 mole equivalent) of TPS to remove the traces of water usually present in the Tr-dT powder. The solutions of Tr-dT and B type derivative were mixed in dry box and the disappearance of the B signal was followed. The initial concentrations used were: B derivative of pdT-Ac 0.1M; BD derivative of pdTpdT-Ac 0.05M; Tr-dT 0.2M.

The reactivity of the C type derivatives.

To study the reaction of C1 derivative (Scheme I) with alcohols the solution of 2.2 ml of pdT-Ac (0.18M) and TPS (0.35M) in pyridine was kept for 2 hours. Then the mixture was added to 0.38 mmoles of Tr-dTpdT-Ac, the total volume being 2.65 ml. ^{31}P NMR spectrum was taken to estimate the C1 content. The aliquotes (1.3 ml) were added to the solutions (dried as mentioned above) of either 0.39 ml of 2-cyanoethanol (0.97M) or 0.57 ml of Tr-dT (0.68 M) in absolute pyridine and the consumption of C was followed.

To perform the separation of the C type dinucleotide derivatives C1 and C2 the solution of 500 mg of pdTpdT-Ac (pyridinium salt) in 3 ml dimethylformamide (DMF) was added dropwise to the solution of 600 mg of dicyclohexylcarbodiimide (DCC)

in 3 ml DMF at 30°C. The reaction mixture was stirred about 40 min. and ^{31}P NMR spectrum was recorded to check completion of the reaction. The mixture was diluted by 64 ml of water (t = 1°C) and immediatly applied to a column (25 x 170 mm) with Molselect-DEAE 25 ion-exchange resin in HCO_3^- form with the flow rate 70 ml/h. A glass filter was used to remove DCC and dicyclohexylurea (DCU) precipitates. The elution was performed at 4°C with a linear gradient of ammonium bicarbonate pH 7.5 (0.01-0.80M), the eluent volume was 1000ml, the rate of elution was adjusted to 120 ml/h. The fractions corresponding to the substances with one and two ionized groups were gathered separatly, evaporated at 25°C in vacuo to a volume of about 2 ml and ^{31}P NMR spectra of these samples were recorded (fig.3a and 3b).

To study the reactions of Ci and C2 (Scheme II) with alcohols the solution of 0.375 mmoles of pdTpdT-Ac (triethylammonium salt) and 0.56 mmoles of TPS in absolute pyridine (the total volume 3.75 ml) was kept for 2 hours at 30°C. The completion of the reaction was checked by recording ^{31}P NMR spectrum. Then 0.250 mmoles of the hydroxy component (2-cyanoethanol, or Tr-dT) in 0.15 ml of the absolute pyridine was added to the above solution (1.25 ml aliquote) in dry box. The reactions proceeded at 30°C. To calculate the integral intensities of all main components of the reaction mixture (the reaction with Tr-dT is taken as an example) the following intensities were measured (fig.4): a) i_a - total intensity of the signals of the phosphodiester groups; b) i_b - total intensity of the signals of all pyrophosphate groups; c) i_1 intensity of peak 1, the resonance of P_α atom of Tr-dTp$_\alpha$dTp$_\beta$dT-Ac (see Table 1); d) i_2- intensity of the resonance of disubstituted pyrophosphate group of O(pdTpdT-Ac)$_2$; e) $i_{3,4}$- intensity of the sum of the peaks 3 and 4 signals of the high field part of spin AB-system related to the resonance of internucleotide P atom of Ci (one of two diastereomers, existing approximatly in the equal concentrations)[7]. Then intensities of the signals of the main components were estimated as follows: 1) intensity of Ci as $4xi_{3,4}$ 2) intensity of Tr-dTpdTpdT-Ac as 2 x i_1; 3) intensity of sum of O(pdTpdT-Ac)$_2$ and C3 (see in Results) as 2 x (i_a - 2i_1);

4) intensity of C2 as $i_b - 4i_{3,4} - (i_a - 2i_1)$; 5) intensity of $O(pdTpdT\text{-}Ac)_2$ as $2 \times i_2$; 6) intensity of C3 as $2 \times (i_a - 2i_1) - 2 \times i_2$. The kinetic curves obtained in this way are shown in fig.5. The calculations for the reactions of Ci and C2 with 2-cyanoethanol were performed in similar manner.

The reaction mixture of Ci and C2 with Tr-dT (see above) was kept for 7 days at room temperature, treated with water, trityl and acetyl residues removed according to [12] and dTpdTpdT formed was isolated by ion-exchange chromatography in 65% yield.

The reactivity of the D type derivatives.

The conditions of the reaction of the D derivative of Tr-dTpdT-Ac (scheme I) with 2-cyanoethanol are given in the legend to fig.6. The rate constant of the reaction was calculated by equation

$$k = [\text{triester}] \Big/ \int_0^t [D](0.23 - [\text{triester}])\, dt$$

using the kinetic curves both of the disappearance of D and of the accumulation of the triester in the reaction mixture containing 0.077M Tr-dTpdT-Ac, 0.16M TPS and 0,23M 2-cyanoethanol, the latter being added 20 min. after mixing of the two former components.

To study the reaction of Di derivative of Tr-dTpdTpdT-Ac with 2-cyanoethanol the solution (1.36 ml) of Tr-dTpdTpdT-Ac (0.05M) and TPS (0.10M) in pyridine in NMR tube was kept for 2 hours and ^{31}P NMR spectrum was recorded to check the completion of the reaction. The spectrum is similar to that presented in fig.7. 0.1 ml of 2-cyanoethanol was added and the reaction was followed by the disappearance of the signals at 15-19 ppm.

RESULTS

Kinetic characteristics of the reactions of TPS in pyridine with pdT-Ac and its derivatives.

The time course of all steps (Scheme I, process (1)) of conversion of pdT-Ac in the reaction with TPS in pyridine may be easily studied following either the disappearance of the phosphorus containing reagent or the accumulation of the reaction product. In the same way the conversion of Tr-dTpdT-Ac to D derivative, (process (3), scheme I) was studied. In no

case the signals of the arylsulfonyl residue containing intermediates were seen in the ^{31}P NMR spectra of the reaction mixtures. That means that the mixed anhydrides of arylsulfonic acid and phosphoester derivative commonly accepted as primary products of the attack of phosphate oxygen on the electrophilic S atom are extremely reactive and present in low steady state concentration, the formation of these mixed anhydrides being the rate limiting step. This is in agreement with previously found second order of the intermediate steps of conversion of pdT-Ac to B derivative [16] although the stoichiometry of the reactions differs from bimolecular, e.g.

$$3\ O(pdT\text{-}Ac)_2 + TPS \longrightarrow 2A$$

The temperature dependences of the second order rate constants for all three steps of conversion of pdT-Ac to B derivative and of conversion of Tr-dTpdT-Ac to D derivative are presented in fig.1.

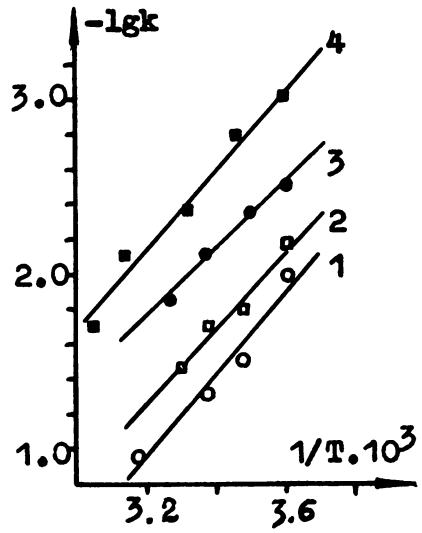


Fig.1. The Arrhenius plots of the temperature dependence of the rate constants of the reactions in pyridine: 1 - pdT-Ac + TPS, the concentration used at 5° were 0.1-0.3M of pdT-Ac and 0.04-0.15M of TPS, at higher temperatures 0.03M pdT-Ac + 0.015M TPS were used and the pdT-Ac consumption was followed by paper chromatography; 2 - dinucleoside pyrophosphate (0.04-0.3M) + TPS (0.05-0.15M); 3 - Tr-dTpdT-Ac (0.075-0.17M) + TPS (0.05-0.15M); 4 - trinucleoside tripolyphosphate A + TPS (see Materials and Methods).

The Arrhenius parameters of all above mentioned reactions as well as $\Delta H_o^{\neq}$ and $\Delta S_o^{\neq}$ values and the rate constants at 5° are given in table 2.

Table 2

Kinetic characteristics of the reactions of TPS in pyridine with pdT-Ac and its derivatives

Reaction	E kcal/mole	lg k_o (k_o $M^{-1}s^{-1}$)	$\Delta H_o^{\neq}$ kcal/mole	$\Delta S_o^{\neq}$ e.u.	$k \cdot 10^3$ ($M^{-1}s^{-1}$) at 5°
pdT-Ac + TPS	10.4±1.4	6.3±1.2	9.8	-31.7	11.0
O(pdT-Ac)$_2$+ TPS	10.5±0.2	6.1±0.1	9.9	-32.6	7.0
A + TPS	10.3±1.0	5.1±0.2	9.7	-37.2	1.1
Tr-dTpdT-Ac + TPS	9.1±0.2	4.6±0.1	8.5	-39.4	3.1

It is seen that the reactivity of phosphodiester group is only four times lower than that of phosphomonoester group. Taking into account that the activation of phosphomonoester groups is a stepwise process with a rather slow final step we may conclude that the accumulation of the phosphorylating intermediates of the B type derived from monoester proceeds even slower than that of the D type derived from diester. Due to small difference in the activation energies the reactivity ratio should not change significantly with temperature.

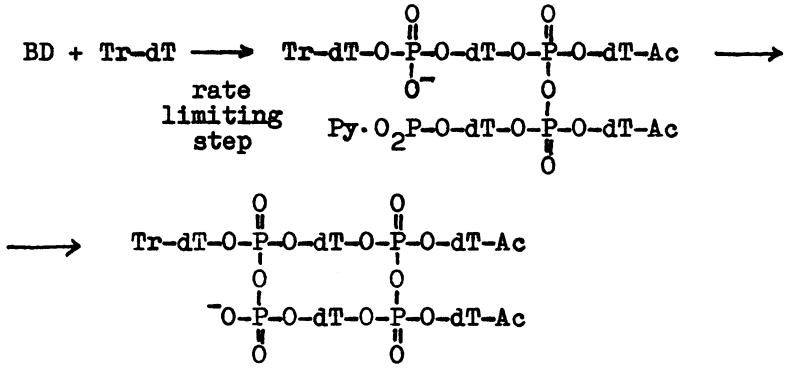
Earlier it was demonstrated that there is no difference neither between disappearance rates of mononucleotides nor between accumulation rates of the B type derivatives in the mixture of equimolar amounts of pdT-Ac and MMTr-dTp with TPS [17]. That means that there is no difference in reactivities of 3'- and 5'-phosphates in the reaction with TPS.

The reactivity of the B type derivatives.

The derivative of pdT-Ac B (Scheme I) and derivative of pdTpdT-Ac BD (Scheme II) were taken as representatives of this type of active phosphorylating intermediates. The reactivity was estimated using the reaction with Tr-dT which results in formation of a new internucleotide bond.

As it was demonstrated earlier phosphodiester group forming in the rate-limiting step of the reaction (2) of B with Tr-dT reacts readily with the second B molecule, compound C1 being

the product accumulating in the reaction mixture [5]. Therefore two B molecules are consumed per one molecule of Tr-dT. Similar stoichiometry should be expected for the reaction of BD with Tr-dT, e.g.



The initial reaction rates of the rate limiting steps of reactions of B and BD with Tr-dT were measured following the disappearance of the typical ^{31}P NMR signal of the B type group. The rate constants were found to be $0.8 \times 10^{-3} M^{-1}s^{-1}$ at 2° and $7.3 \times 10^{-3} M^{-1}s^{-1}$ at 30° with B derivative and $6.3 \times 10^{-3} M^{-1}s^{-1}$ at 30° with BD derivative. It is seen that the reactivities of both derivatives don't differ significantly.

The reactivity of the C type derivatives.

Compounds C1 (Scheme I), C2 and Ci (Scheme II) were taken as representatives of this type of phosphorylating intermediates. Reactivity was estimated using Tr-dT and 2-cyanoethanol as nucleophilic compounds. C1 derived by mixing of equimolar amounts of Tr-dTpdT-Ac and B derivative of pdT-Ac was found to react readily in pyridine with both alcohols. The main new signals appearing in the ^{31}P NMR spectra (over 90% with 2-cyanoethanol and 70% with Tr-dT) are the signals of phosphodiester groups thus proving the esterification of alcohol to be the predominating reaction. Besides this small amounts of $O(pdT\text{-}Ac)_2$ accumulate in the reaction mixture. Measuring the disappearance of C1 in the presence of Tr-dT and 2-cyanoethanol the rate constants at 30° were calculated and were found to be respectively 0.9×10^{-4} and $4.7 \times 10^{-4} M^{-1}s^{-1}$. Thus the compound C was demonstrated to be reactive to alcohols, however its reactivi-

ty is two orders of magnitude lower than that of the B type derivative.

Trisubstituted pyrophosphates were found to be the main products of reaction of dinucleotides (pdTpdT-Ac) either with moderate excess of TPS or with DCC in pyridine as well as in DMF [7,9]. The complicated ^{31}P NMR spectrum of the reaction mixture was assigned to two main compounds - Ci and C2 (Scheme II).

Being easily hydrolyzed in the presence of pyridine these compounds were found to be stable enough in aqueous DMF to be separated by ion-exchange chromatography. In fig.2 the elution profile in Tomlinson-Tener system is represented for reaction mixture obtained by treatment of pdTpdT-Ac with DCC in DMF. The peaks corresponding to compounds with one (Ci) and two negative charges (C2) are seen as well as the peaks of more negatively charged compounds.

Using preparative scale ion-exchange chromatography the fractions containing separated single-charged and double-charged derivatives were obtained. The ^{31}P NMR spectra of both fractions are given in fig.3. The spectrum of the first fracti-

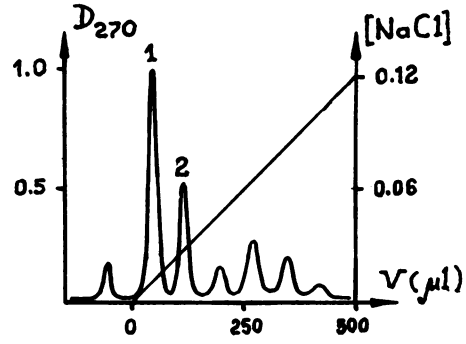
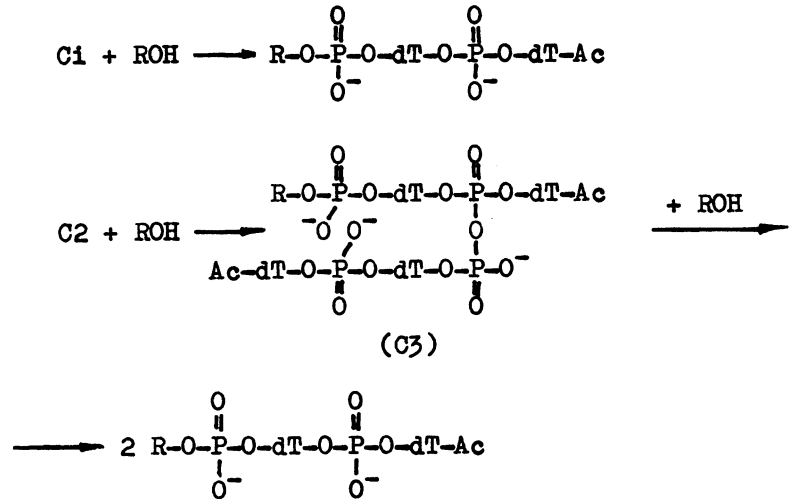


Fig.2. The elution profile of the microcolumn ion-exchange chromatography [18] of the mixture of the C type derivatives of pdTpdT-Ac. 0.1M pdTpdt-Ac + 0.5M DCC in DMF were kept for 50 min at room temperature, treated with water and immediately applied to the column with DEAE-cellulose (50µl, 60mm height) in HCO_3^- form and eluted with the linear gradient of NaCl (0.0-0.12M) in 7M urea, 0.02M Tris- HCl pH 7,5 with the flow rate 600µl/h. 1) th e peak of single-charged derivative; 2) the peak of double-charged derivative.

on is represented by two spin AB systems in accordance with the existence of two diastereoisomers Ci. The anomalous high J_{POP} values (23 and 25 Hz) are typical of the eight-member pyrophosphate cycle [7]. The spectrum of the second fraction is represented by several overlapping spin AB systems in the range 11-14 ppm typical of trisubstituted pyrophosphates with nucleoside residues.The complexity of the spectrum is most probably due to possibility of the formation of three diastereoisomers.

The reaction of Ci and C2 with alcohols should result in the formation of new phosphodiester groups according to reaction scheme



The main final product of the reaction of the mixture of Ci and C2 with Tr-dT in pyridine was shown to be trinucleoside diphosphate. After the removal of the blocking groups dTpdTpdT was isolated in 65% yield. The typical ^{31}P NMR spectrum of the reaction mixture recorded in the course of the process is presented in fig.4. The main new signals appear in the phosphodiester groups region.

These groups besides Tr-dTpdTpdT-Ac are present in C3. Signal at 10.6 ppm appears in the spectrum thus indicating that some amount of disubstituted pyrophosphate most probably $O(pdTpdT\text{-}Ac)_2$ forms in the reaction. Assuming Ci, C2, C3, Tr-dTpdTpdT-Ac and $O(pdTpdT\text{-}Ac)_2$ to be the main P containing

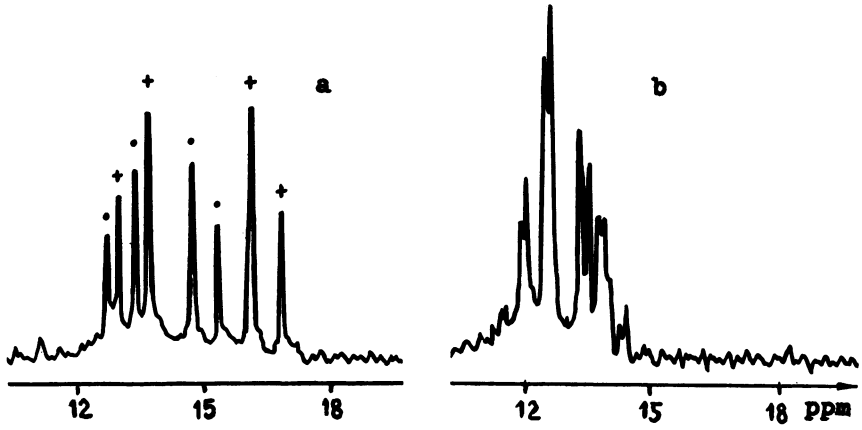


Fig.3. ^{31}P NMR spectra of Ci (a) and C2 (b) ; (•) and (+) indicate the signals of two spin AB systems of Ci

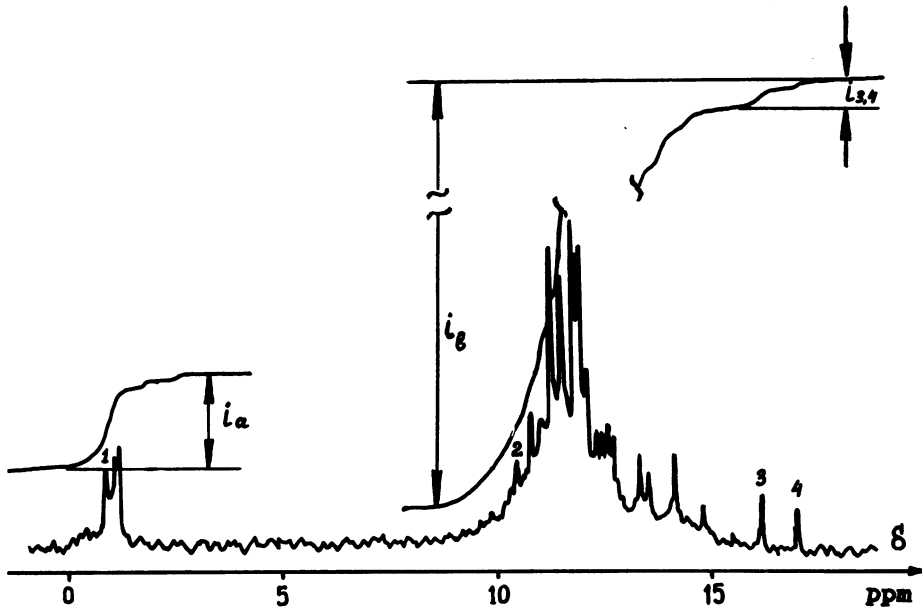


Fig.4. The ^{31}P NMR spectrum and the integral intensities of the reaction mixture of Ci + C2 with Tr-dT (for details see Materials and Methods).

components of the reaction mixture their concentrations were calculated from the spectra using the procedure described in Materials and Methods. The kinetic curves for all five components are presented in fig.5. The rate constants of the consumption of Ci and C2 were calculated and were found to be 2.8×10^{-4} and $0.6 \times 10^{-4} M^{-1} s^{-1}$ respectively. It is seen that the intramolecular cyclic derivative Ci is several times more reactive than the macrocyclic derivative C2

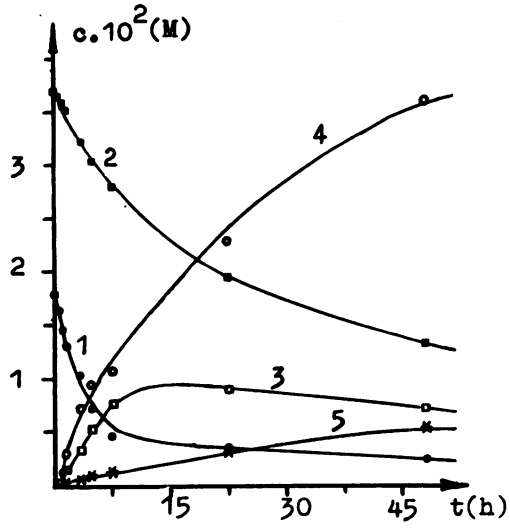
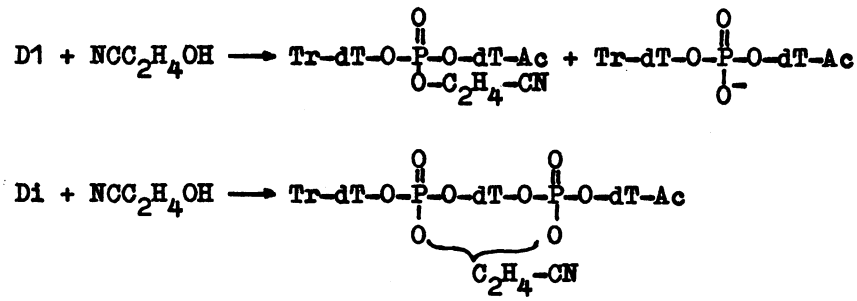


Fig.5. The kinetic curves of the reaction of the mixture of Ci and C2 derivatives of pdTpdT-Ac with Tr-dT at 30° in pyridine: 1 - Ci; 2 - C2; 3 - C3; 4 - Tr-dTpdTpdT-Ac; 5 - $O(pdTpdT\text{-}Ac)_2$

In a similar way the reaction of Ci and C2 with 2-cyanoethanol was studied. The rate constants at 30° were found to be 5.8×10^{-4} and 2.0×10^{-4} $M^{-1} s^{-1}$ respectively. In this case the intramolecular C type compound Ci is also more reactive than the intermolecular one.

The reactivity of the D type derivatives.

D1 derivative of Tr-dTpdT-Ac (Scheme I) and Di derivative of Tr-dTpdTpdT-Ac (Scheme II) were taken as representatives of this type of phosphorylating intermediates. The reaction with 2-cyanoethanol resulting in formation of corresponding triesters



(the mixture of isomers)

was studied to estimate reactivities of the D type derivatives. The typical kinetics of the reaction of D with 2-cyanoethanol is given in fig.6. To escape complications due to side reacti-

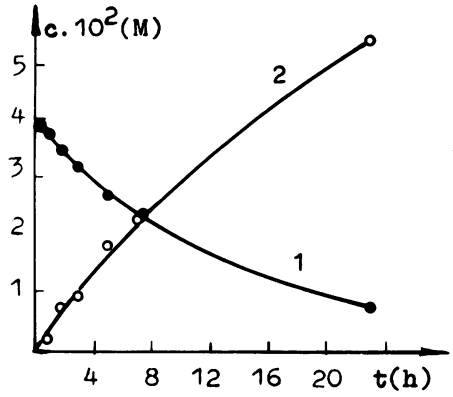


Fig.6. The kinetic curves of the reaction of the D1 with 2-cyanoethanol (0.23M) at 30°. D1 was prepared by treatment of Tr-dTpdT-Ac (0.077M) with TPS (0.16M). 1 - D1; 2 - 2-cyanoethyl ester of Tr-dTpdT-Ac.

ons with nucleophilic impurities including the traces of water D1 was obtained with a 2 fold excess of TPS. Therefore, Tr-dTpdT-Ac formed in equimolar amount to the triester in the reaction of D1 with 2-cyanoethanol is converted rapidly back to D1. The method of calculation of the rate constant is presented in Materials and Methods. The rate constant at 30° was found to be $1.5 \times 10^{-4} M^{-1} s^{-1}$. That means that the reactivity of D1 does not differ significantly from that of C1 and C2. Di derivative was obtained by treatment of Tr-dTpdTpdT with TPS. The ^{31}P NMR spectrum of the reaction mixture is presented in fig.7. Almost all components of four spin AB systems expec-

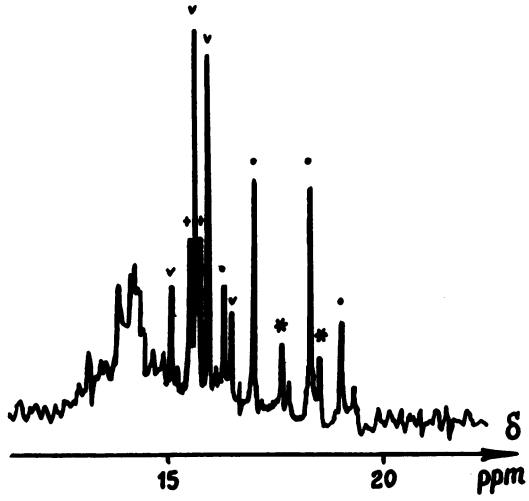


Fig.7. The pyrophosphate range of the ^{31}P NMR spectrum of the reaction mixture of Tr-dTpdTpdT (0.03M) with TPS (0.12M) in pyridine in 30 min. after beginning of the reaction. (•) (v) (+) and (*) are the components of four spin AB system.

ted for the mixture of four diastereoisomers of Di are seen in the range 15-19 ppm upfield to the typical range of tetrasubstituted pyrophosphates. This upfield shift as well as the enhanced J_{POP} values (20.5-27 Hz) prove the formation of the eight-member cycle.

Table 3

The rate constants of the reactive phosphorylating derivatives of mono and oligonucleotides with alcohols in pyridine at 30°

Phosphorylating derivative		$k \cdot 10^4$ $M^{-1}s^{-1}$ with Tr-dT	$k \cdot 10^4$ $M^{-1}s^{-1}$ with 2-cyanoethanol
B derivative of pdT-Ac		73	-
B group of the BD derivative of pdTpdT-Ac		63	-
Tr-dTpdT-Ac pdT-Ac	(C1)	0.9	4.7
pdTpdT-Ac Ac-dTpdTp	(C2)	0.6	2.0
pdTpdT-Ac	(Ci)	2.8	5.8
Tr-dTpdT-Ac Tr-dTpdT-Ac	(D1)	-	1.5
Tr-dTpdTpdT-Ac	(Di)	-	4.7

The rate constants are given with the accuracy of 20%.

The reactivity of the Di type derivative was studied using the reaction of the mixture of Tr-dTpdTpdT-Ac derivatives (containing besides Di derivative a mixture of the intermolecular tetrasubstituted pyrophosphates) with 2-cyanoethanol. The rate constant was found to be $4.7 \times 10^{-4} M^{-1} s^{-1}$. This value is several times greater than that with D1 derivative of Tr-dTpdT-Ac thus proving the enhanced reactivity of the eight-member pyrophosphate cycles.

The whole set of the rate constants determined in this paper is presented in the table 3.

DISCUSSION

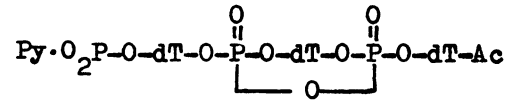
The main result of the present investigation is the quantitative estimate of the reactivity towards alcohols of all main types of the reactive phosphorylating intermediates forming by treatment of oligonucleotides with TPS. The reactivity was found to decrease in the series

$$B \gg Ci \approx Di > C \approx D$$

The data obtained permit to estimate the role of the side reactions touching phosphodiester groups of the nucleotide and nucleoside components in the phosphodiester approach to oligonucleotide synthesis. The most undesirable by-process seems to be the phosphorylation of hydroxy group of the nucleoside component by activated phosphodiester groups that is of the D type, especially Di intermediates. The intermediates of the C type were previously demonstrated to react with nucleophiles (amines, orthophosphate [19], pyrophosphate [20] forming only the phosphomonoester group derivatives.

The essential result of this work seems to be the establishment of the reactivity of the C type derivatives in the oligonucleotide synthesis. These derivatives are formed by preactivation of dinucleotide with the moderate excess of condensing reagents. The esterification of hydroxy groups by these derivatives proceeds rather slowly. Probably the reaction rate may be enhanced raising the temperature. Due to rather small difference in the reactivity of phosphomono- and phosphodiester groups the formation of the D type derivates cannot be excluded in the course of activation of longer oligonucleotides.

The BD derivatives of dinucleotides contain activated phosphodiester group and may react forming some amount of triesters thus giving raise to the branching of the oligonucleotide chain with the obvious undesirable consequences to the oligonucleotide synthesis. However the reactivity of the D groups being nearly the same as that of the C groups should be two orders of magnitude lower than that of the B group. Taking into account that only one phosphodiester group is attacked per two activated phosphomonoester groups in the BD derivative of dinucleotide we may conclude that less than 1 triester residue forms per 200 new diester residues.Therefore, BD derivatives of dinucleotides may be recommended to be used as phosphorylating reagents in the oligonucleotide synthesis.In the same time the use of trinucleotides as nucleotide components is connected not only with the increase of the number of activated phosphodiester groups but also with the possibility of the formation of intramolecular tetrasubstituted pyrophosphates with several times enhanced reactivity.For example the derivative of trinucleotide



should react with hydroxy group forming di- and triesters in the ratio 1 : 30 (assuming the equal reactivities of Ci and Di derivatives towards 3'-OH group) thus introducing up to 3% of impurities in the each step of the blocked synthesis.

The contact of the nucleoside component with the condensing reagents may result in the D type derivatives formation. The partial protection of phosphodiester groups decrease the number of the neighbouring unprotected phosphates amenable to form more reactive eight-member cyclic derivatives to a greater extent than the total number of unprotected phosphodiester groups.Therefore,even the incomplete protection of internucleotide phosphates may reduce significantly the side reactions.

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