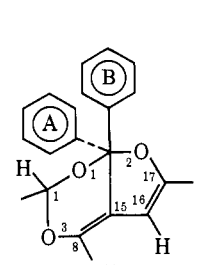
coordinated phosphorus. Probably, one of the main reasons for the stability of this oxyphosphorane **4** is the ability of the flat double bond system and of the five aromatic rings to arrive at an accommodation of their mutual steric interactions to minimize the intramolecular crowding in the bipyramid.

The phosphorus–phenyl bond distances are 1.82 Å, which should be compared with the mean distance of 1.85 Å reported for the equatorial phosphorus–phenyl rings in pentaphenylphosphorane.[20] The third P–C distance in **4** was also 1.82 Å. This is normal for a P–C single bond[21] (1.82 Å). The apical P–O bond distances are 1.79 Å for the bond in the five-membered ring, and 1.74 Å for the bond in the six-membered ring. These correspond to P–O single bonds (1.76 Å), and we surmise that the π bond character of these apical bonds is nearly zero.

It is interesting to speculate on the possible motions of the atoms in the bicyclic oxyphosphorane **4a** in solution. The groups attached to pentacoordinated phosphorus can exchange positions by an intramolecular bond-bending process (pseudorotation[4,15,16,18,22,23]) and by a bond rupture–recombination mechanism,[4] depending on the stability of the molecule and on the experimental conditions. The consequences of one pseudorotation of oxyphosphorane **4a** using the phenyl ring A as pivot would be[22c] the bipyramid **11**. For steric reasons alone this pseudorotation **4a** → **11** seems unlikely. One pseudorotation of **4a** using ring B as pivot would have analogous consequences. One pseudorotation of **4a** using C15 as pivot would give bipyramid **12**. Again, for steric reasons alone the



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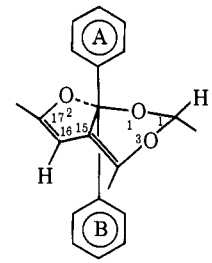

12

pseudorotation **4a** → **12** seems unlikely. Moreover, note that in both cases pseudorotation moves both oxygens to equatorial positions. We conclude that **4a** is essentially incapable of pseudorotation. In fact, the molecule slowly falls apart in solution on standing at 20°; this decomposition prevents studies of variable temperature ^{1}H nmr at higher temperatures.

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(23) NOTE ADDED IN PROOF. It has been recently suggested that the positional exchange of the ligands in pentacoordinated phosphorus can take place by the *turnstile rotation* mechanism. It was claimed that cyclic phosphoranes can undergo permutational isomerizations only by the turnstile rotation, while acyclic phosphoranes can do so, in many cases, by both mechanisms, *turnstile rotation* and *Berry pseudorotaton.*

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Polymerization of Unprotected 2′-Deoxyribonucleoside 5′-Phosphates at Elevated Temperature[1a,b]

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Abstract: Polymerization of unprotected thymidine 5′-phosphate and 2′-deoxyribonucleoside 5′-phosphate (disodium salts) in *N,N*-dimethylformamide at reflux temperature for 30 min has been observed and studied. This reaction is catalyzed by proton(s) or a proton donor and is involved with P^1,P^2-dinucleosidyl 5′-pyrophosphate as a key intermediate. The main products are two series of oligomers with structural formulas of $(pN)_n$ and $(pN)_np$; 5–10% of the oligonucleotides consist of at least one 5′–5′ phosphodiester linkage (or less likely pyrophosphate linkage) as indicated by their resistance to spleen phosphodiesterase; no 3′–3′ phosphodiester linkage has been found since all materials are hydrolyzable by venom diesterase. With the best catalysts (β-imidazolyl-4(5)-propanoic acid or triethylamine hydrochloride), the yield of dimer to hexamer of thymidine oligonucleotides ranges from **12** to 5% of each species. The mechanisms of the synthetic and degradative processes are discussed. The study of this polymerization process may provide additional understanding about the prebiotic synthesis of polynucleotides.

In recent years, chemical polymerization of deoxynucleotides has been successfully achieved in two laboratories.[2–4] The synthetic procedure generally adopted involves the activation of the phosphate moiety of the suitably protected nucleotides in the polymerization mixture. This procedure inadvertently yields pyrophosphates and cyclic phosphates as side products. The pyrophosphates could be eliminated by an improvement of the reaction conditions as well as by a subsequent degradation which involves treatment with car-

(1) (a) This work was supported in part by a contract from the Atomic Energy Commission (AT-30(1)-3538) and a grant from the National Science Foundation (GB-8500). (b) Presented in part at the 159th National Meeting of the American Chemical Society, New York, N. Y., 1969.

(2) H. G. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Amer. Chem. Soc.*, **80**, 6223 (1958); H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *ibid.*, **83**, 1983 (1961); R. K. Ralph, W. J. Connors, H. Scholler, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).

(3) (a) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," Wiley, New York, N. Y., 1961; (b) F. Cramer, *Angew. Chem.*, **78**, 640 (1966).

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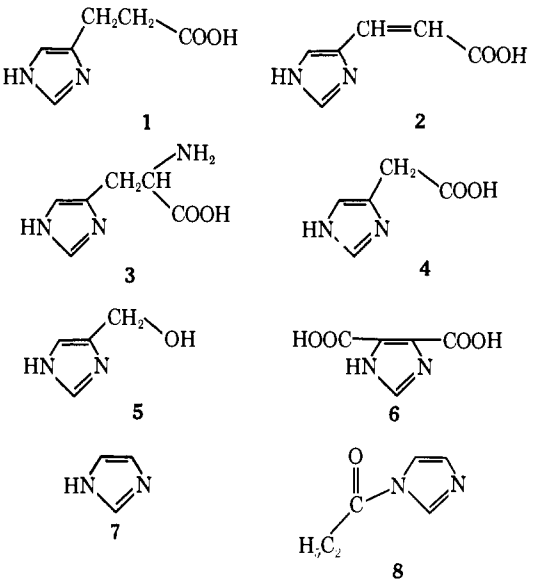
boxylic anhydrides and chlorides.[5-7] In a further study, it was found that pyrophosphates can be used directly for the synthesis of oligonucleotides.[8] Activation of P^1,-P^2-dithymidine 5′-pyrophosphate by benzoic anhydride or aryl acid chlorides was shown to result in the formation of (3′–5′) internucleotide bonds with suitably protected nucleoside or oligonucleotide receptors.

In a previous communication,[9] we have reported an interesting observation about the polymerization of thymidine 5′-phosphate. In this process, the nucleotides are polymerized at an elevated temperature with β-imidazolyl-4(5)-propanoic acid as catalyst. No significant amount of cyclic phosphates or pyrophosphates was detected in the analysis of the end products. In search of a mechanism for this rather uncommon reaction, we first investigated the possibility that the course of the polymerization reaction was due to a unique feature of β-imidazolyl-4(5)-propanoic acid as a catalyst. This was found not to be the case. In the experiments described below, the polymerization of the thymidine 5′-phosphate at elevated temperatures was found to be catalyzed by proton(s) or a proton donor. Upon the investigation of the time course of this polymerization, we observed the immediate formation of pyrophosphate which subsequently decayed as the reaction proceeded. This observation suggests that the pyrophosphate species could be the reactive intermediate in this polymerization procedure. This suggestion is strongly supported by the following two experiments: (1) heating a solution of P^1,P^2-dideoxynucleoside 5′-pyrophosphate results in the formation of deoxyoligonucleotides; (2) heating a solution of P^1,-P^2-dithymidinine 5′-pyrophosphate with 5′-*O*-acetylthymidine results in the formation of thymidylyl-(3′–5′)-thymidine after removal of the acetyl group.

Results

The Use of Imidazole Derivatives for the Polymerization of Thymidine 5′-Phosphate. As previously reported,[9] the polymerization of the thymidine 5′-phosphate at elevated temperature can be catalyzed by β-imidazolyl-4(5)-propanoic acid. In the present study, we tested other imidazolyl derivatives as well as imidazole itself as possible catalysts for this reaction. The results are shown in Table I. All the imidazole derivatives which could function as a proton donor catalyzed the polymerization of thymidine 5′-phosphate. This result shows that the reported polymerization reaction is not due to a unique feature of β-imidazolyl-4(5)-propanoic acid, although for reasons not entirely obvious at present, this compound gave the best yields in oligonucleotide formation. As indicated in Table I, some of the imidazole derivatives have been used as free acids and some as salts. All the imidazolyl compounds which have a protonated nitrogen (**1**–**7**) could



be used as a catalyst in the polymerization. *N*-Carboxyethylimidazole, which does not have a protonated nitrogen, could not be used as a polymerization catalyst. No reaction product was found when this compound was refluxed with thymidine 5′-phosphate. These data suggest that the role of the imidazole derivatives as catalysts in the observed polymerization of thymidine 5′-phosphate is that of a proton donor. To confirm this suggestion, we studied the ability of the triethylamine hydrochloride to catalyze the polymerization of thymidine 5′-phosphate.

Polymerization of Thymidine 5′-Phosphate in the Presence of Triethylamine Hydrochloride. A solution of thymidine 5′-phosphate in *N*,*N*-dimethylformamide was refluxed for 30 min in the presence of hydrogen chloride (~2 equiv) and triethylamine (5 equiv). The resulting reaction mixture was separated on a diethylaminoethyl (DEAE)-cellulose column in the bicarbonate form. The elution pattern is shown in Figure 1 and the distribution of the nucleotide material is described in Table II. As shown, the yield of oligonucleotide material was as high as that obtained in the polymerization reaction of thymidine 5′-phosphate catalyzed by the β-imidazolyl-4(5)-propanoic acid. Apparently, the polymerization of thymidine 5′-phosphate can be achieved by means of a proton catalysis. This catalysis leads to the formation of two main series of polymer products. One series consists of the thy-

Table I. Polymerization of Thymidine 5′-Phosphate in the Presence of Different Imidazolyl Compounds[a]

Compd	% oligonucleotide material
1	55–60[c]
2	25[c]
3	20[d]
4[b]	12[c]
5[b]	10[d]
6	11[d]
7	15[c]
8	0[d]

[a] The procedure is described in detail in the Experimental Section. [b] These two compounds were used in the form of hydrogen chloride salt; others were used as free acids. [c] The products have been separated by DEAE-cellulose (bicarbonate) chromatography using a linear triethylammonium bicarbonate gradient and investigated by enzymatic degration studies as described in the Experimental Section. [d] The products have been investigated by paper chromatography in solvent system C and by paper electrophoresis at pH 7.5.

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(8) M. W. Moon and H. G. Khorana, *ibid.*, **88**, 1805 (1966).
(9) O. Pongs and P. O. P. Ts'o, *Biochem. Biophys. Res. Commun.*, **36**, 576 (1969).

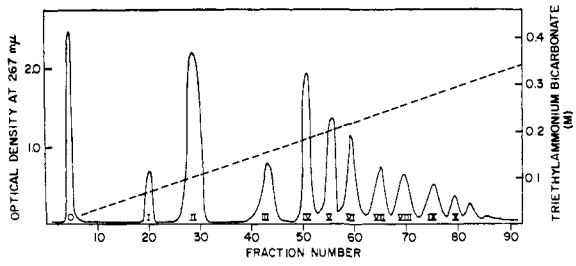

Figure 1. Chromatography on a DEAE-cellulose (bicarbonate) column of the total reaction mixture obtained after heating thymidine 5′-phosphate in the presence of triethylammonium chloride in *N,N*-dimethylformamide. The broken line shows the triethylammonium bicarbonate gradient. The product distribution is given in Table II.

midine oligomers with a free 3′-OH end $(pT)_n$, and the other consists of the thymidine oligonucleotides with phosphorylated 3′-OH end $(pT)_np$.

Table II.[a] Polymerization of Thymidine 5′-Phosphate by Triethylammonium Chloride

Peak no.	Fraction no.	Composition[c]	Yield[b]
0	4–7	T	11.0
I	19–21	TpT	3.0
II	26–31	pT	26.0
III	42–45	$(pT)_2$	9.1
IV	49–51	$(pT)_3$; pTp	13.3
V[d]	54–56	$(pT)_4$; $(pT)_2p$	9.8
VI[d]	58–60	$(pT)_5$; $(pT)_3p$	9.1
VII[d]	63–66	$(pT)_6$; $(pT)_4p$	6.0
VIII[d]	68–71	$(pT)_7$; $(pT)_5p$	4.8
IX[d]	73–75	$(pT)_8$; $(pT)_6p$	4.1
X[d]	77–80	$(pT)_9$; $(pT)_7p$	3.0

[a] The procedure is described in detail in the Experimental Section. [b] The calculation of yield is based on the numbers of OD units in every peak of Figure 1. It was not corrected for hypochromicity. [c] After alkaline phosphatase treatment the $(pT)_n$ products can be separated from $(pT)_np$ products by paper chromatography in solvent system C. The relative distribution of each $(pT)_n$ and $(pT)_np$ product in the different peaks is 60–70% and 30–40%, respectively. For complete product characterization see Experimental Section. [d] About 5% of the oligonucleotides from peak V to peak X contain at least one 5′–5′ phosphodiester linkage as shown by their resistance to the spleen diesterase after monoesterase treatment. See Experimental Section.

Formation of Pyrophosphate during the Polymerization Reaction. The next step of our investigations was a kinetic study of the polymerization reaction. Thymidine 5′-phosphate was refluxed in *N,N*-dimethylformamide together with β-imidazolyl-4(5)-propanoic acid for varying time intervals. The analysis of the reaction products by means of paper chromatography and paper electrophoresis is shown in Figure 2. In the first 15 min of the reaction a relatively large amount of pyrophosphate was formed, which then decreased rapidly and could no longer be detected after 30 min. The oligonucleotide material was formed mainly in the latter 15 min of the 30-min reaction period. After 30 min the amount of oligonucleotide material formed did not significantly increase. Therefore, the necessary reaction time for a maximum yield of oligonucleotide material is 30 min for this reaction under the present conditions. These data suggest that in the course of the polymerization reaction, pyrophosphate appears to be formed not as a side product, but as a reaction

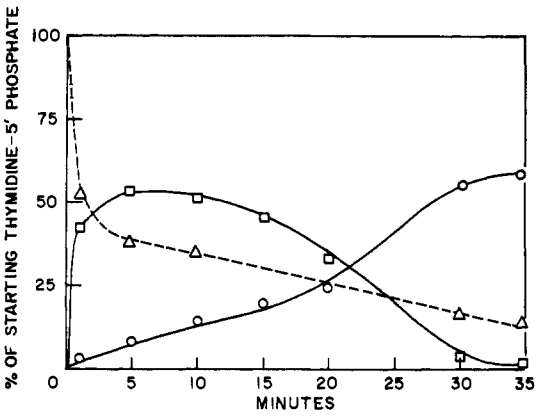

Figure 2. The time dependence of P^1,P^2-dithymidine 5′-pyrophosphate formation and of thymidine oligonucleotide formation during the heating of thymidine 5′-phosphate in the presence of β-imidazolyl-4(5)-propanoic acid in *N,N*-dimethylformamide: □, pyrophosphate formation; ○, oligonucleotide formation; △, remaining mononucleotide.

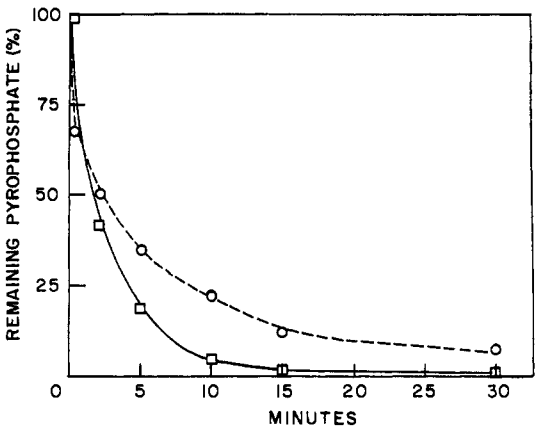

Figure 3. The time dependence of degradation of P^1,P^2-dithymidine 5′-pyrophosphate refluxed in anhydrous pyridine (○) and refluxed in *N,N*-dimethylformamide (□).

intermediate. Degradation of this intermediate leads to the formation of oligonucleotides. Therefore, it should be possible to use P^1,P^2-dithymidine pyrophosphate itself as starting material for the formation of thymidine oligonucleotides.

Synthesis of Thymidine Oligonucleotides from P^1,P^2-Dithymidine 5′-Pyrophosphate. The kinetics of the degradation of P^1,P^2-dithymidine 5′-pyrophosphate was studied first and is shown in Figure 3. When the pyridinium salt of P^1,P^2-dithymidine 5′-pyrophosphate was refluxed in anhydrous pyridine or in *N,N*-dimethylformamide, it was degraded completely within 30 min. However, the reaction resulted in the formation of thymidine oligonucleotides only when refluxed in *N,N*-dimethylformamide.

P^1,P^2-Dithymidine 5′-pyrophosphate was refluxed in *N,N*-dimethylformamide on a larger scale for 30 min, and the reaction mixture was separated on a DEAE-cellulose column in the bicarbonate form. The elution pattern is shown in Figure 4 and the distribution of the nucleotide material is shown in Table III. The results show that pyrophosphate itself can be used for the formation of oligonucleotides. As in the polymerization reaction with triethylamine hydrochloride, we again observed the formation of two main series

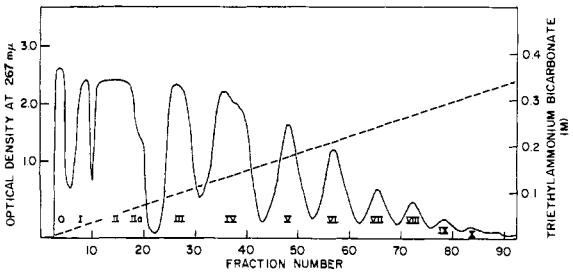

Figure 4. Chromatography on a DEAE-cellulose (bicarbonate) column of thymidine oligonucleotides (total polymeric mixture), obtained after heating P^1,P^2-dithymidine 5′-pyrophosphate in *N,N*-dimethylformamide. The broken line shows the triethylammonium bicarbonate gradient. The product distribution is given in Table III.

of oligonucleotides, $(pT)_n$ and $(pT)_np$. Also, the yield of oligonucleotide material (about 40%) was comparable to the polymerization of thymidine 5′-phosphate with β-imidazolyl-4(5)-propanoic acid. Besides the oligonucleotides of the composition $(pT)_n$ and $(pT)_np$ we also found some other types of oligonucleotides in peak I and III–VI of Figure 4. Peak I contained not only thymidylyl-(3′–5′)-thymidine, but also thymidylyl-(5′–5′)-thymidine to the same extent. In addition, approximately 5–10% of the material of peaks III–VI could be degraded by spleen phosphodiesterase even prior to alkaline phosphatase treatment. This indicates the presence of a small amount of oligonucleotide material which does not contain a terminal 5′-phosphate group and thus has a composition different from the series of $(pT)_n$ and $(pT)_np$, such as $T(pT)_n$. Peak IIa in Figure 4 corresponds to a small amount of P^1,P^2-dithymidine 5′-pyrophosphate remaining in the reaction mixture. It should be noted that the kinetic studies of the pyrophosphate degradation described above were carried out with a 20 times lower concentration of pyrophosphate. This difference in concentration might account for the observation that in the polymerization reaction not all of the pyrophosphate material had degraded.

Table III.[a] Thymidine Oligonucleotide Formation by Heating P^1,P^2-Dithymidine 5′-Pyrophosphate

Peak no.	Fraction no.	Composition[b]	Yield, %[c]
0	3–5	Thymidine	16
I	6–9	TpT[d]	16
II	10–19	pT	44.5
IIa		O<(pT)$_2$	
III	22–30	$(pT)_2$	8.0
IV	31–42	$(pT)_3$; pTp	7.0
V[e]	43–52	$(pT)_4$; $(pT)_2p$	2.5
VI[e]	53–60	$(pT)_5$; $(pT)_3p$	1.6
VII[e]	61–68	$(pT)_6$; $(pT)_4p$	1.9
VIII[e]	69–74	$(pT)_7$; $(pT)_5p$	0.6
IX[e]	75–79	$(pT)_8$; $(pT)_6p$	0.4
X[e]	80–92		0.4

[a] The procedure is described in detail in the Experimental Section. [b] After alkaline phosphatase treatment the $(pT)_n$ products can be separated from the $(pT)_np$ products by paper chromatography in solvent system C. The relative distribution of each $(pT)_n$ and $(pT)_np$ product in the different peaks is approximately 70 and 30%, respectively. For complete product characterization see Experimental Section. [c] The calculation of yield is based on the numbers of OD units in every peak of Figure 4. It was not corrected for hypochromicity. [d] This compound contains an even mixture of 3′–5′- and 5′–5′-dinucleoside monophosphate. [e] About 5% of the oligonucleotides from peak V to X contain at least one 5′–5′ phosphodiester linkage as shown by their resistance to spleen diesterase after monoesterase treatment. See Experimental Section.

Polymerization of Deoxyadenosine 5′-Phosphate with β-Imidazolyl-4(5)-propanoic Acid. It was of interest to see whether the polymerization process reported above could also be applied to the other deoxynucleotides, especially the 2′-deoxypurine nucleoside 5′-phosphates. Thus, unprotected deoxyadenosine 5′-phosphate was refluxed in *N,N*-dimethylformamide for 30 min in the presence of β-imidazolyl-4(5)-propanoic acid. The reaction mixture was chromatographed on a DEAE-cellulose column in the bicarbonate form. The elution pattern is shown in Figure 5 and the distribution of the nucleotide material is shown in Table IV. Compared to the same polymerization procedure for thymidine 5′-phosphate, the yield (33%) of oligonucleotide material was lower by about 40%. A large amount of the starting material (40%) was degraded to adenine and deoxyadenosine during the reaction. Peak VI of Figure 5 was found to contain P^1,P^2-dideoxyadenosine 5′-pyrophosphate. This observation again suggests pyrophosphate as an intermediate in the polymerization reaction, and that the polymerization of deoxyadenosine 5′-phosphate follows the same reaction mechanism as that of thymidine 5′-phosphate. This suggestion was supported by the experiments described in the subsequent section. It is noteworthy that a spectrophotometric investigation of all products did not indicate the formation of phosphoramidates. The materials in peak III and peaks V–X of Figure 5 exhibited uv spectral properties, which are typical for deoxyadenosine oligonucleotides, judging from their absorption maxima and the 280/260 mμ ratio of extinction values. If the materials in the peaks contained phosphoramidates, a red shift of the absorption maxima and an increase of the 280/260 mμ ratio should be

Table IV.[a] Polymerization of Deoxyadenosine 5′-Phosphate by β-Imidazolyl-4(5)-propanoic Acid

Peak no.	Fraction no.	Composition[b]	Yield,[c] %
I	2–6	Deoxyadenosine	24.0
II	9–14	Adenine	16.5
III	17–18	β-Imidazolyl-4(5)-propanoic acid	
IV	19–22	dApdA	1.0
V	29–32	pdA	13.0
VI	33–35	O<$(pdA)_2$	4.0
VII	42–49	$(pdA)_2$	6.5
VIII	51–56	pdAp; $(pdA)_3$	15.4
IX[d]	63–69	$(pdA)_2$; $(pdA)_4$	8.0
X[d]	74–80	$(pdA)_3p$; $(pdA)_5$	3.9
XI[d]	84–90	$(pdA)_4p$; $(pdA)_6$	1.7

[a] The procedure is described in detail in the Experimental Section. [b] After alkaline phosphatase treatment the $(pdA)_n$ products can be separated from the $(pdA)_np$ products by paper chromatography in solvent system C. The relative distribution of each $(pdA)_n$ and $(pdA)_np$ product in the different peaks is approximately 70 and 30%, respectively. For complete product characterization see Experimental Section. [c] The calculation of yield is based on the number of OD units in every peak of Figure 5. It was not corrected for hypochromicity. [d] About 5% of the oligonucleotides from peak IX to X contain at least one 5′–5′ phosphodiester linkage as shown by their resistance to spleen diesterase after monoesterase treatment. See Experimental Section.

expected which, however, was not observed. Also, the paper chromatographic and enzyme degradation studies did not indicate the presence of phosphoramidates.

Synthesis of Deoxyadenosine Oligonucleotides by the Use of P^1,P^2-Di-*N*-benzoyldeoxyadenosine 5′-Pyrophosphate. A solution of the pyridinium salt of P^1,P^2-di-*N*-benzoyldeoxyadenosine pyrophosphate was refluxed for 30 min in *N*,*N*-dimethylformamide. After removal of the *N*-benzoyl group the reaction mixture was subjected to paper chromatographic analysis and enzymatic degradation studies. Formation of deoxyadenosine oligonucleotides was observed, though the yield (10–15%) was not as high as in the polymerization reaction of deoxyadenosine 5′-phosphate with β-imidazolyl-4(5)-propanoic acid.

The Use of Pyrophosphate for Dimer Synthesis. The above study indicates that it would be possible to synthesize dinucleoside monophosphates by this reaction. Therefore, a solution of the pyridinium salt of P^1,P^2-dithymidine 5′-pyrophosphate in *N*,*N*-dimethylformamide was refluxed in the presence of thymidine (2 equiv). The concentration of P^1,P^2-dithymidine 5′-pyrophosphate employed in this reaction was ten times lower than that in the polymerization experiments. Similarly, a solution of P^1,P^2-dithymidine 5′-pyrophosphate in *N*,*N*-dimethylformamide was refluxed in the presence of 5′-*O*-acetylthymidine. In addition, a solution of P^1,P^2-di-3′-*O*-acetylthymidine 5′-pyrophosphate was refluxed in the presence of 5′-*O*-acetylthymidine. The products were analyzed by means of paper electrophoresis, chromatography, and enzymatic degradation. The results are summarized in Table V. As shown, the pyrophosphate can be used for dinucleotide synthesis by simply heating a mixture of pyrophosphate and nucleoside.

Table V. Reaction between P_1,P_2-Dithymidine 5′-Pyrophosphate and Nucleosides[a]

Pyrophosphate	Nucleoside (2 equiv)	Formation[b] of % Dimer	Formation[b] of % Oligonucleotide
P^1,P^2-Dithymidine 5′-pyrophosphate		18[c]	26.4
	Thymidine	33[d]	
	5′-*O*-Acetylthymidine	25	
P^1,P^2-Di(3′-*O*-acetylthymidine) 5′-pyrophosphate	5′-*O*-Acetylthymidine	0[e]	0[e]

[a] The experiments were described in the Experimental Section. [b] The product distribution was calculated by means of paper chromatography in solvent system B and paper electrophoresis as described in detail in the Experimental Section. [c] This figure represents an approximately even mixture of thymidylyl-(3′–5′)-thymidine and thymidylyl-(5′–5′)-thymidine. [d] This figure represents a mixture of 17% thymidylyl-(3′–5′)-thymidine and 16% thymidylyl-(5′–5′)-thymidine. [e] Dimer and oligonucleotides could not be detected in the reaction mixture and about 20% of the starting pyrophosphate was degraded to thymidine 5′-phosphate.

Interestingly, in the case of P^1,P^2-di-3′-*O*-acetylthymidine 5′-pyrophosphate, a condensation reaction was not observed. Also, this compound appeared to be more heat resistant. In addition, reactions were conducted in mixtures of the protected P^1,P^2-di-3′-acetylthymidine 5′-pyrophosphate and the unprotected P^1,P^2-dithymidine 5′-pyrophosphate (1:10; 1:5; 1:2). In all of these cases, the extent of dimer formation corresponded only to the amount of unprotected pyrophosphate present in the starting mixture.

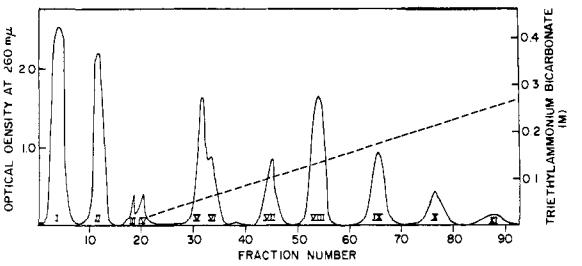

Figure 5. Chromatography on a DEAE–cellulose (bicarbonate) column of deoxyadenosine oligonucleotides (total polymeric mixture), obtained after heating deoxyadenosine 5′-phosphate in the presence of β-imidazolyl-4(5)-propanoic acid in *N*,*N*-dimethylformamide. The broken line shows the triethylammonium bicarbonate gradient. The product distribution is given in Table IV.

Degradation of pTpT in *N*,*N*-Dimethylformamide at Reflux Temperature. The above studies suggest that a degradative process is also occurring in the reaction. In order to characterize this process more precisely, the degradation of pTpT was studied. Two milligrams of pTpT (ammonium salt) was refluxed in 5 ml of *N*,*N*-dimethylformamide for 30 min. Analysis showed that about 40% of the pTpT broke down to thymidine 5′-phosphate. Only faint traces of thymidine, thymidylyl-(3′–5′)-thymidine, and thymidine 3′,5′-diphosphate could be detected by paper chromatography. Thymidine 3′-phosphate was not found.

Discussion

Results in this study indicate that both unprotected thymidine 5′-phosphate and deoxyadenosine 5′-phosphate can be polymerized at elevated temperature, and this process is catalyzed by proton(s) or a proton donor. β-Imidazolyl-4(5)-propanic acid, the compound originally employed,[9] and triethylammonium chloride are the most effective catalysts so far investigated. In studying the time course of the reaction, the immediate formation and the subsequent decay of P^1,P^2-dithymidine 5′-pyrophosphate were observed. The pattern of the formation and decay of this compound in relation to the disappearance of the starting mononucleotide and the appearance of the oligonucleotide products (Figure 2) strongly suggests that this type of P^1,P^2-dinucleoside 5′-pyrophosphate is a key reaction intermediate in this polymerization process. This suggestion is further supported by the following two observations. (1) Heating P^1,P^2-dithymidine 5′-pyrophosphate or P^1,P^2-di-*N*-benzoyldeoxyadenosine 5′-pyrophosphate in *N*,*N*-dimethylformamide led to effective formation of oligonucleotide. (2) Heating of P^1,P^2-dithymidine 5′-pyrophosphate with thymidine or 5′-*O*-acetylthymidine led to the formation of the expected dinucleoside monophosphates. It is not certain whether the solvent *N*,*N*-dimethylformamide plays an additional role in this synthetic reaction, since heating P^1,P^2-dithymidinine 5′-pyrophosphate in anhydrous pyridine leads only to degradation of the pyrophosphate without concomitant formation of oligonucleotides (Figure 3). For example, the reaction product of *N*,*N*-dimethyl-

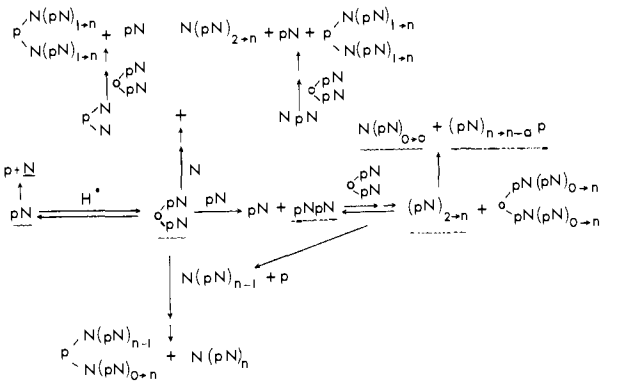

Figure 6. The overall reaction scheme of the nucleoside 5′-phosphate dissolved in *N*,*N*-dimethylformamide, and catalyzed by acid at reflux temperature. Double arrows (→→) mean multiple steps; all the major components (reactants, intermediates, and products) are underlined; the reactant in a particular synthetic reaction is indicated alongside the arrow; the subscript $0 \rightarrow n$ means 0, 2, 3 . . . n, or $n \rightarrow n - a$ means $n, n-1, n-2 \ldots n-a$.

formamide and phosgene[10] has been used as a reagent for oligonucleotide synthesis. On the other hand, we have observed that dioxane can also be used effectively as solvent for the polymerization of thymidine 5′-phosphate catalyzed by β-imidazolyl-4(5)-propanoic acid.[9]

Separation and characterization of the reaction products were done by column chromatography, paper chromatography, and electrophoresis, and by different enzymic degradations, procedures which have been used very effectively by Khorana and his associates. Generally, two main series of oligonucleotides were found: $(pN)_n$ and $(pN)_np$. A common property of all the $(pN)_np$ compounds, which indicates the presence of two phosphomonoester end groups per molecule, is the striking increase in mobility on paper chromatography after treatment with alkaline phosphatase. In fact, this property was used for the separation of the compounds of this series from the $(pN)_n$ compound. For example, while $(pT)_3$ and pTp were eluted together from the column (Table II, peak IV), the two compounds can be separated and identified as $T(pT)_2$ and T after enzymic removal of the phosphomonester groups. In addition, the presence of a small amount (5–10% at peaks IV–VI in Figure 4) of $N(pN)_n$ (or less likely $N(pN)_np$) is indicated by the fact that a small amount of the oligonucleotides could be degraded by spleen phosphodiesterase even prior to alkaline phosphatase treatment. About 90% of these oligonucleotide series contain only the (3′–5′) linkages. About 10% or less of these oligonucleotides contain at least one (5′–5′) phosphodiester linkage (or pyrophosphate linkage); Therefore they are resistant to spleen phosphodiesterase. The presence of oligonucleotides containing at least one (3′–3′) phosphodiester linkage has not been detected, since no material has been found to be resistant to snake venom phosphodiesterase.

In addition to the synthetic process, degradative processes also take place under conditions of high temperature. Two processes are most obvious and prominent. The first one is breakage of the (3′–5′) internucleotidyl linkage best illustrated by heating pTpT in the *N*,*N*-dimethylformamide. In this process, the breakage can occur at the P–O(3′) bond to give pT, and at the P–O(5′) bond to give T and pTp. In this experiment, the data indicate that this first route, *i.e.*, the breakage of the P–O(3′) bond, is much more prominent. However, in view of the occurrence of the $(pN)_np$ series, breakage of P–O(5′) bond must also occur in the $(pN)_n$ series during the polymerization reaction. The formation of thymidine 3′-phosphate is not detected in the degradation of pTpT since it requires the breakage of two P–O bonds. The second degradative process is the dephosphorylation of the 5′-phosphate indicated by the formation of nucleosides (Table III and Table IV), and the formation of the $N(pN)_n$ series. These observations show that under the present reaction condition, both the P–O(3′) and the P–O(5′) bond are vulnerable to cleavage. Since the degradative products can and do participate in the synthetic reaction, their contributions as reactants in the overall process have to be carefully considered.

From the above discussion, the scheme shown in Figure 6 is proposed to describe the various reactions in the overall process at high temperature. The main components in the reactions, either as starting material (pN), or as key intermediate $O<(pN)_2$, or as products (pNpN, $(pN)_{2\rightarrow n}$, $(pN_{n-1}p)$, are underlined. The dominant synthetic reaction is the formation of the P^1,P^2-dinucleosidyl 5′-pyrophosphate from the starting unprotected nucleoside 5′-phosphate catalyzed by protons, and the subsequent reaction of this pyrophosphate intermediate either with itself or with nucleoside 5′-phosphate to yield oligonucleotides. According to this scheme, only two types of internucleotidyl linkages can be formed: the naturally occurring (3′–5′) phosphodiester linkage, and the (5′–5′) pyrophosphate linkage. Since the pyrophosphate linkage is much more reactive and less stable, the oligonucleotides containing the (3′–5′) phosphodiester linkage should be the predominant species as observed. Besides the synthetic reactions, there are two major degradative processes. The first one is the breakdown of the (3′–5′) phosphodiester linkage of the oligonucleotides $(pN)_{2\rightarrow n}$; this degradation can take place at the P–O(3′) bond to give a series of pN, pN_{n-1}, pN_{n-2}, etc., or at the P–O(5′) bond to give $(pN_{n-1})p + N$ (at the end) or $(pN)_{n-aP} + N(pN)_{n-1}$ (at the interior). As discussed above, the breakage of the P–O(3′) bond is much more prevalent but the breakage of the P–O(5′) bond does occur and the products of this process can accumulate. The second degradative process is the breakage of the P–O(5′) bond of the nucleoside 5′-phosphate to give the nucleoside. Since the nucleoside 5′-phosphate is the starting material, this formation of nucleoside can be substantial. In the case of purine deoxynucleotide, the adenine deoxynucleotide can be degraded to adenine as well, due to the presence of protons, since the glycosyl bond of this compound is sensitive to acid hydrolysis. At present, the dephosphorylation reaction has been substantially reduced as compared to the earlier report; this was achieved by reduction of the acidity and moisture in the reaction mixture.

The nucleoside (N) and series of $N(pN)_n$ compounds formed in the degradation possess an unprotected 5′-hydroxyl group. These compounds, therefore, upon reaction with the P^1,P^2-dinucleoside pyrophosphate can

(10) T. M. Jacob and H. G. Khorana, *J. Amer. Chem. Soc.*, **86**, 1630 (1964).

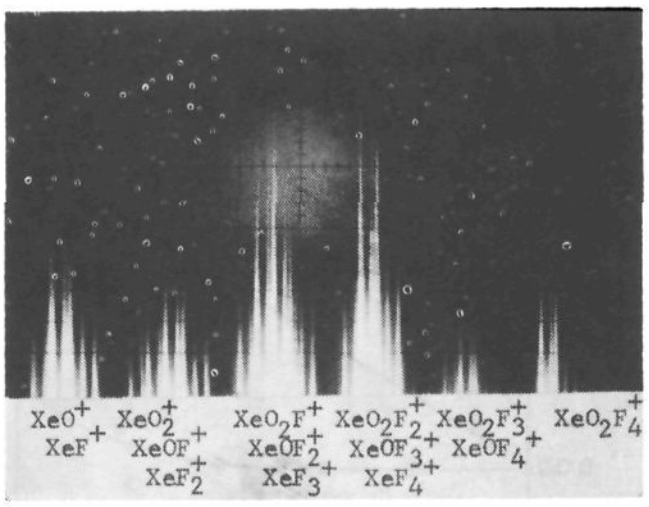

Figure 1. The mass spectrum of XeO_2F_4; source pressure, 2×10^{-6} Torr; ionizing electron energy, 70 eV.

made by the reaction $XeO_3F_2 + XeF_6 \rightarrow XeOF_4 + XeO_2F_4$ and identified by mass spectroscopy. It is the most volatile of the known xenon compounds and may therefore possess the symmetrical, nonpolar, D_{4h} symmetry predicted by Gillespie.[1] An attempt will be made to verify this supposition by obtaining the compound's vibrational spectra.

XeO_3Fe, which originally was made by the reaction of XeF_6 with Na_4XeO_6,[2] is made[3] in much better yield by the reaction of XeF_6 (in a Kel-F system) with XeO_4. It is destroyed by more than brief contact with the XeF_6, being converted to $XeOF_4$. While thus preparing XeO_3F_2 for a study of its vibrational spectra,[4] an extremely small xenon pattern corresponding to $XeO_2F_4^+$ was observed in its mass spectrum; apparently XeO_3F_2 is fluorinated by XeF_6 to XeO_2F_4 which then decomposes rapidly in the presence of XeF_6 to $XeOF_4$ and O_2. By conducting this reaction in solvent $XeOF_4$ it was possible to increase sufficiently the steady-state amount of XeO_2F_4 to allow a sufficient quantity to be separated and identified (Figure 1) by its qualitative mass spectrum.

In addition to the parent molecule ion all fragment ions to be expected from XeO_2F_4 are observed, and no other, permitting unambiguous identification of the compound. A notable feature of the spectrum is the relative abundance of the molecule ion which, unlike the molecule ions of all other xenon compounds, is more intense than the fragment ions formed by removal of one F or one O. The smallest ion in the spectrum is XeF_4^+ and the largest is $XeOF_3^+$.

The yield of XeO_2F_4 from this reaction is quite small but no other fluorinating agents have been found to make the compound. ClF_3 and ClF_5 react as reducing agents with XeO_3F_2, being oxidized to ClO_3F with formation of lower xenon compounds. SbF_5 and IF_7 apparently form solid adducts with XeO_3F_2, but no XeO_2F_4. *FSO_2OH was not observed to react with XeO_4.*

Solid samples of XeO_3F_2 and XeO_2F_4 give mass spectra of comparable intensity when the XeO_2F_4 is at a temperature about 20° lower than the temperature of the XeO_3F_2, showing the greater volatility of XeO_2F_4. This difference in volatility is sufficient to allow separation by fractional distillation. Residual XeO_3F_2 can then be combined with XeF_6 and XeO_4 to make more XeO_2F_4. A background of $XeOF_4$ was observed in the Kel-F line after distillation of XeO_2F_4, indicating some decomposition of XeO_2F_4 to $XeOF_4$, even in the absence of XeF_6.

(1) R. J. Gillespie in "Noble Gas Compounds," H. Hyman, Ed., University of Chicago Press, Chicago, Ill., 1963, p 334.
(2) J. L. Huston, *Inorg. Chem. Lett.*, **4**, 29 (1967).
(3) J. L. Huston, Abstracts, 160th National Meeting of the American Chemical Society, Chicago, Ill., Sept 1970, No. INOR 149.
(4) H. H. Claassen and J. L. Huston, *J. Chem. Phys.*, **55**, 1505 (1971).

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Oxidation of Organic Compounds with Cerium(IV). XIV. Formation of α-Azido-β-nitratoalkanes from Olefins, Sodium Azide, and Ceric Ammonium Nitrate[1]

Sir:

It has been known for some time that ceric salts oxidize metallic azides to nitrogen quantitatively.[2] The azido radical has been suggested as an intermediate[2c] and there seems to be little doubt that the azido radical exists since it has been observed spectroscopically during

$$Ce^{IV} + N_3^- \longrightarrow Ce^{III} + N_3\cdot$$

$$2N_3\cdot \longrightarrow 3N_2$$

flash photolysis of aqueous sodium azide solutions.[3] The possibility of the intermediacy of the azido radical during the reaction of cerium(IV) and azide ion led us to attempt to trap the azido radical by carrying out the reaction in the presence of olefins.

The oxidation of sodium azide by ceric ammonium nitrate (CAN) in acetonitrile has been shown to yield quantitatively stochiometric amounts of nitrogen.[4] The addition of an olefin causes an almost complete cessation of gas evolution and the products isolated have azide and nitrate functional groups as inferred from their ir spectra (2110 and 1645 cm^{-1}, respectively[5]). Cyclohexene, 1-methylcyclohexene, 1,3,5-cycloheptatriene, norbornene, α-pinene, α-methylstyrene, stilbene, 2,4-dimethylbutadiene, and 1,4-diphenylbutadiene are some of the olefins which suppress gas evolution. Gas evolution is not suppressed by diethyl fumarate, maleic anhydride, 4,4-dimethylcyclohexenone, phenylacetylene, 2-methyl-3-butyn-2-ol, *trans*-cinnamic acid, or cholesterol (perhaps due to low solubility in the reaction mixture). These unsaturated compounds can be recovered intact.

(1) (a) Part XIII: W. S. Trahanovsky, M. D. Robbins, and D. Smick, *J. Amer. Chem. Soc.*, **93**, 2086 (1971). (b) This work was partially supported by Public Health Service Grant No. GM 13799 from the National Institute of General Medical Sciences and Grant No. GP-18031 from the National Science Foundation. We are grateful for this support. (c) Based on work by M. D. R. in partial fulfillment of the requirements for the Ph.D. degree at Iowa State University.
(2) (a) E. Sommer and H. Pincas, *Chem. Ber.*, **48**, 1963 (1915); (b) J. Martin, *J. Amer. Chem. Soc.*, **49**, 2133 (1927); (c) L. F. Audrieth, *Chem. Rev.*, **15**, 169 (1934); (d) J. W. Arnold, *Ind. Eng. Chem. Anal. Ed.*, **17**, 215 (1945); (e) Y. Mizushima and S. Nagayama, *Kogyo Kayaku Kyokai-shi*, **17**, 113 (1956); (f) E. L. Grove, R. S. Braman, H. F. Combs, and S. R. Nicholson, *Anal. Chem.*, **34**, 682 (1962).
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(4) G. A. Ward and C. M. Wright, *J. Electroanal. Chem.*, **8**, 302 (1964).
(5) K. Nakanishi, "Infrared Absorption Spectroscopy," Holden-Day, San Francisco, Calif., 1962.

yield of dimer to hexamer of pyrimidine oligonucleotides is reasonable (12–5% of each species, Table II). The prospect of this thermally activated system as a synthetic procedure for purine homodeoxyoligonucleotides starting from the unprotected 5′-mononucleotides is not as good, since the glycosyl bond in the purine nucleotides is more susceptible to the acid-catalyzed hydrolysis at high temperature.

Finally, as mentioned in our previous communication, the study of this polymerization process may provide additional understanding about prebiotic synthesis of polynucleotides. Under simple conditions of heat and acid in a medium of organic solvent, significant amounts of oligonucleotides with (3′–5′) linkage can be formed from the unprotected 5′-mononucleotides. This observation points to a possible way for a primitive system for prebiotic polynucleotide synthesis.

Experimental Section

General Methods. Column chromatography was carried out with Isco and LKB fraction collectors. The elution profiles were recorded by the Isco ultraviolet analyzer Model UA-2 which gives linear response to absorbance less than 2.5. Fractions in these peaks were pooled and measured in a uv spectrophotometer. Paper chromatography was carried out by the descending technique on S and S paper 2043b. The solvent systems were: solvent A, isopropyl alcohol–concentrated ammonium hydroxide–water (7:1:2, v/v); solvent B, ethanol–1 *M* ammonium acetate (7:3, v/v); solvent C, 1-propanol–concentrated ammonium hydroxide–water (55:10:35, v/v); solvent D, 1-propanol–2 *N* HCl (3:1, v/v). Thin-layer chromatography was carried out by the ascending technique with Eastman chromatogram sheets 6060 (silica gel with fluorescent indicator). Paper electrophoresis was performed using Whatman 3MM paper in a Savant Flat Plate electrophoresis chamber with a Savant Model HV power supply operated at 2000 V for 1 hr; the solution was buffered at pH 8.0 with 0.002 *M* triethylammonium bicarbonate. Nucleosides and their derivatives were observed on paper strips and thin-layer sheets under ultraviolet light. Compounds containing the *p*-dimethoxytrityl group were detected by spraying the papers or thin-layer sheets with 10% perchloric acid and drying them in a stream of warm air.[12] Silica gel (60–200 mesh) from Baker Chemical Co. was used for column chromatography. The eluent was monitored by thin-layer chromatography.

Ultraviolet spectra were obtained on a Cary 15 recording spectrophotometer. The term OD unit refers to the extinction of the nucleotide material in 1 ml of neutral solution in a quartz cell with a 1-cm light path.

Reagents and Chemicals. Reagent grade pyridine was distilled from *p*-toluenesulfonyl chloride, redistilled from potassium hydroxide, and stored over Fisher Type 5A molecular sieves. Dimethylformamide (Fisher, infrared spectranalyzed) was stored over Fisher Type 5A molecular sieves. Nucleosides and nucleotides were purchased from Sigma Chemical Co. 5′-*O*-Acetylthymidine,[13] 5′-*O*-dimethoxytritylthymidine,[13] 3′-*O*-acetylthymidine 5′-phosphate,[14] *N*-benzoyldeoxyadenosine 5′-phosphate,[15] P_1,P_2-dithymidine 5′-pyrophosphate,[16] P^1,P^2-di(3′-*O*-acetylthymidine 5′-pyrophosphate,[16] and P_1,P_2-di(*N*-benzoyldeoxyadenosine) 5′-pyrophosphate[16] were prepared as described in the literature.

The nucleotides were used in the form of their disodium salts and the pyrophosphates in the form of their pyridinium salts. Histidine (free base, A grade), dihydrourocanic acid (A grade), urocanic acid·H_2O (A grade), imidazoleacetic acid (hydrogen chloride, A grade), and 4-hydroxymethylimidazole (hydrogen chloride, A grade) were purchased from Calbiochem, Los Angeles, Calif. 1-Carbethoxyimidazole was purchased from Aldrich Chemical Co., Milwaukee, Wis., and 4,5-imidazoledicarboxylic acid and imidazole from Eastman Chemicals, Rochester, N. Y.

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(13) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958).
(14) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).
(15) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2932 (1961).
(16) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **81**, 4660 (1959).

Oligonucleotide Synthesis. The reactions were carried out in dry *N,N*-dimethylformamide under reflux conditions. A small Soxhlet extraction apparatus (25-ml flask) was used. The extraction thimble (10 × 50 mm) was filled with Fisher Type 5A molecular sieves which were used to remove the water formed due to the synthesis of the phosphodiester bonds. A drying tube filled with calcium chloride was attached on top of the apparatus in order to exclude moisture from the outside.

Polymerization of Thymidine 5′-Phosphate with Imidazole Derivatives. The disodium salt of thymidine 5′-phosphate (0.25 m*M*) was suspended in 10 ml of dry *N,N*-dimethylformamide together with 2 equiv of the imidazole derivative (0.5 m*M*). The mixture was refluxed for 30 min in the Soxhlet apparatus. The reaction flask was then chilled in an ice bath. A pyridine–ice mixture (10 ml) was added and the solution was evaporated under reduced pressure. The residue was taken up in water (2 ml), adjusted to pH 8 with 2 *N* ammonium hydroxide, and subjected to the product analysis by means of paper chromatography and/or DEAE-cellulose chromatography (see Table I). The material was applied on top of a DEAE-cellulose column (15 × 30 cm) in the bicarbonate form. The column was washed with water (100 ml) and then eluted with 4 l. of triethylammonium bicarbonate salt gradient (0.0–0.4 *M*). Fractions (18 ml) were collected at approximately 15-min intervals. The fractions of the different peaks were pooled, and were evaporated to a gum under reduced pressure and temperature. In order to remove the residual triethylammonium bicarbonate, the residue was dissolved in water and the solution reevaporated. This process was repeated several times. The remaining material as triethylammonium salts was passed through small columns of Dowex 50-X resin in ammonium form for conversion to the ammonium salts. These final solutions were concentrated, lyophilized, and characterized.

Polymerization of Thymidine 5′-Phosphate with Triethylamine Hydrochloride. The disodium salt of thymidine 5′-phosphate (0.2 m*M*) was suspended in 10 ml of dry *N,N*-dimethylformamide. Freshly distilled triethylamine (0.5 ml) was added together with 0.05 ml of anhydrous dioxane previously saturated with dry hydrogen chloride. The mixture was refluxed for 30 min in the Soxhlet apparatus described above. The reaction flask was then chilled in an ice bath. A pyridine–ice mixture (10 ml) was added and the resulting solution was evaporated under reduced pressure. The residue was taken up in pyridine–water and reevaporated. The residual gum was taken up in water (2 ml), adjusted to pH 8 with 2 *N* ammonium hydroxide, and applied on top of a DEAE-cellulose column (1.5 × 30 cm) in the bicarbonate form. The column was washed with water (200 ml). Elution was carried out using a linear salt gradient of triethylammonium bicarbonate buffer pH 7.4. The mixing vessel contained 2 l. of water and the reservoir contained 2 l. of 0.5 *M* buffer. Fractions (18 ml) were collected at approximately 15-min intervals. The elution pattern is shown in Figure 1. The pooled fractions containing different peaks were evaporated to a gum under reduced pressure and at low temperature. The removal of the residual buffer and the conversion of the products to ammonium salt were carried out as described in the previous section. These final solutions were concentrated, lyophilized, and characterized. The distribution of the nucleotide material in the different peaks of the elution pattern is shown in Table II.

Polymerization of P^1,P^2-Dithymidine 5′-Pyrophosphate. The pyridinium salt of P^1,P^2-dithymidine 5′-pyrophosphate (0.4 m*M*) was dissolved in 10 ml of dry *N,N*-dimethylformamide. The solution was refluxed for 30 min in the Soxhlet apparatus, and then chilled in an ice bath. A pyridine–ice mixture (10 ml) was added and the solution was evaporated under reduced pressure. This procedure was repeated twice. Finally, the residue was taken up in 2 ml of water, adjusted to pH 8 with 2 *N* ammonium hydroxide, and applied on top of a DEAE-cellulose column (1.5 × 30 cm) in the bicarbonate form. The elution and work-up was carried out as described above. The elution pattern obtained is shown in Figure 4. The distribution of nucleotide material in the different peaks of the elution pattern is shown in Table III.

Polymerization of Deoxyadenosine 5′-Phosphate with β-Imidazolyl-4(5)-propanoic Acid. The disodium salt of deoxyadenosine 5′-phosphate (0.25 m*M*) was suspended in 10 ml of dry *N,N*-dimethylformamide together with 2 equiv of β-imidazolyl-4(5)-propanoic acid (0.5 m*M*). The mixture was refluxed for 30 min in the Soxhlet apparatus and then chilled in an ice bath. A pyridine–ice mixture (10 ml) was added and the solution was evaporated under reduced pressure. This procedure was repeated twice. Finally, the residue was taken up in water (2 ml) adjusted to pH 8 with 2 *N* ammonium hydroxide, and applied on top of a DEAE-

cellulose column (1.5 × 30 cm) in the bicarbonate form. Elution and work-up were carried out as described above. The elution pattern obtained is shown in Figure 5. The distribution of nucleotide material in the different peaks is shown in Table IV.

Polymerization of P^1,P^2-Di-*N*-benzoyldeoxyadenosine 5′-Pyrophosphate. The pyridinium salt of P^1,P^2-di-*N*-benzoyldeoxyadenosine 5′-pyrophosphate (0.1 m*M*) was dissolved in 10 ml of dry *N,N*-dimethylformamide. The solution was refluxed for 30 min in the Soxhlet apparatus, and then chilled in an ice bath. A pyridine–ice mixture (10 ml) was added and the solution was evaporated under reduced pressure. The residue was taken up in 4 ml of concentrated ammonia and kept at room temperature for 48 hr. The solution was evaporated to dryness and the residue was taken up in 2 ml of water. It was subjected to paper chromatographic and paper electrophoretic analysis.

Reaction of P^1,P^2-Dithymidine 5′-Pyrophosphate with Thymidine. The pyridinium salt of P^1,P^2-dithymidine 5′-pyrophosphate (0.04 m*M*) was dissolved in 10 ml of dry *N,N*-dimethylformamide. Thymidine (0.08 m*M*) was added and the mixture was refluxed for 30 min in the Soxhlet apparatus and then chilled in an ice bath. A pyridine–ice mixture was added (10 ml) and the reaction mixture was evaporated under reduced pressure. The residue was taken up in water (2 ml) and analyzed by paper chromatography in solvent system B, by paper electrophoresis, and by enzymatic degradation studies.

Reaction of P^1,P^2-Dithymidine 5′-Pyrophosphate with 5′-*O*-Acetylthymidine. The pyridinium salt of P^1,P^2-dithymidine 5′-pyrophosphate (0.04 m*M*) was dissolved in 10 ml of dry *N,N*-dimethylformamide. 5′-*O*-Acetylthymidine (0.08 m*M*) was added and the mixture was refluxed for 30 min in the Soxhlet apparatus, then chilled in an ice bath. A pyridine–ice mixture was added (10 ml) and the reaction mixture was evaporated under reduced pressure. The residue was dissolved in 3 ml of 1 *N* sodium hydroxide and kept for 1 hr at room temperature to remove the acetyl group. Dowex 50-X resin (pyridinium form) was then added until the pH dropped to neutrality. The solution was then filtered from the resin and the latter washed thoroughly with water. The total aqueous solution was concentrated at low temperature under reduced pressure to ~2 ml. The material was investigated by paper chromatography in solvent system B, by paper electrophoresis, and by enzymatic degradation studies. The results are reported in Table V.

Reaction of P^1,P^2-Di(3′-*O*-acetylthymidine) 5′-Pyrophosphate with Thymidine. The pyridinium salt of P^1,P^2-di(3′-*O*-acetylthymidine) 5′-pyrophosphate (0.04 m*M*) was dissolved in 10 ml of dry *N,N*-dimethylformamide. Thymidine (0.08 m*M*) was added and the mixture was refluxed for 30 min in the Soxhlet apparatus and then chilled in an ice bath. A pyridine–ice mixture was added (10 ml) and the reaction mixture was evaporated under reduced pressure. The residue was dissolved in 3 ml of 1 *N* sodium hydroxide and kept for 1 hr at room temperature to remove the acetyl group. Dowex 50-X resin (pyridinium form) was added until the pH was lowered to neutrality. The solution was then filtered from the resin and the latter washed thoroughly with water. The total aqueous solution was concentrated at low temperature to ~2 ml. The material was investigated by paper chromatography in solvent system B, by paper electrophoresis, and by enzymatic degradation studies. The results are given in Table V.

Time Dependence of the Reaction of Thymidine 5′-Phosphate with β-Imidazolyl-4(5)-propanoic Acid. The reaction mixtures consisted of the disodium salt of thymidine 5′-phosphate (0.1 m*M*) and β-imidazolyl-4(5)-propanoic acid (0.2 m*M*) in 10 ml of dry *N,N*-dimethylformamide. The reaction mixtures were refluxed in the usual way for 1, 5, 10, 15, 20, 30, and 35 min. After these time intervals they were chilled in an ice bath and worked up as described above. The product formation (pyrophosphate and oligonucleotides) was investigated by paper chromatography in solvent system B and by paper electrophoresis; the results are summarized in Figure 2.

Time Dependence of the Degradation of P^1,P^2-Dithymidine 5′-Pyrophosphate. The reaction mixtures consisted of a solution of the pyridinium salt of P^1,P^2-dithymidine 5′-phosphate (0.02 m*M*) in anhydrous pyridine (10 ml) and *N,N*-dimethylformamide (10 ml). The reaction mixtures were refluxed in the usual way for 1, 2, 5, 10, 15, and 30 min. After these time intervals they were chilled in an ice bath and worked up as described above. The decrease of the starting material was investigated by paper chromatography in solvent system B and by paper electrophoresis. The results are summarized in Figure 3.

Degradation of pTpT in *N,N*-Dimethylformamide at Reflux Temperature. Two milligrams of pTpT (ammonium salt) was refluxed in 5 ml of *N,N*-dimethylformamide for 30 min. The reaction mixture was cooled and evaporated to dryness. Then it was taken up in a small amount of water and subjected to paper chromatography in solvent system C. The spot, corresponding to thymidine 5′-phosphate, was cut out. The paper strip was eluted with water and the resulting solution was then subjected to paper chromatography in solvent system D. In system D, thymidine 3′-phosphate has a relative mobility to thymidine 5′-phosphate of 0.88, and therefore can be separated from the 5′-nucleotide.

Enzyme Assays. In general, the procedure described in a previous communication was followed.[9] (a) For the removal of terminal phosphate by bacterial alkaline phosphatase, lyophilized bacterial alkaline phosphatase (5 mg) obtained from Worthington (BAPF quality) was dissolved in 50 ml of 0.05 *M* Tris buffer, pH 8. An aliquot of this solution (5 μl) was added to the substrate containing 0.1 μmol of terminal phosphate in 0.03–0.04 ml of 0.05 *M* Tris buffer, pH 8. The mixture was incubated at 37° for 4 hr and was then analyzed by paper chromatography developed in solvent C for 2–4 days. Nucleotide bands were cut out, eluted with buffer, and subjected to phosphodiesterase degradation.

(b) **Spleen Phosphodiesterase.** Lyophilized spleen phosphodiesterase (15 units) obtained from Worthington was dissolved in 1 ml of water. An aliquot of this solution (10 μl) was added to 10 μl of an oligonucleotide solution containing 0.1 μmol of nucleoside unit and 0.25 *M* sodium succinate, pH 6.5. The mixture was incubated at 37° for 8 hr and then analyzed by paper chromatography in solvent A. Nucleotide bands were cut out, eluted with water, and diluted to a volume of 2 ml. Absorbances were read at the extinction maximum of each nucleotide material. The absorbance readings were corrected by appropriate blank readings.

(c) **Snake Venom Phosphodiesterase.** Lyophilized snake venom diesterase (5 mg) obtained from Worthington was dissolved in 1 ml of water. An aliquot of this solution (20 μl) was added to the oligonucleotide solution containing 0.1 μmol of nucleoside units in 10 μl of 1 *M* ammonium carbonate buffer, pH 9.0. The mixture was incubated at 37° for 8 hr. The solution was then worked up in the same manner as described for the spleen enzyme.

Characterization of Products. All compounds synthesized in this report have already been described in the literature. Therefore, the products can be sufficiently characterized by means of paper chromatography and electrophoresis, ultraviolet absorption spectroscopy, melting point, if possible, and by enzymatic degradation. All peaks of Figures 1, 4, and 5, which amounted to more than 1% of the overall yield, have been subjected to enzymatic degradation studies. For the enzymatic degradation studies 5 OD of material or more, if possible, was used. The degradation studies were generally carried out as follows: (a) treatment with alkaline phosphatase and then with snake venom phosphodiesterase; (b) treatment with alkaline phosphatase and then with spleen phosphodiesterase; (c) treatment with snake venom phosphodiesterase and then with alkaline phosphatase; (d) spleen phosphodiesterase. The paper chromatographic mobilities of all compounds of the $(pN)_n$ and $N(pN)_n$ series have been reported previously.[9] The methods of product characterization and chain-length determination have been well described in the literature and in our previous paper.[9] Therefore, only illustrative examples are given here.

Oligonucleotides of the general composition $(pN)_n$ were degraded by alkaline phosphatase to $N(pN)_{n-1}$, and the oligonucleotides of the general composition $(pN)_nP$ were degraded by alkaline phosphatase to $N(pN)_{n-1}$ also. However, in the chromatographic separation, $(pN)_n$ is usually located in the same area as $(pN)_{n-2}p$. Thus, after the alkaline phosphatase treatment of the materials in this same peak, the resulting products of $N(pN)_{n-1}$ and $N(pN)_{n-3}$ were readily separated. The separated oligomers were further degraded by phosphodiesterases to nucleoside and mononucleotide. The resulting mononucleotide–nucleoside ratio gave the information for the chain length of the oligomer. The $(pN)_n$, $N(pN)_n$, and $(pN)_nN$ compounds, which only contain (3′–5′) phosphodiester linkages, are completely degraded after alkaline phosphatase treatment by both the phosphodiesterases. All compounds containing other linkages are not, *i.e.*, compounds with (5′–5′) and (3′–3′) phosphodiester linkages, or compounds with pyrophosphate linkages and cyclic phosphates. Oligonucleotides with even just one (3′–3′) linkage[17] are totally resistant to the venom diesterase

(17) If an oligonucleotide contains both (3′–3′) and (5′–5′) linkages, then such an oligonucleotide will only be partially resistant but not

since this exonuclease requires a free 3′-hydroxyl end.[18,19] Such oligonucleotides are not resistant to spleen diesterase until this 5′ exonuclease reaches the dinucleotide containing the 3′–3′ linkage.[19] Similarly, oligonucleotides with one (5′–5′) linkage[17] (or one 5′–5′ pyrophosphate linkage) are totally resistant to the spleen diesterase since this exonuclease requires a free 5′-hydroxyl end.[20] Therefore, after the action of phosphomonoesterase, these two enzyme treatments are very sensitive procedures for the detection of the occurrence of at least one 3′–3′ linkage or 5′–5′ linkage of the oligonucleotides.

I. Characterization of Peaks III and IV of Figure 4, Table III. Peak III had an original R_f value of 0.86[21] in solvent system; after alkaline phosphatase treatment it had 1.26 and it degraded to a nucleoside:mononucleotide ratio of 0.91. Therefore, the material of this peak represented $(pT)_2$.[22] Peak IV had an original R_f[21] value of 0.70 in solvent system C; after alkaline phosphatase treatment it gave two spots of R_f[21] 1.60 and 1.12. The first spot had the same R_f as thymidine. The second spot (R_f 1.12) was degraded by phosphodiesterase to a mononucleotide:nucleoside ratio of 1.86. Therefore, the material of this peak represented $(pT)_3$[23] and pTp.

II. Characterization of the Mixture of (3′–5′)-TpT and (5′–5′)-TpT in Peak I of Figure 4. Treatment by spleen phosphodiesterase degraded only half of the material (R_f[21] in solvent system C, 1.36) to thymidine and thymidylic acid, while treatment with snake venom phosphodiesterase degraded the material completely. The thymidine:thymidylic acid ratio was in both cases close to 1. The electrophoresis at pH 7.5 showed two spots, which had a relative mobility to thymidine 5′-phosphate of 0.40 and 0.56, respectively. The slower moving spot was degradable by spleen phosphodiesterase, the faster moving spot by snake venom phosphodiesterase. These data indicated that peak I of Figure 4 corresponded to a mixture of (3′–5′)-TpT and (5′–5′)-TpT. Similar facts and conclusions were derived for peak I of Figure 1 and the data given in Table V.

III. Search for Oligonucleotides with (3′–3′) Phosphodiester Linkages. The (3′–3′) phosphodiester linkage is resistant to snake venom phosphodiesterase.[17] After alkaline phosphatase treatment all the oligonucleotide material was subjected to snake venom phosphodiesterase. Subsequent paper chromatographic analysis in solvent systems A and C did not show material which was resistant to snake venom phosphodiesterase. Therefore, the presence of oligonucleotides containing at least one (3′–5′) phosphodiester linkage, if any, must be below 2%, the present level of detection.

IV. Investigation of Oligonucleotides with (5′–5′) Phosphodiester Linkages. The oligonucleotides containing at least one (5′–5′) phosphodiester linkage (or (5′–5′) pyrophosphate linkage) are resistant to spleen phosphodiesterase. After alkaline phosphatase treatment all the oligonucleotide material was subjected to spleen phosphodiesterase. Subsequent paper chromatographic analysis in solvent systems A and C showed that approximately 5% of the material in peaks V–X of Figure 1, peaks V–X of Figure 4, and peaks IX–XI of Figure 5 were resistant to spleen phosphodiesterase. These observations indicated that about 5% of the oligonucleotides did contain at least one 5′–5′ phosphodiester linkage or one 5′–5′ pyrophosphate linkage. Since the pyrophosphate linkage is less stable at high temperature, therefore a majority of the 5′–5′ linkage formed in the reaction mixture under reflux condition is more likely to be the phosphodiester linkage.

V. Detection of P^1,P^2-Dithymidine 5′-Pyrophosphate. The presence of about 5% of the P^1,P^2-dithymidine 5′-pyrophosphate has been detected in polymerization experiments with thymidine 5′-phosphate. The pyrophosphate can be identified by its relative mobility to thymidine 5′-phosphate in paper chromatography and paper electrophoresis. The values are: 1.8 (solvent system A); 2.0 (solvent system B); 1.15 (solvent system C); 0.9 (paper electrophoresis, pH 7.5).

VI. The Presence of Unidentified Brownish Yellow Material(s) in the *N*,*N*-Dimethylformamide Reaction Mixture of Nucleoside 5′-Phosphate and β-Imidazolyl-4(5)-propanoic Acid. This colored material did not form if *N*,*N*-dimethylformamide was refluxed for 30 min in the presence of β-imidazolyl-4(5)-propanoic acid, and such a preheated mixture can be used for a successful polymerization of thymidine 5′-phosphate. In the absence of β-imidazolyl-4(5)-propanoic acid, such colored material also did not occur in the other heated reaction mixture.

Determination of Yields. When a DEAE-cellulose chromatography was carried out, the total number of OD units at the extinction maximum of the nucleotide material and the number of OD units in each pooled fraction were determined. From these figures the apparent yield of each product was calculated based on the percentage of absorbancy found in each fraction without correction for hypochromicity, since the extinction coefficients of these oligonucleotides have not been accurately determined. Therefore, the real yield of the oligonucleotide materials is probably a few per cent higher, especially for the adenine compounds. The recovery of total amount of added nucleotides after the reaction and column fractionating as indicated by optical density units at 260 m*M* has been quantitative, *i.e.*, above 90%. When a DEAE-cellulose chromatography was not carried out, an aliquot of the reaction mixture was analyzed by paper chromatography and/or electrophoresis (see footnotes of Tables I–V). Nucleoside and nucleotide bands were cut out, eluted with water, and diluted to a volume of 2 ml. Absorbances were read at a proper wavelength. The absorbance readings were corrected by appropriate blank readings. From these figures the yield of each product was calculated.

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totally resistant to venom or spleen diesterase. However, the occurrence of such an oligonucleotide is undoubtedly very rare.

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(21) All the R_f values reported here are relative to pT in the same run.

(22) The R_f of $(pT)_2$ relative pT in solvent C was cited to 0.90 in ref 14.

(23) The R_f of $(pT)_3$ relative pT in solvent C was cited to 0.76 in ref 14.