Studies on Polynucleotides. LV.[1] The Use of Mesitoyl Chloride in the Synthesis of Internucleotide Bonds[2]

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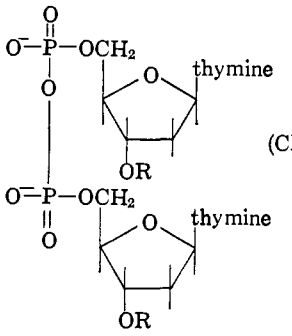
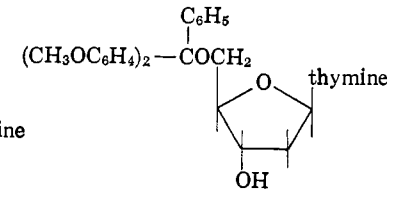
Abstract: The reaction of P^1,P^2-dithymidine-5′ pyrophosphate with benzoic anhydride or benzoyl chloride was shown to result in the formation of thymidine oligonucleotides. Following this discovery, the use of mesitoyl chloride for the stepwise synthesis of deoxyribooligonucleotides was investigated in detail. Condensation of equivalent amounts of 5′-O-dimethoxytritylthymidine and 3′-O-acetylthymidine-5′ phosphate, which was used as the pyrophosphate, gave a high yield of thymidylyl(3′→5′)thymidine. Condensation of 5′-O-tritylthymidine with the protected dinucleotide, 5′-O-phosphorylthymidylyl(3′→5′)-3′-O-acetylthymidine (pTpT-OAc) gave the trinucleotide in 43% yield whereas the condensation of pTpT-OAc with 5′-O-tritylthymidylyl(3′→5′)thymidine gave the expected tetranucleotide in 33% yield. In the corresponding experiment using 5′-O-tritylthymidylyl(3′→5′)-thymidylyl(3′→5′)thymidine and the dinucleotide, pTpT-OAc (fourfold excess), the yield of the pentanucleotide was 30%.

A study of the mechanism of the degradation of nucleotide pyrophosphates with carboxylic anhydrides and chlorides demonstrated the rapid initial formation of polyphosphate species.[1] It appeared possible that the intermediates thus generated would be sufficiently reactive so as to be of use in the synthesis of internucleotide bonds. This possibility has now been tested with considerable success and the results, which form the subject of this paper, show that a hindered carboxylic acid chloride such as mesitoyl chloride can serve as an alternative activating agent[3] in polynucleotide synthesis.

In initial experiments, P^1,P^2-dithymidine-5′ pyrophosphate (I) was treated with benzoic anhydride in anhydrous pyridine. Chromatographic analysis of the



I; R = H
II; R = acetyl



III

products after an alkaline treatment (*cf.* ref 1) did indeed show the formation of some thymidine dinucleotide, pTpT,[4] and higher homologs (Table I). The extent of polymerization was not significantly altered when the excess of benzoic anhydride used was varied. When the mononucleotide thymidine-5′ phosphate was directly treated with benzoic anhydride in anhydrous pyridine, the dinucleotide, pTpT, was again produced but the yield (6%) was much lower than when the corresponding pyrophosphate (I) was used. In further exploratory experiments, when the pyrophosphate (I) was treated with 2.5 equiv of benzoyl chloride, the final yield (18%) of the dinucleotide and higher homologs was somewhat higher and the reaction proceeded at a higher rate than when benzoic anhydride was used (Table I).

When in the above type of experiments acetic anhydride was used, only a very small amount (3%) of the dinucleotide, pTpT, was detected and it appeared probable that the variation in the results was due to the difference in the rates of acylation on the one hand and of phosphorylation on the other. Thus, in the case of acetic anhydride, probably the 3′-hydroxyl group of the nucleotide was very rapidly blocked by acetylation whereas with benzoic anhydride the rate of benzoylation of the hydroxyl group was slower[5] and therefore a higher degree of phosphorylation was achieved. It therefore followed that in order to promote internucleotide bond synthesis the use of a hindered carboxylic acid derivative was advisable. The use of trimethylacetyl chloride (3 equiv) in a polymerization experiment did in fact give a marked increase in the yield of the polymers (Table I). For further studies described below on internucleotide bond synthesis between a nucleotidic component and a second hydroxyl group bearing component, the reagent mesitoyl chloride was chosen, it being preferred over trimethylacetyl chloride because of the much slower rates of reaction observed with the latter reagent.

The results of experiments involving condensation between 5′-O-dimethoxytritylthymidine (III; DMTr-T) and P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate

(1) Paper LIV: M. W. Moon and H. G. Khorana, *J. Am. Chem. Soc.*, 88, 1798 (1966).

(2) This work has been supported by grants from the National Science Foundation, (Grant No. GB-3342), the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service (Grant No. CA-05178), and the Life Insurance Medical Research Fund (Grant No. G-62-54).

(3) Methods for the synthesis of internucleotide bonds have formed the subject of extended investigations in this laboratory, and several previous papers in this series have been devoted to this aspect of the over-all problem of polynucleotide synthesis. For pertinent references see H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, 87, 2954 (1965).

(4) The system of abbreviations for the specification of oligonucleotides is as has been defined in previous papers in this series; for example, see T. M. Jacob and H. G. Khorana, *ibid.*, 87, 2971 (1965); S. A. Narang and H. G. Khorana, *ibid.*, 87, 2981 (1965); S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, 87, 2988 (1965).

(5) This conclusion was verified experimentally. Thus with a 20-fold excess of acetic anhydride, thymidine-5′ phosphate was almost completely acetylated in 2 hr, while with the same excess of benzoic anhydride only 50% benzoylation of the 3′-hydroxyl group was found in 15 hr.

Table I. Polymerization of P^1,P^2-Dithymidine-5′ Pyrophosphate Using Carboxylic Acid Derivatives[a]

Reagent	Equiv of reagent	Reaction time, hr	Distribution of products, %		
			O<pT pT	pT	pTpT and higher polymers
Benzoic anhydride	2.1	24	17.5	66.5	14.0[b]
Benzoic anhydride	20.0	24	7.8	71.6	17.5[c]
Benzoyl chloride	1.0	0.5	36.9	51.5	11.6
Benzoyl chloride	1.0	5.0	43.2	42.6	14.2
Benzoyl chloride	2.5	0.5	30.6	55.3	14.1
Benzoyl chloride	2.5	5.0	30.7	50.9	18.4
Trimethylacetyl chloride	1.0	5.0	42.8	43.8	13.4
Trimethylacetyl chloride	1.0	24	47.9	42.0	10.1
Trimethylacetyl chloride	3.0	5.0	17.4	51.2	31.4

[a] For methodology of work-up see the Experimental Section. [b] This material was further characterized as 10.5% of dinucleotide and 3.5% of trinucleotide and higher polymers. A small amount of cyclic mononucleotide was also formed in the reaction. [c] This material consisted of 14.3% dinucleotide and 3.2% higher polymers.

Table II. The Synthesis of 5′-O-Dimethoxytritylthymidylyl(3′→5′)thymidine from 5′-O-Dimethoxytritylthymidine and P^1,P^2-Di(3′-O-acetylthymidine-5′) Pyrophosphate (II)[a]

Mesitoyl chloride, equiv	Distribution of nucleotidic material, %				Yield (%) of TpT[b]
	pT	Ms-pT	O<pT pT	TpT	
1	6.1	24.1	67.4	2.4	4.8
2	12.9	37.2	22.4	27.5	55.0
3	14.3	40.9	11.0	33.8	67.6
4	17.6	36.7	8.0	37.6	75.2
5	20.9	28.4	10.1	40.6	81.2

[a] Using equimolar amounts of the two components and mesitoyl chloride as the condensing agent. For details see the Experimental Section. [b] As based on the protected nucleoside.

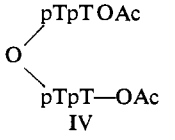
Table III. Yields of Thymidylyl(3′→5′)thymidine from 5′-O-Tritylthymidine and 3′-O-Acetylthymidine-5′ Phosphate[a]

Time, days	Yield[b] (%) using 3′-O-acetylthymidine-5′ phosphate		Yield[b] (%) using P^1,P^2-di(3′-O-acetylthymidine-5′) pyrophosphate	
	1 equiv	2 equiv	1 equiv[c]	2 equiv[c]
1	18.0	24.8	43.0	65.6
2	30.0	41.0	52.8	70.0
4	44.4	58.0	57.0	74.4
7	54.3			

[a] Or its pyrophosphate (II) with mesitoyl chloride (5 equiv) as condensing agent. For details see the Experimental Section. [b] The yields (per cent) are based on the nucleosidic component, 5′-O-tritylthymidine. They are, however, calculated by measuring the distribution of the nucleotidic products, since some of the nucleoside is removed by mesitoylation and cannot therefore be measured accurately. [c] The amounts are based on thymidine-5′ phosphate content.

(II) are shown in Table II. In these experiments equimolar amounts of II and III were used and the amount of the reagent, mesitoyl chloride, was varied. It is seen that a yield of as high as 81.2% (as based on the nucleoside) of thymidylyl(3′→5′)thymidine (TpT) was obtained using 5 equiv of the reagent. It is noteworthy that the yield (4.8%) using 1 equiv of mesitoyl chloride was very low whereas the use of 2 molar equiv brought about a dramatic increase in the yield. Further increases in the reagent caused relatively small effects. Table III shows the results of further experiments in which the use of the protected nucleotide 3′-O-acetylthymidine-5′ phosphate itself was compared with that of the corresponding pyrophosphate and, furthermore, the influence of the amount of the nucleotidic component on the yield of the internucleotide bond was investigated. An excess (fivefold) of the condensing agent was used in these experiments. As seen in Table III, when the nucleotide was used in the form of the pyrophosphate, the reaction proceeded at a faster rate and the ultimate yields obtained were higher than when the mononucleotide itself was used.

Earlier work on the synthesis of polynucleotides by using preformed di- and trinucleotide blocks showed only moderate success[6] and recently a reinvestigation of this general approach has been undertaken. Concurrently with the investigation of other condensing agents such as mesitylenesulfonyl chloride[7] and triisopropylbenzenesulfonyl chloride,[8] the use of mesitoyl chloride in the condensation of oligonucleotide blocks was investigated. In the first experiments the condensation of the acetylated thymidine dinucleotide (pTpT-OAc)[4] with 5′-O-tritylthymidine (Tr-T) was studied and the results are given in Table IV. As is seen, using equimolar amounts of pTpT-OAc and of Tr-T, the reaction leveled off in about 4 days to give a yield of 43% of the condensation product (analysis for thymidylylthymidylylthymidine after removal of the protecting groups). When the dinucleotide, pTpT-OAc, was converted to the corresponding pyrophosphate (abbreviated formulation IV) by treatment with dicyclohexylcarbodiimide[1] and this was used in the condensation reaction, a similar yield (45%) was obtained (Table IV). Thus, in contrast with the findings reported above (*e.g.*, Table III), there was little advantage in preforming the pyrophosphate of the dinucleotide pTpT-OAc.



IV

Block condensations using mesitoyl chloride were further investigated in the synthesis of tetra- and pentanucleotides. The components bearing free 3′-hydroxyl end group were 5′-O-tritylthymidylyl(3′→5′)thymidine (Tr-TpT)[4] and the next homolog, 5′-O-tritylthymidylyl-(3′→5′)thymidylyl(3′→5′)thymidine (Tr-TpTpT). The

(6) See, for example, H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3841 (1963).
(7) T. M. Jacob and H. G. Khorana, *ibid.*, **86**, 1630 (1964).
(8) R. Lohrmann and H. G. Khorana, *ibid.*, **88**, 829 (1966).

Table IV. Yield of Thymidylyl(3′→5′)thymidyl(3′→5′)thymidine Formed from 5′-O-Tritylthymidine and 5′-O-Phosphorylthymidylyl(3′→5′)-3′-O-acetylthymidine[a]

Time, days	Yield[b] of TpTpT, % Using pTpT-OAc	Using O<pTpT-OAc / pTpT-OAc
1	21.8	28.0
2	35.7	38.4
3	41.2	45.5
4	42.1	45.4
8	45.0	47.4

[a] Or its pyrophosphate using mesitoyl chloride as condensing reagent. For details see the Experimental Section. [b] The yield was calculated by paper chromatographic analysis of TpTpT and of pyrophosphate found unreacted.

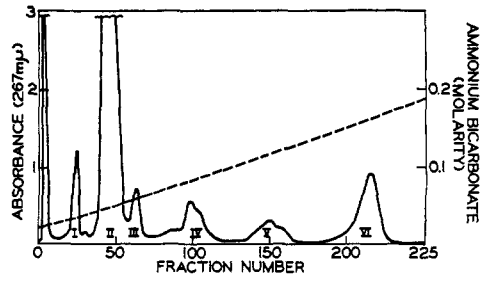


Figure 1. Chromatography on a DEAE-cellulose (bicarbonate) column of the products formed on reaction of 5′-O-tritylthymidylylthymidylylthymidine with thymidine dinucleotide (pTpT-OAc) in the presence of mesitoyl chloride. For details see text.

results of the experiment involving condensation between Tr-TpT and the dinucleotide pTpT-OAc are shown in Table V. Thus, the yield of the tetranucleotide, Tr-TpTpTpT, using equivalent amounts of the two reactants, was 30% after a 4-day reaction period. The results of the condensation of Tr-TpTpT with an excess of the dinucleotide, pTpT-OAc, are given in Figure 1 and Table VI. The yield of the expected product, Tr-TpTpTpTpT, isolated by column chromatography was 30% using 4 equiv of the dinucleotide component. As a large excess of mesitoyl chloride was used in this reaction, it is possible that mesitoylation of the 3′-hydroxyl group caused lowering of the yield.

Table V. Yield of 5′-O-Tritylthymidylyl(3′→5′)thymidylyl-(3′→5′)thymidylyl(3′→5′)thymidine in Reaction of 5′-O-Tritylthymidylyl(3′→5′)thymidine and 5′-O-Phosphorylthymidylyl(3′→5′)-3′-O-acetylthymidine[a]

Reaction time, days	1	2	3	4	8
Yield[b] of TrTpTp-TpT, %	16.2	25.4	29.4	30.5	33.0

[a] Using mesitoyl chloride as condensing reagent. For details see the Experimental Section. [b] The yield was calculated by paper chromatographic analysis of Tr-TpTpT and of the amount of pTpT-OAc (or its pyrophosphate) found unreacted.

Table VI. Identity of Products Formed in Synthesis of 5′-O-Tritylthymidylyl(3′→5′)thymidylyl-(3′→5′)thymidylyl(3′→5′)thymidylyl(3′→5′)thymidine[a]

Peak	Fractions pooled	OD_{267} units eluted	Identity
I	20–28	...	Mesitoic acid
II	38–55	675	NH_2-pTpT[b]
III	55–70	56	TrTpTpT and pTpT
IV	90–105	82	Unidentified
V	140–165	77	O<pTpT / pTpT and an unidentified trityl containing product
VI	200–225	190	Pure TrTpTpTpTpT

[a] The peak numbers refer to the elution pattern given in Figure 1. [b] The amidate of the dinucleotide pTpT. This product was formed during the ammoniacal treatment given in the work-up.

Discussion. The present work has demonstrated that reasonably powerful phosphorylating species can be generated by reaction of nucleotide pyrophosphates and oligonucleotides with carboxylic acid anhydrides and chlorides. The condensations proceed under mild conditions and the reagents offer, like the aromatic sulfonyl chlorides,[7] the advantage that trialkylammonium salts, which aid the attainment of homogeneous reaction mixtures, may be used. The kinetics of condensation reactions, on the other hand, are more similar to those characteristics of reactions mediated by dicyclohexylcarbodiimide. In both cases, it seems certain that poly- or metaphosphate species serve as the reactive species.

While the results obtained on the block condensation reactions were promising, parallel work to be published separately using mesitylenesulfonyl chloride and triisopropylbenzenesulfonyl chloride has shown that the latter class of reagents is superior. Other features, which further detract from the use of mesitoyl chloride, are that some mesitoylation of the 3′-hydroxyl group present in one of the reactants occurs and furthermore that the mono- or oligonucleotide containing the phosphomonoester end group is recovered mainly as its mesitoyl derivative, the mixed anhydride between the phosphomonoester group and mesitoic acid. The latter derivatives are exceptionally resistant to hydrolysis and therefore complicate the recovery of the parent oligonucleotide component. A great deal of chemical work was in fact carried out with the mixed anhydride between mesitoic acid and thymidine-5′ phosphate (Ms-pT) which was rapidly formed on reaction of mesitoyl chloride and thymidine-5′ phosphate. While the properties observed are described in the Experimental Section, attention may be drawn to one highly interesting finding. On treatment of Ms-pT with concentrated ammonia at room temperature for 2 days, the major product (94% yield) was the amidate of thymidine-5′ phosphate. This result provides a simple and efficient method for the preparation of the general class of nucleoside phosphoroamidates[9] which have found wide application in the synthesis of nucleotide coenzymes and related compounds.[10]

Experimental Section

General Methods. Paper chromatography was performed by the descending technique using Whatman No. 1 paper. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent B, 1-propanol–concentrated

(9) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 649 (1961).
(10) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961, Chapter 4.

ammonia–water (55:10:35, v/v); solvent C, ethyl alcohol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v); solvent D, ethyl alcohol–0.5 *M* ammonium acetate, pH 3.8 (7:3, v/v); solvent E, *n*-butyl alcohol–acetic acid–water (5:2:3, v/v). The R_f values of different compounds are given in Table VII. Paper electrophoresis was carried out in a commercially available apparatus capable of giving a potential of 5000 v using potassium phosphate buffer (0.03 *M*, pH 7.1). The mobilities of different compounds are given in Table VII.

Table VII. Paper Chromatography of Thymidine-Containing Compounds

	Solvent A	Solvent B	Paper electrophoretic mobility[a] (pH 7.1)
TrT	0.85		
TrTpT	0.72	0.86	
TrTpTpT	0.49	0.81	0.58
TrTpTpTpT	0.27	0.72	0.79
TrTpTpTpTpT	0.14	0.64	0.89
pT	0.12	0.43	1.0
Ms-pT	0.72		0.50
$O(pT)_2$	0.22		
pTpT	0.06	0.37	
Ms-pTpT	0.49		
pTpTpT	0.03		
T	0.66		
TpT	0.41		
TpTpT	0.23		

[a] Mobility relative to pT.

3′-O-Acetylthymidine-5′ phosphate[11] and 5′-O-tritylthymidylylthymidine[12] were used as the pyridinium salts. The pyrophosphate P^1,P^2-dithymidine-5′ pyrophosphate and the corresponding acetylated derivative (I and II),[13] and the pyrophosphate,[1] $O(pTpT\text{-}OAc)_2$, were prepared in the form of their triethylammonium salts. 5′-O-tritylthymidylyl(3′→5′)thymidylyl(3′→5′)thymidine[12] was used as the triethylammonium salt throughout.

Polymerization of P^1,P^2-Dithymidine 5′-Pyrophosphate. A. Using Benzoic Anhydride. The pyrophosphate (0.05 mmole) was rendered anhydrous and dissolved in anhydrous pyridine (1 ml). The required amount of benzoic anhydride was added, and samples (about 0.1 ml) were removed from the solution at time intervals up to 1 day. The samples were precipitated in ether (2 ml), and water (0.5 ml) was used to extract the nucleotidic products. NaOH (1 *N*; 0.3 ml) was added to the aqueous layer and after 15 min at room temperature pyridinium Dowex-50 resin was added to remove sodium ions. An aliquot of the solution was chromatographed in solvent A, the spots corresponding to the pyrophosphate, mononucleotide, the dinucleotide, pTpT, and that containing the trinucleotide, and higher polymers were eluted, and their relative intensities were determined. Using 20 molar equiv of benzoic anhydride, the extent of polymerization was determined as a function of time to be: 15 min, 6%; 30 min, 7.5%; 1 hr, 12%; 2 hr, 14%; 4 hr, 16%; 8 hr, 18%. (See also Table I where the results are given for 24-hr reaction periods.)

B. Using Benzoyl Chloride. A stock solution of the pyrophosphate (0.5 mmole) in anhydrous pyridine (5 ml) was prepared. Aliquots of this solution (0.1 ml, 0.01 mmole of pyrophosphate) were added to varying amounts of benzoyl chloride in 0.1 ml of anhydrous pyridine. Samples were removed after 0.5 and 5.0 hr, water was added, and benzoic acid was removed by ether extraction. The products were treated with alkali and then analyzed as described above under A. The results are shown in Table I.

C. Using Trimethylacetyl Chloride. The experiments were similar to those using benzoyl chloride except that samples were removed after 5 and 24 hr. The results are included in Table I.

Rate of 3′-O-Benzoylation of Thymidine-5′ Phosphate. Anhydrous pyridinium thymidine-5′ phosphate (0.1 mmole) was dissolved in pyridine (2 ml), and benzoic anhydride (452 mg, 2 mmoles) was added. Samples (about 0.1 ml) were removed, water (0.5 ml) was added, and the aqueous layer was extracted with cyclohexane. The product was chromatographed in solvent E, the spots corresponding to the mixed anhydride of thymidine-5′ phosphate and benzoic acid (Bz-pT) and its 3′-O-benzoylated product (Bz-pT-OBz) were cut out and eluted with 0.1 *N* NaOH solution, and their relative intensities were determined. Allowance was made for the contribution of benzoic acid to the extinction of the thymidine-5′ phosphate (5% contribution per benzoyl group) in calculating the rate of benzoylation; the small amount of pTpT (<6%) formed during the reaction was ignored. The extent of 3′-O-benzoylation as a function of time was thus determined to be as follows (mean of two determinations): 4.5 hr, 20.0%; 9 hr, 38%; 15.5 hr, 55%; 29 hr, 72%; 38 hr, 75%.

Influence of the Amount of Mesitoyl Chloride on the Synthesis of 5′-O-Dimethoxytritylthymidylyl(3′→5′)thymidine. Mesitoyl chloride (0.4 mmole) was made up to 1 ml with anhydrous pyridine. P^1,P^2-Di(3′-O-acetylthymidine-5′) pyrophosphate (0.01 mmole in 0.1 ml of anhydrous pyridine) and 5′-O-dimethoxytritylthymidine (0.01 mmole in 0.05 ml of pyridine) were mixed and treated with the appropriate amount of the mesitoyl chloride solution. After 2 days at room temperature, water (0.2 ml) was added and the product was hydrolyzed with 2 *N* NaOH solution (0.2 ml) for 10 min. Sodium ions were removed in the usual way and the product was evaporated to a gum and dissolved in 80% acetic acid (0.5 ml) for 30 min, after which time the material was again evaporated to a gum. The gum was dissolved in water and was extracted with ether. An aliquot of the product was chromatographed in solvent A; the spots corresponding to Ms-Pt, TpT, $O(pT)_2$,pT were eluted and from their intensities the yield of the dinucleoside phosphate (TpT) was calculated (Table II).

Rate of Internucleotide Bond Synthesis Using 3′-O-Acetylthymidine-5′ Phosphate and 5′-O-Tritylthymidine. A. 5′-O-tritylthymidine (0.1 mmole) and 3′-O-acetylthymidine-5′ phosphate (0.1 mmole) were rendered anhydrous in the usual way by evaporation of pyridine. Mesitoyl chloride (0.5 mmole) in 1 ml of pyridine was then added and the solution was thoroughly mixed. Samples (about 0.1 ml) were removed after 1, 2, 4, and 7 days. The samples were evaporated to a gum and treated with 80% acetic acid (3 ml) at 100° for 30 min; then acetic acid was removed. The gum was dissolved in pyridine (0.3 ml) and concentrated ammonia solution (1.0 ml) was added. After 1 hr an aliquot of the solution was removed and analyzed by chromatography in solvent A. The spots corresponding to thymidine, thymidylyl(3′→5′)thymidine (TpT), dithymidine pyrophosphate (trace), and thymidine-5′ phosphate were eluted and the product distribution was calculated. The yield of TpT is shown in Table III.

B. The above experiment was repeated using 5′-O-tritylthymidine (0.05 mmole), 3′-O-acetylthymidine-5′ phosphate (0.1 mmole), and mesitoyl chloride (0.5 mmole). The results are shown in Table III.

Rate of Internucleotide Bond Synthesis Using P^1,P^2-Di(3′-O-acetylthymidine-5′) Pyrophosphate. A. An anhydrous solution of 5′-O-tritylthymidine (0.1 mmole), the pyrophosphate (0.05 mmole), and mesitoyl chloride (0.5 mmole) in anhydrous pyridine (1 ml) was prepared. Samples (about 0.1 ml) removed after 1, 2, and 4 days were evaporated to a gum and treated with 80% acetic acid (3 ml) at 100° for 30 min. Acetic acid was then removed, and the product was dissolved in pyridine (0.3 ml) and treated with concentrated ammonium hydroxide solution for 1 hr. An aliquot was analyzed by paper chromatography in solvent A, the yield of TpT being calculated from the distribution of the four products thymidine, thymidine-5′ phosphate, TpT, and the pyrophosphate $O(pT)_2$ (Table III).

B. The above experiment was repeated using 5′-O-tritylthymidine (0.05 mmole), the pyrophosphate (0.05 mmole), and mesitoyl chloride (0.5 mmole); the results are shown in Table III.

Formation of Thymidylyl(3′→5′)thymidylyl(3′→5′)thymidine from 5′-O-Tritylthymidine and 5′-O-Phosphorylthymidylyl-(3′→5′)-3′-O-acetylthymidine (pTpT-OAc). An anhydrous solution of 5′-O-tritylthymidine (0.05 mmole), the dinucleotide (0.05 mmole), and mesitoyl chloride (0.3 mmole) in pyridine (1 ml) was prepared. Samples removed after 1, 2, 4, and 8 days were evaporated to a gum and treated with 80% acetic acid (3 ml) at 100° for 30 min. After evaporation of acetic acid, the sample was dissolved in pyridine (0.3 ml) and treated with concentrated ammonia solution (1 ml) for 1 hr. An aliquot was then analyzed by paper chromatography in solvent A, spots corresponding to T, TpTpT, and pTpT were eluted, and the product distribution was

(11) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 675 (1961).
(12) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965).
(13) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **81**, 4606 (1959).

calculated to give the yield of trinucleoside diphosphate (Table IV).

Formation of Thymidylyl(3′→5′)thymidylyl(3′→5′)thymidine from 5′-O-Tritylthymidine and the Dinucleotide Pyrophosphate O(pTpT-OAc)$_2$. An anhydrous solution of 5′-O-tritylthymidine (0.05 mmole), the pyrophosphate (0.025 mmole, equivalent to 0.05 mmole of dinucleotide), and mesitoyl chloride (0.3 mmole) in anhydrous pyridine (1.0 ml) was prepared. Samples were removed after 1, 2, 4, and 8 days and the work-up conditions used in the preceding experiment were followed. The yield of trinucleoside diphosphate is shown in Table IV.

Formation of 5′-O-Tritylthymidylyl(3′→5′)thymidylyl(3′→5′)-thymidylyl(3′→5′)thymidine from 5′-O-Tritylthymidylyl(3′→5′)-thymidine and 5′-O-Phosphorylthymidylyl(3′→5′)-3′-O-acetyl-thymidine (pTpT-OAc). 5′-O-Tritylthymidylyl(3′→5′)thymidine (0.05 mmole) and the dinucleotide pTpT-OAc (0.05 mmole) were dissolved in pyridine and rendered anhydrous by evaporation of pyridine. Mesitoyl chloride (0.3 mmole) in pyridine (1 ml) was added to the mixture. Samples (about 0.1 ml) were removed after 1, 2, 3, 4, and 8 days and were diluted with an equal volume of water. After 2 hr concentrated ammonium hydroxide solution (0.5 ml) was added for 30 min. Ammonia was removed and the gum was dissolved in aqueous pyridine, an aliquot of the solution being analyzed by paper chromatography in solvent A. The distribution of optical density in the products TrTpT, Ms-pTpT, TrTpTpTpT, and pTpT spots enabled the yield to be calculated (Table V).

Synthesis of 5′-O-Tritylthymidylyl(3′→5′)thymidylyl(3′→5′)-thymidylyl(3′→5′)thymidylyl(3′→5′)thymidine. 5′-O-Tritylthymidylyl(3′→5′)thymidylyl(3′→5′)thymidine (375 OD_{267} units) and 5′-O-phosphorylthymidylyl(3′→5′)-3′-O-acetylthymidine (0.05 mmole) were rendered anhydrous by evaporation of pyridine. A solution of mesitoyl chloride (0.3 mmole) in pyridine (1 ml) was added to the gum and the reaction was allowed to stand for 5 days at room temperature and concentrated ammonium hydroxide solution (2.0 ml) was then added to the mixture. After 2 days the solution was evaporated to a gum, dissolved in aqueous pyridine, and extracted with ether. The aqueous layer was applied to the top of a DEAE–cellulose column (1 × 50 cm, bicarbonate form) which was eluted with a linear salt gradient (4 l. of 0.02 *M* ammonium bicarbonate in the mixing vessel; 4 l. of 0.4 *M* ammonium bicarbonate in the reservoir); 15-ml fractions were collected. The elution pattern is shown in Figure 1 and the identification of products in Table VI. The product TrTpTpTpTpT was eluted in fractions 200–225 and amounted to 190 OD_{267} units (31% yield).

Preparation and Properties of the Mixed Anhydride Mesitoylthymidine-5′ Phosphate (Ms-pT). Diammonium thymidine-5′ phosphate (1.0 mmole) was suspended in pyridine (10 ml) and rendered anhydrous by evaporation of three portions of pyridine. To the compound in pyridine (10 ml) was added mesitoyl chloride (5.0 mmoles). After shaking the reaction solution for 10 min, water (10 ml) was added, the mixture was extracted with ether to remove mesitoic acid, and the aqueous layer was evaporated to half the volume and transferred with water to a 25-ml graduated flask. Ms-pT was the sole nucleotidic product (R_f 0.72 in solvent A).

A. Hydrolysis with Ammonia. The standard solution (1.0 ml) of Ms-pT was treated with concentrated ammonium hydroxide solution (1.0 ml). Samples were removed at different intervals and analyzed by paper chromatography in solvent A. The product formed was identified as thymidine-5′ phosphoramidate (NH_2-pT) and the rate of hydrolysis was: 4 hr, 22%; 12 hr, 48.7%; 24 hr, 76.4%; 48 hr, 96%; 96 hr 100%. In the 96-hr product, a small amount (6%) of the free nucleotide, pT, was also present.

B. Hydrolysis and Rearrangement with Alkali. The standard solution (1.0 ml) was treated with 1.0 ml of 2 *N* NaOH solution. Aliquots were removed after various times. Sodium ions were removed using pyridinium Dowex-50 resin and the products formed were determined by paper chromatography in solvent A. The results are given in Table VIII.

Table VIII. Products Formed on Treatment of the Mixed Anhydride (Ms-pT) with 2 *N* NaOH[a]

Time, hr	Ms-pT	pT-OMs[b]	pT
2	61.5	18.4	20.1
4	43.6	26.2	29.2
6	29.0	34.2	36.8
12	21.0	32.0	47.0
24	10.1	40.2	49.7
48	0	26.7	73.3

[a] For details see text. [b] This is abbreviation for 3′-O-mesitoylthymidine-5′ phosphate.

The product identified as the 3′-O-mesitoyl ester of thymidine-5′ phosphate (pT-OMs) had the following properties: its paper electrophoretic mobility, relative to pT, was pH 7.1, 0.79, pH 2.7, 0.63. On paper chromatography, the R_f values were: solvent A, 0.42; solvent B, 0.74; solvent C, 0.72; solvent D, 0.80. It was resistant to the action of the 5′-nucleotidase in crude snake venom (*Crotalus adamanteus*). This behavior would be expected for a 3′-O-substituted nucleotide. It was however dephosphorated by bacterial alkaline phosphatase to a product (3′-O-mesitoylthymidine) with R_f 0.82 in solvent A. The spectral characteristics of the latter product and the starting material at neutral and alkaline pH were just similar to those of thymidine. The nucleotide as expected for pT-OMs was not acetylated by acetic anhydride in pyridine. It was hydrolyzed in 5 *N* sodium hydroxide to pT and mesitoic acid.