Studies on Polynucleotides. XXXVII.[1] The Synthesis of Specific Deoxyribopolynucleotides.[2] Further Examination of the Approach Involving Stepwise Synthesis[3]

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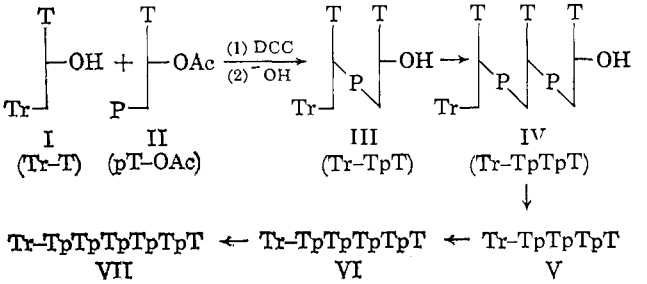
The stepwise synthesis of deoxyribopolynucleotides involving chain elongation by one unit at a time has been further investigated. The reaction sequence studied was: 5'-O-tritylthymidylyl-(3'→5')-thymidine → 5'-O-tritylthymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidine → 5'-O-tritylthymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidine → 5'-O-tritylthymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidine → 5'-O-tritylthymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidine. The condensing agents used were dicyclohexylcarbodiimide and mesitylenesulfonyl chloride. The yield of the desired product using stoichiometric amounts of the two components, the oligonucleotide bearing the free 3'-hydroxyl group and 3'-O-acetylthymidine 5'-phosphate, decreased as the chain length of the oligonucleotide component increased. However, high yields of the desired products could be sustained by increasing the excess of the mononucleotide component with an increase in the size of the oligonucleotide component. The probable side reactions which lead to the degradation of the polynucleotide chains have been elucidated by a study of several of the side products formed during the synthetic reactions. A procedure for the large scale preparation of 5'-O-tritylthymidylyl-(3'→5')-thymidine is described.

The stepwise synthesis of deoxyribopolynucleotides may be carried out either by the condensation of preformed blocks of oligonucleotides or by the successive addition of mononucleotide units to the hydroxyl end of a "growing" oligonucleotide chain. The former approach has previously been investigated in the synthesis of several tetra- and pentanucleotides.[2c,f,g] The yields of the desired products using "economical" proportions of the two components have been low (15–25%) in this approach, using the condensation methods that have so far been available. The alternative approach involving the oligonucleotide chain elongation by one unit at a time[2b] has now been given a careful study and the results are reported in this paper. Because the main objective of this study was the evaluation of the approach in regard to the repetitive synthesis of the internucleotidic linkages, attention was confined in the present work to the synthesis of thymidine homooligonucleotides, where the problem of the protection of the heterocyclic ring does not arise. The results show that satisfactory yields with respect to the "growing" chain can be maintained provided an increasing excess of the mononucleotide component is used as the chain length in the oligonucleotide component increases. The nature of several of the side products formed during the synthetic reactions has been investigated and this study has thrown light on the probable reactions responsible for the side-product formation. Brief reports of parts of this work have previously been made.[4,5] Subsequent papers will describe the application of this approach in the synthesis of deoxyribopolynucleotides containing specific nucleotide sequences.[6,7]

The individual steps investigated in the present work for the synthesis of the hexanucleotide, Tr-TpTpTpTpTpT,[8] are shown in Chart I.[8] From the previous work,

Chart I. The Stepwise Synthesis of 5'-O-Tritylthymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidylyl-(3'→5')-thymidine (VII)



[a] The steps, III → IV, IV → V, V → VI, VI → VII, each involved (a) condensation with the mononucleotide, II, in the presence of DCC or mesitylenesulfonyl chloride, and (b) alkaline treatment.

dicyclohexylcarbodiimide (DCC) and the aromatic sulfonyl chlorides, for example, mesitylenesulfonyl

(1) Paper XXXVI: D. Söll and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 360 (1965).

(2) (a) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958); (b) P. T. Gilham and H. G. Khorana, *ibid.*, **81**, 4647 (1959); (c) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962); (d) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963); (e) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963); (f) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963); (g) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963).

(3) This work has been supported by grants from the National Cancer Institute (Grant No. CA-05178) of the National Institutes of Health, the National Science Foundation (Grant No. GB-976), and Life Insurance Medical Research (Grant No. G-62-54).

(4) T. M. Jacob, E. Ohtsuka, M. Moon, S. A. Narang, and H. G. Khorana, *Federation Proc.*, 531 (1964).

(5) T. M. Jacob and H. G. Khorana, Abstracts of the Sixth International Congress of Biochemistry, Vol. I, New York, N. Y., 1964, p. 62.

(6) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, in press.

(7) S. A. Narang and H. G. Khorana, *ibid.*, in press (1965).

(8) With a view to economizing space and for convenience, we are using in this paper the system of abbreviations and the diagrammatic representations for oligonucleotides which have been described elsewhere[9] and which are currently in use in *Journal of Biological Chemistry*. Some additional abbreviations used are: Tr-T for 5'-O-tritylthymidine; pT-OAc for 3'-O-acetylthymidine 5'-phosphate; Tr-Tp for 5'-O-tritylthymidine 3'-phosphate; Tr-TpT for 5'-O-tritylthymidylyl-(3'→5')-thymidine, and similarly Tr-TpTpT, Tr-TpTpTpT, etc. for homologous members; Tr-TpTp, Tr-TpTpTp, etc. for corresponding compounds bearing 3'-phosphomonoester end groups.

(9) 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 5.

chloride, have emerged as the most satisfactory reagents.[10] In the present work, both of these reagents were used and further compared for their effectiveness as reagents in the stepwise synthesis of oligonucleotides of increasing chain length (Table I).

Table I. Yields of Homologous Oligonucleotides in Stepwise Synthesis[a]

Starting oligonucleotide	Excess (molar equiv.) of mononucleotide (pT-OAc)	Reagent	Product	Yield,[b] %
Tr-T	None	DCC	Tr-TpT	86
Tr-TpT	None	DCC	Tr-TpTpT	74[c]
Tr-TpT	1	DCC	Tr-TpTpT	71
Tr-TpT	3	DCC	Tr-TpTpT	96
Tr-TpT	3	DCC	Tr-TpTpT	92[e]
Tr-TpT	1	$MsSO_2Cl$[d]	Tr-TpTpT	73
Tr-TpTpT	None	DCC	Tr-TpTpTpT	65
Tr-TpTpT	4	DCC	Tr-TpTpTpT	83
Tr-TpTpT	4	$MsSO_2Cl$[d]	Tr-TpTpTpT	83
Tr-TpTpTpT	None	DCC	Tr-TpTpTpTpT	32
Tr-TpTpTpT	4	DCC	Tr-TpTpTpTpT	61
Tr-TpTpTpT	4	$MsSO_2Cl$[d]	Tr-TpTpTpTpT	75
Tr-TpTpTpTpT	14	$MsSO_2Cl$[d]	Tr-TpTpTpTpTpT	66
Tr-TpTpTpTpT	13	DCC	Tr-TpTpTpTpTpT	60

[a] Involving condensation of 3′-O-acetylthymidine 5′-phosphate with oligonucleotides of increasing chain length. All reactions were in anhydrous pyridine except where noted. For further details see Experimental. [b] Based on the oligonucleotide used as starting material. [c] Mean of two experiments. [d] $MsSO_2Cl$ is the abbreviation for mesitylenesulfonyl chloride. [e] A mixture of dimethylformamide and pyridine was used as the medium.

5′-O-Tritylthymidylylthymidylylthymidine[2b] (*IV*). The starting material Tr-TpT (III) was prepared by the condensation of Tr-T[8] and pT-OAc[8]; the isolation procedure now developed involved solvent extraction instead of column chromatography.[2a] Its condensation with pT-OAc,[8] using stoichiometric amounts of the two components in dry pyridine at room temperature, gave Tr-TpTpT in 74% yield. When 100% excess of the mononucleotide was used the yield of the desired product was again similar (Table I). The elution pattern obtained on chromatography of the total product on a DEAE–cellulose column is shown in Figure 1 and the distribution of the nucleotidic material in different peaks and the identification is given in Table II. The result is similar to that obtained in earlier work.[2b] The lack of significant difference in the yield when 1 or 2 *M* proportions of the nucleotide component are used[11] is not understood. However, there was a marked increase in the yield of the desired product when a larger excess of the mononucleotide was used. Thus, with a threefold excess of pT-OAc, the yield of Tr-TpTpT was 96%. DCC was the reagent in this experiment and the time of reaction was only one day at room temperature. It is also worthy of note that in a repeat of the same experiment using a mixture of dimethylformamide and pyridine as the solvent, the yield was not significantly impaired. Dimethylformamide is a powerful solvent for oligonucleotides and its use is frequently necessary.

It is also noted from Table I that both reagents, DCC and mesitylenesulfonyl chloride, gave practically identical yields. Experiments were carried out to test the effect of separate activation, prior to mixing, of one or both of the components with the reagent. No significant difference in the yields was detected. The prior brief treatment of Tr-TpT with DCC to form the corresponding fully protected pyrophosphate might have had a favorable effect (76% yield in place of 72%).

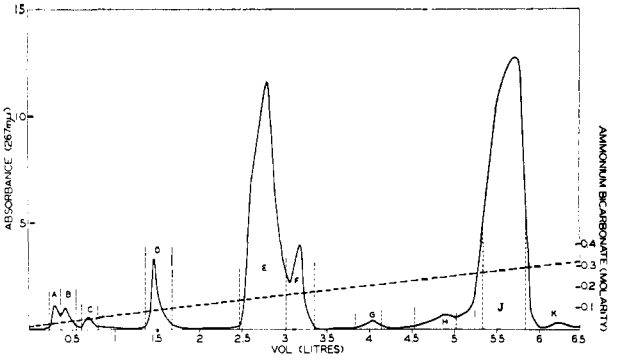


Figure 1. Chromatography of 35% of the reaction mixture in the preparation of Tr-TpTpT (IV) (experiment I) on a DEAE–cellulose (carbonate) column (43 × 4 cm.). For details, see text. Optical density was read at 267 mμ; ---------, salt gradient.

Table II. Distribution of Nucleotidic Material in the Different Products Obtained in the Condensation of Tr-TpT and pT-OAc[a]

Peak No.[b]	$O.D._{267\ m\mu}$	Trityl test	Identification remarks
A	72	—	5′-C-Pyridiniumthymidine (VIII) + unidentified
B	95	—	5′-C-Pyridinium-TpT
C	55	—	Unidentified
D	372	—	Thymidine-3′,5′-cyclic phosphate
E	3810	—	pT
F	756	+	Tr-TpT + pyrophosphate of pT
G	80	—	Unidentified
H	265	+	Tr-TpTpT + trityl-negative material[c]
I	420	+	Tr-TpTpT + trityl-negative material[c]
J	5544	+	Tr-TpTpT
K	244	+	Tr-TpTpT + trace of Tr-TpTpTpT

[a] Using 100% excess of pT-OAc. Elution pattern is shown in Figure 1. [b] The fractions pooled for each peak were as shown by the vertical dotted lines in Figure 1. [c] This accounts for about 10% of the material present in peaks H and I and is probably pTpT derived from the self-condensation of pT, the latter, presumably, being an impurity in the pT-OAc sample used.

5′-O-Tritylthymidylylthymidylylthymidylylthymidine (*V*). The reaction between Tr-TpTpT and pT-OAc was carried out under the standard conditions except that both components were separately treated for a short period of time with DCC before being brought together. Figure 2a and 2b show the elution patterns of the products obtained using, respectively, stoichiometric amount and a fourfold excess of pT-OAc in this reaction. The yield of V in the former experiment was 65% after a four-day reaction, a few side products being detected. In the latter experiment, the yield of V isolated after chromatography (Figure 2b) was 83% as based on the amount of the Tr-TpTpT originally used.

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

(11) In the condensation of Tr-Tp with β-cyanoethyl thymidine 3′-phosphate to form Tr-TpTp,[2c] the yield of the latter was again similar when 1:1 or 1:2 *M* proportions of the two components were used.

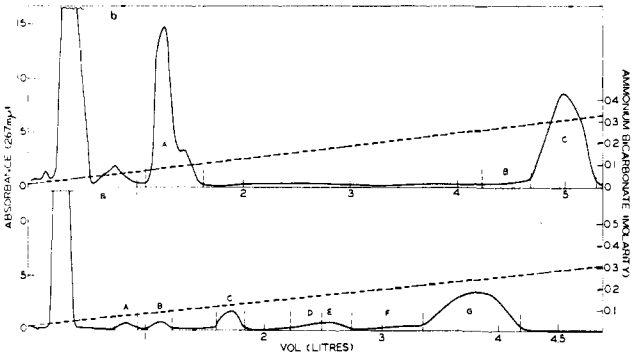


Figure 2. (a) Chromatography of the reaction mixture in the preparation of Tr-TpTpTpT (V) (experiment I) on a DEAE–cellulose (carbonate) column (44 × 4 cm.) using a linear gradient of ammonium bicarbonate in 20% alcohol. Fractions (15 ml.) were collected every 5 min.; – – – – salt gradient. Peak G is of the desired Tr-TpTpTpT. The contents of peaks C through F also gave a positive test for the trityl group. Unreacted pT was present in peak C. (b) Chromatography of the reaction mixture in the preparation of Tr-TpTpTpT as described in experiment II. Conditions of column chromatography were similar to that in (a) except for slower flow rate (1.25 ml./min.). In both (a) and (b) the first major peak (preceding peak A) was that of pyridine.

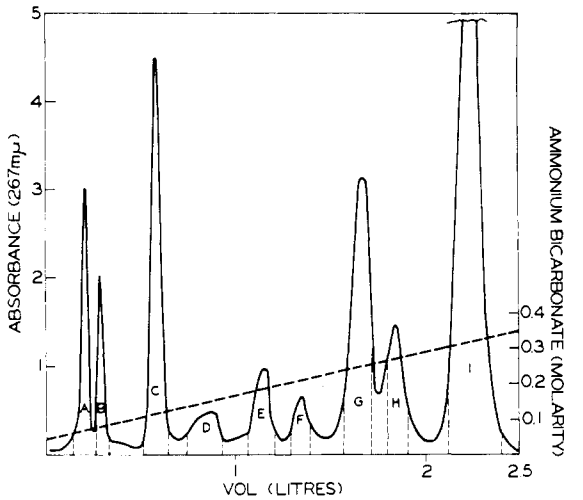


Figure 3. Chromatography of the reaction mixture in the preparation of Tr-TpTpTpTpT (VI) (experiment I) on a DEAE–cellulose (carbonate) column (40 × 2.5 cm. i.d.) using a linear gradient of ammonium bicarbonate containing 20% alcohol. Fractions (15 ml.) were collected every 15 min.; – – – – salt gradient.

(The yield as based on the total amount of the oligonucleotide components recovered is higher.) In a parallel experiment in which mesitylenesulfonyl chloride was used as the reagent, the yield after a 4-hr. reaction period (room temperature) was again 83% (Table I).

It is interesting that the yield using stoichiometric amounts of the two components (Figure 2a), while consistent with the notion of a downward trend with an increase in the chain length of the oligonucleotide component, was rather high. The use of an excess of the mononucleotide component resulted in an appreciable increase in the yield of V (Figure 2b).

5′-O-Tritylthymidylylthymidylylthymidylylthymidylylthymidine (VI). Using stoichiometric amounts of the two components, Tr-TpTpTpT (V) and pT-OAc (both separately treated with DCC prior to the condensation reaction), the yield of VI was only 32% after a 5-day reaction period. The elution pattern of the total products from a chromatographic column is shown in Figure 3 and the distribution of the ultraviolet absorbing material in the different peaks is in Table III. A rather large number of side peaks were obtained and each of these contained more than one and, often, several compounds. A detailed study of some of these side products is given later.

The yield of VI, using a fourfold excess of pT-OAc, was 61% or better when either of the two condensing agents (Table I) was used.

5′-O-Tritylthymidylylthymidylylthymidylylthymidylylthymidylylthymidine (VII). In the condensation of Tr-TpTpTpTpT with pT-OAc, a 13-fold excess of the latter was used. A typical elution pattern obtained on column chromatography of the reaction mixture is shown in Figure 4. The pure desired product present in peak H (edges discarded) was obtained in 60% yield in the DCC reaction and in 66% yield in the mesitylenesulfonyl chloride reaction. A number of minor side products were present, there being, in the experiment using DCC, about 15% of unreacted starting material (peak G of Figure 4).

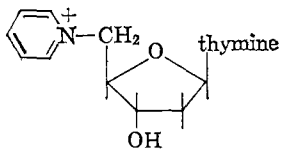
The Side Products. Side products were formed in small amounts in all condensation reactions. In the synthesis of Tr-TpTpTpTpT from Tr-TpTpTpT and pT-OAc using stoichiometric amounts of the two components (Figure 3, Table III) the side products were present in rather substantial amounts. Several of the pooled peaks (Figure 3) were each found to have more than one product. Although several of the minor products formed remain unidentified, two series of homologous compounds were positively identified. The first series

Table III. Distribution of Nucleotidic Material in the Different Products Obtained in the Condensation of Tr-TpTpTpT and pTOAc[a]

Peak no.	O.D.$_{267\ m\mu}$	Trityl test	Identification remarks[b]
A	136.5	–	Thymidine-3′,5′-cyclic phosphate + probably thymidylyl-N-(N,N′-dicyclohexylurea)
B	69	–	5′-C-Pyridinium-TpTpT[c] + trace TpT
C	299	–	Mostly pT + some 5′-C-pyridinium-TpTpTpT[d]
D	79	+	Tr-Tp + two unidentified trityl-negative products[e]
E	111	+	Unidentified[f]
F	65	+	Mainly Tr-TpTp
G	370	+	Mainly Tr-TpTpTpT
H	138	+	Mostly Tr-TpTpTp
I	956	+	Tr-TpTpTpTpT + trace Tr-TpTpTpTpTpT[g]

[a] Stoichiometric amounts. Elution pattern is shown in Figure 3. [b] The paper chromatographic and paper electrophoretic mobilities of the identified side products are given in Table IV. [c] Degraded by venom phosphodiesterase to pT and 5′-C-pyridinium-T. [d] Degraded completely to pT + 5′-C-pyridinium-T by venom phosphodiesterase. [e] One of these, probably, is cyclic thymidine dinucleotide resulting from the polymerization of pT (see footnote *g* below). [f] Contains one main product, trityl-positive and containing a phosphomonoester group. [g] The formation of this product shows that the sample of pT-OAc used contained some pT. The latter condensed with Tr-TpTpTpT to give Tr-TpTpTpTpT bearing free 3′-hydroxyl group and the latter again reacted with pT-OAc.

corresponded to Tr-Tp, Tr-TpTp, and Tr-TpTpTp (Table III), and the second series corresponded to 5′-C-pyridinium-T (VIII), 5′-C-pyridinium-TpT, and homologs (Tables II and III). Thus, compounds of the first



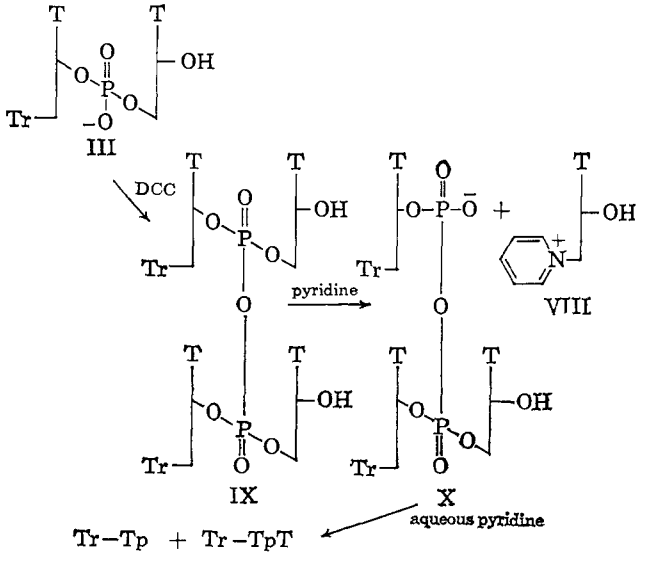
VIII

series all contained phosphomonoester groups and after treatment with the bacterial phosphomonoesterase were converted to Tr-T, Tr-TpT, and Tr-TpTpT, respectively. Compounds of the second series were isolated from several experiments and characterized. For example, 5′-C-pyridinium-T and 5′-C-pyridinium-TpT were identified among the products encountered in the synthesis of Tr-TpTpT and, similarly, the homologous 5′-C-pyridinium-TpTpT and 5′-C-pyridinium-TpTpTpT were identified among the products in the synthesis of Tr-TpTpTpTpT (Table III, Figure 3).

A further control experiment provided supporting evidence for the formation of the above series of side products. When Tr-TpT was treated with DCC in anhydrous pyridine for six days at room temperature and the reaction products were subjected directly to column chromatography, 5′-C-pyridinium-T (VIII) accounted for approximately 2.5% of the starting material. It should be emphasized that chromatography of the starting material under identical conditions on the same material showed no contamination by any impurity or side product of type VIII.

The reaction leading to the formation of a fragment bearing a 3′-phosphomonoester group and, simultaneously, another fragment bearing a 5′-C-pyridinium group may be envisaged to proceed as shown in Chart II for the simplest compound Tr-TpT. The initial step

Chart II. Postulated Scheme for the Cleavage of an Internucleotidic Linkage.



is the formation of a tetrasubstituted pyrophosphate on reaction with the reagent and in the case of longer oligonucleotides this step may readily occur intra-

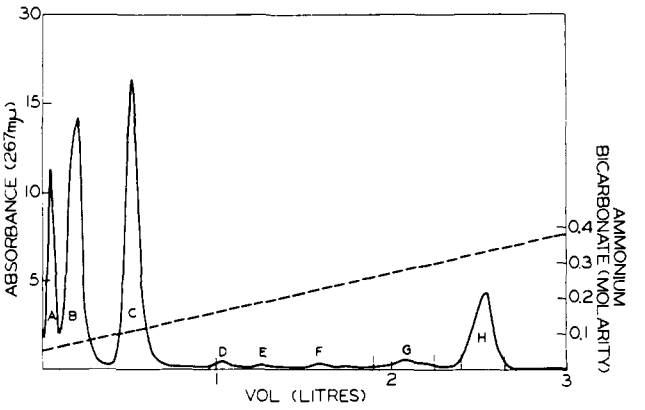


Figure 4. Chromatography of the reaction mixture in the preparation of Tr-TpTpTpTpTpT (VII) (experiment I) on a DEAE-cellulose (carbonate) column (38 × 2.5 cm.) using a linear gradient of ammonium bicarbonate containing 20% alcohol. Fractions (20 ml.) were collected every 20 min.; – – – – salt gradient.

molecularly. In the latter event, the fragmentation of the molecule would again occur so as to form one fragment with a 3′-phosphomonoester group and the second bearing a 5′-C-pyridinium group. As judged by the result of the experiment cited above with Tr-TpT, the contribution of this reaction per diester bond is at least about 5% in a 6-day reaction with DCC. In the experiment with Tr-TpTpTpT and pT-OAc where side products were numerous the contribution from this reaction alone would be in the range of 15%.

The second side reaction which may be postulated involves, as the first step, the formation of a tertiary (neutral) phosphate ester group.[2c] While this reaction is discussed in detail in an accompanying paper,[12] the point relevant to the present work is that this side reaction *can* lead to the formation of a $C_{3'}$–$C_{3'}$ internucleotidic linkage. Samples of the synthetic products (IV–VII) obtained in the present work were therefore carefully checked for complete susceptibility toward the sensitive analytical reagents, the venom and spleen phosphodiesterases. Complete degradation occurred in every case, thus excluding the possibility of any contamination of the desired products by side products containing any unnatural internucleotidic linkage.

Concluding Remarks. The approach to the stepwise synthesis of deoxyribopolynucleotides involving addition of one unit at a time has been given careful scrutiny in this paper and appears to offer attractive features. As the oligonucleotide chain increases, an increasing excess of the mononucleotide component is used to maintain satisfactory yields with respect to the increasingly valuable oligonucleotide component. In the work reported in the succeeding papers, the yield of the isolated desired products at every step has averaged to be 70%.

The side products reported are minor provided an excess of the mononucleotide component is used, thereby not only increasing the yield of the desired product but also reducing the time of exposure of the internucleotidic linkages to the degradative processes. Furthermore, it has become clear that a major side reaction involves the attack of the fully substituted pyrophosphate (X) by pyridine. The latter solvent has been

(12) E. Ohtsuka, M. Moon, and H. G. Khorana, *J. Am. Chem. Soc.*, in press.

used in most of the work carried out so far and it seems likely that if another suitable solvent can be found which is free from the high degree of nucleophilicity characteristic of pyridine then the side products resulting from this type of reaction may be eliminated or much reduced.

Experimental

General Methods. Paper chromatography was performed by the descending technique using mostly Whatman paper No. 1 and, occasionally, No. 40 or 44. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water, 7:1:2; solvent B, ethyl alcohol–1 *M* ammonium acetate (pH 7.5) (7:3, v./v.); solvent C, *n*-propyl alcohol–concentrated ammonia–water (55:10:35). Paper electrophoresis was performed using Whatman 3 MM paper and 0.03 *M* phosphate buffer at a potential of about 60 v./cm. The paper chromatographic and electrophoretic mobilities of different compounds are listed in Table IV.

Table IV. R_f's of Different Compounds on Paper Chromatography and Paper Electrophoresis

Compound	R_f,[a] (paper chromatograph) Solvent A	Solvent C	Mobility,[b] (paperelectrophoresis, pH 7.1)
Tr-TpT	7.4	2.14	
Tr-TpTpT	4.5	2	0.58
Tr-TpTpTpT	3	1.86	0.77
Tr-TpTpTpTpT	1.16	1.7	0.89
Tr-TpTpTpTpTpT	0.55	1.5	0.95
Tr-Tp	3.75		0.69
Tr-TpTp	2.1		0.89
Tr-TpTpTp	1.08		1
5′-C-Pyridinium-T	2.75		−0.9
5′-C-Pyridinium-TpT	1.8		0.0
5′-C-Pyridinium-TpTpT	0.85		0.21
5′-C-Pyridinium-TpTpTpT	0.45		0.48

[a] R_f's relative to that of pT. [b] Mobility relative to that of pT.

The trityl groups in compounds were detected by spraying the chromatograms with 10% aqueous perchloric acid and drying them in warm air. The trityl-containing compounds appeared yellow.

Tr-T was prepared by the method of Weimann and Khorana.[2c] Mesitylenesulfonyl chloride and anhydrous pyridine were prepared as described previously.[10] pT-OAc was prepared as described previously [13a] except that the product was precipitated twice from a mixture of pyridine and dry ether.[6]

Anhydrous reaction mixtures or components thereof refer to the following standard treatment. A solution of the components in pyridine or aqueous pyridine was evaporated *in vacuo* using a Dry Ice–acetone trap. Fresh dry pyridine was added to dissolve the residue and the evaporation was repeated. The whole procedure was repeated several times, the reaction vessel being opened to the dry atmosphere of a large desiccator (phosphorus pentoxide) each time.

In experiments where the reaction mixture was set up by prior separate treatment of one or both of the components with the reagents, the components were in two separate flasks with male and female joints. After the "preactivation," the components were mixed by opening the two flasks into each other and shaking.

The preparation of DEAE–cellulose in the carbonate form and the packing of the columns was as described elsewhere.[13b]

The oligonucleotidic products were isolated from the pooled chromatographic fractions by evaporation to remove alcohol and prolonged lyophilization to remove ammonium bicarbonate. The values of the molar extinction coefficients used for calculation of the concentrations of the different oligonucleotides are as follows. Each value is at 267 mμ at neutral pH: Tr-TpT, 18,500; Tr-TpTpT, 25,800; Tr-TpTpTpT, 34,000; Tr-TpTpTpTpT, 42,500; Tr-TpTpTpTpTpT, 51,000. The abbreviation $O.D._{267}$ refers to the extinction of a nucleotidic solution at neutral pH at 267 mμ in 1 ml. of solution using a 1-cm. light path quartz cell.

Enzymatic experiments, involving bacterial alkaline phosphatase and the snake venom phosphodiesterase, were performed as described previously.[13a]

Preparation of Tr-TpT. To an anhydrous mixture of Tr-T (3.92 g., 7 mmole),[14] pT-OAc (7.5 mmole), and pyridinium Dowex-50 ion-exchange resin (15 g.) in dry pyridine (50 ml.) was added DCC (7.7 g., 37.5 mmole) and the sealed reaction mixture was shaken in the dark at room temperature for eight days. Water (20 ml.) was then added and the mixture was kept further for two days at room temperature. Paper chromatography in solvent A at this stage showed one major product (trityl-containing, R_f 0.72) and a faint spot corresponding to Tr-T (R_f 0.85). The reaction mixture was filtered through glass wool, the solid being washed with 50% aqueous pyridine. The total filtrate was made up to 300 ml. with 50% aqueous pyridine and extracted with ether three times (250 ml., 150 ml., and 150 ml.). The combined ether extract was back-extracted with water (50 ml.) and this extract combined with the aqueous pyridine solution. Paper chromatography at this stage showed that the extraction of Tr-T was complete and, further, that the ether extract contained only Tr-T. The aqueous pyridine solution was concentrated *in vacuo* to about 40 ml. and the residue was extracted gently[15] with chloroform[16] (250 ml.). The chloroform extraction was repeated twice. The total chloroform extracts contained only the desired product, Tr-TpT-OAc, as judged by paper chromatography. The aqueous layer contained small amounts of Tr-TpT-OAc, Tr-TpT, and traces of other compounds. The chloroform solution was evaporated *in vacuo* after addition of some pyridine and the residue dissolved in about 50 ml. of pyridine. Sodium hydroxide (70 ml. of 1 *N*) was added and after 1 hr. at room temperature the solution was extracted with chloroform to remove any residual dicyclohexylurea. The aqueous alkaline solution was treated with pyridinium Dowex-50 ion-exchange resin until the pH dropped to neutrality. The total mixture was transferred to a column, the supernatant solution being

(13) (a) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, 83, 675 (1961); (b) H. G. Khorana and W. J. Connors, *Biochem. Prepn.*, in press.

(14) This calculation of molecular weight includes 1 mole of benzene which is present in the crystalline preparations of Tr-T.[2a]

(15) Violent agitation must be avoided, otherwise a stable emulsion results.

(16) The addition of some triethylamine before partial evaporation and extraction with chloroform would be advisable particularly if the more sensitive, mono and dimethoxytrityl derivatives are used.

slowly passed through the resin, and the latter was washed with 30% aqueous pyridine. The total eluate was concentrated in the presence of added pyridine and the dry residue was dissolved in 50 ml. of anhydrous pyridine. The solution was added dropwise to an excess (1 l.) of dry ether and the precipitate of pyridinium Tr-TpT was collected by centrifugation. For use in condensation reactions, the total product was dissolved in 50 ml. of dry pyridine and the concentration of this stock solution was determined by removing aliquots, evaporating the latter repeatedly with added ammonia, and making the final residue to a known volume for spectrophotometric analysis. The yield of Tr-TpT was thus determined to be 5.8 mmoles (83% as based on the Tr-T originally used).

Tritylthymidylylthymidylylthymidine (IV). A. Using DCC. Experiment I. An anhydrous pyridine solution (10 ml.) of Tr-TpT (1 mmole) and pT-OAc (2 mmole) containing pyridinium Dowex-50 ion-exchange resin (4 g.) was treated with DCC (10 mmole, 2.06 g.), and the sealed mixture was shaken for four days at room temperature. Water (20 ml.) and pyridine (10 ml.) were then added and the mixture was kept overnight. After filtration to remove the resin and dicyclohexylurea (aqueous pyridine wash), the clear solution was extracted with petroleum ether and then made up to 100 ml. with 50% aqueous pyridine. A part (35 ml.) of this solution was concentrated *in vacuo* to about 10 ml. and then treated with 20 ml. of concentrated ammonium hydroxide for 24 hr. Pyridine and ammonia was then evaporated after the addition of 1 mmole of ammonium bicarbonate and the residual solution was diluted to about 100 ml. with 50% aqueous ethyl alcohol. This solution was applied to the top of a DEAE–cellulose (carbonate) column (43 × 4 cm. i.d.) pre-equilibrated with 0.01 *M* ammonium bicarbonate. Elution was carried out using a linear gradient, the mixing vessel containing, initially, 2.5 l. of 0.01 *M* ammonium bicarbonate in 10% ethyl alcohol and the reservoir an equal volume of 0.25 *M* ammonium bicarbonate in 20% ethanol. After the total solution had passed through, the mixing vessel was supplied with 1.5 l. of 0.25 *M* ammonium bicarbonate (20% ethanol) and the reservoir with 1.5 l. of the same salt (0.4 *M*) in 20% ethanol. Fractions of about 15-ml. volume were collected at 10-min. intervals. The elution pattern is given in Figure 1 and the distribution of the nucleotidic material in different peaks is given in Table II. Peak J contained pure Tr-TpTpT, while peaks H, I, and K also contained, mainly, the same product, there being about 10% contamination from the other compounds. The total yield of pure Tr-TpTpT was 6380 $O.D._{267}$ (71%). Analysis of a 5% portion of the total reaction mixture by paper chromatography in solvent A after alkaline removal of the acetyl group also gave identical result with respect to the yield of Tr-TpTpT. Tr-TpT recovered unchanged (peak F) amounted to about 12%. There were no other trityl-containing products in any significant amount.

Experiment II. Pyridinium Tr-TpT (0.15 mmole) in dry pyridine (0.75 ml.) was treated with DCC (0.085 g.) for 15 min. Dry pyridinium Dowex-50 ion-exchange resin (0.35 g.) was added followed by the addition of a dry pyridine solution (1 ml.) of pyridinium pT-OAc (0.15 mmole) preactivated with DCC (0.1 g.) for 5 min. The total mixture was shaken at room temperature for five days and then worked up as described above in Experiment I. The yield of pure Tr-TpTpT was 2645 $O.D._{267}$ (76%).

Experiment III. This was similar to experiment II except for the difference that Tr-TpT was not pretreated with DCC. Instead the total amount (0.185 mg., 0.9 mmole) of DCC used in the experiment was added to the pyridine solution of pyridinium pT-OAc and after 5 min. the nucleotide solution was combined with the Tr-TpT solution. The yield of Tr-TpTpT was 2523 $O.D._{267}$ (72%).

Experiment IV. Pyridinium pT-OAc (0.4 mmole) in 2 ml. of dry pyridine was treated with DCC (0.406 g., 2 mmoles) for 5 min. at room temperature. The mixture was then combined with an anhydrous pyridine solution of pyridinium Tr-TpT (0.1 mmole) containing pyridinium Dowex-50 ion-exchange resin (0.3 g.). The yield of Tr-TpTpT after a 25 hr. reaction period was 2483 $O.D._{267}$ (96%).

Experiment V. This was similar to experiment IV except that the solvent for the reaction was a mixture of freshly distilled dimethylformamide (1 ml.) and pyridine (0.5 ml.). The yield of Tr-TpTpT was 2385 $O.D._{267}$ (92%).

B. Using Mesitylenesulfonyl Chloride. To an anhydrous pyridine solution (1 ml.) of triethylammonium Tr-TpT (0.1 mmole) and pT-OAc (0.2 mmole) was added mesitylenesulfonyl chloride (0.130 g., 0.6 mmole) and the sealed mixture was kept at room temperature for 5.5 hr. Water (1 ml.) was added under cooling with ice and the aqueous pyridine solution kept at room temperature for a few hours. It was then treated with an excess of concentrated ammonium hydroxide for 24 hr., the ammonia was removed by evaporation, and the total mixture was analyzed by chromatography on a standard DEAE–cellulose column. The yield of Tr-TpTpT was 1874 $O.D._{267}$ (73%).

5′-O-Tritylthymidylylthymidylylthymidylylthymidine (V). A. Using DCC as the Reagent. Experiment I. An anhydrous pyridine (1 ml.) solution of Tr-TpTpT (0.1 mmole) was treated with DCC (0.075 g.) for 25 min. Pyridinium pT-OAc (0.1 mmole) in dry pyridine (1 ml.) was separately treated with DCC (0.1 g.) for 5 min. The two components were mixed and after the addition of pyridinium Dowex-50 resin (0.5 g.) the mixture was kept at room temperature for four days. Water (1 ml.) was then added and after a further two-day period the mixture was given a standard work-up and the total applied on top of a DEAE–cellulose (carbonate) column (44 × 4 cm. i.d.) pre-equilibrated with 0.01 *M* ammonium bicarbonate. The elution was carried out with a linear gradient of ammonium bicarbonate in 20% ethyl alcohol. The conditions and the elution pattern are shown in Figure 2a. The pure product, Tr-TpTpTpT, was in peak G, the yield being 2219 $O.D._{267}$ (65%). The tail of this peak contained an additional 156 $O.D._{267}$ of almost pure material.

Experiment II. This was identical with experiment I except that 0.5 mmole (5 molar equiv.) of pT-OAc was used. The elution pattern is shown in Figure 2b. The yield of Tr-TpTpTpT was 2831 $O.D._{267}$ (83%), not including an additional amount (about 4%) of the almost pure product present in the tail end of the main peak.

B. Using Mesitylenesulfonyl Chloride as the Reagent. An anhydrous pyridine (1 ml.) solution of Tr-TpTpT (0.1 mmole) was prepared separately. Anhydrous pyridinium pT-OAc (0.5 mmole) in 1 ml. of pyridine was treated with mesitylenesulfonyl chloride (0.219 g., 1 mmole) and after 10 min. the solutions were mixed under exclusion of moisture. After 4 hr. at room temperature the mixture was worked up and analyzed by chromatography on a DEAE–cellulose column by the standard method. The yield of Tr-TpTpTpT was 2835 $O.D._{267}$ (83%).

5'-O-Tritylthymidylylthymidylylthymidylylthymidylylthymidine (VI). A. Using DCC. Experiment I. Pyridinium Tr-TpTpTpT (0.07 mmole) in dry pyridine (1 ml.) was treated with DCC (0.041 g., 0.2 mmole) for 25 min. Pyridinium pT-OAc (0.07 mmole) was separately treated in 1 ml. of pyridine with DCC (0.05 g.) for 15 min. The two solutions were mixed, pyridinium Dowex-50 resin (0.4 g.) was added, and the sealed reaction mixture was kept at room temperature for five days. After a standard work-up, the total mixture was applied to the top of a DEAE–cellulose (carbonate) column (40 × 2.5 cm. i.d.) pre-equilibrated with 0.01 *M* ammonium bicarbonate containing 20% ethyl alcohol. The conditions for chromatography and the elution pattern are shown in Figure 3, while Table III gives the distribution of ultraviolet absorbing material in different peaks and the nature of their components. The total recovery of the optical density from the column was 82% of the total used in the starting materials. The various peaks were isolated by lyophilization and examined by paper chromatography (solvent A), and the multiple bands that were thus obtained were further analyzed by paper electrophoresis, dephosphorylation with phosphomonoesterase, and rechromatography in solvent A. The yield of pure Tr-TpTpTpTpT was 956 $O.D._{267}$ (32%).

Experiment II. The experiment was set up exactly as described above in experiment I except that 5 *M* proportions of pT-OAc were used. After chromatography on a DEAE–cellulose column, the yield of Tr-TpTpTpTpT was 1827 $O.D._{267}$ (61%), 2380 $O.D._{267}$ of the starting material, Tr-TpTpTpT, having been used as in experiment I above. The recovery of total ultraviolet absorbing material from the column was essentially quantitative (5530 $O.D._{267}$ out of 5775 $O.D._{267}$ applied to the column).

B. Using Mesitylenesulfonyl Chloride. An anhydrous pyridine solution (1 ml.) of Tr-TpTpTpT (2550 $O.D._{267}$, 0.075 mmole) was treated with a previously prepared anhydrous mixture of pT-OAc (0.375 mmole) and mesitylenesulfonyl chloride (0.75 mmole, 0.165 g.) in 1 ml. of anhydrous pyridine. The total reaction mixture was kept at room temperature for 6 hr., then cooled to 0° and treated with water (2 ml.) for about 12 hr. at 2°. Concentrated ammonium hydroxide (10 ml.) was then added and after a further 4 hr. at room temperature the solution was evaporated. The total product was then chromatographed on a DEAE–cellulose column (44 × 4 cm., i.d.) under the standard conditions. The yield of Tr-TpTpTpTpT, as contained in the pure peak, was 2146 $O.D._{267}$ (67%). The preceding and the succeeding peaks from the column also contained mainly the desired product and, including the amount of the product present in these peaks, the yield was 2402 $O.D._{267}$ (75%).

5'-O-Tritylthymidylylthymidylylthymidylylthymidylylthymidylylthymidine (VII). A. Using DCC. An anhydrous mixture of pyridinium Tr-TpTpTpTpT (960 $O.D._{267}$, 0.0226 mmole), pyridinium pT-OAc (0.324 mmole), and pyridinium Dowex-50 ion-exchange resin (1 g.) in 3 ml. of dry pyridine was treated with DCC (0.32 g.) and the mixture was kept at room temperature for three days. Water (2 ml.) was then added, the excess of DCC was extracted with pentane, and the aqueous pyridine solution was kept at room temperature for two days. After removal of the resin (wash with 50% aqueous pyridine) by filtration, the solution was partly evaporated and chromatographed on a DEAE–cellulose (carbonate) column (38 × 2.5 cm. i.d.). The conditions of elution and the elution pattern are shown in Figure 4. Peak H contained the desired product, the yield being 694 $O.D._{267}$ (60%).

B. Using Mesitylenesulfonyl Chloride. An anhydrous pyridine (1 ml.) solution of pT-OAc (0.48 mmole) was treated with mesitylenesulfonyl chloride (0.25 g., 1.12 mmole) at room temperature with exclusion of moisture and after 3 min. the solution was added to an anhydrous pyridine (1 ml.) solution of pyridinium Tr-TpTpTpTpT (1380 $O.D._{267}$, 0.032 mmole). The sealed reaction mixture was kept at 5° for 20 hr. and then treated with 2 ml. of ice–water. This solution was kept at 2° for one day, then treated with concentrated ammonium hydroxide (10 ml.) for one day. After evaporation, the mixture was chromatographed as described in the experiment of Figure 4. The yield of the desired product (VII) was 1090 $O.D._{267}$ (65.8%).

Reaction of Tr-TpT with DCC in Pyridine. Isolation of 5'-C-Pyridiniumthymidine (VIII). To pyridinium Tr-TpT (0.05 mmole) in 0.5 ml. of dry pyridine was added DCC (0.2 mmole, 0.103 g.) and the solution was kept stoppered at room temperature for six days. Water (0.5 ml.) was then added and the mixture was kept overnight. The solvent was evaporated after adding ammonium bicarbonate (0.1 *M*, 2 ml.). The residue was dissolved in alcohol–water and chromatographed on a DEAE–cellulose (carbonate) column (23.5 × 1.5 cm., i.d.) using a linear gradient of ammonium bicarbonate. A peak (about 30 O.D. units at 267 mμ) appeared at the front after the hold-up volume. It consisted mainly of a compound which on chromatography in solvent A had an R_f of 0.27. On electrophoresis at pH 7.1 this compound had a mobility of −0.9 compared to pT. Chromatography of the starting material Tr-TpT (0.05 mmole) itself under identical conditions using the same column failed to reveal any peak corresponding to the above products. The compound showed a spectrum composite of the quaternary pyridinium cation and thymidine chromophore.