Studies on Polynucleotides. LX.[1] The Use of Preformed Dinucleotide Blocks in Stepwise Synthesis of Deoxyribopolynucleotides[2]

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Abstract: The condensation of preformed thymidine dinucleotide bearing a 5′-phosphomonoester group (pTpT-OAc) with thymidine oligonucleotides bearing a free 3′-hydroxyl group has been systematically investigated. Aromatic sulfonyl chlorides proved to be the most active and rapid condensing agents. The rate of internucleotide bond synthesis was slower than that found previously using mononucleotides as the condensing unit. The yield of the tetranucleotide, Tr-TpTpTpT, formed by the condensation of pTpT-OAc and 5′-O-tritylthymidylylthymidine (Tr-TpT) using roughly stoichiometric amounts of the two components, was 65–80%. The yield in the formation of the hexanucleotide from the tetranucleotide, Tr-TpTpTpT, using two- to fourfold excess of pTpT-OAc, was 38–57%, while the yield in the synthesis of the octanucleotide from the hexanucleotide, Tr-TpTpTpTpTpT, and pTpT-OAc (fivefold excess) was 24–38%. It is concluded that the use of preformed dinucleotides in deoxyribopolynucleotide synthesis is feasible provided an increasing excess of the dinucleotides is used with an increase in the chain length of the second component.

The principle used in recent work on the stepwise synthesis of specific deoxyribopolynucleotides has involved the successive addition of mononucleotide units to the 3′-hydroxyl end of a growing oligonucleotide chain.[4] The alternative approach, which theoretically is more attractive, involves the use of preformed di- and higher oligonucleotide blocks in repetitive condensation steps. Some investigations of this approach were reported several years ago[5] but the yields then obtained of the desired condensation products, in general, were low and, consequently, no use was made of this approach in more recent synthetic work. With continued interest in the construction of relatively long deoxyribopolynucleotide chains of specific nucleotide sequences, a more systematic investigation of the approach involving preformed oligonucleotide blocks as the condensing units has now been carried out. The experiments reported in this paper deal with the synthesis of thymidine oligonucleotides by the use of preformed thymidine dinucleotide as the condensing unit. Accompanying papers describe further investigations of this general approach involving oligonucleotide blocks in the stepwise synthesis of deoxyribopolynucleotides containing specific sequences.[6,7]

Synthesis of the Trinucleotide Tr-TpTpT.[8] The synthesis of Tr-TpTpT from Tr-T[8] and pTpT-OAc[8] was investigated using mesitylenesulfonyl chloride (MS)[9] and triisopropylbenzenesulfonyl chloride (TPS)[10] as the condensing agents. Figure 1 shows the kinetics of formation of Tr-TpTpT using equimolar amounts of the two reaction components and 5 molar equiv, as based on pTpT-OAc, of the condensing agents. The amount of the product reaches a maximum in about 24 hr in both cases, and the most significant finding is that the rate of reaction is much slower than that of the condensation of the mononucleotide, pT-OAc, and Tr-T.[9] In addition, the final yields obtained in the present study were lower than those obtained previously in the experiments with pT-OAc.

Table I shows the results of further comparative experiments on the synthesis of Tr-TpTpT. In the first experiments equivalent amounts of Tr-T and pTpT-OAc were used and the amount of the condensing agents was varied. The use of 2.5 and 5 molar equiv[11]

Table I. Yields of the Trinucleotide, Tr-TpTpT, Formed by Condensation of Tr-T with pTpT-OAc[a]

Tr-T, μmoles	pTpT-OAc, equiv, based on Tr-T	Reagent	Reagent, equiv, based on pTpT-OAc	Volume of reacn soln, ml	Yield of Tr-TpTpT, %
12.5	1.0	MS	2.5	0.25	46[b]
12.5	1.0	MS	5.0	0.25	48[b]
54.0	1.0	MS	5.0	0.25	52[b]
12.5	1.0	MS	7.5	0.25	33[b]
12.5	1.0	TPS	5.0	0.25	53[b]
12.5	1.0	TPS	7.5	0.25	53[b]
25.0	0.25	MS	5.0	0.55	67[b]
3.1	4.0	MS	5.0	0.17	65[c]

[a] The reaction mixtures were set up according to the general procedure in the text. The reaction period was 24 hr at room temperature throughout, and the analyses were carried out by paper chromatography in solvent A. [b] Yields based on pTpT-OAc. [c] Yield based on the minor, Tr-T, component.

(1) Preceding paper in this series: A. R. Morgan, R. D. Wells, and H. G. Khorana, *Proc. Natl. Acad. Sci. U. S.*, **56**, 1899 (1966).

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(3) H. K. wishes to acknowledge the receipt of a postdoctoral fellowship (1964–1966) from the Deutscher Akademischer Austauschdienst, Bad Godesberg, Germany.

(4) (a) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 368 (1965); (b) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 2971 (1965); (c) S. A. Narang and H. G. Khorana, *ibid.*, **87**, 2981 (1965); (d) S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, **87**, 2988 (1965).

(5) (a) G. Weimann and H. G. Khorana, *ibid.*, **87**, 419 (1962); (b) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963); (c) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963).

(6) H. Kössel, H. Büchi, and H. G. Khorana, *ibid.*, **89**, 2185 (1967).

(7) E. Ohtsuka and H. G. Khorana, *ibid.*, **89**, 2195 (1967).

(8) Abbreviations used are as have been defined in earlier papers. See, for example, ref 4a and 5c.

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

(10) R. Lohrmann and H. G. Khorana, *ibid.*, **88**, 829 (1966).

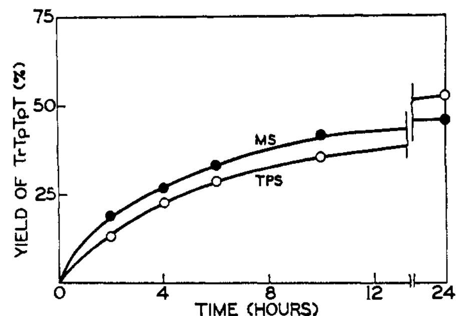

Figure 1. Rate of formation of the trinucleotide, Tr-TpTpT, by condensation of equimolar quantities of Tr-T with pTpT-OAc in anhydrous pyridine using the reagents MS or TPS (both were used at a concentration of 5 molar equiv based on the dinucleotide). Work-up and estimation of yield was as in the Experimental Section.

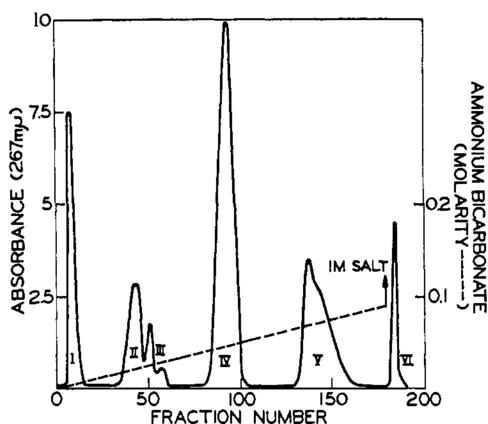

Figure 2. Analysis of the reaction mixture in the preparation of the trinucleotide, Tr-TpTpT, from Tr-T (54 μmoles) and pTpT-OAc (54 μmoles) in the presence of MS (270 μmoles) (line 3 of Table I). Following work-up (see Experimental Section) the total mixture was fractionated on a DEAE-cellulose column (50 × 1 cm, bicarbonate form in 50% aqueous ethyl alcohol). The column was eluted at room temperature with a linear salt gradient (2 l. of 50% aqueous ethyl alcohol in the mixing vessel and 2 l. of 0.2 M ammonium bicarbonate in 50% aqueous ethyl alcohol in the reservoir). Fractions of 8 ml were collected every 15 min. The identification of the different ultraviolet-absorbing peaks was as follows: peak I, pyridine, 5′-pyridiniumthymidine, and 5′-pyridiniumthymidylyl-(3′→5′)-thymidine; peak II, mesitylenesulfonic acid; peak III (130 OD_{267} units), Tr-TpT and an unidentified component; peak IV (660 OD_{267} units), pure Tr-TpTpT; peak V (398 OD_{267} units), pTpT; peak VI (65 OD_{267} units), mainly symmetrical pyrophosphate of pTpT.

of MS gave maximal yields, whereas a further increase in MS (to 7.5 molar equiv) caused a decrease, presumably due either to direct sulfonylation of Tr-T or to an increase in degradation of Tr-TpTpT-OAc, the product of condensation. The use of 5–7.5 molar equiv of TPS also gave equally good yields. In further experiments, the effect of varying the ratio of Tr-T to pTpT-OAc was studied. As seen in Table I (bottom two lines), the use of an excess of either one of the two components increased the yield with respect to the minor component, but the yield with respect to this component was not quantitative. As seen below, an important consequence of the use of an excess of the component bearing the phosphomonoester group seems to be to increase the rate of reaction.

(11) With equivalent amounts of the two components, pTpT-OAc and Tr-T, the use of less than 2 molar equiv (based on pTpT-OAc) of the condensing agents gave little condensation.

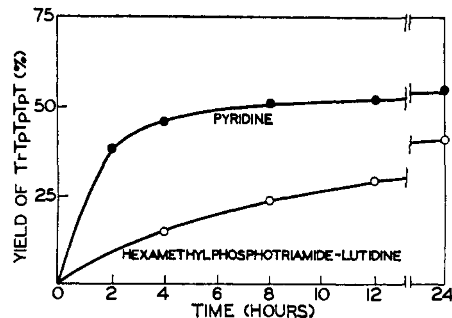

Figure 3. Rate of formation of the tetranucleotide, Tr-TpTpTpT, by condensation of Tr-TpT (42 μmoles) with pTpT-OAc (58 μmoles) in anhydrous pyridine or in a mixture of three parts of hexamethylphosphotriamide and one part of 2,6-lutidine (total volume 0.6 ml). In both cases, TPS (5 equiv as based on pTpT-OAc) was used as the condensing agent. For work-up see Experimental Section. Yields are based on pTpT-OAc.

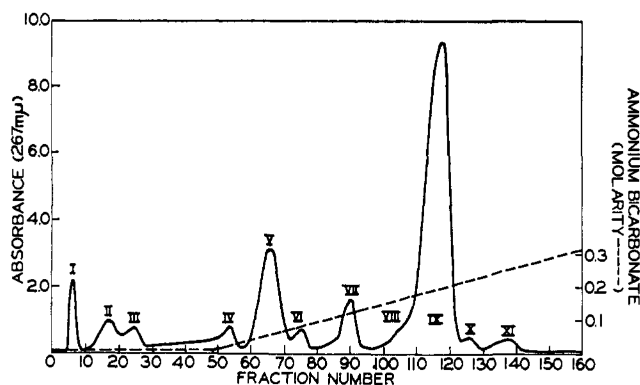

Figure 4. Chromatographic analysis of the reaction mixture in the preparation of the tetranucleotide, Tr-TpTpTpT, from Tr-TpT (84 μmoles) and pTpT-OAc (58 μmoles) in the presence of TPS (150 μmoles). Following work-up (see Experimental Section) the bulk of the product was fractionated on a DEAE-cellulose column (50 × 1 cm, bicarbonate form in 20% ethyl alcohol). The column was first eluted at room temperature with 0.01 M ammonium bicarbonate in 20% ethyl alcohol, fractions of 10–11 ml being collected every 14 min. After 48 fractions had been collected, elution was continued using a linear salt gradient (750 ml of 20% ethyl alcohol in the mixing vessel and 750 ml of 0.4 M ammonium bicarbonate in 20% ethyl alcohol in the reservoir). Identification of the products is given in Table II.

While paper chromatography was mainly used to determine the extent of reaction in the above experiments, one analysis was performed by DEAE-cellulose column chromatography. The elution pattern obtained and the identification of the various products formed are shown in Figure 2. It is thus seen that the results of the two methods of analysis were in good agreement and, further, the results of Figure 2 confirmed that, in the synthesis of Tr-TpTpT, side-product formation was not serious.

Synthesis of the Tetranucleotide, Tr-TpTpTpT, from Tr-TpT and pTpT-OAc. Results of a kinetic study of the formation of Tr-TpTpTpT using a slight excess (38%) of pTpT-OAc over Tr-TpT are shown in Figure 3. The final yield (77%) of the product was high, and it was reached within a 12-hr reaction period. It is very significant that the rate of reaction is higher than that observed above for the condensation of Tr-T with pTpT-OAc.[12]

(12) This finding demonstrates the participation of the phosphodiester anion in Tr-TpT in forming a mixed anhydride linkage with the

Table II. Identification of Products Formed in the Synthesis of Tr-TpTpTpT[a,b]

Peak	Fraction	OD_{267} units	Identification
I	5–8	51	5′-Pyridinium T and 5′-pyridinium TpT
II	12–21	70	Sulfonic acid, TpT, 5′-pyridinium-TpTpT
III	22–27	41	Sulfonic acid and TpT or cyclo-pTpT[c]
IV	48–57	50	Sulfonic acid
V	60–71	216	Mainly Tr-TpT
VI	72–77	30	TpTpT?
VII	84–93	86	Mainly pTpT
VIII	100–106	38	Unidentified
IX	107–122	782	Tr-TpTpTpT (89%); $O(pTpT)_2$ (11%)
X	123–129	23	Unidentified
XI	131–140	23	Unidentified

[a] In another similar experiment the elution pattern of which is not shown, nucleotidic material was eluted at a salt concentration of 0.45–0.65 *M*, which amounted to 14% of the total nucleotidic material applied to the column. By paper chromatography in solvent A at least five components could be detected, the two major bands (Tr positive) moving with R_f's of 0.38 (46%) and 0.12 (41%). Upon treatment with 1 *N* alkali for 1 hr at 100° both of these bands were completely degraded. Each gave rise to the same five products with R_f values of 0.70 (Tr positive), 0.43 (Tr positive), 0.36 (Tr negative), 0.23 (Tr positive), and 0.09 (Tr positive). [b] For details of reaction and column chromatography see Figure 4. [c] As shown by resistance to alkaline phosphomonoesterase.

Table III. Yields of the Tetranucleotide, Tr-TpTpTpT, Formed by Condensation of Tr-TpT with pTpT-OAc[a]

Tr-TpT, μmoles	pTpT-OAc, equiv, based on Tr-TpT	Reagent	Reagent, equiv, based on pTpT-OAc	Reaction conditions: Volume, ml	Reaction conditions: Time, hr	Yield of Tr-Tp-TpTpT, %
9.4	1.4	MS	4	0.25	24	74[b]
9.4	1.4	TPS	4	0.25	24	77[b]
4.7	2.8	MS	4	0.20	6	80[b]
4.7	2.8	TPS	4	0.20	6	81[b]
4.7	2.8	TPS	8	0.20	6	83[b]
2.3	5.5	MS	4	0.17	6	87[b]
2.3	5.5	TPS	4	0.17	6	91[b]
2.3	5.5	TPS	8	0.17	6	87[b]
42	1.4	TPS	2.6	0.5	24	77[b]
42	1.4	TPS	4.3	0.5	24	75[b]
84	0.7	TPS	2.6	0.5	24	66[c]
84	0.7	TPS	4.3	0.5	24	76[c]
94	0.7	TPS	3.8	0.5	11	52[c]
50	1.5	DCC[d]	6.0	1.0	120	66[b]
21	1.4	Picryl chloride	3.0	1.0	48	51[c]

[a] The reaction mixtures were set up according to the general procedure in the text. Analysis of the small-scale experiments (1–8) was carried out by paper chromatography in solvent A. The larger scale experiments (9–15) were analyzed by chromatography on DEAE-cellulose columns using conditions as described in Figure 4. [b] Yields based on Tr-TpT. [c] Yields based on pTpT-OAc. [d] The reaction mixture contained also 200 mg of dry pyridinium Dowex 50X2 ion-exchange resin. Both of the reaction components were used in the pyridinium form.

Detailed analysis by DEAE-cellulose column chromatography of reaction products obtained in the condensation of Tr-TpT and pTpT-OAc (using a slight excess of Tr-TpT) is shown in Figure 4 and Table II. As is seen, a number of side products were formed in small amounts but the major peaks corresponded to the desired product (peak IX), unreacted pTpT (peak VII), and unreacted Tr-TpT (peak V). The yield of pure Tr-TpTpTpT in this experiment was 76%.

Table III shows the results of experiments on the synthesis of Tr-TpTpTpT in which variations in reaction conditions were tried. The following points are worthy of comment. (1) The use of MS and TPS as the reagents in two sets of experiments gave essentially identical yields. (2) The yield of Tr-TpTpTpT was uniformly high when approximately equal proportions of the two components were used. (3) The use of an increasing excess of pTpT-OAc increased the yield of the product and a further major effect of this variation was an increase in the rate of reaction, the reactions being essentially complete in 2–4 hr. (4) The use of DCC as the condensing agent gave also a high yield (66%) of Tr-TpTpTpT when stoichiometric amounts of the two components, Tr-TpT and pTpT-OAc, were used. (5) The use of picryl chloride[13] gave a lower yield of Tr-TpTpTpT, the time of reaction necessary being much longer than those used for the reactions with sulfonyl chlorides.

The nucleophilic attack by pyridine, the standard medium of reaction, on activated phosphate esters leads to the formation of side products during condensations experiments, and attempts have been made from time to time in this laboratory to find alternative solvent systems. In one experiment, a mixture of lutidine and the very polar solvent, hexamethylphosphorotriamidate, was used as the medium. The rate of synthesis of Tr-TpTpTpT (Figure 3) in this solvent was substantially lower than in pyridine.

Synthesis of the Pentanucleotide, Tr-TpTpTpTpT. When equivalent amounts of Tr-TpTpT and pTpT-OAc were used in the presence of TPS, Tr-TpTpTpTpT was obtained in 26% yield (Table IV). By using an excess of pTpT-OAc, better yields were obtained (Table IV), the maximum yield (60%) being obtained in presence of 4 equiv of pTpT-OAc. The results of this experiment are presented in more detail in Figure 5 and Table V, which show the distribution of products on anion-exchange chromatography. The side products in this experiment were minor.

Table IV. Yields of Pentanucleotide, Tr-TpTpTpTpT, Formed in the Reaction of Tr-TpTpT with pTpT-OAc[a]

Tr-TpTpT, μmoles	pTpT-OAc, equiv, based on Tr-TpTpT	Reagent	Reagent, equiv, based on pTpT-OAc	Yield of Tr-TpTp-TpTpT, %
17.3	1.0	TPS	6	26
17.3	2.0	TPS	6	49
10.4	4.1	TPS	6	60
6.9	7.0	TPS	6	58
13.0	4.1	Picryl chloride	3	50

[a] The reaction volume was 1.0 ml and the reaction time 12 hr. Work-up was as described in the Experimental Section. Analysis was carried out by chromatography on a DEAE-cellulose column.

second component pTpT-OAc. This prior interaction between the two components would be expected to facilitate the phosphorylation of the 3′-hydroxyl group in Tr-TpT. A similar conclusion has been drawn previously: footnote 36 in G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 4329 (1962).

(13) F. Cramer, R. Wittmann, K. Daneck, and G. Weimann, *Angew. Chem.*, **75**, 92 (1963).

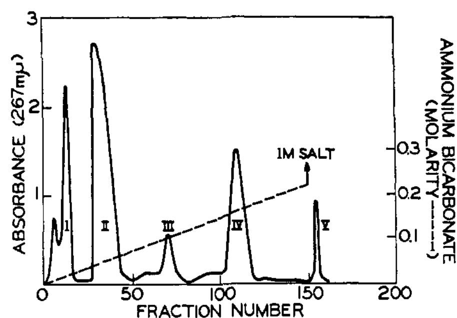

Figure 5. Chromatographic analysis of the reaction mixture in the preparation of the pentanucleotide, Tr-TpTpTpTpT, from Tr-TpTpT (10.4 μmoles) and pTpT-OAc (42.6 μmoles) in the presence of TPS (255 μmoles). Following work-up (see Experimental Section) the total product was fractionated on a DEAE-cellulose column (45 × 1 cm, bicarbonate form). The column was eluted at room temperature with a linear salt gradient (3 l. of 0.2 *M* ammonium bicarbonate in the mixing vessel and 3 l. of 0.3 *M* ammonium bicarbonate in the reservoir). Fractions of 15 ml were collected every 20 min. For identification of the products see Table V.

When Tr-TpTpT and the dinucleotide, pTpT-OAc (4 equiv), were condensed in the presence of picryl chloride the yield (50%) of the pentanucleotide was lower than when TPS was used as the condensing reagent. Also the reaction was slower using picryl chloride.

Table V. Identification of Products Formed in the Synthesis of the Pentanucleotide, Tr-TpTpTpTpT (Experiment of Figure 5)

Peak	Fraction	OD_{267} units	Identification
I	8–16	...	Sulfonic acid
II	28–44	650	pTpT and Tr-TpTpT
III	67–75	45	Mainly $O(pTpT)_2$
IV	102–120	306	Tr-TpTpTpTpT (95% pure)
V	152–160	32	Unidentified (1 *M* salt fraction)

Synthesis of the Hexanucleotide, Tr-TpTpTpTpTpT, from Tr-TpTpTpT and pTpT-OAc. Results of three experiments on the condensation of Tr-TpTpTpT and pTpT-OAc are given in Table VI. Using 2 equiv of

Table VI. Yields of the Hexanucleotide, Tr-TpTpTpTpTpT, Formed by Condensation of Tr-TpTpTpT with pTpT-OAc[a]

Tr-TpTpTpT, μmoles	12.5	12.5	9.25
pTpT-OAc, equiv, based on Tr-TpTpTpT	2.0	4.0	4.0
TPS, equiv, based on pTpT-OAc	5.0	5.0	2.0
Vol., ml	0.5	0.5	0.3
Yield of hexanucleotide, %	38	52	38
Recovery of Tr-TpTpTpT, % of theor	24	14	43
Recovery of pTpT, % of theor	83	61	97

[a] The time of reaction was 6 hr. Analysis was carried out by chromatography on DEAE-cellulose columns using conditions as described for Figure 6.

pTpT-OAc, the yield of the hexanucleotide was 38%, and by using a larger amount (4 equiv) of the dinucleotide, the yield increased to 52%. The products obtained in the former experiment were analyzed by chromatography on a DEAE-cellulose column, and the elution pattern and identification of the different peaks obtained are given in Figure 6 and Table VII. It is seen that several side products, which remain unidentified, were obtained. In an attempt to limit side-product formation, a condensation reaction (last column of Table VI) was carried out using a smaller amount (2 as opposed to 5 equiv based on the dinucleotide component) of the condensing agent, TPS. This appeared successful in that a better recovery of the unused tetranucleotide component was obtained, but the yield of the desired product was also reduced.

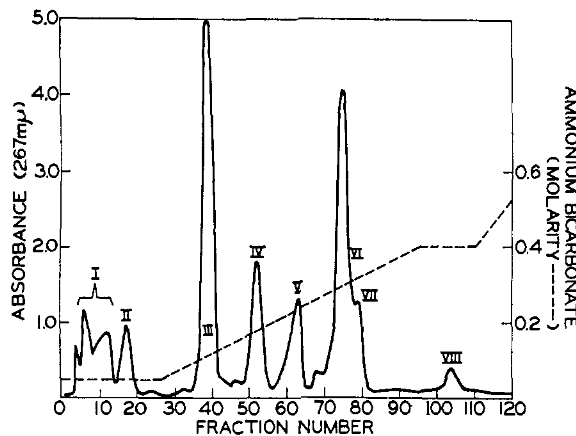

Figure 6. Chromatographic analysis of the reaction mixture in the preparation of the hexanucleotide, Tr-TpTpTpTpTpT, from Tr-TpTpTpT (12.5 μmoles) and pTpT-OAc (25 μmoles) in the presence of TPS (100 μmoles). Following work-up (see Experimental Section) the total mixture was fractionated on a DEAE-cellulose column (50 × 1 cm, bicarbonate form in 20% ethyl alcohol). The column was eluted at room temperature with 0.01 *M* ammonium bicarbonate in 20% ethyl alcohol; fractions of 18 ml were collected every 15 min. After 25 fractions had been collected elution was continued using a linear salt gradient (750 ml of 20% ethyl alcohol in the mixing vessel and 750 ml of 0.5 *M* ammonium bicarbonate in 20% ethyl alcohol in the reservoir). For the identification of the products see Table VII.

Table VII. Identification of Products Formed in the Synthesis of the Hexanucleotide, Tr-TpTpTpTpTpT (Experiment of Figure 6)

Peak	Fraction	OD_{267} units	Identification
I	5–14	137	Sulfonic acid and unidentified products
II	15–20	54	Unidentified
III	36–42	298	pTpT
IV	49–55	112	Tr-TpTpTpT (57%); $O(pTpT)_2$ (23%) and unidentified product
V	58–65	92	Unidentified[a]
VI	70–78	307	Tr-TpTpTpTpTpT (89%) + compound of peak VII (11%)
VII	79–82	58	Unidentified
VIII	102–106	26	Unidentified

[a] This product (R_f 0.07 in solvent A) gave a trityl-positive reaction and was sensitive to alkali. After treatment with 1 *N* NaOH for 1 hr at 100° followed by chromatography in solvent A, three bands (R_f 0.07, 0.24, and 0.35) in about equal amounts were obtained.

Synthesis of the Octanucleotide, Tr-TpTpTpTpTpTpTpT. The octanucleotide was synthesized from Tr-TpTpTpTpTpT and pTpT-OAc in two experiments.

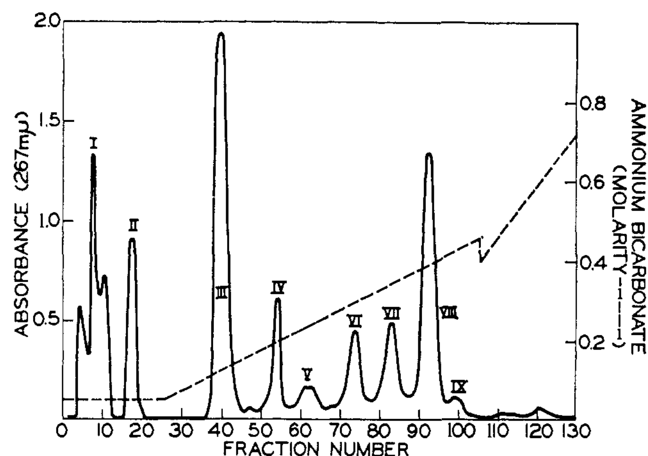

Figure 7. Chromatographic analysis of the reaction mixture in the preparation of the octanucleotide, Tr-TpTpTpTpTpTpTpT, from Tr-TpTpTpTpTpT (3.1 μmoles) and pTpT-OAc (16 μmoles). Following work-up (see Experimental Section) the total mixture was fractionated on a DEAE-cellulose column (50 × 1 cm, bicarbonate form in 20% ethyl alcohol). The column was eluted at room temperature with 0.01 *M* ammonium bicarbonate in 20% ethyl alcohol. Fractions of 18 ml were collected every 15 min. After 25 fractions had been collected elution was continued using a linear salt gradient (750 ml of 20% ethyl alcohol in the mixing vessel and 750 ml of 0.5 *M* ammonium bicarbonate in 20% ethyl alcohol in the reservoir). For identification of the products see Table IX.

The same excess of pTpT-OAc (4 equiv) was used in both experiments, the difference in the reactions being the quantity of sulfonyl chloride reagent used. The results are given in Table VIII. Limiting the amount of the condensing reagent appeared to reduce side-product formation, as shown by better recovery of the unused hexanucleotide component. However, the yield of the octanucleotide was lower. Detailed analysis of one of the reaction mixtures is shown in Figure 7 and identification of the different ultraviolet-absorbing products is shown in Table IX.

Table VIII. Yields of the Octanucleotide Tr-TpTpTpTpTpTpTpT Formed by Condensation of Tr-TpTpTpTpTpT with pTpT-OAc[a]

Tr-TpTpTpTpTpT, μmoles	3.1	2.35
pTpT-OAc, equiv, based on Tr-TpTpTpTpTpT	5.16	5.0
TPS, equiv, based on pTpT-OAc	4.0	2.0
Yield of octanucleotide, %	38	24
Recovery of Tr-TpTpTpTp-TpT, % of theor	24	50
Recovery of pTpT, % of theor	80	88

[a] The time of reaction was 6 hr and the volume 0.2 ml. Analysis was carried out by chromatography on DEAE-cellulose columns according to Figure 7.

Table IX. Identification of Products Formed in the Synthesis of the Octanucleotide Tr-TpTpTpTpTpTpTpT (Experiment of Figure 7)

Peak	Fraction	OD_{267} units	Identification
I	4–11	63	Mainly sulfonic acid
II	15–19	34	Unidentified[a]
III	37–45	205	pTpT
IV	51–55	24	$O(pTpT)_2$
V	60–65	14	Two unidentified products[b]
VI	70–77	31	Mainly Tr-TpTpTpTpTpT
VII	79–86	36	Unidentified[c]
VIII	88–96	88	Pure Tr-TpTpTpTpTpTpTpT
IX	97–100	7	Unidentified

[a] R_f 0.13 in solvent A. [b] One trityl positive (R_f 0.07 in solvent A) and a second trityl negative (R_f 0.00 in solvent A). [c] Trityl positive (R_f 0.12 in solvent A).

Discussion

The main conclusion from the present experiments is that reasonably high yields can be obtained by using a protected dinucleotide for condensation with the 3′-hydroxyl end of a growing oligonucleotide chain. A decrease in yield with an increase in the chain length of the component bearing a 3′-hydroxyl group was observed. This decrease was partly overcome by increasing the excess of the dinucleotide component. The use of more than 2 molar equiv of aromatic sulfonyl chlorides with respect to the dinucleotide component increased appreciably the yields in most cases but at the same time increased the extent of side reactions leading to degradation of the oligonucleotide chain. Further, the seriousness of degradative reactions, the possible mechanisms of which have been discussed previously,[4a,14] increased with an increase in length of the oligonucleotide chain. From these results it seems advisable[6] to use a generously large excess of the dinucleotide component on the one hand and to use, on the other hand, the minimum amount (about two molar proportions as based on the dinucleotide) of the sulfonyl chloride. The use of a large excess of the dinucleotide component would be expected to reduce the time of reaction, and this factor, together with the low concentration of the sulfonyl chloride, would minimize the side reactions and permit better recoveries both of the condensation products and of the unused starting materials. The latter would be expected to be sufficiently pure for reuse.

Aromatic sulfonyl chlorides and dicyclohexylcarbodiimide have previously been found to be the most efficient reagents for polynucleotide synthesis. In the present study of block condensations, highest yields were obtained with the aromatic sulfonyl chloride although the yield obtained using DCC was not significantly inferior in the experiment in which a comparison was made. The aromatic sulfonyl chlorides, besides giving the most rapid rates of reaction, have the advantage that the condensation reactions can be carried out in the presence of trialkylamines which are often desirable components of reaction mixtures in polynucleotide synthesis.

Experimental Section

General Methods. Paper chromatography was performed by the descending technique using Whatman No. 1 paper. The solvent system used were: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent B, *n*-propyl alcohol–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). The R_f values of the different compounds are listed in Table X.

Paper electrophoresis was carried out using a commercially available apparatus capable of giving a potential of 5000 v using potassium phosphate (0.03 *M*, pH 7.1) buffer.

(14) E. Ohtsuka, M. W. Moon, and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 2956 (1965).

Table X. Paper Chromatography of Thymidine-Containing Deoxyribopolynucleotides

Compd	R_f in solvent A	B	C	D	Electrophoretic mobility at pH 7	$\epsilon_{230}/\epsilon_{267}$ at pH 7
Tr-T	0.90					1.05
Tr-TpT	0.73	0.86	0.83			0.82
Tr-TpTpT	0.49	0.81	0.72		0.58	0.60
Tr-TpTpTpT	0.27	0.72	0.59		0.68	0.52
Tr-TpTpTpTpT	0.14	0.64	0.44		0.85	
Tr-TpTpTpTpTpT	0.06	0.54				0.43
Tr-TpTpTpTpTpTpTpT	0.01	0.37			1.01	0.38
pT	0.14		0.36	0.48	1.0	0.28
pTpT	0.05	0.37	0.28	0.29		
$O(pTpT)_2$				0.09		
pT-OAc			0.49			
pTpT-OAc				0.39		
T	0.66					
TpT	0.41					
TpTpT	0.23					
TpTpTpT	0.07					
TpTpTpTpT		0.39				
TpTpTpTpTpT		0.31				
TpTpTpTpTpTpTpT		0.18				
Triisopropylsulfonic acid	0.82					
Mesitylenesulfonic acid	0.77					

All reaction mixtures were prepared in a drybox which was maintained under a slightly positive nitrogen pressure and contained phosphorus pentoxide to ensure removal of moisture. Enzymic degradation of the synthetic products using spleen phosphodiesterase and purified venom phosphodiesterase were performed as described previously.[15] The trityl group in compounds was detected by spraying the chromatograms with 10% aqueous perchloric acid and drying them in warm air; the trityl-containing products appeared yellow. The preparation of MS,[9] TPS,[10,16] Tr-T,[5a] Tr-TpT,[4a] and Tr-TpTpT[4a] has been described previously. Picryl chloride was obtained commercially. Pyridine was purified by fractional distillation from chlorosulfonic acid and then redistilled from sodium hydroxide and stored over molecular sieves. In thymidine oligonucleotides, hypochromicity was neglected and, for calculation of molar extinction coefficients, a value of 9600 was used for each thymidine residue of the molecule at 267 mμ at neutral pH.

5′-O-Phosphorylthymidylyl-(3′→5′)-thymidine. An aqueous pyridine solution of pyridinium β-cyanoethylthymidine 5′-phosphate (5 mmoles) and pyridinium 3′-O-acetylthymidine 5′-phosphate (10 mmoles) was rendered anhydrous by repeated evaporation of pyridine. Pyridine (40 ml), anhydrous pyridinium Dowex 50 ion-exchange resin (1 g), and DCC (6 g) were added to the residual gum, and the resulting solution was shaken for 4 days at room temperature. Water (40 ml) was added and the solution was extracted with cyclohexane (two 100-ml portions). The aqueous pyridine solution was treated at room temperature with 2 *N* sodium hydroxide solution (30 ml). After 20 min, an excess of pyridinium Dowex 50 ion-exchange resin was added to remove sodium ions, and the resin was removed by filtration and washed with water. The filtrate and washings were evaporated to a gum, dissolved in water (40 ml), and applied to the top of a DEAE-cellulose column (90 × 5 cm, bicarbonate form). Elution was carried out at 0° using a linear salt gradient (8 l. of water in the mixing vessel and 8 l. of 0.2 *M* triethylammonium bicarbonate in the reservoir). The dinucleotide was eluted at 0.1 *M* salt concentration and amounted to 60,000 OD_{267} units (63%). The fractions containing the product were evaporated in the presence of pyridine and rendered anhydrous by repeated evaporation of pyridine. The anhydrous gum was dissolved in anhydrous pyridine (25 ml) and precipitated in ether (700 ml) to afford 2.5 g of the dinucleotide. The product was homogeneous by paper chromatography and by paper electrophoresis.

5′-O-Phosphorylthymidylyl-(3′→5′)-3′-O-acetylthymidine, pTpTOAc. 5′-O-Phosphorylthymidylyl-(3′→5′)-thymidine (150 mg) was dissolved in 10 ml of anhydrous pyridine, and the pyridine was evaporated. The dry gum was dissolved in pyridine (5 ml), and acetic anhydride (0.6 ml) was added. After 7 hr at room temperature water (15 ml) was added, and the product was left at room temperature for 6 hr. The solution was evaporated to a gum which was dissolved in water (200 ml) and lyophilized. The yield of the acetylated dinucleotide was 154 mg. On chromatography in solvent D two products were observed, the dinucleotide pTpT-OAc (R_f 0.39) and its pyrophosphate $O\text{-}(pTpT\text{-}OAc)_2$ (R_f 0.22). The sample contained 17% of the pyrophosphate.

General Methods for Kinetic Studies of Condensation Reactions. (a) Condensation of TrT or Pyridinium Tr-TpT with Triethylammonium pTpT-OAc. Reactions on 3–15-μmole Scale. Stock solutions of the nucleoside and nucleotide components were separately prepared by dissolving the components in anhydrous pyridine. After repeated evaporation of pyridine the gum obtained was dissolved in anhydrous pyridine (1 ml). The concentrations of the solutions were TrT, 0.12 *M;* Tr-TpT, 0.094 *M;* and pTpT-OAc, 0.12 *M*. A stock solution of the sulfonyl chloride reagents MS (0.6 *M*) and TPS (0.6 *M*) were similarly prepared in anhydrous pyridine. Appropriate aliquots from the nucleoside, nucleotide, and sulfonyl chloride solutions were removed using micropipets and transferred to 3-ml test tubes which were immediately sealed; complete details of the composition of the mixtures are presented in Tables I and III. Samples were removed from the reaction solutions after 2, 4, 6, 10, and 24 hr. They were diluted with water (0.2 ml) and allowed to stand at room temperature overnight. Concentrated ammonia solution (0.5 ml) was added for 1 hr, and the products were analyzed by paper chromatography in solvent A.

(b) Condensation of Tr-TpT with pTpT-OAc on a Large Scale. Pyridinium Tr-TpT (89 mg, 84 μmoles) and triethylammonium pTpT-OAc (98 mg, 116 μmoles) were dissolved in anhydrous pyridine (2 ml) and dried by repeated evaporation of pyridine. The dry gum was dissolved in anhydrous pyridine (1 ml) and TPS (152 mg, 500 μmoles) was added. Samples were removed from the reaction solution after 2, 4, 8, 12, and 24 hr. They were diluted with an equal volume of water and allowed to stand at room temperature overnight. Concentrated ammonia solution (twice the original sample volume) was added for 2 hr. Aliquots of the samples were analyzed by paper chromatography in solvent A. The bulk of the 24-hr sample was evaporated, dissolved in 20% ethanol, and analyzed by DEAE-cellulose column chromatography (see below). Results are given in Tables II and III and in Figures 3 and 4.

General Method for Individual Condensation Experiments. The 3′-hydroxyl-containing compound (TrT, Tr-TpT, Tr-TpTpT, Tr-TpTpTpT, or Tr-TpTpTpTpTpT) and 5′-O-phosphorylthymidylyl-(3′→5′)-3′-O-acetylthymidine (pTpT-OAc) were mixed and rendered anhydrous by repeated evaporation of pyridine. Anhydrous pyridine was readded to the residual gum, and the sulfonyl chloride reagent was added as a solid to the reaction mixture, which was immediately sealed. In all cases homogeneous solutions were obtained. Complete details of the quantity of reagents taken and reaction times are shown in Tables I, III, IV,

(15) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961).
(16) TPS was generously provided by Dr. R. Lohrmann.

VI, and VIII for the preparation of the tri-, tetra-, penta-, hexa-, and octanucleotides, respectively. After completion of the reaction an equal volume of water was added to the reaction solution, which was set aside for 18 hr. Concentrated ammonia solution (an equal volume) was then added for 1 hr, after which time the solution was evaporated to a gum, dissolved in aqueous pyridine, and chromatographed on a DEAE-cellulose column under the conditions described in the text for the individual reactions studied.

Synthesis of the Tetranucleotide Tr-TpTpTpT, Using Picryl Chloride. Pyridinium Tr-TpT (22 mg, 20.8 μmoles) and triethylammonium pTpT-OAc (25 mg, 29.6 μmoles) were dissolved in anhydrous pyridine (2 ml) and dried by repeated evaporation of pyridine. The residual gum was dissolved in anhydrous pyridine (1 ml), and picryl chloride (28 mg, 0.11 mmole) was added. The picryl chloride was only partly soluble at the start of reaction but had dissolved after 1 day. Samples (about 0.2 ml) were removed after 6, 24, and 48 hr. Each sample was diluted with water (0.5 ml) and extracted with ether (two 2.5-ml portions). A 2 N sodium hydroxide solution (0.3 ml) was added to the aqueous layer and after 15 min at room temperature an excess of pyridinium Dowex 50 ion-exchange resin was added. An aliquot of the sample was analyzed following paper chromatography in solvent A. The yield of Tr-TpTpTpT estimated in this way was: 6 hr, 24%; 24 hr, 44%; and 48 hr, 51%.

Synthesis of the Pentanucleotide Tr-TpTpTpTpT Using Picryl Chloride. Triethylammonium Tr-TpTpT (375 OD_{267} units) and triethylammonium pTpT-OAc (45 mg, 53 μmoles) were dissolved in anhydrous pyridine and rendered anhydrous by evaporation of pyridine. The dry gum was dissolved in anhydrous pyridine (1 ml) and picryl chloride (36 mg, 0.14 mmole) was added. The sealed reaction mixture was shaken at room temperature for 5 days. Water (3 ml) was added and the mixture was set aside at room temperature overnight. The aqueous layer was extracted with ether (three 10-ml portions) and treated with concentrated ammonium hydroxide solution (2 ml) for 1 hr. Ammonia was evaporated, and the product was dissolved in aqueous pyridine and applied to the top of a DEAE-cellulose column (45 × 1 cm, bicarbonate form) which was eluted with a linear salt gradient (3 l. of 0.02 M ammonium bicarbonate in the mixing vessel and 3 l. of 0.3 M salt in the reservoir); 15-ml fractions were collected every 20 min. The pure pentanucleotide was eluted in fractions 165–190 and amounted to 312 OD units (50% of theoretical).

Characterization of Products. The trityl-containing products were characterized as pure by chromatography in at least two solvent systems (Table X) and by electrophoresis. Their chromatographic behavior was also compared with authentic samples previously prepared[4a] at pH 7.1 (Table X). Following detritylation they were again shown to be pure on paper chromatography (Table X). The tetra-, hexa-, and octanucleotides obtained by detritylation were completely characterized by degradation with venom phosphodiesterase and spleen phosphodiesterase (Table XI).

Table XI. Characterization of Products by Enzymic Degradation

Compd	Enzyme	OD_{267} units degraded	Nucleoside: nucleotide	Theor ratio
TpTpTpT	Venom	4.7	1:3.5	1:3
TpTpTpT	Spleen	3.75	1:3.1	1:3
TpTpTpTpTpT	Venom	11.5	1:5.3	1:5
TpTpTpTpTpT	Spleen	5.5	1:4.5	1:5
TpTpTpTpTpTpTpT	Venom	14.5	1:6.8	1:7

Studies on Polynucleotides. LXI.[1] Polynucleotide Synthesis in Relation to the Genetic Code. General Introduction[2]

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Abstract: An approach to the study of the genetic code has previously been developed which involves the use of ribopolynucleotides containing known repeating nucleotide sequences as messengers in the cell-free protein-synthesizing system. With the aim of a rather systematic extension of this approach for further studies of the genetic code, chemical synthesis of a variety of deoxyribopolynucleotides containing repeating tri- and tetranucleotide sequences has been undertaken. This introductory paper examines the general considerations underlying the synthetic work which is described in a series of accompanying papers. Biochemical accomplishments and experiments made possible by the availability of DNA-like and RNA-like polymers containing known sequences are briefly reviewed.

The approaches to the study of the genetic code which have been developed in this laboratory[4-7] have comprised investigations along the following lines: (1) chemical synthesis of several series of short-chain deoxyribopolynucleotides containing repeating di- and trinucleotide sequences;[8-11] (2) use of chemically synthesized deoxyribonucleotides with repeating nucleotide sequences as templates for DNA-dependent RNA polymerase and characterization of the products as high molecular weight ribopolynucleotides containing repeating nucleotide sequences;[5,12,13] (3) use of chem-

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