Studies on Polynucleotides. LXVI.[1] The Synthesis of Deoxyribopolynucleotides Containing Repeating Tetranucleotide Sequences (3). A Further Study of the Synthetic Approach Involving Condensation of Preformed Oligonucleotide Blocks[2]

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Abstract: The synthesis of a hexadecadeoxyribonucleotide containing the repeating tetranucleotide sequence thymidylylthymidylyldeoxyadenylyldeoxycytidylyl has been accomplished by stepwise condensation starting with preformed protected tetranucleotide blocks. The synthetic steps used were as follows: condensation of 5′-O-monomethoxytritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-N^6-benzoyldeoxyadenylyl-(3′→5′)-N^6-anisoyldeoxycytidine (d-MMTr-TpTpA^{Bz}pC^{An}) with the protected tetranucleotide, 5′-O-phosphorylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-N^6-benzoyldeoxyadenylyl-(3′→5′)-N^6-anisoyl-3′-O-acetyldeoxycytidine (d-pTpTpA^{Bz}pC^{An}OAc), gave the protected octanucleotide d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An}-OAc. After selective removal of the acetyl group on the terminal 3′-hydroxyl function, the protected octanucleotide was condensed with the same protected tetranucleotide (d-pTpTpA^{Bz}pC^{An}-OAc) to give the fully protected dodecanucleotide. The hexadecanucleotide was obtained by a repeat of the condensation between the protected tetranucleotide and dodecanucleotide. Triisopropylbenzenesulfonyl chloride was used as the condensing reagent and the yields in different steps were 20–40%. Initial to the above block condensations, the condensation of a protected trinucleotide, 5′-O-phosphorylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-N^6-benzoyl-3′-O-acetyldeoxyadenosine (d-pTpTpA^{Bz}), with the protected tetranucleotide, d-MMTr-TpTpA^{Bz}pC^{An}, as well as the condensation of the trinucleotide, pTpTpT-OAc, with the protected trinucleotide, Tr-TpTpT, were investigated. The general approach to polynucleotide synthesis using preformed oligonucleotide blocks is discussed in the light of the present results.

Selected deoxyribopolynucleotides containing repeating tetranucleotide sequences were required as templates for the DNA polymerase catalyzed synthesis of DNA-like polymers containing repeating tetranucleotide sequences.[3] In the two preceding papers,[1,4] chemical syntheses of several series of deoxyribopolynucleotides of this general type have been described. The present paper records the synthesis of a dodeca- and a hexadecanucleotide containing the tetranucleotide sequence thymidylylthymidylyldeoxyadenylyldeoxycytidylate. The synthesis of an octanucleotide containing the complementary sequence thymidylyldeoxyadenylyldeoxyadenylyldeoxyguanylate has been recorded in the preceding paper.[1] Both the dodecanucleotide and the homologous hexadecanucleotide now described, when tested with the DNA polymerase, in combination with the octanucleotide previously described, directed the synthesis of a DNA-like polymer containing the appropriate repeating tetranucleotide sequences. Considerations which led to the choice of the repeating tetranucleotide sequences contained in this polymer have been outlined in an accompanying paper.[3] Brief announcements of the work reported in this paper have been made earlier.[5,6]

While the specific objective of the work described in this paper was the synthesis of the deoxypolynucleotides containing the above-mentioned repeating sequence, a fundamentally important aspect has been the further study of the general approach to deoxypolynucleotide synthesis involving condensation of preformed blocks. Recently, the use of preformed dinucleotide blocks in stepwise synthesis was investigated, and the method has been used successfully in the syntheses described in the preceding paper.[1] Preformed oligonucleotide blocks containing 5′-phosphomonoester groups, larger than the dinucleotides, have, however, not been investigated.[7] It is clearly of interest for further advance in polynucleotide synthesis to try to use preformed oligonucleotide blocks of longer chain length. The present paper describes the first experiments on the use of protected tri- and tetranucleotide blocks in synthetic work and the stepwise synthesis, using a protected tetranucleotide as the condensing unit, of a hexadecanucleotide, which is the largest size polynucleotide prepared so far by the stepwise method.

(1) Preceding paper in this series is by H. Kössel, H. Büchi, and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2185 (1967).

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

(3) H. G. Khorana, H. Büchi, T. M. Jacob, H. Kössel, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **89**, 2154 (1967).

(4) T. M. Jacob, S. A. Narang, and H. G. Khorana, *ibid.*, **89**, 2177 (1967).

(5) H. G. Khorana, "Proceedings of the 3rd Meeting of the Federation of the European Biochemical Societies, Warsaw, April 1966," in press.

(6) H. G. Khorana, H. Büchi, H. Ghosh, N. Gupta, T. M. Jacob, H. Kössel, R. Morgan, S. A. Narang, E. Ohtsuka, and R. D. Wells, *Cold Spring Harbor Symp. Quant. Biol. (June 1966)*, in press.

(7) In early work [G. Weimann, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3835 (1963); H. Schaller and H. G. Khorana, *ibid.*, **85**, 3841 (1963)] syntheses of two pentanucleotides were described in which trinucleotides bearing 5′-phosphate end groups were one of the two components. The reagent most investigated then was dicyclohexylcarbodiimide. As noted in accompanying papers, aromatic sulfonyl chlorides are the preferred reagents in syntheses involving block condensations.

Table I. Summary of Conditions and Yields Obtained in Deoxyribopolynucleotide Synthesis Using Oligonucleotide Blocks

3′-OH-bearing component	Amount, μmoles	5′-Phosphate-bearing component	Amount, μmoles	Pyridine, ml	TPS, μmoles	Time, hr	Yield, %
Tr-TpTpT	6.8	pTpTpT-OAc	13.8	0.4	83	12	40
d-MMTr-TpTpA^{Bz}pC^{An}	21	d-pTpTpA^{Bz}-OAc	60	3	360	12	37
d-MMTr-TpTpA^{Bz}pC^{An}	16	d-pTpTpA^{Bz}pC^{An}-OAc	63	1	473	11	40
d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An}	4	d-pTpTpA^{Bz}pC^{An}-OAc	40	0.3	300	9	40
d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$pTpA^{Bz}pC^{An}	0.68	d-pTpTpA^{Bz}pC^{An}-OAc	12	0.1	90	4	20

Protected Oligonucleotides. The new protected deoxyribooligonucleotide blocks used in the present work were the protected tetranucleotide, d-MMTr-TpTpA^{Bz}-pC^{An},[8] the protected trinucleotide bearing a 5′-phosphate end group, d-pTpTpA^{Bz}-OAc[8], and the protected tetranucleotide, d-pTpTpA^{Bz}pC^{An}-OAc.[8] The oligonucleotide d-MMTr-TpTpA^{Bz}pC^{An} was prepared by the stepwise procedure shown in Chart I. The products were purified at each step by DEAE-cellulose chromatography. The protected trinucleotide d-pTpTpA^{Bz}-OAc and the tetranucleotide d-pTpTpA^{Bz}pC^{An}-OAc were also prepared by the stepwise method, the steps being shown in Chart II. Thus the starting material was the dinucleotide, pTpT. The phosphomonoester group was protected with the β-cyanoethyl group by condensing with β-hydracrylonitrile in the presence of dicyclohexylcarbodiimide (DCC). After treatment of the reaction mixture at pH 9 the monocyanoethyl compound was obtained, which was brought into reaction with d-pA^{Bz}-OAc in the presence of triisopropylbenzenesulfonyl chloride (TPS).[9] After an alkaline treatment the product, d-pTpTpA^{Bz}, was isolated by column chromatography and was subsequently treated with hydracrylonitrile and DCC. Although, in addition to the monocyanoethyl ester, di- or higher cyanoethyl esters may have been present, the pH 9 treatment was omitted and instead the protected trinucleotide was directly condensed with d-pC^{An}-OAc using TPS as the condensing agent. The protected tetranucleotide was isolated pure by column chromatography after an alkaline treatment. The 3′-O-acetyl derivatives, d-pTpTpA^{Bz}-OAc and d-pTpTpA^{Bz}pC^{An}-OAc, were prepared by acetylation with acetic anhydride in pyridine.

Chart I. Stepwise Synthesis of the Protected Tetranucleotide 5′-O-Monomethoxytritylthymidylylthymidylyl-N-benzoyldeoxyadenylyl-N-anisoyldeoxycytidine, d-MMTr-TpTpA^{Bz}pC^{An}

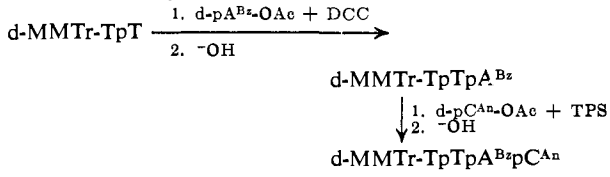
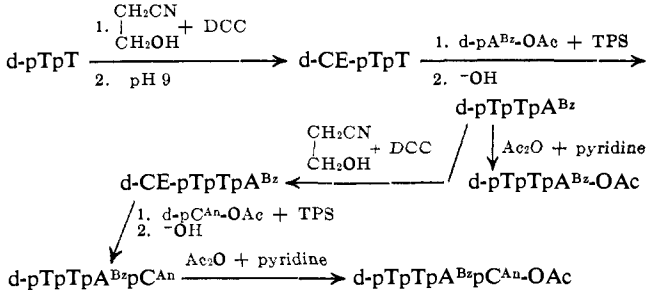


Chart II. Stepwise Synthesis of the Protected Tetranucleotide 5′-O-Phosphorylthymidylylthymidylyl-N-benzoyldeoxyadenylyl-N-anisoyl-3′-O-acetyldeoxycytidine, d-pTpTpA^{Bz}pC^{An}-OAc



Initial Block Condensations. The condensation of the protected thymidine trinucleotide, pTpTpT-OAc,[10] with the protected trinucleotide, Tr-TpTpT,[11] was first studied. The trinucleotide, pTpTpT-OAc, was used in onefold excess over the hydroxyl-containing component and TPS was used as the condensing agent (Table I). The products were analyzed by chromatography on a DEAE-cellulose column; the elution pattern is in Figure 1 and the identification of some of the peaks obtained is in Table II. The desired hexanucleotide, Tr-TpTpTpTpTpT, was obtained in 40% yield. Evidently side products were formed and the recovery of the unreacted starting component Tr-TpTpT was less than theoretical. One of the side products (peak X of Figure 1) was identified as Tr-TpTpTpTp. Compounds of this type have been encountered and identified previously.[11]

Table II. Chromatography of the Products Obtained in the Synthesis of the Hexanucleotide, Tr-TpTpTpTpTpT (Figure 1)

Peak no.	Fractions pooled	Total OD units at 267 mμ	Identification
III	41–45	18.5	Tr-negative compound
IV	46–50	25.2 (12.6)[a]	Tr-TpTpT (essentially pure)
VII	102–121	171.5	pTpTpT (essentially pure)
X	161–172	37.6	Mainly Tr-TpTpTpTp
XI	173–210	160.5 (40)[a]	Tr-TpTpTpTpTpT (essentially pure)

[a] The numbers in parentheses are percentage yields as based on the amount of the starting material, Tr-TpTpT.

The condensation of the protected trinucleotide d-pTpTpA^{Bz}-OAc with a larger block bearing a 3′-hydroxyl group, d-MMTr-TpTpA^{Bz}pC^{An}, was studied next. About 3 molar equiv of the trinucleotide, d-pTpTpA^{Bz}-OAc, were used, the reaction conditions being included in Table I. The elution pattern obtained on chromatography of the reaction products is shown in Figure 2 and the identification of the different peaks is in Table III. The heptanucleotide d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz} was obtained in peak VII. The preceding peak, while contaminated with a tritylnegative material, also contained mainly the heptanucleotide. The total yield of the heptanucleotide was 37%.

(8) The system of abbreviations is as has been described and used in the preceding papers.

(9) R. Lohrmann and H. G. Khorana, *J. Am. Chem. Soc.*, **88**, 829 (1966).

(10) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).

(11) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965).

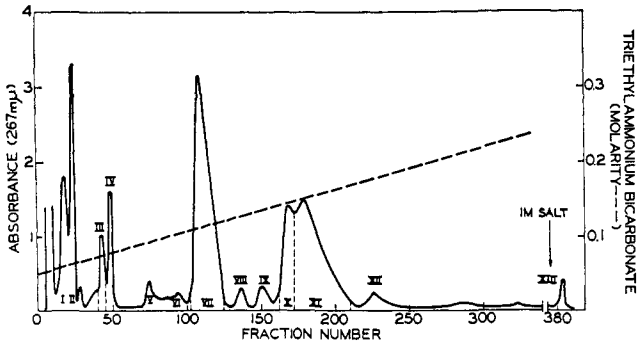


Figure 1. Chromatography of the products obtained in the synthesis of the hexanucleotide Tr-TpTpTpTpTpT on a DEAE-cellulose (bicarbonate) column (1.1 × 50 cm) preequilibrated with 0.05 *M* triethylammonium bicarbonate in 40% ethyl alcohol. Elution was carried out using a linear salt gradient of triethylammonium bicarbonate in 40% ethyl alcohol (1 l. of 0.05 *M* salt in the mixing vessel and an equal volume of 0.3 *M* salt in the reservoir). Fractions of about 5 ml were collected every 20 min. Fractions were pooled as shown by the vertical broken lines. For identification of different peaks see Table II.

Stepwise Synthesis of the Hexadecanucleotide with Repeating Tetranucleotide Sequence. The results of the condensations described above were encouraging in that the yields obtained using trinucleotide blocks were not significantly lower than those obtained recently using dinucleotide blocks and, furthermore, the yields were comparable when the hydroxyl-bearing component was increased in size from a tri- (Tr-TpTpT) to a tetranucleotide (d-MMTr-TpTpA^{Bz}pC^{An}). It was, therefore, decided to undertake the synthesis of the hexadecanucleotide containing the required repeating sequence by condensations using protected *tetranucleotide blocks* (Chart III). In addition, it may be noted that while for enzymatic experiments the dodecanucleotide, d-T(pTpApCpT)$_2$pTpApC, would have sufficed, the block condensation method was extended to the synthesis of the hexadecanucleotide from the protected dodecanucleotide.

Table III. Chromatography of the Products Obtained in the Synthesis of the Heptanucleotide d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz} (Figure 2)

Peak no.	Fractions pooled	Total OD units at 280 mμ	Identification
I	101–110	39.5	Unidentified
II	155–164	57.0	Unidentified
III	191–201	64.0 (6)[a]	d-MMTr-TpTpA^{Bz}pC^{An} (about 90% pure)
IV	238–267	885.0	d-pTpTpA^{Bz} (about 90% pure)
VI	451–477	150.0	Mainly d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz} (68%)
VII	478–517	510.0 (30)[a]	d-MMTr-TpTpA^{Bz}pC^{An}-pTpTpA^{Bz} (more than 90% pure)
IX	603–615	162.0	1 *M* fraction

[a] The numbers in parentheses are percentage yields as based on the amount of the starting material, d-MMTr-TpTpA^{Bz}pC^{An}.

In the first step, d-MMTr-TpTpA^{Bz}pC^{An} was condensed with d-pTpTpA^{Bz}pC^{An}-OAc using 4 molar equiv of the latter (Table I). The desired octanucleotide was isolated by chromatography on a DEAE-cellulose column, the elution pattern obtained being in Figure 3. The distribution of ultraviolet-absorbing material in different peaks is shown in Table IV. Ammoniacal treatment of the product gave d-MMTr-TpTpApCpTpTpApC which was purified from a small amount of impurity by chromatography in solvent D. The fully unprotected octanucleotide was obtained by removal of the methoxytrityl group in pyridine–acetate buffer (see below). Any impurities in the unprotected octanucleotide were removed by further chromatography in solvent E. The contamination of the octanucleotide from the impurities varied in different parts of the main peak (cuts shown as peaks VI–VIII in Figure 3). The yield of the total pure unprotected octanucleotide in the above condensation was estimated to be about 40%. The results of the spleen phosphodiesterase degradation of the unprotected octanucleotide are described in the Experimental Section.

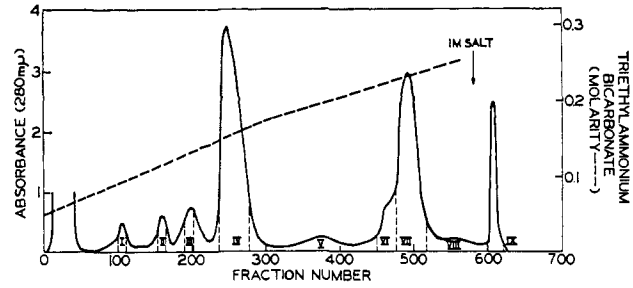


Figure 2. Chromatography of the product obtained in the synthesis of the heptanucleotide d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz} on a DEAE-cellulose (bicarbonate) column (2.5 × 60 cm) preequilibrated with 0.05 *M* triethylammonium bicarbonate in 40% ethyl alcohol at 4°. Elution was carried out using a linear salt gradient of triethylammonium bicarbonate in 40% ethyl alcohol (3 l. of 0.05 *M* salt in the mixing vessel and an equal volume of 0.3 *M* salt in the reservoir). Fractions of 7–10 ml were collected every 15 min. Fractions were pooled as shown by the vertical broken lines. For identification of different peaks see Table III.

Chart III. Synthesis of the Hexadecanucleotide d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_3$pTpA^{Bz}pC^{An} by Stepwise Condensation Using Protected Tetranucleotide Blocks

d-MMTr-TpTpA^{Bz}pC^{An} —(1. d-pTpTpA^{Bz}pC^{An}-OAc + TPS; 2. $^-$OH)→ d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An} octanucleotide

↓ 1. d-pTpTpA^{Bz}pC^{An}-OAc + TPS; 2. $^-$OH

d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$pTpA^{Bz}pC^{An} dodecanucleotide

↓ 1. NH$_3$; 2. H$^+$

d-T(pTpApCpT)$_2$pTpApC unprotected dodecanucleotide

1. d-pTpTpA^{Bz}pC^{An}-OAc + TPS; 2. $^-$OH

d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_3$pTpA^{Bz}pC^{An} hexadecanucleotide

↓ 1. NH$_3$; 2. H$^+$

d-T(pTpApCpT)$_3$pTpApC unprotected hexadecanucleotide

In the next step, the protected octanucleotide was brought into reaction with an excess (10 molar equiv) of the protected tetranucleotide. The reaction conditions are included in Table I. The elution pattern obtained on chromatography of the reaction mixture is shown in Figure 4 and the identification of the different peaks is in Table V. Peak VIII (Figure 4) contained

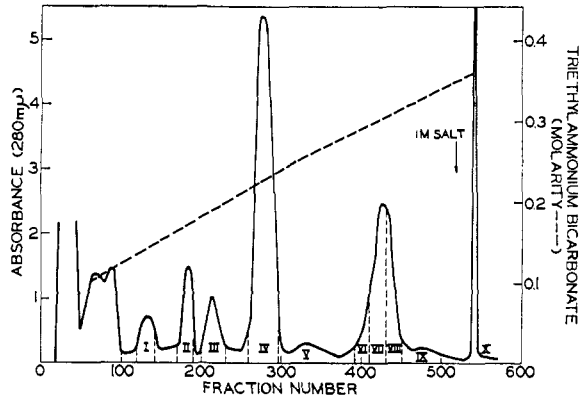


Figure 3. Chromatography of the products obtained in the synthesis of the octanucleotide d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An} on a DEAE-cellulose (bicarbonate) column (2.5 × 60 cm) preequilibrated with 0.05 *M* triethylammonium bicarbonate in 40% ethyl alcohol at 4°. Elution was carried out using a linear salt gradient of triethylammonium bicarbonate in 40% ethyl alcohol (3 l. of 0.1 *M* salt in the mixing vessel and an equal volume of 0.4 *M* salt in the reservoir). Fractions of 9.5–11 ml were collected every 15 min. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table IV.

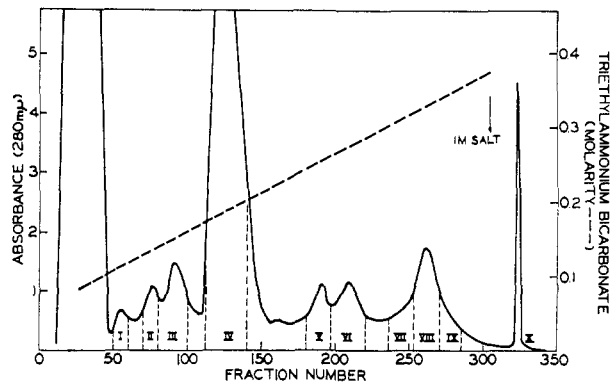


Figure 4. Chromatography of the products obtained in the synthesis of the dodecanucleotide d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$-pTpA^{Bz}pC^{An} on a DEAE-cellulose (bicarbonate) column (1.5 × 60 cm) preequilibrated with 0.05 *M* triethylammonium bicarbonate in 40% ethyl alcohol at 4°. Elution was carried out using a linear salt gradient of triethylammonium bicarbonate in 40% ethyl alcohol (1.5 l. of 0.1 *M* salt in the mixing vessel and an equal volume of 0.5 *M* salt in the reservoir). Fractions of 5.2–6.4 ml were collected every 20 min. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table V.

essentially pure protected dodecanucleotide and the amount corresponded to 31% of the theoretical yield. Peaks VII and IX also contained mainly the desired product, and this could be purified by paper chromatography after removal of the protecting groups. The total yield of the dodecanucleotide was close to 40%, but the recovery of the unreacted octanucleotide was only 32% of theoretical.

For the synthesis of the hexadecanucleotide, the protected dodecanucleotide was treated with 18 molar equiv of the protected tetranucleotide (reaction conditions in Table I). The elution pattern obtained on chromatography of the products after work-up is shown in Figure 5 and the identification of the different peaks is in Table VI. The hexadecanucleotide was present in peak V and, when examined by paper chromatography in solvent D after an ammoniacal treatment, was homogeneous. The fully unprotected hexadecanucleotide obtained after removal of the methoxytrityl group was essentially pure, a trace of an impurity being removed by chromatography in solvent E. R_f readings of the protected and unprotected compounds are listed in Table VII. The yield of the pure hexadecanucleo-

Table IV. Chromatography of the Products Obtained in the Synthesis of the Octanucleotide d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An} (Figure 3)

Peak no.	Fractions pooled	Total OD units at 280 mμ	Identification
I	131–141	70	Two MMTr-negative compounds
II	180–188	116	d-MMTr-TpTpA^{Bz}pC^{An} (20%)[a] and MMTr-negative compound (80%)
III	200–222	176	Three MMTr-negative compounds
IV	260–294	1397	d-pTpTpA^{Bz}pC^{An} (essentially pure)
VI	391–410	109	d-MMTr-TpTpA^{Bz}pC^{An}-pTpTpA^{Bz}pC^{An} and MMTr-negative compound
VII	411–430	388 (25)[b]	d-MMTr-TpTpA^{Bz}pC^{An}-pTpTpA^{Bz}pC^{An} (almost pure)
VIII	431–450	248 (16)[b]	d-MMTr-TpTpA^{Bz}pC^{An}-pTpTpA^{Bz}pC^{An} (almost pure)
X	540–544	114	1 *M* fraction

[a] This amount corresponds to 3% of the starting material, d-MMTr-TpTpA^{Bz}pC^{An}. [b] The numbers in parentheses are percentage yields as based on the amount of the starting material, d-MMTr-TpTpA^{Bz}pC^{An}.

Table V. Chromatography of the Products Obtained in the Synthesis of the Dodecanucleotide d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$TpA^{Bz}pC^{An} (Figure 4)

Peak no.	Fractions pooled	Total OD units at 280 mμ	Identification
I	51–60	43	Unidentified
II	71–80	52	Two MMTr-negative compounds
III	81–100	135	Three MMTr-negative compounds
IV	117–140	919	d-pTpTpA^{Bz}pC^{An} (essentially pure)
V	186–196	74 (19)[a]	d-MMTr-TpTpA^{Bz}pC^{An}-pTpTpA^{Bz}pC^{An} (essentially pure)
VI	197–220	160	O(pTpTpA^{Bz}pC^{An})$_2$
VII	236–252	70	Mainly d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$pTpA^{Bz}pC^{An}
VIII	253–270	178 (31)[a]	d-MMTr-T(pTpA^{Bz}pC^{An}-pT)$_2$pTpA^{Bz}pC^{An} (almost pure)
IX	271–285	55 (10)[a]	Mainly d-MMTr-T(pTpA^{Bz}-pC^{An}pT)$_2$pTpA^{Bz}pC^{An}
X	323–326	45	1 *M* fraction

[a] The numbers in parentheses are percentage yields as based on the amount of the starting material d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An}.

Table VI. Chromatography of the Products Obtained in the Synthesis of the Hexadecanucleotide d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_3$pTpA^{Bz}pC^{An} (Figure 5)

Peak no.	Fractions pooled	Total OD units at 280 mμ	Identification
I	121–140	70	MMTr-negative compound
II	157–170	282	d-pTpTpA^{Bz}pC^{An} (essentially pure)
III	246–260	18.2	O(pTpTpA^{Bz}pC^{An})$_2$
IV	316–335	23.3 (18)[a]	d-MMTr-T(pTpA^{Bz}pC^{An}-pT)$_2$pTpA^{Bz}pC^{An} (almost pure)
V	376–410	27.0 (20)[a]	d-MMTr-T(pTpA^{Bz}pC^{An}-pT)$_3$pTpA^{Bz}pC^{An} (almost pure)
VI	458–465	3.4	1 *M* fraction

[a] The numbers in parentheses are percentage yields as based on the amount of the starting material d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$pTpA^{Bz}pC^{An}.

tide in the condensation was 20%. Although the reaction time used was less (4 hr, Table I), the recovery of unused dodecanucleotide (Figure 5) was low (23% of theoretical).

The customary method for the removal of methoxytrityl groups has been treatment with 80% acetic acid at room temperature. In the present work, aqueous acetic acid buffered with some pyridine was investigated and has been used. Although, as expected, the rate of the trityl group removal is reduced, the modified medium seems to be safer in regard to the possibility of depurination reaction.

General Comments. The general approach to the synthesis of polynucleotides involving use of preformed oligonucleotide blocks clearly offers many advantages over the alternative one in which mononucleotides are used as the condensing agents. This paper has reported first experiments on the use of preformed tetranucleotide blocks in the synthesis of a hexadecanucleotide, and the results on the whole are encouraging. Probably with further experimentation yields in the condensation steps can be improved. The most important problem, the seriousness of which increases with an increase in the chain length of the component bearing the 3′-hydroxyl group, is the damage to the preformed internucleotide bonds during the condensation reactions. Conditions which minimize the side reactions leading to destruction of the polynucleotide chains must be found if much longer deoxyribopolynucleotide chains are to be synthesized.

Experimental Section

General Methods. Paper chromatography was performed by the descending technique using mostly Whatman No. 40 or Whatman No. 1 paper. The solvent systems used were: solvent A, isopropyl alcohol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethyl alcohol–1 *M* ammonium acetate, pH 7.5 (7:3, v/v); solvent C, ethyl alcohol–0.5 *M* ammonium acetate, pH 3.8 (7:3, v/v; solvent D, *n*-propyl alcohol–concentrated ammonia–water (55:10:35, v/v/v); solvent E, isobutyric acid–concentrated ammonia–water (66:1:33, v/v/v). The R_f readings of different polynucleotides are given in Table VII.

Paper electrophoresis was performed in a high-voltage apparatus in which the paper was immersed in a high-boiling petroleum fraction (Varsol). Potassium phosphate buffer (0.03 *M*, pH 7.1) was used.

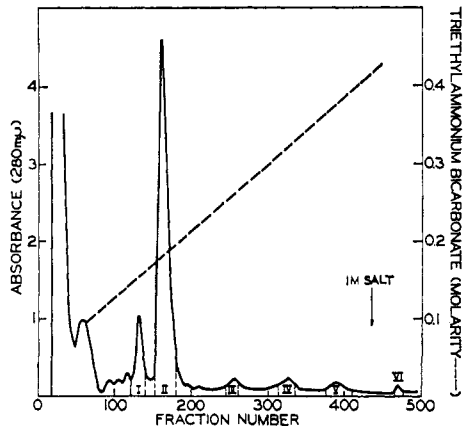


Figure 5. Chromatography of the products obtained in the synthesis of the hexadecanucleotide d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_3$pTpA^{Bz}pC^{An} on a DEAE-cellulose (bicarbonate) column (1.5 × 60 cm) preequilibrated with 0.05 *M* triethylammonium bicarbonate in 40% ethyl alcohol at 4°. Elution was carried out using a linear salt gradient of triethylammonium bicarbonate in 40% ethyl alcohol (1.5 l. of 0.1 *M* salt in the mixing vessel and an equal volume of 0.5 *M* salt in the reservoir). Fractions of 5.0–6.5 ml were collected every 20 min. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table VI.

The trityl group and monomethoxytrityl group in compounds were detected by spraying the chromatograms with 10% aqueous perchloric acid and drying them in warm air. The preparation of TPS,[12] Tr-TpTpT,[13] and pTpTpT[13] has been described previously.

For use in condensations with DCC as reagent, pyridine was treated with a small amount of chlorosulfonic acid before fractional distillation and was redistilled from potassium hydroxide and stored over Molecular Sieves (4A) obtained from the Linde Co. For condensation reactions using TPS, chlorosulfonic acid treatment of pyridine was omitted. All reaction mixtures at the start of condensation reactions were made anhydrous by repeated evaporation of added dry pyridine *in vacuo*, the last three times the flask being opened inside of a box in which an anhydrous atmosphere was maintained by a positive pressure of dry nitrogen gas and phosphorus pentoxide. The molar extinction values used for the mononucleotides are as follows: pT, 9600 (267 mμ) and 6340 (280 mμ); d-pA^{Bz}, 18,300 (280 mμ); d-pC^{An}, 22,450 (302 mμ) and 18,000 (280 mμ).

For removal of the N-protecting groups the compounds were treated with a large excess of concentrated ammonia for 2 days. Monomethoxytrityl groups were removed using either 80% aqueous acetic acid (40 min) or a mixture of acetic acid, pyridine, and water (14:1:3, v/v/v) for 24 hr at room temperature.

Enzymic degradation of the synthetic products using bacterial alkaline phosphomonoesterase and spleen phosphodiesterase were performed as described previously.[14]

The abbreviation OD refers to the extinction of a nucleotidic solution in a 1-ml volume using a 1-cm light-path quartz cell. The wavelength used for this measurement is indicated as a subscript after this abbreviation.

Acetylation of 3′-Hydroxyl End Groups of Oligonucleotides. Acetylation of the 3′-hydroxyl groups in pTpTpT, d-pTpTpA^{Bz}, and d-pTpTpA^{Bz}-pC^{An} was carried out by the following procedure described for pTpTpT. Triethylammonium pTpTpT (1000 OD$_{267}$ units, 0.035 mmole) was rendered anhydrous with pyridine and allowed to react with acetic anhydride (0.3 ml) in pyridine (0.6 ml). A precipitate which appeared immediately after the addition of acetic anhydride dissolved after 4 hr. The mixture was kept at room temperature overnight. Methyl alcohol (0.5 ml) was added under cooling and after 15 min at room temperature, 20% aqueous pyridine (1 ml) was added and the solution set aside at room temperature for 8 hr. The solution was evaporated and rendered anhydrous with added pyridine. The residue in pyridine (2 ml) was added to ether

(12) TPS was generously provided by Dr. R. Lohrmann.

(13) These compounds were synthesized and generously provided by Dr. M. W. Moon.

(14) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961).

Table VII. Paper Chromatography of Deoxyribopolynucleotides

Compound	R_f relative to pT: Solvent B	Solvent D	Solvent E
Oligonucleotides Bearing 5′-Phosphate End Groups			
pTpT	0.76		
pTpTpT	0.49		
pTpTpT-OAc	0.72		
d-pTpTpA	0.27	0.66	
d-pTpTpA^{Bz}	0.66		
d-pTpTpA^{Bz}-OAc	0.83		
d-pTpTpApC		0.50	0.60
d-pTpTpA^{Bz}pC^{An}	0.40		
d-pTpTpA^{Bz}pC^{An}-OAc	0.45		
O(d-pTpTpApC)(d-pTpTpApC)		0.21	0.55
O(d-pTpTpA^{Bz}pC^{An})(d-pTpTpA^{Bz}pC^{An})	0.12		
Polynucleotides Bearing Free 5′-Hydroxyl Groups			
d-TpTpA	1.03	1.23	
d-TpTpApC	0.52	0.93	1.10
d-TpTpApCpTpTpA		0.42	0.62
d-TpTpApCpTpTpApC		0.24, 1.00[a]	0.59, 1.00[a]
d-T(pTpApCpTp)$_2$-TpApC		0.23[a]	0.32[a]
d-T(pTpApCpTp)$_3$-TpApC		0.07[a]	0.08[a]
Polynucleotides Bearing 5′-Trityl Groups			
Tr-TpTpT[b]	2.3		
Tr-T(pT)$_2$pT[b]	2.1		
Tr-T(pT)$_4$pT[b]	0.85		
d-MMTr-TpTpA	2.0		
d-MMTr-TpTpA^{Bz}	2.3		
d-MMTr-TpTpApC	1.4		
d-MMTr-TpTpA^{Bz}pC^{An}	2.1		
d-MMTr-TpTpApCp-TpTpA	0.20	1.21	
d-MMTr-TpTpA^{Bz}-pC^{An}TpTpA^{Bz}	0.90		
d-MMTr-TpTpApCp-TpTpApC	0.06	0.91	
d-MMTr-TpTpA^{Bz}-pC^{An}pTpTpA^{Bz}pC^{An}	0.44		
d-MMTr-T(pTpAp-CpT)$_2$pTpApC		0.30	
d-MMTr-T(pTpA^{Bz}-pC^{An}pT)$_2$pTpA^{Bz}-pC^{An}	0.07		
d-MMTr-T(pTpApCp-pT)$_3$pTpApC		0.09	

[a] R_f relative to the octanucleotide d-TpTpApCpTpTpApC. [b] R_f readings of these compounds were also measured in solvent A and were as follows: Tr-TpTpT, 0.51; Tr-T(pT)$_2$pT, 0.35; and Tr-T(pT)$_4$pT, 0.06.

(100 ml). The precipitate was collected by centrifugation and washed with ether three times. The powder was kept over phosphorus pentoxide *in vacuo* and reprecipitated from pyridine–ether just before use.

For the acetylation of d-pTpTpA^{Bz}, triethylammonium d-pTpTpA^{Bz} (2000 OD_{280} units, 0.066 mmole) was treated with acetic anhydride (0.9 ml) in pyridine (1.8 ml) for 9 hr. In acetylation of the tetranucleotide, d-pTpTpA^{Bz}pC^{An}, the composition of the acetylation mixture was as follows: triethylammonium d-pTpTpA^{Bz}pC^{An} (3100 OD_{280} units, 0.063 mmole), acetic anhydride (1 ml), and pyridine (3 ml). R_f readings of the acetylated oligonucleotides are in Table I.

β-Cyanoethyl Ester of pTpT (CE-pTpT). This was prepared by a procedure similar to that described in an accompanying paper. A mixture of pyridinium pTpT (1.25 mmoles), β-hydracrylonitrile (5 ml, 72 mmoles), and pyridine (15 ml) was allowed to react with DCC (6 g, 29 mmoles) at room temperature for 24 hr. Paper electrophoresis (pH 7.1) at this stage showed complete disappearance of the starting material. The main spots were located at 0.66, 0.27, and 0.0 relative to pT. Water (30 ml) was added and the mixture was extracted with cyclohexane. After 2 days the solution was adjusted to pH 9 with 2 *N* ammonia and kept at room temperature for 16 hr. Ammonium ion was removed by passing the solution through a column of pyridinium Dowex 50 ion-exchange resin. The effluent and washings were evaporated with added pyridine. The dried pyridine solution (15 ml) was added to ether (500 ml), and the gummy precipitate which formed was collected by centrifugation and dissolved in pyridine and a minimum amount of water. The solution was made anhydrous by evaporation with pyridine. The dried residue was dissolved in pyridine (15 ml) and reprecipitated with ether (500 ml). The yield was 19,300 OD_{267} units, 80%.

Preparation of the Trinucleotide d-pTpTpA^{Bz}. A mixture of pyridinium CE-pTpT(19,300 OD_{267} units, 1 mmole) and pyridinium d-pA^{Bz}-OAc (4 mmoles) was allowed to react with TPS (6 mmoles) in pyridine (15 ml) for 7.5 hr at room temperature. Aqueous pyridine (15 ml of 50%) was added with cooling and the mixture kept at room temperature. After 12 hr the solution was treated with 2 *N* sodium hydroxide (40 ml) in an ice bath for 20 min and neutralized with pyridinium Dowex 50 ion-exchange resin. The resin was removed by filtration and washed with 20% aqueous pyridine. The filtrate and washings (200 ml) were diluted with pyridine (200 ml) and applied on a column (5 × 75 cm) of DEAE-cellulose (bicarbonate) preequilibrated with 0.05 *M* triethylammonium bicarbonate in 10% ethyl alcohol at 4°. The column was washed with the same buffer and eluted with a linear gradient salt concentration using 0.05 *M* triethylammonium bicarbonate in 10% ethyl alcohol (6 l.) in the mixing vessel and 0.25 *M* triethylammonium bicarbonate in 10% ethyl alcohol (6 l.) in the reservoir. Various fractions of the resulting main peak were checked by paper chromatography before and after ammonia treatment (for R_f readings see Table I). The fractions containing pure product were pooled and evaporated below 10° with added pyridine. The yield was 18,400 OD_{280} units (0.59 mmole, 59%). The spectral properties at pH 8 were λ_{max} 274 mμ, λ_{min} 238 mμ, and $\epsilon_{302}/\epsilon_{280} = 0.25$.

β-Cyanoethylation of d-pTpTpA^{Bz}. A mixture of pyridinium d-pTpTpA^{Bz} (7700 OD_{280} units, 0.25 mmole), β-hydracrylonitrile (2 ml, 28 mmoles), and DCC (5 g, 24 mmoles) in pyridine (10 ml) was kept for 2 days. An aliquot of the reaction mixture was then treated with aqueous pyridine and checked by paper electrophoresis at pH 7.1. No starting material was detected, there being four nucleotidic spots. Water (10 ml) was added, and the mixture was extracted with cyclohexane. After 16 hr at room temperature the aqueous pyridine solution was evaporated with added pyridine. The dried residue was dissolved in pyridine (5 ml) and added to ether (500 ml). The resulting precipitate was collected by centrifugation, washed with ether three times, and dried over phosphorus pentoxide *in vacuo*. The yield was 7200 OD_{280} units (0.23 mmole, 92%). This product was used without purification.

Preparation of the Tetranucleotide d-pTpTpA^{Bz}pC^{An}. A mixture of pyridinium β-cyanoethyl esters of d-pTpTpA^{Bz} (0.23 mmole) and tri-*n*-hexylammonium d-pC^{An}-OAc (1.8 mmoles) was allowed to react with TPS (2.7 mmoles) in pyridine (7 ml) for 8 hr. Aqueous pyridine (10 ml of 50%) was added with cooling and the mixture kept at room temperature. After 14 hr the mixture was treated with 2 *N* sodium hydroxide (30 ml) in an ice bath for 20 min with added water to get a homogeneous solution. The solution was neutralized with pyridinium Dowex 50 ion-exchange resin. The resin was removed by filtration and washed with 30% aqueous pyridine. The filtrate and washings (200 ml) were chromatographed at 4° by a column (4 × 45 cm) of DEAE-cellulose (bicarbonate) using conditions similar to those used in the case of d-pTpTpA^{Bz} preparations except that the volume of the starting buffer in the mixing vessel was 4 l. and the reservoir contained 4 l. of 0.4 *M* buffer. The yield was 5750 OD_{280} units, 0.117 mmole, 55%. Purity was tested by paper chromatography before and after removing protecting groups; R_f readings are shown in Table I. The spectral properties of the protected tetranucleotide were λ_{max} 277 mμ, λ_{min} 234 mμ, and $\epsilon_{302}/\epsilon_{280} = 0.58$ in 0.01 *M* triethylammonium bicarbonate, those of d-pTpTpApC being λ_{max} 263 mμ, λ_{min} 233 mμ, and $\epsilon_{280}/\epsilon_{260} = 0.56$ in water.

The compound was checked by spleen diesterase (a commercially available sample) for its susceptibility. The tetranucleotide was completely degraded by the enzyme.

Preparation of the Trinucleotide d-MMTr-TpTpA^{Bz}. Pyridinium MMTr-TpT (0.7 mmole) and pyridinium d-pA^{Bz}-OAc (1.1 mmoles) were allowed to react with DCC (10 mmoles) in pyridine (10 ml) for 4 days. After aqueous pyridine treatment (16 hr) and cyclohexane extraction, the mixture was treated with 2 *N* sodium hydroxide (10 ml) in an ice bath for 20 min and neutralized

with pyridinium Dowex 50 ion-exchange resin. One-half of the mixture was applied to a DEAE-cellulose (bicarbonate) column (4 × 45 cm). The elution was carried out at 4° using a gradient of 0.05 *M* triethylammonium bicarbonate in 10% ethyl alcohol (4 l.) and 0.35 *M* triethylammonium bicarbonate in 30% ethyl alcohol (4 l.). The product was obtained in a broad peak, the beginning part of the peak being contaminated with a side product. The fractions containing the pure product were pooled (4300 OD_{280} units, 0.15 mmole) and concentrated in the presence of an excess of pyridine at low temperature (<10°). The product was stored as its solution in anhydrous pyridine at −20°. The ultraviolet absorption characteristics at pH 8 were λ_{max} 274 mμ, λ_{min} 247 mμ, and $\epsilon_{302}/\epsilon_{280}$ = 0.24. The product was homogeneous by paper chromatography (Table I) before and after removing protecting groups.

Preparation of the Tetranucleotide d-MMTr-TpTpA^{Bz}pC^{An}. Triethylammonium d-MMTr-TpTpA^{Bz} (1940 OD_{280} units, 0.061 mmole) was condensed with tri-*n*-hexylammonium d-pC^{An}-OAc (0.35 mmole) using TPS (0.53 mmole) in pyridine (2 ml). After a 6-hr reaction period at room temperature, 50% aqueous pyridine (2 ml) was added; the solution was kept for 12 hr at room temperature. After the usual sodium hydroxide treatment, the mixture was chromatographed on a DEAE-cellulose (bicarbonate) column (2.5 × 60 cm) preequilibrated with 0.05 *M* triethylammonium bicarbonate in 40% ethyl alcohol at 4°. For elution the mixing vessel contained 0.05 *M* triethylammonium bicarbonate in 40% ethyl alcohol (3 l.), and the reservoir contained 0.2 *M* triethylammonium bicarbonate in 40% ethyl alcohol (3 l.). Fractions of about 10-ml volume were collected every 20 min. Fractions 300–330 contained the product and a side product. Fractions 331–400 contained the pure product (1900 OD_{280} units, 0.04 mmole), and were pooled and evaporated at 10° or below in the presence of pyridine. The spectral properties of the protected tetranucleotide were λ_{max} 276 mμ, λ_{min} 243 mμ, and $\epsilon_{302}/\epsilon_{280}$ = 0.58 in 0.01 *M* triethylammonium bicarbonate. After removal of the N-protecting groups, the characteristics of d-MMTr-TpTpApC were λ_{max} 263 mμ, λ_{min} 247 mμ, and $\epsilon_{280}/\epsilon_{260}$ = 0.59 in water. R_f readings are shown in Table I.

The Hexanucleotide Tr-TpTpTpTpTpT. Condensation of Tr-TpTpT and pTpTpT-OAc. A mixture of triethylammonium Tr-TpTpT (200 OD_{267} units, 6.8 μmoles) and pTpTpT-OAc (400 OD_{267} units, 13.8 μmoles) was rendered anhydrous by evaporating with added pyridine, and the residue was dissolved in pyridine (0.4 ml). TPS (25 mg, 83 μmoles) was added to the mixture. After 12 hr 50% aqueous pyridine (0.5 ml) was added, and the solution was kept 24 hr at room temperature. Concentrated ammonia (1 ml) was then added to the mixture and after 2 hr at room temperature most of ammonia was removed by evaporation, pH of the resulting solution being about 8. The solution was diluted to 10 ml with 40% ethyl alcohol and applied on a DEAE-cellulose (carbonate) column. Elution was carried out using a linear gradient of triethylammonium bicarbonate. The conditions used and the elution pattern obtained are shown in Figure 1 and the distribution and the identification of the ultraviolet-absorbing material are shown in Table II. Pyridine and triisopropylbenzenesulfonic acid were eluted in the first two peaks. Peak XI contained the essentially pure product, Tr-TpTpTpTpTpT, which was homogeneous in solvents A, B, C, and D. Peak X contained two side products. The main side product in this peak was alkaline phosphatase sensitive and the dephosphorylated compound showed the same mobility as an authentic sample of Tr-TpTpTpT in solvent A.

The Heptanucleotide d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}. Condensation of d-MMTr-TpTpA^{Bz}pC^{An} and d-pTpTpA^{Bz}-OAc. A mixture of triethylammonium d-MMTr-TpTpA^{Bz}pC^{An} (1040 OD_{280} units, 0.021 mmole) and triethylammonium d-pTpTpA^{Bz}-OAc (1800 OD_{280} units, 0.06 mmole) was allowed to react with TPS (109 mg, 0.36 mmole) in pyridine (3 ml) under exclusion of moisture for 12 hr at room temperature. Aqueous pyridine (3 ml of 50%) was added under cooling, and the solution was kept for 8 hr at room temperature. Then the solution was treated with 2 *N* sodium hydroxide (10 ml) and water (5 ml) for 10 min in an ice bath and neutralized with pyridinium Dowex 50 ion-exchange resin. The resin was removed by filtration and washed with 50% aqueous pyridine. The filtrate and washings (80 ml) were applied to a DEAE-cellulose (carbonate) column. A linear salt gradient was used for the elution. The condition and the elution pattern are shown in Figure 2. The distribution and the identification of the ultraviolet-absorbing material are shown in Table III. The product, d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}, obtained in peak VII, gave a single spot in solvents B and C. Peak VI also contained the same product (68%) but in addition a trityl-negative side product. The spectral properties of the product at pH 8 were λ_{max} 275 mμ, λ_{min} 239 mμ, and $\epsilon_{302}/\epsilon_{280}$ = 0.42. The unprotected heptanucleotide, d-TpTpApCpTpTpA showed λ_{max} 264 mμ, λ_{min} 233 mμ, and $\epsilon_{280}/\epsilon_{260}$ = 0.50 in water. R_f values are given in Table I.

The Octanucleotide d-MMTr-TpTpA^{Bz}pC^{An}TpTpA^{Bz}pC^{An}. Condensation of d-MMTr-TpTpA^{Bz}pC^{An} and d-pTpTpA^{Bz}pC^{An}-OAc. Triethylammonium d-pTpTpA^{Bz}pC^{An}-OAc (3100 OD_{280} units, 0.063 mmole) was condensed with triethylammonium d-MMTr-TpTpA^{Bz}pC^{An} (780 OD_{280} units, 0.016 mmole) in pyridine (1 ml) and tri-*n*-hexylamine (0.082 ml, 0.025 mmole) using TPS (143 mg, 0.47 mmole) as the condensing reagent. After 11 hr at room temperature 50% aqueous pyridine (3 ml) was added with cooling. A further amount of pyridine (2 ml) was added to dissolve the precipitate (presumably the sulfonic acid) and after 3 hr at room temperature the clear solution was treated with 2 *N* sodium hydroxide (5 ml) in an ice bath for 10 min. Sodium ions were removed by pyridinium Dowex 50 ion-exchange resin. The resin was removed by filtration and washed with 50% aqueous pyridine. The filtrate and washings (110 ml) were applied on the top of a DEAE-cellulose (carbonate) column. The conditions of elution and the pattern are shown in Figure 3. The distribution and the identification of the ultraviolet-absorbing material are shown in Table IV. Peak VII and VIII contained almost pure d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An} which traveled as a single spot in solvent B and had an absorption ratio $\epsilon_{302}/\epsilon_{280}$ of 0.58. Ammonia treatment (2 days) followed by 80% acetic acid treatment (40 min) gave d-TpTpApCpTpTpApC which was homogeneous in solvent D but showed three trace impurities in solvent E. The spectral properties of d-TpTpApCpTpTpApC were λ_{max} 263 mμ, λ_{min} 234 mμ, and $\epsilon_{280}/\epsilon_{260}$ = 0.59 in water. The spleen diesterase degradation of the octanucleotide followed by chromatography (solvent C) gave Tp, d-Ap, d-Cp, and deoxycytidine in a ratio of 4:1.9:0.8:0.8, theoretical being 4:2:1:1. The presence of ultraviolet-absorbing material (about 13% of total) was detected at the origin of the paper chromatogram.[15]

The Dodecanucleotide d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$pTpA^{Bz}pC^{An}. Condensation of d-MMTr-TpTpA^{Bz}pC^{An}pTpTpA^{Bz}pC^{An} and d-pTpTpA^{Bz}pC^{An}-OAc. Triethylammonium d-pTpTpA^{Bz}pC^{An}-OAc (1960 OD_{280} units, 0.04 mmole) was condensed with the octanucleotide (peak VII of Figure 3, 380 OD_{280} units, 0.039 mmole) in pyridine (0.3 ml) and tri-*n*-hexylamine (0.054 ml, 0.16 mmole) using TPS (91 mg, 0.3 mmole). After 9 hr, pyridine (1 ml) and 50% aqueous pyridine (3 ml) was added under cooling. The mixture was left for 1 hr in an ice bath and then treated with 2 *N* sodium hydroxide (3.5 ml) for 10 min in an ice bath. Sodium ions were removed by pyridinium Dowex 50 ion-exchange resin. The resin was removed by filtration and washed with 50% aqueous pyridine, and the filtrate and washings were applied on a DEAE-cellulose (carbonate) column. The elution conditions and pattern are shown in Figure 4. The distribution and the identification of the ultraviolet-absorbing material are shown in Table V. Peak VIII contained the dodecanucleotide ($\epsilon_{302}/\epsilon_{280}$ = 0.58) and was homogeneous in solvent B. After ammonia treatment the monomethoxytrityl product showed a single spot in solvent D. Treatment with 80% acetic acid (40 min at room temperature) gave d-T(pTpApCpT)$_2$pTpApC as the main product (solvent D), which was further purified in solvent E from trace of impurities. R_f values are shown in Table I.

The Hexadecanucleotide d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_3$pTpA^{Bz}pC^{An}. Condensation of d-MMTr-T(pTpA^{Bz}pC^{An}pT)$_2$pTpA^{Bz}pC^{An} and d-pTpTpA^{Bz}pC^{An}-OAc. Freshly precipitated triethylammonium d-pTpTpA^{Bz}pC^{An}-OAc (590 OD_{280} units, 12 μmoles) was allowed to react with the protected dodecanucleotide (peak VIII of Figure 4, 100 OD_{280} units) using TPS (27 mg, 90 μmoles) in pyridine (0.1 ml) and tri-*n*-hexylamine (0.017 ml, 50 μmoles). After 4 hr, 50% aqueous pyridine (1 ml) and pyridine (1 ml) were added under cooling. Sodium hydroxide (2 ml, 2 *N*) then was added to the mixture, some water (1.5 ml) being added to get a clear solution. After 10 min in an ice bath the solution was neutralized with pyridinium Dowex 50 ion-exchange resin. The filtrate and washings (40 ml) were applied on a DEAE-cellulose (carbonate) column. The elution conditions and pattern are shown in Figure 5. The distribution and identification of the ultraviolet-absorbing material are shown in Table VI. Peak V contained the hexadecanucleotide ($\epsilon_{302}/\epsilon_{280}$ = 0.58). After ammonia treatment (2 days at room temperature) the product gave a single spot in solvent D. The compound was eluted from the chromatogram with 30% aqueous pyridine using descending technique. The aqueous pyridine was evaporated and the residue was treated with a mixture of acetic acid, pyridine,

(15) The octanucleotide was degraded completely by spleen diesterase in a different run.

and water (14:1:3, v/v/v) for 24 hr at room temperature. The unprotected hexadecanucleotide showed a trace of faster traveling impurity in solvent E (R_f values are shown in Table I). The spectral properties of d-T(pTpApCpT)$_3$pTpApC were λ_{max} 263 mμ, λ_{min} 234 mμ, and $\epsilon_{280}/\epsilon_{260}$ = 0.60 in water.

Removal of Monomethoxytrityl Group from d-MMTr-TpTpApC Using Acetic Acid–Pyridine Buffer. Ammonium d-MMTr-TpTpApC (70 OD_{260} units) was dissolved in 0.5 ml of a mixture of acetic acid, pyridine, and water (14:1:3). Aliquots (0.05 ml) were taken at different time intervals and evaporated with pyridine. The residue was analyzed by paper chromatography in solvent A. Half-life of the compound was found to be 5 hr at room temperature After 24 hr d-TpTpApC was practically the only nucleotidic compound detected in solvent A. The spot was eluted, treated with concentrated ammonia at 37° for 12 hr, and chromatographed in solvent E. Again a single spot was observed. When the above acetic acid–pyridine treatment was prolonged to 5 days, again only a single product corresponding to d-TpTpApC (solvents A and E) was observed, and no evidence of depurination was obtained. After 10 days at room temperature, the formation of new minor ultraviolet-absorbing products corresponding, presumably, to the removal of adenine was observed (solvents A and E).

Allosteric Linkage

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Abstract: For any system there exists a binding potential, $\mathcal{J}$, which is a function of the chemical potentials, μ, of all the components present save one, the reference component. This has the property that $\partial\mathcal{J}/\partial\mu_i = n_i$, where n_i is the amount of component i per unit of reference component. In the case of a macromolecule which exists in several allosteric conformations all in equilibrium with one another, $\mathcal{J}$ assumes the special form given by eq 1.5 or 1.6, becoming what we call the allosteric binding potential. This considerably simplifies the discussion of the macromolecule and predicts various key features of its behavior in its reactions with ligands. It leads at once to the concept of allosteric linkage, a type of linkage which arises exclusively from the prevalence of equilibrium between the various conformations, independently of whether these, by themselves, show any linkage effects at all. Allosteric linkage, when heterotropic, may be either positive or negative; when homotropic, it is always positive (or cooperative). In the case where the macromolecule contains only a single site for a ligand X, equilibrium between the various conformations has the result that the ligand equilibrium curve for X necessarily assumes the form of a simple titration curve. When the number of sites is greater than one, the situation is of course more complex. In such cases, however, the median ligand activity of the macromolecule as a whole, which gives the total work of saturating it with ligand, may be expressed very simply in terms of the median ligand activities of the various forms by eq 4.3 or 4.4 The introduction of the allosteric binding potential clarifies the whole concept of homotropic linkage in an allosteric macromolecule and leads to a sharper distinction between the true and apparent interaction free energy. It likewise clarifies the concept of heterotropic linkage and the regulation to which it can give rise. In particular, it shows that the potential fineness of allosteric control of an enzyme by its activators and inhibitors increases with the number of sites for them in the macromolecule (eq 6.4; see also section 10c). Moreover it brings out the fact that whenever the interactions are allosteric in origin the shape of the ligand equilibrium curve for a given ligand cannot be invariant for changes of the ligand activity of the control ligands, nor, in general, will the curve be symmetrical. (An exception is of course the one- or two-site case.) Finally, the introduction of the binding potential leads to an expression for the heat of combination of the macromolecule with a ligand, which shows how this quantity depends on the heats of the various allosteric transitions (eq 9.3). An analysis of the ligand equilibria of hemoglobin in the light of these principles shows that they are not inconsistent with the idea that the various interactions displayed, both the heterotropic and homotropic ones, are predominantly allosteric in origin. This leads, almost perforce, to the conclusion that the major part of the interactions arises within the $\alpha\beta$ subunits. The interactions between these subunits, whether in the same or different molecules, though of decisive importance in producing the observed values of the Hill interaction parameter, n, are much smaller.

Previous discussions of linkage[1-3] have been pitched on the most general note, without heed for mechanism. Now, in view of mounting interest in allosteric transitions as a possible source of regulation in enzymes, and indeed in working proteins generally, the time would seem ripe for a more detailed analysis, directed specifically at those linkage effects, commonly known as allosteric effects, which arise from the prevalence of equilibrium between different conformational forms in a macromolecule. The task is greatly lightened by the introduction of an expression for the binding potential of the macromolecule, which we shall call the *allosteric binding potential*. It is with this that we begin; we end, as usual, with a discussion of hemoglobin, which provides an incomparable test body for all such ideas.

1. The Allosteric Binding Potential

In order to introduce the concept of the allosteric binding potential a few words about the binding potential in general are called for by way of orientation. The binding potential is a thermodynamic concept which applies to any system but is particularly relevant to a macromolecule containing a number of interacting sites for several different ligands.[3] It is closely related to the grand canonical partition function of statistical

(1) J. Wyman, *Advan. Protein Chem.*, 4, 407 (1948).
(2) J. Wyman, *ibid.*, 19, 223 (1964).
(3) J. Wyman, *J. Mol. Biol.*, 11, 631 (1965).