Studies on Polynucleotides. XLIV.[1] The Synthesis of Dodecanucleotides Containing the Repeating Trinucleotide Sequence Thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxycytidine[2,3]

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The synthesis of the dodecanucleotide containing the repeating trinucleotide sequence thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxycytidine (d-TpTpCpTpTpCpT-pTpCpTpTpC) has been accomplished. The synthetic approach used involved the stepwise condensation of a suitably protected mononucleotide to the 3'-hydroxyl end of a growing oligonucleotide chain. The starting materials were 5'-O-tritylthymidine and the protected mononucleotides N-anisoyl-3'-O-acetyldeoxycytidine 5'-phosphate (d-pC^{An}-OAc) and 3'-O-acetylthymidine 5'-phosphate (pTOAc). The condensing agents used were dicyclohexylcarbodiimide or mesitylenesulfonyl chloride. After each condensation step, the terminal 3'-O-acetyl group was selectively removed from the protected oligo- or polynucleotides by a mildly alkaline treatment, and the latter products were purified by chromatography on DEAE-cellulose anion-exchanger columns. By using an increasing excess of the protected mononucleotide with an increase in the chain length of the oligonucleotide, high yields (70–80%) with respect to the latter component could be maintained. All of the intermediate oligo- and polynucleotides, protected and unprotected, have been isolated pure and characterized.

The over-all considerations in undertaking the synthesis of deoxyribopolynucleotides containing known repeating di- and trinucleotide sequences have been stated separately[4] and, in an accompanying paper,[1] the synthesis of several series of deoxyribopolynucleotides containing repeating dinucleotide sequences has been reported. The present paper records the synthesis of deoxyribopolynucleotides, up to the dodecanucleotide, containing the repeating trinucleotide sequence thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxycytidine.[5] Two accompanying papers describe parallel series of experiments which have accomplished the synthesis of the dodecanucleotide containing the repeating sequence thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxyinosine,[6] and of the two hexanucleotides containing the repeating sequences deoxycytidylyl-(3'→5')-deoxyadenylyl-(3'→5')-deoxyadenosine and deoxyguanylyl-(3'→5')-deoxyadenylyl-(3'→5')-deoxyadenosine, respectively.[7]

The approach used for the synthesis of deoxyribopolynucleotides containing repeating dinucleotide sequences involved the polymerization of suitably protected dinucleotides.[1] The alternative approach of stepwise synthesis involving the addition of one protected nucleotide unit at a time to the 3'-hydroxyl end of a growing deoxypolynucleotide chain has been used in the present work.[8] This approach is illustrated, with structural formulas (Chart I), for the first two steps leading to the synthesis of the protected trinucleotide 5'-O-tritylthymidylyl-(3'→5')-thymidylyl-(3'→5')-N-anisoyldeoxycytidine (d-TrTpTpC^{An})[11] (V) and then, by removal of the protecting groups to VI, d-TpTpC.[11] 5'-O-Tritylthymidine (I) served as the starting protected nucleoside, the use of the more acid-labile, *p*-methoxytrityl groups[12] being unnecessary because of the adequate acidic stability of the pyrimidine glycosyl bonds in the present series of compounds. The protected mononucleotides used were 3'-O-acetylthymidine[13] (pT-OAc)[11] and N-anisoyl-3'-O-acetyl-

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

(2) For references to the previous papers which deal directly with this topic see footnote 2 in ref. 1.

(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 the Life Insurance Medical Research Fund (Grant No. G-62-54).

(4) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965).

(5) Brief reports of this work have already appeared: T. M. Jacob, E. Ohtsuka, M. W. Moon, S. A. Narang, and H. G. Khorana, *Federation Proc.*, **23**, 531 (1964); T. M. Jacob and H. G. Khorana, Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., July 1964, Section 1, p. 62.

(6) S. A. Narang and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 2981 (1965).

(7) S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, **87**, 2988 (1965).

(8) Polymerization of suitably protected trinucleotides should, in principle, provide the simpler approach to the synthesis of deoxyribopolynucleotides containing a repeating trinucleotide sequence. However, because of the limited success so far in polynucleotide synthesis involving trinucleotides, with phosphomonoester groups as the starting materials,[9] and because of the very encouraging results obtained in stepwise addition of mononucleotide units,[10] the latter approach was preferred. In particular, the stepwise approach appeared to be the more promising for the synthesis of deoxyribopolynucleotides in the chain length range 15-20, and at the time this work was undertaken, the minimal chain length adequate for template function in DNA-polymerase and DNA-dependent RNA-polymerase reactions could not be predicted. The polymerization of preformed suitably protected trinucleotides clearly is deserving of further study, and work along these lines is now in progress.

(9) See for a fuller discussion H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.* **85**, 3841 (1963).

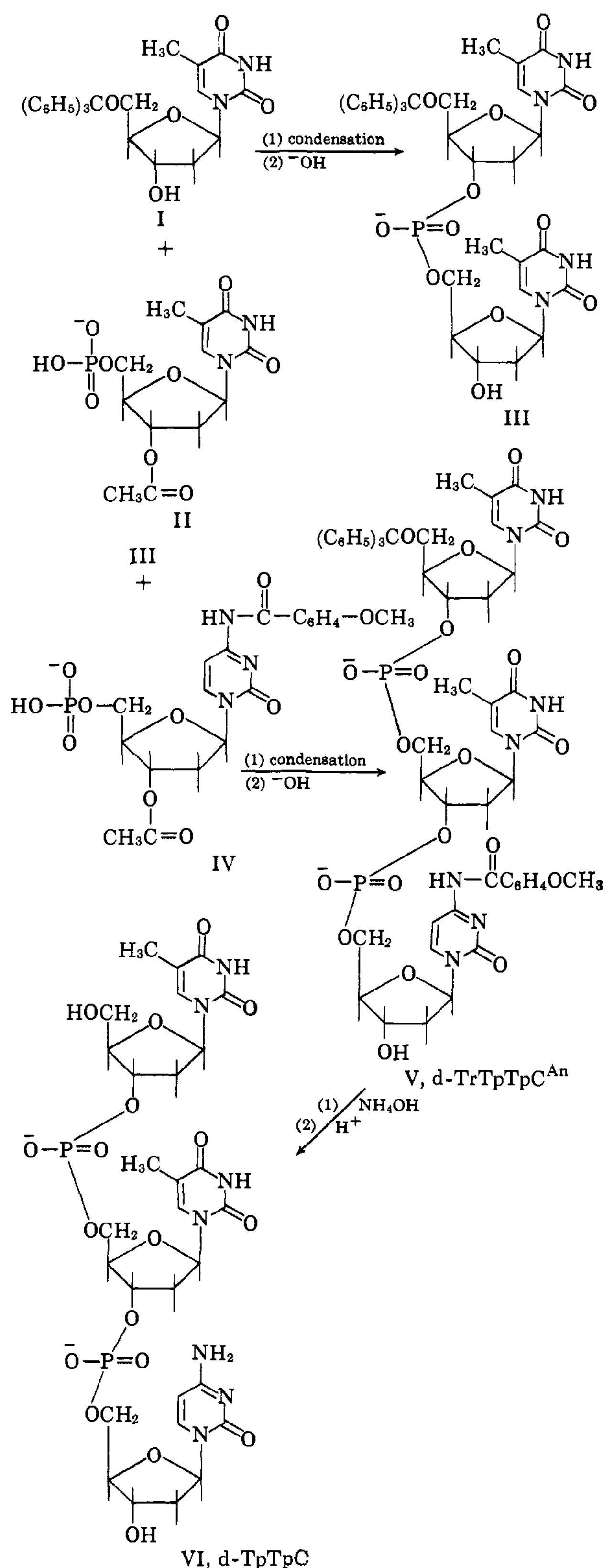
(10) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 368 (1965).

(11) For convenience and for economy of space, abbreviations are used extensively for both the protected and unprotected series of compounds described in this paper. The basic system of abbreviations is as has been described elsewhere (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) and is in use in *J. Biol. Chem.* The abbreviations for protected nucleotides have also been defined previously.[9] Thus, Tr is the abbreviation for trityl; the presence of an anisoyl group on the cytosine ring in deoxycytidine is represented by C^{An}. Thus, 5'-O-tritylthymidylyl-(3'→5')-thymidylyl-(3'→5')-N-anisoyldeoxycytidine (V) is abbreviated to d-TrTpTpC^{An}, and similarly for the higher homologs. The presence of a 3'-O-acetyl group on a nucleotide or polynucleotide is shown by the abbreviation OAc added to the right. Thus, 3'-O-acetylthymidine 5'-phosphate is pT-OAc.

(12) See *e.g.*, H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3821 (1963).

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

Chart I. Synthesis of 5′-O-Tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-N-anisoyldeoxycytidine (V)



deoxycytidine 5′-phosphates[14] (d-pC^{An}-OAc)[11] which, because of the fact that they were used in large excess, were checked carefully for the presence of the parent nucleotide (pT or d-pC^{An}) bearing a free 3′-hydroxyl group.[15] The condensing agent used most in the present work was mesitylenesulfonyl chloride, the reactions being run at room temperature in anhydrous pyridine for periods of 2-4 hr. in accordance with the results reported previously.[10] After the condensation reaction an alkaline treatment, which was completely selective for the removal of the terminal 3′-O-acetyl group from the desired protected oligo- or polynucleotide, was given, and the reaction products were then separated by anion-exchange chromatography on a DEAE-cellulose column.

In the early phase of the work the possibility was considered that, during the extended period of multistep synthesis, which included prolonged exposure of the protected intermediates to eluents during chromatographic purification, partial loss of the anisoyl group might occur[16] or, alternatively, that the deliberate removal of the anisoyl group from the cytosine residues might be necessary as an aid to purification at the intermediate steps.[17] A procedure was therefore developed for the anisoylation of the cytosine ring in an oligonucleotide, for example, 5′-O-tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-deoxycytidine (VI), prepared from V by treatment with concentrated ammonium hydroxide. No such need arose, however. By anion-exchange chromatography using DEAE-cellulose in the acetate form and by using triethylammonium acetate (pH 6.5) in 50% aqueous ethyl alcohol as the eluent it was possible to isolate all of the intermediate fully protected deoxyribopolynucleotides (full list in Table I) in pure form and to use them for the next step directly. The use of a high proportion of ethyl alcohol in the eluent has a marked advantage in sharpening up the peaks and in enhancing the resolution of products, carrying large organic protecting groups.[17]

As the chain length of the protected oligonucleotide component of the condensation reaction increases, the more valuable this component becomes. The most attractive feature of the present approach, as established in the previous work,[10] is that high yields with respect to the oligonucleotide component can be sustained by using an increasing excess of the protected mononucleotide which is readily available in quantity. In Table I are listed the molar proportions[18] of the mononucleotides actually used in the successive condensation steps and the yields, with respect to the oligo- or polynucleotide component, that were obtained after isolation of the pure desired products by anion-exchange chromatography. As is seen, the yield at all steps except the last ranged between 70 and 80%.

The actual separations of the reaction products on anion-exchanger columns are shown in Figures 1-8 for many of the steps. As can be seen, the resolution of the desired product from the starting oligo- or

(14) H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3828 (1963).

(15) The presence of this leads to the self-polymerization of the mononucleotide and therefore to the appearance of trace amounts of homopolymers.

(16) Thus, in the original publication on N-anisoyldeoxycytidine 5′-phosphate [H. G. Khorana, A. F. Turner, and H. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 686 (1961)], the utilization of this protected derivative within a week of its preparation was recommended.

(17) Thus, poor resolution was obtained in an attempt at direct separation of N-anisoyldeoxycytidine oligonucleotides (footnote 10, ref. 9) by anion-exchange chromatography. This result may now be ascribed to the use of predominantly aqueous (as against aqueous alcoholic now used) buffers then used.

(18) The molar excess of the mononucleotides used was arbitrary. No information is available concerning the *minimum* excess of the mononucleotide which would be necessary to sustain high yields.

Table I. Stepwise Synthesis of Deoxyribopolynucleotides Containing the Repeating Sequence Thymidylthymidylyldeoxycytidine

Oligonucleotide component	Mono-nucleo-tide	Mono-nucleo-tide, molar excess	Product	Yield, %
d-TrTpT	pC^{An}-OAc	2	d-TrTpTpC^{An}	82
d-TrTpTpC^{An}	pTOAc	9	d-TrTpTpC^{An}pT	79
d-TrTpTpC^{An}pT	pTOAc	13	d-TrTpTpC^{An}pTpT	77
d-TrTpTpC^{An}pTpT	pC^{An}OAc	17	d-TrTpTpC^{An}pTpTpC^{An}	74
d-TrTpTpC^{An}pTpTpC^{An}	pTOAc	29	d-TrTpTpC^{An}pTpTpC^{An}pT	75
d-TrTpTpC^{An}pTpTpC^{An}pT	pTOAc	39	d-TrTpTpC^{An}pTpTpC^{An}pTpT	73
d-TrTpTpC^{An}pTpTpC^{An}pTpT	pC^{An}OAc	84	d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}	75
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}	pTOAc	119	d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pT	70
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pT	pTOAc	184	d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpT	70
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpT	pC^{An}OAc	224	d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpTpC^{An}	56

polynucleotide was satisfactory throughout. In most runs trace amounts of products evidently derived from self-condensation of the mononucleotide bearing a free 3′-hydroxyl group were encountered. The latter type of reaction, however, presumably gave cyclic dinucleotide and only very short chain oligonucleotides, and the possibility of their contaminating the desired products existed only for the early synthetic steps. On the other hand, with increasing chain length of the synthetic products the possibilities were considered of trace contaminations from the starting material (lower homolog) and from any of the "higher homolog,"

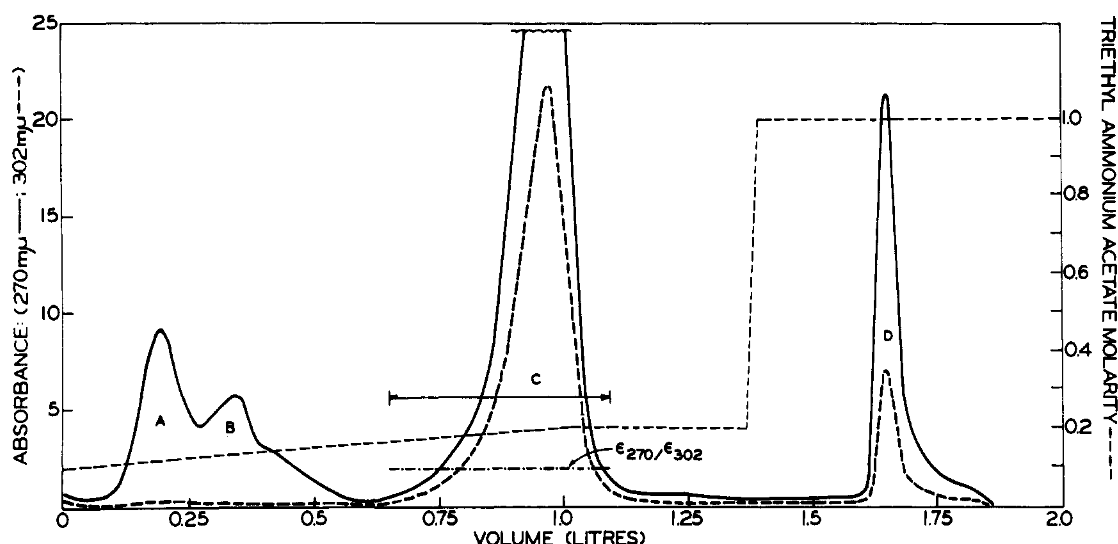


Figure 1. Chromatography of the reaction products in the preparation of the tetranucleotide d-TrTpTpC^{An}pT on a DEAE–cellulose (acetate) column. Fractions of 9 ml. were collected every 0.5 hr. d-TrTpTpC^{An}pT is in peak C.

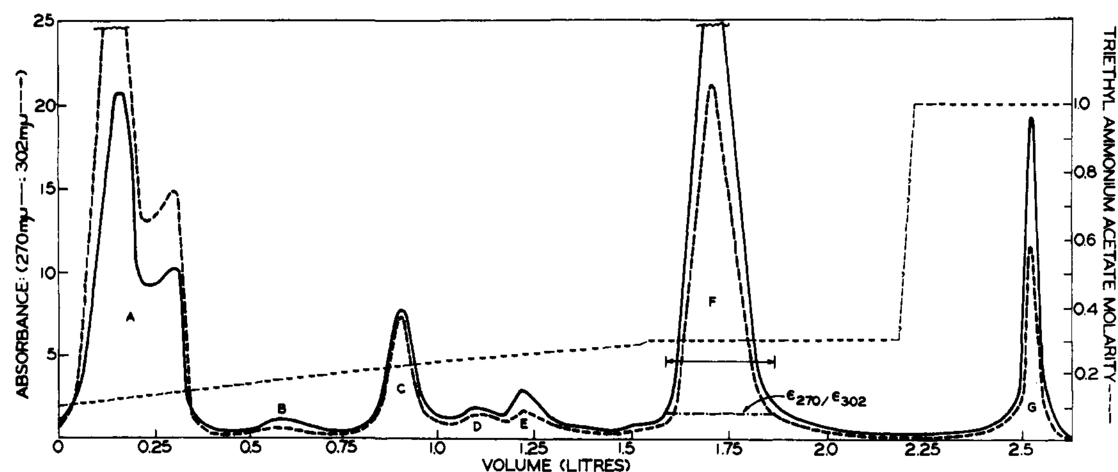


Figure 2. Chromatography of the reaction products in the preparation of the hexanucleotide d-TrTpTpC^{An}pTpTpC^{An} on a DEAE–cellulose (acetate) column. Fractions of 11 ml. were collected every 0.5 hr. Peak F contains the hexanucleotide d-TrTpTpC^{An}-pTpTpC^{An}.

Table II. Characterization and Properties of Polynucleotides[a]

Compound	$\epsilon_{270}/\epsilon_{302}$[b]	R_f[c] Solvent B	Solvent D
d-TrTpTpC^{An}	1.5	1.97	
d-TrTpTpC^{An}pT	1.9	1.59	2.07
d-TtTpTpC^{An}pTpTp	2.3	1.24	1.86
d-TrTpTpC^{An}pTpTpC^{An}	1.5	0.86	1.67
d-TrTpTpC^{An}pTpTpC^{An}pT	1.7	0.57	1.17
d-TrTpTpC^{An}pTpTpC^{An}pTpT	1.9	0.33	0.86
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}	1.5	0.23	0.73
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pT	1.65	0.12	
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpT	1.8	0.066	
d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpTpC^{An}	1.5	0.03	

[a] TrTpTpC^{An} and higher members with repeating sequence. [b] The values listed were those that were found experimentally at pH 6.5 in aqueous buffer. These are identical with those theoretically derived by simple summation of the molar extinctions of the mononucleotides at the two wave lengths. For d-pC^{An}, the value used for ϵ_{270} is 15,000 and that for ϵ_{302} is 22,450 (ref. 17). For pT, the value used for ϵ_{270} is 9300, the ϵ_{302} being nil. [c] R_f relative to that of pT.

which might arise by the addition of a mononucleotide unit with a free 3′-hydroxyl group and, then, of another unit with a 3′-O-acetyl group. Therefore, in the pooling of fractions for isolation of the desired product (Figures 1-8) the tail end and the leading edge of the main peak were not included. It should be added further that measurement of the ratio of extinction at 302 mμ (due to N-anisoyldeoxycytidine) and that at 270 mμ provided throughout (Table II) a dependable guide for the location of the peak of the desired product, and that the constancy of this ratio in the peak material reflected the purity of this product. (Only the fractions in which this ratio was constant were pooled.)

Characterization of Synthetic Products. The formation of discrete peaks, well resolved from the starting oligo- or polynucleotide components, the expected $\epsilon_{270}/\epsilon_{302}$ ratio, and its constancy in the column fractions corresponding to the desired product all indicated that the products isolated after each synthetic step were pure. After isolation, as described in the Ex-

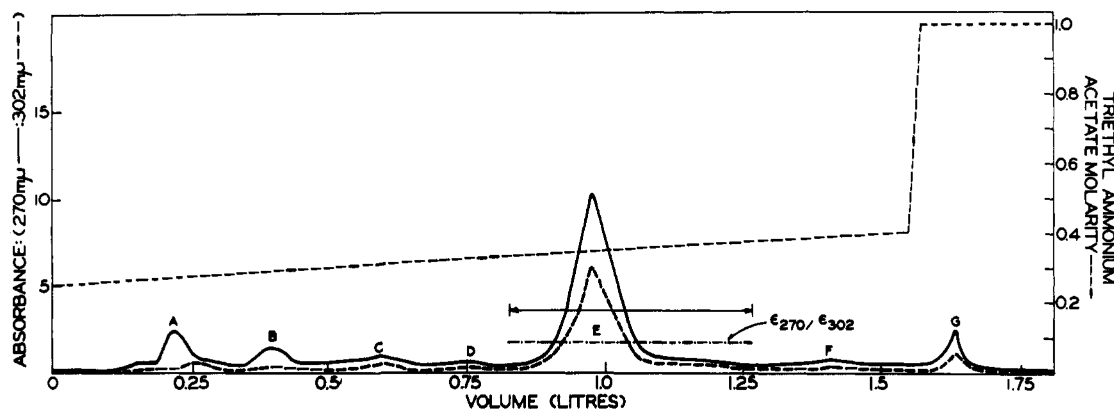


Figure 3. Chromatography of the reaction products in the preparation of the heptanucleotide d-TrTpTpC^{An}pTpTpC^{An}pT on a DEAE–cellulose (acetate) column. Peak E contains the heptanucleotide.

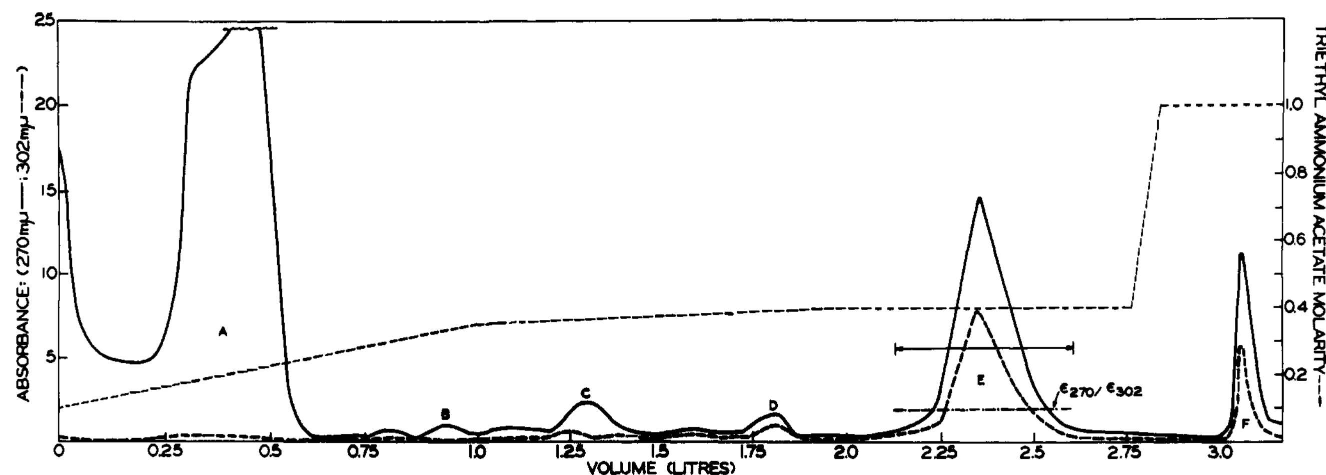


Figure 4. Chromatography of the reaction products in the preparation of the octanucleotide d-TrTpTpTpC^{An}pTpTpTpC^{An}pTpT on a DEAE–cellulose (acetate) column. The octanucleotide was in peak E.

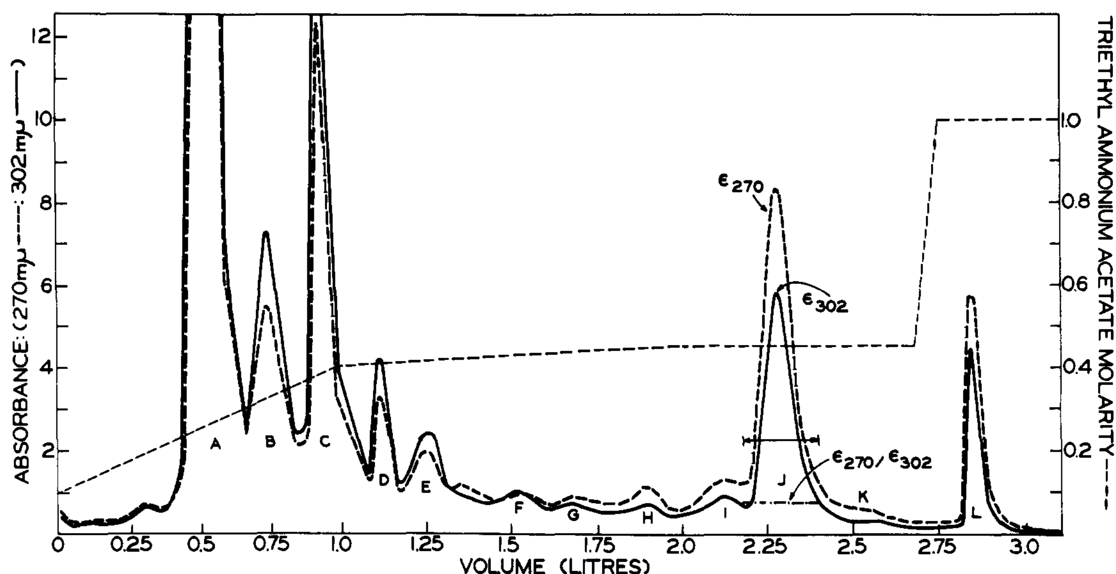


Figure 5. Chromatography of the reaction products in the preparation of the nonanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An} on a DEAE-cellulose (acetate) column. Peak J contains the nonanucleotide.

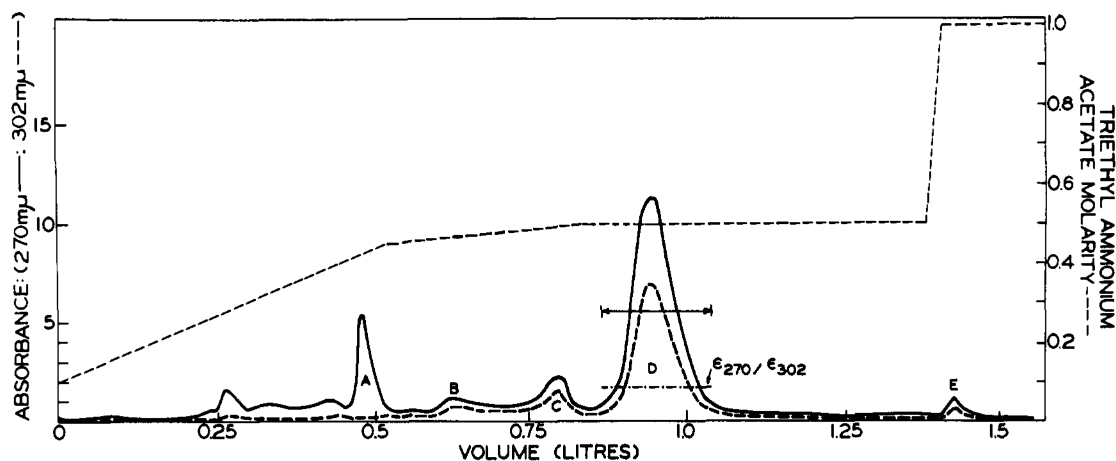


Figure 6. Chromatography of the reaction products in the preparation of the decanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pT on a DEAE-cellulose (acetate) column. After absorption of material, the column was washed first with 450 ml. of 0.05 *M* triethylammonium acetate (pH 6.5) in 50% ethanol and then with 275 ml. of the same buffer with concentration of 0.1 *M*. The salt gradient then used for the elution is shown (--------). Every fraction was measured for its optical density at 270 and 302 mμ. Peak D contains the decanucleotide.

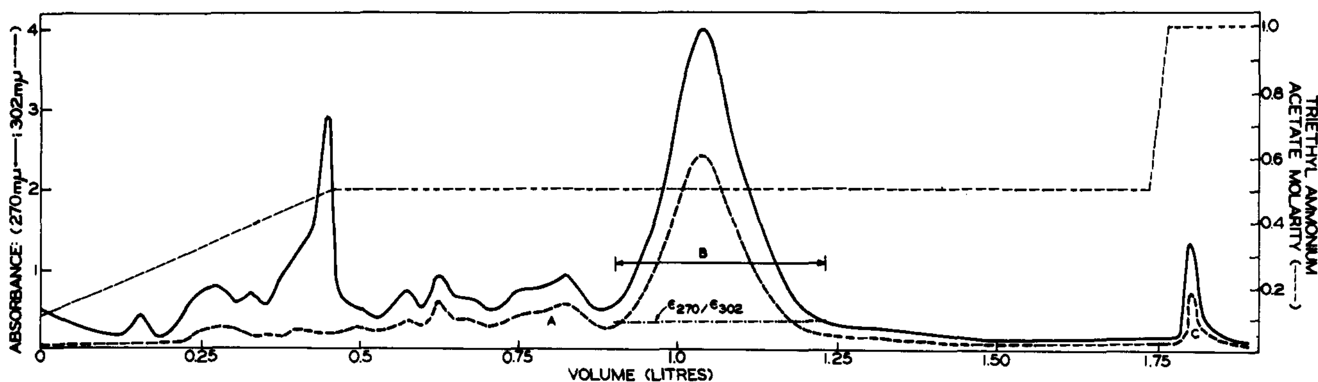


Figure 7. Chromatography of the reaction products in the preparation of the undecanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pTpT on a DEAE-cellulose (acetate) column. After absorption of the nucleotidic material the column was washed first with 280 ml. of 0.05 *M* triethylammonium acetate (pH 6.5) in 50% ethanol and then 250 ml. of the same buffer (0.1 *M*). Subsequent salt gradient used for elution is shown (--------). Fractions of 6 ml. were collected every 15 min. The undecanucleotide is in peak B.

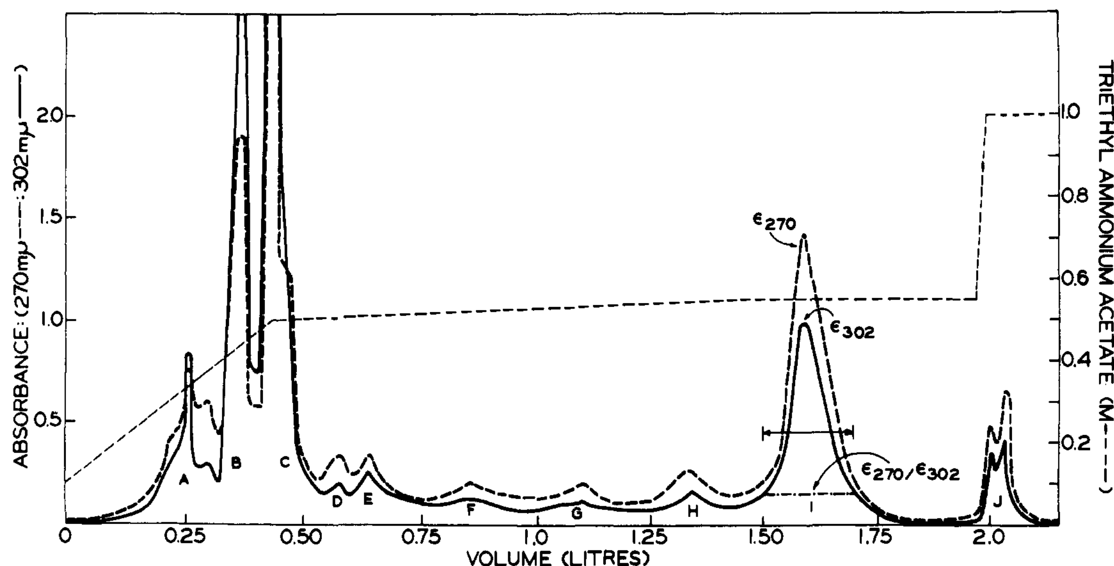


Figure 8. Chromatography of the reaction products in the preparation of the dodecanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}-pTpTpC^{An} on a DEAE-cellulose (acetate) column. Chromatography was started by washing the column first with 200 ml. of 0.05 *M* triethylammonium acetate (pH 6.5) in 50% ethanol and then with 600 ml. of the same buffer (0.1 *M*). Then a gradient of the same buffer was used as shown (---------). The desired dodecanucleotide is in peak I.

perimental section, all the members were characterized as pure by paper chromatography (a) of the fully protected derivatives (d-TrTpTpC^{An} and homologs) in two solvent systems (R_f values are given in Table II), (b) of the products obtained after removal of the anisoyl groups by ammoniacal treatment (d-Tr-TpTpC and homologs), and (c) of the oligo- or polynucleotides obtained after acidic removal of the trityl group (R_f values are given in Table III). Finally selected members, obtained at intermediate steps, were checked for structure and purity by analysis of the products formed on degradation with spleen phosphodiesterase. The results are given in Table IV. Thus, the degradation of d-TpTpCpT gave, as expected, Tp:d-Cp:T in the ratio 2:1:1; d-TpTpCpTpTpCpTp-TpC after similar degradation gave Tp:d-Cp:d-C in the ratio 6:2:1; and finally the undecanucleotide d-TpTp-CpTpTpCpTpTpCpTpT gave Tp:d-Cp:d-T in the ratio 7:3:1.

Table III. Characterization of Polynucleotides[a]

Compound	$\epsilon_{280}/\epsilon_{260}$[b]	R_f[c] (solvent C)
d-TrTpTpC		1.9
d-TrTpTpCpT		1.79
d-TrTpTpCpTpT		1.59
d-TrTpTpCpTpTpC		1.35
d-TrTpTpCpTpTpCpT		1.2
d-TrTpTpCpTpTpCpTpT		1.03
d-TrTpTpCpTpTpCpTpTpC		0.89
d-TrTpTpCpTpTpCpTpTpCpT		0.61
d-TrTpTpCpTpTpCpTpTpCpTpT		0.49
d-TrTpTpCpTpTpCpTpTpCpTpTpC		0.36
d-TpTpC	1.1	
d-TpTpCpT	0.99	1.03
d-TpTpCpTpT	0.92	0.76
d-TpTpCpTpTpC	1.1	0.58
d-TpTpCpTpTpCpT	0.987	0.47
d-TpTpCpTpTpCpTpT	0.980	0.32
d-TpTpCpTpTpCpTpTpC	1.1	0.24
d-TpTpCpTpTpCpTpTpCpT	0.97	0.17
d-TpTpCpTpTpCpTpTpCpTpT	0.96	0.13
d-TpTpCpTpTpCpTpTpCpTpTpC	1.1	0.11

[a] d-TrTpTpC and higher members; d-TpTpC and higher members. [b] Spectra taken at pH 2.5. [c] R_f relative to that of pT.

Side Products. There was evidence for the formation in trace amounts of a number of side products which gave a positive reaction for the trityl group. These products emerged from the columns mostly after the mononucleotide and before the desired products. Although their amounts were too small to permit further work on them, these products are undoubtedly similar to the series of side products investigated during the previous systematic study of the stepwise approach to the synthesis of polynucleotides.[10]

Stripping the anion-exchanger columns with 1 *M* salt at the end of gradient elution invariably gave 5-20% of the total nucleotidic material (see Figures 1-8). The formation of this series of "side products" with, apparently, higher number of charges than those in the desired product appears to be more serious. While the nature of the products present in these fractions is under further study, they seem to arise by the phosphorylation (by the activated mononucleotide) of the heterocyclic rings.[19] Thus, prolonged ammoniacal treatment of the 1 *M* fractions from the chromatographic runs shown in Figures 2-5 followed by paper chromatography showed the release of the free mononucleotide and the 5'-O-trityl derivative of the appropriate oligo- or polynucleotide as the major product. Furthermore, as expected, the mononucleotide released was the one that had been used in that synthetic step (see Experimental). It should be added that the ring phosphorylation leading to these side products is evidently slow and occurs in the presence

(19) Thymine and uracil rings in the corresponding nucleosides undergo ring benzoylation on reaction with an excess of benzoyl chloride [J. J. Fox, *et al.*, *J. Am. Chem. Soc.*, **81**, 178 (1959); R. Lohrmann and H. G. Khorana, *ibid.*, **86**, 4118 (1964)]. Analogously, phosphorylation of the same function by a powerful phosphorylating agent (obtained in the presence of the sulfonyl chlorides) may be postulated.

Table IV. Splenic Phosphodiesterase Degradation of Synthetic Polynucleotides[a]

Compound	Products, O.D.$_{267}$ d-Cp[b]	d-Tp[b]	d-C[b]	d-T[b]	Ratio[b]	Theor.	Found
d-TpTpCpT	1.55	2.96	. .	1.53	Tp:d-Cp:T	2:1:1	1.94:1:1
d-TpTpCpTpTpCpTpTpC	1.34	3.93	0.66	. .	Tp:d-Cp:d-C	6:2:1	5.95:2:1
d-TpTpCpTpTpCpTpTpCpTpT	2.6	6.0	. .	0.85	Tp:d-Cp:T	7:3:1	7:3:1

[a] Degradation of the compounds with the phosphodiesterase was carried out as described previously.[16] The products were separated by chromatography in solvent E. Their concentrations were determined by elution with water and spectrophotometric analysis at pH 2 using appropriate blanks. [b] For calculation of the molar amounts of the products the value of ϵ_{267} used for Tp and T is 9700; the value used for d-C and d-Cp at the same wave length is 9700 (G. H. Beaver, E. R. Holiday and E. A. Johnson, "Nucleic Acids," Vol. I, E. Chargaff and J. N. Davidson, Ed., Academic Press Inc., New York, N. Y., 1955, p. 493).

of a large excess of the mononucleotide. This side reaction would be expected to occur after the virtual completion of the mononucleotide addition at the 3′-hydroxyl end of the oligo- or polynucleotide component.

Concluding Remarks. The approach used in the present work, although involving the maximum possible number of steps for the synthesis of a polynucleotide, has the merit of giving consistently high yields and is free from any ambiguity in the identification of the desired product after every step. The same conclusion is reinforced by the parallel studies reported in accompanying papers on the use of the stepwise approach. Furthermore, the 1 *M* fractions (Figures 1-8) also represent essentially the desired products and, if the formation of these side products can be circumvented, then the average yields in the individual steps would be 90% or better.

Finally, it seems reasonable to expect that the same methodology can be used successfully to build polynucleotide chains certainly up to the size 20 or even a little higher. Satisfactory resolution of the desired products from the immediately lower homologs was obtained throughout the present work and no difficulty should be expected in extending this work by several steps.[20]

The synthetic polynucleotides described in this paper have been successfully used as templates for the DNA-dependent RNA polymerase and the characterization of the resulting large ribopolynucleotide with repeating adenylyladenylylguanosine sequence has been described separately.[21]

Experimental

General Methods. Paper chromatography was carried out by the descending technique using Whatman No. 40 or Whatman No. 1 paper. 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); solvent D, isopropyl alcohol-water (70:30) with ammonia in the vapor phase; solvent E, ethyl alcohol-0.5 *M* ammonium acetate (pH 3.8). Paper electrophoresis was performed using Whatman 3 MM paper and 0.03 *M* phosphate buffer (pH 7.1) at a potential of about 60 v./cm. The paper chromatographic and electrophoretic mobilities of different compounds are listed in Tables II and III.

(20) By using thinner and longer columns, if necessary.

(21) S. Nishimura, T. M. Jacob, and H. G. Khorana, *Proc. Natl. Acad. Sci. U. S.*, **52**, 1494 (1964).

The presence of the trityl group in different compounds was detected by spraying the chromatograms with 10% aqueous perchloric acid and drying in warm air. The trityl-containing compounds appeared yellow.

Pyridinium 3′-O-acetylthymidine 5′-phosphate and pyridinium 3′-O-acetyl-N^6-anisoyldeoxycytidine 5′-phosphate were prepared as described previously[13,14] except for the removal of pyridinium acetate. The total acetylation product was rendered anhydrous by evaporation of added dry pyridine. The residual sirup was dissolved in dry pyridine (2-10 ml.) and the solution was added dropwise to an excess (about 20-50-fold) of anhydrous ether. The nucleotidic material, which separated as a fine white powder, was collected by centrifugation and washed thoroughly with fresh dry ether. The purity of the nucleotides was checked, prior to use, by chromatography in solvent B. Contamination of d-pC^{An}-OAc by any anisic acid was further checked by paper electrophoresis at pH 7.1.

The ϵ_{max} value used for pT-OAc at 267 mμ is 9600 and that for d-pC^{An}-OAc at 302 mμ is 22,450. The abbreviations O.D.$_{267}$, O.D.$_{302}$, etc., refer to the extinction of the nucleotidic solutions at neutral pH in 1-ml. of solution using a 1-cm. light path quartz cell, the number in subscript being the wave length used.

Isolation of Protected Oligonucleotides. Following chromatography on a DEAE-cellulose (acetate) column using triethylammonium acetate as the eluent, the appropriate fractions containing the desired oligo- or polynucleotides (containing 5′-O-trityl and N-anisoyl groups) were pooled and the solvent was removed by evaporation at 20° or below under reduced pressure. Pyridine was added at intervals during evaporation. The sirupy residue was rendered anhydrous by evaporation of added pyridine and was then taken up in dry pyridine (10-15 ml.). This solution was added dropwise to an excess (25-100-fold) of dry ether. The fine white precipitate of the nucleotidic material was collected by centrifugation and washed with ether by resuspension in the centrifuge tube. In general, the loss in the ethereal layer was negligible. (The ether layer was frequently checked for nucleotidic material either by evaporation and chromatography or by addition of an excess of pentane.)

Removal of Protecting Groups. The removal of N-anisoyl groups from protected oligonucleotides was effected by treating a pyridine solution of the compound with an excess (2-3-fold) of concentrated ammonium hydroxide for 1-2 days at room temperature. The trityl group was removed (only after the removal of the

anisoyl group) by treating the lyophilized product (ammonium or pyridine salt) with an excess of 80% acetic acid for 1 day at room temperature. The acetic acid was then removed by evaporation at room temperature *in vacuo*, followed by an ether wash of the residue.

General Method of Condensation. The condensation of the protected mononucleotides (pyridinium 3′-O-acetylthymidine 5′-phosphate or 3′-O-acetyl-N-anisoyl-deoxycytidine 5′-phosphate) with the protected oligonucleotides bearing a free 3′-hydroxyl group was carried out by the following general method. The protected mononucleotide (calculated amount, large excess) and the oligonucleotide component (known amount) were combined and the mixture was freshly precipitated (immediately prior to use) by adding their concentrated solution in pyridine to an excess of ether in a centrifuge tube.[22] The solid white powder of the two components thus obtained was dissolved in pyridine and the mixture was rendered anhydrous by repeated evaporation of added dry pyridine *in vacuo*, each time the flask being opened to dry air of a large desiccator containing phosphorus pentoxide. During the last evaporation of pyridine, the minimal amount (2-5 ml.) of the solvent necessary for complete solubilization of the reaction components was allowed to remain and mesitylenesulfonyl chloride (1.5-2 *M* proportions based on the mononucleotide) was added inside a box in which a dry nitrogen atmosphere was maintained. The sealed reaction mixture was kept for 1.25-4 hr. at room temperature or for 20 hr. at 5°. An excess of water was then added under cooling and this was followed, after some minutes, by the addition of 2 *N* sodium hydroxide sufficient to give over-all molarity of 1 *M*.[23] The alkaline mixture was kept for 10 min. at 0° and the sodium ions were then removed by the rapid addition of an excess of pyridinium Dowex 50 ion-exchange resin. The resin was removed and washed thoroughly with 20-50% aqueous pyridine. The total aqueous pyridine solution was applied directly to a DEAE-cellulose (acetate) column pre-equilibrated with triethylammonium acetate buffer (0.01-0.05 *M*) in 50% ethyl alcohol. The column was washed with the same buffer to remove pyridine and then eluted with appropriate gradients of triethylammonium acetate in 50% ethyl alcohol.

5′-O-Tritylthymidylyl-(3′→5′)-thymidylyl-(3′→5′)-deoxycytidine (d-TrTpTpC). An anhydrous solution of triethylammonium d-TrTpT (0.5 mmole)[10,11] and triethylammonium d-pC^{An}-OAc (1.5 mmoles) in dry pyridine (5 ml.) was treated with mesitylenesulfonyl chloride (0.980 g., 4.2 mmoles) for 20 hr. at 5°. Water (10 ml.) was then added and was followed, after 4 hr., by the addition of an excess of concentrated ammonium hydroxide. This solution was kept sealed for 2 days at room temperature, and most of ammonia was then removed by suction *in vacuo*. The total solution was applied to the top of a DEAE-cellulose (bicarbonate) column (40 × 4.2 cm. diameter) pre-equilibrated with 0.01 *M* ammonium bicarbonate in 20% aqueous ethanol. After a wash with the same buffer, elution was carried out using a linear gradient, 4 l. of 0.01 *M* ammonium bicarbonate in the mixing vessel and an equal volume of 0.4 *M* ammonium bicarbonate in the reservoir, both solutions in 20% ethyl alcohol. Fractions of 12 ml. were collected every 5 min. and the concentration of the nucleotidic material in the effluent was determined spectrophotometrically at 270 mμ. Fractions 16-31 contained anisic acid; fraction 73-90 contained mainly mesitylenesulfonate. Fractions 73-151 contained deoxycytidine 5′-phosphate, its pyrophosphate, and a small amount of TrTpT. The desired product, d-TrTpTpC, was in fractions 202-301 (total volume, 1200 ml.). The yield as determined spectrophotometrically was 82% (12,200 $O.D._{267}$ units at pH 2.5).

(22) Examination of the ether supernatant showed negligible loss of the nucleotidic material.

(23) If a two-phase mixture results on the addition of alkali, homogenization is effected by adjusting the pyridine-water ratio.

The Trinucleotide d-TrTpTpC^{An}. (A) Via d-TrTpTpC. The trinucleotide d-TrTpTpC (0.2 mmole) in dry pyridine (2 ml.) was treated with anisoyl chloride (2 mmoles, 0.28 ml.) at room temperature for 2 hr. The formation of a crystalline precipitate, presumably pyridine hydrochloride, was observed and this pre-precipitate was removed by filtration of the solution in a dry nitrogen atmosphere. Water (5 ml.) was added to the pyridine solution and, after a few hours, the aqueous pyridine solution was treated with 5 ml. of 2 *N* sodium hydroxide at 0° for 20 min. Sodium ions were removed by the addition of an excess of pyridinium Dowex 50 ion-exchange resin and the clear solution and washings, after removal of the resin, were extracted with ether repeatedly to remove anisic acid (as its pyridine salt). The aqueous pyridine solution was concentrated with added pyridine and the product again taken up in 10 ml. of dry pyridine. This solution was added dropwise to an excess (500 ml.) of dry ether. Pyridinium d-TrTpTpC^{An} precipitated as a white powder and was collected by centrifugation. The yield as determined spectrophotometrically was essentially quantitative.

(B) By Condensation of d-TrTpT with d-pC^{An}OAc. A mixture of pyridinium d-TrTpT (0.5 mmole), pyridinium d-pC^{An}OAc (1.5 mmoles), pyridinium Dowex 50 ion-exchange resin (2 g.), and DCC (7.5 mmoles) in dry pyridine (5 ml.) was shaken at room temperature for 4 days. Water (5 ml.) was then added and after a few hours the resin and dicyclohexylurea were removed by filtration (50% aqueous pyridine wash). The aqueous pyridine solution was extracted with ether to remove unreacted DCC and then kept at room temperature for a further 8 hr. After subsequent concentration to about 5 ml. and dilution with water to 10 ml., the solution was treated with 10 ml. of 2 *N* sodium hydroxide for 10 min. at 0°. After removal of the sodium ions in the usual way, the total product was applied to the top of a DEAE-cellulose (acetate) column (56 cm. × 2.5 cm. diameter) pre-equilibrated with 0.05 *M* triethylammonium acetate (pH 6.5) in 50% ethyl alcohol. For elution, the mixing vessel contained 2 l. of 0.05 *M* triethylammonium acetate in 50% ethyl alcohol and the reservoir contained 2 l. of 0.25 *M* salt. Fractions of 16 ml. were collected every 15 min. and elution was followed by measurement of the optical density at 302 and at 270 mμ and by testing for the presence of trityl group. Fractions 7-25 contained mostly d-TrTpT. Fractions 43-85 contained d-pC^{An} and the product, d-TrTpTpC^{An}, the

latter being mostly in fractions 55–77. (The pyrophosphate of d-pC^{An} was in fractions 85–100.) Fractions 55–77 were pooled (17,925 O.D.$_{270}$ units) and evaporated *in vacuo* with added pyridine, and the nucleotidic product was freed from the salt by precipitation from pyridine–ether mixture. The precipitate was dissolved in 25 ml. of pyridine and 1% of this product was rechromatographed on a DEAE-cellulose (acetate) column (25 × 1.5 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate in 20% ethyl alcohol. Elution was performed using a linear salt gradient, there being 200 ml. of 0.01 *M* triethylammonium acetate in 20% ethyl alcohol in the mixing vessel, and an equal volume of the same buffer (0.2 *M*) in the reservoir. Fractions of 7 ml. were collected every 5 min. Fractions 49–58 contained d-pC^{An}. All the eluent had passed through the column and further elution was performed by using more of the 0.2 *M* buffer in 20% ethyl alcohol. The product, d-TrTpTpC^{An}, appeared in fractions 61–79 free from d-pC^{An} and in all fractions having a constant $\epsilon_{270}/\epsilon_{302}$ of 1.5. The yield of pure product was 50 O.D.$_{302}$ units. The total yield in the experiment, 5000 O.D.$_{302}$, corresponded to 45%.[24]

The Tetranucleotide d-TrTpTpC^{An}pT. An anhydrous pyridine solution (5 ml.) of the protected trinucleotide d-TrTpTpC^{An} (3500 O.D.$_{302}$ units, 0.156 mmole), pyridinium d-pT-OAc (1.5 mmoles), and mesitylenesulfonyl chloride (0.779 g., 3.5 mmoles) was kept at room temperature for 4 hr. Water (10 ml.) was then added and after a few minutes the aqueous pyridine solution was treated with 20 ml. of 2 *N* sodium hydroxide for 7 min. at 0°. The subsequent work-up was as described under General Methods. The total mixture was applied to the top of a DEAE-cellulose column (77 × 2 cm.) pre-equilibrated with 0.05 *M* triethylammonium acetate (pH 6.5) in 50% ethanol. The column was first washed with the same buffer concentration (300 ml.) and then with 500 ml. of 0.1 *M* buffer. These washes removed the excess of mononucleotide, its pyrophosphate, and also the unreacted starting material, d-TrTpTpC^{An}. Elution was next carried out using a salt gradient. The conditions of chromatography and the elution pattern obtained are shown in Figure 1. Peaks A and B contained residual pT, its pyrophosphate, and also some unreacted d-TrTpTpC^{An}. Peak C had a constant $\epsilon_{270}/\epsilon_{302}$ of 1.9 and contained pure d-TrTpTpC^{An}pT. The yield of this product in the pooled peak (405 ml.) was 2767 O.D.$_{302}$ units (79%). Subsequent to this product, elution with 1 *M* salt gave, as seen in Figure 1, peak D which contained 248 O.D.$_{302}$ units.

The Pentanucleotide d-TrTpTpC^{An}pTpT. An anhydrous pyridine solution (3 ml.) of the protected tetranucleotide (2500 O.D.$_{302}$ units, 0.111 mmole) as its triethylammonium salt, pyridinium pT-OAc (1.5 mmoles), and mesitylenesulfonyl chloride (0.743 g.) was kept under exclusion of moisture for 2.5 hr. at room temperature. The work-up including alkaline treatment was as under General Methods. The total mixture in aqueous pyridine was applied to the top of a DEAE-cellulose (acetate) column (36 × 2 cm.) pre-equilibrated with 0.05 *M* triethylammonium acetate in 50% ethyl alcohol. The column was washed first with 270 ml. of the same buffer and then with 930 ml. of 0.1 *M* concentration of the same buffer. A part of the nucleotidic material, including the desired product, appeared in the second wash and was rechromatographed as described later. Elution of the column was carried out with a linear salt gradient, the mixing vessel containing 500 ml. of 0.15 *M* triethylammonium acetate in 50% ethanol and the reservoir an equal volume of 0.2 *M* concentration of the same buffer. Fractions of 6 ml. were collected every 12 min. The product, d-TrTpTpC^{An}pTpT, appeared as a broad peak in fractions 18–102, the $\epsilon_{270}/\epsilon_{302}$ being constant (2.3) throughout these fractions. The total yield of the product in 502 ml. of the pooled fraction was 1469 O.D.$_{302}$ units. Subsequent elution of the column with 1 *M* salt gave another peak which contained 148 O.D.$_{302}$ units.

The 0.1 *M* buffer wash obtained above was diluted with an equal volume of 50% ethyl alcohol and the total solution was slowly absorbed on top of a larger DEAE-cellulose (acetate) column (77 × 2 cm.). The conditions for gradient elution of this column were similar to those described above. In this way, a small peak (72 O.D.$_{302}$ units) (mainly the starting material, d-TrTpTpC^{An}pT) was first obtained and this was followed by elution of the pentanucleotide d-TrTpTpC^{An}-pTpT (455 O.D.$_{302}$ units), the fractions containing this product having a constant $\epsilon_{270}/\epsilon_{302}$ of 2.3. Only a negligible amount of material was eluted on subsequent wash of the column with 1 *M* salt.

The combined yield of the desired pentanucleotide was 1924 O.D.$_{302}$ units, corresponding to 77%.

The Hexanucleotide d-TrTpTpC^{An}pTpTC^{An}. An anhydrous pyridine solution (4 ml.) of the pentanucleotide d-TrTpTpC^{An}pTpT, as its triethylammonium salt (1800 O.D.$_{302}$ units, 0.0802 mmole), pyridinium d-pC^{An}OAc (1.5 mmoles), and mesitylenesulfonyl chloride (0.689 g.) was kept at room temperature for 1.25 hr. Water (5 ml.) was then added and the subsequent work-up was as described under General Methods. The total mixture was applied on top of a DEAE-cellulose (acetate) column (73 × 2 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate in 50% ethyl alcohol. The column was first washed with 250 ml. of 0.05 *M* triethylammonium acetate and then with 550 ml. of 0.1 *M* concentration of the same buffer. These washes removed pyridine, mesitylenesulfonate, and most of d-pC^{An}. Elution was then carried out using a linear salt gradient. The elution pattern obtained is shown in Figure 2. Peaks D and E (190 O.D.$_{302}$ units) contained partly the unreacted starting material. Peak F (325 ml.) had a constant ratio of $\epsilon_{270}/\epsilon_{302}$ (1.5) throughout its fractions and contained pure desired product, d-TrTpTpC^{An}pTpTpC^{An}. The yield of the pooled fractions, excluding the edges, was 2650 O.D.$_{302}$ units (73.6%).

The Heptanucleotide d-TrTpTpC^{An}pTpTC^{An}pT. An anhydrous pyridine solution (1 ml.) of the hexanucleotide d-TrTpTpC^{An}pTpTpC^{An}, as its triethylammonium salt (625 O.D.$_{302}$ units, 0.014 mmole), pyridinium d-pT-OAc (0.45 mmole), and mesitylenesulfonyl chloride (0.218 g., 1 mmole) was kept at room temperature

(24) The recovery of nucleotidic material was not quantitative in either of the two column chromatographic runs. The over-all yield of d-TrTpTpC^{An} can probably be improved considerably by modifying the alcohol content in the eluents used, as has been done in more recent work (see accompanying paper, ref. 7).

for 4 hr. The subsequent work-up was as described above under General Methods. The total aqueous pyridine solution of the nucleotidic material was applied to the top of a DEAE-cellulose (acetate) column (47 × 1.5 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate (pH 6.5) in 50% ethyl alcohol. The column was first washed with 280 ml. of 0.05 *M* concentration and then with 550 ml. of 0.1 *M* concentration of the same buffer. These washes were followed by elution with a gradient (0.1 and 0.25 *M*, 250 ml. of each), and when this solution had passed through the column elution was carried out as shown in Figure 3. Peak C (71 ml.) contained 25 $O.D._{302}$ units (mainly the unreacted hexanucleotide) while peak E contained the desired heptanucleotide. The ratio of $\epsilon_{270}/\epsilon_{302}$ was constant (1.7) in all the fractions of this peak and the yield as determined spectrophotometrically was 466 $O.D._{302}$ units (75%). The other peaks (A to G) contained side products (positive reaction for the trityl group) which were not investigated further.

The Octanucleotide d-TrTpTpC^{An}pTpTC^{An}pTpT. An anhydrous pyridine solution (2 ml.) of the heptanucleotide d-TrTpTpC^{An}pTpTpC^{An}pT, as its triethylammonium salt (1568 $O.D._{302}$ units, 0.035 mmole), pyridinium pT-OAc (1.5 mmoles), and mesitylenesulfonyl chloride (0.66 g., 3 mmoles) was kept at room temperature for 1.5 hr. After the usual work-up, including the alkaline treatment, the total mixture was applied to the top of a DEAE-cellulose (acetate) column (72 × 2 cm.). The initial washes with triethylammonium acetate buffer in 50% alcohol were similar to that in the preceding experiment. Subsequent elution with salt gradient was as shown in Figure 4. Peak A (Figure 4) contained dithymidine 5'-pyrophosphate. Peak D (130 ml.) contained 65 $O.D._{302}$ units (mainly the unreacted heptanucleotide). Peak E (410 ml.) had throughout its fractions a constant ratio (1.9) of $\epsilon_{270}/\epsilon_{302}$ and contained pure octanucleotide. The yield excluding the edges was 1140 $O.D._{302}$ units (73%). Other peaks which contained side products were not investigated further.

The Nonanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}. An anhydrous pyridine solution (2 ml.) of triethylammonium d-pC^{An}OAc (1.75 mmoles) was allowed to react with mesitylenesulfonyl chloride (0.758 g., 3.5 mmoles) under exclusion of moisture for 3 min. at room temperature. This mixture was added, under exclusion of moisture, to an anhydrous pyridine solution (2 ml.) of the triethylammonium salt of the octanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpT (906 $O.D._{302}$ units, 0.0202 mmole), and the sealed reaction mixture was kept at room temperature for 1.25 hr. After the usual work-up and alkaline treatment, the total mixture was applied to the top of a DEAE-cellulose (acetate) column (72 × 2 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate (0.01 *M*) in 50% ethyl alcohol. The column was washed first with 450 ml. of 0.05 *M* concentration, then with 800 ml. of 0.1 *M* concentration of the same buffer. This was followed by elution using a gradient, as shown in Figure 5, the flow rate being 60 ml./hr. The elution pattern obtained is shown in Figure 5. From the absorption patterns it is evident that peaks A, B, C, and D arose from d-pC^{An} only, peak A being the symmetrical pyrophosphate. The unreacted starting material was in peak H (140 ml., 49 $O.D._{302}$ units). Peak J (350 ml.) had, as shown, a constant $\epsilon_{270}/\epsilon_{302}$ of 1.5 and contained pure nonanucleotide. The yield after discarding the edges (designated I and K) was 861 $O.D._{302}$ units. The edges, I and K, also contained essentially pure desired product. The total yield of the nonanucleotide thus was 1014 $O.D._{302}$ units (75%). Peak L eluted with 1 *M* salt contained 263 $O.D._{302}$ units.

The Decanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpT-pC^{An}pT. An anhydrous pyridine solution (3 ml.) of the nonanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}, as its triethylammonium salt (826 $O.D._{302}$ units, 0.0123 mmole), pyridinium pT-OAc (1.5 mmoles), and mesitylenesulfonyl chloride (0.622 g., 2.8 mmoles) was kept at room temperature for 1 hour and 50 min. Subsequent work-up was in the usual way, and the total mixture in aqueous pyridine was applied to the top of a DEAE-cellulose (acetate) column (50 × 1.5 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate (pH 6.5) in 50% ethyl alcohol. The conditions of chromatography and the elution pattern obtained are given in Figure 6. Peak C (98 ml., 64 $O.D._{302}$ units) contained the unreacted starting material. Peak D (218 ml.) had a constant $\epsilon_{270}/\epsilon_{302}$ ratio of 1.65 and contained pure decanucleotide. The yield, not including the front and the tail edges of this peak, was 575 $O.D._{302}$ units (70%). Other peaks in the figure contained side products which were not investigated further.

The Undecanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTp-C^{An}pTpT. An anhydrous pyridine solution (3 ml.) of the decanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpTpC^{An}pT, as the triethylammonium salt (510 $O.D._{302}$ units, 0.0076 mmoles), pyridinium pT-OAc (1.4 mmoles), and mesitylenesulfonyl chloride (0.444 g., 2 mmoles) was kept at room temperature for 2.5 hr. and then at 5° for 23 hr. After the standard work-up, the mixture was applied to the top of a DEAE-cellulose (acetate) column (48 × 1.5 cm.) pre-equilibrated with 0.07 *M* triethylammonium acetate (pH 6.5) buffer in 50% ethyl alcohol. The conditions of chromatography and the elution pattern obtained are shown in Figure 7. Peak A (150 ml., 62 $O.D._{302}$ units) contained unreacted decanucleotide. The desired undecanucleotide was in peak B. The fractions in this peak had a constant $\epsilon_{270}/\epsilon_{302}$ of 1.8. The total amount of the product in the peak (265 ml.) and in the tail end (415 ml.), which also contained pure product, was 358 $O.D._{302}$ units and 645 $O.D._{270}$ units (70%). Other peaks contained byproducts which were not investigated further.

The Dodecanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpT-pC^{An}pTpTpC^{An}. An anhydrous pyridine solution of the undecanucleotide d-TrTpTpC^{An}pTpTpC^{An}pTpT-pC^{An}pTpT, as its triethylammonium salt (150 $O.D._{302}$ units, 0.0022 mmole), triethylammonium d-pC^{An}OAc (0.5 mmole), and mesitylenesulfonyl chloride (0.244 g., 1.1 mmoles) was kept at room temperature for 2 hr. under exclusion of moisture. After the standard subsequent work-up, the total aqueous pyridine solution was applied to the top of a DEAE-cellulose (acetate) column (48 × 1.5 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate (pH 6.5) in 50% ethyl alcohol. The conditions of elution and the elution pattern obtained are shown in Figure 8. Peaks

D, E, F, G, and J contained side products which remain unidentified. The unreacted starting material was found in peak H (14 O.D.$_{302}$ units). The desired dodecanucleotide was in peak I, and all the fractions in it had a constant $\epsilon_{270}/\epsilon_{302}$ of 1.5. The yield in the pooled fractions (158 ml.) was 89 O.D.$_{302}$ units, and the tail contained an additional 24 O.D.$_{302}$ units of the same product, the total yield thus being 113 O.D.$_{302}$ units (56%).

Properties of 1 M Fractions. Portions (10-15 O.D.$_{302}$ units) of materials obtained in 1 *M* fractions in Figures 2-5 were kept in concentrated ammonium hydroxide for 2-3 days at room temperature and the products formed were examined by paper chromatography.

The product derived from the 1 *M* fraction in Figure 2 (preparation of the hexanucleotide) was chromatographed in solvent A. Several nucleotidic bands were detected. A major band corresponded to deoxycytidine 5'-phosphate (identified by comparison with authentic material in two solvents) and another major band (trityl positive) contained the hexanucleotide TrTpTpCpTpTpC.

The products derived from the 1 *M* fractions in Figures 3-5 were chromatographed in solvent C. In each case the formation of a mononucleotide as one of two major products was noted. Thus thymidine 5'-phosphate was present in the products from the 1 *M* fraction in Figures 3 and 4, while deoxycytidine 5'-phosphate was present in the products from the 1 *M* fraction in Figure 5. The products from Figure 4 were not separable in solvent C but were clearly resolved on rechromatography in solvent B. The other major product in the 1 *M* fraction from Figure 3 was the heptanucleotide TrTpTpCpTpTpCpT, that from Figure 4 was the octanucleotide TrTpTpCpTpTpCpTpT, and that from Figure 5 was the nonanucleotide TrTpTpCpTpTpCpTpTpC.

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Studies on Polynucleotides. XLV.[1] The Synthesis of Dodecanucleotides Containing the Repeating Trinucleotide Sequence Thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxyinosine[2]

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The synthesis of the dodecanucleotide containing the repeating trinucleotide sequence thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxyinosine (d-TpTpIpTpTpIpTpTpIpTpTpI) has been accomplished. The synthetic approach used involved the stepwise condensation of a suitably protected mononucleotide to the 3'-hydroxyl end of a growing oligonucleotide chain. The starting materials were 5'-O-di-p-methoxytritylthymidine and the protected mononucleotide, 3'-O-acetyldeoxyinosine 5'-phosphate and 3'-O-acetylthymidine 5'-phosphate. The condensing agents used were dicyclohexylcarbodiimide or mesitylenesulfonyl chloride. After each condensation step, the terminal 3'-O-acetyl group was removed from the protected oligo- or polynucleotides by ammonia treatment and the latter products were purified by chromatography on a DEAE-cellulose anion-exchange column. By using an increasing excess of the protected mononucleotide with an increase in the chain length of the oligonucleotide, a high yield (65-80%) with respect to the latter component could be maintained. All the intermediate oligo- and polynucleotides, protected and unprotected, have been isolated pure and characterized.

The work described in the present paper was carried out in parallel with that reported in the preceding paper[1] and deals with the synthesis of the dodecanucleotide containing the repeating trinucleotide sequence thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxyinosine.[3] While the over-all considerations for the synthetic work described in the present series of papers have been presented separately,[4] an additional consideration in the synthesis of deoxyribopolynucleotides by the stepwise approach was that of protection of the heterocyclic rings. In the first phase of this work on the synthesis of deoxyribopolynucleotides containing repeating trinucleotide sequences, those nucleotide combinations were chosen which required the minimum of protection on the purine or pyrimidine rings. Thus in the work reported in the preceding paper only the amino group in the cytosine ring needed protection.[1] In the present work a trinucleotide sequence consisting of two pyrimidine (thymine) units and a purine (a keto base such as hypoxanthine) was chosen. The hypoxanthine ring does not require protection and there is ample evidence, both physicochemical and enzymatic, that

(1) Paper XLIV: T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 2971 (1965).

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(3) A brief report of a part of this work has already appeared: S. A. Narang and H. G. Khorana, Abstracts, Sixth International Congress of Biochemistry, New York, N. Y., 1964, Section 1, p. 76.

(4) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965).