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.

Acknowledgment. The technical assistance of Mr. H. Radloff throughout this work is acknowledged with appreciation.

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

S. A. Narang and H. G. Khorana

Contribution from the Institute for Enzyme Research of the University of Wisconsin, Madison, Wisconsin. *Received March 22, 1965*

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

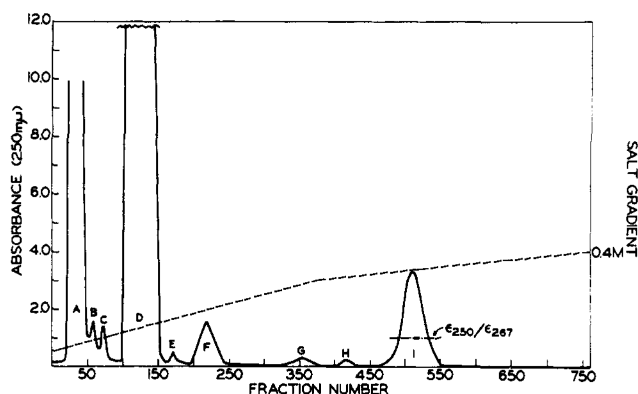


Figure 1. Chromatography of the reaction products in the preparation of the hexanucleotide d-DMTr-TpTpIpTpTpI on DEAE cellulose (carbonate) column (83 × 3 cm.), using a linear gradient of ammonium bicarbonate (0.05–0.3 M, 2 l., each containing 20% alcohol) followed by the next gradient (0.3–0.4 M, 2 l. each, containing 20% ethyl alcohol) for elution. Fractions containing 10 ml. were collected every 15 min. Peak I contains the hexanucleotide d-DMTr-TpTpIpTpTpI; $\epsilon_{250}/\epsilon_{267}$, —·—·—·; salt gradient, ---------.

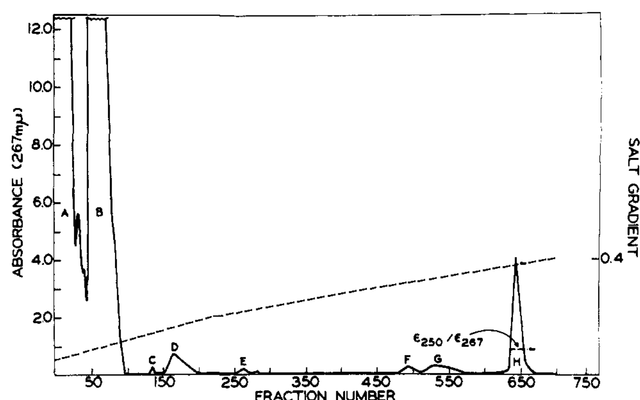
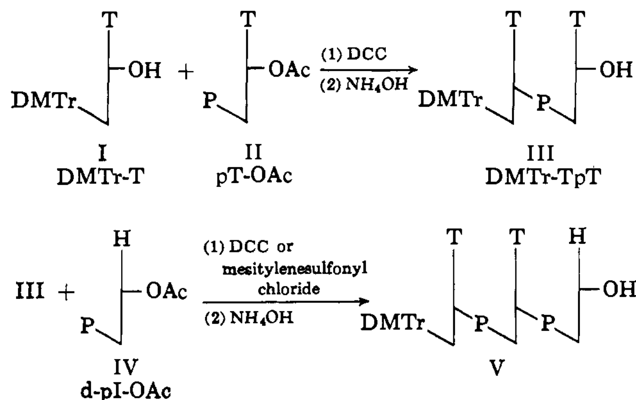


Figure 2. Chromatography of the reaction products in the preparation of the heptanucleotide d-DMTr-TpTpIpTpTpIpT on DEAE–cellulose (carbonate) column (88 × 1.8 cm.) by using linear gradients of 0.05–0.2, 0.2–0.3, and 0.3–0.4 M, respectively, 1 l. each of ammonium bicarbonate containing 30% ethyl alcohol. Fractions containing 8 ml. were collected every 15 min. Peak H contains the heptanucleotide d-DMTr-TpTpIpTpTpIpT; $\epsilon_{250}/\epsilon_{267}$ —·—·—·; salt gradient, ---------.

it can fully substitute for guanine in a Watson–Crick type base-pairing mechanism.[5]

As in the previous work,[1] the synthetic approach involved the stepwise condensation of a mononucleotide unit with the 3′-hydroxyl end group of a growing oligonucleotide chain. The typical operations used are shown with respect to the first two steps in the simplified structural representations I–V.[6] 5′-O-Di-



methoxytritylthymidine[7] was used as the protected nucleoside in place of 5′-O-tritylthymidine because of the much milder acidic treatment which is adequate for removal of the dimethoxytrityl group. Thus, it was ensured early at the trinucleotide (V) stage that this protecting group could be removed without causing any cleavage of the purine glycosyl bond. The required mononucleotide, deoxyinosine 5′-phosphate, was prepared in excellent yield from the commercially available deoxyadenosine 5′-phosphate by nitrous acid deamination.[8] For condensation, both dicyclohexylcarbodiimide (DCC) and mesitylenesulfonyl chloride[9] were used as the reagents. The latter reagent offers the particular advantage that trialkylammonium salts can be used to aid solubilization of the nucleotidic components in the reaction medium, and in several steps described in the present work the use of trialkylammonium salts was in fact necessary.

After each condensation step an ammoniacal treatment was given to remove the 3′-O-acetyl group and the products were separated by anion-exchange chromatography on DEAE–cellulose columns using the volatile salt ammonium bicarbonate as the eluent. Excellent separations were thus obtained, the elution patterns for several of the steps being shown in Figures 1–7. The separation and sharpening of the peaks was often aided by varying the ethyl alcohol content of the eluting salt solution.

With increasing chain length of the oligonucleotide component an increasing excess of the mononucleotide was used (Table I), and in this way satisfactory yields of the desired products were obtained at all of the synthetic steps (Table I). From Table I it is also seen that the two condensing agents have comparable yields wherever a comparison was made.

From the elution patterns given in the figures it is seen that often a considerable amount of nucleotidic product was eluted when the columns were stripped with 1 M salt. These products appear to form, as concluded in the preceding paper,[1] by the phosphorylation of the heterocyclic ring(s). Prolonged ammonia treatment[10] apparently cleaves off the nucleotidic

(5) See, *e.g.*, M. J. Bessman, I. R. Lehman, J. Adler, S. B. Zimmerman, E. S. Simms, and A. Kornberg, *Proc. Natl. Acad. Sci. U. S.*, **44**, 633 (1958); R. B. Inman and R. L. Baldwin, *J. Mol. Biol.*, **8**, 452 (1964); F. M. Kahan and J. Hurwitz, *J. Biol. Chem.*, **237**, 3778 (1962).

(6) With a view to economizing space and for convenience, we are using the system of abbreviations and the diagrammatic representations which have already been introduced and used in the preceding papers [ref. 1 and E. Ohtsuka, M. W. Moon, and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 2956 (1965)]. Additional abbreviations used here are: DMTr is the abbreviation for di-*p*-methoxytrityl (di-*p*-methoxyphenyl, phenylmethyl); 5′-O-di-*p*-methoxytritylthymidine, DMTr-T; deoxyinosine 5′-phosphate, d-pI, while hypoxanthine in the same nucleotide is shown by H.

(7) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963).

(8) The procedure used is similar to that of E. P. Geiduscheck, *Proc. Natl. Acad. Sci. U. S.*, **47**, 950 (1961).

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

(10) In the present work prolonged ammonia treatment was permissible at every step because of the absence of the protecting groups on the heterocyclic rings and the 1 M fractions could have been eliminated or much reduced with consequent gain in the yield of the desired product. Unfortunately, the properties of the 1 M fraction were not investigated until most of the work in this and the preceding paper was complete.

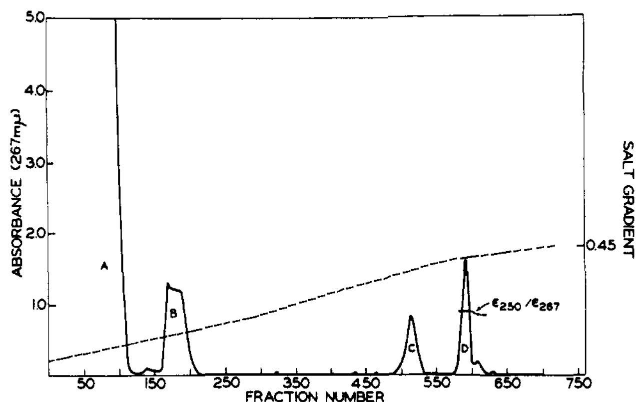


Figure 3. Chromatography of the reaction products in the preparation of the octanucleotide d-DMTr-TpTpIpTpTpIpTpT on DEAE-cellulose (carbonate) column (88 × 1.8 cm.) by using the linear gradient of ammonium bicarbonate, 0.05–0.2 *M*, 1 l. each; 0.2–0.3 *M*, 500 ml. each; 0.3–0.4 *M*, 500 ml. each; and 0.4–0.45 *M*, 500 ml. each. All the solutions contained 40% ethyl alcohol. Fractions containing 6.5 ml. were collected every 15 min. Peak D contains the octanucleotide d-DMTr-TpTpIpTpTpIpTpT, the unchanged d-DMTr-TpTpIpTpTpIpT being in peak C; $\epsilon_{250}/\epsilon_{267}$, –·–·–·; salt gradient, ---------.

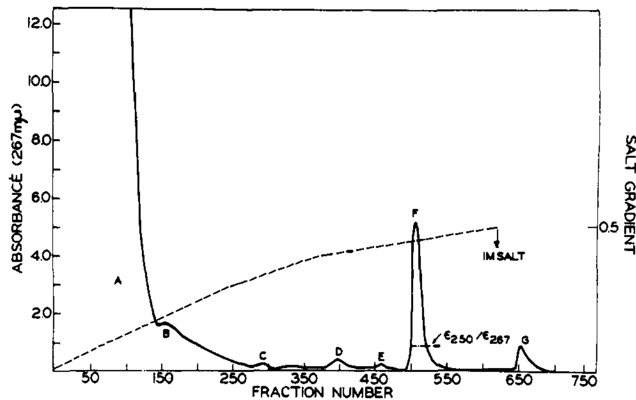


Figure 5. Chromatography of the reaction products in the preparation of the decanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpT on DEAE-cellulose (carbonate) column (74 × 1.0 cm.) by using linear gradients of ammonium bicarbonate, 0.01–0.3 *M*, 500 ml. each; 0.3–0.4 *M*, 250 ml. each; and 0.4–0.5 *M*, 500 ml. each. All solutions contained 50% ethyl alcohol. Fractions containing 4 ml. were collected every 15 min. Peak F contains the decanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpT and peak G contains the 1 *M* fraction; $\epsilon_{250}/\epsilon_{267}$, –·–·–·; salt gradient, ---------.

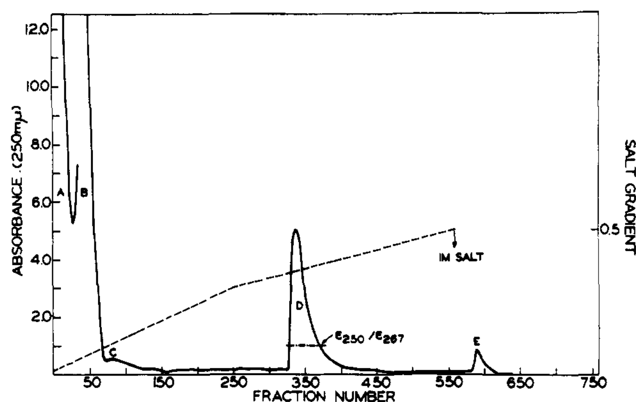


Figure 4. Chromatography of the reaction products in the preparation of the nonanucleotide d-DMTr-TpTpIpTpTpIpTpTpI on DEAE-cellulose (carbonate) column (74 × 1.8 cm.) by using linear gradient of ammonium bicarbonate, 0.01–0.3 and 0.3–0.5 *M*, 1 l. of each solution containing 50% ethyl alcohol. Fractions containing 6.0 ml. were collected every 15 min. Peak D contains the nonanucleotide d-DMTr-TpTpIpTpTpIpTpTpI, and peak E contains 1 *M* fraction; $\epsilon_{250}/\epsilon_{267}$, –·–·–·; salt gradient, ---------.

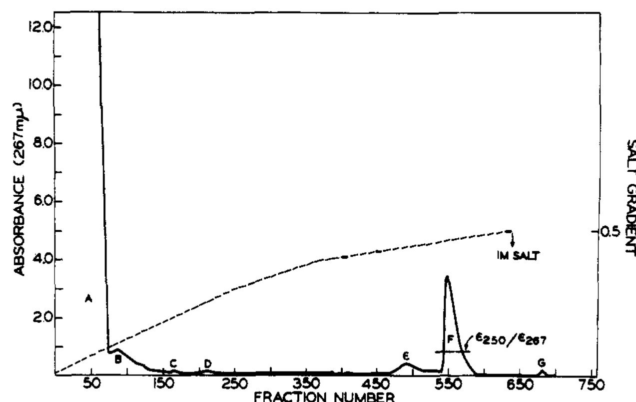


Figure 6. Chromatography of the reaction products in the preparation of undecanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpTpT on DEAE-cellulose (carbonate) column (74 × 1.0 cm.) by using linear gradients of ammonium bicarbonate, 0.01–0.3 *M*, 500 ml. each; 0.3–0.4 *M*, 250 ml. each; and 0.4–0.5 *M*, 500 ml. each. All solutions contained 50% alcohol. Fractions containing 3.5 ml. were collected every 15 min. Peak F contains the undecanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpTpT; $\epsilon_{250}/\epsilon_{267}$, –·–·–·; salt gradient, ---------.

group on the heterocyclic ring(s) and thus in the synthesis of the dodecanucleotide (Figure 7), when the duration of ammonia treatment was 2 days in place of the usual 1 day, none of the 1 *M* fraction was detected.

While the present method of synthesis ensures unambiguity in the structures of the desired products, several lines of additional evidence pointed to the purity of the homologous polynucleotides. First, the position of elution of the major product after every condensation reaction was as expected. Second, there was excellent agreement between the calculated and the experimentally determined values of $\epsilon_{250}/\epsilon_{267}$[11] for every synthetic product (Table II). Furthermore, as shown in Table III, the isolated synthetic products were homogeneous on paper chromatograms, both before and after the removal of the dimethoxytrityl group. It should be noted that both series of compounds, one containing the dimethoxytrityl group and the second unprotected, had patterns of R_f values consistent with the chain length of each member (Table III). Finally, selected members of the series were checked for structure and purity by degradation with spleen phosphodiesterase. These degradations went to completion and gave the expected products (Table IV). Thus, the degradation of d-TpTpIpT gave Tp:d-Ip:T in the ratio of 2:1:1; d-TpTpIp-TpTpIpT gave Tp:d-Ip:T in the ratio of 4:2:1; and finally the nonanucleotide d-TpTpIpTpTpIpTpTpI gave Tp:d-Ip:d-I in the ratio of 6:2:1.

(11) The particular wave lengths chosen correspond to the λ_{max} for the two chromophores, thymidine and deoxyinosine. While the agreement between the calculated and the experimentally determined values might indicate the absence of pronounced stacking interaction between the heterocyclic rings, it has not been determined whether some hypochromicity develops in the synthetic products with increasing chain length.

Table I. Yields at Individual Steps in the Synthesis of Deoxyribopolynucleotides Containing the Repeating Trinucleotide Sequence Thymidylyl-(3'→5')-thymidylyl-(3'→5')-deoxyinosine

Starting oligonucleotide	Molar excess of mono-nucleotide	Reagent	Product	Yield,[a] %
d-DMTr-T	None	DCC	d-DMTr-TpT	80
d-DMTr-TpT	3.1	DCC	d-DMTr-TpTpI[b]	70
d-DMTr-TpT	3.7	$MsSO_2Cl$[c]	d-DMTr-TpTpI	80
d-DMTr-TpTpI	6.1	DCC	d-DMTr-TpTpIpT	63
d-DMTr-TpTpI	6	$MsSO_2Cl$	d-DMTr-TpTpIpT	65
d-DMTr-TpTpIpT	15	DCC	d-DMTr-TpTpIpTpT	66
d-DMTr-TpTpIpT	16	$MsSO_2Cl$	d-DMTr-TpTpIpTpT	74
d-DMTr-TpTpIpTpT	20	DCC	d-DMTr-TpTpIpTpTpI[b]	52
d-DMTr-TpTpIpTpT	30	$MsSO_2Cl$	d-DMTr-TpTpIpTpTpI	76
d-DMTr-TpTpIpTpTpI	66	DCC	d-DMTr-TpTpIpTpTpIpT	65
d-DMTr-TpTpIpTpTpI	77	$MsSO_2Cl$	d-DMTr-TpTpIpTpTpIpT	70
d-DMTr-TpTpIpTpTpIpT	120	$MsSO_2Cl$	d-DMTr-TpTpIpTpTpIpTpT	80
d-DMTr-TpTpIpTpTpIpTpT	150	$MsSO_2Cl$	d-DMTr-TpTpIpTpTpIpTpTpI	85
d-DMTr-TpTpIpTpTpIpTpTpI	200	$MsSO_2Cl$	d-DMTr-TpTpIpTpTpIpTpTpIpT	70
d-DMTr-TpTpIpTpTpIpTpTpIpT	330	$MsSO_2Cl$	d-DMTr-TpTpIpTpTpIpTpTpIpTpT	78
d-DMTr-TpTpIpTpTpIpTpTpIpTpT	500	$MsSO_2Cl$	d-DMTr-TpTpIpTpTpIpTpTpIpTpTpI	70

[a] As based on the oligonucleotidic components. [b] The solvent used was a mixture of dimethylformamide and pyridine. [c] Abbreviation for mesitylenesulfonyl chloride.

Experimental

General Methods. Paper chromatography and paper electrophoresis were performed as described in the preceding paper.[1] Solvents A–C for paper chromatography were as described there.[1] Solvent D is isobutyric acid–concentrated ammonia–water (577:385:38, v./v.); solvent E, ethyl alcohol–0.5 *M* ammonium acetate (pH 3.8) (700:300, v./v.).

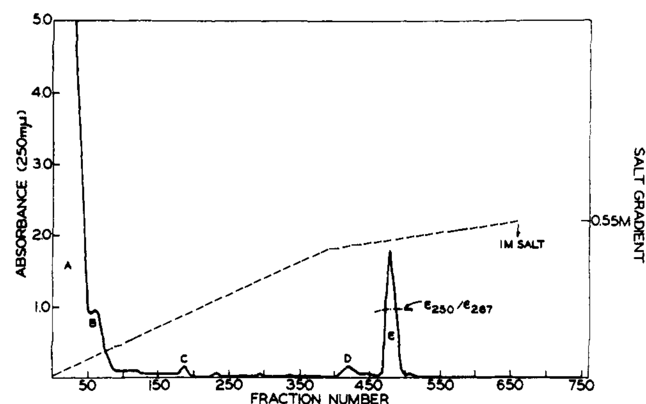


Figure 7. Chromatography of the reaction products in the preparation of dodecanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpTpTpI on DEAE-cellulose (carbonate) column (74 × 1.0 cm.) by using linear gradients of ammonium bicarbonate; 0.01–0.3 *M*, 500 ml. each; 0.3–0.45 *M*, 250 ml. each; and 0.45–0.55 *M*, 500 ml. each. All solutions contained 50% alcohol. Fractions of 3.5 ml. volume were collected every 15 min. Peak E contains the dodecanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpTpTpI; $\epsilon_{250}/\epsilon_{267}$, —·—·—·; salt gradient, ---------.

The di-*p*-methoxytrityl-containing compounds were made visible as orange-red spots by spraying the paper chromatograms with 10% aqueous perchloric acid and drying them in warm air.

5'-O-Dimethoxytritylthymidine was prepared by the method of Schaller, *et al.*[7] Mesitylenesulfonyl chloride and anhydrous pyridine were prepared as described earlier.[9] Pyridinium 3'-O-acetylthymidine 5'-phosphate was prepared as described in the preceding paper, freshly prepared batches being used as far as possible in the individual condensation steps.

Table II. The Ratio of Molecular Extinction (250 and 267 mμ) of Homologous Compounds at pH 7.1

Compounds	$\epsilon_{250}/\epsilon_{267}$[a] Found	Calcd.
d-DMTr-TpT	0.76	0.70
d-DMTr-TpTpI	1.07	1.02
d-DMTr-TpTpIpT	0.91	0.92
d-DMTr-TpTpIpTpT	0.84	0.84
d-DMTr-TpTpIpTpTpI	1.0	1.0
d-DMTr-TpTpIpTpTpIpT	0.88	0.92
d-DMTr-TpTpIpTpTpIpTpT	0.84	0.86
d-DMTr-TpTpIpTpTpIpTpTpI	0.99	0.99
d-DMTr-TpTpIpTpTpIpTpTpIpT	0.92	0.94
d-DMTr-TpTpIpTpTpIpTpTpIpTpT	0.89	0.90
d-DMTr-TpTpIpTpTpIpTpTpIpTpTpI	0.98	0.99

[a] The calculated values are derived by simple summation of the molar extinctions of the mononucleotides at 250 and 267 mμ at neutral pH. For d-pI, the value used for ϵ_{250} is 12,200 and that for ϵ_{267} is 5100; for DMTr-T, the value used for ϵ_{250} is 8500, that for ϵ_{267} being 11,500; and for d-pT, the value used for ϵ_{250} is 5700, ϵ_{267} being 9600.

The ϵ_{max} values used are 9600 for pT-OAc at 267 mμ and 12,200 for pI-OAc at 250 mμ. The abbreviations $O.D._{267}$ and $O.D._{250}$ refer to the extinction of the nucleotidic solution 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.

Removal of Protecting Group. The dimethoxytrityl group was removed by treating the lyophilized product (ammonium or pyridine salt) with an excess of 80% acetic acid for 15 min. at room temperature. The acetic acid was then removed by evaporation at room temperature *in vacuo* followed by an ether wash of the residue.

Anhydrous reaction mixtures or components thereof were prepared by the following standard treatment. A solution of the components in pyridine or aqueous pyridine was evaporated *in vacuo* using a Dry Ice–acetone trap. Fresh dry pyridine was added to dissolve the residue and the evaporation was repeated. The whole procedure was repeated several times, the reaction

Table III. R_f Values of Homologous Compounds on Paper Chromatography

Compound[b]	Solvent A	Solvent C	Solvent D
Pyridinium deoxyinosine 5′-phosphate			0.40
Pyridinium 3′-O-acetyldeoxyinosine 5′-phosphate			0.64
DMTr-TpT	0.78		
DMTr-TpTpI	0.51		
DMTr-TpTpIpT	0.30	1.70[a]	
DMTr-TpTpIpTpT	0.14	1.54[a]	
DMTr-TpTpIpTpTpI	0.07	1.40[a]	
DMTr-TpTpIpTpTpIpT		1.13[a]	
DMTr-TpTpIpTpTpIpTpT		0.93[a]	
DMTr-TpTpIpTpTpIpTpTpI		0.82[a]	
DMTr-TpTpIpTpTpIpTpTpIpT		0.54[a]	
DMTr-TpTpIpTpTpIpTpTpIpTpT		0.39[a]	
DMTr-TpTpIpTpTpIpTpTpIpTpTpI		0.23[a]	
TpTpI	0.256	1.17[a]	
TpTpIpT	0.21	0.87[a]	
TpTpIpTpT		0.76[a]	
TpTpIpTpTpI		0.59[a]	
TpTpIpTpTpIpT		0.43[a]	
TpTpIpTpTpIpTpT		0.37[a]	
TpTpIpTpTpIpTpTpI		0.28[a]	
TpTpIpTpTpIpTpTpIpT		0.14[a]	
TpTpIpTpTpIpTpTpIpTpT		0.11[a]	
TpTpIpTpTpIpTpTpIpTpTpI		0.092[a]	

[a] R_f relative to that of pT. [b] Pyridinium or ammonium salt of the homologous compounds were used for paper chromatography.

through a column (40 × 3 cm.) of pyridinium Dowex 50 ion-exchange resin. The column was washed with a total of 1 l. of 10% aqueous pyridine. The total aqueous pyridine effluent was concentrated to about 500 ml. *in vacuo* and this solution was lyophilized, the process of dissolution in water and lyophilization being repeated twice. Pyridinium deoxyinosine 5′-phosphate was thus obtained as a finely divided powder.

Pyridinium 3′-O-Acetyldeoxyinosine 5′-Phosphate. Redistilled acetic anhydride (17.5 ml.) was added to a suspension of pyridinium deoxyinosine 5′-phosphate (6.0 mmoles, 73,200 O.D.$_{250}$ units) in 50 ml. of dry pyridine. The sealed flask was shaken for 1 hr. until a homogeneous solution resulted, and the latter was kept at room temperature for 6 hr. in the dark. Water (280 ml.) was next added and the solution was kept for another 2 hr. The solution was now evaporated under reduced pressure and the residue was dissolved in dry pyridine (50 ml.). The desired acetylated product was isolated by adding the pyridine solution dropwise to an excess of dry ether (1 l.) with shaking. The resulting finely dispersed powder was collected by centrifugation and washed with ether (six 200-ml. portions) to ensure completely the removal of pyridinium acetate. The nucleotide was finally stored as a solution in pyridine containing 10% water in a 50-ml. flask. The

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

Compound	Products, O.D.$_{267}$ d-Ip	d-Tp	d-I	T		Ratio Theor.	Found[c]
d-TpTpIpT	0.42	1.60	..	0.79	Tp:d-Ip:T	2:1:1	2.02:1:1
d-TpTpIpTpTpIpT	0.61	2.40	..	0.60	Tp:d-Ip:T	4:2:1	3.94:1.94:1
d-TpTpIpTpTpIpTpTpI	1.02	6.00	0.50	..	Tp:d-Ip:d-I	6:2:1	6.04:2:1

[a] Degradation of the compounds with the phosphodiesterase was carried out as described previously.[12] The products were separated by chromatography in solvent E.[b] Their concentrations were determined by elution with water and spectrophotometric analysis at pH 7.1 using appropriate blanks. [b] R_f values: d-Ip, 0.21; Tp, 0.36; T, 0.81; d-I, 0.60. [c] For calculation of the molecular amounts of the products the value of $\epsilon_{267m\mu}$ used for Tp and T is 9600; the value used for d-I and d-Ip at the same wave length is 5100.

vessel being opened to the dry atmosphere of a large desiccator containing phosphorus pentoxide each time.

Pyridinium Deoxyinosine 5′-Phosphate. An aqueous solution (50 ml.) of diammonium deoxyadenosine 5′-phosphate (2.50 g., 6.70 mmoles) was added to a mixed solution of sodium nitrite (50 ml. of 2.5 *M*) and sodium acetate (50 ml. of 0.6 *M*, pH 4.20), and the resulting clear solution was kept at 37° for 4 hr. The reaction was terminated by raising the pH of the solution to 7.0 by adding 2 *N* ammonium hydroxide, and the solution was diluted to 600 ml. with water. This solution was applied on the top of a column (35 × 5 cm.) of Dowex 1 X8 ion-exchange resin (acetate form) at room temperature. A small amount of adenine and hypoxanthine (500 O.D.$_{250}$ units) was first eluted with 1 l. of 0.05 *M* ammonium acetate (pH 3.8). Subsequent elution with 1 l. of 0.2 *M* ammonium acetate (pH 3.8) first removed the unreacted deoxyadenosine 5′-phosphate (500 O.D.$_{260}$ units) and then elution with 1 l. more of the same buffer gave deoxyinosine 5′-phosphate (74,000 O.D.$_{250}$ units, 92%). For isolation of the product, the pooled fraction (1 l.) was first evaporated to about 100 ml., pyridine (100 ml.) was then added, and the total solution was passed

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

yield of the acetylated product as determined spectrophotometrically (71,000 O.D.$_{250}$ units) was quantitative.

5′-O-Di-p-methoxytritylthymidylyl-(3′→5′)-thymidine. An anhydrous pyridine solution (5 ml.) of 5′-O-di-*p*-methoxytritylthymidine (1.0 mmole), pyridinium 3′-O-acetylthymidine 5′-phosphate (1.1 mmoles), and pyridinium Dowex 50 ion-exchange resin (500 mg.) was treated with DCC (500 mg.), and the sealed mixture was shaken for 4 days at room temperature. Water (1.5 ml.) was then added and the mixture was kept further for a day at room temperature. The reaction mixture was filtered through glass wool, the solid being washed with 50% aqueous pyridine (three 5-ml. portions), and the total filtrate was extracted with ether three times (20 ml. each). Paper chromatography in solvent A at this stage showed that the extraction of unreacted dimethoxytritylthymidine was complete and that the ether extract contained only the latter compound. The aqueous pyridine solution was next concentrated to 5-ml. in the presence of an excess of pyridine, and the solution was diluted with chloroform (60 ml.). The chloroform–pyridine solution was washed carefully with 0.1 *M* triethylammonium bicarbonate (pH 7.8, five 30-ml. portions) and finally with water (15 ml.). The chloroform solution was evap-

orated *in vacuo* after addition of an excess of pyridine and the residue was dissolved in pyridine (10 ml.). Ammonium hydroxide (9 *N*, 5 ml.) was added and after 24 hr. at room temperature the solution was concentrated *in vacuo* in the presence of an excess of pyridine. The pyridine solution was passed through a column (20 × 2 cm.) of Dowex 50 ion-exchange resin (pyridinium form) and the column was washed with 40% aqueous pyridine. The total aqueous pyridine effluent was concentrated in the presence of pyridine and the residue was dissolved in 10 ml. of anhydrous pyridine. The solution was added dropwise to an excess (200 ml.) of dry ether and the precipitate of pyridinium DMTr-TpT was collected by centrifugation. For use in the condensations, the total product was dissolved in 10 ml. of dry pyridine and the concentration of this stock solution was determined by removing aliquots, evaporating the latter repeatedly with added ammonia, and making the final residue to a known volume with aqueous buffer (pH 7.5) for spectrophotometric analysis. The yield of pyridinium DMTrTpT was thus determined to be 16,400 $O.D._{267}$ units (80%).

5'-O-Dimethoxytritylthymidylylthymidylyldeoxyinosine. (A) Using DCC. A mixture of pyridinium 5'-O-di-*p*-methoxytritylthymidylylthymidine (0.128 mmole, 2700 $O.D._{267}$ units), pyridinium 3'-O-acetyldeoxyinosine 5'-phosphate (0.40 mmole), pyridinium Dowex 50 ion-exchange resin (400 mg.), and DCC (400 mg., 1.9 mmoles) in anhydrous pyridine (4 ml.) and dimethylformamide (1.2 ml.) was shaken at room temperature for 4 days in the dark. Aqueous pyridine (2 ml. of 50%) was then added and the reaction mixture was filtered through glass wool, the solid being washed with 50% aqueous pyridine (two 2-ml. portions). The total filtrate was kept overnight at room temperature and then extracted with petroleum ether (b.p. 30–60°, two 5-ml. portions). Sodium hydroxide (2 *N*, 1.5 ml.) was added and after 1 hr. at room temperature an excess of pyridinium Dowex 50 ion-exchange resin was added to remove sodium ions. The resin was removed by filtration and the clear solution and the resin wash were applied on top of a column (40 × 2.5 cm.) of DEAE–cellulose (carbonate form) pre-equilibrated with 0.02 *M* ammonium bicarbonate in 20% ethyl alcohol. Elution was carried out using a linear gradient of ammonium bicarbonate (0.02–0.2 *M*, 2 l. each of the solution in 20% ethyl alcohol in the mixing vessel and reservoir, respectively). Fractions of about 10 ml. were collected at a flow rate of 1 ml./min., and the elution was followed spectrophotometrically at 250 mμ. The desired d-DMTr-TpTpI was eluted in fractions 310–356 at a salt concentration of about 0.19 *M*. The pooled fractions (2250 $O.D._{267}$ units, 70%) were concentrated at room temperature *in vacuo* in the presence of a few drops of 1-octanol to prevent foaming. Lyophilization of the concentrated solution yielded a finely dispersed powder. This was dissolved in 50% aqueous pyridine (5 ml.) and passed through a freshly prepared column (20 × 2 cm.) of pyridinium Dowex 50 ion-exchange resin followed by an elution with 200 ml. of 50% aqueous pyridine. The total aqueous pyridine effluent was evaporated at low temperature *in vacuo* in an excess of pyridine. The product was stored at low temperature as its pyridine solution.

(B) Using Mesitylenesulfonyl Chloride. To an anhydrous solution of pyridinium 3'-O-acetyldeoxyinosine 5'-phosphate (1.5 mmoles), pyridinium 5'-O-di-*p*-methoxythymidylylthymidine (0.427 mmole, 9000 $O.D._{267}$ units), and tri-*n*-hexylamine (3.0 mmoles, 1.11 ml.) in dry pyridine (5 ml.) was added slowly in the cold mesitylenesulfonyl chloride (3.5 mmoles, 780 mg.). After 15 min., the dark brown reaction mixture was kept at room temperature for 2.5 hr. Water (1.5 ml.) was then added under cooling and the resulting solution was allowed to stand for 1 hr. Concentrated ammonia (4 ml.) was added and after 24 hr. the ammonia was removed by evaporation, pyridine being added toward the end of evaporation. The reaction mixture was next diluted with 0.02 *M* ammonium bicarbonate in 20% ethyl alcohol to 500 ml., and this solution was applied on the top of a column (55 × 4.5 cm.) of DEAE–cellulose (carbonate form). Elution was first carried out by using a linear gradient of ammonium bicarbonate solution in 20% ethyl alcohol with 4 l. of 0.02 *M* salt in the mixing vessel and an equal volume of 0.2 *M* salt in the reservoir. Elution was then continued with the linear gradient, 0.2–0.3 *M* containing 20% ethyl alcohol, there being 2 l. of each solution in the mixing vessel and reservoir. Fractions, 10 ml. each, were collected at every 15-min. interval and elution was followed spectrophotometrically at 250 mμ. The main product was eluted in fractions 770–830 at a salt concentration of about 0.25 *M*. The total yield of the d-DMTr-TpTpI was 8400 $O.D._{267}$ units (78%). The product was isolated as its pyridinium salt by the procedure described above and stored as its solution in pyridine.

The Tetranucleotide d-DMTr-TpTpIpT. (A) Using DCC. An anhydrous mixture of pyridinium d-DMTr-TpTpI (1960 $O.D._{267}$ units, 0.082 mmole), pyridinium 3'-O-acetylthymidine 5'-phosphate (0.50 mmole), and pyridinium Dowex 50 ion-exchange resin (500 mg.) in 3 ml. of dry pyridine was treated with DCC (0.500 mg.), and the mixture was shaken for 3 days at room temperature. After the usual work-up, the product was chromatographed on DEAE–cellulose (carbonate) column (40 × 2.5 cm.). Elution was carried out using a linear gradient of ammonium bicarbonate (0.02–0.3 *M* in 20% ethyl alcohol, 2 l. each of the solution being in the mixing vessel and in the reservoir, respectively). Fractions of about 10 ml. were collected at a flow rate of 1 ml./min. The desired d-DMTr-TpTpIpT (1500 $O.D._{267}$ units, 63%) was eluted in fractions 220–280 at a salt concentration of about 0.25 *M*, and the starting material d-DMTr-TpTpI was recovered from fractions 162–180 (200 $O.D._{267}$ units). The products were isolated by the methods described above.

(B) Using Mesitylenesulfonyl Chloride. To an anhydrous solution of pyridinium 3'-O-acetylthymidine 5'-phosphate (0.6 mmole) and pyridinium d-DMTr-TpTpI (0.105 mmole, 2500 $O.D._{267}$ units) in 3 ml. of dry pyridine was added slowly in the cold mesitylenesulfonyl chloride (1.4 mmoles, 308 mg.). After 15 min. the sealed reaction mixture was kept at room temperature for 2.5 hr. Water (1.5 ml.) was then added and after the usual work-up the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column (40 × 2.5 cm.). Elution was carried out by using a linear gradient (0.02–0.35 *M* of ammonium

bicarbonate containing 30% ethyl alcohol, fractions of 10 ml./15 min. being collected). The desired tetranucleotide d-DMTr-TpTpIpT (2270 O.D.$_{267}$ units, 67%) was present in fractions 326–370.

The Pentanucleotide d-DMTr-TpTpIpTpT. (*A*) *Using DCC.* An anhydrous mixture of pyridinium d-DMTrTpTpIpT (1400 O.D.$_{267}$ units, 0.0391 mmole), pyridinium 3′-O-acetylthymidine 5′-phosphate (0.60 mmole), and pyridinium Dowex 50 ion-exchange resin (300 mg.) in dry pyridine (3 ml.) was shaken with DCC (500 mg.) for 3 days at room temperature. After usual work-up, the reaction mixture was chromatographed on a DEAE-cellulose (carbonate) column (40 × 2.5 cm.). Elution was begun by using a linear gradient of ammonium bicarbonate (0.02-0.3 *M* in 20% ethyl alcohol, with 2 l. each of the salt solution in mixing vessel and the reservoir, respectively). This was followed by elution with another gradient of the same salt (0.3–0.4 *M*, 1 l.) of each of the solutions. Fractions of about 11 ml. were collected at a flow rate of 1 ml./min. The desired protected pentanucleotide (1000 O.D.$_{267}$ units, 66%) was eluted in fractions 324–344 at a salt concentration of about 0.35 *M*, being preceded by a peak of the starting protecting tetranucleotide (200 O.D.$_{267}$ units) in fractions 300–316.

(*B*) *Using Mesitylenesulfonyl Chloride.* To an anhydrous solution of pyridinium d-DMTr-TpTpIpT (2000 O.D.$_{267}$ units, 0.056 mmole), pyridinium 3′-O-acetylthymidine 5′-phosphate (1.0 mmole) in dry pyridine (3 ml.) was added in the cold mesitylenesulfonyl chloride (500 mg.) under anhydrous condition and the sealed reaction mixture was kept for 2.5 hr. at room temperature. After usual work-up, the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column (83 × 3 cm.). Elution was carried out by using a linear gradient, 0.05–0.3 *M*, 2 l. each, of ammonium bicarbonate containing 40% ethyl alcohol, followed by the next gradient, 0.30–0.35 *M*, 1 l. each, of the same salt solution in 40% ethyl alcohol, fractions of about 10 ml. being collected at intervals of 15 min. The pentanucleotide d-DMTr-TpTpIpTpT (1600 O.D.$_{267}$ units, 74%) was present in fractions 425–460, 300 O.D.$_{267}$ units of the starting material being recovered from the preceding peak.

The Hexanucleotide d-DMTr-TpTpIpTpTpI. (*A*) *Using DCC.* To an anhydrous mixture of pyridinium d-DMTr-TpTpIpTpT (900 O.D.$_{267}$ units, 0.02 mmole), pyridinium 3′-O-acetyldeoxyinosine 5′-phosphate (0.40 mmole), and pyridinium Dowex 50 ion-exchange resin (300 mg.) in dimethylformamide (1 ml.) and pyridine (2.5 ml.) was added DCC (500 mg.), and the reaction mixture was shaken for 3 days at room temperature. After usual work-up, the product was chromatographed on a DEAE-cellulose (carbonate) column (40 × 2.5 cm.). Elution was carried out using a linear gradient of ammonium bicarbonate (0.02–0.2 *M*, 500 ml. of each containing 20% ethyl alcohol) followed by another gradient of ammonium bicarbonate (0.2–0.5 *M*, 2 l. of each of the solutions in 20% ethyl alcohol). Fractions of 12 ml. were collected at intervals of 15 min. The hexanucleotide d-DMTr-TpTpIpTpTpI (420 O.D.$_{267}$ units, 52%) was eluted in fractions 240–320 at a salt concentration of about 0.40 *M*; unreacted starting material, d-DMTr-TpTpIpTpT, was recovered from fractions 160–200 (160 O.D.$_{267}$ units).

(*B*) *Using Mesitylenesulfonyl Chloride.* To an anhydrous solution of pyridinium d-DMTr-TpTpIpTpT (1200 O.D.$_{267}$ units, 0.026 mmole) and tri-*n*-hexylammonium 3′-O-acetyldeoxyinosine 5′-phosphate (0.80 mmole) in dry pyridine (3 ml.) was added in the cold mesitylenesulfonyl chloride (400 mg.). After 15 min. a gum separated out and the reaction mixture was kept for another 2 hr. After usual work-up, the reaction mixture was chromatographed on a DEAE–cellulose column. The conditions used and the elution pattern obtained are shown in Figure 1. The yield of the isolated hexanucleotide was 1000 O.D.$_{267}$ units (76%).

The Heptanucleotide d-DMTr-TpTpIpTpTpIpT. (*A*) *Using DCC.* To an anhydrous mixture of pyridinium d-DMTr-TpTpIpTpTpI (380 O.D.$_{267}$ units, 0.0075 mmole), pyridinium 3′-O-acetylthymidine 5′-phosphate (0.50 mmole), and pyridinium Dowex 50 ion-exchange resin (300 mg.) in 2 ml. of pyridine was added DCC (0.32 g.), and the reaction mixture was shaken for 3 days at room temperature. After usual work-up the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column (40 × 2.5 cm.). Elution was carried out by using a linear gradient of ammonium bicarbonate (0.02–0.2 *M*, 500 ml. each of the solution containing 20% ethyl alcohol) followed by the next gradient (0.3–0.5 *M*, 1 l. each of the solution containing 20% ethyl alcohol). Fractions of about 10 ml. were collected at intervals of 15 min. The heptanucleotide d-DMTr-TpTpIpTpTpIpT (250 O.D.$_{267}$ units, 65%) was eluted in fractions 206–280 at a salt concentration of 0.45 *M*.

(*B*) *Using Mesitylenesulfonyl Chloride.* To an anhydrous solution of pyridinium d-DMTr-TpTpIpTpTpI (450 O.D.$_{267}$ units, 0.0089 mmole), pyridinium 3′-O-acetylthymidine 5′-phosphate (0.80 mmole) in dry pyridine (1.5 ml.) was added in the cold mesitylenesulfonyl chloride (440 mg.). The sealed reaction mixture was kept for 3 hr. at room temperature. After the usual work-up the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column. The conditions used and the elution pattern obtained are shown in Figure 2. The heptanucleotide d-DMTr-TpTpIpTpTpIpT (300 O.D.$_{267}$ units, 70%) was obtained from fractions 634–660, 90 O.D.$_{267}$ units of the starting material being recovered from the preceding peak.

The Octanucleotide d-DMTr-TpTpIpTpTpIpTpT. To an anhydrous solution of pyridinium d-DMTr-TpTpIpTpTpIpT (0.00415 mmole, 250 O.D.$_{267}$ units) and pyridinium 3′-O-acetylthymidine 5′-phosphate (0.50 mmole) in dry pyridine (1.5 ml.) was added in the cold mesitylenesulfonyl chloride (300 mg.). The sealed reaction mixture was kept for 3 hr. After the usual work-up the reaction mixture was chromatographed on a DEAE–cellulose column. The condition used and the elution pattern obtained are shown in Figure 3. The octanucleotide d-DMTr-TpTpIpTpTpIpTpT (136 O.D.$_{267}$ units) was present in fractions 578–600, 105 O.D.$_{267}$ units of the starting material being recovered from the preceding peak.

The Nonanucleotide d-DMTr-TpTpIpTpTpIpTpTpI. To an anhydrous solution of pyridinium d-DMTrTpTpIpTpTpIpTpT (0.00688 mmole, 480 O.D.$_{267}$ units), and the tri-*n*-hexylammonium salt of 3′-O-acetyldeoxy-

inosine 5′-phosphate (1.0 mmole) in dry pyridine (2 ml.) was added mesitylenesulfonyl chloride (440 mg.). The sealed reaction mixture was shaken for 2.5 hr. at room temperature. After the usual work-up, the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column. The condition used and the elution pattern obtained are shown in Figure 4. The nonanucleotide d-DMTr-TpTpIpTpTpIpTpTpI (444 O.D.$_{267}$ units, 85%) was obtained from fractions 328–378.

The Decanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpT. To an anhydrous solution of the tri-*n*-hexylammonium salt of d-DMTr-TpTpIpTpTpIpTpTpI (350 O.D.$_{267}$ units, 0.0048 mmole) and pyridinium 3′-O-acetylthymidine 5′-phosphate (1.0 mmole) in dry pyridine (1.5 ml.) was added mesitylenesulfonyl chloride (440 mg.). The sealed reaction mixture was shaken for 3.5 hr. After the usual work-up, the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column. The conditions used and the elution pattern obtained are shown in Figure 5. The decanucleotide d-DMTr-TpTpIpTpTpIpTpTpIpT was isolated from fractions 500–530. The yield was 280 O.D.$_{267}$ units (70%).

The Undecanucleotide d-DMTr-TpTpIpTpTpIpTpTp IpTpT. To an anhydrous solution of tri-*n*-hexylammonium d-DMTr-TpTpIpTpTpIpTpTpIpT (250 O.D.$_{267}$ units, 0.003 mmole) and pyridinium 3′-O-acetylthymidine 5′-phosphate (1.0 mmole) in dry pyridine (1.5 ml.) was added mesitylenesulfonyl chloride (440 mg.). The sealed reaction mixture was shaken for 3.5 hr. at room temperature. After usual work-up, the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column. The conditions used and the elution pattern obtained are shown in Figure 6. The undecanucleotide d-DMTr-TpTpIpTpTpIpTpTp-IpTpT (220 O.D.$_{267}$ units, 78%) was isolated from fractions 549–570.

The Dodecanucleotide d-DMTr-TpTpIpTpTpIpTpTp-IpTpTpI. To an anhydrous solution of the tri-*n*-hexylammonium salt of d-DMTr-TpTpIpTpTpIpTpTpIpTpT (200 O.D.$_{267}$ units, 0.0021 mmole) and the tri-*n*-hexylammonium salt of 3′-O-acetyldeoxyinosine 5′-phosphate (1.0 mmole) in dry pyridine (1.5 ml.) was added mesitylenesulfonyl chloride (440 mg.). The sealed reaction mixture was shaken for 3.5 hr. In the work-up, the duration of ammoniacal treatment was prolonged to 48 hr. at room temperature. One-half of the reaction mixture was chromatographed on a DEAE–cellulose (carbonate) column. The conditions used and the elution pattern obtained are shown in Figure 7. The dodecanucleotide d-DMTr-TpTpIp-TpTpIpTpTpIpTpTpI (73 O.D.$_{267}$ units, 70%) was isolated from fractions 458–498.

Studies on Polynucleotides. XLVI.[1] The Synthesis of Hexanucleotides Containing the Repeating Trinucleotide Sequences Deoxycytidylyl-(3′→5′)-deoxyadenylyl-(3′→5′)-deoxyadenosine and Deoxyguanylyl-(3′→5′)-deoxyadenylyl-(3′→5′)-deoxyadenosine[2]

S. A. Narang, T. M. Jacob, and H. G. Khorana

Contribution from the Institute for Enzyme Research of the University of Wisconsin, Madison, Wisconsin. *Received March 22, 1965*

The syntheses of the hexanucleotides containing the repeating trinucleotide sequences deoxyguanylyl-(3′→5′)-deoxyadenylyl-(3′→5′)-deoxyadenosine and deoxycytidylyl-(3′→5′)-deoxyadenylyl-(3′→5′)-deoxyadenosine are described. The synthetic approach involved the stepwise condensation of a suitably protected mononucleotide to the 3′-hydroxyl end group of a growing oligonucleotide chain. The protected nucleosides used as starting materials were 5′-O-monomethoxytrityl N-acetyldeoxyguanosine and 5′-O-monomethoxytrityl-N-anisoyldeoxycytidine; the protected mononucleotides used as starting materials were N-anisoyl-3′-O-acetyldeoxycytidine 5′-phosphate, N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate, and N,3′-O-diacetyldeoxyguanosine 5′-phosphate. The condensing agent used was dicyclohexylcarbodiimide. After each condensation step, the terminal 3′-O-acetyl group was removed from the protected oligonucleotide by a brief alkaline treatment and the latter was purified by chromatography on a DEAE–cellulose column in the acetate or bicarbonate form. All of the oligonucleotides, up to the hexanucleotide in both series, have been isolated pure and characterized.

In two accompanying papers[1,3] syntheses of the dodecanucleotides containing the repeating trinucleotide sequences thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-

(1) Paper XLV: S. A. Narang and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 2981 (1965).

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(3) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 2971 (1965).