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′)-

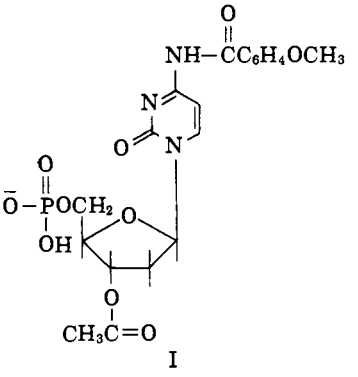
(1) Paper XLV: S. A. Narang and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 2981 (1965).

(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).

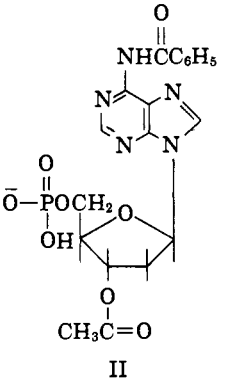
(3) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 2971 (1965).

deoxycytidine and thymidylyl-(3′→5′)-thymidylyl-(3′→5′)-deoxyinosine have been recorded. The present paper describes extension of these studies to the synthesis of the hexanucleotides containing the repeating trinucleotide sequences deoxycytidylyl-(3′→5′)-deoxyadenylyl-(3′→5′)-deoxyadenosine and deoxyguanylyl-(3′→5′)-deoxyadenylyl-(3′→5′)-deoxyadenosine. While the over-all objectives of the synthetic work contained in the present series of papers[1,3,4] have been described separately,[5] two additional considerations, which determined the selection of the nucleotide sequences now put together, were as follows. First, from the chemical standpoint, the present syntheses were expected to further the suitability of the various protecting groups, since all of the nucleosides and mononucleotide derivatives used as intermediates required full protection. Second, the nucleotide sequences now chosen are complementary, in the Watson–Crick base-pairing sense, to those whose syntheses have been described in the preceding papers, and work with the DNA polymerase has so far necessitated the use of deoxyribopolynucleotides containing short segments of both of the complementary strands.[6,7] Thus, the DNA polymerase catalyzed synthesis of DNA-like polymer containing alternating thymidylate and deoxyguanylate units in one strand and alternating deoxycytidylate and deoxyadenylate units in the complementary strand requires as templates a mixture of the deoxyribooligonucleotides containing both types of repeating dinucleotide sequences.[7] In the hope of preparing DNA-like polymers containing repeating trinucleotide sequences, it was, therefore, considered necessary to have available sets of synthetic deoxyribopolynucleotides containing complementary trinucleotide sequences.

The general approach used in the present work is the same as that used in the two preceding papers[1,8] and involves the stepwise synthesis of an oligonucleotide chain by the repetitive condensation of a protected mononucleotide with the 3′-hydroxyl end group of a growing oligonucleotide chain (starting with a protected nucleoside bearing a 3′-hydroxyl group). The protected mononucleotides used as starting materials were N-anisoyl-3′-O-acetyldeoxycytidine 5′-phosphate (I, d-pC^{An}-OAc),[8] N-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate (II, d-pA^{Bz}-OAc),[8] and N,3′-O-diacetyldeoxyguanosine 5′-phosphate (III, d-pG^{Ac}-OAc).[8] Methods for their preparation have been described previously[9–11]; the chief modification now used involved the careful precipitation of the pyridinium salts from pyridine–ether mixtures shortly before use in condensation reactions. The protected nucleosides used were 5′-O-monomethoxytrityl-N-acetyldeoxyguanosine (IV, d-MMTr-G^{Ac})[8] and 5′-O-



I



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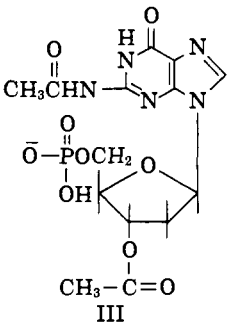
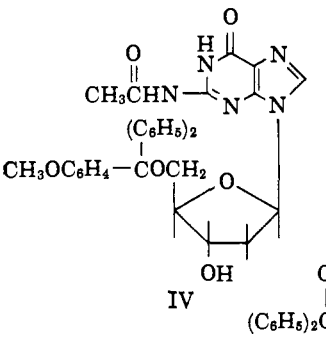
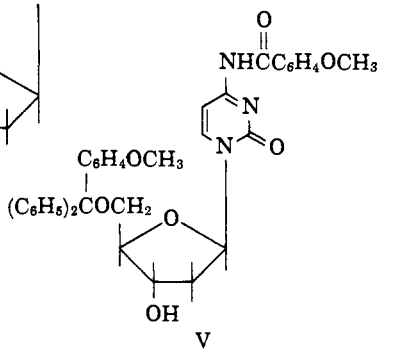


III

monomethoxytrityl-N-anisoyldeoxycytidine (V, d-MMTr-C^{An}).[8] The starting material for the preparation of IV was N,O$^{3'}$,O$^{5'}$-triacetyldeoxyguanosine which was prepared by the prolonged treatment of deoxyguanosine with an excess of acetic anhydride–pyridine mixture in the presence of tetraethylammonium hydroxide.[12] Selective alkaline hydrolysis of the ester groups in the triacetyldeoxyguanosine gave N-acetyldeoxyguanosine which was converted to IV by reaction with monomethoxytrityl chloride. 5′-O-Monomethoxytrityl-N-anisoyldeoxycytidine (V) was prepared similarly from N-anisoyldeoxycytidine.[13]



IV



V

The condensing agent used in all the present work has been dicyclohexylcarbodiimide (DCC). All of the protected starting materials and the synthetic intermediates were soluble as pyridinium salts in dry pyridine which was throughout the medium of reaction. While aromatic sulfonyl chlorides are also powerful condensing agents,[14] DCC remains still the

(4) E. Ohtsuka, M. W. Moon, and H. G. Khorana, *ibid.*, **87**, 2956 (1965).

(5) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *ibid.*, **87**, 2954 (1965).

(6) C. Byrd, E. Ohtsuka, M. W. Moon, and H. G. Khorana, *Proc. Natl. Acad. Sci. U. S.*, **53**, 79 (1965).

(7) R. D. Wells, E. Ohtsuka, and H. G. Khorana, *J. Mol. Biol.*, in press.

(8) The system of abbreviations is as has been defined in an accompanying paper.[4]

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

(10) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3835 (1963).

(11) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963).

(12) This procedure which results in the complete acetylation of the amino group was developed in this laboratory by Dr. H. Schaller.

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

(14) T. M. Jacob and H. G. Khorana, *ibid.*, **86**, 1630 (1964).

Table I. Stepwise Synthesis of Deoxyribooligonucleotides Containing the Repeating Sequence Guanylyladenylyladenosine and Cytidylyladenylyladenosine

Oligonucleotide component	Mono-nucleotide	Mono-nucleotide, molar excess	Product	Yield, %
d-MMTr-G^{Ac}	pA^{Bz}-OAc	0.6	d-MMTr-$G^{Ac}pA^{Bz}$	60
d-MMTr-$G^{Ac}pA^{Bz}$	pA^{Bz}-OAc	2.3	d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}$	56
d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}$	pG^{Ac}-OAc	16	d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}$	54
d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}$	pA^{Bz}-OAc	19	d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}$	31
d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}$	pA^{Bz}-OAc	65	d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}pA^{Bz}$	23
d-MMTr-C^{An}	pA^{Bz}-OAc	None	d-MMTr-$C^{An}pA^{Bz}$	80
d-MMTr-$C^{An}pA^{Bz}$	pA^{Bz}-OAc	7	d-MMTr-$C^{An}pA^{Bz}pA^{Bz}$	70
d-MMTr-$C^{An}pA^{Bz}pA^{Bz}$	pC^{An}-OAc	14	d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}$	73
d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}$	pA^{Bz}-OAc	25	d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz}$	70
d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz}$	pA^{Bz}-OAc	62	d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz}pA^{Bz}$	66

safest reagent as it can be used in large excess without any danger.[15] After each condensation step, an alkaline treatment was given to remove selectively the 3′-O-acetyl group. As before,[1,3,16] an increasing excess of the mononucleotidic component was used with increasing chain length of the oligonucleotidic component (Table I).

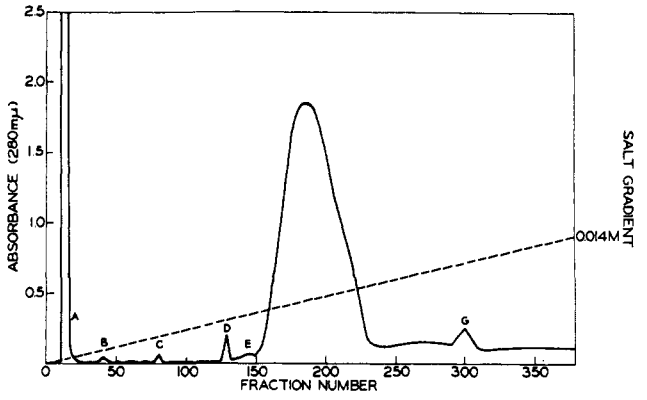


Figure 1. Chromatography of the reaction products in the preparation of the dinucleotide d-MMTr-$C^{An}pA^{Bz}$ on a DEAE-cellulose (carbonate) column (3 × 40 cm.). For elution by using linear gradient, the mixing vessel contained 2 l. of methyl alcohol and the reservoir contained 24 ml. of 1 *M* triethylammonium bicarbonate (pH 7.5) in 2 l. of methyl alcohol for elution. Peak F contains the dinucleotide d-MMTr-$C^{An}pA^{Bz}$; salt gradient, ---------.

Two types of procedures were used for isolation of the condensation products bearing the sensitive protecting groups. In the series d-MMTr-$C^{An}pA^{Bz}$ and homologs, DEAE-cellulose anion-exchanger columns in the carbonate form were used. The eluent was ethanolic triethylammonium bicarbonate and chromatography was routinely performed at about 4°. With appropriate choice of the proportion of ethyl alcohol in the eluent and of the salt gradient, satisfactory separations were obtained at every step, the elution patterns obtained being shown in Figures 1–5. Neither during chromatography nor in subsequent removal of the buffer was any loss of the protecting groups observed. The second procedure, which was used for d-MMTr-$G^{Ac}pA^{Bz}$ and homologous members, involved the use of DEAE-cellulose columns in the acetate form; the eluent was aqueous ethanolic triethylammonium acetate (pH 6.5) and chromatography was performed at room temperature (Figures 6–8). No detectable loss of any protecting group occurred during chromatography but during the subsequent isolation of the products, despite care,[17] detectable loss of the monomethoxytrityl group sometimes was observed.[18]

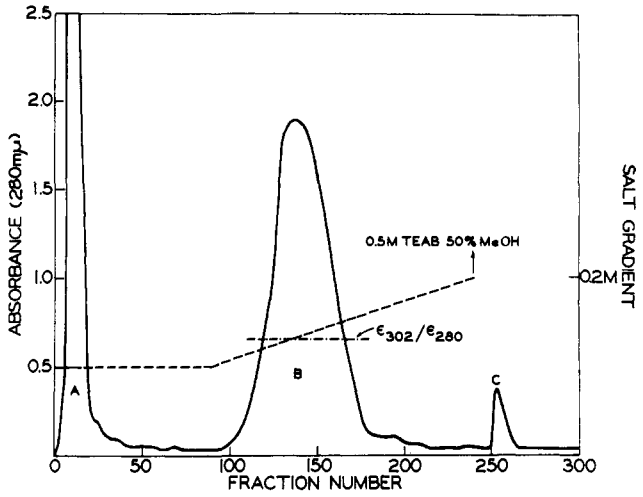


Figure 2. Chromatography of the reaction products in the preparation of trinucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}$; on DEAE-cellulose (carbonate) column (1 × 55 cm.), first eluting with 0.1 *M* triethylammonium bicarbonate (500 ml.) containing 15% methyl alcohol and then by using the linear gradient of 0.1–0.2 *M* triethylammonium bicarbonate, 500 ml. each of the solution containing 60% methanol. Peak B contains the trinucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}$; $\epsilon_{302}/\epsilon_{280}$, -·-·-·; salt gradient, ---------.

As seen in Table I, the yields in the series d-CpApA and homologs were in the range of 65–80% throughout; however, in the series d-GpApA and homologs reduced yields were obtained at the last two steps. All of the synthetic products were characterized in a variety of ways. In addition to the expected position of elution from columns and the characteristic spectrophoto-

(15) Furthermore, at the dinucleotide stage (*e.g.*, d-MMTr-$C^{An}pA^{Bz}$) the use of aromatic sulfonyl chloride has the disadvantage that the sulfonate salt formed as a by-product coincides in its elution position with the desired product.

(16) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 368 (1965).

(17) Thus, in order to prevent lowering of the pH during evaporation some tri-*n*-butylamine was routinely added to the combined eluate.

(18) From the total of experience, the use of triethylammonium bicarbonate–alcohol mixture as eluent at low temperature is preferred to the triethylammonium acetate.

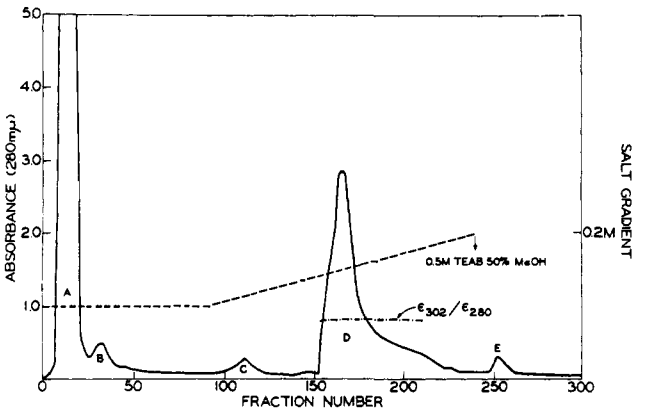


Figure 3. Chromatography of the reaction products in the preparation of tetranucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}$ on a DEAE-cellulose (carbonate) column (1 × 55 cm.) first eluting with 0.1 *M* triethylammonium bicarbonate (500 ml.) containing 15% methyl alcohol and the by using a linear gradient of 0.1–0.2 *M* triethylammonium bicarbonate (500 ml.) each of the solution containing 60% methyl alcohol. Peak D contains the trinucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}$; $\epsilon_{302}/\epsilon_{280}$, -·-·-·; salt gradient, ---------.

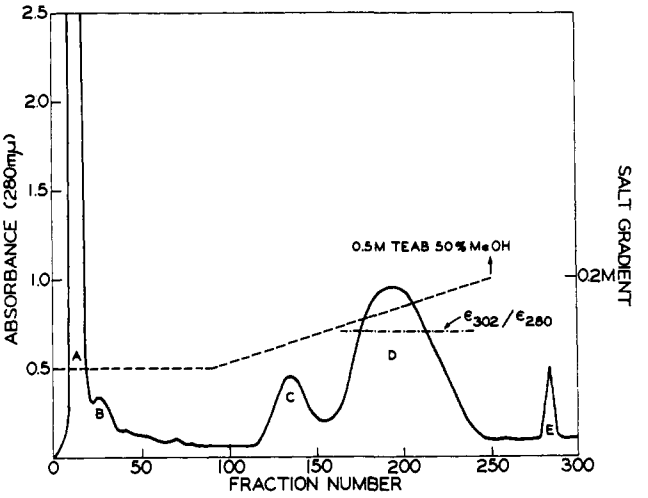


Figure 4. Chromatography of the reaction products in the preparation of pentanucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz}$ on a DEAE-cellulose (carbonate) column (1 × 55 cm.), first eluting with 0.1 *M* triethylammonium bicarbonate (500 ml.) containing 15% methyl alcohol and then by using a linear gradient of 0.1–0.3 *M* triethylammonium bicarbonate (500 ml.) each of the solution containing 60% methyl alcohol. Peak D contains the pentanucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz}$; $\epsilon_{302}/\epsilon_{280}$, -·-·-·; salt gradient, ---------.

metric properties, the homologous compounds were homogeneous by paper chromatography (R_f values in Tables II and III). It should be noted that the removal of all of the protecting groups by successive treatments with ammonia and then aqueous acetic acid (1 hr. at room temperature) was unattended by the formation of any detectable side products, the resulting oligonucleotides being again homogeneous by paper chromatography. Finally, the hexanucleotides d-CpApApCpApA and d-GpApApGpApA were further characterized by degradation with the spleen phosphodiesterase.

The cause of the low yield and of the formation of considerable amounts of side products in the synthesis of, for example, the hexanucleotide d-MMTr-$G^{Ac}pA^{Bz}$-

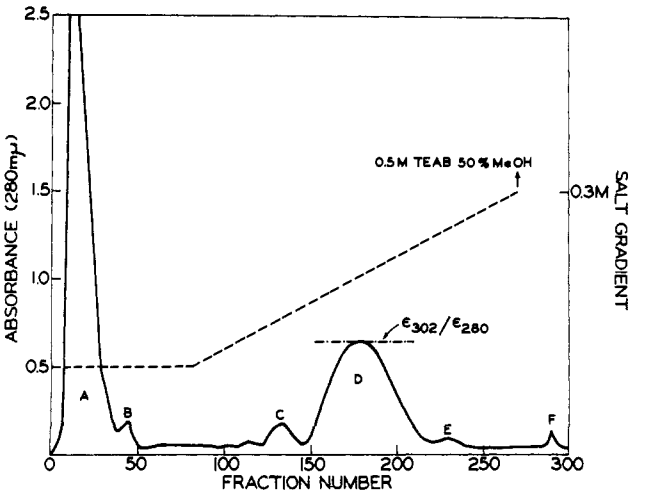


Figure 5. Chromatography of the reaction products in the preparation of hexanucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz}pA^{Bz}$ on a DEAE-cellulose (carbonate) column (1 × 55 cm.) first using 0.1 *M* triethylammonium bicarbonate (500 ml.) containing 15% methyl alcohol and then by using the linear gradient of 0.1–0.3 *M* triethylammonium bicarbonate (500 ml.) each of the solution containing 70% methyl alcohol. Peak D contains the hexanucleotide d-MMTr-$C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz}pA^{Bz}$; $\epsilon_{302}/\epsilon_{280}$, -·-·-·; salt gradient, ---------.

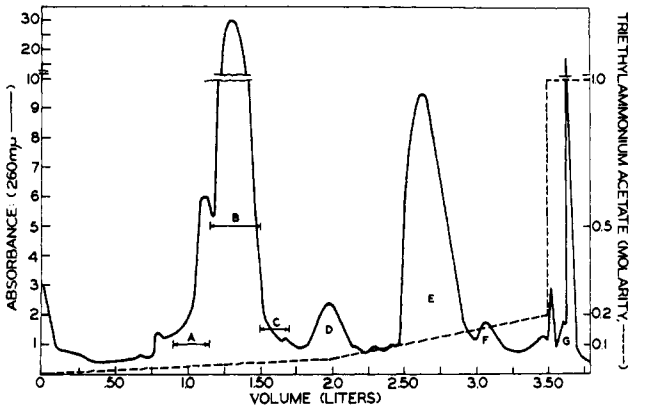


Figure 6. Chromatography of the reaction products in the preparation of the dinucleotide d-MMTr-$G^{Ao}pA^{Bz}$ on a DEAE-cellulose (acetate) column (2 × 85 cm.). d-MMTr-$G^{Ac}pA^{Bz}$ is in peak B.

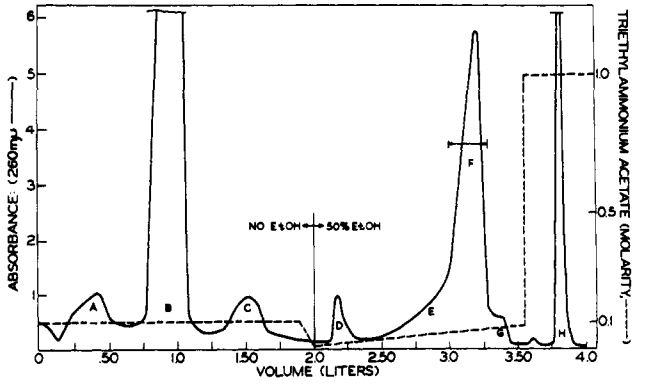


Figure 7. Chromatography of the reaction products in the preparation of the trinucleotide d-MMTr-$G^{Ao}pA^{Bz}pA^{Bz}$ on a DEAE-cellulose (acetate) column (2 × 76 cm.). d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}$ is in peak F.

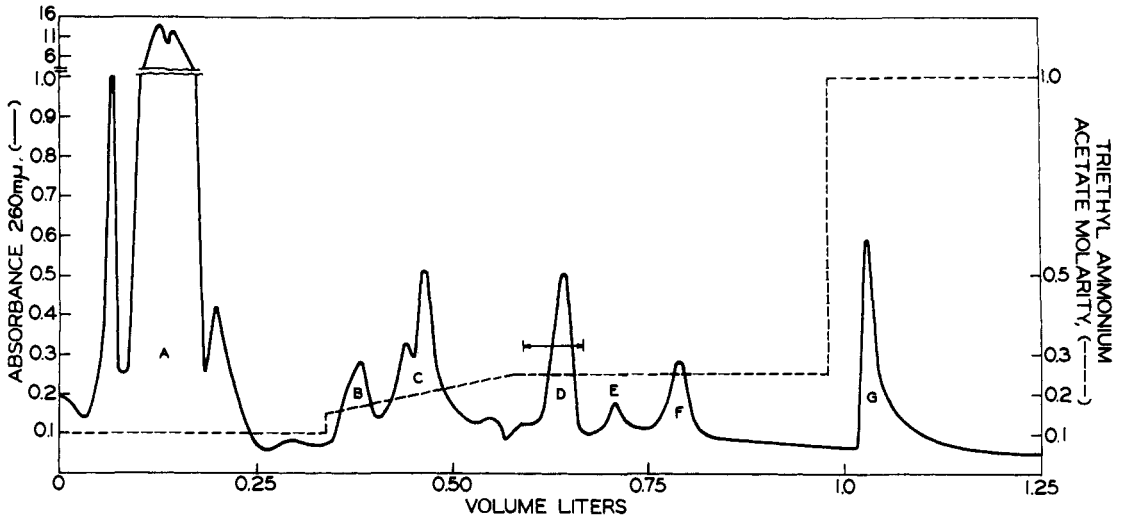


Figure 8. Chromatography of the reaction products in the preparation of the hexanucleotide d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}pA^{Bz}$ on a DEAE-cellulose (acetate) column (1 × 47 cm.). d-MMTr$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}pA^{Bz}$ is in peak D.

$pA^{Bz}pG^{Ac}pA^{Bz}pA^{Bz}$ requires further study. However, the successful syntheses reported in this paper and those reported in the accompanying papers demonstrate that the total methodology now available is satisfactory for the synthesis of short deoxyribopolynucleotides of any defined sequence.

Table II. Characterization of Polynucleotides[a]

Compound	R_f[b] Solvent A	Solvent B	Solvent C
d-MMTr-$G^{Ac}pA^{Bz}$		3.8	
d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}$		3.5	
d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}$		3.17	
d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}$		2.7	
d-MMTr-$G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}$-$pA^{Bz}pA^{Bz}$		1.98	
d-MMTr-GpA	7.29	3	1.97
d-MMTr-GpApA	3.56	2.05	1.8
d-MMTr-GpApApG	0.74	0.97	1.49
d-MMTr-GpApApGpA	0.27	0.4	1.39
d-MMTr-GpApApGpApA	0.09	0.15	1.19
d-GpA		2.17	1.09
d-GpApA		0.7	0.86
d-GpApApG		0.22	0.48
d-GpApApGpA		0.07	0.36
d-GpApApGpApA			0.27

[a] d-MMTr-$G^{Ac}pA^{Bz}$ and higher members; d-MMTr-GpA and higher members; d-GpA and higher members. [b] R_f relative to that of d-pA.

Table III. R_f Values of Homologous Compounds on Paper Chromatography

Compounds[b]	Solvent[a] A	Solvent[c] C
d-CpA	3.6	1.30
d-CpApA	0.85	0.96
d-CpApApC	0.23	0.80
d-CpApApCpA	..	0.76
d-CpApApCpApA	..	0.55

[a] R_f relative to that of d-pA. [b] Ammonium salt of the homologous compounds were used for paper chromatography.

Experimental

General Methods. Paper chromatography and paper electrophoresis were performed as described earlier.[1] Solvents A–D for paper chromatography were as described there. Solvent E is *n*-butyl alcohol–5 *N* acetic acid (2:1, v./v.). The presence of monomethoxytrityl group in different compounds was detected by spraying the paper chromatograms with 10% perchloric acid followed by drying in warm air. Monomethoxytrityl-containing compounds appeared as orange-yellow. The R_f values of different compounds are given in Tables II and III. Thin layer chromatography was carried out by using silica gel G or aluminum oxide G[19] by using the solvents ethyl acetate–methanol or chloroform–methanol.

Pyridinium d-pC^{An}-OAc, d-pA^{Bz}-OAc, and d-pG^{Ac}-OAc were prepared as described previously[9,11] except for the ether-precipitation step which was carried out as described separately.[3] The ϵ_{max} values used for the protected mononucleotides are as follows: d-pC^{An}-OAc: 22,450 (302 mμ) and 18,000 (280 mμ); d-pA^{Bz}-OAc: 6100 (302 mμ), 18,300 (280 mμ), and 11,450 (260 mμ); d-pG^{Ac}-OAc: 16,700 (260 mμ) and 10,900 (280 mμ).

Isolation of Protected Oligonucleotides. Following chromatography on DEAE-cellulose columns (bicarbonate or acetate form), the appropriate fractions were pooled and evaporated under reduced pressure at temperatures below 10° (when triethylammonium bicarbonate was the eluent) or 20° (when triethylammonium acetate was the eluent). These evaporations were carried out in the presence of added pyridine and in the case of triethylammonium acetate eluates some drops of tri-*n*-butylamine were also added. The sirupy residues obtained finally were rendered anhydrous by evaporation of added anhydrous pyridine and were then taken up in 5–10 ml. of dry pyridine. The resulting solutions were added dropwise to anhydrous ether (25–100-fold excess in volume). The oligonucleotidic materials separated as fine white precipitates. These were collected by centrifugation

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and washed with fresh ether by resuspension in the centrifuge tube. The ethereal layer was checked for nucleotidic material by the further addition of an excess of pentane.

Removal of Protecting Groups. The removal of the anisoyl, benzoyl, and acetyl groups from the amino groups of the oligonucleotides was effected by treating a pyridine solution of the compound with an excess (2–3 × volume of pyridine) of concentrated ammonium hydroxide for 2–3 days at room temperature. The monomethoxytrityl group was removed (only after the removal of the protecting group on amino groups) by treating the product (ammonium salt) with an excess of 80% acetic acid for 1 hr. 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 Using DCC. The protected oligonucleotide, *completely* free from triethylammonium salts, was converted to the pyridinium salt by passage through a column of pyridinium Dowex 50 ion-exchanger (at least 20 × the theoretical amount necessary) column. The total effluent and wash (with 50% aqueous pyridine) were evaporated *in vacuo* at low temperature and the residue was rendered anhydrous by repeated addition of added pyridine. The pyridinium salt of the oligonucleotide, thus obtained, and the pyridinium salt of the protected mononucleotide (the appropriate amount) were together freshly precipitated from dry ether and then dissolved in dry pyridine. Dry pyridinium Dowex 50 ion-exchange resin was added and the total mixture was rendered anhydrous by repeated evaporation of added pyridine. During the last evaporation a portion of dry pyridine was retained to serve as the medium of reaction. DCC (at least 5 equiv. as based on the mononucleotide) was added and the sealed reaction mixture was shaken in the dark for 2–3 days at room temperature. An equal volume of water was then added and the reaction mixture was filtered to remove dicyclohexylurea and the insoluble resin. The total filtrate and 50% aqueous pyridine wash were extracted twice with petroleum ether to remove unreacted DCC. The resulting clear solution was kept for 8–16 hr. at room temperature and then treated with 2 *N* sodium hydroxide (over-all molarity of the final solution about 1 *N* with respect to sodium hydroxide) at 0° for 10 min. The sodium ions were then removed by the addition of an excess of pyridinium Dowex 50 ion-exchange resin and the total solution after removal of the resin (wash with 50% aqueous pyridine) was applied directly to DEAE-cellulose anion-exchanger columns.

N^6-Anisoyl-5′-O-mono-p-methoxytrityldeoxycytidine. N^6-Anisoyldeoxycytidine (0.120 mg., 0.372 mmole) was treated in dry pyridine (2 ml.) with freshly crystallized mono-*p*-methoxytrityl chloride (0.140 mg., 0.440 mmole) for 18 hr. Ethyl alcohol was then added to destroy any of the unreacted reagent and the solution was evaporated to a gum. This was taken up in 2 ml. of benzene and 0.5 ml. of methanol and the solution was applied on a column (bed volume 25 ml.) of alumina (10% deactivated with water). Elution was carried out successively with benzene (50 ml.), benzene + 5% methanol (100 ml.), and then benzene + 10% methanol (100 ml.). Five-milliliter fractions were collected at a flow rate of about 0.5 ml./min. Fractions 3–6 contained a small amount of monomethoxytrityl-containing material and fractions 12–19 contained pure N^6-anisoyl-5′-O-monomethoxytrityldeoxycytidine. By evaporation and drying under vacuum, 480 mg. of the product was recovered. This material was homogeneous in thin layer chromatography (3.3 cm./10 cm., silica gel G, 3% methanol in ethyl acetate).

$N,O^{3'},O^{5'}$-Triacetyldeoxyguanosine. Deoxyguanosine (2.85 g., 10 mmoles) was treated with 17.5 ml. of 10% tetraethylammonium hydroxide and the resulting salt was rendered anhydrous by repeated evaporation of added pyridine. The residue was treated with 175 ml. of dry pyridine and 50 ml. of acetic anhydride for 4 days in the dark at room temperature. The excess of acetic anhydride was destroyed by addition of ethyl alcohol in the cold and the resulting mixture was evaporated under reduced pressure. The residual gum was diluted with water and lyophilized. The product was treated with an excess of pyridinium Dowex 50 ion-exchange resin and the aqueous pyridine solution was again lyophilized. The resulting powder (2.5 g.) was pure $N,O^{3'},O^{5'}$-triacetyldeoxyguanosine. Thus it was homogeneous by paper chromatography in two solvent systems: R_f in solvent B, 0.79; R_f of deoxyguanosine, 0.59, of 3′,5′-di-O-acetylguanosine, 0.73; R_f in solvent E, 0.74, of deoxyguanosine, 0.32, of 3′,5′-di-O-acetyldeoxyguanosine, 0.63. The ultraviolet absorption characteristics were similar to those of d-pG^{Ac}-OAc.

N-Acetyldeoxyguanosine. $N,O^{3'},O^{5'}$-Triacetyldeoxyguanosine as obtained above was dissolved in ethyl alcohol (20 ml.) and the solution was treated with 20 ml. of 2 *N* sodium hydroxide for 10 min. at 0°. An excess of pyridinium Dowex 50 ion-exchange resin was then added to remove sodium ions. The filtrate from the resin and washings (aqueous pyridine) were concentrated and the dilute aqueous pyridine solution was lyophilized twice. The white powder (2 g.) thus obtained was homogeneous in two solvents: R_f in solvent B, 0.68 (deoxyguanosine, 0.59); R_f in solvent E, 0.43 (deoxyguanosine, 0.32).

5′-O-Monomethoxytrityl-N-acetyldeoxyguanosine. An anhydrous pyridine solution (5 ml.) of N-acetyldeoxyguanosine (0.24 g.) was treated with monomethoxytrityl chloride (0.29 g.) at room temperature for 20 hr. The mixture was then poured into an excess of ice–water. The product was extracted into chloroform and the extract after concentration was added to a large excess of ether. The precipitate of the desired product was collected by centrifugation and dried (0.39 g.). Thin layer chromatography on aluminum oxide G (chloroform–methanol, 81:15) showed one major trityl-containing product and a trace of monomethoxytrityl alcohol. The sample was used in further work without purification.

5′-O-Monomethoxytrityl-N^6-anisoyldeoxycytidylyl-(3′→5′)-N^6-benzoyldeoxyadenosine. An anhydrous mixture of pyridinium d-pA^{Bz}-OAc (0.02 mmole), 5′-O-monomethoxytrityl-N^6-anisoyldeoxycytidine (0.02 mmole, 480 $O.D._{302}$ units), anhydrous pyridinium Dowex 50 ion-exchange resin (8% cross linked, 200 mg.), and DCC (150 mg.) in anhydrous pyridine (2

ml.) was shaken at room temperature for 3 days. Water (1 ml.) was then added and the insoluble dicyclohexylurea was removed by filtration and next washed with 50% aqueous pyridine (5 ml.). The total filtrate was extracted twice with petroleum ether (b.p. 60–90°). The pyridine solution was kept overnight at room temperature and then treated with 5 ml. of 2 *N* sodium hydroxide for 5 min. at 2°. An excess of pyridinium Dowex 50 ion-exchange resin was added to neutralize the alkali. The resin was removed by filtration and washed with aqueous pyridine (5 ml. of 50%). It was then concentrated to 1 ml. and this solution after dilution with methanol (5 ml.) was applied on a DEAE-cellulose (bicarbonate) column at 4°. The conditions used and the elution pattern obtained are shown in Figure 1. Fractions 158–228 were combined and concentrated in the presence of pyridine at 0–10° under reduced pressure. The yield of the isolated product was 598 O.D.$_{280}$ units (80% based on starting nucleotide). The ultraviolet absorption characteristics at pH 7.5 were: λ_{max} at 282 mμ; $\epsilon_{302}/\epsilon_{280}$ = 0.82. The R_f of the product after removal of the protecting groups is given in Table III.

The Protected Trinucleotide d-MMTr-C^{An}pA^{Bz}pA^{Bz}. An anhydrous mixture of pyridinium N^6-benzoyl-3′-O-acetyldeoxyadenosine 5′-phosphate (0.1 mmole), pyridinium d-MMTr-C^{An}pA^{Bz} (0.0135 mmole, 500 O.D.$_{280}$ units), and anhydrous pyridinium Dowex 50 ion-exchange resin (200 mg.) in dry pyridine (2 ml.) was treated with DCC (250 mg.) and the sealed mixture was shaken at room temperature for 3 days. After usual work-up including alkaline treatment, the total product was chromatographed on a DEAE-cellulose (bicarbonate) column at 4°. The conditions of elution and the chromatographic pattern are shown in Figure 2. The desired product, d-MMTr-C^{An}pA^{Bz}pA^{Bz}, was isolated from the pooled fractions 110–170 (494 O.D.$_{280}$ units). The ultraviolet absorption characteristics at pH 7.5 were λ_{max} 280 mμ, $\epsilon_{302}/\epsilon_{280}$ = 0.67. The R_f of the product after removal of the protecting groups is given in Table III.

The Protected Tetranucleotide d-MMTr-C^{An}pA^{Bz} pA^{Bz}pC^{An}. An anhydrous mixture of pyridinium d-MMTr-C^{An}pA^{Bz}pA^{Bz} (400 O.D.$_{280}$, 0.0072 mmole), pyridinium d-pC^{An}-OAc (0.1 mmole), pyridinium Dowex 50 ion-exchange resin (200 mg.), and DCC (200 mg.) in dry pyridine (2 ml.) was shaken in the dark at room temperature for 3 days. After the standard work-up, the total product was chromatographed on a DEAE-cellulose (bicarbonate) column (1 × 55 cm.) at 4°.

The conditions of elution and the elution pattern obtained are shown in Figure 3. Fractions 154–212 contained the desired tetranucleotide (340 O.D.$_{280}$ units). The ultraviolet absorption spectrum showed λ_{max} 280 mμ (pH 7.5) with $\epsilon_{302/280}$ = 0.81. The product was homogeneous, both before and after removal of the protecting groups, as judged by paper chromatography (R_f values given in Table III).

The Protected Pentanucleotide d-MMTr-C^{An}pA^{Bz} pA^{Bz}pC^{An}pA^{Bz}. An anhydrous mixture of pyridinium d-MMTr-C^{An}pA^{Bz}pA^{Bz}pC^{An} (380 O.D.$_{280}$ units, 0.004 mmole), pyridinium d-pA^{Bz}-OAc (0.1 mmole), pyridinium Dowex 50 ion-exchange resin (200 mg.), and DCC (200 mg.) in dry pyridine (2 ml.) was shaken at room temperature for 3 days. After the standard work-up, the product was chromatographed on a DEAE-cellulose (carbonate) form at 4°. The conditions used and the elution pattern are shown in Figure 4. The pentanucleotide (210 O.D.$_{280}$ units) was isolated from fractions 166–230. The ultraviolet absorption spectrum showed λ_{max} 280 mμ (pH 7.5) with $\epsilon_{302}/\epsilon_{280}$ = 0.72.

The Protected Hexanucleotide d-MMTr-C^{An}pA^{Bz}pA^{Bz}-pC^{An}pA^{Bz}pA^{Bz}. An anhydrous mixture of pyridinium d-MMTr-C^{An}pA^{Bz}pA^{Bz}pC^{An}pA^{Bz} (150 O.D.$_{280}$ units, 0.0016 mmole), pyridinium d-pA^{Bz}-OAc (0.1 mmole), pyridinium Dowex 50 ion-exchange resin (200 mg.), and DCC (200 mg.) in dry pyridine (1.5 ml.) was shaken at room temperature for 3 days. After the standard work-up, the product was chromatographed on a DEAE-cellulose (carbonate) form at 4°. The condition used and the elution pattern are shown in Figure 5. The hexanucleotide (120 O.D.$_{280}$ units) was isolated from fraction 152–210. The ultraviolet absorption spectrum showed λ_{max} 280 mμ (pH 7.5) with $\epsilon_{302}/\epsilon_{280}$ = 0.64.

5′-O-Monomethoxytrityl-N-acetyldeoxyguanylyl-(3′→5′)-N-benzoyldeoxyadenosine. An anhydrous mixture of 5′-O-monomethoxytrityl-N-acetyldeoxyguanosine (0.28 g., 0.5 mmole), pyridinium d-pA^{Bz}-OAc (0.8 mmole), Dowex 50 ion-exchange resin (1 g.), and DCC (0.83 g., 4 mmoles) in dry pyridine (5 ml.) was shaken in the dark for 4 days. After standard work-up and alkaline treatment, the total mixture was applied to the top of a DEAE-cellulose (acetate) column (2 × 85 cm.) preequilibrated with 95% ethyl alcohol. After a wash with 95% ethyl alcohol to remove pyridine, elution of the column was carried out with a linear salt gradient of triethylammonium acetate in 95% ethanol. Fractions of about 20-ml. were collected every half hour and each fraction was measured for its absorbance at 260 and 280 mμ. The elution pattern and the salt gradient are shown in Figure 6. Peak B contained pure d-MMTr-G^{Ac}pA^{Bz}, with a constant $\epsilon_{280}/\epsilon_{260}$ ratio of 1.04. Peaks A and C also contained mostly the same product but evidently contained some impurities as indicated by inconstant $\epsilon_{280}/\epsilon_{260}$ ratios. The total yield of the desired product was 9500 O.D.$_{280}$ units (60%). d-pA^{Bz} is in peak E.

The Trinucleotide d-MMTr-G^{Ac}pA^{Bz}pA^{Bz}. A mixture of pyridinium d-MMTr-G^{Ac}pA^{Bz} (2000 O.D.$_{280}$ units, 0.06 mmole), pyridinium d-pA^{Bz}-OAc (0.2 mmole), and Dowex 50 (pyridinium) ion-exchange resin (0.5 g.) in dry pyridine (3 ml.) was shaken with DCC (0.42 g., 2 mmoles) for 5 days. The work-up including the alkaline treatment was as given above. The total mixture in aqueous pyridine was applied to the top of a DEAE-cellulose (acetate) column (2 × 76 cm.). The column was first washed with 2 l. of 0.01 *M* triethylammonium acetate containing no alcohol. Elution was then carried out first with 2 l. of the same 0.1 *M* eluent and then with a linear gradient starting from 0.01 *M* concentration of the same salt in 50% ethyl alcohol. Fractions (10 to 20 ml.)[20] were collected every 0.5 hr. and the absorbance was measured at 260 and 280 mμ. The elution pattern

(20) On changing from a purely aqueous eluent to one containing 50% ethyl alcohol, there was observed a drop in flow rate.

and the salt gradient are given in Figure 7. Peak B contained the mononucleotide d-pA^{Bz}. Peak F contained pure d-MMTr-G^{Ac}pA^{Bz}pA^{Bz} ($\epsilon_{280}/\epsilon_{260}$ = 1.2). The tail end of peak E also contained the same material. The same product was present in peaks C, D, and G but contained impurities. The yield of the pure product from peaks E and F was 1778 O.D.$_{280}$ units (56%).

The Tetranucleotide d-MMTr-G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}. A mixture of pyridinium d-MMTr-G^{Ac}pA^{Bz}pA^{Bz} (880 O.D.$_{280}$ units, 0.0176 mmole), pyridinium d-pG^{Ac}-OAc (0.17 mmole), and Dowex 50 (pyridinium) ion-exchange resin (0.4 g.) in dry pyridine (1.5 ml.) was shaken with DCC (0.3 g., 1.5 mmoles) for 2.5 days at room temperature. After the standard work-up, the total mixture in aqueous pyridine was applied to the top of a DEAE-cellulose (acetate) column (2 × 33 cm.), pre-equilibrated with 0.01 *M* triethylammonium acetate (pH 6.5) containing 50% ethanol. The column was first washed with the same eluent until most of the pyridine was removed. Elution was then carried out using a linear gradient. Initially, the mixing vessel contained 1 l. of 0.05 *M* triethylammonium acetate (50% alcohol) and the reservoir contained 1 l. of 0.125 *M* solution of the same salt. Fractions (about 20 ml.) were collected every 15 min. Fractions 40–70 contained the mononucleotide d-pG^{Ac} and its symmetrical pyrophosphate. The tetranucleotide d-MMTr-G^{Ac}pA^{Bz}-pA^{Bz}pG^{Ac} appeared as a broad peak (fractions 75–100). The yield was 582 O.D.$_{280}$ units (54%). This product was slightly contaminated by d-pG. It was further purified by preparative paper chromatography (two 9-in. wide strips of Whatman paper No. 3) using solvent B. The major band (trityl positive) was eluted with 0.01 *M* triethylammonium acetate containing 50% ethyl alcohol. The recovery of the pure product was 542 O.D.$_{280}$ units. The absorption spectrum showed $\epsilon_{280}/\epsilon_{260}$ = 1.06.

The Pentanucleotide d-MMTr-G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}. A mixture of pyridinium d-MMTr-G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac} (300 O.D.$_{280}$ units, 0.0049 mmole), pyridinium d-pA^{Bz}-OAc (0.1 mmole), Dowex 50 (pyridinium) ion-exchange resin (0.4 g.), and DCC (0.12 g.) in dry pyridine (1 ml.) was shaken for 1 day at room temperature. After the work-up including the alkaline treatment, the total mixture in aqueous pyridine was applied to the top of a DEAE-cellulose (acetate) column (1 × 47 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate containing 50% alcohol. The column was washed first with the same 0.01 *M* buffer until the pyridine was removed. Elution was carried out first with 0.1 *M* solution of the same eluent. Fractions (about 8 ml.) were collected every 0.5 hr. After fraction 44 (300 ml. of eluent used) a linear gradient of the same eluent was used. The mixing vessel contained 280 ml. of 0.15 *M* triethylammonium acetate containing 50% ethanol and the reservoir contained 280 ml. of 0.25 *M* of the same buffer. Fractions 57–69 (99 ml.) contained d-MMTr-G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz} (122 O.D.$_{280}$ units, 31%; $\epsilon_{280}/\epsilon_{260}$ = 1.13). Fractions 33–55 contained the starting material (161 O.D.$_{280}$ units).

The Hexanucleotide d-MMTr-G^{Ac}pA^{Bz}pA^{Bz}pG^{Ac}pA^{Bz}-pA^{Bz}. A mixture of pyridinium d-MMTr-G^{Ac}pA^{Bz}-pA^{Bz}pG^{Ac}pA^{Bz} (60 O.D.$_{280}$ units, 0.76 μmole), pyridinium d-pA^{Bz}-OAc (0.05 mmole), Dowex 50 (pyridinium) ion-exchange resin (0.5 g.), and DCC (0.15 g.) in dry pyridine (1 ml.) was shaken for 2.5 days at room temperature. After the work-up the total mixture in aqueous pyridine was applied to the top of a DEAE-cellulose (acetate) column (1 × 47 cm.) pre-equilibrated with 0.01 *M* triethylammonium acetate containing 50% ethanol. The column was first washed with 0.01 *M* triethylammonium acetate containing 50% ethanol and elution was then carried out using a linear gradient of the same salt. The conditions of elution and the elution pattern are shown in Figure 8. Peak A contained the mononucleotide d-pA^{Bz}; peak C contained the starting oligonucleotide and peak D contained the desired hexanucleotide (17 O.D.$_{280}$ units; 23%; $\epsilon_{280}/\epsilon_{260}$ = 1.2).

Enzymatic Degradation of Hexanucleotides. The hexanucleotide d-CpApApCpApA (3.5 O.D.$_{260}$ units) was incubated with spleen phosphodiesterase.[21] The products, which were separated by paper chromatography in solvent D, were found to be deoxyadenosine, deoxycytidine 3′-phosphate, and deoxyadenosine 3′-phosphate, their molar concentrations, respectively, being 1:1.99:3.15 (theoretical 1:2:3). Similarly the degradation of the hexanucleotide d-GpApApGpApA (1.6 O.D.$_{260}$ units) with the same enzyme preparation gave as the products deoxyadenosine, deoxyguanosine 3′-phosphate, and deoxyadenosine 3′-phosphate; their molar ratios were, respectively, 1:2:2.9 (theoretical 1:2:3).

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