

CV. Total Synthesis of the Structural Gene for an Alanine Transfer Ribonucleic Acid from Yeast. Chemical Synthesis of an Icosadeoxyribonucleotide Corresponding to the Nucleotide Sequence 31 to 50

H. BÜCHI AND H. G. KHORANA

The icosanucleotide having the sequence, d-G-C-T-C-C-T-T-A-G-C-A-T-G-G-G-A-G-A-G- has been chemically synthesized. This and the icosanucleotide described in the preceding paper (CIV) are the longest polydeoxynucleotides with specific non-repeating sequences which have been chemically synthesized to date. The present icosanucleotide covers the nucleotide sequence 31 to 50 of one of the strands in the DNA duplex representing the gene for yeast alanine transfer RNA. The nucleotide sequence and the polarity of the DNA strand, of which the icosanucleotide herein described forms a part, is the same as that of the tRNA itself (Fig. 1). The synthesis involved the following steps, using fully protected intermediates. The nucleoside d-MMTr-G^{1B} was condensed successively with d-pC^{An}OAc and d-pT-OAc to give d-MMTr-G^{1B}pC^{An}pT. The latter was condensed successively with the three dinucleotide blocks, d-pC^{An}pC^{An}OAc, d-pC^{An}pT-OAc and d-pTpA^{Bz}OAc, to give the protected nonanucleotide, d-MMTr-G^{1B}pC^{An}pTpC^{An}pC^{An}pC^{An}pTpTpA^{Bz}. Then followed condensations with the following trinucleotide and tetranucleotide blocks in the order that the blocks are written: d-pG^{1B}pC^{An}pA^{Bz}OAc, d-pTpG^{1B}pG^{1B}pG^{1B}OiB and d-pA^{Bz}pG^{1B}pA^{Bz}pG^{1B}OAc. All the blocks, the intermediate polydeoxynucleotides and the final product were characterized in a variety of ways, both in the protected and the unprotected form.

1. Introduction

The present paper describes the synthesis of the icosadeoxynucleotide, d-G-C-T-C-C-C-T-T-A-G-C-A-T-G-G-G-A-G-A-G, which covers the nucleotide sequence 31 to 50 of one of the strands of the DNA duplex corresponding to the yeast alanine transfer RNA (paper CIII). This work was carried out concurrently with the synthesis of the icosanucleotide described in the preceding paper (CIV). The two icosanucleotides belong to the complementary strands of the DNA duplex, show an overlap of ten nucleotide units (Fig. 1) and together span the nucleotide sequence 21 to 50 of the gene. The two icosanucleotides proved crucial for initial studies of the DNA-joining enzymes, studies which provided the theoretical basis for planning the total synthesis of the gene (paper CIII). In the final plan, the hexadecanucleotide, the immediate precursor in the chemical synthesis of the icosanucleotide herein described, was in fact used, the relevant part of the plan being shown in Figure 2. As may be seen, the hexadecanucleotide forms segment 8 in this scheme. In addition, the two icosanucleotides shown in Figure 1 have proved extremely useful in studies of the DNA and RNA polymerases (Gupta & Khorana, 1968; Kleppe, Ohtsuka, Kleppe, Molineux & Khorana, 1971; Terao, Kleppe & Khorana, unpublished work), studies which are a prerequisite to the replication and transcription of the gene synthesized in the present series of papers.

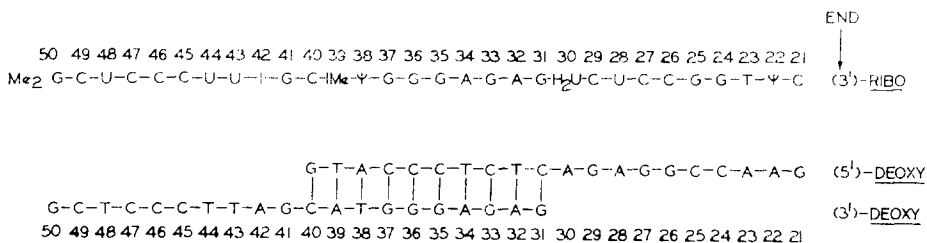


FIG. 1. Structures of the two partly complementary icosanucleotides representing the nucleotide sequence 21 to 50 of the double-stranded DNA corresponding to the gene for the yeast alanine transfer RNA. Note the opposite polarities of the two deoxypolynucleotide chains and their relation to the polarity of the transfer RNA sequence. As explained in the preceding paper, in the derivation of the DNA sequence from that of the alanine transfer RNA sequence, the assumption has been made that the minor bases are formed by modification of the four common ribonucleotide units; only the latter are believed to be used in transcription by the RNA polymerase.

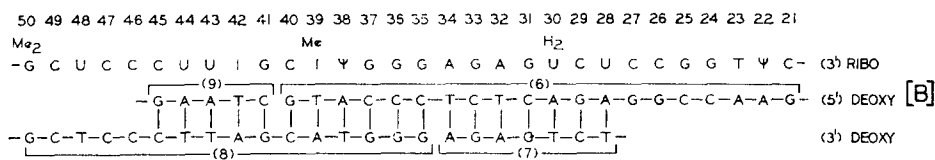


FIG. 2. Chemically synthesized deoxyribopolynucleotides corresponding to sequence 21 to 50 of the gene for the yeast alanine transfer RNA. The chemically synthesized segments are as shown within brackets. The hexadecanucleotide, whose synthesis is described herein, is segment 8 in the plan for the middle [B] part of the gene.

2. Discussion of Experimental Methods

(a) *The protection of the guanine ring and the synthetic plan*

The general methodology used for building the polydeoxynucleotide chain has been reviewed in the preceding paper (CIV). As mentioned there, the question of the protection of the guanine ring in deoxyguanosine and its 5'-nucleotide was re-examined. This resulted in the introduction of the isobutyryl group. Preparation of the *N*-protected isobutyryl derivative of deoxyguanosine 5'-phosphate on a 10 m-mole scale is described in the Experimental Methods section. Because deoxyguanosine constitutes the 5'-terminus of the icosanucleotide, a method for the preparation of *N*-isobutyryl-5'-*O*-monomethoxytrityl deoxyguanosine on a large scale was also worked out. At least in regard to its susceptibility towards alkali, the *N*-isobutyryl group showed, as might have been expected, considerably higher stability than the *N*-acetyl group (see Experimental Methods).

The plan adopted for the synthesis of the icosanucleotide is shown in Chart 1. As may be seen, except for the synthesis of the protected trinucleoside diphosphate, d-MMTr-G¹⁸pC⁴⁰pT (I), where mononucleotides were used in stepwise condensation, chain elongation involved preformed blocks of di-, tri and tetranucleotides.

The synthesis up to the nonanucleotide (IV) stage was carried out using dinucleotide blocks. There were several reasons for this. Because of the many steps involved, the scale of the work at the initial stages was especially large, and therefore, very large amounts of the blocks were required. Previous experience has shown that higher yields are obtained using dinucleotide blocks than when, say, tetranucleotide blocks

CHART 1

Steps in the synthesis of the icosanucleotide, d-G-C-T-C-C-C-T-T-A-G-C-A-T-G-G-A-G-A-G

Protected form	Unprotected form
d-MMTTr-G ^{1B}	
(1) d-pC ^{An} .OAc + DCC (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An}	
(1) d-pT ^O .OAc + MS (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An} pT (I)	(1) NH ₄ OH (2) H ⁺ → d-G-C-T
(1) d-pC ^{An} pC ^{An} .OAc + MS (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} (II)	(1) NH ₄ OH (2) H ⁺ → d-G-C-T-C-C (The pentanucleotide)
(1) d-pC ^{An} pT ^O .OAc + MS (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} pC ^{An} pT (III)	(1) NH ₄ OH (2) H ⁺ → d-G-C-T-C-C-C-T (The heptanucleotide)
(1) d-pTpA ^{Bz} .OAc (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} pC ^{An} pTpTpA ^{Bz} (IV)	(1) NH ₄ OH (2) H ⁺ → d-G-C-T-C-C-C-T-T-A (The nonanucleotide)
(1) d-G ^{1B} pC ^{An} pA ^{Bz} .OAc + MS (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} pC ^{An} pTpTpA ^{Bz} pG ^{1B} pC ^{An} pA ^{Bz} (V)	(1) NH ₄ OH (2) H ⁺ → d-G-C-T-C-C-C-T-T-A-G-C-A (The dodecanucleotide)
(1) d-TpG ^{1B} pG ^{1B} pG ^{1B} .O _i B + MS (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} pC ^{An} pTpTpA ^{Bz} pG ^{1B} pC ^{An} pA ^{Bz} pTpG ^{1B} pG ^{1B} pG ^{1B} (VI)	(1) NH ₄ OH (2) H ⁺ → d-G-C-T-C-C-C-T-T-A-G-C-A-T-G-G-G (The hexadecanucleotide)
(1) d-pA ^{Bz} pG ^{1B} pA ^{Bz} pG ^{1B} .OAc + MS (2) $\bar{O}H$	
d-MMTTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} pC ^{An} pTpTpA ^{Bz} pG ^{1B} pC ^{An} pA ^{Bz} pTpG ^{1B} pG ^{1B} pG ^{1B} pA ^{Bz} pG ^{1B} pA ^{Bz} pG ^{1B} (VII)	(1) NH ₄ OH (2) H ⁺ → d-G-C-T-C-C-C-T-T-A-G-C-A-T-G-G-A-G-A-G (The icosanucleotide)

are used (Kössel, Büchi & Khorana, 1967; Ohtsuka & Khorana, 1967; Hachmann & Khorana, 1969). Further, the separations on anion-exchange columns were expected to be adequate at the level of the relatively short chains when the net negative charge in the product increased by two over the starting material. In later stages longer blocks were used. Thus, the nonanucleotide was taken to the dodecanucleotide (V) and the latter was taken to the hexadecanucleotide (VI) and then to the icosanucleotide (VII) by using tetranucleotide blocks.

The required protected dinucleotide blocks, $d\text{-pC}^{\text{An}}\text{pC}^{\text{An}}\text{-OAc}$, $d\text{-pC}^{\text{An}}\text{pT-OAc}$ and $d\text{-pTpA}^{\text{Bz}}\text{-OAc}$ were prepared by the previously described methods, except that the preparations were increased to a several-millimole scale. This entailed the use of considerably larger DEAE-cellulose anion-exchange columns. The trinucleotide block $d\text{-pG}^{\text{IB}}\text{pC}^{\text{An}}\text{pA}^{\text{Bz}}\text{-OAc}$ was prepared by the step-wise addition of the protected mononucleotides $d\text{-pC}^{\text{An}}\text{-OAc}$ and $d\text{-pA}^{\text{Bz}}\text{-OAc}$ to the cyanoethyl derivatives of $d\text{-pG}^{\text{IB}}$ and $d\text{-pG}^{\text{IB}}\text{pC}^{\text{An}}$, respectively. The product was purified by chromatography, the pattern obtained being shown in Figure 3. The tetranucleotide block $d\text{-pTpG}^{\text{IB}}\text{pG}^{\text{IB}}\text{-pG}^{\text{IB}}\text{-OiB}$

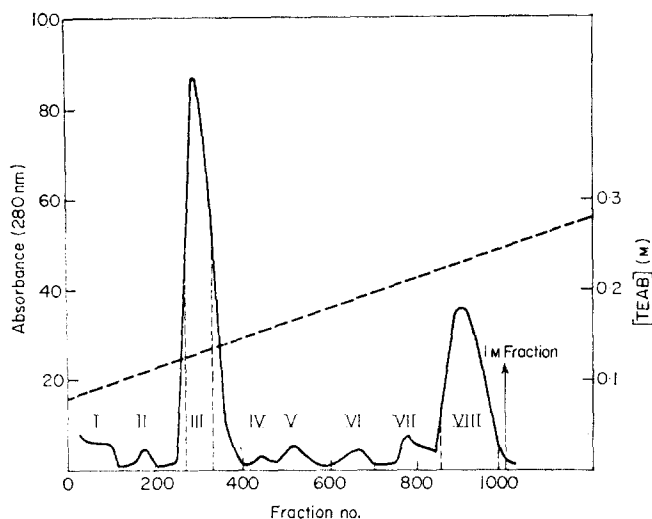


Fig. 3. Chromatography of the reaction mixture obtained in the synthesis of the trinucleotide $d\text{-pG}^{\text{IB}}\text{pC}^{\text{An}}\text{pA}^{\text{Bz}}$ on a DEAE-cellulose column (bicarbonate form, 5.5 cm $\times$ 99 cm) pre-equilibrated to 0.05 M-TEAB-10% ethanol. After pyridine had been eluted, a linear salt gradient was started with 12 l. of 0.08 M-TEAB-10% ethanol in the mixing vessel and an equal volume of 0.28 M-TEAB-10% ethanol in the reservoir. Fractions of 20 ml. were collected every 5 min. Fractions were pooled as shown by the vertical broken lines. Peak VIII contained the product. (----) Concn TEAB; (—) absorbance.

was prepared by the repeated condensation of the 5'-cyanoethyl mono- or oligonucleotide with $d\text{-pG}^{\text{IB}}\text{-OiB}$. This route to the tetranucleotide was preferred to the alternative, which would involve the preparation of $d\text{-pG}^{\text{IB}}\text{pG}^{\text{IB}}\text{-OAc}$ (or -OiB). The preparation of the latter in pure form presents the technical problem of insufficient separation on anion-exchange columns from the contaminating pyrophosphate of $d\text{-pG}^{\text{IB}}$. Furthermore, as pointed out in accompanying papers, the yields obtained in condensations using purine (especially guanine) dinucleotide blocks are usually low.

The chromatographic pattern obtained in the preparation of $d\text{-pTpG}^{\text{IB}}\text{pG}^{\text{IB}}$ is shown in Figure 4, while that obtained in the preparation of the tetranucleotide,

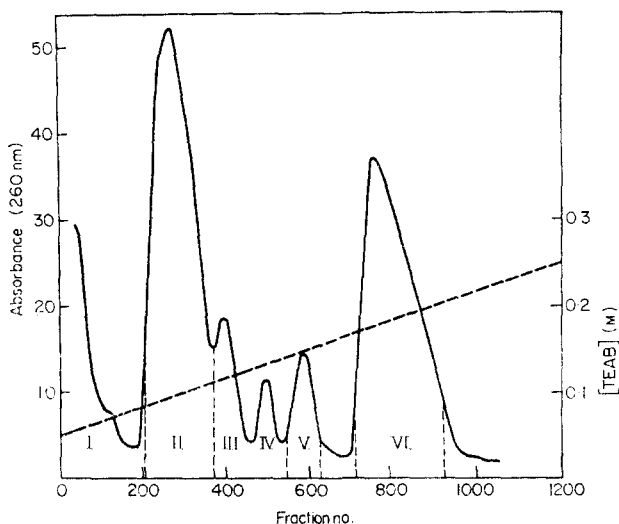


FIG. 4. Chromatography of the reaction mixture obtained in the synthesis of the trinucleotide d-pTpG^{1b}pG^{1b} on a DEAE-cellulose column (bicarbonate form, 5.5 cm × 99 cm) pre-equilibrated to 0.05 M-TEAB-10% ethanol. After pyridine had been eluted, a linear salt gradient was started with 12 l. of 0.05 M-TEAB-10% ethanol in the mixing vessel and 12 l. of 0.25 M-TEAB-10% ethanol in the reservoir. Fractions of 20 ml. were collected every 5 min. Fractions were pooled as shown by the vertical broken lines. Peak VI contained the product. (-----) Concn TEAB; (—) absorbance.

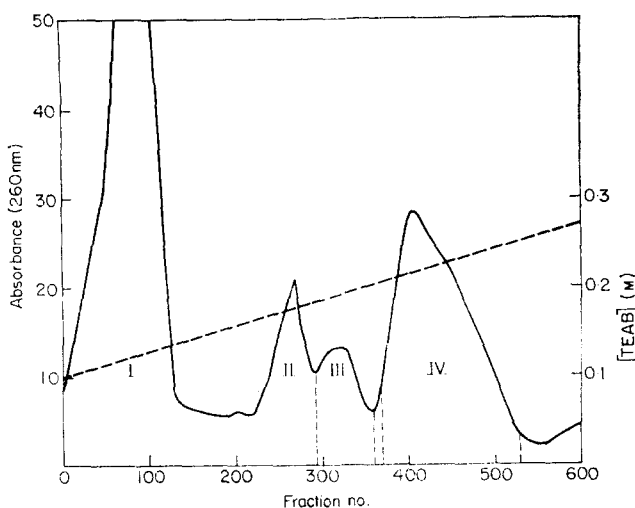


FIG. 5. Chromatography of the reaction mixture in the preparation of the tetranucleotide d-pTpG^{1b}pG^{1b}pG^{1b} on a DEAE-cellulose column (bicarbonate form, 4 cm × 84 cm) pre-equilibrated to 0.05 M-TEAB-10% ethanol. After pyridine was eluted with the same buffer, a linear salt gradient was started with 6 l. of 0.10 M-TEAB-10% ethanol in the mixing vessel and 6 l. of 0.27 M-TEAB-10% ethanol in the reservoir. Fractions of 20 ml. were collected every 3 to 4 min. Fractions were pooled as shown by the vertical broken lines. Peak IV contained the product. (-----) Concn TEAB; (—) absorbance.

d-pTpG^{1b}pG^{1b}pG^{1b}, is shown in Figure 5. Because of the cumulating deoxyguanosine residues and detectable loss of the isobutyryl groups, the tetranucleotide block had to be purified by further chromatography (Fig. 6).

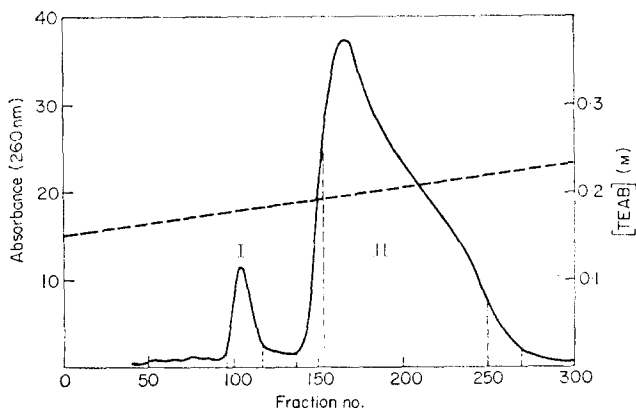


FIG. 6. Rechromatography of the tetranucleotide $d\text{-pT(pG}^{1\text{B}})_2\text{pG}^{1\text{B}}$ on a DEAE-cellulose column (bicarbonate form, $3.2 \text{ cm} \times 103 \text{ cm}$) pre-equilibrated to $0.05 \text{ M-TEAB-20\% ethanol}$. After pyridine was eluted, a linear salt gradient of $0.15 \text{ M-TEAB-20\% ethanol}$ in the mixing vessel and $0.26 \text{ M-TEAB-20\% ethanol}$ in the reservoir was started. Fractions of 20 ml. were collected every 15 min. Fractions were pooled as shown by the vertical broken lines. (----) Concn TEAB; (—) absorbance.

For the preparation of the tetranucleotide block, $d\text{-pA}^{\text{Bz}}\text{pG}^{1\text{B}}\text{pA}^{\text{Bz}}\text{pG}^{1\text{B}}\text{-OAc}$, the method involving condensation of dinucleotide blocks was used, this was because of the symmetrical nature of the tetranucleotide. The dinucleotide $d\text{-pA}^{\text{Bz}}\text{pG}^{1\text{B}}$ was prepared in a large quantity. A part was cyanoethylated to give $d\text{-CE-pA}^{\text{Bz}}\text{pG}^{1\text{B}}$, while another part was acetylated to give $d\text{-pA}^{\text{Bz}}\text{pG}^{1\text{B}}\text{-OAc}$. Condensation followed by chromatography gave $d\text{-pA}^{\text{Bz}}\text{pG}^{1\text{B}}\text{pA}^{\text{Bz}}\text{pG}^{1\text{B}}$, which was purified by chromatography (Fig. 7) and finally acetylated to protect the 3'-hydroxyl group.

(b) Synthesis of the icosanucleotide

5'-O-Monomethoxytrityl-N-isobutyryl-deoxyguanosine, the preparation of which on a rather large scale is described in the Experimental Methods section, was condensed

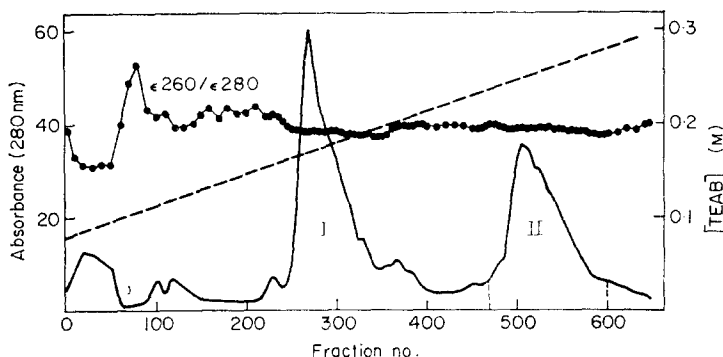


FIG. 7. Chromatography of the products obtained by condensing the dinucleotide $d\text{-pA}^{\text{Bz}}\text{pG}^{1\text{B}}\text{-OAc}$ and $d\text{-CE-pA}^{\text{Bz}}\text{pG}^{1\text{B}}$ on a DEAE-cellulose column (bicarbonate form, $1.9 \text{ cm} \times 70 \text{ cm}$) which had been pre-equilibrated to 0.03 M-TEAB in $10\% \text{ ethanol}$. The elution was carried out using a linear gradient of 0.08 M-TEAB in $10\% \text{ ethanol}$ (6 l.) in the mixing vessel and $0.28 \text{ M-TEAB-10\% ethanol}$ (6 l.) in the reservoir. Fractions of 20 ml. each were collected every 12 min. Peaks I and II were identified as unreacted di- and desired tetranucleotide, respectively. The peaks were pooled as shown by vertical broken lines. (----) Concn TEAB; (—) absorbance.

TABLE I

Summary of reaction conditions, recoveries and yields in block condensations

Exp. no.	Starting chain (3'-OH component) (A)	Amount (m-mole)	Oligonucleotide block (5'-phosphate end) (B)	Amount in m-mole (B)/(A)	MS† (eq./P)	Block recovered‡ (%)	Reaction- time (hr)	Starting chain recovered‡ (%)	Product	Yield (%)
1	The trinucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT	1.00	d-pC ^{Aa} pC ^{Aa} -OAc	6.14 (6.14)	0.64	50	1½	—	The pentanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pTpC ^{Aa} pC ^{Aa}	63¶
2		0.74		3.70 (5.0)	0.90§	45	2	—		
1	The pentanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pTpC ^{Aa} . pC ^{Aa}	0.80	d-pC ^{Aa} pT-OAc	6.95 (8.7)	0.65	61	1½	10	The heptanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT(pC ^{Aa}) ₃ pT	79
2		0.21		1.68 (8.0)	0.65	56	1½	—		66
1	The heptanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT- (pC ^{Aa}) ₃ pT	0.144	d-pTpA ^{Bz} -OAc	1.45 (10)	0.55	60	1½	17	The nonanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT(pC ^{Aa}) ₃ - (pT) ₂ pA ^{Bz}	48
2		0.306		3.44 (11.2)	0.77	76	1½	12		65
	The nonanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT- (pC ^{Aa}) ₃ (pT) ₂ pA ^{Bz}	0.233	d-pG ¹⁸ pC ^{Aa} . pA ^{Bz} -OAc	1.31 (5.6)	0.80	67	2¼	48	The dodecanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT(pC ^{Aa}) ₃ - (pT) ₂ pA ^{Bz} pG ¹⁸ pC ^{Aa} pA ^{Bz}	32
	The dodecanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT(pC ^{Aa}) ₃ - (pT) ₂ pA ^{Bz} pG ¹⁸ pC ^{Aa} pA ^{Bz}	0.057	d-pT(pG ¹⁸) ₂ - pG ¹⁸ -O-iB	0.69 (12.1)	0.65	61	2½	63	The hexadecanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT(pC ^{Aa}) ₃ - (pT) ₂ pA ^{Bz} pG ¹⁸ pC ^{Aa} pA ^{Bz} pT- (pG ¹⁸) ₂ pG ¹⁸	17
	The hexadecanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT(pC ^{Aa}) ₃ - (pT) ₂ pA ^{Bz} pG ¹⁸ pC ^{Aa} pA ^{Bz} . pT(pG ¹⁸) ₂ pG ¹⁸	0.0092	d-pA ^{Bz} pG ¹⁸ . pA ^{Bz} pG ¹⁸ -OAc	0.59 (64)	0.65	56	2½	49	The icosanucleotide d-MMTr-G ¹⁸ pC ^{Aa} pT(pC ^{Aa}) ₃ - (pT) ₂ pA ^{Bz} pG ¹⁸ pC ^{Aa} pA ^{Bz} pT- (pG ¹⁸) ₃ pA ^{Bz} pG ¹⁸ .pA ^{Bz} pG ¹⁸	21

† Mesitylenesulfonylchloride, equivalents per phosphate group dissociation in the two components.

‡ % of the original amount for further condensations without additional purification.

§ The condensing agent used in this case was TPS.

¶ Over-all yield from the two condensations after rechromatography of impure product fractions.

with d-pC^{An}-OAc using dicyclohexylcarbodiimide as the condensing agent. The required product, d-MMTr-G^{IB}pC^{An}-OAc, was isolated by an extraction procedure and, after removal of the acetyl group, was carried through to the next step (condensation with d-pT-OAc). The trinucleoside diphosphate, d-MMTr-G^{IB}pC^{An}pT (I), was first enriched from the reaction mixture by solvent extraction and then purified by chromatography on a DEAE-cellulose column (Fig. 8).

In the preparation of the pentanucleotide, d-MMTr-G^{IB}pC^{An}pTpC^{An}pC^{An} (II), from d-MMTr-G^{IB}pC^{An}pT and d-pC^{An}pC^{An}-OAc, difficulty was encountered in the separation of the required pentanucleotide from the symmetrical pyrophosphate of the dinucleotide, although a gradient of ethanol was superimposed on the salt gradient (Fig. 9 and

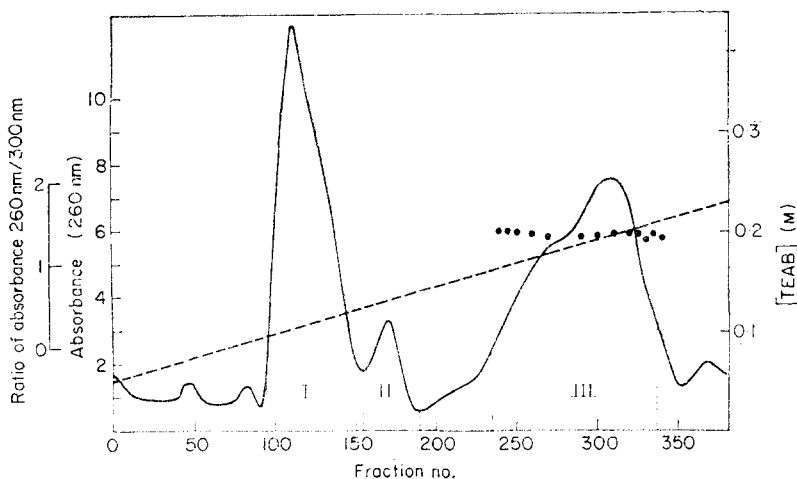


FIG. 8. Chromatography of the reaction products in the preparation of the trinucleotide d-MMTr-G^{IB}pC^{An}pT on a DEAE-cellulose column (bicarbonate form, 2.4 cm × 87 cm) pre-equilibrated with 0.05 M-TEAB-30% ethanol. After pyridine was eluted with the same buffer a linear salt gradient was started with 3.5 l. of 0.05 M-TEAB-30% ethanol in the mixing vessel and 3.5 l. of 0.38 M-TEAB-40% ethanol in the reservoir. Fractions of 10 ml. were collected. Fractions were pooled as shown by the vertical broken lines. Peak III contained the product. (----) Concn TEAB; (—) absorbance; (●), ratio of absorbance at 260 nm to that at 300 nm.

Table 2). Both compounds have the same net negative charge and both possess features which retard their elution from the column. Thus, the pentanucleotide has the methoxytrityl group, the presence of which is normally taken advantage of for delaying elution of this class of compounds until the blocks have been eluted. However, in the above case, the pyrophosphate of d-pC^{An}pC^{An} was also greatly retarded, because of the presence of four C^{An} groups. (Of the protected mononucleotides, d-pC^{An} is the last to be eluted from anion-exchange columns.) However, a rechromatography of the mixture of d-MMTr-G^{IB}pC^{An}pTpC^{An}pC^{An} and the pyrophosphate of d-pC^{An}pC^{An} using a more sophisticated change in ethyl alcohol concentration gave the pentanucleotide in pure form. The separation pattern obtained is shown in Figure 10.

Condensation of the above pentanucleotide (II) with the dinucleotide d-pC^{An}pT-OAc proceeded well and the chromatographic pattern obtained is shown in Figure 11. Analysis of the different peaks is given in Table 3. Similarly, the condensation of the protected heptanucleotide, d-MMTr-G^{IB}pC^{An}pTpC^{An}pC^{An}pC^{An}pT, with the dinucleo-

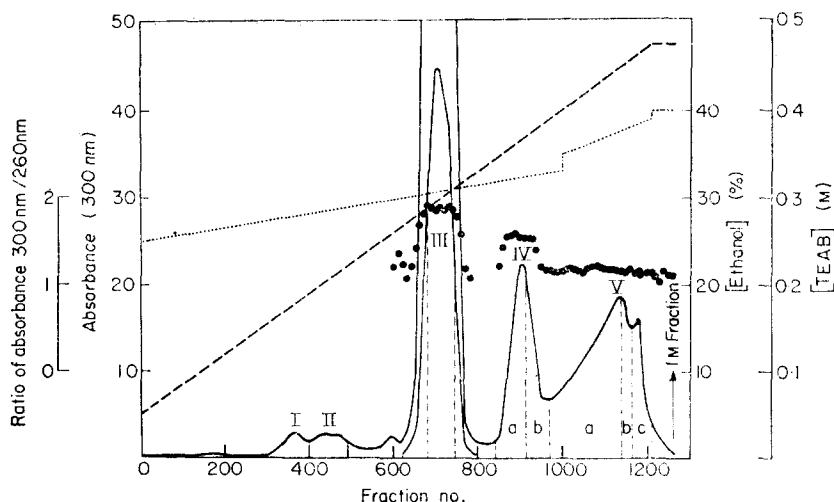


FIG. 9. Chromatography of the reaction products in the preparation of the pentanucleotide d-MMTr-G¹BpC^{An}pTpC^{An}pC^{An} on a DEAE-cellulose column (bicarbonate form, 5.5 cm × 99 cm) pre-equilibrated to 0.05 M-TEAB-25% ethanol. After pyridine was eluted with the same buffer a linear salt gradient was started with 10 l. of 0.05 M-TEAB-25% ethanol in the mixing vessel and 10 l. of 0.40 M-TEAB-33% ethanol in the reservoir. The gradient was continued with 2 l. of 0.40 M-TEAB-35% ethanol in the mixing vessel and 2 l. of 0.475 M-TEAB-39% ethanol in the reservoir. Finally, the column was washed with 0.475 M-TEAB-40% ethanol. Fractions of 20 ml. were collected every 10 min. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table 2. Peak III is also shown in a third of the original scale. (----) Concn TEAB; (....) concn ethanol; (—) absorbance; (●), ratio of absorbance at 300 nm to that at 260 nm.

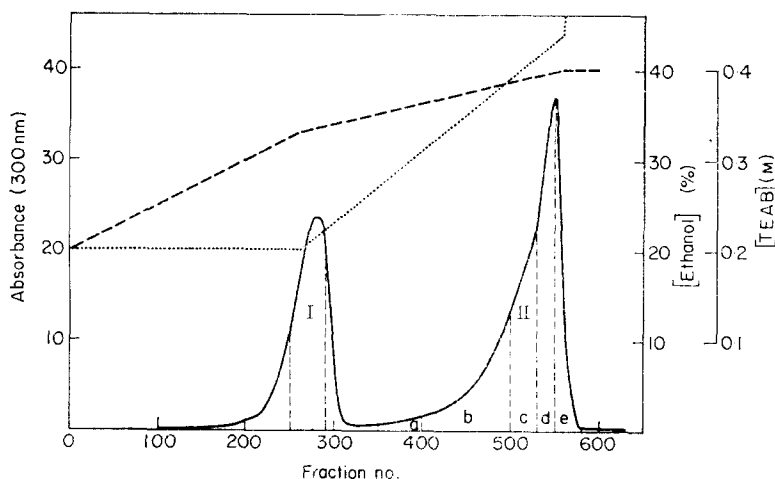


FIG. 10. Rechromatography of a mixture of d-MMTr-G¹BpC^{An}pTpC^{An}pC^{An} with the symmetrical pyrophosphate of d-pC^{An}pC^{An} on a DEAE-cellulose column (bicarbonate form, 3.2 cm × 103 cm) pre-equilibrated to 0.05 M-TEAB-20% ethanol. After pyridine was eluted with the same buffer a linear salt gradient was started with 2.65 l. of 0.20 M-TEAB-20% ethanol in the mixing vessel and 2.65 l. of 0.33 M-TEAB-20% ethanol in the reservoir. The gradient was continued with 3 l. of 0.33 M-TEAB-20% ethanol in the mixing vessel and 3 l. of 0.40 M-TEAB-44% in the reservoir. Fractions of 20 ml. were collected. (----) Concn TEAB; (....) concn ethanol; (—) absorbance.

TABLE 2

Chromatography of the products obtained in the synthesis of the pentanucleotide, d-MMTr-G^{1B}pC^{An}pTpC^{An}pC^{An} (Fig. 9)

Peak no.	Fractions pooled	Total o.d. units at 300 nm	Identification
I	301-400	3800	Unidentified
II	401-490	5000	Mainly d-MMTr-G ^{1B} pC ^{An} pT
III	681-745	137,000	Pure d-pC ^{An} pC ^{An}
IVa	840-914	17,300	Symmetrical pyrophosphate of d-pC ^{An} pC ^{An}
IVb	915-970	11,800	d-O-(pC ^{An} pC ^{An}) ₂ and d-MMTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An}
Va	971-1140	30,200	d-MMTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} (pure)
Vb	1141-1163	7200	d-MMTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} (95% pure)
Vc	1164-1210	8200	d-MMTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An} (70% pure)
	1261-	740	Eluted with 1 M-ammonium bicarbonate solution.

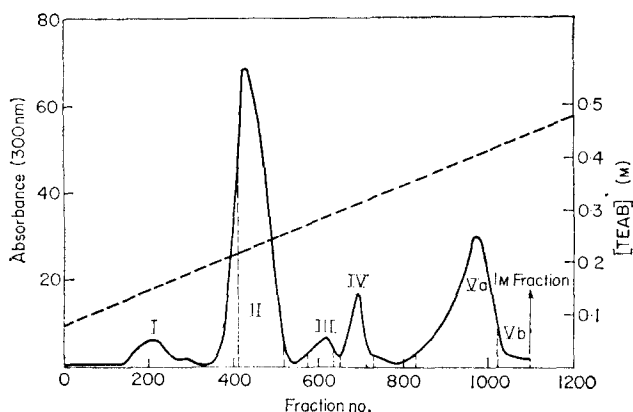


FIG. 11. Chromatography of the reaction products in the preparation of the heptanucleotide d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃pT on a DEAE-cellulose column (bicarbonate form, 5.5 cm × 99 cm) pre-equilibrated to 0.05 M-TEAB-32% ethanol. After pyridine was eluted with the same buffer a linear salt gradient was started with 12 l. of 0.08 M-TEAB-40% ethanol in the mixing vessel and 12 l. of 0.48 M-TEAB-40% ethanol in the reservoir. Fractions of 20 ml. were collected. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table 3. (-----) Concn TEAB; (—) absorbance.

TABLE 3

Chromatography of the products obtained in the synthesis of the heptanucleotide, d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃pT (Fig. 11)

Peak no.	Fractions pooled	Total o.d. units at 300 nm	Identification
I			Unidentified
II	412-520	96,000	Pure d-pC ^{An} pT
III	576-636	6200	Pure d-MMTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An}
IV	650-730	12,900	Symmetrical pyrophosphate of d-pC ^{An} pT
Va	830-1023	61,400	Pure product, d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ pT
Vb	1024-1060	2640	Mainly the heptanucleotide
	1101-	2820	Eluted with 1 M-ammonium bicarbonate solution

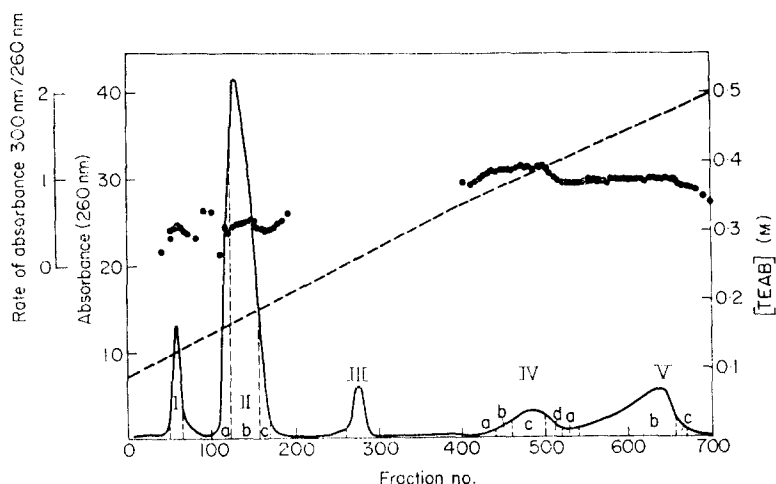


FIG. 12. Chromatography of the reaction products in the preparation of the nonanucleotide $d\text{-MMTr-G}^{18}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3(\text{pT})_2\text{pA}^{Bz}$ on a DEAE-cellulose column (bicarbonate form, $3.2\text{ cm} \times 103\text{ cm}$) pre-equilibrated to $0.05\text{ M-TEAB-35\% ethanol}$. After pyridine was eluted with the same buffer a linear salt gradient was started with $7\text{ l. of }0.09\text{ M-TEAB-35\% ethanol}$ in the mixing vessel and $7\text{ l. of }0.50\text{ M-TEAB-35\% ethanol}$ in the reservoir. Fractions of 20 ml. were collected every 10 min. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table 4. (---) Concn TEAB; (—) absorbance; (●), ratio of absorbance at 300 nm to that at 260 nm .

TABLE 4

Chromatography of the products obtained in the synthesis of the nonanucleotide, $d\text{-MMTr-G}^{18}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3(\text{pT})_2\text{pA}^{Bz}$ (Fig. 12)

Peak no.	Fractions pooled	Total O.D. units at 280 nm	Identification
I	50–60	3490	Mainly cyclic $d\text{-pTpA}^{Bz}$
II	122–156	23,900	Pure $d\text{-pTpA}^{Bz}$
III	260–290	2330	Symmetrical pyrophosphate of $d\text{-pTpA}^{Bz}$
IV	a 421–440	314	Mainly $d\text{-MMTr-G}^{18}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3\text{pT}$
	b 441–460	655	Mostly $d\text{-MMTr-G}^{18}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3\text{pT}$
	c 461–500	2350	Pure $d\text{-MMTr-G}^{18}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3\text{pT}$
	501–520	633	Mixed fraction
V	a 521–540	420	Mixed fraction
	b 541–656	8390	Pure $d\text{-MMTr-G}^{18}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3(\text{pT})_2\text{pA}^{Bz}$
	c 657–670	465	Impure nonanucleotide
	701–	158†	Eluted with $1\text{ M-ammonium bicarbonate solution}$.

† At 300 nm .

tide block $d\text{-pTpA}^{Bz}\text{-OAc}$, gave a satisfactory yield of the nonanucleotide (IV) which separated very well from the unreacted heptanucleotide on chromatography (Fig. 12 and Table 4).

Table 1 summarizes the conditions used in the condensation reactions and the yields obtained in the present work. As may be seen, the isolated yields in the above-described three dinucleotide block condensations were remarkably satisfactory. This

was chiefly because pyrimidine dinucleotide blocks were involved. Further, a six- to tenfold excess of the blocks over the 3'-hydroxylic component was used and the amount of the condensing agent, mesitylenesulfonylchloride, was in the carefully determined range of 0.6 to 0.8 mole/mole of phosphoryl group dissociation. This range is optimal for high yield of the condensation product and minimal breakdown of the polynucleotide chains (present work and work reported in previous papers; see e.g. Hachmann & Khorana, 1969).

As pointed out in the preceding paper (CIV), the technique of paper chromatography was of limited value in ascertaining homogeneity of higher deoxypolynucleotides. In the present work, the purity of the nonanucleotide (IV) and the higher polynucleotides was checked after complete deprotection, by chromatography on an analytical DEAE-cellulose column in the presence of 7 M-urea. The pattern obtained for a sample of the nonanucleotide, G-C-T-C-C-C-T-T-A, is shown in Figure 13.

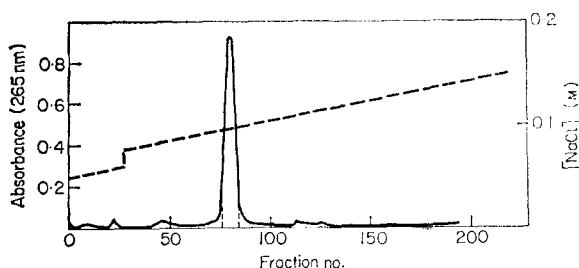


Fig. 13. Analytical chromatography of the nonanucleotide d-G-C-T-C-C-C-T-T-A on an acidic DEAE-cellulose column (Whatman DE23, chloride form, 7 mm $\times$ 950 mm) pre-equilibrated to 7 M-urea, 0.05 M-sodium chloride, pH 3.5 (hydrochloric acid). Immediately after applying the sample a linear salt gradient was started with 500 ml. of 0.05 M-sodium chloride in the mixing vessel and 500 ml. of 0.15 M-sodium chloride in the reservoir, both in 7 M-urea adjusted to pH 3.5 with hydrochloric acid. Fractions of 3.8 ml. were collected every 14 min. (----) Concn NaCl ; (—) absorbance.

Condensation of the protected nonanucleotide (IV) with the protected trinucleotide block (d-pG^{IB}pC^{An}pA^{Bz}.OAc) was carried out using only about a fivefold excess of the latter. (The scale of the condensation reaction at this stage of the synthesis was unusually high.) The chromatographic pattern of the reaction products is shown in Figure 14 and the analysis of the different peaks is given in Table 5. The yield of the isolated product (peak VII) was fair (32%) and the unreacted product (VI) was recovered in an essentially pure form. It should also be added that in most of the present condensations (an exception is given below) complete separation of all of the components present in the reaction mixtures could be accomplished in a single chromatographic run. In particular, the oligonucleotide blocks, which were used in excess, were recovered in good yield in high purity (Table 1), thus making it possible to re-use them in condensation reactions.

The dodecanucleotide obtained in Figure 14 was further checked for purity, after deprotection, by chromatography on a DEAE-cellulose column in the presence of 7 M-urea. This analysis was carried out using buffered salt gradients; the pattern obtained at pH 7.5 is shown in Figure 15, while the pattern obtained in chromatography at pH 3.5 is shown in Figure 16.

TABLE 5

Chromatography of the products obtained in the synthesis of the dodecanucleotide, d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz} (Fig. 14)

Peak no.	Fractions pooled	Total o.d. units at 280 nm	Identification
I			Mainly mesitylenesulfonic acid
II	47-87	7700	Derivative of the tri-block d-pG ^{1B} pC ^{An} pA ^{Bz}
III	87-140	41,700	Essentially pure d-pG ^{1B} pC ^{An} pA ^{Bz}
IV	170-236	3150	Symmetrical pyrophosphate of d-pG ^{1B} pC ^{An} pA ^{Bz}
V	237-315	3430	Unidentified
VI	316-390	15,500	Pure nonanucleotide, d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ (pT) ₂ pA ^{Bz}
VIIa	401-462	12,500	Pure dodecanucleotide, d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ (pT) ₂ pA ^{Bz} pG ^{1B} pC ^{An} pA ^{Bz}
VIIb	463-480	660	Mainly dodecanucleotide
	531-	680	Eluted with 1 M-ammonium bicarbonate solution

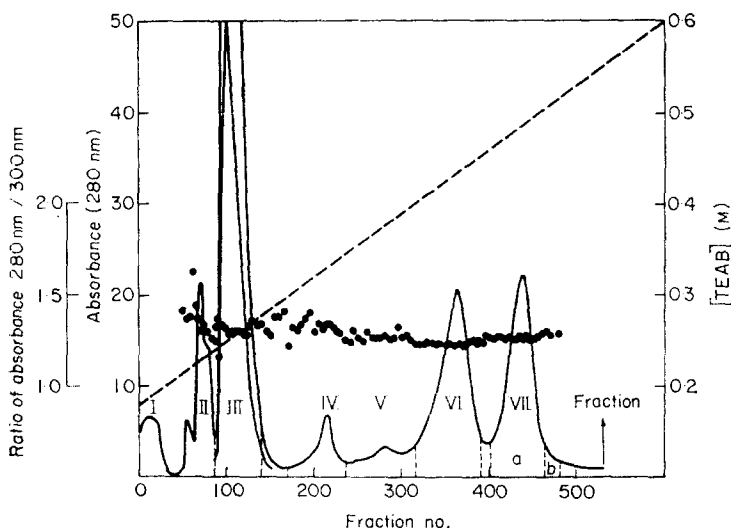


Fig. 14. Chromatography of the reaction products in the preparation of the dodecanucleotide d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz} on a DEAE-cellulose column (bicarbonate form, 3.2 cm × 103 cm) pre-equilibrated to 0.05 M-TEAB-35% ethanol. After pyridine was eluted with the same buffer, a linear salt gradient was started with 6 l. of 0.18 M-TEAB-35% ethanol in the mixing vessel and 6 l. of 0.60 M-TEAB-35% ethanol in the reservoir. Fractions of 20 ml. were collected every 15 min. Fractions were pooled as shown by the vertical broken lines. Peak III is also given in half of the original absorbance scale. For identification of peaks, see Table 5. (----) Concn TEAB; (—) absorbance; (●), ratio of absorbance at 280 nm to that at 300 nm.

Condensation of the protected dodecanucleotide (V) with the tetranucleotide block, d-TpG^{1B}pG^{1B}pG^{1B}-O_iB, was carried out using a 12-fold excess of the latter. The chromatographic pattern obtained is shown in Figure 17 and analysis of the different peaks is in Table 6. As may be seen, the hexadecanucleotide (peak IV) was largely, but not completely, resolved from the starting dodecanucleotide. The yield of the protected product collected as shown in the Figure was 17%, but 63% of the unreacted dodecanucleotide was also recovered in essentially pure form. The unused tetra-

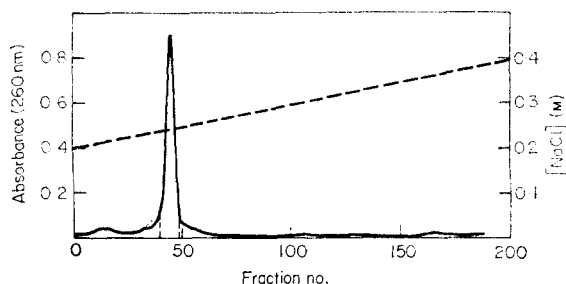


FIG. 15. Analytical chromatography of the dodecanucleotide d-G-C-T-C-C-C-T-T-A-G-C-A on a neutral DEAE-cellulose column (Whatman DE23, chloride form, 5 mm $\times$ 990 mm) pre-equilibrated to 7 M-urea, 0.05 M-sodium chloride, 0.02 M-Tris-HCl (pH 7.5). Immediately after applying the sample a linear salt gradient was started with 300 ml. of 0.20 M-sodium chloride in the mixing vessel and 300 ml. of 0.40 M-sodium chloride in the reservoir, both in 7 M-urea, 0.02 M-Tris-HCl. Fractions of 3 ml. were collected every 15 min. (----) Concn of NaCl; (—) absorbance.

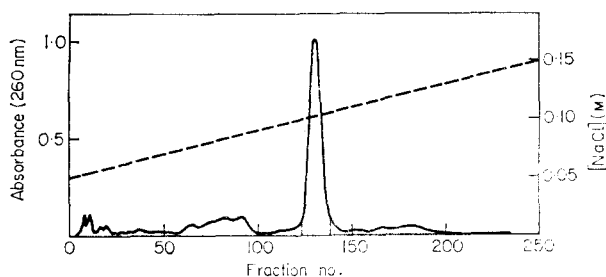


FIG. 16. Analytical chromatography of the dodecanucleotide d-G-C-T-C-C-C-T-T-A-G-C-A on an acidic DEAE-cellulose column (Whatman DE23, chloride form, 7 mm $\times$ 895 mm) pre-equilibrated to 7 M-urea, 0.05 M-sodium chloride and 0.02 M-ammonium formate (titrated to pH 3.5 with hydrochloric acid). Immediately after applying the sample a linear salt gradient was started with 500 ml. of 0.05 M-sodium chloride in the mixing vessel and 500 ml. of 0.15 M-sodium chloride in the reservoir, both in 7 M-urea, 0.02 M-ammonium formate (pH 3.5). Fractions of 4 ml. were collected every 17 min. (----) Concn of NaCl; (—) absorbance.

TABLE 6

Chromatography of the products obtained in the synthesis of the hexadecanucleotide, MMTr-G^{1B} pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B} pC^{An}pA^{Bz}pT(pG^{1B})₂pG^{1B} (Fig. 17)

Peak no.	Fractions pooled	Total o.d. units at 280 nm	Identification
Ia	30-57	6600	Mainly d-pT(pG ^{1B}) ₂ pG ^{1B}
Ib	58-90	16,700	Pure d-pT(pG ^{1B}) ₂ pG ^{1B}
II			Symmetrical pyrophosphate of d-pT(pG ^{1B}) ₂ pG ^{1B}
IIIa	201-230	950	Unidentified
IIIb	231-267	5960	d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ (pT) ₂ pA ^{Bz} pG ^{1B} pC ^{An} pA ^{Bz}
IIIc	277-280	880	Mixed fractions between dodecanucleotide and hexadecanucleotide
IV	281-310	2000	The hexadecanucleotide product
	335-	240†	Eluted with 0.9 M-ammonium bicarbonate-10% ethanol

† At 300 nm.

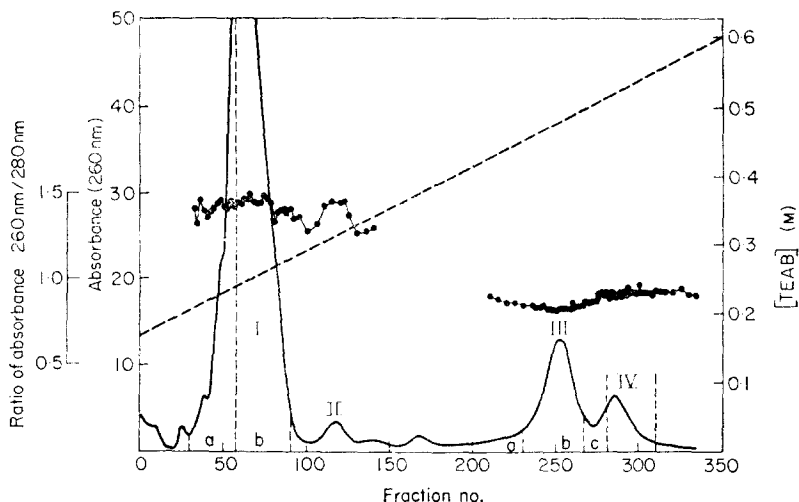


FIG. 17. Chromatography of the reaction products in the preparation of the hexadecanucleotide, $d\text{-MMTr-G}^{18}\text{pC}^{A2}\text{pT(pC}^{A2})_3\text{(pT)}_2\text{pA}^{B2}\text{pG}^{18}\text{pC}^{A2}\text{pA}^{B2}\text{pT(pG}^{18})_2\text{pG}^{18}$, on a DEAE-cellulose column (bicarbonate form, $2.5\text{ cm} \times 84\text{ cm}$) pre-equilibrated to $0.05\text{ M-TEAB-40\% ethanol}$. After pyridine was eluted with the same buffer, a linear salt gradient was started with $3.5\text{ l. of } 0.17\text{ M-TEAB-40\% ethanol}$ in the mixing vessel and $3.5\text{ l. of } 0.60\text{ M-TEAB-40\% ethanol}$ in the reservoir. Fractions of 20 ml. were collected every 20 min. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table 6. (----) Concn of TEAB; (—) absorbance; —●—●—, ratio of absorbance at 260 nm to that at 280 nm .

nucleotide block was also recovered in a good (61%) yield (Table 1). The protected hexadecanucleotide thus obtained was used directly for the synthesis of the icosanucleotide (VII), although a sample recovered unchanged from the latter condensation was subjected to analysis at the fully unprotected stage (see below). The condensation of the hexadecanucleotide with the tetranucleotide block containing only the purines ($d\text{-pA}^{B2}\text{pG}^{18}\text{pA}^{B2}\text{pG}^{18}\text{-OAc}$) was carried out using a very large excess of the latter (Table 1). For isolation of the product, the total reaction mixture was first chromatographed so as to recover the tetranucleotide block and its pyrophosphate. The pattern obtained is shown in Figure 18 and the analysis of the peaks is in Table 7. The larger products, mainly the hexadeca- and the icosanucleotides, were eluted from the column with high salt and rechromatographed on a thinner anion-exchange column. The pattern obtained is shown in Figure 19. As may be seen, the major product was the unreacted hexadecanucleotide which, despite the losses incurred in discarding the fore and the tail end of the peak, was recovered in 49% yield. This sample was subjected to analytical chromatography in 7 M-urea after removal of the protecting groups. The pattern reproduced in Figure 20 showed that the hexadecanucleotide was pure.

The icosanucleotide was present in peak IIIa of Figure 19. After deprotection, a sample was checked for purity by chromatography on a DEAE-cellulose column in the presence of 7 M-urea . The results of two such analyses are shown in Figure 21. The shoulder seen in the pattern, especially in Figure 21(b), has been detected in two repetitions of the separation on a larger scale. One pattern obtained, which is not different from that shown in Figure 21, is shown in Figure 22†. The peak was cut into

† We are grateful to Dr E. Ohtsuka for the preparative fractionation (Fig. 22) of the icosanucleotide.

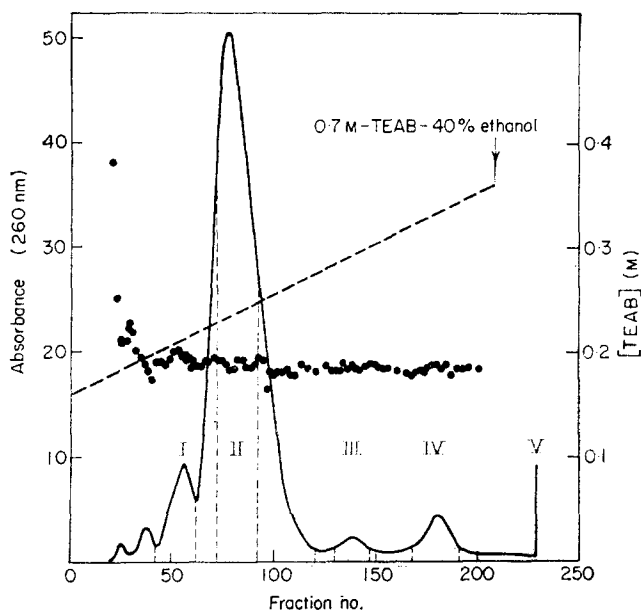


Fig. 18. Chromatography of the shorter oligonucleotides of the reaction mixture in the preparation of the icosanucleotide, $d\text{-MMTr-G}^{1B}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3(\text{pT})_2\text{pA}^{Bz}\text{pG}^{1B}\text{pC}^{An}\text{pA}^{Bz}\text{pT}(\text{pG}^{1B})_3\text{pA}^{Bz}\text{pG}^{1B}\text{pA}^{Bz}\text{pG}^{1B}$, on a DEAE-cellulose column (bicarbonate form, $2.5 \text{ cm} \times 84 \text{ cm}$) pre-equilibrated to $0.05 \text{ M-TEAB-40\% ethanol}$. After pyridine was eluted with the same buffer, a linear salt gradient was started with $2.5 \text{ l. of } 0.16 \text{ M-TEAB-40\% ethanol}$ in the mixing vessel and $2.5 \text{ l. of } 0.40 \text{ M-TEAB-40\% ethanol}$ in the reservoir. Fractions of 20 ml. were collected every 20 min. Fractions were pooled as shown by the vertical broken lines. For identification of peaks, see Table 7. (----) Concn TEAB; (—) absorbance.

TABLE 7

Chromatography to separate short oligonucleotides obtained in the synthesis of the icosanucleotide, $d\text{-MMTr-G}^{1B}\text{pC}^{An}\text{pT}(\text{pC}^{An})_3(\text{pT})_2\text{pA}^{Bz}\text{pG}^{1B}\text{pC}^{An}\text{pA}^{Bz}\text{pT}(\text{pG}^{1B})_3\text{pA}^{Bz}\text{pG}^{1B}\text{pA}^{Bz}\text{pG}^{1B}$ (Fig. 18)

Peak no.	Fractions pooled	Total O.D. units at 280 nm	Identification
I	42-51	890	Derivatives of $d\text{-pA}^{Bz}\text{pG}^{1B}\text{pA}^{Bz}\text{pG}^{1B}$
IIa,c	52-71 93-120	11,400	Impure $d\text{-pA}^{Bz}\text{pG}^{1B}\text{pA}^{Bz}\text{pG}^{1B}$
IIb	72-92	19,600	Pure $d\text{-pA}^{Bz}\text{pG}^{1B}\text{pA}^{Bz}\text{pG}^{1B}$
III	130-147	730	Unidentified
IV	168-191	1430	Probably symmetrical pyrophosphate of $d\text{-pA}^{Bz}\text{pG}^{1B}\text{pA}^{Bz}\text{pG}^{1B}$
V	227-260		Mixture of the hexadecanucleotide (starting material) and the desired product together with impurities

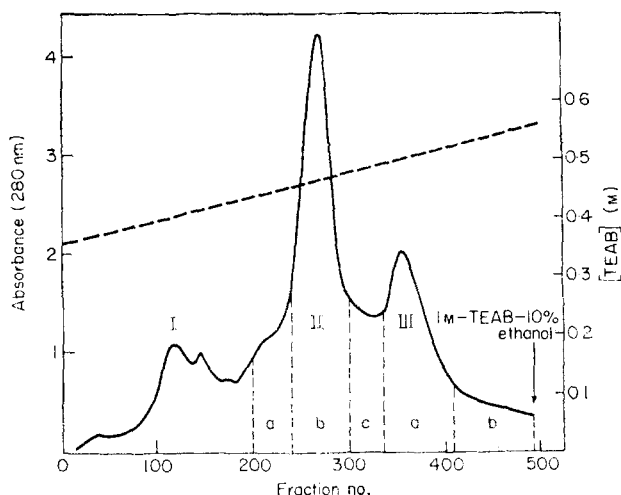


FIG. 19. Chromatography of the higher oligonucleotides of the reaction mixture in the preparation of the icosanucleotide, $d\text{-MMTr-G}^{18}\text{pC}^{\text{An}}\text{pT}(\text{pC}^{\text{An}})_3(\text{pT})_2\text{pA}^{\text{Bz}}\text{pG}^{18}\text{pC}^{\text{An}}\text{pA}^{\text{Bz}}\text{pT}(\text{pG}^{18})_3\text{pA}^{\text{Bz}}\text{pG}^{18}\text{pA}^{\text{Bz}}\text{pG}^{18}$, on a DEAE-cellulose column (bicarbonate form $1.5 \text{ cm} \times 84 \text{ cm}$) pre-equilibrated to $0.05 \text{ M-TEAB-40\% ethanol}$. After pyridine was eluted with the same buffer, a linear salt gradient was started with $1.5 \text{ l. of } 0.35 \text{ M-TEAB-40\% ethanol}$ in the mixing vessel and $0.60 \text{ M-TEAB-40\% ethanol}$ in the reservoir. Fractions of 5 ml. were taken every 16 min. Peak IIIa contained the product. (----) Concn TEAB; (—) absorbance.

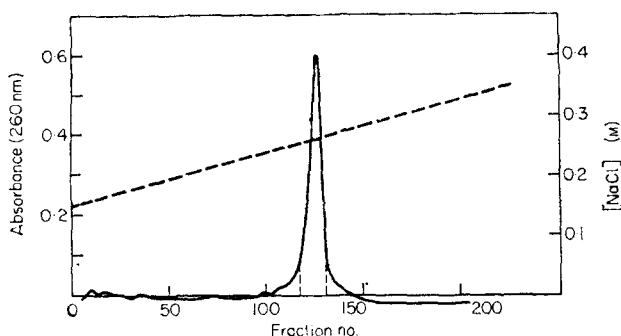


FIG. 20. Analytical chromatography of the recovered hexadecanucleotide $d\text{-G-C-T-C-C-C-T-T-A-G-C-A-T-G-G-G}$ from the preparation of the icosanucleotide $d\text{-MMTr-G}^{18}\text{pC}^{\text{An}}\text{pT}(\text{pC}^{\text{An}})_3(\text{pT})_2\text{pA}^{\text{Bz}}\text{pG}^{18}\text{pC}^{\text{An}}\text{pA}^{\text{Bz}}\text{pT}(\text{pG}^{18})_3\text{pA}^{\text{Bz}}\text{pG}^{18}\text{pA}^{\text{Bz}}\text{pG}^{18}$. Chromatography was carried out on a neutral DEAE-cellulose column (Whatman DE23, chloride form, $7 \text{ mm} \times 950 \text{ mm}$) pre-equilibrated to 7 M-urea , $0.05 \text{ M-sodium chloride}$, $0.02 \text{ M-Tris-HCl (pH 7.5)}$. Immediately after applying the sample, a linear salt gradient was started with $450 \text{ ml. of } 0.15 \text{ M-sodium chloride}$ in the mixing vessel and $450 \text{ ml. of } 0.35 \text{ M-sodium chloride}$ in the reservoir, both solutions 7 M in urea and 0.02 M in Tris-HCl, pH 7.5. Fractions of 4 ml. were collected every 15 min. (----) Concn NaCl; (—) absorbance.

four portions as shown. The material in pooled fraction III gave, on enzymic degradation, analytical values which were in good agreement with the theoretical values for the icosanucleotide (Table 13). Further tests carried out during use of the purified icosanucleotide in enzymic joining reactions showed it to be at least 95% pure. In

more recent work, the icosanucleotide has been purified by anion-exchange chromatography at 65°C in the presence of 7 M-urea and improved resolution has been obtained†.

The oligonucleotide blocks containing 5'-phosphate group and the oligo- and polynucleotides belonging to the icosanucleotide chain were characterized as thoroughly as possible by the techniques of paper chromatography, extensive spectrophotometric analysis, analytical anion-exchange chromatography after removal of the protecting groups (see above) and by quantitative analysis of the products obtained on enzymic degradation. The paper chromatographic analyses of the various protected, partially

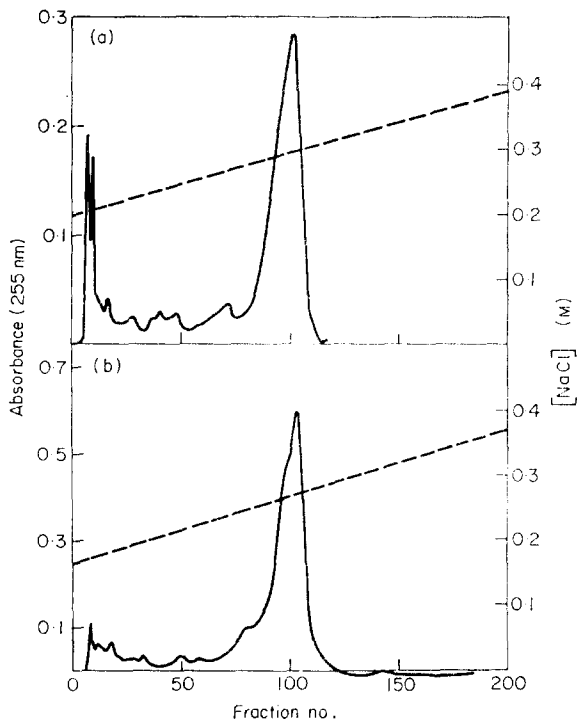


FIG. 21. Analytical chromatography of the icosanucleotide d-G-C-T-C-C-C-T-T-A-G-C-A-T-G-G-G-A-G-A-G on a neutral DEAE-cellulose column (Whatman DE23, chloride form 5 mm × 920 mm) pre-equilibrated to 7 M-urea, 0.05 M-sodium chloride, 0.02 M-Tris·HCl, pH 7.5. Immediately after applying the sample, a linear salt gradient was started with 315 ml. of 0.20 M-sodium chloride in the mixing vessel and 315 ml. of 0.40 M-sodium chloride in the reservoir, both solutions 7 M in urea and 0.02 M in Tris·HCl, pH 7.5. Fractions of 3 ml. were collected every 16 min. (----) Concn NaCl; (—) absorbance. (a) and (b) are the results of two such analyses.

deprotected and fully deprotected oligonucleotides are assembled in Table 8. Tables 9 to 12 give comprehensive spectrophotometric data of the various series of protected and unprotected oligo- and polynucleotides encountered in the present work. Thus, Table 9 assembles the calculated and observed spectral characteristics of the protected mono- and oligonucleotides, while Table 10 does the same for the corresponding unprotected compounds. In Tables 11 and 12 are compiled the spectrophotometric characteristics (calculated and observed) for all of the compounds belonging to the icosanucleotide chain itself, both in the protected (Table 11) and in the unprotected

† Unpublished work of Dr T. Terao in this laboratory.

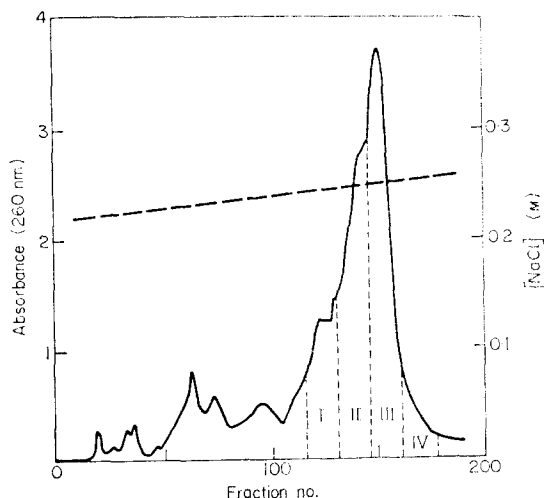


FIG. 22. Large scale purification of the icosanucleotide d-G-C-T-C-C-C-T-T-A-G-C-A-T-G-G-G-A-G-A-G on a DEAE-cellulose column in the presence of 7 M-urea. Approximately 60 o.d.₂₆₀ units of the fully deprotected icosanucleotide product were applied to a DEAE-cellulose column (0.5 cm × 80 cm) in the chloride form. The column had been pre-equilibrated with 0.02 M-Tris-HCl buffer (pH 7.5), 0.05 M-sodium chloride, 7 M-urea. The column was washed with 20 ml. of the above pre-equilibration solution and then subjected to a linear salt gradient elution. The mixing vessel contained 125 ml. of 0.22 M-sodium chloride, 0.02 M-Tris-HCl (pH 7.5), 7 M-urea, while the reservoir contained 0.3 M-sodium chloride plus the buffer and urea. Fractions of 0.66 ml. were collected every 30 min. The main peak containing the nucleotidic product was cut into 4 portions as shown by vertical broken lines. Each pooled portion was combined and desalted by passage through a Biogel-P2 column (1.5 cm × 50 cm) pre-equilibrated with 0.001 M-Tris-HCl, pH 7.6. Pooled fraction I contained a total of 9.4 o.d.₂₆₀ units ($\epsilon_{280}/\epsilon_{260} = 0.61$); pooled fraction II contained a total of 20.8 o.d.₂₆₀ units ($\epsilon_{280}/\epsilon_{260} = 0.58$); pooled fraction III contained a total of 24.2 o.d.₂₆₀ units ($\epsilon_{280}/\epsilon_{260} = 0.55$), while the pooled fraction IV contained a total of 3.2 o.d.₂₆₀ units ($\epsilon_{280}/\epsilon_{260} = 0.63$). (----) Concn NaCl; (—) absorbance.

(Table 12) form. As far as feasible, the oligo- and polynucleotides were characterized by quantitative analysis of the products obtained by degradation with the snake venom and spleen phosphodiesterases (Table 13). Further, it may be added that the successful use (and subsequent analysis of the products) of the hexadecanucleotide and the icosanucleotide in extensive joining reactions mediated by the joining enzymes provides additional characterization of the synthetic products.

Finally, it may be noted that the icosanucleotide herein described represents the polydeoxynucleotide analogue of the alanine transfer RNA segment, which corresponds to the stem and the anticodon loop of the transfer RNA. That the present icosanucleotide can adopt a similar secondary structure (as shown in Fig. 23) has been demonstrated in recent results of nucleotide incorporation studies carried out with DNA polymerases (Kleppe *et al.*, 1971).

3. Experimental Section

General methods and materials

Most of the general methods and reagents used were as described in the preceding papers. Additions or differences worthy of note are as follows.

Paper chromatography was carried out by the descending technique on Whatman no. 1 paper or DEAE-paper (Whatman DE81) in the case of solvent system F. The solvent

TABLE 8
Paper chromatography of deoxyribooligonucleotides

Solvents	$R_F(d\cdot T)^\dagger$		$R_F(d\cdot pT)^\ddagger$		F§
	A	B	C	D	
The growing oligonucleotide chain					
d-G ^{1B}		2.4			
d-MMTr-G ^{1B}	1.49	2.4			
d-MMTr-G ^{1B} pC ^{An}	1.25	2.4			
d-MMTr-GpC	0.90	1.41	1.93		
d-GpC	0.21		1.0	1.37	
d-MMTr-G ^{1B} pC ^{An} pT	0.97	2.0			
d-MMTr-GpCpT	0.46	1.80			
d-GpCpT			0.78	0.85	
d-MMTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An}		1.6			
d-MMTr-GpCpTpCpC			1.35		
d-GpCpTpCpC			0.60	0.50	
d-MMTr-GpCpT(pC) ₃ pT			0.96		
d-GpCpT(pC) ₃ pT			0.26		
d-MMTr-GpCpT(pC) ₃ (pT) ₂ pA			0.70		
d-GpCpT(pC) ₃ (pT) ₂ pA			0.32		
d-MMTr-GpCpT(pC) ₃ (pT) ₂ pApGpCpA			0.53		
The blocks					
d-pC ^{An} pC ^{An} .OAc		1.18			
d-O-(pC ^{An} pC ^{An} .OAc) ₂		1.65			
d-pC ^{An} pC ^{An}		1.02			
d-O-(pC ^{An} pC ^{An}) ₂		0.90			
d-pCpC			0.68	1.06	
d-O-(pCpC) ₂			0.55		
d-pC ^{An} pT.OAc		1.20			
d-pC ^{An} pT		0.99			
d-O-(pC ^{An} pT) ₂		0.94			
d-pCpT			0.85	0.84	
d-O-(pCpT) ₂			0.75	0.32	
d-pTpA ^{Bz} .OAc		1.39			
d-pTpA ^{Bz}		1.07			
d-O-(pTpA ^{Bz}) ₂		0.94			
d-pTpA			0.84		
cyclic d-pTpA ^{Bz}		1.70			
cyclic d-pTpA			1.43		
d-pG ^{1B} pC ^{An}		1.22			
d-pGpC			0.56	0.52	
d-pG ^{1B} pC ^{An} pA ^{Bz} .OAc		1.19			
d-O-(pG ^{1B} pC ^{An} pA ^{Bz} .OAc) ₂		1.48			
d-pGpCpA			0.47	0.62	
d-pTpG ^{1B}		1.16			0.55
d-pTpG			0.61	0.60	
d-pTpG ^{1B} pG ^{1B}		1.11			0.25
d-pTpGpG			0.47	0.15	
d-pT(pG ^{1B}) ₂ pG ^{1B} .OiB		1.30			
d-O-(pTpG ^{1B} pG ^{1B} pG ^{1B} .OiB) ₂		1.69			
d-pT(pG ^{1B}) ₂ pG ^{1B}		1.03			
d-pA ^{Bz} pG ^{1B}		1.28			0.39
d-pApG			0.62	0.94	
d-pA ^{Bz} pG ^{1B} pA ^{Bz}		1.03			0.60
d-pApGpA			0.47	1.00	
d-pA ^{Bz} pG ^{1B} pA ^{Bz} pG ^{1B} .OAc		1.30			
d-pA ^{Bz} pG ^{1B} pA ^{Bz} pG ^{1B}		1.12			0.10
d-pApGpApG			0.44	0.32	

† R_F values relative to d-T. R_F of d-T in solvent A was 0.61.

‡ R_F values relative to d-pT. R_F values of d-pT were: solvent B, 0.54; solvent C, 0.43; solvent D, 0.32.

§ Paper chromatograms in solvent F were run on DEAE-paper (Whatman DE81).

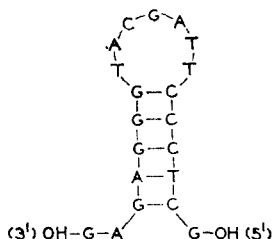


Fig. 23. A plausible secondary structure model for the synthetic icosanucleotide.

systems used were: solvent A, isopropanol/conc. ammonia/water (7:1:2, by vol.); solvent B, ethanol-1M-ammonium acetate (7: 3, by vol., pH 7.5); solvent C, *n*-propanol/conc. ammonia/water (55 : 10 : 35, by vol.); solvent D, isobutyric acid/conc. ammonia/water (66 : 1 : 33, by vol.); solvent E, isobutyric acid/conc. ammonia/water (557 : 38 : 385, by vol.); solvent F, 7 M-urea-0.33 M-ammonium bicarbonate. Thin-layer chromatography was carried out on coated plates of Silicagel having an inorganic fluorescence indicator (Brinkmann F254). The solvent systems used were mixtures of chloroform/ethanol in the following proportions (v/v): solvent G, 95 : 5; solvent H, 5 : 1; solvent I, 1 : 1.

The paper chromatographic mobilities of different compounds (Table 8) were measured usually relative to d-pT, R_F (d-pT), and rel. to d-T in solvent system A, R_F (d-T). For analysis and purity check by paper chromatographic methods usually 10 o.d.₂₆₀ units of the various preparations were applied. The impurities referred to as traces in the text are never more than 5% of the major product, usually 1 to 2%, or less. Chloroform was freed from acid and traces of water by passing it through a short column of aluminium oxide (basic, activity 1). The triethylammonium salts of d-pC^{An}pC^{An} and d-pTpA^{Bz} were prepared according to previously described procedures (Narang, Jacob & Khorana, 1967). The preparation of d-pC^{An}pT is described in the preceding paper.

The calculations of yields in syntheses of protected oligo- and polynucleotides were based on u.v. absorption spectra as theoretically derived by summation of the individual spectra of the protected mononucleotides. Any hypochromicity was ignored. The molar extinctions used for protected mononucleotides are given in the preceding paper. Those used for different oligo- and polynucleotides at selected wavelengths are given in Table 9.

Isolation of the protected oligonucleotides

The general methods of condensation and of column chromatography were described in the preceding paper. The method for the isolation of the protected oligonucleotides was also described previously. Additional details were as follows. Before pooling, fractions from columns were stored at the lowest possible temperature (usually about -20°C) at which they would not freeze. The yield was usually determined from the optical density of the pooled fractions of the product, measured against a blank of distilled water. The buffer was evaporated in the presence of an equal amount of pyridine under a vacuum (0.1 to 0.05 Torr) using a rotary evaporator with coolant liquid (-15°C) circulated through the condenser. The bath temp. never exceeded 30°C . As soon as the buffer solution had evaporated, dry pyridine was fed into the evaporator. Finally, the compounds were dissolved in dry pyridine (about 5 ml./m-mole phosphorus) and added dropwise to anhydrous ether (about 300 ml./m-mole phosphorus) with stirring and exclusion of moisture. The precipitate was collected by centrifugation and washed at least three times by resuspension in fresh ether. After decantation of the supernatant, the nucleotidic material was dried under vacuum at room temp. for at least 1 hr. For storage, all products were kept in precipitated form over calcium chloride at -20°C .

Characterization of polynucleotides by urea column chromatography

Small amounts (20 to 40 o.d. units) of the protected forms of the oligonucleotides (d-pT(pG)₂pG, the nonanucleotide d-GpCpT(pC)₃(pT)₂pA and compounds of longer nucleotide chain-length) were deprotected by successive treatments with ammonia and acetic

TABLE 9

Calculated and observed spectral characteristics of protected mononucleotides and oligonucleotide blocks

Compound	$\lambda_{\max}$ (nm) ($\epsilon_{\max}$ calc.)†	$\lambda_{\min}$ (nm)	Observed absorbance ratio		Calculated ϵ -values‡		
			260 nm/280 nm	280 nm/300 nm	260 nm	280 nm	300 nm
d-pA ^{Bz}	280 (18,300)	245	0.61	2.64	10,800	18,300	5900
d-pC ^{An}	302 (22,300)	236 (252)§	0.69	0.76	12,800	17,700	22,300
d-pG ^{1B}	259 (16,700)	227 (278)§	1.43	2.54	16,700	11,500	4600
d-pC ^{An} pC ^{An}	301 (44,600)	237 (255)§	0.60	0.78	25,600	35,400	44,600
d-pC ^{An} pT	277 (24,100)	295	0.84	1.06	21,600	24,100	22,500
	302 (22,500)	237					
d-pTpA ^{Bz}	276 (25,300)	237 (260)§	0.82	3.15	19,600	24,700	6100
d-pG ^{1B} pC ^{An}	260 (29,500)						
	289 (29,300)	234 (300)§	1.06	0.99	29,500	29,200	26,900
		272					
d-pG ^{1B} pC ^{An} pA ^{Bz}	285 (46,800)	268	0.85	1.33	40,400	47,500	32,900
	262 (40,400)	233					
d-pTpG ^{1B}	261 (25,000)	230	1.43	3.6	25,500	17,900	4800
d-pTpG ^{1B} pG ^{1B}	259 (42,500)	228	1.46	1.80	42,200	29,400	9400
d-pT(pG ^{1B}) ₂ pG ^{1B}	260 (58,900)	227 (280)§	1.43	2.75	58,900	40,900	14,000
d-pA ^{Bz} pG ^{1B}	279 (30,000)	230	0.68	2.45	27,500	29,800	10,500
	262 (27,900)	267					
d-pA ^{Bz} pG ^{1B} pA ^{Bz}	280 (48,100)	230 (262)§	0.79	2.50	38,300	48,100	27,200
d-pA ^{Bz} pG ^{1B} pA ^{Bz} pG ^{1B}	260 (55,000)	228	0.94	2.56	55,000	59,600	21,000
	278 (59,500)	268					

† Interpolation of calculated values on the basis of the observed spectrum.

‡ ϵ -values of oligonucleotides at 260, 280 and 300 nm were calculated by superposition, neglecting hypochromicity.

§ Numbers in brackets are wavelengths (nm) for shoulders.

TABLE 10
Calculated and observed spectral characteristics of unprotected oligonucleotide blocks

Compound	$\lambda_{\max}$ (nm) ($\epsilon_{\max}$ calc.)†	$\lambda_{\min}$ (nm)	Observed absorbance ratio		Calculated ϵ -values‡		
			250nm/260 nm	260 nm/280 nm	260 nm	280 nm	300 nm
d-pCpC	270 (18,200)	251		1.09	14,600	14,600	200
d-pCpT	268 (18,400)	238	0.75	1.22	16,100	13,700	300
d-pTpA	261 (24,200)	231	0.75	2.70	24,200	8600	200
d-pGpC	255 (19,000)	225 (267)§	1.02	1.38	19,000	15,300	500
d-pGpCpA	258 (34,800)	227	0.94	1.97	34,400	17,500	500
d-pTpG	255 (21,400)	230 (268)§	0.99	1.53	20,500	14,400	600
d-pTpGpG	254 (34,200)	227 (270)§	1.05	1.62	32,200	22,400	1000
d-pT(pG) ₂ pG	253 (47,800)	227 (270)§	1.07	1.64	43,900	30,400	1400
d-pApG	255 (28,000)	225	1.01	2.48	27,100	10,200	400
d-pApGpA	257 (42,600)	226	0.92	0.32	42,500	12,400	400
d-pApGpApG	254 (28,000)	225	1.05	2.51	54,200	20,400	800

† Interpolation of calculated values on the basis of the observed spectrum.

‡ ϵ -Values of oligonucleotide blocks at 260, 280 and 300 nm were calculated by superposition, neglecting hypochromicity. The following ϵ -values of mononucleotides (aqueous solution at neutral pH) at 260, 280 and 300 nm, respectively, were used: A (15,400, 2200, 0); C (7300, 7300, 100); G (11,700, 8000, 400); T (8800, 6400, 200).

§ Numbers in brackets are wavelengths (nm) for shoulders.

TABLE 11
Calculated and observed spectral characteristics of protected polydeoxynucleotides

Compound	$\lambda_{\max}$ (nm) ($\epsilon_{\max}$ calc.)†	$\lambda_{\min}$ (nm)	Observed absorbance ratio		Calculated ϵ -values‡		
			260 nm/280 nm	280 nm/300 nm	260 nm	280 nm	300 nm
d-MMTr-G ^{1B} pC ^{An}	288 (30,300)	271 (301)§	1.01	1.03	29,500	30,100	28,600
	263 (29,800)	242 (257)§					
d-MMTr-G ^{1B} pC ^{An} pT	263 (38,800)	240 (280)§	1.08	1.28	38,300	35,600	27,100
d-MMTr-G ^{1B} pC ^{An} pTpC ^{An} pC ^{An}	290 (71,700)	240 (300)§	0.87	0.99	63,900	71,000	71,700
d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ pT	285 (95,400)	239 (300)§	0.87	1.03	85,500	95,100	94,200
d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ (pT) ₂ - pA ^{Bz}	280 (105,000)	243	0.86	1.21	105,000	120,000	100,000
d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ (pT) ₂ - pA ^{Bz} pG ^{1B} pC ^{An} pA ^{Bz}	280 (167,000)	238 (263)§	0.87	1.26	146,000	167,000	133,000
d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ (pT) ₂ - pA ^{Bz} pG ^{1B} pC ^{An} pA ^{Bz} pT(pG ^{1B}) ₂ - pG ^{1B}	278 (208,000)	240			204,000	208,000	147,000
	263 (206,000)	268	0.97	1.39			
d-MMTr-G ^{1B} pC ^{An} pT(pC ^{An}) ₃ (pT) ₂ pA ^{Bz} pG ^{1B} - pC ^{An} pA ^{Bz} pT(pG ^{1B}) ₃ pA ^{Bz} pG ^{1B} - pA ^{Bz} pG ^{1B}	278 (259,000)	243					
	263 (262,000)	268	0.96	1.52	259,000	268,000	168,000

† Interpolation of calculated values on the basis of the observed spectrum.

‡ ϵ -values of oligonucleotides at 260, 280 and 300 nm were calculated by superposition, neglecting hypochromicity.

§ Numbers in brackets are wavelength (nm) for shoulders.

TABLE 12

Calculated and observed spectral characteristics of polydeoxynucleotides without N-protecting groups

Compound	$\lambda_{\max}$ (nm)	$\lambda_{\min}$	Observed absorbance ratio		Calculated ϵ -values†		
	($\epsilon_{\max}$ calc.)†	(nm)	250 nm/260 nm	260 nm/280 nm	260 nm	280 nm	300 nm
d-MMTr-GpC	233	226 (257)§ (272)§	1.07	1.36	19,000	15,300	500
d-GpC	255 (19,900)	223 (270)§	1.02	1.35	19,000	15,300	500
d-MMTr-GpCpT	257 (28,100)	244 (268)§		1.33	27,800	21,700	700
	233	228					
d-GpCpT	265 (broad) (28,200)	229	0.91	1.41	27,800	21,700	700
d-MMTr-GpCpTpCpC	269 (46,400)	246 (232)§	0.92	1.22	42,400	36,300	900
d-GpCpTpCpC	268 (46,400)	231	0.90	1.25	42,400	36,300	900
d-MMTr-GpCpT(pC) ₃ pT	267 (60,500)	243 (233)§	0.91	1.30	58,500	50,000	1200
d-GpCpT(pC) ₃ pT	266 (60,500)	236	0.90	1.33	58,500	50,000	1200
d-GpCpT(pC) ₃ (pT) ₂ pA	264 (86,700)	231	0.84	1.46	82,700	58,600	1400
d-GpCpT(pC) ₃ (pT) ₂ pApGpCpA	262 (118,000)	230	0.87	1.61	117,000	76,100	1900
d-GpCpT(pC) ₃ (pT) ₂ pApGpCpA- pT(pG) ₂ pG	257 (163,000)	230	0.95	1.69	161,000	106,000	3300
d-GpCpT(pC) ₃ (pT) ₂ pApGpCpA- pT(pG) ₃ pApGpApG	257 (217,000)		0.95	1.76	215,000	127,000	4100

† Interpolation of calculated values on the basis of the observed spectrum.

‡ ϵ -values of oligonucleotide blocks at 260, 280 and 300 nm were calculated by superposition, neglecting hypochromicity. The following ϵ -values of mononucleotides (aqueous solution at neutral pH) at 260, 280, and 300 nm, respectively, were used: A (15,400, 2200, 0); C (7300, 7300, 100); G (11,700, 8000, 400); T (8800, 6400, 200).

§ Numbers in brackets are wavelengths (nm) for shoulders.

TABLE 13

Characterization by enzymic degradation of intermediates in the synthesis of the icosanucleotide, d-GpCpT(pC)₃(pT)₂pApGpCpApT(pG)₃pApGpApG

Snake venom diesterase

Compound	Nucleoside and nucleotides				
	d-G	d-pA	d-pC	d-pG	d-pT
d-pGpCpA (D)†					
o.d. units‡		1.72	1.04	1.57	
Observed molar ratio		0.98	1.00	1.01	
Theoretical molar ratio		1	1	1	
d-pT(pG) ₂ pG (B)					
o.d. units‡				3.00	0.75
Observed molar ratio				3.00	1.07
Theoretical molar ratio				3	1
d-pApGpA (D)					
o.d. units‡		6.37		2.96	
Observed molar ratio		1.91		1.00	
Theoretical molar ratio		2		1	
d-pApGpApG (D)					
o.d. units‡		7.92		8.16	
Observed molar ratio		0.86		1.00	
Theoretical molar ratio		1		1	
d-GpCpT(pC) ₃ (pT) ₂ pA (A and D)§					
o.d. units‡	1.06	1.36	3.16		2.22
Observed molar ratio	0.93	1.06	4.18		2.76
Theoretical molar ratio	1	1	4		3
d-GpCpT(pC) ₃ (pT) ₂ pApGpCpA (D and A)¶					
o.d. units‡	1.84	4.44	7.12	2.16	4.28
Observed molar ratio	1.00	2.15	4.95	1.00	2.82
Theoretical molar ratio	1	2	5	1	3

Spleen diesterase

Compound	Nucleoside and nucleotides				
	d-C	d-T	d-Cp	d-Gp	d-Tp
d-GpCpTpCpC (D)					
o.d. units‡	0.98		1.80	1.40	0.85
Observed molar ratio	1.06		1.94	1.00	0.87
Theoretical molar ratio	1		2	1	1
d-GpCpT(pC) ₃ pT (A)					
o.d. units‡		0.83	3.29	1.15	0.68
Observed molar ratio		1.00	4.31	0.98	0.83
Theoretical molar ratio		1	4	1	1

Icosanucleotide fraction III of Fig. 22||

	Nucleotides			
	d-Cp	d-Tp	d-Ap	d-Gp
Found (molar ratio)	5.1	4.0	3.6	5.8
Theoretical (molar ratio)	5	4	4	6

† The solvent system(s) in which the mononucleotides and mononucleosides were separated are shown in brackets.

‡ At $\lambda_{\max}$ at neutral pH. The following $\epsilon_{\max}$ were used: A, 15,400; C, 9100; G, 13,700; T, 9600.

§ d-G was separated from the nucleotides in solvent A and the latter were then separated from each other by following-up chromatography, after drying the paper, in the reverse direction in solvent D.

¶ The mixture of d-pA and d-G obtained in solvent D was separated in solvent A.

|| The degradation was carried out by micrococcal nuclease and then by spleen phosphodiesterase as described in accompanying papers. The analysis was done in the nucleic-acid analyzer on a microscale.

acid, as described in the preceding paper. The dry residue obtained after evaporation of the acetic acid solution was taken up in 0.2 M-TEAB buffer (1 ml.) and extracted three times with ethylacetate (1 ml.). The aqueous phase was evaporated and the residue dissolved in a small amount (0.1 to 0.3 ml.) of the buffer with which the urea column had been pre-equilibrated, and applied on top of the column.

Analytical urea columns were set up in the following manner: DEAE-cellulose (Whatman DE23) pretreated with acid and alkali was stirred in 7 M-urea-0.5 M-sodium chloride solution, which contained the appropriate buffer (0.02 M-Tris-HCl, pH 7.5, or ammonium formate, brought to pH 3.5 by HCl). The column was packed with this slurry and, after a thorough wash with 7 M-urea-0.5 M-sodium chloride solution, the column was pre-equilibrated with 7 M-urea-0.05 M-sodium chloride solution, which contained the appropriate buffer. Linear salt gradients in sodium chloride dissolved in 7 M-urea were started immediately after applying the samples. The absorbancy of individual fractions was measured against a blank of 7 M-urea (the first fraction eluted from the column was used for this purpose). Removal of urea and salt, after pooling of appropriate fractions, was effected by passing the urea solution through a column of Biogel-P2 as described under individual experiments.

3'-O-Acetylation of di- and higher oligonucleotides

The general method was similar to that described in the preceding paper. Thus, for the protected dinucleotides, an anhydrous pyridine solution (28 ml.) of 1 m-mole of the triethylammonium salt of the dinucleotide (e.g. d-pC^{An}pC^{An}) was treated with acetic anhydride (7 ml.) for 4 hr in a closed container. To the clear solution, methanol (14 ml.) was added with cooling and the mixture kept at room temp. for 15 min. Water (28 ml.) was added with cooling and the mixture kept at 10°C overnight. The solvent was evaporated with frequent addition of dry pyridine and the product (e.g. d-pC^{An}pC^{An}-OAc) precipitated twice into ether, washed carefully and collected by centrifugation as usual. The trinucleotide d-pG^{1B}pC^{An}pA^{Bz} (1.3 m-moles) was acetylated by a mixture of acetic anhydride (10 ml.) and pyridine (40 ml.) for 4 hr. The work-up was as described above. The tetranucleotide d-pA^{Bz}pG^{1B}pA^{Bz}pG^{1B} (0.67 m-mole) was acetylated by a mixture of acetic anhydride (8 ml.) and pyridine (40 ml.). The work-up was as described above.

N,3,5'-Tri-isobutyryldeoxyguanosine

Deoxyguanosine (5.70 g, 20 m-moles) was treated with a 10% aqueous solution of tetraethylammonium hydroxide (35 ml.). The resulting clear solution was rendered anhydrous by repeated addition and evaporation of dry pyridine. The colorless solid residue was shaken with dry pyridine (400 ml.) and isobutyric anhydride (100 ml.) in the dark at room temperature for 48 hr. At this stage a sample on thin-layer chromatography in solvent G showed essentially one u.v.-absorbing spot with $R_F = 0.31$ and no material at the origin corresponding to deoxyguanosine. Ethanol (140 ml.) was added with cooling, the reaction mixture kept at room temp. overnight and concentrated to a gum *in vacuo*. Pyridine (250 ml.) was added and the solution concentrated to a volume of about 50 ml. and then taken up in chloroform (1 l.). The chloroform solution was washed three times with 2 M-potassium bicarbonate solution (400 ml.) with addition of ice, then once with saturated sodium chloride solution (200 ml.). The aqueous phases were back-washed with chloroform (2 × 1 l.). The organic layer was rendered anhydrous by filtration through sodium sulfate and the solvent evaporated. The crude product was obtained as an oil and showed on thin-layer chromatography in solvent G one main spot with $R_F = 0.31$ and some additional traces. In solvent H the R_F value of the major spot was 0.58 and in solvent I it was 0.89. The crude product was chromatographed on a column of Silicagel H (Brinkmann), 4 cm × 46 cm, with chloroform containing 9% ethanol (v/v) as eluant. Fractions of 13 ml. were collected every 30 min. Fractions 29 to 34 were pooled and contained pure (checked by thin-layer chromatography) N,3,5'-tri-isobutyryldeoxyguanosine. Nuclear magnetic resonance spectrum, τ -values (protons): 8.76 (18); 7.4 (5); 5.55 (3); 4.58 (1); 3.76 (1); 2.20 (1).

The ultraviolet absorption properties in ethanol were: $\lambda_{\max}$ at 255 to 260 nm (broad) and 280 nm and $\lambda_{\min}$ at 273 nm and at 255 nm; $\epsilon_{260}/\epsilon_{280} = 1.35$, $\epsilon_{280}/\epsilon_{300} = 1.90$.

N-Isobutyryldeoxyguanosine (d-G^{1B})

The crude *N*,3',5'-tri-isobutyryldeoxyguanosine obtained from 10 m-moles of deoxyguanosine was dissolved in ethanol (100 ml.) and 2 M-sodium hydroxide solution (100 ml.) was added at 0°C. The slightly turbid, but homogeneous solution was kept at this temperature for 15 min. Pyridinium Dowex-50 ion-exchange resin (200 ml.) was added with vigorous shaking and the pH checked by paper (about 7). The resin was removed by filtration and washed slowly with ethanol (2 l.). The total filtrate and washings were concentrated *in vacuo* and rendered anhydrous by repeated addition and evaporation of dry pyridine. The residue was taken up in pyridine (25 ml.) and precipitated into a mixture of pentane/ether (2 : 1, v/v). The precipitate was collected by centrifugation and washed once with ether/pentane mixture and three times with pentane. After storage in a vacuum dessicator, *N*-isobutyryldeoxyguanosine was obtained as a fine colorless powder, which showed on thin-layer chromatography in ethanol one spot with $R_F = 0.58$ plus a trace at $R_F = 0.35$. In solvent G the product *N*-isobutyryldeoxyguanosine stayed at the origin. The yield in the above experiment was 3.51 g (quantitative). The spectral properties in ethanol were: $\lambda_{\max}$ at 255 to 260 nm (broad), $\epsilon_{260} = 16,200$ and $\lambda_{\max}$ at 281 nm, $\epsilon_{281} = 11,900$; $\lambda_{\min}$ at 273 nm, $\epsilon_{260}/\epsilon_{280} = 1.37$, $\epsilon_{280}/\epsilon_{300} = 1.96$.

5'-O-Monomethoxytrityl-N-isobutyryldeoxyguanosine (d-MMTr-G^{1B})

N-isobutyryldeoxyguanosine (706 mg, 2 m-moles) was dissolved in pyridine and the solution rendered anhydrous by repeated addition and evaporation of dry pyridine. The residue was taken up in dry pyridine (8 ml.) and monomethoxytritylchloride (800 mg, 2.6 m-moles) added to the reaction solution, which was shaken at room temp. in the dark for 18 hr. Ethanol (1 ml.) was added, the solution kept for 4.5 hr at room temp. and then taken up in chloroform (300 ml.), which had been washed with 0.1 M-ammonium bicarbonate solution. The chloroform solution was extracted with 0.1 M-ammonium bicarbonate solution (3 $\times$ 100 ml.) with addition of ice. The aqueous phase was back-washed with chloroform (1 $\times$ 300 ml.). The organic layers were combined, filtered through a layer of sodium sulfate and evaporated *in vacuo*, using a water aspirator.

The crude product on thin-layer chromatography in solvent H showed three main spots, one with $R_F = 0.43$, which corresponded to the desired product, and other spots with R_F values of 0.62 and 0.73, presumably monomethoxytritanol and monomethoxyphenyldiphenylethoxymethane, respectively. The product was purified by separation on a Silicagel H column (Brinkmann), 4 cm $\times$ 46 cm, with chloroform containing 9% ethanol (v/v) as the eluant. Fractions of 13 ml. were collected every 30 min. Fractions around tube no. 26 contained the material with R_F values of 0.62 and 0.73, whereas the product was collected within the fractions 45 to 58. Later fractions contained, in addition to the product, a trityl-positive substance ($R_F = 0.34$), which was very probably r-MMTr-G^{1B} due to a slight impurity of r-G^{1B} in the starting material.

The fractions containing the product were pooled, the solvent evaporated with addition of pyridine towards the end. The residue was taken up in dry pyridine (10 ml.) and precipitated by drop-wise addition to an excess of pentane (800 ml.). The precipitate was collected by centrifugation, washed three times with pentane and kept in a vacuum dessicator. The yield was 930 mg (17,700 O.D.₂₈₂ units) corresponding to 1.49 m-moles (75%). The spectral properties in ethanol were: $\lambda_{\max}$ at 282, 255 and 235 nm; $\lambda_{\min}$ at 273, 243 and 225 nm with a shoulder at 261 nm; $\epsilon_{260}/\epsilon_{280} = 1.31$, $\epsilon_{280}/\epsilon_{300} = 2.08$.

Pyridinium N-isobutyryl-3'-O-isobutyryldeoxyguanosine 5'-phosphate

Diammonium deoxyguanosine 5'-phosphate (3.9 g, approximately 10 m-moles) was converted to the pyridinium salt as follows.

The nucleotide was dissolved in 120 ml. water and shaken in the dark with 200 ml. of pyridinium Dowex 50W (X8) resin. The total mixture was then run through a column (2.5 cm $\times$ 40 cm) of fresh pyridinium Dowex 50 resin at a speed of about 1 ml. per min. The column was washed with three times the bed volume of 5% aqueous pyridine. The total effluent was made up with pyridine to a 70% aqueous pyridine solution and evaporated to about 30 ml. in a rotary evaporator. The solution was rendered anhydrous by three evaporations following additions of dry pyridine (50 ml.). Finally, the residue was taken

up in 100 ml. of dry pyridine and to the solution was added 60 ml. of reagent-grade distilled isobutyric anhydride. The mixture was shaken in the dark at room temp. for 5 days. The solution at this stage was practically clear and paper chromatography showed the absence of all of the starting material. Methanol (50 ml.) was then added with cooling (bath temp. -20°C). After a further 20 min at room temp. 100 ml. of 20% pyridine was added and the mixture was allowed to stand for 16 hr in the dark at room temp. Concentration and evaporation were carried out in the usual manner and the pyridinium salt of the nucleotide was precipitated as a dry powder.

Preparation of pyridinium N-isobutyryl deoxyguanosine 5'-phosphate

Approximately 5 g of d-pG^{1B}-OiB was dissolved in 150 ml. of 50% pyridine. Then 150 ml. of cooled 2 M-NaOH was added while the flask was being cooled in an ice bath. It was important that the solution be homogeneous. If it was not, the addition of a measured amount of water was added to obtain a clear solution. After 7 min reaction at 0°C , 200 ml. of pyridinium Dowex 50W (X8) resin were added to neutralize to pH 6.7 to 7.0. The resin-nucleotide mixture was passed through a column (4 ml./min) of 50 ml. fresh pyridinium Dowex 50W (X8) resin and the resin washed with three times the bed volume or 750 ml. of 30% pyridine. The total effluent was concentrated with added pyridine as usual. The syrupy solution of d-pG^{1B} was precipitated as a fine powder from a pyridine/ether mixture in the usual manner.

Comparison of the stabilities of N-acyldeoxyguanosine 5'-phosphates in 1 N-sodium hydroxide

The protected nucleotide, d-pG^{Ac}-OAc or d-pG^{1B}-OiB, (40 mg) was dissolved in a mixture of pyridine (0.4 ml.) and water (0.6 ml.) at 4°C . Sodium hydroxide (1 ml. of 2 M), which had been cooled to 4°C , was added with vigorous agitation. From the homogeneous reaction mixtures, samples of 0.10 ml. were taken at intervals and added to 10% aqueous pyridine (0.30 ml.), which contained Dowex-50 ion-exchange resin in the pyridinium form (0.2 ml. of the resin). The neutralized mixture was stirred vigorously and samples of 50 μl . representing about 10 o.d. units were applied to paper chromatograms. The system used for chromatography was solvent B.

In the case of *N,O*-diacetyl-deoxyguanosine 5'-phosphate, 25 sec after the start of the alkaline treatment the neutralized mixture showed the following ultraviolet-absorbing spots: (R_F values rel. to d-pT) d-pG (0.55), d-pG^{Ac} (0.87), d-pG^{Ac}-OAc (1.10) and an additional, unidentified spot ($R_F = 1.61$; λ_{max} at 240 nm, probably non-nucleotidic). In the case of *N,O*-di-isobutyryldeoxyguanosine 5'-phosphate, paper chromatography showed spots with the following R_F values: d-pG^{1B} (1.43), d-pG (0.49) and d-pG^{1B}-OiB (2.07).

The spots were eluted with water and their concentrations determined spectrophotometrically using appropriate blanks.

The concentrations (expressed as the percentage of total for samples removed at each time interval) of the different compounds were found to be as follows.

Time (min)		20	40	60	120
A. d-pG ^{Ac} -OAc	d-pG ^{Ac} (%)	94	91	89	82
	d-pG (%)	6	9	11	18
B. d-pG ^{1B} -OiB	d-pG ^{1B} (%)	98	97	96.5	95
	d-pG (%)	2	3	3.5	5

The dinucleotide, d-pG^{1B}pC^{An}

An anhydrous pyridine solution (45 ml.) of pyridinium d-CE-pG^{1B} (11.9 m-moles, 199,000 o.d.₂₅₉ units), pyridinium d-pC^{An}-OAc (10.5 m-moles, 236,000 o.d.₃₀₂ units), containing *N,N*-di-isopropylethylamine (21 m-moles) was treated with MS (4.32 g, 19.7 m-

moles) for 1 hr with exclusion of moisture. A 1 M-solution of di-isopropylethylamine in pyridine (18.4 ml.) and very shortly afterwards water (50 ml.) was added with cooling. After standing overnight at 10°C the alkaline treatment (20 min at 0°C) was carried out as described in the General Methods and Materials section. The crude reaction mixture was purified on a DEAE-cellulose column (bicarbonate form, 5.5 cm × 99 cm) pre-equilibrated with 0.05 M-TEAB-35% ethanol. After pyridine had been eluted with the same buffer a linear salt gradient was started with 5.2 l. of 0.05 M-TEAB-35% ethanol in the mixing vessel and 5.2 l. of 0.137 M-TEAB-35% ethanol in the reservoir. The gradient was continued in a similar way with 6 l. of 0.137 M-TEAB-35% ethanol and 6 l. of 0.26 M-TEAB-20% ethanol. Fractions of 20 ml. were collected every 10 min. The dinucleotide was eluted between fractions 570 and 780 (salt concentration 0.15 to 0.18 M). After the product was eluted completely, the column was washed with 1 M-ammonium bicarbonate. A total of 15,000 o.d.₂₆₀ units was obtained in the 1 M-fraction. Only the second half of the product peak had a constant ratio $\epsilon_{300}/\epsilon_{260}$ of 0.96 and was homogeneous on paper chromatography in solvent B. The fractions 635 to 764 were pooled to give d-pG^{1B}pC^{An} in the amount of 81,000 o.d.₂₆₀ units (2.75 m-moles, 26%). The volatile salt was evaporated in the presence of excess pyridine and the product collected by precipitation from ether as usual. After ammonia treatment, sample showed on paper chromatography in solvent C one main spot and a very faint trace with an R_F of 0.77 relative to d-pT. In solvent H only one spot was observed.

The cyanoethylated dinucleotide, d-CE-pG^{1B}pC^{An}

Triethylammonium d-pG^{1B}pC^{An} (2.75 m-moles, 81,000 o.d. units) was dissolved in 30% pyridine (50 ml.) and the solution was passed through a column of pyridinium Dowex W50 (X8) ion-exchange resin (100 ml.). The resin was washed with 50% pyridine (300 ml.) and the total effluent evaporated in the presence of more pyridine. The residue was rendered anhydrous by addition and evaporation of dry pyridine and finally dissolved in dry pyridine (30 ml.). β -Cyanoethanol (10 ml.), dry pyridinium Dowex W50 (X8) ion-exchange resin (2 g) and DCC (5.7 g, 27.5 m-moles) were added and the reaction mixture was shaken for 22 hr. Paper electrophoresis at pH 7.1 at this stage showed no starting material, but two major spots with an electrophoretic mobility of 0.26 and 0.71 rel. to d-pG^{1B}pC^{An}. Water (50 ml.) was added and the reaction mixture extracted with cyclohexane (3 × 200 ml.). The aqueous phase was kept at room temp. overnight, made to 20% in pyridine by adding more water. Dicyclohexylurea was removed by filtration and washed with 20% pyridine. The total filtrate was evaporated with an excess of pyridine, the product precipitated twice and collected as usual by centrifugation. The product showed on paper chromatography in solvent B two main spots with R_F values of 1.52 and 1.75 rel. to d-pG^{1B}pC^{An}. The yield, determined by ultraviolet absorption, was 2.56 m-moles (93%).

The trinucleotide, d-pG^{1B}p^{An}CpA^{Bz}

An anhydrous pyridine solution (50 ml.) of pyridinium d-CE-pG^{1B}pC^{An} (2.56 m-moles, 76,000 o.d.₂₆₀ units), pyridinium d-pA^{Bz}-OAc (7.20 m-moles) and MS (3.15 g, 14.4 m-moles) was shaken at room temp. for 2 hr. The work-up of the reaction mixture including an alkaline treatment (20 min at 0°C) was carried out as described above. Column chromatography was carried out as shown in Fig. 3. Peak I contained mesitylene sulfonic acid. The central portion of peak III (fractions 270 to 332) consisted of pure d-pA^{Bz} (63% of the original amount). Peak VI (fractions 601 to 700, 5000 o.d.₂₆₀ units) contained 7% of the original dinucleotide d-pG^{1B}pC^{An} and was impure. Peak VIII (fractions 854 to 986) consisted of pure trinucleotide, all the fractions having a constant ratio of $\epsilon_{280}/\epsilon_{260} = 1.17$. After fraction 1000 the column was washed with 1 M-ammonium bicarbonate solution. A total of 15,000 o.d.₂₆₀ units was thus eluted from the column.

The pooled product fractions (yield, 62,500 o.d.₂₆₀ units, 52%) were evaporated in the presence of pyridine and the trinucleotide finally precipitated from an excess of dry ether as usual. On paper chromatography in solvent B it showed one main spot and, in addition, a very faint fluorescence at R_F (pT) = 0.51. After ammonia treatment, the compound was homogeneous on paper chromatography in solvent C. In solvent D, however, the following very faint traces were detected in addition to the trinucleotide spot with

R_F (d-pT) values of 0.15, 0.31 and 0.77. For enzymic degradation, the pure product obtained after chromatography in solvent D was used.

The dinucleotide, d-pTpG^{1B}

An anhydrous pyridine solution (70 ml.) of d-CE-pT (6.34 m-moles), d-pG^{1B}-OiB (12.4 m-moles) and MS (5.52 g, 25.2 m-moles) was shaken at room temp. for 2 hr. The work-up of the reaction mixture including an alkali treatment (20 min at 0°C) was carried out as usual. The reaction mixture was chromatographed on a DEAE-cellulose column (bicarbonate form, 5.5 cm × 99 cm) pre-equilibrated to 0.05 M-TEAB-10% ethanol. After pyridine had been eluted with the same buffer, a linear salt gradient was started with 12 l. of 0.05 M-TEAB-10% ethanol in the mixing vessel and 12 l. of 0.25 M-TEAB-10% ethanol in the reservoir. Fractions of 20 ml. were collected every 5 min. The dinucleotide started to elute from the column at fraction 470 with a salt concentration of 0.13 M. All tubes (484 to 700) which showed a constant ratio of absorbance at 290 nm/260 nm of 1.45 were pooled to give a yield of 122,000 o.d.₂₆₀ units (4.78 m-moles, 75%).

After complete elution of the product, the column was washed with 0.75 M-ammonium bicarbonate; 17,500 o.d.₂₆₀ units of higher oligonucleotides were obtained. The product peak was evaporated with an excess of pyridine and the dinucleotide d-pTpG^{1B} collected by precipitation. On paper chromatography in solvent B it showed, in addition to the main spot, a faint fluorescence moving ahead of d-pTpG^{1B} and a spot of d-pG at an R_F (d-pT) of 0.50. The impurities were less than 5%.

A sample after ammonia treatment showed on paper chromatography in solvent C a very faint trace at R_F (d-pT) = 1.21, in addition to the main spot. On paper chromatography in solvent D two additional faint spots besides the dinucleotide were observed: R_F (d-pT) values of 0.84 and 1.55.

The cyanoethylated dinucleotide, d-CE-pTpG^{1B}

The cyanoethylation of d-pTpG^{1B} (4.78 m-moles) was carried out in an analogous fashion to the preparation of d-CE-pG^{1B}pC^{An} (see above). The product obtained was free from starting material as checked by paper electrophoresis at pH 7.1.

The trinucleotide, d-pTpG^{1B}pG^{1B}

An anhydrous pyridine solution (70 ml.) of d-CE-pTpG^{1B} (obtained from 4.78 m-moles of d-pTpG^{1B}), pyridinium d-pG^{1B}-OiB (11.9 m-moles) and MS (5.5 g, 25 m-moles) was shaken for 2 hr at room temp. Work-up including an alkaline treatment (10 min at 0°C and 10 min warming up to room temp.), was as described in the General Methods and Materials section. Column chromatography of the reaction mixture was carried out as shown in Fig. 4. Peak I was mainly pyridine and peak II the recovered mononucleotide d-pG^{1B}. Peak V consisted essentially of d-pTpG^{1B}, but showed an additional considerable contaminant with R_F (d-pT) = 0.55 on paper chromatography in solvent D.

Peak VI, containing the trinucleotide product, and the fractions 711 to 920, which showed a constant ratio of $\epsilon_{290}/\epsilon_{260} = 0.54$, were pooled. A total of 93,600 o.d.₂₆₀ units (2.22 m-moles) was obtained corresponding to a yield of 46% calculated on the basis of the dinucleotide d-pTpG^{1B} (2 reaction steps). A purity check on paper chromatography in solvent B revealed two additional spots with R_F (d-pT) values of 0.75 (faint) and 1.83 (very faint).

After ammonia treatment the product showed on paper chromatography in solvent C, besides the main spot, a faint one at an R_F (d-pT) = 0.83. In solvent H this impurity was located at an R_F (d-pT) of 0.78.

The cyanoethylated trinucleotide, d-CE-pTpG^{1B}pG^{1B}

A mixture of pyridinium d-pTpG^{1B} (84,000 o.d.₂₆₀ units, 1.99 m-moles), β -cyanoethanol (16 ml.), dry pyridinium Dowex 50W (X8) ion-exchange resin (3 g) and DCC (20 g, 97 m-moles) was shaken in a sealed flask for 46 hr. At this stage, paper electrophoresis of the sample at pH 7.1 showed no starting material but two spots of nucleotidic material at electrophoretic mobilities of 0.18 and 0.55 rel. to the starting material. The work-up was as described above for d-CE-pG^{1B}pC^{An}. After the first precipitation, high-speed centrifugation

was used to obtain a clear supernatant phase. A second precipitation produced a precipitate which sedimented readily. The yield was quantitative.

The tetranucleotide, d-pT(pG^{1B})₂pG^{1B}

An anhydrous pyridine solution (44 ml.) of pyridinium d-CE-pTpG^{1B}pG^{1B} (2.04 m-moles, 86,000 o.d.₂₆₀ units), pyridinium d-pG^{1B}-OiB (8 m-moles) and MS (3.65 g, 16.6 m-moles) was shaken for 2 hr at room temp. Work-up, including alkali treatment (20 min at room temp.), was as usual. Before column chromatography of the reaction mixture a sample was chromatographed on DEAE-paper. In solvent F the three expected spots of d-pG^{1B}, d-pTpG^{1B}pG^{1B} and of the product were observed. The column chromatography was carried out as shown in Fig. 5. Peak I contained the mononucleotide d-pG^{1B}, peak III (12,800 o.d.₂₆₀ units) mostly d-pTpG^{1B}pG^{1B} as shown by paper chromatography on DEAE-cellulose paper in solvent F. Peak IV containing the product was pooled between fractions 371 to 530, which had a constant ratio of $\epsilon_{260}/\epsilon_{290}$ of 1.76. The yield at this stage was 0.90 m-mole (53,200 o.d.₂₆₀ units, 44%). The product was isolated in solid form by the usual method. Examination of a small portion of the product after removal of the *N*-protecting groups showed an impurity (about 7%) (R_F (d-pT) = 0.53 in solvent C and = 0.46 in solvent D, the tetranucleotide having zero mobility in these systems). The above product was therefore rechromatographed as shown in Fig. 6. Peak I (fractions 96 to 117) contained the impurity, which remains unidentified. Peak II (fractions 153 to 250) contained the pure tetranucleotide d-pTpG^{1B}pG^{1B}pG^{1B} (47,000 o.d.₂₆₀ units, 0.8 m-mole, 39%) and was isolated in the precipitated form in the usual manner. The purity of this preparation was further checked by chromatography of a small portion, after removal of protecting groups, on a cellulose column in 7 M-urea and a sodium chloride gradient.

3'-O-Isobutyrylation of the tetranucleotide, d-pT(pG^{1B})₂pG^{1B}

An anhydrous pyridine solution (50 ml.) of triethylammonium d-pT(pG^{1B})₂pG^{1B} (0.80 m-mole) was treated with isobutyric anhydride (10 ml.) for 48 hr at room temp. in a sealed flask in the dark. Methanol (15 ml.) was added with cooling and the reaction mixture was kept at 0°C for 1 hr. Water (50 ml.) was added with cooling, the solution kept overnight at 10°C and finally evaporated with addition of more pyridine. The tetranucleotide d-pT(pG^{1B})₂pG^{1B}-OiB was collected by two precipitations from anhydrous ether as usual. On paper chromatography in solvent B it gave a very strong spot of the product and an additional spot at an R_F (d-pT) of 1.69 (symmetrical pyrophosphate of d-pT(pG^{1B})₂pG^{1B}-OiB). The yield was 0.69 m-mole (86%).

The dinucleotide, d-pA^{Bz}pG^{1B}

An anhydrous pyridine solution (60 ml.) of pyridinium d-CE-pA^{Bz} (10 m-moles), pyridinium d-pG^{1B}-OiB (11 m-moles) and MS (5.25 g, 24 m-moles) was shaken at room temp. for 130 min. Subsequent work-up including an alkali treatment (20 min at room temp.) was as usual. The reaction mixture was checked by chromatography on DEAE-cellulose paper in solvent F at this stage and contained the product, d-pG^{1B} and a trace of d-pA^{Bz}. The reaction mixture was chromatographed on a DEAE-cellulose column (bicarbonate form, 5.5 cm × 99 cm) pre-equilibrated to 0.05 M-TEAB-10% ethanol. After pyridine had been washed off with the same buffer, a linear salt gradient was started with 12 l. of 0.05 M-TEAB-10% ethanol in the mixing vessel and 0.20 M-TEAB-10% ethanol in the reservoir. Fractions of 20 ml. were collected every 5 min. The dinucleotide started to elute from the column at a salt concentration of 0.135 M and was obtained in fractions 748 to 1010, which had a constant ratio of $\epsilon_{260}/\epsilon_{280}$ = 0.92 (purity checked by paper chromatography in solvent B). The product was isolated by precipitation from an excess of dry ether. The yield was 5.45 m-moles (54% as based on d-CE-pA^{Bz}).

Cyanoethylated d-pA^{Bz}pG^{1B}

An anhydrous pyridine solution (50 ml.) of pyridinium d-pA^{Bz}pG^{1B} (2.56 g, 2.68 m-moles), β -cyanoethanol (8.9 ml., 134 m-moles) and DCC (5.36 g, 26.8 m-moles) was kept for 20 hr at room temp. Paper electrophoresis of a sample at this stage showed complete absence of the starting material. The work-up including treatment with water, extraction with cyclo-

hexane and precipitation from an excess of ether was as described in the above cyanoethylations. The product was obtained as a fine precipitate after a second precipitation from ether in the yield of 76,200 o.d.₂₈₀ units (2.56 m-moles, 95%).

The tetranucleotide, d-pA^{Bz}pG^{1B}pA^{Bz}pG^{1B}†

An anhydrous pyridine solution (about 30 ml.) of d-CE-pA^{Bz}pG^{1B} (2.56 m-moles) and d-pA^{Bz}pG^{1B}.OAc (2.64 m-moles) was treated with freshly crystallized MS (2.57 g; 11.7 m-moles) for 2 hr at room temp. in the dark. The mixture was then treated in the cold with *N,N*-di-isopropyl *N*-ethylamine (23.4 ml. of 1 M) in pyridine and 30 ml. of water, and kept overnight at -5°C . The work-up, including an alkaline treatment, was by the standard method and the total product was chromatographed on a DEAE-cellulose column (3.2 cm $\times$ 103 cm). The elution pattern is shown in Fig. 7. Peak I contained unreacted d-pA^{Bz}pG^{1B} and peak II contained essentially pure tetranucleotide. However, for complete purification, fractions 471 to 600 were combined and the product, after the usual isolation, was rechromatographed on the same DEAE-cellulose column (3.2 cm $\times$ 103 cm), but using a shallower salt gradient. Pure tetranucleotide (d-pA^{Bz}pG^{1B}pA^{Bz}pG^{1B}) was obtained in a yield of 39,700 o.d.₂₈₀ units (0.67 m-mole). The purity was checked by chromatography in solvent B and in solvents C and D after removal of protecting groups.

The dinucleotide, d-MMTr-G^{1B}pC^{An}

5'-O-Monomethoxytrityl-*N*-isobutyryldeoxyguanosine (4.02 g, 7.30 m-moles) and pyridinium d-pC^{An}.OAc (9.44 m-moles) were dissolved in dry pyridine (85 ml.) and the solution treated with dry pyridinium Dowex-50 (X2) ion-exchange resin (2 g) and DCC (13.6 g, 66 m-moles) and the mixture shaken in the dark with exclusion of moisture for 98 hr. At this stage a sample of the reaction mixture showed, on thin-layer chromatography in solvent H, trityl-positive material streaking up from the origin, a behavior characteristic of the expected product‡, and two additional spots with R_F values of 0.58 (d-MMTrG^{1B}) and 0.72 (methoxytritanol?). After addition of water (40 ml.) and ether (40 ml.) with cooling, the reaction mixture was kept overnight. The aqueous phase was made to a vol. of 200 ml. with pyridine/water (1 : 1, v/v) and extracted with ether (3 $\times$ 500 ml.). The ether phase was back-washed with 50% pyridine (200 ml.) followed by 40% pyridine (2 $\times$ 200 ml.).

The ether phase showed on thin-layer chromatography in solvent H only two spots with R_F values of 0.58 (d-MMTr-G^{1B}) and 0.72 and no trityl-positive material at the origin.

The aqueous phase was concentrated with frequent addition of pyridine and the residual gum, which contained only very little pyridine, was taken up in chloroform (500 ml.) which had been washed with 0.2 M-TEAB. The organic layer was washed with 0.2 M-TEAB (3 $\times$ 50 ml.) and the TEAB portions back-washed with chloroform (3 $\times$ 400 ml.). The TEAB solution on paper chromatography in solvent D contained only d-pC^{An}.OAc (R_F = 1.51 rel. to d-pT) and the symmetrical pyrophosphate of d-pC^{An}.OAc (R_F = 1.77 rel. to d-pT). The combined chloroform phase was filtered through sodium sulfate and evaporated *in vacuo* with addition of pyridine. The residue showed on thin-layer chromatography in solvent I one main trityl-positive spot with an R_F of 0.45 and some additional traces. This product was dissolved in pyridine (50 ml.) and water (50 ml.) and the solution cooled to 0°C . At this temp. cooled 2 M-sodium hydroxide solution (100 ml.) and ethanol (100 ml.) were added. The clear solution was kept at 0°C for 10 min and then neutralized with pyridinium Dowex-50 (X8) (200 ml.) and poured onto an additional amount of resin (100 ml.) packed in a suitable column. The resin was washed with 50% pyridine (1 l.) and the total effluent concentrated *in vacuo* with the addition of dry pyridine to a final vol. of 20 ml. This product was precipitated from an excess of ether, the precipitate collected by centrifugation and resuspended and washed three times with ether and dried in a high vacuum. Yield was 5.35 g (4.78 m-moles, 66%). The product showed on thin-layer chromatography in solvent I one main spot with an R_F of 0.21 and a trace of an impurity.

† We are grateful to Dr M. Poonian for this preparation.

‡ The mononucleotide component also stays at the origin on thin-layer chromatography in solvent H. The purpose of thin-layer chromatography in this analysis was to estimate the extent of reaction by determining the amount of unreacted d-MMTr-G^{1B} (R_F = 0.58).

The trinucleotide, d-MMTr-G¹⁸pC^{An}pT

An anhydrous pyridine solution (4 ml.) of pyridinium d-MMTr-G¹⁸pC^{An} (0.23 m-mole), pyridinium d-pT-OAc (0.68 m-mole) and TPS (617 mg, 0.03 m-mole) was shaken at room temp. for 3 hr. Water (2 ml.) and a 1 M-solution of *N,N*-di-isopropylethylamine in pyridine (4.1 ml.) was added with cooling and the solution kept overnight at 10°C. The mixture was treated with 1 M-sodium hydroxide solution for 10 min at 0°C and then neutralized. Column chromatography of the reaction mixture was carried out as shown in Fig. 8. Peak I contained d-pT (3700 o.d.₂₆₇ units), and peak II an unidentified trityl-positive product. Peak III contained the desired trinucleotide d-MMTr-G¹⁸pC^{An}pT. Fractions 235 to 337 were pooled according to a constant ratio of $\epsilon_{260}/\epsilon_{300} = 1.37$ to give a yield of 5300 o.d.₂₆₃ units (0.14 m-mole, 62%) and were evaporated with pyridine. The product showed essentially a single spot on paper chromatography in the solvents A and B. A sample, after removal of the *N*-protecting groups, showed essentially 1 spot (trityl-positive) on paper chromatography in solvent C; after treatment with acetic acid the product was again homogeneous, as shown by chromatography in solvents C and D.

*The pentanucleotide, d-MMTr-G¹⁸pC^{An}pTpC^{An}pC^{An}**With mesitylenesulfonylchloride as condensing agent*

An anhydrous pyridine solution (40 ml.) of triethylammonium d-MMTr-G¹⁸pC^{An}pT (1.00 m-mole), triethylammonium d-pC^{An}pC^{An}-OAc (6.14 m-moles) and MS (2.80 g, 12.8 m-moles) was shaken at room temp. for 1.5 hr. The work-up involving an alkaline treatment (10 min at 0°C) was as described in the General Methods and Materials section. Column chromatography of the reaction mixture was carried out as shown in Fig. 9. The distribution and identification of the u.v.-absorbing compounds is shown in Table 2. Peak III, the pure portion of d-pC^{An}pC^{An} (50% of the starting material), having a constant ratio of $\epsilon_{260}/\epsilon_{300} = 0.47$, showed one spot on paper chromatography in solvent B and proved also to be essentially pure, after removal of the protecting groups, on paper chromatography in solvents C and D. Peak Va consisted of essentially pure d-MMTr-G¹⁸pC^{An}pTpC^{An}pC^{An}, as judged by paper chromatography in solvent C. In addition, peaks IVb, Vb and Vc also contained mainly the pentanucleotide and were evaporated separately and used for rechromatography as described below. The estimates given in Table 2 for the purity of the various peak fractions were made after treatment with ammonia and paper chromatography in solvent C. The pooled fractions of peak Va (30,800 o.d.₂₉₀ units, 0.43 m-mole, corresponding to a yield of 43%) were evaporated with pyridine and precipitated from ether as usual.

With tri-isopropylbenzenesulfonyl chloride as condensing agent

In a similar experiment (reaction conditions are given in Table 1) a pure portion of the desired pentanucleotide corresponding to a yield of only 30% was obtained. Fractions from this experiment containing both d-MMTr-G¹⁸pC^{An}pTpC^{An}pC^{An} and the symmetrical pyrophosphate of d-pC^{An}pC^{An} and peak-portions IVb, Vb and Vc from the preceding experiment were taken together (total 68,200 o.d.₃₀₀ units) dissolved in 30% pyridine and applied to a DEAE-cellulose column for rechromatography, which was carried out as shown in Fig. 10. Peak I consisted of the symmetrical pyrophosphate of d-pC^{An}pC^{An} (23,600 o.d.₃₀₀ units). Peak II, which was strongly colored at the front end, was divided into 5 arbitrary portions (a to e), which were checked for purity on paper chromatography in solvent C after removal of *N*-protecting groups. Only sections b, c and d were considered sufficiently pure for further use. The combined material amounted to 29,500 o.d.₂₉₀ units (0.41 m-mole) and was precipitated from ether and collected by centrifugation as usual. After removal of the *N*-protecting groups from a sample, the product consisted of 98% of d-MMTr-GpCpTpCpC by paper chromatography in solvent C. The impurities were located in two spots with *R_F* values of 0.35 and 0.15 rel. to d-pT. An over-all yield of the two condensations leading to the pentanucleotide d-MMTr-G¹⁸pC^{An}pTpC^{An}pC^{An} is given in Table 1.

The heptanucleotide, d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃pT

An anhydrous pyridine solution (50 ml.) of triethylammonium d-MMTr-G^{1B}pC^{An}pTpC^{An}.pC^{An} (0.80 m-mole), triethylammonium d-pC^{An}pT-OAc (6.95 m-moles) and MS (3.44 g, 15.7 m-moles) was shaken at room temp. for 1.5 hr. The work-up of the reaction and alkaline treatment (8 min at 0°C) was as described in the General Methods and Materials section. Column chromatography of the reaction mixture was carried out as shown in Fig. 11. The distribution and identification of the u.v.-absorbing material is shown in Table 3. In peak II, the central portion with a constant ratio of $\epsilon_{260}/\epsilon_{300} = 0.88$ contained pure unreacted d-pC^{An}pT (61%) and showed one spot on paper chromatography in solvent B. Peak III, the recovered pentanucleotide (10% of the starting material) showed, after ammonia treatment, only one spot on paper chromatography in solvent C. The fractions of the product, peak Va, were pooled according to a constant ratio of $\epsilon_{260}/\epsilon_{300} = 0.90$ and were evaporated with an excess of pyridine, and the heptanucleotide d-MMTr-G^{1B}pC^{An}pT-(pC^{An})₃pT finally collected by precipitation from anhydrous ether. The yield was 63,700 o.d.₂₈₅ units (0.67 m-mole, 79%). The purity of the product was checked after ammonia treatment by paper chromatography in solvent C, a single spot being observed. Another sample of the product was treated to remove all of the protecting groups. Paper chromatography in solvent C again showed a single spot. Chromatography in solvent D gave only a streak up from the origin, therefore indicating that the limit in use of this solvent in the present series had been reached.

The nonanucleotide, d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}

An anhydrous pyridine solution (10 ml.) of triethylammonium d-MMTr-G^{1B}pC^{An}pT-(pC^{An})₃pT (0.144 m-mole), triethylammonium d-pTpA^{Bz}-OAc (1.45 m-moles) and MS (629 mg, 2.87 m-moles) was shaken at room temp. for 1.5 hr. The work-up including an alkaline treatment (9 min at 0°C) was as described in the General Methods and Materials section. Column chromatography of the reaction mixture was carried out as shown in Fig. 12. The distribution and identification of the u.v.-absorbing material is shown in Table 4.

The central portion of peak I showed a u.v. spectrum similar to that of the block d-pTpA^{Bz} ($\lambda_{\max} = 277$ nm). On paper chromatography in solvent B the pooled peak I consisted of one main spot with an R_F of 1.70 and two additional minor spots. The main spot, after treatment with ammonia, had an R_F (d-pT) of 1.43 on paper chromatography in solvent C and an R_F (d-pT) of 1.41 on paper chromatography in solvent E. The u.v. spectrum of this deprotected compound at neutral and acidic pH was similar to that of d-pTpA. The compound was resistant to bacterial alkaline phosphatase and to spleen phosphodiesterase. With snake venom phosphodiesterase, however, the compound was completely degraded to a 1 : 1 mixture of d-pT and d-pA, which were separated by paper chromatography in solvent D. All these observations are consistent with the assumption that peak I contained mainly cyclic d-pTpA.

Peak II, 24,400 o.d.₂₇₆ units corresponded to 60% of the starting block d-pTpA^{Bz} and showed one spot on paper chromatography in solvent B. A sample after ammonia treatment gave one spot with an R_F (d-pT) of 1.13 on paper chromatography in solvent E and one main spot on paper chromatography in solvent C. Both edges of peak II combined (7350 o.d.₂₇₆ units) contained, besides d-pTpA^{Bz} (93%), an additional spot with an R_F of 0.84 rel. to d-pT.

Peaks IV and V were cut into portions according to the observed ratio of $\epsilon_{300}/\epsilon_{260}$ (1.14 for the heptanucleotide d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃pT and 1.00 for the nonanucleotide product). The portions were evaporated separately.

Portion IVc, the bulk of the peak material was pure heptanucleotide. However, portion IVb was slightly contaminated by impurities with R_F (d-pT) values of 0.15, 0.28 and 0.39 (less than 10% of the total u.v.-absorbing material of this portion).

Peak IVc corresponded to 17% of the starting heptanucleotide and was precipitated from dry ether and collected by centrifugation as usual.

Peak Vb, the bulk of the nonanucleotide peak was homogeneous on paper chromatography in solvent C after ammonia treatment, amounted to a total yield of 8390 o.d.₂₈₀ units (64 μ moles, 48%) and was precipitated from ether as usual. Peaks Va and Vc showed a ratio $\epsilon_{300}/\epsilon_{260}$ of less than 1 and were impure according to paper chromatography after removal of the *N*-protecting groups.

For further characterization, a portion of the MMTr-GpCpT(pC)₃(pT)₂pA was eluted from paper (chromatography in solvent C) and treated with acetic acid as usual. The completely unprotected product was again homogeneous on paper chromatography in solvent C. The single band was eluted from this chromatogram and subjected to analytical column chromatography on an acidic urea column at pH 3.5. The elution pattern and conditions of chromatography are shown in Fig. 13. Fractions 76 to 84 containing the pure nonanucleotide were pooled, neutralized and the nucleotidic material freed from urea and salt by a Biogel column. The solution of the nonanucleotide thus obtained was lyophilized and the residue analysed by enzymic degradation.

In another repeat of the synthesis of the nonanucleotide (conditions and recovery in Table 1), a yield of 65% of the desired pure product was obtained.

The dodecanucleotide, d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz}

An anhydrous pyridine solution (25 ml.) of triethylammonium d-MMTr-G^{1B}pC^{An}pT-(pC^{An})₃(pT)₂pA^{Bz} (233 m-moles), triethylammonium d-pG^{1B}pC^{An}pA^{Bz}-OAc (1.31 m-moles) and MS (1.24 g, 5.68 m-moles) was shaken at room temp. for 2.25 hr. The work-up including an alkaline treatment (10 min at 0°C) was as described in the General Methods and Materials section. Column chromatography of the reaction mixture was carried out as shown in Fig. 14. The distribution and identification of the u.v.-absorbing material is shown in Table 5.

Peak II showed a u.v.spectrum ($\lambda_{\max}$ at 285 and 261 nm, $\lambda_{\min}$ at 267 nm) similar to that of d-pG^{1B}pC^{An}pA^{Bz}, both in the small peak to the left and in the middle portion. Peak III corresponded to 67% of the starting block d-pG^{1B}pC^{An}pA^{Bz} and showed a single spot on paper chromatography in solvent B. Samples were treated with ammonia as usual and checked by paper chromatography. In solvent C there were, besides the main spot, two very faint traces with R_F (d-pT) values of 0.61 and 0.82. In solvent H these impurities were located at an R_F (d-pT) of 0.38 and 2.0.

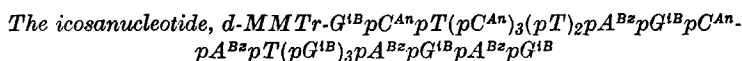
Peak VI corresponded to 48% of the starting nonanucleotide and was pure, as shown by paper chromatography in solvent C after ammonia treatment. Peak VIIa contained the pure dodecanucleotide. The pooled fractions (75 m-moles, 32% yield) were evaporated in the presence of excess pyridine and the material precipitated from excess dry ether and collected by centrifugation. A sample of the product was treated with ammonia and showed on paper chromatography in solvent C, in addition to the product, three faint bands (R_F values rel. to d-MMTr-GpCpTpCpC : 0.07, 0.27 and 0.61). The main band was eluted with 1% ammonia and treated with acetic acid to remove the methoxytrityl group. After paper chromatography in solvent C, the nucleotide material was eluted (single band) and subjected to analytical column chromatography at pH 7.5 as shown in Fig. 15. For further characterization by chromatography at acidic pH, a sample (63 o.d.₃₀₀ units) of the fully protected dodecanucleotide was deprotected and chromatographed as shown in Fig. 16. The main peak (31.5 o.d.₂₆₀ units, 57% of the expected amount) of dodecanucleotide was neutralized with ammonium bicarbonate solution and freed from urea and salt by passage through Biogel-P2. For degradation with snake venom phosphodiesterase a sample (14 o.d.₂₆₀ units) of this material was used with the results given in Table 13.

*The hexadecanucleotide, d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz}.
pT(pG^{1B})₂pG^{1B}*

An anhydrous pyridine solution (11 ml.) of triethylammonium d-MMTr-G^{1B}pC^{An}pT-(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz} (57 m-moles), triethylammonium d-pT(pG^{1B})₂pG^{1B}-OiB (0.69 m-mole) and MS (586 mg, 2.67 m-moles) was shaken at room temp. for 2.5 hr. The work-up of the reaction mixture including an alkaline treatment (12 min at 0°C) was as described in the General Methods and Materials section. Column chromatography of the reaction mixture was carried out as shown in Fig. 17. The distribution and identification of the u.v.-absorbing material is shown in Table 6.

The recovered block d-pT(pG^{1B})₂pG^{1B} (peak Ib) was checked on paper chromatography in solvent B and proved to be homogeneous (61% of the starting material). Pooled peak IIIa had a $\lambda_{\max}$ at 280 nm and its u.v. spectrum was only slightly different from that of the starting dodecanucleotide. Peak IIIb corresponded to 63% recovery of the original amount

of the dodecanucleotide. Peak IV consisted of the desired hexadecanucleotide d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz}pT(pG^{1B})₂pG^{1B}. The 2000 o.d. units at 280 nm corresponded to a calculated yield of 17% (9.6 m-moles). This material was directly used for the condensation to the icosanucleotide. Peak IV was evaporated with excess pyridine and the product collected by precipitation from dry ether as usual. Characterization by urea column chromatography, after removal of protecting groups from the hexadecanucleotide recovered from the next condensation, is given below.



An anhydrous pyridine solution (8 ml.) of triethylammonium d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz}pT(pG^{1B})₂pG^{1B} (9.2 m-moles, 1916 o.d.₂₈₀ units), triethylammonium d-pA^{Bz}pG^{1B}pA^{Bz}pG^{1B}-OAc (0.586 m-mole, 34,900 o.d.₂₈₀ units) and MS (434 mg, 1.98 m-moles) was shaken at room temp. for 2.5 hr. The work-up including an alkaline treatment (10 min at 0°C) was as described in the General Methods and Materials section. Column chromatography was first performed to separate the shorter oligonucleotides and the separation pattern is shown in Fig. 18. The distribution and identification of the u.v.-absorbing material is shown in Table 7. Peak IIb contained the pure unreacted tetranucleotide (56% of the original amount) and the purity was checked both before and after the removal of the protecting groups.

At fraction 208 the column was stripped with 0.7 M-TEAB-40% ethanol. The buffer solution of the eluted nucleotidic material was evaporated in the presence of excess pyridine and the final pyridine solution diluted with 0.05 M-TEAB-40% ethanol and chromatographed as shown in Figure 19.

Peak I contained oligonucleotides with chain-length smaller than 16. Peak II material was divided into fractions as follows: peaks IIa (234 o.d.₂₈₀ units), IIc (234 o.d.₂₈₀ units) and IIb (195 o.d.₂₈₀ units). The material in peak IIb (935 o.d.₂₈₀ units) was the recovered hexadecanucleotide d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz}pT(pG^{1B})₂pG^{1B} and corresponded to 49% of the original amount. (After fraction 497, the column was washed with 1 M-TEAB-10% ethanol. An additional amount of 89 o.d.₂₈₀ units was isolated.)

Peak IIIa of Fig. 17 contained the icosanucleotide d-MMTr-G^{1B}pC^{An}pT(pC^{An})₃(pT)₂pA^{Bz}pG^{1B}pC^{An}pA^{Bz}pT(pG^{1B})₃pA^{Bz}pG^{1B}pA^{Bz}pG^{1B} in an amount of 510 o.d.₂₈₀ units (1.91 m-moles, 21%).

The pooled peaks IIb and IIIa were evaporated in the presence of excess pyridine and precipitated from dry ether. From small portions, all of the protecting groups were removed and the samples of the hexadecanucleotide (22 o.d.₂₈₀ units) and the icosanucleotide (16.8 o.d.₂₅₅ units) were subjected to analytical column chromatography in the presence of 7 M-urea. Elution pattern of the hexadecanucleotide is shown in Fig. 20. The central part of the peak, fractions 118 to 131 (as shown by the vertical broken lines), contained 17.2 o.d.₂₈₀ units (75% of the original o.d. units at 260 nm). Salt and urea were removed by Biogel-P2 as described above.

Urea column chromatography of the icosanucleotide sample is shown in Fig. 21 (a) and (b). The main part of the peak (fractions 76 to 112) was pooled and freed from salt and urea in the usual way.

A larger portion of the deprotected icosanucleotide was subjected to purification by chromatography as shown in Fig. 22. The pooled fraction III of the major peak was subjected to enzymic degradation. The results are given in Table 13. The material in this fraction has been used in enzymic work and was found to be at least 95% pure.

REFERENCES

- Gupta, N. K. & Khorana, H. G. (1968). *Proc. Nat. Acad. Sci., Wash.* **61**, 215.
 Hachmann, J. & Khorana, H. G. (1969). *J. Amer. Chem. Soc.* **91**, 2749.
 Kleppe, K., Ohtsuka, E., Kleppe, R., Molineux, I. J. & Khorana, H. G. (1971). *J. Mol. Biol.* **56**, 341.
 Kössel, H., Büchi, H. & Khorana, H. G. (1967). *J. Amer. Chem. Soc.* **89**, 2185.

- Narang, S. A., Jacob, T. M. & Khorana, H. G. (1967). *J. Amer. Chem. Soc.* **89**, 2158.
Ohtsuka, E. & Khorana, H. G. (1967). *J. Amer. Chem. Soc.* **89**, 2195.