Studies on Polynucleotides. XCII.[1] The Synthesis of a Deoxyribododecanucleotide Containing Specific Amino Acid Codons[2]

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Abstract: High molecular weight deoxyribopolynucleotides with repeating nucleotide sequences have previously been shown to direct the synthesis of polypeptides in the bacterial cell-free protein synthesizing system. With the aim of synthesizing polypeptides containing specific amino acid sequences *via* nucleic acid templates, a deoxyribododecanucleotide has now been synthesized. The synthetic polynucleotide, d-A-T-G-C-A-C-T-C-T-T-A-G, contains at the appropriate 5′ end the trinucleotide sequence A-T-G, which stands for formylmethionine and initiates the synthesis of peptide chain, and at the 3′ end the sequence T-A-G, which should terminate and release the polypeptide chain. The codons selected for internal positions were C-A-C (histidine) and T-C-T (serine). The protected trinucleotide blocks d-MMTr-A^{Bz}pTpG^{iBu},[3] d-pC^{An}pA^{Bz}pC^{An}-OAc, d-pTpC^{An}pT-OAc,[3] and d-pTpA^{Bz}-pG^{iBu}-OAc[3] were prepared by stepwise methods using the protected nucleoside and nucleotides. The blockwise condensations of the protected trinucleotides to form the dodecanucleotide were carried out using mesitylenesulfonyl chloride as the condensing agent, yields in the individual steps being 30–50%.

The sequences of the trinucleotide codons for different amino acids in the nucleic acid directed synthesis of proteins are now known.[4] Certain trinucleotides can signal the biological initiation or termination of protein chains, and the sequences of these trinucleotides are also known.[4-6] One can, therefore, derive the nucleotide sequence of a polynucleotide chain which will direct the synthesis of a polypeptide containing a predetermined amino acid sequence. The synthesis of specific polynucleotides with this object in view, therefore, becomes an important direction for further work. Conversely, it is clear that the availability of polynucleotides containing defined trinucleotide codons will enable further precise studies of the protein-synthesizing system, studies which are largely carried out at present using the viral RNAs as messengers.[4]

Normally, an RNA serves as the messenger in protein synthesis. However, recently it has been found that in the presence of certain aminoglycoside antibiotics, single-stranded deoxyribopolynucleotides can also direct protein synthesis.[7] In a further study with deoxyribopolynucleotides containing repeating nucleotide sequences, it has been shown[8] that the amino acid incorporations directed by them are the same as previously found for the corresponding ribopolynucleotides.[9] These results, therefore, justify the synthesis of deoxyribopolynucleotides as messengers, and this conclusion is of practical importance in that the synthesis of relatively long deoxyribopolynucleotides is more advanced at present than the synthesis of corresponding ribopolynucleotides. In the present work a start has been made in the above direction by the synthesis of a deoxyribododecanucleotide. From the extensive previous work[4] on the successful use of short polynucleotides as messengers, chains as short as the dodecanucleotides would be expected to adequately stimulate amino acid incorporation in the protein synthesizing systems.

The nucleotide sequence selected for synthesis was d-A-T-G-C-A-C-T-C-T-T-A-G; the reasons were as follows. At the 5′ end there is the codon A-T-G, which recognizes the initiator formylmethionyl-tRNA.[10] At the 3′ end there is the codon T-A-G, a chain-terminator codon.[4,6] The codons in the interior of the above chain correspond to the amino acids histidine (C-A-C) and serine (T-C-T). Thus, the expected tripeptide would be formylmethionylhistidylserine. This peptide sequence lends itself well to characterization by mild acidic degradation: the formyl group and serine[11] would be readily cleaved and the resulting dipeptide and free serine could be readily characterized.

A further consideration for the above selection was that the total sequence is pyrimidine rich, and so would

(1) Paper XCI: U. L. RajBhandary and H. P. Ghosh, *J. Biol. Chem.*, in press.

(2) This work has been supported by grants from National Cancer Institute of the National Institutes of Health (Grant No. CA-05178), the National Science Foundation (Grant No. GB-7484X), and the Life Insurance Medical Research Fund (Grant No. 65-44).

(3) The system of abbreviations used is as has been proposed and used in earlier papers of this series; see, *e.g.*, H. Schaller and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3841 (1963). An abbreviation not defined and encountered previously in this series is *i*Bu for the isobutyryl group. The use of this acyl group for protection of the amino group in guanine ring has been developed recently in this laboratory (unpublished work of H. Buchi and H. Weber).

(4) The Genetic Code, *Cold Spring Harbor Symp. Quant. Biol.*, **31**, 1 (1966).

(5) This is true strictly for synthesis in the microorganism *Escherichia coli*.[4,6]

(6) H. G. Khorana, Harvey Lectures, Series 62, Academic Press, New York, N. Y., 1968, p 79; *Biochem J.*, in press.

(7) B. J. McCarthy and J. J. Holland, *Proc. Natl. Acad. Sci. U. S.*, **54**, 880 (1965).

(8) A. R. Morgan, R. D. Wells, and H. G. Khorana, *J. Mol. Biol.*, **26**, 477 (1967).

(9) D. S. Jones, S. Nishimura, and H. G. Khorana, *ibid.*, **16**, 454 (1966).

(10) The ribonucleotide codon established for this tRNA is A-U-G. The deoxy counterpart, A-T-G, has been verified to recognize the same tRNA. Thus, A-T-G was found to stimulate the binding of formylmethionyl-tRNA to ribosomes (unpublished experiments of Drs. H. P. Ghosh and A. R. Morgan).

(11) S. Nishimura, D. S. Jones, and H. G. Khorana, *J. Mol. Biol.*, **13**, 302 (1965).

be expected from past experience to be a substrate for DNA-dependent RNA polymerase,[12] and it therefore might be of interest for a study of the initiation site for the action of the RNA polymerase.

The synthesis of the dodecanucleotide began with the synthesis of the trinucleoside diphosphate at the 5′ end as the protected derivative d-MMTr-A^{Bz}pTpG^{iBu}. The latter was synthesized by the reaction sequence shown below in Chart I. The intermediate d-MMTr-A^{Bz}pT could be isolated pure by a rapid extraction procedure, while d-MMTr-A^{Bz}pTpG^{iBu} was purified by chromatography on a DEAE-cellulose column.

Chart I. Synthesis of d-MMTr-A^{Bz}pTpG^{iBu}

$$\text{d-MMTr-A}^{Bz} \xrightarrow[\text{2. Extraction with organic solvents}\\ \text{3. OH}^-]{\text{1. pT-OAc + MsCl}} \text{d-MMTr-A}^{Bz}\text{pT}$$

$$\text{d-MMTr-A}^{Bz}\text{pT} \xrightarrow[\text{2. OH}^-\\ \text{3. Chromatography}]{\text{1. pG}^{iBu}\text{-O-}i\text{-Bu + MsCl}} \text{d-MMTr-A}^{Bz}\text{pTpG}^{iBu}$$

Further condensations at the 3′-hydroxyl end of the growing chain used protected trinucleotide blocks corresponding to the individual amino acid codons. This general approach offers flexibility for introducing variations at specific sites in the codon sequences of the synthetic deoxypolynucleotides.[13] Further, it was of interest in this work to experiment with the use of protected trinucleotide blocks as a method for the synthesis of deoxyribopolynucleotides.

The method used for the synthesis of the protected trinucleotides was that developed previously[14] and is illustrated in an abbreviated form for the trinucleotide d-pC^{An}pA^{Bz}pC^{An} in Chart II.[3]

The protected trinucleoside diphosphate d-MMTr-A^{Bz}pTpG^{iBu} was condensed with d-pC^{An}pA^{Bz}pC^{An}-OAc using onefold excess of the latter block and mesitylenesulfonyl chloride (MsCl) as the condensing agent (Table I.) After the condensation step the extent of reaction could be analyzed by chromatography on DEAE-cellulose paper. After a brief alkali treatment to remove the 3′-O-acetyl group, the reaction product was separated on a DEAE-cellulose column. The hexanucleotide, d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An} thus obtained was condensed with d-pTpC^{An}pT-OAc, using an eightfold excess of the latter, and the resulting nonanucleotide was isolated as usual by DEAE-cellulose column chromatography. The protected nonanucleotide thus obtained was then condensed with d-pTpA^{Bz}pG^{iBu}-OAc (50-fold excess) and the dodecanucleotide was isolated by DEAE-cellulose column chromatography and further purified by preparative paper chromatography. The amounts of the various components used in the blockwise synthesis and the yields of the products are shown in Table I. Purity of the products after condensations was checked by paper chromatography after removal of the N-protecting groups with concentrated ammonia and of monomethoxytrityl groups with 80% acetic acid. The products were further characterized by their ultraviolet absorption spectrum at the protected and the unprotected stage and by degradation to the constituent nucleoside and nucleotides on treatment with venom phosphodiesterase followed by paper chromatography. The analytical results are given in the Experimental Section.

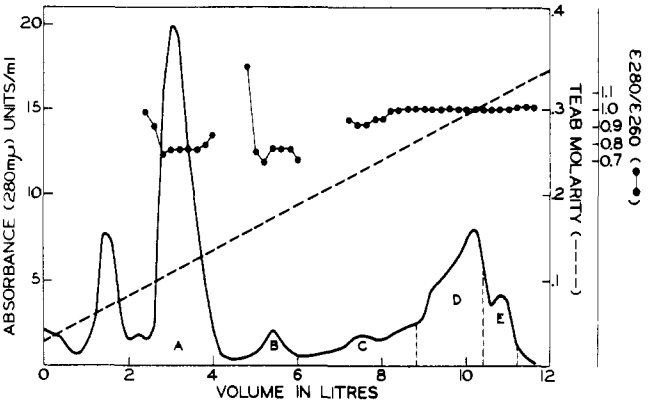


Figure 1. Condensation of d-MMTr-A^{Bz}pT with d-pG^{iBu}. Separation of the reaction products was on a DEAE-cellulose column (bicarbonate) (3.5 cm diameter × 60 cm) preequilibrated at 4° with 0.05 M $(C_2H_5)_3NH^+HCO_3^-$ in 10% ethanol. The $(C_2H_5)_3NH^+$-HCO_3^- gradient used is shown by the dashed line, using 0.05 M $(C_2H_5)_3NH^+HCO_3^-$ in 10% ethanol (6 l.) in the mixing vessel and 0.35 M $(C_2H_5)_3NH^+HCO_3^-$ in 30% ethanol (6 l.) in the reservoir. Peaks were pooled as shown by vertical dotted lines. For identification of peaks, see text.

Results of eazymatic experiments carried out with the dodecanucleotide herein described will be reported upon in a later publication.

Experimental Section

General Methods. Paper chromatography was performed by the descending technique using Whatman No. 40 or No. 1 paper. Solvent systems used were: solvent A, 2-propanol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethanol–ammonium acetate (1 M) (pH 7.5) (7:3, v/v); solvent C, 1-propanol–concentrated ammonia–water (55:10:35, v/v); solvent D, isobutyric acid–concentrated ammonia–water (66:1:33, v/v); solvent E, 0.25 M ammonium acetate–7 M urea; solvent F, 0.15 M sodium chloride–0.02 M Tris hydrochloride buffer (pH 7.5)–7 M urea. DEAE-cellulose paper was used for the last two solvent systems. Paper chromatographic mobilities are given in Tables II and III.

Thin layer chromatography (tlc) was performed on precoated silica gel plates using chloroform with varying proportions of ethanol as the solvent; the plates were viewed in ultraviolet light.

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

Monomethoxytrityl groups were detected by spraying the chromatograms with 10% aqueous perchloric acid and drying them in warm air.

Pyridine was treated with a small amount of chlorosulfonic acid before distillation, redistilled from potassium hydroxide and stored

(12) Thus oligothymidylates and oligonucleotides containing repeating T-C and T-T-C sequences serve as templates for the polymerase [A. Falaschi, J. Adler, and H. G. Khorana, *J. Biol. Chem.*, **238**, 3080 (1963); S. Nishimura, T. M. Jacob, and H. G. Khorana, *Proc. Natl. Acad. Sci., U. S.*, **52**, 1494 (1964)]. However, deoxypolynucleotides of comparable size containing purines, *e.g.*, $d(T\text{-}G)_{5-6}$ and $d(A\text{-}G)_{5-6}$ elicited no response from the polymerase (unpublished experiments of Drs. Mehrotra and Nishimura).

(13) Projecting into the future, it already seems not inconceivable that laboratory synthesis of specific proteins will be carried out using nucleic acid templates. For this purpose, protected trinucleotides representing different codons will be made in quantity and on a commercial basis and these will be used in the synthesis of nucleic acid templates for proteins, the approach offering flexibility and selectivity in amino acid substitutions at the template level.

(14) S. A. Narang, T. M. Jacob, and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2158 (1967).

Table I. Summary of Conditions and Yields Obtained in the Synthesis of d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An}pTpC^{An}pTpTpA^{Bz}pG^{iBu}-OH[a]

Condensation product	3′-OH-bearing component	Amt, mmol (μmol)	5′-Phosphate-bearing component	Amt, mmol (μmol)	Pyridine, ml	MsCl, mmol (μmol)	Time, hr	Yield, %
MMTr-d-A^{Bz}pTpG^{iBu}	MMTr-d-A^{Bz}pT	(700)	d-pG^{iBu}-O*i*Bu	1.5	5	3	2	60
d-pC^{An}pA^{Bz}	d-CEpC^{An}	2	d-pA^{Bz}-OAc	3	8	6	3	70
d-pC^{An}pA^{Bz}pC^{An}	d-CEpC^{An}pA^{Bz}	(500)	d-pC^{An}-OAc	1.5	10	3	2.5	50
d-pTpC^{An}	d-CEpT	5	d-pC^{An}-OAc	6	15	12	2.5	50
d-pTpC^{An}pT	d-CEpTpC^{An}	2.1	d-pT-OAc	5	10	7	2	45
MMTr-d-A^{Bz}pTpG^{iBu}pC^{An}-pA^{Bz}pC^{An}	MMTr-d-A^{Bz}pTpG^{iBu}	(400)	d-pC^{An}pA^{Bz}pC^{An}-OAc	(800)	5	3	2	40
MMTr-d-A^{Bz}pTpG^{iBu}pC^{An}-pA^{Bz}pC^{An}pTpC^{An}pT	MMTr-d-A^{Bz}pTpG^{iBu}-pC^{An}pA^{Bz}pC^{An}	(150)	d-pTpC^{An}pT-OAc	1.2	3	4	2	35
MMTr-d-A^{Bz}pTpG^{iBu}pC^{An}-pA^{Bz}pC^{An}pTpC^{An}-pTpTpA^{Bz}pG^{iBu}	MMTr-d-A^{Bz}pTpG^{iBu}pC^{An}-pA^{Bz}pC^{An}pTpC^{An}pT	(5)	d-pTpA^{Bz}pG^{iBu}-OAc	(250)	1	(900)	2	25

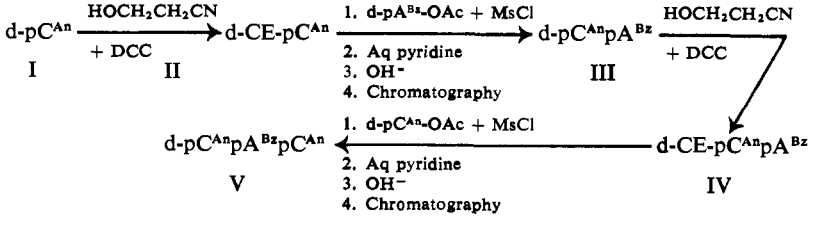
[a] For the exceptional use of *i*Bu see ref 3.

Table II. Spectral and Chromatographic Properties of Fully Protected Polynucleotides

Compound	λ_{max}, mμ	$\varepsilon_{280}/\varepsilon_{260}$	Solvent B R_f	Solvent[a] E R_f	Solvent[a] F R_f
d-pC^{An}pA^{Bz}	285	1.6	0.54	0.64	0.34
d-pC^{An}pA^{Bz}-OAc	285	1.6	0.60	...	...
d-pC^{An}pA^{Bz}pC^{An}	287	1.54	0.47	0.46	0.24
d-pC^{An}pA^{Bz}pC^{An}-OAc	287	1.54	0.52	...	...
d-pTpC^{An}	272 302	1.14	0.36	0.66	0.38
d-pTpC^{An}-OAc	272 302	1.14	0.40	...	...
d-pTpC^{An}pT	270 302	1.0	0.30	0.48	0.25
d-pTpC^{An}pT-OAc	270 302	1.0	0.41	...	...
d-pTpA^{Bz}	276	1.2	0.53	0.64	0.36
d-pTpA^{Bz}-OAc	276	1.2	0.58	...	...
d-pTpA^{Bz}pG^{iBu}	262 275	0.94	0.55	0.52	0.25
d-pTpApG^{iBu}-OAc	262 275	0.94	0.62	...	...
d-MMTr-A^{Bz}pT	277	1.23	0.88	...	...
d-MMTr-A^{Bz}pTpG^{iBu}	262 275	0.94	0.78	...	...
d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An}	280	1.3	...	...	0.40
d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An}pTpC^{An}pT	280	1.16	...	...	0.27
d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An}pTpC^{An}pTpTpA^{Bz}pG^{iBu}	277	1.08	...	...	...

[a] With solvents E and F, DEAE-cellulose paper was used for chromatography.

Chart II. Synthesis of d-pC^{An}pA^{Bz}pC^{An}



over molecular sieves obtained from the Linde Co., and then used in condensation reactions.

All the condensation reactions were carried out by repeated evaporation *in vacuo* of a solution of the components with added dry pyridine (at least three times), and every time the reaction flask, which was in a vacuum, was opened into a drybox maintained anhydrous with phosphorus pentoxide and a positive pressure of dry nitrogen gas. The molar extinction values of protected mononucleotides used are as follows: d-pT, 9600 (267 mμ); d-pA^{Bz}, 18,300 (280 mμ);[15] d-pC^{An}, 22,430 (302 mμ)[16] and 18,000 (280 mμ); and d-pG^{iBu}, 16,700 (259 mμ).[17]

(15) R. K. Ralph and H. G. Khorana, *J. Am. Chem. Soc.*, 83, 2926 (1961).

Table III. Chromatographic Properties of Unprotected Polynucleotides

Compound	Solvent C R_f	Solvent D R_f
d-MMTr-ApT	0.72[a]	
d-MMTr-ApTpG	0.62[a]	
d-pTpC	0.34	0.40
d-pTpCpT	0.34	0.18
d-pCpA	0.47	0.55
d-pCpApC	0.33	0.36
d-pTpA	0.48	
d-pTpApG	0.35	0.22
d-MMTr-ApTpGpCpApC	1.2[b]	0.8[b]
d-ApTpGpCpApC	0.44[b]	0.25[b]
d-MMTr-ApTpGpCpApCpTpCpT	0.7[b]	0.5[b]
d-ApTpGpCpApCpTpCpT	0.17[b]	0.11[b]
d-MMTr-ApTpGpCpApCpTpCpTpTpApG	0.2[b]	0.12[b]
d-ApTpGpCpApCpTpCpTpTpApG	0.08[b]	0.04[b]

[a] These values were measured in solvent A. [b] R_f indicated is with reference to pT.

For removal of the N-protecting groups the compounds were treated at room temperature with a large excess of concentrated ammonia for 2 days. Monomethoxytrityl groups were removed with 80% aqueous acetic acid at room temperature for 40 min.

Enzymic degradation of the synthetic products was performed using 5–10 OD_{260} of the material with venom phosphodiesterase as described previously.[16] The abbreviation OD refers to the extinction of a nucleotidic solution in a 1-ml volume using a 1-cm light-path quartz cell. The wavelength used for this measurement is indicated as a subscript after this abbreviation.

β-Cyanoethylation of the 5′-phosphomonoester groups in mono- and oligonucleotides was done using tenfold excess of dicyclohexylcarbodiimide (DCC) and 20–30-fold excess of β-hydracrylonitrile in dry pyridine solution by shaking for 18 hr in the dark.[18] To an aliquot (20 μl) of the reaction mixture water (20 μl) was added which was then extracted three times with 2–3 ml of cyclohexane. The aqueous pyridine solution was checked for completion of reaction by paper electrophoresis at pH 7.1. If no trace of the starting material was observed, then the whole mixture was treated with water and the aqueous pyridine solution was kept at 2–4° for 24 hr. It was then filtered and extracted with cyclohexane. The solution was mixed with 1 *M* triethylammonium bicarbonate solution to obtain pH 9.0, and the reaction mixture was kept for 4 hr at this pH. More pyridine was then added to make the concentration of pyridine at least 50%, and the solution was then concentrated on a rotary evaporator under vacuum below 20°. The cyanoethylated compound was finally isolated by precipitation from pyridine and an excess of ether. Acetylations of the 3′-hydroxyl end groups in the protected trinucleotides were carried out by the general procedure described previously.[18]

Preparation of d-MMTr-A^{Bz}. This compound was prepared from deoxyadenosine (10 mmol) according to the procedure previously described.[19] N-Benzoyl-5′-O-monomethoxytrityldeoxyadenosine was finally purified by column chromatography on silica gel (grade G, 150 g) using a chloroform–ethanol mixture (95:5) as solvent. Fractions were checked by tlc using the same solvent, and fractions 45–61 which contained the desired compound were pooled together, concentrated, and precipitated into pentane from a chloroform solution.

Preparation of d-MMTr-A^{Bz}pT. A mixture of d-MMTr-A^{Bz} (1 mmol) and pyridinium d-pT-OAc (1.5 mmol) was treated with MsCl (3 mmol) in 5 ml of dry pyridine. The clear solution was kept for 2 hr in the dark at room temperature and was then cooled in a Dry Ice–alcohol bath. Ten milliliters of 1 *M* diisopropylethylamine solution in pyridine was then added followed by 5 ml of water, and the solution was kept overnight in a refrigerator. It was then diluted with water (50 ml) and extracted three times with ether (100-ml portions). The aqueous layer was concentrated to 20 ml and extracted with chloroform (three times using 100 ml). Tlc of the ethereal layer, the chloroform layer, and the aqueous layer using chloroform–ethanol (50:50) as the solvent indicated that monomethoxytritanol and unreacted d-MMTr-A^{Bz} were present in the ether extract, the chloroform extract contained a single trityl-positive compound identified as d-MMTr-A^{Bz}pT, and the aqueous layer contained traces of a trityl-positive compound and two trityl-negative nucleotidic compounds. The chloroform layer was concentrated with addition of pyridine, the residue taken up in 20 ml of pyridine, and the solution treated with 25 ml of 2 *N* sodium hydroxide solution for 10 min in an ice bath. The sodium ions were then removed with an excess of pyridinium Dowex-50 ion-exchange resin; the resin was removed by filtration. The filtrate and washings were concentrated in the presence of pyridine and the product was precipitated as a powder by pouring a dry pyridine solution into an excess of ether. The product was checked to be pure by paper chromatography in solvents B and C after removal of the protecting groups. The protected compound (ammonium salt) has λ_{max} 277 mμ and $\varepsilon_{280}/\varepsilon_{260}$ 1.20; the yield was 60%.

Preparation of d-MMTr-A^{Bz}pTpG^{iBu}. An anhydrous pyridine solution (5 ml) of d-MMTr-A^{Bz}pT (0.7 mmol) and d-pG^{iBu}-O*i*Bu (1.5 mmol) was treated with MsCl (3.5 mmol) for 2 hr in the dark. The solution was then cooled to −20° and 5 ml of water was added followed by 5 ml of a 1 *M* pyridine solution of diisopropylethylamine. After keeping the reaction mixture for 4 hr at room temperature, 10 ml of water was added and the mixture was left overnight in the refrigerator. It was then treated with 30 ml of 2 *N* sodium hydroxide in an ice bath for 10 min. The removal of the sodium ions with ion-exchange resin was as described above. The final aqueous pyridine solution (500 ml) was applied to a DEAE-cellulose column. The conditions for chromatography and the elution pattern are shown in Figure 1. Representative fractions from peaks A, B, C, D, and E were checked by paper chromatography in solvent A. Peak A contained d-pG^{iBu}; peak B contained the symmetrical pyrophosphate of d-pG^{iBu} (characterized by paper chromatography and uv spectrum); peak C contained unreacted d-MMTr-A^{Bz}pT; peak D contained pure trinucleotide, d-MMTr-A^{Bz}pTpG^{iBu}; and peak E contained the trinucleotide, d-MMTr-A^{Bz}pTpG, in which the N-isobutyryl protecting group had been lost. Peak E was pooled and the material in it was reisobutyrylated with isobutyric anhydride–pyridine. The total yield of the trinucleoside diphosphate, d-MMTr-A^{Bz}pTpG^{iBu}, isolated by concentration of the peak areas and precipitation into ether from pyridine solution, was 60%, λ_{max} 275 and 262 mμ, $\varepsilon_{280}/\varepsilon_{260}$ 0.94.

Preparation of the Dinucleotide d-pC^{An}pA^{Bz}. A mixture of pyridinium d-CE-pC^{An} (2 mmol) and d-pA^{Bz}-OAc (3 mmol) was allowed to react in the presence of 6 mmol of MsCl in 10 ml of dry pyridine for 2.5 hr. After treatment with water and storage of the aqueous pyridine solution for 24 hr at room temperature the reaction mixture was saponified with 50 ml of 2 *N* NaOH for 25 min at room temperature, the alkali was removed with ion-exchange resin, and the reaction products were chromatographed on a DEAE-cellulose column (60 × 4.5 cm diameter) equilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ containing 20% ethanol. The column was eluted using a linear gradient of 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ containing 20% ethanol and 0.35 *M* $(C_2H_5)_3NH^+HCO_3^-$ containing 20% ethanol (four 4-l. portions). Peak C contained the desired dinucleotide which was checked by paper chromatography using DEAE-cellulose paper, and, after removing the protecting groups, in solvents C and D. The yield and R_f values of the compound are given in Tables I and II; λ_{max} 285 mμ and $\varepsilon_{280}/\varepsilon_{260}$ 1.6.

Preparation of the Cyanoethyl Dinucleotide d-CEpC^{An}pA^{Bz}. An anhydrous mixture of pyridinium d-pC^{An}pA^{Bz} (1 mmol), β-hydracrylonitrile (3 ml), pyridinium Dowex-50 resin (2 g), and DCC (10 mmol) in dry pyridine (5 ml) was shaken in the dark at room temperature for 18 hr. An aliquot was checked by paper electrophoresis at pH 7.1 for complete conversion to the cyanoethyl derivative. Water (20 ml) was then added to the reaction mixture, and the aqueous pyridine solution was filtered from the resin and extracted with cyclohexane (three 100-ml portions). The aqueous pyridrine solution was concentrated and the product was precipitated from a pyridine–ether mixture, the yield being 75%.

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

(17) Unpublished work of Drs. H. Buchi and H. Weber of this laboratory.

(18) See, *e.g.*, E. Ohtsuka and H. G. Khorana, *J. Am. Chem. Soc.*, **89**, 2195 (1967).

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

On paper electrophoresis, the desired product (R_f 0.75 relative to d-pC^{An}pA^{Bz}) was found to contain a slower moving compound (R_f 0.3 relative to d-pC^{An}pA^{Bz}), presumably the dicyanoethylated product.

Preparation of the Trinucleotide d-pC^{An}pA^{Bz}pC^{An}. Pyridinium d-CE-pC^{An}pA^{Bz} (1.5 mmol) and d-pC^{An}-OAc (3.5 mmol) were dissolved in a mixture of pyridine (20 ml), diisopropylethylamine (5 ml), and a few drops of water. This mixture was evaporated to dryness four times after adding 10 ml of dry pyridine each time. Finally, a pyridine solution (10 ml) of the mixture was treated with 6 mmol of MsCl and the solution kept for 2.5 hr at room temperature. After the standard work-up including an alkaline treatment, the aqueous pyridine solution was applied to a DEAE-cellulose column. The conditions for chromatography and the elution pattern are shown in Figure 2. Representative fractions from different parts of the peaks were checked by DEAE-cellulose paper chromatography using 0.6 *M* ammonium bicarbonate containing 20% ethanol as the solvent system. (Because of similar R_f values, d-pC^{An}, d-pC^{An}pA^{Bz}, and d-pC^{An}pA^{Bz}pC^{An} could not be well separated on chromatography in solvent B.)

Peak A contained d-pC^{An}, peak B contained unreacted d-pC^{An}-pA^{Bz} mixed with the pyrophosphate of d-pC^{An}, and peak C contained pure trinucleotide d-pC^{An}pA^{Bz}pC^{An}, the yield being 40%. The purity of the product was checked by paper chromatography in solvents C and D after removal of the protecting groups. The protected trinucleotide has λ_{max} 287 mμ, $\varepsilon_{280}/\varepsilon_{300}$ 1.08.

3′-O-Acetylation of d-pC^{An}pA^{Bz}pC^{An}. The trinucleotide (20,000 OD_{280}) was treated in dry pyridine (10 ml) with acetic anhydride (4 ml) for 4 hr. After work-up and precipitation from dry ether, the 3′-O-acetyl derivative was obtained in a yield of 94%.

The Dinucleotide d-pTpC^{An}. A mixture of pyridinium d-CE-pT (2 mmol) and d-pC^{An}-OAc (3 mmol) in 10 ml of pyridine was shaken in the dark with 8 mmol of MsCl for 2 hr. After the standard work-up, the products were chromatographed on a DEAE-cellulose column (50 × 2.5 cm diameter) equilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 20% ethanol. After pyridine was washed off, a linear gradient of 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 20% ethanol and 0.35 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 20% ethanol was used for elution (four 4-l. portions). The dinucleotide, which was present in the second peak, was obtained in a yield of 50%. It was checked by paper chromatography (Tables I and II). It had λ_{max} 272 and 302 mμ, $\varepsilon_{280}/\varepsilon_{260}$ 1.14.

Preparation of the β-Cyanoethyl Dinucleotide d-CE-pTpC^{An}. The dinucleotide d-pTpC^{An} (1.2 mmol) was treated with β-hydracrylonitrile (3 ml), DCC (2 g), and pyridinium Dowex resin (1 g) in 10 ml of dry pyridine for 18 hr in the dark. After the standard work-up the cyanoethyl derivative was obtained in a yield of 75%.

Preparation of the Trinucleotide d-pTpC^{An}pT. A mixture of d-CE-pTpC^{An} (2.1 mmol) and d-pT-OAc (5 mmol) in dry pyridine (15 ml) was condensed using 8 mmol of MsCl for 2.5 hr. Aqueous pyridine (15 ml of 50%) was added after cooling and the solution kept overnight. After the standard sodium hydroxide treatment, the reaction products were applied on a DEAE-cellulose column. The chromatographic pattern and the conditions are shown in Figure 3. Various fractions were checked by paper chromatography in solvent system B and on DEAE-cellulose paper using 0.6 *M* ammonium bicarbonate containing 20% ethanol as the solvent. Peak A was d-pT and mesitylenesulfonic acid; peak B was the symmetrical pyrophosphate of d-pT mixed with some unidentified material; peak C was unreacted dinucleotide d-pTpC^{An}; and peak D contained pure trinucleotide d-pTpC^{An}pT, the yield being 50%. The compound has λ_{max} 270 and 302 mμ, $\varepsilon_{270}/\varepsilon_{290}$ 1.35.

Acetylation of d-pTpC^{An}pT. Acetylation of pTpC^{An}pT was done in 10 ml of pyridine with 4 ml of acetic anhydride using the standard procedure. The yield of the acetylated product was 95%.

The Dinucleotide d-pTpA^{Bz}. A mixture of d-CE-pT (5 mmol) and d-pA^{Bz}-OAc (6 mmol) in pyridine (10 ml) was allowed to react with MsCl (12 mmol) for 2 hr in the dark. After usual work-up the reaction mixture was applied on a DEAE-cellulose column (40 × 4.5 cm) equilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ buffer containing 10% ethanol. The salt gradient used for the elution was 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ containing 10% ethanol and 0.35 *M* $(C_2H_5)_3NH^+HCO_3^-$ containing 10% ethanol, 4-l. volume in each vessel. Selected fractions were checked by paper chromatography in solvent system B and on DEAE-cellulose paper using 0.6 *M* ammonium bicarbonate containing 20% ethanol. Pure d-pTpA^{Bz} was isolated by concentration of peak B. It had

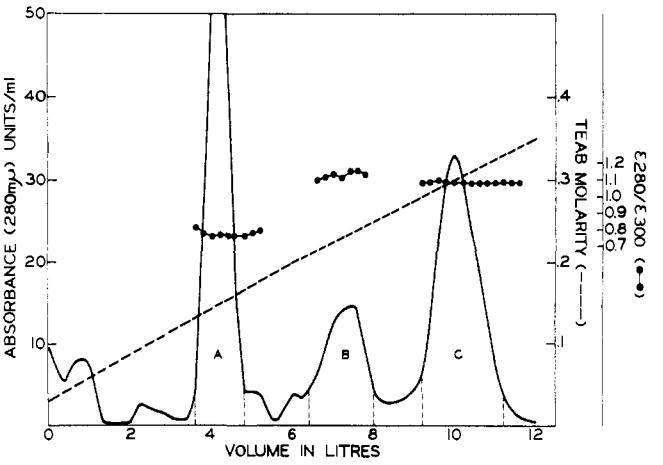


Figure 2. Condensation of d-CE-pC^{An}pA^{Bz} with d-pC^{An}-OAc. Separation of the reaction products on a DEAE-cellulose column (bicarbonate) (4 cm diameter × 60 cm) equilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 10% ethanol. The salt gradient elution was as shown by the dashed line. Peaks were pooled as shown by vertical dotted lines. For identification of peaks, see text.

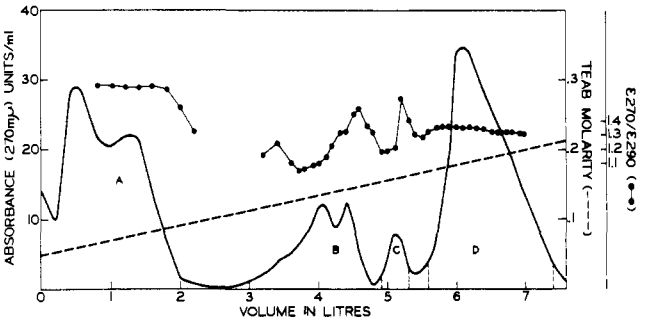


Figure 3. Condensation of d-CE-pTpC^{An} with d-pT-OAc. Separation of the reaction products on a DEAE-cellulose column (bicarbonate) (4.5 cm diameter × 60 cm) preequilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 10% ethanol. The gradient used was as shown by the dotted line. Peaks were pooled as shown by vertical dotted lines. For identification of peaks, see text.

λ_{max} 276 mμ, $\varepsilon_{280}/\varepsilon_{260}$ 1.24. The yield was 50%.

β-Cyanoethyl Dinucleotide d-CE-pTpA^{Bz}. The dinucleotide (2.2 mmol) was cyanoethylated with β-hydracrylonitrile (8 ml) and DCC (6 g) in dry pyridine (20 ml) in the presence of pyridinium Dowex 50 (2 g) in the dark for 18 hr. After the usual work-up as described earlier, the compound was isolated by precipitation from ether, the yield being 75%.

Preparation of the Trinucleotide d-pTpA^{Bz}pG^{iBu}. The protected dinucleotide d-CE-pTpA^{Bz} (1.5 mmol) and d-pG^{iBu}-O*i*Bu (4 mmol) were allowed to react using 6 mmol of MsCl in 10 ml of pyridine for 2 hr. After the usual work-up including the aqueous pyridine and sodium hydroxide treatments, the reaction product was chromatographed on a DEAE-cellulose column. The chromatographic pattern and conditions for elution are shown in Figure 4. Peak C contained the desired trinucleotide, whereas peak B contained the unreacted dinucleotide. Selected fractions were checked in solvents C and D after removal of the protecting groups; R_f values are given in Table II. d-pTpA^{Bz}pG^{iBu} showed λ_{max} 262 and 275 mμ, $\varepsilon_{280}/\varepsilon_{260}$ 0.94.

Acetylation of d-pTpA^{Bz}pG^{iBu}. The trinucleotide (12,000 OD_{280}) was acetylated with acetic anhydride (4 ml) and pyridine (10 ml) for 4 hr. The reaction mixture was worked up as described earlier and the acetylated trinucleotide was precipitated from ether as a white powder. The yield was 90%.

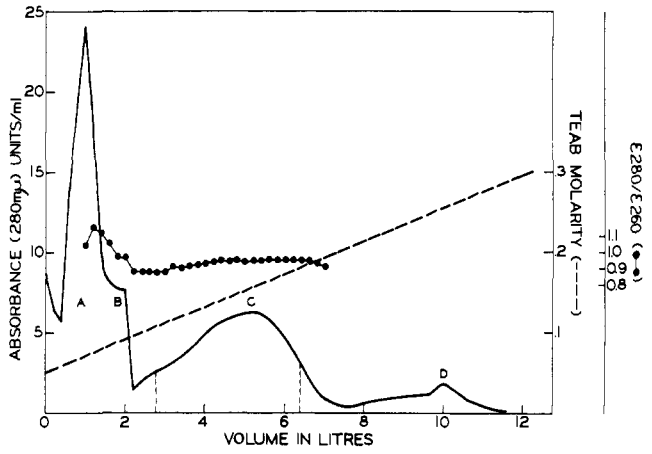


Figure 4. Condensation of d-CE-pTpA^{Bz} with d-pG^{iBu}-O*i*Bu. Separation of the reaction products on DEAE-cellulose column (bicarbonate) (60 cm × 3.5 cm diameter) preequilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 10% ethanol. Peaks were pooled as shown by vertical dotted lines. For identification of peaks, see text.

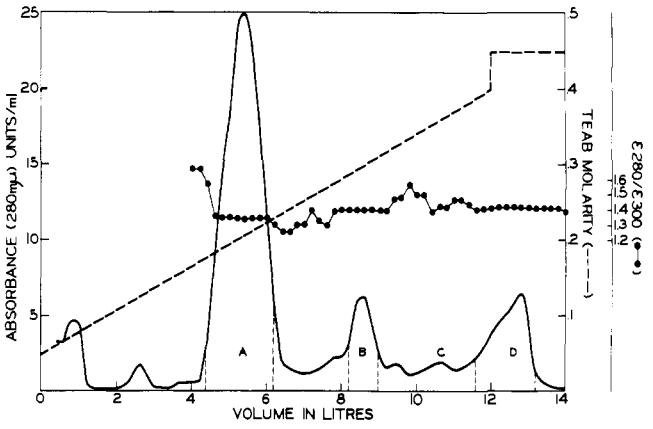


Figure 6. Condensation of d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An} with d-pTpC^{An}pT-OAc. Separation of the reaction products on a DEAE-cellulose column (bicarbonate) (60 cm × 3.5 cm diameter) preequilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 30% ethanol. Elution was carried out using a gradient of $(C_2H_5)_3NH^+HCO_3^-$ indicated by the dashed line. Peaks were pooled as shown by vertical dotted lines. For identification of peaks, see text.

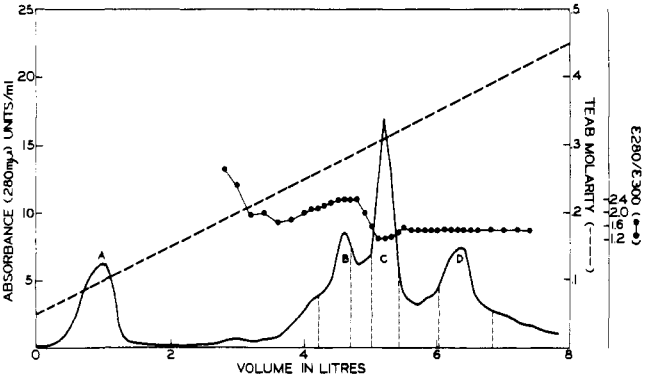


Figure 5. Condensation of d-MMTr-A^{Bz}pTpG^{iBu} with d-pC^{An}-pA^{Bz}pC^{An}-OAc. Separation of the reaction products on a DEAE-cellulose column (bicarbonate) (2.5 cm diameter × 60 cm) (pre-equilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 20% ethanol. The salt gradient is shown by the dashed line. Peaks were pooled as shown by vertical dotted lines. For identification of peaks, see text.

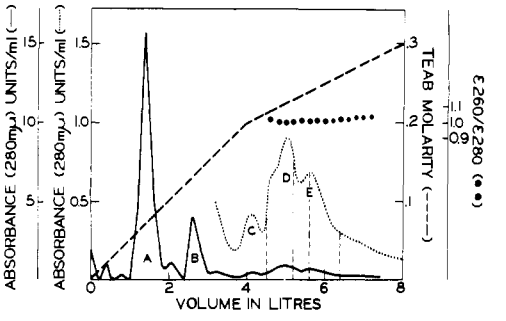


Figure 7. Condensation of d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An}-pTpC^{An}pT with d-pTpA^{Bz}pG^{iBu}-OAc. Separation was on a DEAE-cellulose column (1.5 cm diameter × 70 cm) preequilibrated with 0.05 *M* $(C_2H_5)_3NH^+HCO_3^-$ in 10% ethanol. Peaks were pooled as shown by vertical dotted lines. For identification of peaks, see text.

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

Compound	Degradation product	Theor ratio	Obsd ratio
d-ApTpGpCpApC	d-A:d-pT:d-pC:d-pG:d-pA	1:1:2:1:1	1:1.1:2.3:0.8:1
d-ApTpGpCpApCpTpCpT	d-A:d-pT:d-pC:d-pG:d-pA	1:3:3:1:1	1:3.2:2.8:0.8:1.1
d-ApTpGpCpApCpTpCpTpTpApG	d-A:d-pT:d-pC:d-pG:d-pA	1:4:3:2:2	1:4.2:2.8:2.1:2

[a] The enzymic degradation was carried out as described previously. The mononucleotides and nucleosides produced were separated by chromatography in solvent D, appropriate markers being used along side.

Preparation of the Hexanucleotide d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}-pA^{Bz}pC^{An}. An anhydrous mixture of the trinucleotide d-MMTr-A^{Bz}pTpG^{iBu} (300 μmol) and d-pC^{An}pA^{Bz}pC^{An}-OAc (600 μmol) in dry pyridine (5 ml) was allowed to react with MsCl (3 mmol) by shaking for 3 hr at room temperature. The reaction was terminated by addition of a 1 *M* diisopropylethylamine solution in pyridine (6 ml) and water (5 ml). After storage in aqueous pyridine and a sodium hydroxide treatment, the reaction products were applied on a DEAE-cellulose column. The conditions and the chromatographic pattern are shown in Figure 5. Selected fractions were checked for purity by DEAE-cellulose paper chromatography and after removal of the protecting groups in solvents C and D. Peak A contained mesitylenesulfonic acid; peak B contained unreacted d-MMTr-A^{Bz}pTpG^{iBu}; peak C contained excess d-pC^{An}pA^{Bz}pC^{An}; and peak D contained hexanucleotide. R_f values are given in Tables I and II. The protected hexanucleotide had λ_{max} 280 mμ, $\varepsilon_{280}/\varepsilon_{260}$ 1.32. The yield was 40% as based on d-MMTr-A^{Bz}pTpG^{iBu}.

Preparation of the Nonanucleotide d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}-pA^{Bz}pC^{An}pTpC^{An}pT. An anhydrous mixture of the hexanucleotide d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}pA^{Bz}pC^{An} (150 μmol), d-pTpC^{An}-pT-OAc (1.2 mmol), and MsCl (4 mmol) in dry pyridine (7 ml) was kept for 2.5 hr at room temperature. After the standard work-up, including a sodium hydroxide treatment, the reaction

products were chromatographed on a DEAE-cellulose column. The chromatographic pattern and the conditions are given in Figure 6. Selected fractions were checked by DEAE-cellulose paper chromatography as described above and in solvent system C after removal of the N-protecting groups. Peak A contained excess trinucleotide d-pTpC^{An}pT; peak B contained the unreacted hexanucleotide; peak C contained the symmetrical pyrophosphate of the trinucleotide; and peak D contained the desired nonanucleotide, which had λ_{max} 280 mμ, $\varepsilon_{280}/\varepsilon_{260}$ 1.16. The yield of the nonanucleotide was 35%. R_f values of the unprotected nonanucleotide are given in Table III and the results of snake venom phosphodiesterase degradation are given in Table IV.

Preparation of the Dodecanucleotide, d-MMTr-A^{Bz}pTpG^{iBu}pC^{An}-pA^{Bz}pC^{An}pTpC^{An}pTpTpA^{Bz}pG^{iBu}. An anhydrous mixture of the nonanucleotide (5 μmol, 600 OD_{280}), pyridinium d-pTpA^{Bz}pG^{iBu}-OAc (300 μmol), and MsCl (0.900 mmol) was kept in pyridine (1 ml) for 2 hr at room temperature. After the usual work-up, the reaction mixture was applied on a DEAE-cellulose column. The condition and chromatographic properties are given in Figure 7. Selected fractions were checked in solvent C after taking off the N-protecting groups. Peak A contained excess trinucleotide d-pTpA^{Bz}pG^{iBu}; peak B contained the symmetrical pyrophosphate of the trinucleotide; peak C was not identified; peak D contained unreacted nonanucleotide; and peak E contained the dodecanucleotide. The fractions shown by dotted line in Figure 7 were pooled, concentrated, and further purified by preparative paper chromatography using solvent systems C and D. The yield of the dodecanucleotide was 25%. R_f values and results of enzymatic degradation are given in Tables III and IV. The protected dodecanucleotide had λ_{max} 277 mμ, $\varepsilon_{280}/\varepsilon_{260}$ 1.08.

Studies on Polynucleotides. XCIII.[1] A Further Study of the Synthesis of Deoxyribopolynucleotides Using Preformed Oligonucleotide Blocks[2]

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Abstract: In a further study of polynucleotide synthesis by the use of preformed oligonucleotide blocks, the condensation between the hexanucleotide Tr-TpTpTpTpTpT and the thymidine di-, tri-, tetra-, and pentanucleotides carrying 5′-phosphate end groups (pTpT-OAc and homologs) has been investigated. Mesitylenesulfonyl chloride was used as the condensing agent. The molar excess of the oligonucleotide blocks bearing phosphomonoester groups, the proportion of the condensing agent, and the reaction time were varied. The rates of condensation reaction as well as the *isolated* yields of the desired products were determined. In addition, attention was paid to the extent of polynucleotide chain degradation during the activation process and the nature of the resulting side products. The use of a 10:1 ratio of the oligonucleotide block to Tr-T_6P_5, a lower amount of the condensing agent, and shorter reaction times than those used previously gave the best yields with minimal side reactions. The condensations using di- and trinucleotide blocks showed almost the same rate, and the optimal yields of the desired polynucleotides were, respectively, 78 and 75%. The condensations using the tetra- and pentanucleotides also proceeded at about the same rate, but this rate was lower than that observed for the di- and trinucleotide reactions; the maximum yields obtained in these condensations were close to 50%.

The stepwise synthesis of deoxyribopolynucleotides containing specific sequences may be approached in two alternative ways. In the first approach, suitably protected mononucleotides may be added one at a time to a developing polynucleotide chain. In the second approach, preformed oligonucleotides bearing phosphomonoester end groups may be used in condensation reactions with oligo- or polynucleotidic components bearing hydroxyl end groups. The first approach was developed[3] and used successfully in the earlier work on synthetic deoxyribopolynucleotides.[4] The second approach is more attractive for at least two reasons. If each of the successive block condensations proceed in reasonably good yield, it is possible to increase the overall yield of the desired polynucleotide as based on the increasingly valuable developing chain. Thus, the growing polynucleotide chain is subjected to fewer condensation steps, and even though the yields in the individual reactions may be lower than those for mononucleotide condensations, the yield of the final product can be higher.[5] The second feature of the block approach is

(1) Paper XCII: A. Kumar and H. G. Khorana, *J. Am. Chem. Soc.*, **91**, 2743 (1969).

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

(4) T. M. Jacob and H. G. Khorana, *ibid.*, **87**, 2971 (1965); S. A. Narang and H. G. Khorana, *ibid.*, **87**, 2981 (1965); S. A. Narang, T. M. Jacob, and H. G. Khorana, *ibid.*, **87**, 2988 (1965).

(5) The oligonucleotidic blocks can be prepared usually in satisfactory yields; however, the steps involved in their preparation and the question of yields, which are well below 100% at individual steps, are being omitted from the present argument for over-all efficiency in the synthesis of relatively long deoxyribopolynucleotide chains.