conversion of the starting materials to faster traveling products, whose R_f's are also recorded in Table II. The contaminants in the larger oligonucleotides were also converted to products traveling proportionately higher, and for analysis by venom phosphodiesterase degradation the major bands obtained after dephosphorylation with the phosphomonoesterase were used.

TABLE II

R_f VALUES OF DIFFERENT POLYNUCLEOTIDES IN SOLVENT D ON WHATMAN PAPER 44

Compound	R_f
Polynucleotides bearing -5′ phosphate end groups	
pT (or d-pA)	0.55
d-pTpA	.49
Cyclo-d-pTpA	.58
d-pTpApTpA	.35
d-pTpApTpApTpA	.23[a]
d-pTpApTpApTpApTpA	.145[b]
Larger homologs	.03–0.10
Polynucleotides lacking the terminal -5′ phosphate groups	
d-TpApTpApTpA	0.36
d-TpApTpApTpApTpA	.24
d-TpApTpApTpApTpApTpA	.20
d-TpApTpApTpApTpApTpApTpA	.10
Higher than dodecanucleotide	0–0.06

[a] R_f of the faster traveling impurity in the original peak, 0.35. [b] R_f of the impurity in the original peak, 0.23.

Synthesis of 5′-O-Di-*p*-methoxytrityldeoxyadenylyl-(3′ → 5′)-thymidine-3′ Phosphate, and Deoxyadenylyl-(3′ → 5′)-thymidine-3′ Phosphate.—A dry pyridine (1 ml.) solution of 5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate (0.075 mmole) was treated with DCC (103 mg., 0.5 mmole) for 1 hr. Separately an anhydrous pyridine (1 ml.) solution of β-cyanoethyl thymidine-3′ phosphate (0.1 mmole) was prepared and this solution was added to the first solution. The mixture was kept at room temperature for 4 days. Water (5 ml.) was then added and the reaction mixture left overnight; DCC was then extracted with petroleum ether (3 × 25 ml.) and the solution was then treated with 1 ml. of 1 *N* sodium hydroxide. The mixture was lyophilized. To the resulting powder a further amount (2 ml.) of 1 *N* sodium hydroxide was added and the solution heated at 100° for 10 min. The pH was brought to neutrality by addition of pyridinium Dowex-50 resin and the resin and dicyclohexylurea removed by filtration. Paper chromatography in solvent A showed the main product to be 5′-O-di-*p*-methoxytrityldeoxyadenylyl-(3′ → 5′)-thymidine-3′ phosphate (R_f 0.33), there being, in addition, four weak ultraviolet-absorbing spots. The mixture was applied to the top of a DEAE-cellulose (carbonate form) column (23 × 2.5 cm.) and elution was carried out with a linear gradient, there being 4 l. of water in the mixing vessel and an equal volume of 0.25 *M* triethylammonium bicarbonate in the reservoir; 16–18-ml. fractions were collected at 10-min. intervals. The desired product, 5′-O-di-*p*-methoxytrityldeoxyadenylyl-(3′ → 5′)-thymidine-3′ phosphate, was eluted in fractions 165–213. The combined fractions were evaporated *in vacuo* with all the precautions taken above for the preparation of the 5′-O-di-*p*-methoxytrityldeoxyadenosine-3′ phosphate. The total residue was passed through a column of ammonium Dowex-50 resin and the total effluent lyophilized. Detritylation occurred to the extent of 3–5% during lyophilization, but the product was homogeneous as shown by removal of the di-*p*-methoxytrityl group. Deoxyadenylyl-(3′ → 5′)-thymidine-3′ phosphate thus prepared was homogeneous by paper chromatography and on paper electrophoresis both in acidic and neutral pH range. Incubation of a sample with spleen phosphodiesterase under the standard conditions described previously caused complete degradation, deoxyadenosine-3′ phosphate and thymidine-3′ phosphate being formed in approximately equal amounts. The yield of the isolated dinucleotide was estimated spectrophotometrically to be 34%, using an extinction value at 263 mμ of 20,000.

[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, THE UNIVERSITY OF WISCONSIN, MADISON 6, WIS.]

Studies on Polynucleotides. XXVII.[1] The Stepwise Synthesis of Specific Deoxyribopolynucleotides (7).[2] The Synthesis of Polynucleotides Containing Deoxycytidine and Deoxyguanosine in Specific Sequences and of Homologous Deoxycytidine Polynucleotides Terminating in Thymidine[3]

BY H. SCHALLER AND H. G. KHORANA

RECEIVED MAY 20, 1963

The following protected deoxyribodinucleotides were prepared: 5′-O-cyanoethylphosphoryl-N-anisoyldeoxycytidylyl-(3′ → 5′)-N-acetyldoxyadenosine, 5′-O-phosphorylthymidylyl-(3′ → 5′)-N-acetyldeoxyguanosine, the corresponding 3′-O-acetyl derivative, 5′-O-phosphoryl-N-acetyldeoxyguanylyl-(3′ → 5′)-N,3′-O-diacetyldeoxycytidine, and 5′-O-phosphorvl-N-anisoyldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine. The treatment of the last-mentioned protected dinucleotide with dicyclohexylcarbodiimide (DCC) in anhydrous pyridine–dimethylformamide followed by appropriate work-up gave homologous tetra-, hexa-, octa-, and a small amount of the decanucleotide containing deoxycytidine and deoxyguanosine nucleosides in alternating sequence. The pentanucleotide, deoxycytidylyl-(3′ → 5′)-deoxyguanylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine, was prepared by condensation of 5′-O-dimethoxytrityl-N-anisoyldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine with 5′-O-phosphoryl-N-acetyldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxycytidylyl-(3′ → 5′)-N-3′-O-diacetyldeoxycytidine followed by removal of the protecting groups and ion-exchange chromatography. A new approach to the synthesis of homopolynucleotides containing a different nucleoside at one terminus was developed which involved the treatment of the total mixture obtained by polymerization of a protected mononucleotide with an excess of a suitably protected nucleoside in the presence of a condensing agent. Thymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine was also prepared by condensation of 5′-O-phosphoryl-N-anisoyldeoxycytidylyl-(3′ → 5′)-N-anisoyldeoxycytidylyl-(3′ → 5′)-N-anisoyl-3′-O-acetyldeoxycytidine with 5′-O-dimethoxytritylthymidine.

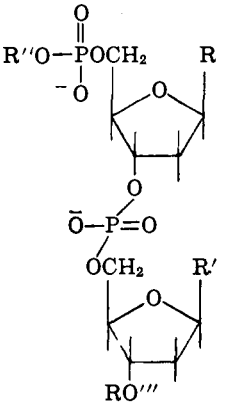
As part of a study of the synthesis of oligonucleotide chains by condensation of suitably protected di- and trinucleotides, the preceding paper described the synthesis of the pentanucleotide thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine and of polynucleotides containing thymidine and deoxyadenosine in alternating sequence.[1] The present paper deals, partly, with a parallel investigation of the synthesis of polynucleotides containing deoxycytidine and deoxyguanosine and, partly, with the evaluation of the available protecting groups in the general problems of polynucleotide synthesis. In addition, a new approach to the synthesis of homopolynucleotides containing a different nucleoside at one terminus is described. A brief report of a part of this work has already appeared.[4]

(1) Paper XXVI: G. Weimann, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 3835 (1963).

(2) Earlier papers in this series which deal with this topic: (a) P. T. Gilham and H. G. Khorana, *ibid.*, **80**, 6212 (1958); (b) *ibid.*, **81**, 4647 (1959); (c) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962); (d) H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *ibid.*, **85**, 3821 (1963); (e) H. Schaller and H. G. Khorana, *ibid.*, **85**, 3828 (1963); (f) ref. 1.

(3) This work has been supported by grants from the National Science Foundation, Washington, D. C., and the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service.

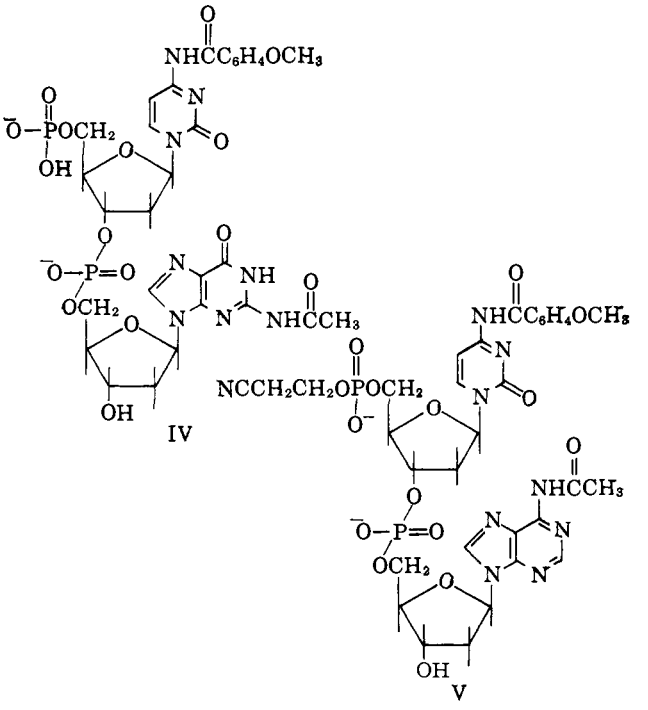
Protected Dinucleotides.—For the synthesis of polynucleotides by condensation of the 5′-phosphomonoester group of one oligonucleotide with the 3′-hydroxyl group of a second oligonucleotide, the protected dinucleotides required are of three general types. The first one is that in which the 5′-phosphomonoester group is free but the 3′-hydroxyl end group is protected (I) and the second type is that in which the 3′-hydroxyl group is free but the 5′-phosphomonoester group is protected by preparation of a diester, for example, the cyanoethyl phosphate ester II. The third type which has both the 3′-hydroxyl group and the 5′-phosphomonoester group free (III) would serve as suitable starting materials for polymerization by self-condensation. Three types of approaches are now



I–III, R,R′ = protected purine or pyrimidine
I, R″ = H; R‴, acyl, *e.g.*, acetyl
II, R″ = *e.g.*, CH_2CH_2CN; R‴ = H
III, R″ = R‴ = H

available for the synthesis of the protected dinucleotides. The first involves the phosphorylation of a protected dinucleoside phosphate containing a free 5′-hydroxyl group. Examples of syntheses using this approach and resulting in compounds of the type III have been provided in earlier papers.[2a,2c,2e] The second approach is provided by a variant of the polymerization reaction[5,6] in which a mixture of an excess of a protected mononucleotide and another mononucleotide containing free 3′-hydroxyl group may be polymerized. A major product would be expected to be a dinucleotide of the type I. An application of this approach is provided in the present work by the reaction of N-acetyldeoxyguanosine-5′ phosphate[7] and N,3′-O-diacetyldeoxycytidine-5′ phosphate with dicyclohexylcarbodiimide (DCC) in dry pyridine. The dinucleotide 5′-O-phosphoryl-N-acetyldeoxyguanylyl-(3′ → 5′)-N,3′-O-diacetyldeoxycytidine d($pG^{Ac}pC^{Ac}$-OAc)[7] was isolated pure in about 25% yield. (The unprotected dinucleotide d-pGpC[7] was subsequently obtained by an ammoniacal treatment.)

The third approach for the preparation of protected dinucleotides involves the condensation of an N-protected deoxyribonucleoside-5′ β-cyanoethyl phosphate with another N,3′-O-protected nucleoside-5′ phosphate. Although this approach was developed early in our studies,[2c] an outstanding example of its use was described in the preceding paper: this involved the preparation of 5′-O-phosphorylthymidylyl-(3′ → 5′)-N-benzoyldeoxyadenosine (d-$pTpA^{Bz}$),[7] the starting material for the synthesis of polynucleotides containing alternating thymidine and deoxyadenosine units. In the present work, this approach was applied to the synthesis of several protected dinucleotides. The condensation of N,3′-O-diacetyldeoxyguanosine-5′ phosphate[8] with thymidine-5′ β-cyanoethyl phosphate followed by a mild alkaline treatment gave 5′-O-phosphorylthymidylyl-(3′ → 5′)-N-acetyldeoxyguanosine (d-$pTpG^{Ac}$). Similarly, the condensation of N,3′-O-diacetyldeoxyguanosine-5′ phosphate with N-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate, whose preparation is also described in the Experimental section, gave the fully protected dinucleotide (d-CE-$pC^{An}pG^{Ac}$-OAc)[7] from which by careful alkaline treatment, the dinucleotide IV ($pC^{An}pG^{Ac}$) was prepared. (The polymerization of this dinucleotide is described below.)



IV

V

N-Acetyldeoxyguanosine-5′ β-cyanoethyl phosphate would be analogously useful for preparation of protected dinucleotides containing a terminal 5′-O-phosphorylguanosine unit and the preparation of this derivative from N-acetyldeoxyguanosine-5′ phosphate[8] is described in the Experimental section.

Of the three types of protected dinucleotides (I–III) discussed above, type III can now be prepared in satisfactory yield in any combination of the two mononucleotides. Type I may be prepared readily from type III by a simple acetylation reaction. Dinucleotides of the type II which are of interest in construc-

(4) H. Schaller, G. Weimann, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 355 (1963).

(5) See *e.g.*, H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961).

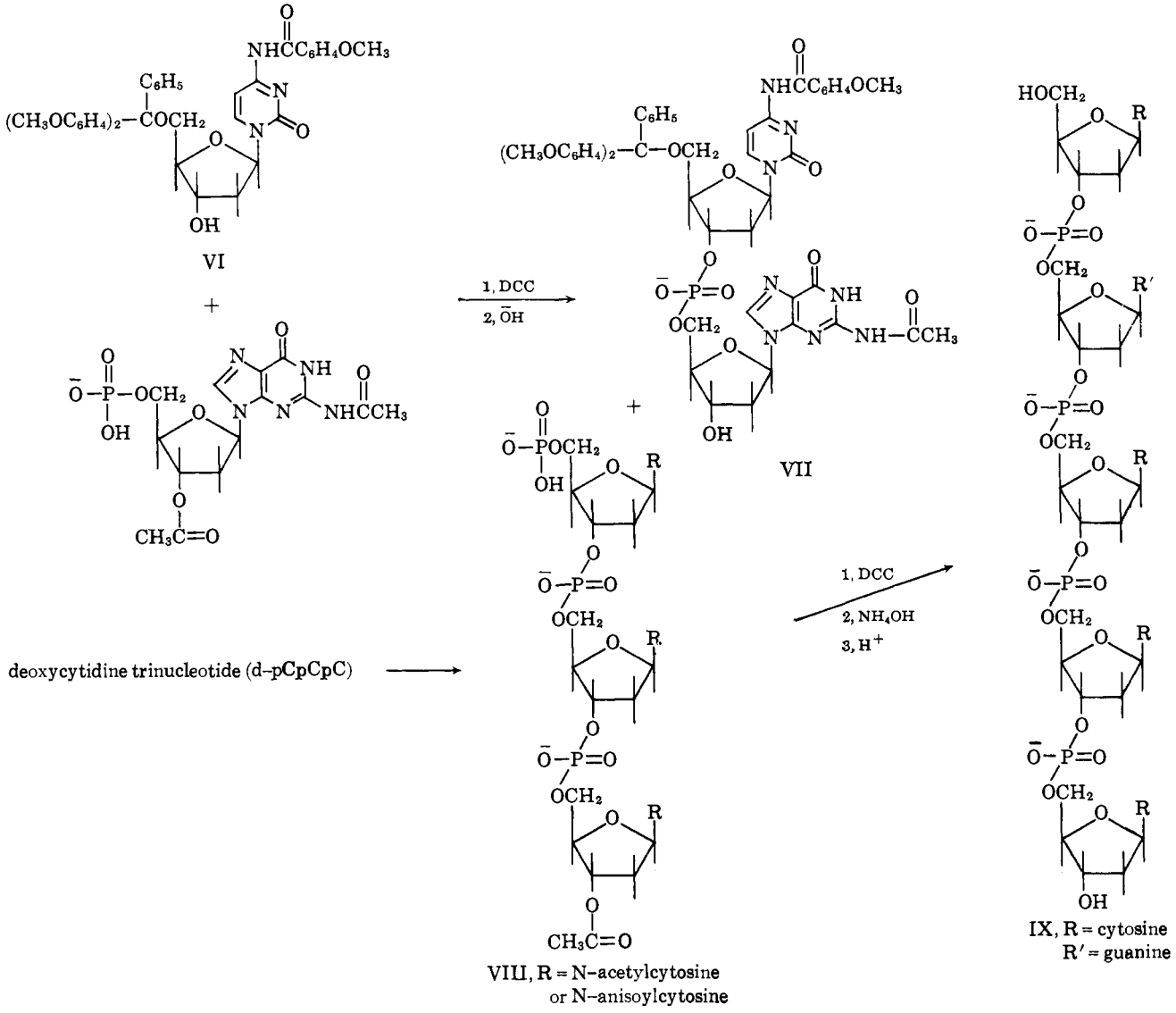
(6) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," John Wiley and Sons, Inc., New York, N. Y., 1961.

(7) The basic system of abbreviations for polynucleotides used here and in earlier papers in this series[8] (*e.g.*, ref. 5) is as has been described elsewhere[2a,6] and is in use in *J. Biol. Chem.* Thus the single letters A, T, C, and G represent the nucleosides of respectively, adenine, thymine, cytosine, and guanine. The letter "p" to the left of the nucleoside initial indicates a 5′-phosphomonoester group and the same letter to the right indicates a 3′-phosphomonoester group. Thus, in going from the left to the right a polynucleotide chain is specified in the C_3'-C_5' direction. In the present and future synthetic work involving protected derivatives of nucleosides and nucleotides, we propose to use additional abbreviations as follows: The protecting groups on the purine or pyrimidine rings will be designated by two letter abbreviations added as superscripts after the nucleoside initial. Thus A^{Bz} for N-benzoyldeoxyadenosine, C^{An} for N-anisoyldeoxycytidine, G^{Ac} for N-acetylguanosine. The cyanoethyl ester of a 5′-phosphomonoester group will be abbreviated to CE-p. Thus, for example, $CEpC^{An}$ for N-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate. The acetyl group at the 3′-hydroxyl group of a nucleoside will be shown by -OAc added after the nucleoside. Thus for example d-pG^{Ac}-OAc for N,3′-O-diacetyldeoxyguanosine-5′ phosphate; d-$pC^{Ac}pC^{Ac}pC^{Ac}$-OAc for 5′-O-phosphoryl-N-acetyldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxycytidylyl-(3′ → 5′)-N,3′-O-diacetyldeoxycytidine.

(8) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1983 (1963).

CHART I

SYNTHESIS OF DEOXYCYTIDYLYL-(3′→5′)-DEOXYGUANYLYL-(3′→5′)-DEOXYCYTIDYLYL-(3′→5′)-DEOXYCYTIDYLYL-(3′→5′)-DEOXYCYTIDINE (IX)



tion of longer polynucleotide chains by elongation from the 3′-hydroxyl end present still somewhat of an outstanding problem. In the present work, N,3′-O-diacetyldeoxyadenosine-5′ phosphate was brought into reaction with N-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate in the presence of DCC. From the initially formed fully protected dinucleotide (d-CE-pC^{An}pA^{Ac}-OAc)[7] it was attempted to remove selectively the 3′-O-acetyl group. Although the desired dinucleotide V was obtained, the yield was not satisfactory because of the concomitant partial elimination of the cyanoethyl group. Earlier experiments on the kinetics of alkaline hydrolysis of 3′-O-acetylthymidine-5′ β-cyanoethyl phosphate also showed comparable rates for the deacetylation and the elimination of the cyanoethyl group.[2e] Further work on this problem is now in progress.

Deoxycytidylyl-(3′→5′)-deoxyguanylyl-(3′→5′)-deoxycytidylyl-(3′→5′)-deoxycytidylyl-(3′→5′)-deoxycytidine (IX).—In parallel with the synthesis of the pentanucleotide[9] described in the preceding paper, the synthesis of the present oligonucleotide[9] was undertaken to investigate further the efficiency of internucleotide bond synthesis when one of the components is a trinucleotide bearing a free 5′-phosphomonoester group.

The steps used in the present synthesis are shown in Chart I. The condensation of N-benzoyl-5′-O-dimethoxytrityldeoxycytidine[2d] (0.35 mmole) (VI) with N,3′-O-diacetyldeoxyguanosine-5′ phosphate[8] (0.7 mmole) in the presence of DCC, followed by a mild alkaline treatment, gave a virtually quantitative yield of VII. The second component (VIII)[10] was prepared from deoxycytidine trinucleotide (d-pCpCpC), which was one of the oligonucleotides obtained by the polymerization of N-anisoyldeoxycytidine-5′ phosphate described previously.[11] Acetylation of the trinucleotide to VIII presented difficulty because of its high insolubility in essentially all solvents. The reaction failed to go to completion even when the powerful reagent acetyl chloride–pyridine was used. The strongly basic tetraalkylammonium hydroxides, *e.g.*,

(9) The synthesis of pentanucleotides containing four pyrimidines and one purine in a definite position was also of interest in continuation of our studies on the specificity and mode of action of deoxyribonucleases such as pancreatic deoxyribonuclease: R. K. Ralph, R. A. Smith, and H. G. Khorana, *Biochem.*, **1**, 131 (1962).

(10) The alternative of obtaining the N-protected trinucleotide from the polymerization mixture[11] directly by ion exchange chromatography did not prove practical because of poor separation of the polymeric products containing N-anisoyl groups on DEAE-cellulose columns.

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

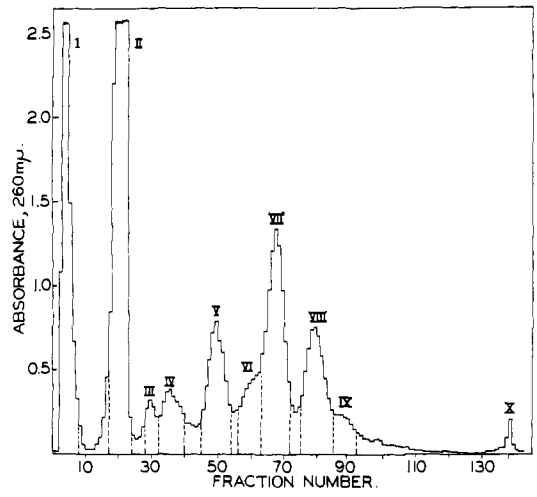


Fig. 1.—Chromatography on a DEAE–cellulose (carbonate) column of the products of condensation of d-pC^{Ac}pC^{Ac}pC^{Ac}OAc and 5′-O-di-*p*-methoxytrityl-N-anisoyldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine. Peak V contains the pentanucleotide d-CpGpCpCpC. For details see text.

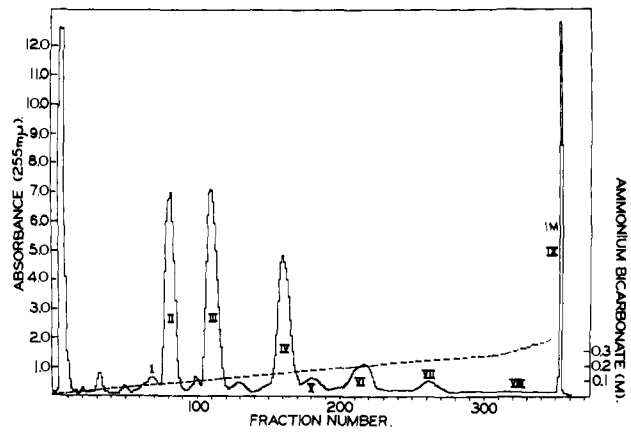


Fig. 2.—Separation of polynucleotides obtained by polymerization of the protected dinucleotide d-pC^{An}pG^{Ac} on a DEAE–cellulose column in the presence of 7 *M* urea. For details see text.

tri-*n*-hexylethylammonium hydroxide,[12] were effective in causing solubilization in dry pyridine and the acetylation with acetic anhydride then went smoothly. Completion of the reaction was determined by the characteristic shift in the ultraviolet absorption spectrum on N-acetylation in this series. In another experiment, the trinucleotide (d-pCpCpC) was converted to the corresponding N-anisoyl derivative (d-pC^{An}pC^{An}pC^{An})[7] by the method described for N-anisoyldeoxycytidine-5′ phosphate. This product was then treated with acetic anhydride in pyridine for acetylation of the terminal 3′-hydroxyl group. (The use of the anisoyl derivative is described below.)

Condensation of the protected trinucleotide d-pC^{Ac}pC^{Ac}pC^{Ac}-OAc (VIII) with the protected dinucleoside phosphate VII was effected in a mixture of pyridine and dimethylformamide using either DCC or 2,5-dimethylbenzenesulfonyl chloride as the condensing agent. The desired product was isolated pure by anion exchange chromatography after removal of the protecting groups. The elution pattern of the total mixture is shown in Fig. 1 and the detailed identification of the different peaks is given in the Experimental section. The desired product IX, d-CpGpCpCpC, was present in peak V, the yield being 15–17%. It was characterized to be pure by chromatography (three solvents) and by degradation with venom phosphodiesterase.[13] Degradation went to completion, the products being deoxycytidine-5′ phosphate, deoxyguanosine-5′ phosphate, and deoxycytidine in the expected molar proportions. The ultraviolet absorption spectral characteristics of the pentanucleotide are given in the Experimental section.

Deoxyribopolynucleotides Containing Deoxycytidine and Deoxyguanosine in Alternating Sequence.—The synthesis of such copolymers was undertaken because of our interest in studying polymers containing purine and pyrimidine nucleosides in alternating sequence,[14] the synthesis of the corresponding deoxyadenylate–thymidylate polymers having been reported in the preceding paper. The starting material used was the protected dinucleotide d-pC^{An}pG^{Ac} (IV) described above. The medium of polymerization was a mixture of dimethylformamide and pyridine, pyridinium Dowex-50 ion exchange resin being present: the reagent used was DCC. Following the standard type of polymerization experiment, acetic anhydride–pyridine treatment[15] was given in the hope of cleaving the pyrophosphate linkages involving the terminal phosphomonoester groups of different oligonucleotides.[16] For separation of the polymeric mixture an attempt was made to chromatograph the products on a DEAE–cellulose column before removal of the protecting groups on the heterocyclic rings.[17] However, the separation was far from satisfactory, similar experience being met previously in attempts to separate N-anisoyldeoxycytidine polynucleotides.[10] After removal of the N-protecting groups, the separation on a DEAE–cellulose column was again not completely satisfactory, only the early peaks being well resolved. Better separation was accomplished by chromatography in the presence of 7 *M* urea according to the procedure developed by Tener and Tomlinson,[18] the technique being applied either directly to the polymeric mixture (Fig. 2) or after prior chromatography on a DEAE–cellulose column by the standard method[5] (Fig. 3). The distribution of the nucleotidic material in the different peaks obtained by chromatography (Fig. 2 and 3) and the composition of the different peaks are shown in Tables I and II.

The first noteworthy feature of these results is the formation of a considerable amount of the cyclic dinucleotide X. A similar amount of the corresponding adenine–thymine member was found in the polymerization[1] of the protected dinucleotide d-pTpA^{Bz}. The apparent ease in the formation of the cyclic dinu-

(12) Prepared by the reaction of tri-*n*-hexylamine with ethyl iodide in ethyl acetate followed by passage of an aqueous solution of the crystalline ethiodide through the hydroxide form of the Dowex-1 ion exchange resin.

(13) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **235**, 2105 (1959).

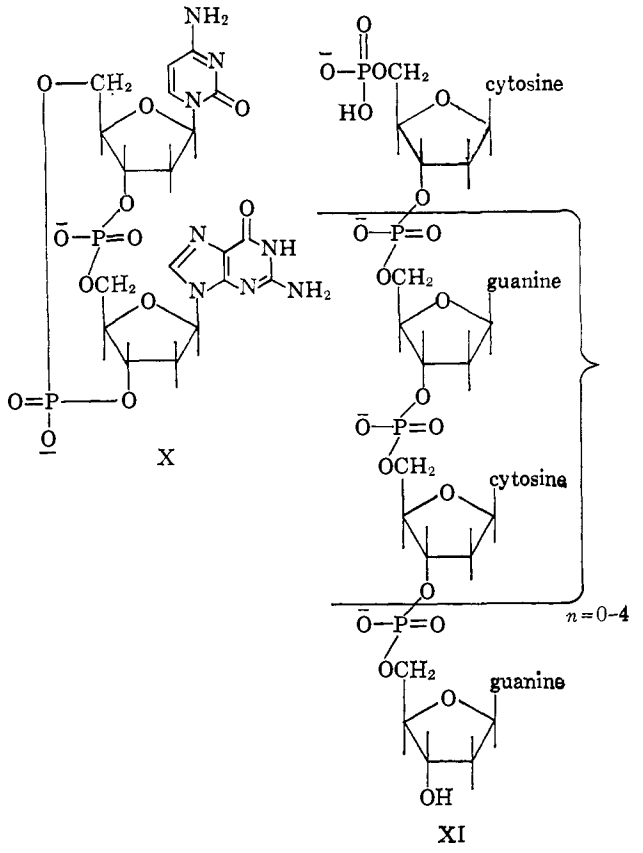
(14) Physicochemical and enzymic studies on such polymers will be reported in subsequent papers. See also the preceding paper[1] for some preliminary results.

(15) H. G. Khorana and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **81**, 4660 (1959).

(16) As found below, substantial amounts of pyrophosphate linked products were still present. Acetic anhydride-pyridine treatment is usually effective in cleaving the pyrophosphate linkages but sometimes it has not given satisfactory results. There seems to be some aspect of this reaction which seems as yet unclear and leads to variable results. It seems possible that the equilibria postulated earlier[15] are influenced greatly by the concentration of acetate ions as found in other more recent work [D. H. Rammler, Y. Lapidot, and H. G. Khorana, *J. Am. Chem. Soc.*, **85**, 1989 (1963)]. Thus, although a very large excess of acetic anhydride is usually employed, the presence of any water may simply have the effect of providing unfavorable concentrations of acetate ions.

(17) The hope here was to avoid any aggregation that might occur and the consequent difficulties in separation and elution from anion exchange columns, in analogy with the experience with deoxyguanosine oligonucleotides.[8] There, the presence of the N-acetyl group on the guanine rings completely eliminated the tendency toward aggregation and well resolved peaks were then obtained on chromatography under usual conditions.

(18) G. M. Tener and N. Tomlinson, *J. Am. Chem. Soc.*, **84**, 2644 (1962).



cleotide from the dinucleotides containing one purine and one pyrimidine nucleotide is interesting in view of the previous results where in the polymerization of purine nucleotides[8,19] the cyclic trinucleotides took the place of the cyclic dinucleotides[5,11] encountered in large amounts during polymerization of pyrimidine mononucleotides.

A considerable amount of the dinucleotide d-pCpG was recovered and a rather large amount of the same was accounted for by the symmetrical pyrophosphate [d-O-(PCpG)$_2$] formed from it by linkage of the phosphomonoester groups. The linear tetranucleotide d-pCpGpCpG) (XI, n = 1) was pure as obtained by column chromatography, but it was necessary to purify further the higher members by preparative paper chromatography both before and after treatment with the bacterial alkaline phosphomonoesterase. The R_f's of the purified products are listed in Table III and further characterization by degradation with venom phosphodiesterase is given in Table IV for the tetranucleotide d-CpGpCpG and the hexanucleotide d-CpGpCpGpCpG. The octanucleotide was purified by similar procedures (R_f in Table III), and there was evidence (prolonged paper chromatography) for the presence of the decanucleotide.

From the results given in Tables I–III it is clear that the extent of polymerization was not as great as was achieved in the parallel study with the dinucleotide d-pTpA^{Bz}.[1] The polymerization reactions still represent aspects that are not clearly understood and, as a result, variations in results are not infrequent. Further intensive studies on the polymerization of di- and trinucleotides are now in progress. However, the short chains of alternating deoxycytidylate–deoxyguanylate polynucleotides are clearly of interest in connection with chemical and enzymic studies, such copolymers having hitherto not been previously obtained by chemical or enzymic methods.

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

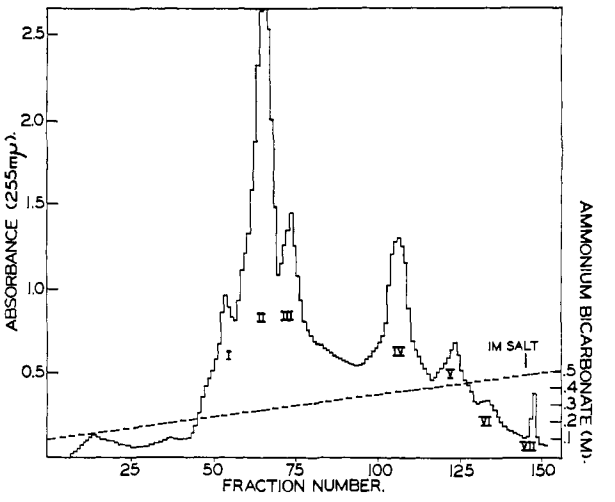
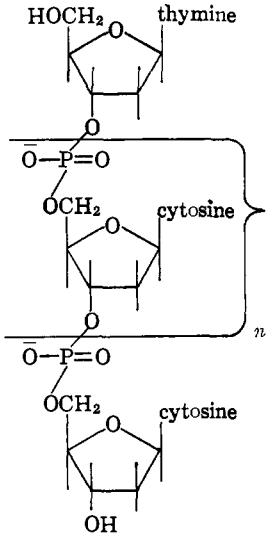


Fig. 3.—Rechromatography of polynucleotides with alternating deoxycytidine–deoxyguanosine units on a DEAE–cellulose column in the presence of 7 *M* urea. The material applied to the column was the 2 *M* fraction obtained by prior chromatography on a DEAE–cellulose column without urea. For further details see text.

Deoxycytidine Polynucleotides Terminating in Thymidine.—Homopolynucleotides[20] with different terminal nucleotides have proved particularly useful in work on the specificity of nucleases[9,21,22] and continue to be of interest in work on sequential analysis of polynucleotides as well as in studies of nucleic acid synthesizing enzymes.[23] In an earlier paper[5] in this series a general method for the preparation of homopolynucleotides containing a different unit at the 3′-hydroxyl end of the chains (*e.g.*, d-pTpTpTpC)[7] was described. The method involved the incorporation of an N,3′-O-diacyldeoxyribonucleoside-5′ phosphate into the nucleoside-5′ phosphate being polymerized. In the present work a complementary approach has been developed in which the different nucleoside unit is added at the opposite end (bearing 5′-hydroxyl group) of the homopolynucleotide. Thus, the polymeric mixture resulting from the polymerization of a suitably protected nucleoside-5′ phosphate is subsequently treated, in the presence of a condensing agent, with an excess of a protected deoxyribonucleoside bearing a free 3′-hydroxyl group. The reaction results in the linkage of the terminal 5′-phosphomonoester groups of the polynucleotides with the free hydroxyl group of the added nucleoside. In the present work, a polymeric mixture of N-anisoyldeoxycytidine polynucleotides was prepared as described previously, and without any work-up it was treated in anhydrous dimethylformamide with an excess of 5′-O-dimethoxytritylthymidine and DCC. After 4 days at room temperature the mixture was worked up so as to remove the protecting groups and the total products chromatographed on a DEAE–cellulose column. The elution pattern is shown in Fig. 4 and the distribution of the total nucleotidic material and the composition of the different peaks are shown in Table V. As can be seen, the procedure was effective in giving as the major products the desired products of the general structure XII. There were present, as expected, the deoxycytidine cyclic oligonucleotides and also some of the unreacted linear members, d-pCpCpC and homologs. Further purification of the desired homologous compounds of the type

(20) Polynucleotides containing only one kind of nucleotide.
(21) W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **235**, 2114 (1959).
(22) W. Fiers and H. G. Khorana, *ibid.*, **238**, 2789 (1963).
(23) For example, A. Falaschi, J. Adler, and H. G. Khorana, *ibid.*, **238**, 3080 (1963).



XII

XII was accomplished by rechromatography on appropriate DEAE–cellulose columns, as exemplified by the preparation of d-TpCpCpCpCpC described in the Experimental section. The characterization of the series of compounds was accomplished as before by degradation with venom phosphodiesterase followed by analysis of the products, thymidine and deoxycytidine-5′ phosphate. The R_f's of the homologous members are given in Table VI.

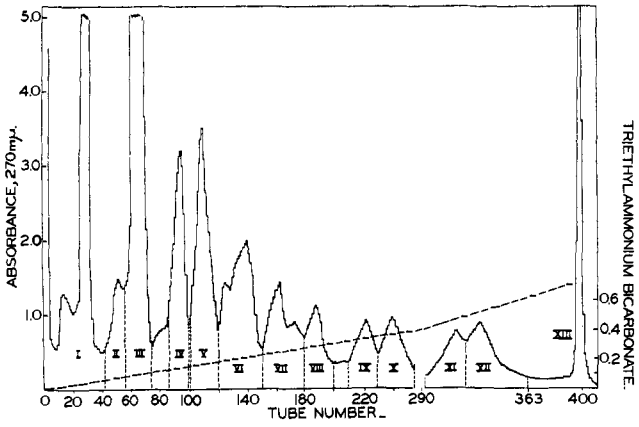


Fig. 4.—The separation of deoxycytidine polynucleotides containing terminal thymidine (d-TpCpCpC and homologs) on a DEAE–cellulose column. For details see text.

The above approach was also examined in the case of a purified oligonucleotide. The N-anisoyldeoxycytidine trinucleotide d-pC^{An}pC^{An}pC^{An}-OAc (VIII) was brought into reaction with about 20-fold excess of 5′-O-di-*p*-methoxytritylthymidine and DCC in a mixture of anhydrous pyridine and dimethylformamide. After a 6-day reaction period, the protecting groups were removed and the products analyzed either by paper chromatography or by column chromatography (Fig. 5). The yield of the pure desired d-TpCpCpC (XII, n = 2) was 59%. It was characterized to be pure by chromatography in two solvent systems and by enzymic degradation. The yield obtained in this synthesis, which started with a purified trinucleotide bearing a free 5′-phosphomonoester group, evidently was less than that obtained in the above experiment in which the total polymeric mixture was used. It is entirely possible that the actual manner of activation of the terminal phosphate groups in the two experiments was different.

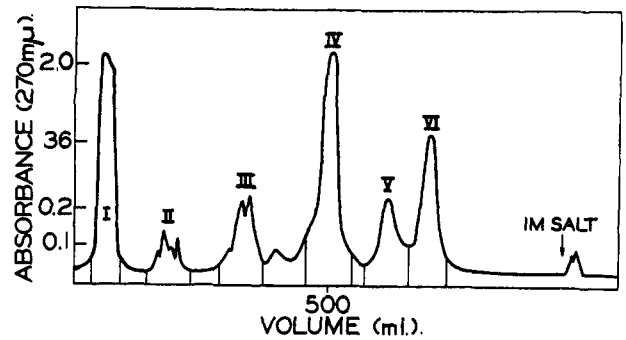


Fig. 5.—Separation of the products obtained by the condensation of 5′-O-dimethoxytritylthymidine with the protected trinucleotide d-pC^{An}pC^{An}pC^{An}-OAc followed by removal of protecting groups. For details see text.

The above principle of esterifying the terminal phosphomonoester group of a polynucleotide chain[24] is of potential use in a number of directions. Thus, it could probably be used in the addition of two new nucleotide units at terminii of polynucleotide chains, when instead of the protected nucleoside used above, protected dinucleoside phosphates (*e.g.*, VIII) are used. Further, an important application has already been made in this Laboratory in which the esterification of the terminal phosphomonoester groups was used to bind the polynucleotides to cellulose.[26]

Discussion

From the total work presented in this and the accompanying three papers,[2d-2f] it is clear that satisfactory methods are available for the preparation of suitably protected derivatives of all of the major deoxyribonucleosides and of the corresponding nucleotides. Further, the method of internucleotide bond synthesis developed[2a] has been proved to be uniformly satisfactory and, also, that all of the possible suitably protected dinucleotides can be readily and satisfactorily prepared. The general approach for the building up of deoxyribopolynucleotide chains which has been the focus of intensive study and emerges as the one of choice is that in which the 5′-phosphomonoester group of one component is condensed with the 3′-hydroxyl group of a second suitably protected component.

So far as the question of suitability of the different available protecting groups is concerned, the only outstanding practical limitation concerns overlapping rates of hydrolysis of the 3′-O-acetyl group present at one end of a chain and that of the cyanoethyl group at the 5′-phosphomonoester end. The problem has been discussed specifically above and is under further investigation.

There are two possible approaches to putting together of "longer" polynucleotide chains. In the first, protected deoxyribonucleoside-5′ phosphates form one of the components and the chain elongation is brought about by the addition of one unit at a time at the 3′-hydroxyl end of the chain. This approach was given a preliminary study much earlier in the synthesis of thymidylyl-(3′ → 5′)-thymidylyl-(3′ → 5′)-thymidine and thymidylyl-(3′ → 5′)-deoxyadenylyl-(3′ → 5′)-deoxycytidine.[2b] The second approach in-

(24) There is the alternative means of obtaining compounds of the type XII, which would involve incorporation of a protected deoxyribonucleoside-3′ phosphate (in the case of XII, 5′-O-acetylthymidine-3′ phosphate[25]) during the polymerization of another N-protected deoxyribonucleoside-3′ phosphate (in the case of XII, N-anisoyldeoxycytidine-3′ phosphate). This approach, however, lacks the scope which, as indicated above, the approach involving esterification of terminal phosphomonoester group possesses.

(25) G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 4329 (1962).

(26) P. T. Gilham, *ibid.*, **84**, 1311 (1962).

volves the condensation of preformed suitably protected di- and trinucleotides. This approach was also investigated earlier in the synthesis of thymidine tri- and tetranucleotides[2c] and has been given closer scrutiny in the preceding and the present paper, the syntheses of the pentanucleotides d-TpApTpTpT and d-CpGpCpCpC having now resulted. The yields using "economical" proportions of the two components were, in each case, in the range of 15%. While it is likely that with further refinements in experimentation the yields could be improved, it is unlikely that there would be a dramatic change. The results support the general conclusion evident from earlier work that an increase in the size of the nucleotide component has an adverse effect on the yield of the internucleotide bond.[2e,25] In the case of activation of the protected trinucleotides (*cf.* pTpTpT-OAc) used in the present work, another complication is likely and the magnitude of this may be expected also to increase with the size of the oligonucleotide component. This would arise from an intramolecular pyrophosphate formation between the terminal phosphomonoester group and an adjacent phosphodiester linkage. The activated intermediate thus formed would probably be not as reactive as the one to be expected on activation of a mononucleotide alone.[25]

The conclusion from all of the present and the earlier results is that the approach involving addition of mononucleotide units to the 3′-hydroxyl end of a growing chain is probably the better approach for the synthesis of longer chains although the steps may be greater in number. This approach is now under careful study and the results will be reported in a forthcoming paper.[27]

An important extension of the earlier studies on polymerization was reported in the preceding and the present paper. This involved the polymerization of preformed protected dinucleotides. Although the results were somewhat variable, they are encouraging in that the method provides rather simply larger numbers of interesting polynucleotides. Work along these lines is deserving of further study and the polymerization of preformed trinucleotides of specific sequences is in active progress.

Subtle differences are apparent in the mechanisms of activation of phosphomonoester groups in the different types of experiments reported in the present papers. Thus the results obtained in the polymerization of the protected dinucleotides, especially that of d-pTpA^{Bz}, are relatively more satisfactory than those usually obtained in stepwise synthesis using oligonucleotide "blocks." Similarly, significant difference was noted in the yields of the compounds of the type d-TpCpCpC when the total polymeric mixture was directly treated with the protected nucleoside thymidine and when the purified protected trinucleotide d-pC^{An}pC^{An}pC^{An}-OAc was brought into reaction with a large excess of the nucleoside. The mechanisms of activation of the phosphomonoester groups in polynucleotide chains are clearly deserving of further study.

Experimental

General Methods.—These including the solvent systems for paper chromatography were as described in an accompanying paper.[2d]

N^6-Benzoyl-5′-O-di-*p*-methoxytrityldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine.—To a pyridine solution (13 ml.) of pyridinium N,O$^{3'}$-diacetyldeoxyguanosine-5′ phosphate (0.7 mmole), N^6-benzoyl-5′-O-dimethoxytrityldeoxycytidine[2d] (220 mg., 0.35 mmole), dimethylformamide (1 ml.), and dry pyridinium Dowex-50 ion exchange resin (200 mg.) were added and the total evaporated to an oil. Pyridine (1 ml.) and DCC (700 mg.) were added and the mixture shaken in the dark at room temperature for 3 days. Water (1 ml.) and pyridine (2 ml.) were then added and the mixture extracted with cyclohexane twice, filtered, and concentrated at below 10°. The concentrate was made up to 25 ml. with pyridine (stock solution) after the addition of 0.13 ml. (1 mmole) of triethylamine. One-fifth of this solution was concentrated *in vacuo* and the residue kept in concentrated ammonium hydroxide (5 ml.) for 2 days at room temperature. After evaporation of the ammoniacal solution, the residue was treated with 5 ml. of 80% acetic acid at room temperature for 1 hr. The total was then applied to three 9-in. wide strips of Whatman 3 MM paper and chromatographed in solvent A. Although other faint bands were present, the major ones were those of deoxyguanosine-5′ phosphate and of deoxycytidylyl-(3′ → 5′)-deoxyguanosine. A small amount of unreacted deoxycytidine was also present. After elution of the bands, the concentrations of the different products were determined spectrophotometrically. Deoxycytidylyl-(3′ → 5′)-deoxyguanosine was recovered in the amount of 1275 optical density units (271 mμ) (62 μmoles). The yield as based on the unreacted deoxycytidine (2.2 μmoles) and that present in the product was calculated to be 97%. The bulk (20 ml.) of the stock solution of the reaction mixture obtained above was treated with 20 ml. of 2 *N* sodium hydroxide for 5 min. at 0° and the total then neutralized with an excess of pyridinium Dowex-50 ion exchange resin. After filtration from the resin and passage through a fresh column of the same resin, the total aqueous pyridine solution (addition of more pyridine) was concentrated at below 20°. The concentrate was applied on 8 strips (9 in. wide each) of Whatman 3 MM paper. The chromatograms were developed in the solvent isopropyl alcohol–concentrated ammonia–water (7:0.4:2.5, v./v.). Since no clear demarcation of the bands was apparent, the papers were cut so as to combine the material with R_f 0.5–0.8 (A) and that with R_f 0.8–1.0 (B). The unreacted nucleotide with R_f less than 0.5 was discarded. The total material with R_f 0.5–0.8 was eluted with a mixture of methyl alcohol–pyridine–water (2:2:1) and after concentration *in vacuo* at 0° gave 2500 optical density units (298 mμ) (0.2 mmole, 80% yield) and it was shown on rechromatography in solvent A to be pure N-benzoyl-5′-O-dimethoxytrityldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine (R_f 0.78; R_f in solvent C, 0.86). The material from the original paper sheets with R_f 0.8–1.0 was contaminated with a little N-benzoyl-5′-O-dimethoxytritydeoxycytidine (R_f 0.95).

Acetylation of Deoxycytidine Trinucleotide (d-pCpCpC): Isolation of VIII.—Pyridinium deoxycytidine trinucleotide (d-pCpCpC, 0.037 mmole, 1000 optical density units at 271 mμ) and tri-*n*-hexylethylammonium hydroxide[28] (0.15 mmole) were rendered anhydrous by repeated evaporation of pyridine solution (3 × 3 ml.). Dry pyridine (2 ml.) and acetic anhydride (0.5 ml.) were added and the sealed clear solution was kept at room temperature for 4 hr. Methyl alcohol (3 ml.) was added and after a further 1 hr. at room temperature the solvents were evaporated *in vacuo*. Evaporation was repeated after addition of water (2 × 2 ml.) and the residue was made up with aqueous pyridine to 10 ml. The yield of the acetylated product as determined spectrophotometrically (960 optical density units at 298 mμ) was quantitative. The spectrum was characteristic of N-acetyldeoxycytidine chromophore with λ_{max} at 298 and 247 mμ. The R_f of the product in solvent C was 0.60 (R_f of d-pCpCpC, 0.46). One-half of the acetylated trinucleotide (0.185 mmole) was evaporated to a gum, extracted with ether (2 × 5 ml.), evaporated *in vacuo*, and finally lyophilized from 5 ml. of water. On repeating this process the product was obtained as a finely divided powder, which was dissolved in anhydrous pyridine. This was used in experiment B described below.

The second half of the material was passed through a column (5 × 1 cm.) of Dowex-50 (pyridinium form) ion exchange resin and the resin washed with water (10 × 1 ml.). By that time the absorbancy of the eluate had dropped to 0.2 at 298 mμ and the total eluate was evaporated to about 1 ml., repeatedly extracted with ether (3 × 5 ml.), and finally the aqueous solution was lyophilized. The resulting finely divided powder was stored in pyridine. This was used in experiment A described below.

Deoxycytidylyl-(3′ → 5′)-deoxyguanylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine (IX). A. Using DCC.—N-Benzoyl-5′-O-dimethoxytritydeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine (720 optical density units at 298 mμ; about 0.057 mmole) and the pyridine salt of the acetylated trinucleotide VIII (0.018 mmole) as obtained above were mixed in pyridine (3 ml.), and pyridinium Dowex-50 ion exchange resin (100 mg.) and dimethylformamide (1 ml.) added. The mixture was made anhydrous by evaporation to 1 ml. *in vacuo*, the process of addition of dry pyridine and concentration being repeated four times. The final evaporation was continued until the volume of the residual mixture was about 0.2 ml.; DCC

(27) T. M. Jacob and H. G. Khorana, paper in preparation.

(28) Prepared by the reaction of tri-*n*-hexylamine with ethyl iodide in ethyl acetate, followed by separation of the insoluble tri-*n*-hexylethylammonium iodide and passage of the latter through the hydroxide form of Dowex-1 ion exchange resin.

(0.5 mmole) was added and the sealed reaction mixture shaken at room temperature for 5 days. Water (1 ml.) was then added and the unreacted DCC extracted with cyclohexane (3 × 5 ml.). The protecting groups were removed in the standard way by successive treatments with concentrated ammonium hydroxide (2 days at room temperature) and 80% acetic acid (25 min. at room temperature) and the bulk (97%) of the product was chromatographed on a DEAE-cellulose (carbonate) column (38 × 1.1 cm. diam.), the elution being carried out with a linear gradient of ammonium bicarbonate (pH 8.5.). The mixing vessel contained 2 l. of water while the reservoir contained an equal volume of 0.5 *M* salt. Fractions of 20 ml. volume were collected at a flow rate of 1 ml./min. The elution was followed spectrophotometrically at 260 mμ. The elution pattern is shown in Fig. 1. Peak I contained nonnucleotidic material (benzamide, etc.). Peak II (fractions 17–24) contained a total of 428 optical density units (253 mμ) of deoxycytidylyl-(3′ → 5′)-deoxyguanosine. Peaks III (fractions 28–32) and IV (fractions 34–40) contained, respectively, 31 and 53 optical density units (270 mμ) of, evidently, deoxycytidine nucleotides. Peak V (fractions 46–54) contained 105 optical density units (257 mμ) of the desired pentanucleotide IX. Subsequent peaks VI–IX[29] contained products with deoxycytidine spectrum, the total recovery of the material containing deoxycytidine only being 334 optical density units (270 mμ).

The yield of the pentanucleotide IX as based on the deoxycytidine trinucleotide used was 13.8%, using an algebraic sum of molar extinctions for the nucleotide residues in the pentanucleotide and ignoring hypochromicity. The yield as based on the total deoxycytidine trinucleotide recovered was 17%.

B. **Using 2′,5′-Dimethylbenzenesulfonyl Chloride.**—An anhydrous solution of N-benzoyl-5′-O-dimethoxytrityldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine (0.057 mmole) and tri-*n*-hexylethylammonium salt of the acetylated deoxycytidine trinucleotide VIII (0.018 mmole) as obtained above and an additional amount of tri-*n*-hexylethylammonium hydroxide (0.057 mmole) in 0.2 ml. of dry pyridine was treated with 2′,5′-dimethylbenzenesulfonyl chloride (0.025 ml. = 30 mg., 0.148 mmole). After 5 min. at room temperature more dry pyridine (0.5 ml.) was added and the resulting homogeneous solution was kept at room temperature for 10 hr. Water (1 ml.) and pyridine (1 ml.) was added and the mixture kept at 2° for 1 week. After removal of the protecting groups, the products were chromatographed on a DEAE-cellulose column exactly as described above under A. The elution pattern was similar to that shown in Fig. 1. The yield of the desired pentanucleotide IX was 11.2% as based on the deoxycytidine trinucleotide recovered.

Characterization of the Pentanucleotide IX.—The ultraviolet absorption characteristics were: pH 7, λ_{max} 257 mμ, λ_{min} 232 mμ, $\epsilon_{280}/\epsilon_{260}$ 0.79; pH 1, λ_{max} 278 mμ, λ_{min} 236 mμ with a shoulder at 270 mμ, $\epsilon_{280}/\epsilon_{260}$ 1.20; pH 12, λ_{max} 268 mμ, λ_{min} 245 mμ with a shoulder at 250–260 mμ, $\epsilon_{2\text{-}0}/\epsilon_{260}$ 0.80.

The compound was completely degraded by venom phosphodiesterase to give deoxycytidine-5′ phosphate, deoxyguanosine-5′ phosphate, and deoxycytidine, the only nucleoside formed, in the ratio of 3.15:1.0:1.11 (theoretical 3:1:1).

The compound was homogeneous on paper chromatograms developed in solvent D (R_f 0.58)[30]; R_f in solvent A, 0.14; R_f in solvent C, 0.38 (R_f of thymidine-5′ phosphate, 0.44).

N⁶-Anisoyldeoxycytidine-5′ β-Cyanoethyl Phosphate.—A mixture of pyridinium N⁶-anisoyldeoxycytidine-5′ phosphate (5.6 mmoles) and hydroacrylonitrile (25 ml., 280 mmoles) was made anhydrous by evaporation of pyridine solution (2 × 20 ml.). Pyridine (25 ml.) and DCC (4.5 g., 21.6 mmoles) were added and the solution kept at room temperature until electrophoresis of an aliquot showed the absence of the mononucleotide or of the corresponding pyrophosphate (19 hr.). Water (25 ml.) was then added and the excess of the reagent was removed by extraction with cyclohexane (3 × 50 ml.). The insoluble material in the aqueous phase was removed by filtration and thoroughly washed with aqueous pyridine (10%). After addition of 10 mmoles of ammonium bicarbonate the solution was evaporated to about 25 ml. and this solution applied to the top of a DEAE-cellulose (carbonate) column (45 cm. × 3.5 cm.). Elution was carried out at 2° with water (300 ml.) followed by a linear gradient of ammonium bicarbonate (pH 7.7, 3 l. of 0.03 *M*) solution in the mixing vessel and 3 l. of 0.3 *M* solution in the reservoir. Fractions of 21–23-ml. volume were collected at a flow rate of 3 ml./min. Selected fractions were examined by paper electrophoresis. Two incompletely separated peaks were obtained. Fractions 20–44 (510 ml.) contained 14000 optical density units (302 mμ) of the neutral nucleotidic product, while fractions 66–146 (1750 ml.) contained 82,000 optical density units (302 mμ) of the desired product. The fractions 45–65 also contained mainly the desired product but in addition some of the neutral product. N⁶-Anisoyldeoxycytidine-5′ β-cyanoethyl phosphate was isolated from fractions 66–146 by evaporation and subsequent lyophilization to remove the salt. The product was homogeneous by paper chromatography in solvent A (R_f 0.70) and solvent C (R_f 0.80). Its mobility on paper electrophoresis at pH 7.1 was 0.55 relative to N⁶-anisoyldeoxycytidine-5′ phosphate.

N-Acetyldeoxyguanosine-5′ β-cyanoethyl phosphate was prepared analogously to the preceding compound by the reaction of N-acetyldeoxyguanosine-5′ phosphate (2 mmole) and hydroacrylonitrile (2 ml.) in pyridine (5 ml.) and dimethylformamide (5 ml.) with DCC (1.6 g.) for 3 days at room temperature. After the standard work-up and separation on a DEAE-cellulose column three main peaks were obtained: peak I corresponded to the neutral compound (3,200 optical density units at 260 mμ), peak II contained the desired product (24,000 optical density units at 260 mμ), while peak III contained the unreacted nucleotide and the corresponding pyrophosphate (6,500 optical density units at 260 mμ). The R_f of the desired product in solvent C was 0.73.

5′-O-Phosphoryl-N⁶-anisoyldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyguanosine (IV).—A mixture of pyridinium-N,O³′-diacetyldeoxyguanosine-5′ phosphate (2.2 g., 4 mmoles), pyridinium N⁶-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate (2 mmoles), pyridinium Dowex-50 ion exchange resin (100 mg.), and DCC (4.0 g., 19 mmoles) in anhydrous pyridine (10 ml.) and dimethylformamide (2 ml.) was shaken at room temperature in the dark for 5 days. Water (5 ml.) was then added and the unreacted reagent was removed by extraction with cyclohexane (2 × 20 ml.). To the aqueous layer, pyridine (5 ml.) was added and the solution kept at room temperature overnight. Water was added to 25 ml. and the solution after being cooled to 0° was treated with an equal volume of precooled 2 *N* sodium hydroxide. After a total of 20 min. at 2°, an excess of pyridinium Dowex-50 ion exchange resin was added to remove sodium ions. The resin was removed by filtration and washed with water. The total filtrate (130 ml.) was treated with 10 ml. of 1 *M* ammonium bicarbonate and after removal of a 1-ml. aliquot for chromatography on a small "analytical" column of DEAE-cellulose, the total solution was evaporated at low temperature. (The analytical column showed several products but the major one, the last peak, was the desired protected dinucleotide IV.) The concentrate (20 ml.) of the total solution was applied on top of a column (45 cm. × 3.5 cm.) of DEAE-cellulose (carbonate form). Elution was carried out at 2° using a linear gradient of ammonium bicarbonate (0.02–0.5 *M*, 4 l. each of the solutions in the mixing vessel and the reservoir, respectively). Fractions of about 20-ml. volume were collected at a flow rate of 2 ml./min. The eluate was checked by its ultraviolet absorption spectrum and mobility on paper electrophoresis (pH 7.1) for pooling the appropriate fractions containing the desired dinucleotide. (The pure product isolated from the analytical column served as the standard.) The product was eluted in fractions 265–304 at a salt concentration of about 0.35 *M*. The pooled fractions (total optical density 27,000 at 285 mμ, yield about 0.9 mmole, 45%) were treated with an excess of Dowex-50 ion exchange resin (pyridinium form), and the total mixture then was evacuated to remove carbon dioxide and finally passed through a fresh column of the above resin (20 × 3 cm.). The total aqueous pyridine effluent was evaporated at low pressure and temperature (0–5°) in the presence of a prepondering amount of pyridine in the solution. The product stored as its solution in pyridine was homogeneous by paper chromatography in solvents A (R_f 0.08) and C (R_f 0.42) and by paper electrophoresis. The ultraviolet absorption characteristics were: λ_{max} 286 and 263 mμ, λ_{min} 269 and 233 mμ, $\epsilon_{286}/\epsilon_{263}$ 1.105, $\epsilon_{269}/\epsilon_{233}$ 2.14.

5′-O-Phosphorylthymidylyl-(3′ → 5′)-N-acetyldeoxyguanosine (d-pTpG^{Ac}).—An anhydrous mixture of pyridinium thymidine-5′ β-cyanoethyl phosphate (1.3 mmoles), pyridinium N,3′-O-diacetyldeoxyguanosine-5′ phosphate (2.25 mmoles, 9500 optical density units at 300 mμ), pyridinium Dowex-50 ion exchange resin (400 mg.), and DCC (1.0 g.) was shaken in dimethylformamide (3 ml.) and pyridine (5 ml.) at room temperature for 3 days. (Longer reaction period showed no further change in the composition of the products.) Water (2 ml.) was added and the subsequent work-up and selective alkaline removal of the 3′-O-acetyl group was as described above for the preparation of d-pC^{An}pG^{Ac}. The total products were separated on a DEAE-cellulose (carbonate) column (55 × 4 cm.), elution being carried out with 3 l. of 0.05 *M* ammonium bicarbonate in the mixing vessel and an equal volume of 0.3 *M* solution of the same salt; 20-ml. fractions were collected every 10 min. The desired dinucleotide d-pTpG^{Ac} was in fractions 105–133, the yield being 22,000 optical density units at 261 mμ. An additional amount was present in fractions 96–105 which contained, mainly, this product, contaminates being the mononucleotides. Lyophilization of the combined fractions gave the ammonium salt of the dinucleotide. The λ_{max} was at 261 mμ and λ_{min} at 231 mμ, there being a weak shoulder at 275 mμ. The ratio of $\epsilon_{260\ m\mu}/\epsilon_{300\ m\mu}$ was 5.25. The

(29) As judged by the result of incubation with bacterial alkaline phosphomonoesterase, peak VI was unreacted trinucleotide d-pCpCpC and peak VII, presumably, the symmetrical pyrophosphate of the trinucleotide (resistance of the compound to the phosphomonoesterase).

(30) This mobility was like that of thymidine-5′ phosphate and this result is rather surprising, in view of the generally lower R_f's of compounds such as d-TpTpTpT and d-TpCpCpC than that of the above mononucleotide.

R_f's of the dinucleotide were: solvent A, 0.08; solvent C, 0.40. Treatment with concentrated ammonia gave 5′-O-phosphorylthymidylyl-(3′ → 5′)-deoxyguanosine (d-pTpG) with R_f's: solvent A, 0.04; solvent D, 0.40 (R_f's of marker, thymidine-5′ phosphate: solvent A, 0.15; solvent C, 0.46; solvent D, 0.52).

5′-O-Phosphoryldeoxyguanylyl-(3′ → 5′)-deoxycytidine and the Corresponding Acetylation Product.—A mixture of pyridinium N-acetyldeoxyguanosine-5′ phosphate (0.5 mmole) and pyridinium N,3′-O-diacetyldeoxycytine-5′ phosphate (1 mmole) was kept in dry pyridine (2 ml., clear solution) in the presence of DCC (600 mg.) at room temperature for 6 days. Water (2 ml.) was then added and the excess of DCC extracted with petroleum ether. The aqueous pyridine solution was evaporated and the residue rendered anhydrous by evaporation of dry pyridine and kept subsequently in a mixture of pyridine (2 ml.) and acetic anhydride (1 ml.) for 3 days. Methyl alcohol was then added after 1 hr. The solution was evaporated. The residue was taken up in a small amount of water and extracted repeatedly with ether. The aqueous solution was then made up to 25 ml. with pyridine.

A 1-ml. portion of this stock solution was treated with concentrated ammonium hydroxide (2 ml.) for 2 days and the resulting products separated on a DEAE-cellulose (carbonate) column (50 × 1 cm.). Elution was carried out with a linear gradient, with 1 l. water in the mixing vessel and an equal volume of 0.6 M ammonium bicarbonate in the reservoir, 10-ml. fractions being collected every 15 min. The elution was followed by measuring ultraviolet absorption at 270 mμ. After the appearance of peaks corresponding to mononucleotides, the peak corresponding to 5′-O-phosphoryldeoxyguanylyl-(3′ → 5′)-deoxycytidine appeared in fractions 34–38 (84 optical density units, about 5 μmoles). This dinucleotide was followed by some deoxyguanosine dinucleotide (d-pGpG) and then by trinucleotides. The yield of the pure desired dinucleotide (d-pGpC) obtained above was about 25%.

Chromatography of the bulk of the stock pyridine solution prior to ammoniacal treatment on a DEAE-cellulose column at 11° gave a similar pattern except that the peak of the desired d-pG^{Ac}pC^{Ac}-OAc was recognized by the ultraviolet absorption characteristics described below. The yield of the pure compound was 1100 optical density units at 300 mμ or 87 μmoles (17.4%) assuming a molar extinction of 12.7×10^3 at this wave length. The ultraviolet absorption characteristics were: λ_{max} 284 and 250 mμ, λ_{min} 273 and 227 mμ, $\epsilon_{250\ m\mu}/\epsilon_{284\ m\mu}$ 1.8 and $\epsilon_{250\ m\mu}/\epsilon_{300\ m\mu}$ 2.35.

5′-(β-Cyanoethylphosphoryl)-N-anisoyldeoxycytidylyl-(3′ → 5′)-N-acetyldeoxyadenosine (V).—An anhydrous mixture of pyridinium N^6-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate (29,000 optical density units at 300 mμ, 1.3 mmoles), pyridinium N,3′-O-diacetyldeoxyadenosine-5′ phosphate (2.3 mmoles), and pyridinium Dowex-50 ion exchange resin (280 mg.) was treated with DCC (1.0 g.) in the solvent dimethylformamide (2 ml.) and pyridine (10 ml.). After 5 days at room temperature, water (3 ml.) was added and after extraction with cyclohexane (2 × 10 ml.) the insoluble solid was removed by filtration and the filtrate and aqueous pyridine washings were made up to 50 ml. with pyridine. Analysis of an aliquot (0.5 ml.) of this stock solution on a DEAE-cellulose column (about 80 × 1 cm.) and following ultraviolet absorption of different peaks at 302 and 273 mμ showed the fourth peak to correspond to the protected dinucleotide CEp-C^{An}pA^{Ac}-OAc; the yield as based on determination at 302 mμ was 59%.

The total of the above stock solution (49.5 ml.) was chromatographed on a DEAE-cellulose (carbonate) column (45 × 3.5 cm.) at 2°. Elution was carried out with a linear salt gradient, 3.5 l. of 0.03 M ammonium bicarbonate, pH 7.7, in the mixing vessel and an equal volume of 0.22 M salt in the reservoir; 20-ml. fractions were collected at a flow rate of 2 ml./min. The fractions were examined for ϵ ratio 275 mμ/300 mμ = 1.45 and by paper electrophoresis (mobility 0.85 of deoxyadenosine-5′ phosphate), these properties being characteristic of the protected dinucleotide (peak IV described above). The pure product was present in fractions 101–153 in the amount of 9300 optical density units (302 mμ) (32% yield as based on N-anisoyldeoxycytidine-5′ β-cyanoethyl phosphate). A further amount (3550 optical density units at 302 mμ, 12%) was present in the succeeding fractions, but the product here was impure. Ammonium bicarbonate was removed by lyophilization. For removal of the 3′-O-acetyl group, the total product was dissolved in the cold (0°) in 10 ml. of 1 N sodium hydroxide; after 2.5 min. at this temperature, the alkali was neutralized by rapid addition of an excess of pyridinium Dowex-50 ion exchange resin. Chromatography of a small portion of the resulting products on an "analytical" DEAE-cellulose column showed a minor peak (4 optical density units at 300 mμ); then the second peak (21 optical density units at 300 mμ) corresponding to CE-p-C^{An}pA^{Ac}, the desired product; and the third peak (22 optical density units at 300 mμ) that of d-pC^{An}pA^{Ac}. The R_f's of the products were: d-pC^{An}pA^{Ac}, solvent A, 0.21; solvent C, 0.42; d-CE-pC^{An}pA^{Ac}, solvent A, 0.65; solvent C, 0.81. The unprotected dinucleotide d-pCpA prepared by an ammoniacal treatment had R_f's: solvent A, 0.06; solvent D, 0.42.

Polymerization of N-Acetyldeoxyguanylyl-(5′ → 3′)-N^6-anisoyldeoxycytidine-5′ Phosphate. Isolation of Oligonucleotides Containing Alternating Sequence of Deoxycytidine and Deoxyguanosine.—The total pyridine solution of the protected dinucleotide (0.9 mmole), as obtained in the preceding experiment was evaporated to a gum. Dowex-50 resin (pyridinium form, 300 mg.), dimethylformamide (1 ml.), and pyridine (10 ml.) were added and the total mixture re-evaporated to a gum. The same amounts of the solvents were again added and the evaporation repeated. Evaporation was continued until the weight of the remaining solvent was 1.4 g. (assuming the weight of the dinucleotide to be 0.80 g.); DCC (680 mg., 3.2 mmoles) was added under rapid agitation, the addition resulting in the precipitation of a gum. The latter hardened to a solid within 1 hr. The sealed mixture was kept in the dark for 7 days at room temperature. Water (1 ml.), triethylamine (0.15 ml.), and pyridine (3 ml.) were added and the mixture shaken until a homogeneous mixture resulted (9 hr.). More triethylamine (0.5 ml.) was added and the solution rendered anhydrous by coevaporation with pyridine (2 × 10 ml.). The residual gum was dissolved in pyridine (5 ml.), acetic anhydride (3 ml.) was added, and the mixture kept at room temperature for 3 days. The reaction was stopped by addition of methanol (10 ml.) followed after 1 hr. by water (2 ml.). Methyl acetate and acetic acid were removed by evaporation at low temperature, followed by extraction with ether. Any solid material in the aqueous layer was removed by filtration. The filtrate was diluted with pyridine to 25 ml. Since chromatography of a small aliquot of this solution on an analytical DEAE-cellulose column showed poor resolution[31] of the oligonucleotides, the bulk (24 ml.) of the solution was treated with concentration ammonia at room temperature for 2 days to remove the protecting groups. The resulting solution was made up to 50 ml. with water. Chromatography of a small aliquot on a DEAE-cellulose column gave peaks corresponding to the starting dinucleotide, d-pCpG, the cyclic dinucleotide preceding d-pCpG, and a subsequent peak at 0.22 M concentration of ammonium carbonate, corresponding to the symmetrical pyrophosphate of the dinucleotide. The rest of the ultraviolet absorbing material failed to separate and could only be eluted with 1–2 M salt concentration. The percentages of the different peaks were: cyclic dinucleotide, 18.3%; dinucleotide d-pCpG, 24.2%; the pyrophosphate (O-(d-pCpG)$_2$), 20.8%; 2 M salt eluate, 21.2%.

Large scale separations were carried out by chromatography (a) on a DEAE-cellulose column in the presence of 7 M urea and (b) on a DEAE-cellulose column by the standard techniques as described above followed by rechromatography of the 2 M salt eluate on another column in the presence of 7 M urea. The procedures used were as described below.

A. **Separation on a DEAE-Cellulose Column in the Presence of Urea.**—Twenty milliliters of the above stock solution was evaporated to dryness. The residue was dissolved in 10 ml. of 6 M urea solution. The solution was applied to the top of a DEAE-cellulose (carbonate) column (43 × 3.5 cm.) which had been packed in 0.2 M ammonium bicarbonate–6 M urea solution and then equilibrated with 0.02 M ammonium bicarbonate–6 M urea solution. Elution was carried out with a linear ammonium bicarbonate (pH 8.6) gradient (3.8 l. of 0.02 M ammonium bicarbonate–6 M urea in mixing vessel, an equal volume of 0.3 M salt–6 M urea in the reservoir). Subsequently, elution was completed with 1 M ammonium bicarbonate–6 M urea. A flow rate of 2 ml./min. was maintained, 22–24-ml. fractions being collected. The elution pattern is shown in Fig. 2 and the distribution of the nucleotidic material in Table I. (The fractions were pooled as shown.) For recovery of the nucleotidic products, the pooled peaks were each diluted with 3 volumes of water and the diluted solutions passed through small DEAE-cellulose (carbonate) columns (10–20-ml. bed volume). Except for the cyclic and the linear dinucleotide, only parts of which were absorbed onto the columns, the retention of the nucleotidic material was complete. Subsequently the products were eluted, with 20–50 ml. of 1 M–2 M ammonium bicarbonate (pH 8.6). The salt from these eluates was removed by direct lyophilization. The identity of the different products is also shown in Table I.

B. **Separation by Rechromatography in Presence of Urea.**—Twenty milliliters of the stock solution obtained above was applied to the top of a DEAE-cellulose (carbonate) column (35 × 3.5 cm. diam.) and elution was carried out with ammonium bicarbonate (pH 8.6), salt gradient being 0.02 M (2 l.) to 0.30 M (2 l.), followed by 2 M ammonium bicarbonate (1 l.). The peaks obtained were as described above for the small column. The 2 M eluate (1860 optical density units at 255 mμ) was lyophilized to remove the salt and the bulk (1520 optical density

(31) Attempts in related work to separate deoxycytidine polynucleotides bearing N-anisoyl groups similarly had given unsatisfactory separation of the homologous members. The separation was better after removal of the protecting groups.

TABLE I

CHROMATOGRAPHY OF DEOXYCYTIDINE-DEOXYGUANOSINE OLIGONUCLEOTIDES ON A DEAE-CELLULOSE COLUMN IN THE PRESENCE OF 6 *M* UREA

Peak no.	Fractions pooled	Total optical density (255 mμ)	% of total optical density recovd.	Identification
I	1–15	4030		Nonnucleotidic (anisic acid)
II[a]	73–90	1050	16.8	Cyclic dinucleotide
III	103–121	1450	23.2	d-pCpG
IV	152–172	1123	18.00	d-(pCpG)(pCpG)O
V	172–189	192	3.07	Unidentified
VI	197–220	527	8.45	d-pCpGpCpG
VII	247–273	214	3.43	Mostly d-(pCpG)(pCpGpCpG)O[b]
VII	274–360	275	4.4	
IX	361–371	547	8.75	d-pCpGpCpGpCpG + higher polynucleotides + pyrophosphates

[a] Peak I was nonnucleotidic (mainly anisic acid or its amide). [b] See text.

units) of the recovered material was reapplied to the top of a DEAE-cellulose (carbonate) column (40 × 2.7 cm. diam.) which had previously been packed in and equilibrated with a 0.1 *M* ammonium bicarbonate solution (pH 8.5) containing 7 *M* urea. The nucleotidic material was applied as a solution in the same mixture and elution begun with a linear gradient of salt (2 l. of 0.1 *M* → 2 l. of 0.6 *M* ammonium bicarbonate) containing 7 *M* urea. Fractions of 20-ml. volume were collected at a flow rate of 2 ml./min. The elution pattern is shown in Fig. 4. The recovery of the oligonucleotides from the urea was as described above under A. The distribution of the nucleotidic material and identification of the different peaks are given in Table II. Further purification of the collected peaks was accomplished by paper chromatography in solvent D, both before and after treatment with alkaline phosphomonoesterase. The R_f's of the different compounds are given in Table III. The results of venom phosphodiesterase on d-CpGpCpG and d-CpGpCpGpCpG are given in Table IV.

TABLE II

RECHROMATOGRAPHY OF 2 *M* ELUATE ON A DEAE-CELLULOSE COLUMN IN THE PRESENCE OF 7 *M* UREA

Peak no.	Fractions pooled	Optical density, 255 mμ	% of total polymeric mixture[a]	Identification
	1–43	72	0.95	
I	44–57	155	2.0	Unidentified
II	58–69	488	6.4	Mostly d-pCpGpCpG
III	71–90	320	4.2	Mainly d-(pCpG)(pCpGpCpG)O
	91–97	63	0.8	
IV	98–116	320	4.2	Mainly d-pCpGpCpGpCpG
V	118–130	110	1.3	d-pCpGpCpGpCpGpCpG + higher polynucleotides + pyrophosphates
VI	131–145	59	0.7	
VII	1 *M* eluate (146–150)	14.4	0.2	

[a] This refers to the total products as obtained in polymerization and as used in the first DEAE column described under B above.

TABLE III

PAPER CHROMATOGRAPHY OF OLIGONUCLEOTIDES CONTAINING DEOXYCYTIDINE AND DEOXYGUANOSINE OLIGONUCLEOTIDES

Compound	R_f, solvent D—Whatman No. 44	R_f, solvent D—Whatman No. 40
d-pTpApTpApTpApTpA[a]	0.17	0.055
Cyclo-d-pCpG	.46	
d-pCpG	.38	
The pyrophosphate d-(pCpG)(pCpG)O	.29	
d-pCpGpCpG	.21	0.095
The pyrophosphate, d-(pCpG)(pCpGpCpG)O	.16	
d-pCpGpCpGpCpG	.10	0.03
d-pCpGpCpGpCpGpCpG	..	0.007
Oligonucleotides lacking 5′-phosphate groups		
d-CpGpCpG	0.35	0.21
d-CpGpCpGpCpG	.27	.10
d-CpGpCpGpCpGpCpG		.04
d-TpApTpApTpApTpA[a]	.28	.145

[a] *Cf.* G. Weimann, H. Schaller, and H. G. Khorana[1], accompanying paper. The R_f's of these polynucleotides are included here for comparison. The markedly lower R_f values of the deoxyguanosine-deoxycytidine oligonucleotides of comparable chain length should be noted.

Synthesis of Deoxycytidine Polynucleotides Terminating in Thymidine Residues (d-TpCpC and Homologs).—An anhydrous solution of N-anisoyldeoxycytidine-5′ phosphate (1.87 mmoles) and N,O[3′]-diacetyldeoxycytidine-5′ phosphate (0.63 mmole) in dry pyridine (2 ml.) was treated with DCC (1.00 g.) and the mixture shaken at room temperature in the presence of some glass beads for 9 days. The solvent was evaporated *in vacuo*, dimethylformamide (4 ml.) was added, and the sealed mixture shaken at room temperature until a homogeneous solution resulted (4 hr.). A part (3.9 ml. out of a total of 5.4 ml. of reaction mixture) was worked up as described previously[11] and the deoxycytidine polynucleotide composition of the polymeric mixture determined by separation on a DEAE-cellulose column. The results were very similar to those obtained previously. The remainder (1.5 ml.) of the above reaction mixture was mixed with 5′-O-dimethoxytritylthymidine[2d] (450 mg., 0.75 mmole) and the total mixture rendered anhydrous by repeated evaporation after addition of pyridine. Finally when the total weight of the reaction mixture was the same as before evaporations with pyridine, more DCC (625 mg.) was added and the solution kept at room temperature for 72 hr. Sodium hydroxide (2 ml. of 1 *N*) was then added and the mixture extracted with cyclohexane (10 ml. × 3). An excess of pyridinium Dowex-50 ion exchange resin was added and after removal and washing of the resin, the total filtrate concentrated to 10 ml. Paper chromatography in solvent A showed the presence of a number of nucleotidic bands (R_f 0.0 to 0.9) which contained dimethoxytrityl group (acid spray). The total mixture of products was treated with an equal volume of concentrated ammonia at room temperature for 2 days and then with 80% acetic acid at room temperature for 2 hr. The resulting mixture was chromatographed on a DEAE-cellulose carbonate column (90 × 2.5 cm. diam.). Elution was carried out with a linear gradient of triethylammonium bicarbonate (0–0.4 *M*, 2.5 l. of water in the mixing vessel and an equal volume of 0.4 *M* salt in the reservoir). Elution was continued with a sharper gradient of the same salt (1 l. of 0.4 *M* salt in mixing vessel, an equal volume of 0.7 *M* in the reservoir). The elution pattern is shown in Fig. 5. The manner of pooling of different fractions, the distribution of nucleotidic material in the pooled fractions, and the composition of the peaks are given in Table V.

The identity of the major peaks was accomplished by paper chromatography in solvent D by comparison with previously characterized deoxycytidine polynucleotides. The R_f's of different compounds are listed in Table VI.

For isolation of pure oligonucleotides further purification was accomplished by rechromatography on smaller DEAE-cellulose (carbonate) columns. Thus peak IX when rechromatographed on a column (55 × 1.2 cm.) gave pure d-TpCpCpCpCpC as the major product. This product was completely degraded by

TABLE IV

VENOM PHOSPHODIESTERASE DEGRADATION OF DEOXYGUANOSINE–DEOXYCYTIDINE OLIGONUCLEOTIDES LACKING 5′-PHOSPHATE END GROUPS

Conditions of incubation were: TRIS buffer, pH 9.2 (0.01 ml. of 0.1 *M*); venom phosphodiesterase preparation, 0.035 ml., and total volume with substrate to 0.1 ml. The incubation was at 37° for 6–9 hr. The total incubation mixtures were then applied to solvent A. After development for about 18 hr., the spots corresponding to dC, d-pC, and d-pG were eluted along with appropriate blanks for spectrophotometric analysis.

Compound	Deoxycytidine (d-C) O.D./spot	Deoxycytidine (d-C) μmole	Deoxycytidine-5′ phosphate (d-pC)[a] O.D./spot	Deoxycytidine-5′ phosphate (d-pC)[a] μmole	Deoxyguanosine-5′ phosphate (d-pG)[b] O.D./spot	Deoxyguanosine-5′ phosphate (d-pG)[b] μmole	Ratio Found	Ratio Calcd.
d-CpGpCpG	3.15	0.239	3.1	0.235	6.35	0.46	1:0.99:1.92	1:1:2
d-CpGpCpGpCpG	1.44	0.109	3.29	0.248	5.075	0.386	1:2.25:3.34	1:2:3

[a] Assuming an extinction of 13.2/μmole at 280 mμ in acid. [b] Assuming an extinction of 13.8/μmole at 252 mμ at pH 7.

TABLE V

CHROMATOGRAPHY OF DEOXYCYTIDINE POLYNUCLEOTIDES TERMINATING IN THYMIDINE RESIDUES

Peak	Fractions pooled	Total optical density (270 mμ)	% of total	Composition
I	12–35	2190	28.0	Mainly d-TpC
II	43–56	366	4.8	pC
III	57–74	1100	14.0	d-pTpCpC + cyclo-d-pCpC
IV	87–102	478	6.1	d-pCpC
V	103–120	673	8.7	d-TpCpCpC
VI	121–150	746	9.7	Cyclo-d-pCpCpC + d-pCpCpC + unidentified component
VII	151–178	590	7.5	Mainly d-TpCpCpCpC
VIII	179–198	359	4.6	Mainly d-pCpCpCpC
IX	210–229	258	3.3	Mainly d-TpCpCpCpCpC
X	230–254	314	4.0	Mainly d-pCpCpCpCpC
XI	293–320	178	2.3	Mainly d-TpCpCpCpCpCpC
XII	321–363	170	2.2	Mainly dpC(pC)$_4$pC
XIII (2 *M* eluate)	394–403	70[a]	0.9[a]	Higher

[a] This figure is believed to be incorrect; actual amount obtained here was probably much greater.

TABLE VI

PAPER CHROMATOGRAPHY OF DEOXYCYTIDINE OLIGONUCLEOTIDES TERMINATING IN THYMIDINE (SOLVENT D)

Compound	R_f	Compound	R_f
d-pC	1	d-TpCpCpCpC	0.84
d-TpC	1.48	d-TpCpCpCpCpC	.65
d-TpCpC	1.25	d-TpCpCpCpCpCpC	.49
d-TpCpCpC	1.02		

venom phosphodiesterase to give thymidine and deoxycytidine-5′ phosphate in the ratio 1:5.28 (theoretical 1:5).

N-Anisoyldeoxycytidine Trinucleotide (d-pC^{An}pC^{An}pC^{An}).—A mixture of trinucleotide d-pCpCpC (2000 optical density units at 270 mμ, 0.074 mmole[32]) and tri-*n*-hexylethylammonium hydroxide (0.3 mmole) was rendered anhydrous by repeated evaporation of pyridine. The residue was dissolved in 2 ml. of dry pyridine, and anisoyl chloride (0.60 ml. 4.2 mmoles) was added and the solution kept at room temperature for 2 hr. Water (10 ml.) was added and the mixture extracted rapidly with chloroform (10 ml. × 3). The combined chloroform extracts were washed once with water (5 ml.), evaporated, and the resulting oil dissolved in 2 ml. of pyridine. Water (2 ml.) and 2 *N* sodium hydroxide (5 ml.) were added. After 15 min. at room temperature, an excess of pyridinium Dowex-50 ion exchange resin was added and the clear supernatant passed through a short column of fresh resin in the pyridine form. The total eluate was diluted with an equal volume of pyridine and the solution evaporated to 2 ml. at low temperature. The concentrate was extracted with ether (3 × 20 ml.) and the residual solution of the product was stored as its solution in pyridine. The yield was 4200 optical density units (302 mμ, 75% assuming the ϵ_{max} previously established for N-anisoyldeoxycytidine-5′ phosphate). This product gave a single spot both in solvents A (R_f 0.10) and C (R_f 0.28). The ultraviolet absorption spectrum was very similar to that of N-anisoyldeoxycytidine-5′ phosphate.

(32) Assuming an extinction of 27/μmole at 270 mμ.

Acetylation of N-Anisoyldeoxycytidine Trinucleotide (VIII).—To the foregoing product was added 1 ml. of dimethylformamide followed by triethylamine (0.025 ml., 0.180 mmole). The solution was rendered anhydrous by evaporation of pyridine (2 × 5 ml.). Finally, pyridine (2 ml.) and acetic anhydride (0.5 ml.) were added, and the solution was kept at room temperature for 3 hr. Methyl alcohol (2 ml.) was added and the resulting solution was kept overnight at room temperature. Pyridine (5 ml.) was then added and the solution evaporated to about 1 ml. at low temperature. The concentrated solution was extracted with ether (3 × 10 ml.) and the residual oil dissolved in water (20 ml.). This solution was lyophilized and the resulting powder was stored as its solution in pyridine.

5′-O-Monomethoxytritylthymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine and Thymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine (XII; n = 2).—A mixture of the foregoing acetylated trinucleotide VIII (420 optical density units at 300 mμ, 5.6 μmoles), 5′-O-monomethoxytritylthymidine (52 mg., 0.1 mmole), and pyridinium Dowex-50 ion exchange resin (50 mg.) was rendered anhydrous by repeated evaporation of pyridine (3 × 5 ml.). Finally, dry pyridine (0.3 ml.) and DCC (18 mg., 0.08 mmole) were added. The sealed reaction mixture was kept for 25 hr. and then dimethylformamide (40 mg.) was added and the mixture kept further for 6 days. Water (1 ml.) and pyridine (4 ml.) were then added and the solution extracted with cyclohexane three times, the extracts being washed back with 10% aqueous pyridine. The total aqueous pyridine solution was divided into two equal parts. One part was treated with concentrated ammonium hydroxide for 2 days, the total product applied to one 9-in. wide strip of Whatman No. 40 paper and chromatographed in solvent A. The following ultraviolet absorbing bands in the order of increasing mobility were detected: band 1, R_f 0.015 (13 optical density units, 270 mμ); band 2, R_f 0.035 (7.5 optical density units, 270 mμ); band 3, R_f 0.090 (4.1 optical density units, 270 mμ); band 4, R_f 0.21 (55 optical density units at 270 mμ), corresponded to the desired product and amounted to 1.5 μmoles, assuming an extinction of 37 optical density units/μmole at 270 mμ. Bands faster than the above corresponded to anisic acid, anisamide, and methoxytritylthymidine. The desired product had ultraviolet absorption characteristics: pH 7, λ_{max} 270 mμ, λ_{min} 253 mμ, shoulder at 230 mμ, $\epsilon_{270m\mu}/\epsilon_{230m\mu}$ 0.77. After evaporation and treatment with 80% acetic acid at room temperature for 3 hr., the aqueous solution contained pure thymidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidylyl-(3′ → 5′)-deoxycytidine, as described below.

The second part was treated with concentrated ammonium hydroxide, followed by 80% acetic acid at room temperature for 3 hr. and the resulting products applied on top of a DEAE–cellulose (carbonate) column (55 × 1.2 cm.). Elution was carried out with a linear gradient (0–0.5 *M*) of ammonium bicarbonate (pH 8.5, 1 l. of solution in each vessel), fractions of 15 ml./10 min. being collected. The desired product (d-TpCpCpC) was present in peak IV (Fig. 2) which was eluted in fractions 28–33. The total amount was 67 optical density units (270 mμ). This product moved as a single spot on paper chromatography and was identical with the sample whose preparation is described above (R_f, solvent C, 0.22; R_f, solvent A, 0.04). The combined yield in the two parts was 122 optical density units (3.3 μmoles, 59% as based on the deoxycytidine trinucleotide used).

The product was completely degraded by venom phosphodiesterase to give thymidine and deoxycytidine-5′ phosphate in the ratio 1:3.13.