Polynucleotides. XII.[1] Chemical Synthesis of Polyriboguanylic Acid[2]

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Abstract: A homologous series of 3′,5′ oligoriboguanylates has been chemically synthesized. Blocked bifunctional monomer, *N*-benzoyl-2′-*O*-tetrahydropyranylguanosine 5′-phosphate, and blocked monofunctional 3′ terminator, *N*-benzoyl-2′,3′-di-*O*-tetrahydropyranylguanosine 5′-phosphate, were converted to pyridinium salts under conditions where the tetrahydropyranyl ether blocking group is stable. Monomer and terminator in a ratio of 4:1 were polymerized using *N,N′*-dicyclohexylcarbodiimide in anhydrous pyridine at 40° over 135 hr. The reaction was stopped with water; *N,N′*-dicyclohexylurea was removed and the crude product mixture treated with acetic anhydride in pyridine to remove any P^1,P^2-di(polynucleotidyl) pyrophosphates. Blocking groups were removed by sequential treatment of the product with base (pH 11.1, 60°) and acid (pH 4.6, 75°), and the deblocked oligoguanylates were fractionated by ion exchange. The major product was tetraguanylate, with lesser quantities of penta-, hexa-, hepta-, and octaguanylates; only a trace of guanosine 5′-phosphate was recovered and no di- or triguanylate was found. Each oligomer was proven homogeneous by ion exchange chromatography and its size determined from the ratio of total phosphorus/IO_4^- reduced. Periodate end group analysis and production of guanosine and guanosine 3′(2′),5′-diphosphate after base hydrolysis demonstrated that all oligomers were linear. Both blocked and unblocked oligomers formed large aggregates, which complicated characterization. Chemically and enzymically synthesized oligoguanylates were found partially resistant to ribonuclease T_1, but proof that all phosphodiester bonds in the chemically synthesized products were $C_3'\rightarrow C_5'$ was obtained using nmr spectroscopy. The synthetic approaches developed appear to have general applicability to polyribonucleotide synthesis.

We describe a chemical synthesis of 5′-phosphoester-terminated oligoguanylates containing $C_3'\rightarrow C_5'$ phosphodiester linkages exclusively. Despite the success of the comparable enzymic synthesis,[3] details of the chemical one are of interest because they introduce reactions and procedures of wide applicability to polynucleotide chemistry. The two earlier chemical polymerizations yielding polyribonucleotides with exclusive natural phosphodiester bonds provide products with the unnatural 3′-phosphate and 5′-hydroxyl termini.[4]

Synthesis of Oligo(G)[5]

The present synthesis (Chart I) involves carbodiimide activated polymerization of a bifunctional Guo-5′-P derivative with blocked 2′-hydroxyl, *N*-Bz-2′-*O*-THP-Guo-5′-P, to prevent $C_2'\rightarrow C_5'$ phosphodiester formation. The 2′-OH blocking group was chosen because of its expected ease of removal from the polymeric product[6] without attendent alteration of the phosphodiester or glycosidic bonds; and the benzoyl substituent on the guanine amino group to avoid N–P bond formation during polymerization,[7] and to overcome the low solubility of guanosine nucleotides in anhydrous pyridine, the solvent of choice for carbodiimide polymerizations.[8] This monomer was polymerized in the presence of a monofunctional Guo-5′-P derivative, *N*-Bz-2′,3′-di-*O*-THP-Guo-5′-P, to provide the 3′-nucleoside terminus, and to minimize formation of macrocyclic oligonucleotides.[9] The polymer product was deblocked and fractionated, yielding a homologous series of oligoguanylates.

Pyridinium *N*-Bz-2′-*O*-THP-Guo-5′-P. The carbodiimide activated esterification of phosphoester and alcohol in the polymerization has stringent acid and solvent requirements[10] that are met by the usual procedure of passing the monomer through a cation exchanger in the pyridinium form and then rendering the pyridinium nucleotide anhydrous by coevaporation with anhydrous pyridine. However, it was found that this procedure results in substantial hydrolysis of the THP ether from the monomer during contact with the ion exchanger even in the presence of a large excess of pyridine over resin sulfonic acid, nearly 10^4 in some experiments. This hydrolysis was avoided by carrying out the ion-exchange step in 50% pyridine at 4°, with resin–nucleotide contact *no more than 1 min.*

Use of a weaker acid cation exchanger than sulfonated poly(styrene–divinylbenzene) for making the desired pyridinium salt did not cause THP ether hydrolysis in 50% pyridine at 4° over 2.5 hr. But the pyridinium salts of phosphocellulose, carboxymethylcellulose, and Amberlite IRC-50 (polymethacrylic acid) were partially soluble even in anhydrous pyridine,

(1) The previous paper in this series was by R. Blake and J. R. Fresco, *Biopolymers*, **12**, 775 (1973).

(2) This work was supported by grants from the National Institutes of Health (GM-07654), the National Science Foundation (GB-18865), and the American Heart Association. D. B. S.: Department of Chemistry, State University of New York at New Paltz, New Paltz, N. Y. 12561.

(3) (a) J. R. Fresco and D-F. Su, *J. Biol. Chem.*, **237**, PC3305 (1962); (b) M. N. Thang, M. Graffe, and M. Grunberg-Manago, *Biochim. Biophys. Acta*, **108**, 125 (1965).

(4) (a) Y. Lapidot and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3857 (1963); (b) C. Coutsogeorgopoulos and H. G. Khorana, *ibid.*, **86**, 2926 (1964).

(5) Abbreviations used: P, when hyphenated, is used for phosphoester (and for the element when unhyphenated); Bz, benzoyl; THP, tetrahydropyranyl; DCC, *N,N′*-dicyclohexylcarbodiimide; DCU, *N,N′*-dicyclohexylurea; DEAE, diethylaminoethyl; Tris, trishydroxymethylaminomethane; dp, polymerization number.

(6) D. B. Straus and J. R. Fresco, *J. Amer. Chem. Soc.*, **87**, 1364 (1965).

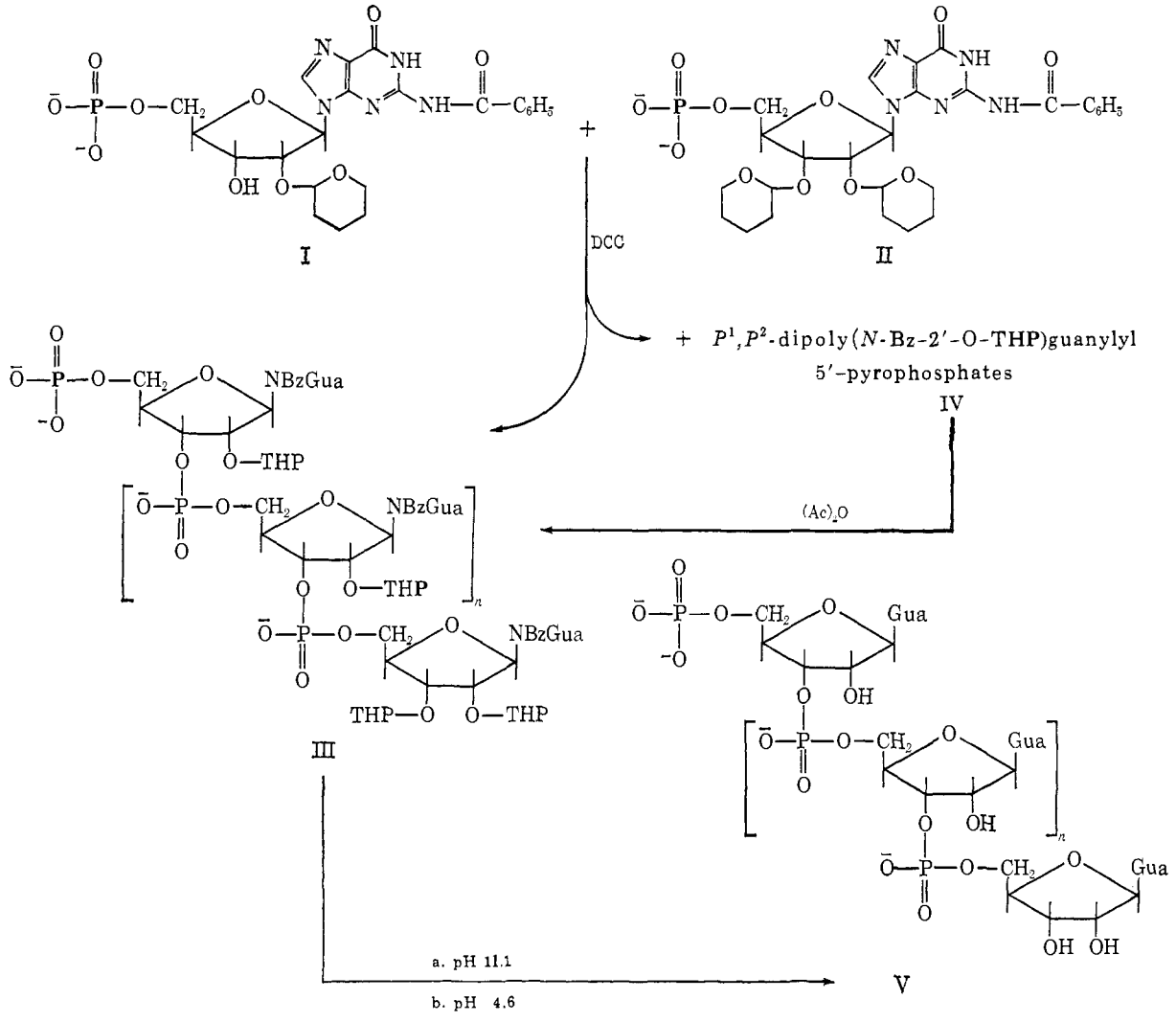
(7) G. M. Tener, *J. Amer. Chem. Soc.*, **83**, 159 (1961).

(8) M. Smith, G. I. Drummond, and H. G. Khorana, *J. Amer. Chem. Soc.*, **83**, 698 (1961).

(9) D. B. Straus, *J. Amer. Chem. Soc.*, **87**, 1375 (1965).

(10) (a) H. G. Khorana, "Some Recent Developments in the Chemistry of Phosphate Esters of Biological Interest," Wiley, New York, N. Y., 1961, pp 93–125; (b) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Amer. Chem. Soc.*, **80**, 6204 (1958).

Chart I



precluding their use in a carbodiimide polymerization. A test (see Experimental Section) devised for determination of pyridinium cation associated with nucleotide gave values of 2.0–3.2 (*cf.* ref 10b) for pyridinium salts formed using either weak or strong acid cation exchangers.

The hydrolysis of the 2′-*O*-THP group from monomer by pyridinium Dowex 50W emphasizes the high sensitivity of this protecting group to acid,[6] though other 2′-*O*-THP nucleotides may not be similarly labile. Thus, for DCC activated reactions of THP-blocked nucleoside 3′-phosphates,[11] strong acid cation exchangers were used to prepare the pyridinium salts without loss of the 2′-*O*-THP group.[11b] But, under conditions designed for quantitative yield of dinucleoside phosphate,[12] the lack of such yield could be interpreted in terms of some loss of 2′-*O*-THP from blocked Up.[11a]

Polymerization. The anhydrous pyridinium salts of the monomer and the 3′ terminator were completely stable in anhydrous pyridine at room temperature for at least 14 days. Monomer (80 μmol) and 3′ terminator (20 μmol) in 0.1 ml of anhydrous pyridine (this 1 *M* nucleotide solution indicates the high solubility of these derivatives) were mixed with 250 μmol of DCC in a total of 0.2 ml of anhydrous pyridine and shaken in the dark at 40°. Additional DCC and pyridine were added during the reaction to maintain excess activating agent and fluidity of the mixture. After the reaction was stopped and DCU removed, the recovered product mixture in anhydrous pyridine was treated with acetic anhydride and tri-*n*-butylamine[13] to decompose any P^1,P^2-di-(polynucleotidyl) pyrophosphates. Parallel treatment of monomer with these reagents caused neither blocking group removal nor acetylation of the 3′-OH.

Analysis of the crude blocked oligo(G) showed a recovery of 88% of total P. Chromatography showed less than 5% of the starting compounds and *N*-Bz-2′-*O*-THP-Guo 3′,5′-cyclic P[6] was absent.

Attempted Fractionation of Blocked Oligo(G). Secondary force interactions of poly(G) are particularly strong[14] and interfere with its fractionation by anion

(11) (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Amer. Chem. Soc.*, **84**, 430 (1962); (b) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (c) F. Cramer and K. H. Scheit, *Angew. Chem.*, **74**, 717 (1962); (d) J. Smrt and F. Sorm, *Collect. Czech. Chem. Commun.*, **27**, 73 (1962); (e) B. E. Griffin and C. B. Reese, *Tetrahedron*, **24**, 2537 (1968).

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

(13) H. G. Khorana, J. P. Vizolyi, and R. K. Ralph, *J. Amer. Chem. Soc.*, **84**, 414 (1962).

(14) (a) J. R. Fresco and J. Massoulie, *J. Amer. Chem. Soc.*, **85**, 1352 (1963); (b) R. K. Ralph, W. J. Connors, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 1983 (1963); (c) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961); (d) R. K. Ralph, W. J. Connors, and H. G. Khorana, *ibid.*, **84**, 2265 (1962).

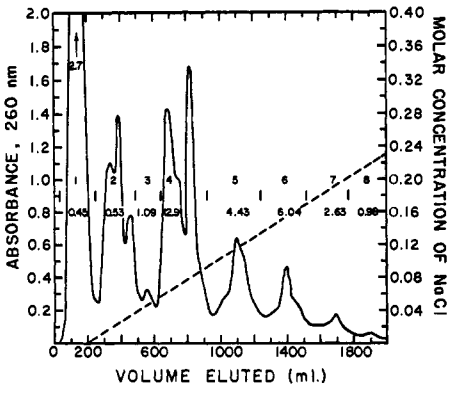


Figure 1. Fractionation of unblocked oligo(G) synthetic product (79 μmol) on DEAE-cellulose (Cl^-). Elution was with a 0.0 → 0.5 *M* NaCl linear gradient in 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2. The fractions (20 ml each) pooled for each peak are indicated by vertical lines; the reference number of each peak and quantity of organic P recovered are given between the vertical lines.

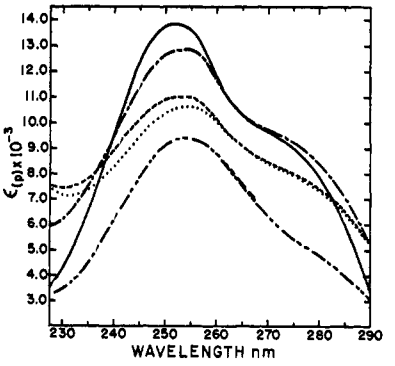


Figure 2. Ultraviolet absorption spectra of synthetic oligoguanylates (— · —), (- - - -), and (· · ·) for fractions 4, 5, and 6, respectively (Figure 1), and, for comparison, Guo-5′-P (—) and enzymically synthesized $(pG)_{\overline{7.2}}$[3a] (— · · —). Spectra determined in H_2O at 22°. The oligo(G)s will exhibit varying degrees of secondary structure;[17] hence, measured extinction coefficients are not absolute.

exchange. This difficulty was previously overcome either by introducing an *N*-acyl blocking group as in the case of poly(*N*-acetyl-dG)[14b] or by elution with 7 *M* urea.[15] However, oligo(*N*-Bz-G) with mixed phosphodiesters aggregates strongly,[6] probably due to the strongly hydrophobic *N*-benzoyl group, and *N*-Bz-Guo-5′-P and *N*-Bz-Guo 3′,5′-cyclic P are retarded relative to the unbenzoylated nucleotides on DEAE-cellulose.[6] Precipitation of poly(*N*-Bz-2′-*O*-THP-G) from aqueous solution on standing several days at 4° was also observed in the present work (though dissolution did occur readily at 100° after which the cycle could be repeated). Furthermore, fractionation of an aliquot (9 μmol of P) of the poly(*N*-Bz-2′-*O*-THP-G) on DEAE-cellulose (HCO_3^-) was quite complex, similar to that of poly(*N*-Bz-G) with mixed phosphodiesters.[6] Recovery of phosphorus was low (~50%) even after elution with concentrated buffer saturated with 1-butanol to overcome hydrophobic interactions. Deblocking was therefore undertaken prior to fractionation of the bulk of the product.

Removal of Blocking Groups. The *N*-Bz group is labile to base and stable to acid, whereas the reverse is true for the 2′-*O*-THP ether.[6] Removal of the Bz substituent in base would not endanger the internucleotide phosphodiester with the THP group in place.[16] Hence, base hydrolysis was followed by acid hydrolysis for deblocking. With the possibility that the susceptible bonds are in significantly different environments in monomer and polymer, influencing their stabilities, combinations of time, temperature, and pH corresponding to 50–100 half-lives for deblocking of monomer[6] were employed. In this manner, 79 μmol of P of blocked product was quantitatively freed of blocking groups.

Ion-Exchange Fractionation of Unblocked Oligo(G). The unblocked product was not completely soluble even in 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2, at 56°, but material dissolved under these conditions was chromatographed on a DEAE-cellulose column eluted with a NaCl gradient in 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2, solvent (Figure 1). Peak fractions were pooled and freed of urea and NaCl and concentrated on short DEAE-cellulose columns. Only 36% of the applied charge was recovered with this solvent; the rest, presumably insoluble aggregate, was eluted at high pH.

Characterization of Oligo(G)

Ultraviolet Absorption Spectra. Spectra of fractions 4, 5, and 6 are shown in Figure 2, together with those of Guo-5′-P and poly(G) (synthesized using polynucleotide phosphorylase,[3a] and having a dp = $\overline{7.2}$). Quantitative evaluation of oligo(G) spectra is difficult due to the slow formation of helical aggregates[14] even in distilled water, which would change spectra with time and make them dependent on the history of the sample.[17] Moreover, the heterogeneity of the enzymic poly(G) will result in its larger components contributing disproportionately to its hypochromicity. Despite these limitations, it is apparent that the fractions in Figure 2 (and also fractions 7, 8, and 9 (not shown)) are progressively hypochromic. It should be noted also that the oligomer spectra contain no indication of residual *N*-Bz-Guo chromophore, which has a prominent λ_{max} at 290 nm.[6]

Chromatography. Figure 1 indicates a series of oligo(G) fractions of progressively increasing charge which, in the absence of macrocyclic products,[4,14b,18] would likely be near-homogeneous. The size and homogeneity of these fractions were assessed by thin layer chromatography on DEAE-cellulose with a 7 *M* urea solvent. Chromatograms run over the pH range 4–8 showed each of the fractions to be homogeneous. However, the limit of detection of a macrocyclic oligo(G) in these chromatograms (in which they should separate from a linear oligomer at pH 4.5) was about

(15) (a) M. N. Lipsett and L. A. Heppel, *J. Amer. Chem. Soc.*, **85**, 118 (1963); (b) M. N. Lipsett, *Biochem. Biophys. Res. Commun.*, **11**, 224 (1963); (c) R. V. Tomlinson and G. M. Tener, *Biochemistry*, **2**, 697 (1963).

(16) D. M. Brown and A. R. Todd, *Annu. Rev. Biochem.*, 24, 311 (1955).

(17) The spectra and measured extinction coefficients (see Table I and Experimental Section) were reproducible in independent determinations for the synthetic oligo(G) fractions between 10^{-5} and 10^{-4} *M* P in water at 22°. Changes in spectra with time were not determined but a time-dependent hypochromicity would be expected, the rate of which would be polynucleotide concentration dependent.[14a]

(18) (a) G. M. Tener, H. G. Khorana, R. Markham, and E. H. Pol, *J. Amer. Chem. Soc.*, **80**, 6223 (1958); (b) H. G. Khorana, A. F. Turner, and J. P. Vizolyi, *ibid.*, **83**, 686 (1961); (c) G. Weimann, H. Schaller, and H. G. Khorana, *ibid.*, **85**, 3885 (1963).

20% of the sample analyzed, so that an appreciable quantity of possible macrocyclic oligo(G) could have escaped detection. This doubt was removed when fraction 4 was rechromatographed on a DEAE-cellulose column at pH 4.5, and yielded a single sharp peak. The homogeneity of the peak originally eluted from DEAE-cellulose at pH 8.2 was, therefore, confirmed.

Polymerization Numbers. Fraction 1 (Figure 1) did not bind to DEAE-cellulose and had the spectrum of pyridinium hydrochloride, with no contribution from a Guo chromophore. Therefore, this fraction could not contain any possible 3′-C-pyridinium-Guo-5′-P side product analogous to 5′-C-pyridinium nucleotides.[4] Fractions 2 and 3 contained negligible amounts of P; their spectra did not correspond to pyridinium ion or to known guanosine derivatives and paper chromatograms of these fractions contained no uv absorbing material except for a trace of Guo-5′-P in fraction 3, which is the deblocked monomer.

Fraction 4 contained 46% of the P recovered from the product mixture. Alkaline hydrolysis of an aliquot yielded Guo-3′(2′)-P, Guo, and Guo-3′(2′),5′-diphosphate. The ratio of total P to Guo-3′(2′),5′-diphosphate (the dp) in the hydrolysate was 4.7, which must be a maximum value since elution of DEAE-cellulose thin layers was sometimes incomplete in controls.

A different end group analysis based on *cis-vic*-glycol oxidation by periodate was developed for dp determination. This procedure (see Experimental Section), based on the work of Dixon and Lipkin,[19] is sensitive to ~1 nmol of *cis-vic*-glycol, gives good precision with 5 nmol, and is rapid and direct.

The results of such end group analyses of fractions 4, 5, and 6 (Table I), together with the identification of both end groups in their alkaline hydrolysates, verify the synthesis of a homologous series of linear oligomers starting with $(pG)_4$. Macrocyclic oligomers would not have reduced IO_4^-, nor given Guo and Guo 3′(2′),5′-diphosphate end groups. The monofunctional 3′ terminator appears to have served its purpose.

Table I. Some Characteristics of Synthesized Oligoguanylates

Oligo(G) fraction[a]	Identification	Length[b]	Yield,[c] %	$10^{-3}\epsilon_P$ at 260 nm
3	pG	1	4	
4	$(pG)_4$	4.0	46	11.7
5	$(pG)_5$	5.2	16	10.2
6	$(pG)_6$	6.2	22	10.1
7	$(pG)_7$	7[d]	9	9.7
8	$(pG)_8$	8[d]	3	9.5

[a] See Figure 1. [b] The ratio organic P/IO_4^- reduced (see Experimental Section). [c] Yield is per cent of total recovered P eluted in fraction. [d] Length inferred from elution position in Figure 1.

Test for Blocking Group Removal. Since both blocking groups are stable on the monomer between pH 7–9,[6] enzymic hydrolysis near neutrality was used to assess blocking group removal. Treatment of $(pG)_4$, $(pG)_5$, and $(pG)_6$ with snake venom phosphodiesterase yielded Guo-5′-P as the only product; none of the partially or completely blocked Guo-5′-P derivatives were detected (sensitive to 1% of the Guo-5′-P). Thus, all blocking groups were quantitatively removed.

It was hoped to confirm this result and also demonstrate the $C_3'\rightarrow C_5'$ internucleotide linkage in the synthetic oligo(G) using spleen phosphodiesterase; but this enzyme requires a free 5′-OH,[20] and even large excesses of *E. coli* alkaline phosphatase were inactive toward the 5′-P of the oligomers. Similar resistance has been observed for the 5′-phosphate terminus of native tRNA.[21] This result emphasizes the strong secondary structure of the G oligomers.

Nature of Phosphodiester Linkage. (a) RNase T_1 Digestion. Initial examination of the linkage utilized the specificity of RNase T_1.[22] When checked against two substrates with exclusive $C_3'\rightarrow C_5'$ linkages, yeast ribosomal RNA[23] and poly (G·C),[24] the P_i released by RNase T_1 plus *E. coli* alkaline phosphatase quantitatively corresponded with the guanosine residue contents as expected. Similar treatment of $(pG)_4$ gave only 54% of total P as P_i. But this result must be viewed in the light of a repeated failure to digest quantitatively poly(G) synthesized with polynucleotide phosphorylase (Table II), which also contains $C_3'\rightarrow C_5'$ linkages exclusively.[25] Neither higher ratios of RNase T_1:poly(G) nor longer incubation increased the P_i/total P ratio appreciably, although different preparations of

Table II. Degradation of Oligoguanylates by RNase T_1[a]

Sample	RNase T_1/ oligo(G)P, $\mu g/\mu mol$	Exposure to T_1, min	P_i/total P
$Poly(G)_A$[b]	50	550	0.72
	38	1200	0.78[c]
$Poly(G)_B$[b]	260	1200	0.67
$Poly(G)_C$[b]	56	1200	0.84
	81	1800	0.83
$(pG)_4$	94	1800	0.54

[a] Reaction mixtures contained 6 μmol of NaCl, 2 μmol of Tris·HCl, pH 7.8, 7–60 nmol of poly(G)P (30–40 nmol in all cases except $poly(G)_A$ 550 min and $poly(G)_B$), 1.5–3.0 μg of RNase T_1, and 10 μg of *E. coli* alkaline phosphatase in a total volume of 0.190 ml. Incubation at 38° was continued for the indicated times; then HCl was added to 0.015 *N* H^+ and incubation continued 120 min when Tris (free base) was added to pH 8.5 followed by 10 μg of *E. coli* alkaline phosphatase and incubation was continued for 60 min. Quadruplicate aliquots were removed for P_i and total P determinations. Values were corrected for P_i at zero time. Digestion and analysis of yeast ribosomal RNA and poly(G·C) were the same except that shorter times and lower ratios of RNase T_1/polymer P were used. [b] $Poly(G)_A$ and $poly(G)_B$ were prepared using *M. luteus* polynucleotide phosphorylase.[3a] $Poly(G)_C$, a gift from Dr. M. Grunberg-Manago, was synthesized using highly purified *A. agile* polynucleotide phosphorylase. [c] Without treatment at pH 2,[a] the P_i/total P for $poly(G)_A$ was 0.54. The other poly(G)s were only slightly affected by exposure to pH 2 after digestion with RNase T_1 plus *E. coli* alkaline phosphatase, their P_i/total P being increased 2–8%.

(19) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).

(20) W. Razzel and H. G. Khorana, *J. Biol. Chem.*, **236**, 3028 (1961).

(21) R. Stern, L. E. Zutra, and U. Z. Littauer, *Biochemistry*, **8**, 313 (1969).

(22) F. Egami and K. Nakamura, "Microbial Ribonucleases," Springer-Verlag, New York, N. Y., 1969, pp 19–33.

(23) (a) A. M. Crestfield, K. C. Smith, and F. W. Allen, *J. Biol. Chem.*, **216**, 185 (1955); (b) W. Guschlbauer, E. G. Richards, K. Beurling, A. Adams, and J. R. Fresco, *Biochemistry*, **4**, 964 (1965).

(24) We thank Dr. Luisa Hirschbein for this material prepared by using RNA polymerase with a poly(C) template and GTP as sole nucleoside 5′-triphosphate substrate.

(25) L. A. Heppel, P. J. Ortiz, and S. Ochoa, *Science*, **123**, 415 (1956).

polynucleotide phosphorylase-synthesized poly(G) were digested to different extents (Table II). It is possible that disordered aggregates or the type of ordered multistranded secondary structure of which oligo- and poly(G) are capable[14a] (which differs from that of poly(G·C)) interferes with its digestion by RNase T_1. In any case, negative conclusions concerning the nature of the internucleotide linkage(s) in the synthetic oligo- (G) based on incomplete RNase T_1 susceptibility are not warranted even though $C_2'\rightarrow C_5'$ linkages are known to be resistant to RNase T_1.[26]

(b) Nmr Spectroscopy. Ts'o, *et al.*,[27] found a significant difference in the chemical shift of the C_1'–H in the nmr spectra of $C_3'\rightarrow C_5'$ and $C_2'\rightarrow C_5'$ dinucleoside phosphates. The dependence of the C_1'–H signal on the position of the internucleotide linkage is explicable in terms of differences in deshielding of this proton by the phosphodiester anionic center. Consequently, nmr spectra can serve to identify and quantitate the internucleotide phosphodiesters in the oligo(G) fractions. Such an analysis has been carried out with Dr. M. P. Schweizer and will be detailed elsewhere, but the results are summarized here.

The nmr spectrum of $(pG)_4$ showed peaks corresponding to $C_3'\rightarrow C_5'$ linkages but no indication of $C_2'\rightarrow C_5'$ phosphodiesters within the limit of the analysis, estimated to be about 10%. For this analysis, the models used to assign the C_1'–H resonance of each type of phosphodiester included $(Gp)_{7\text{-}5}$ (chemically synthesized to contain $C_3'\rightarrow C_5'$ and $C_2'\rightarrow C_5'$ linkages),[28,29] Guo-2'-P, Guo-3'-P, and several guanosine-containing dinucleoside phosphates. Therefore, $(pG)_4$ is at least 90% $C_3'\rightarrow C_5'$, and quite possibly all its phosphodiesters are of this type. A more refined analysis of $(pG)_6$ giving higher detection sensitivity for the $C_2'\rightarrow C_5'$ linkage (to about 4%) again showed no indication of the undesired phosphodiester. Thus, in two homogeneous fractions of the synthesized oligomers, a careful search for the undesired phosphodiester failed to demonstrate its presence. Hence, all internucleotide phosphodiesters appear to be the planned $C_3'\rightarrow C_5'$ linkage.

General Conclusions

This work is the first chemical polymerization of a 5'-ribonucleotide. The synthesis is unique in several respects when compared to other nucleotide polymerizations: (1) monomer incorporation is very high; (2) the two lowest members of the homologous series of oligomers are absent; and (3) the three classes of side product characteristic of nucleotide polymerizations are absent. The high incorporation of monomer is indicated by the very low recovery of Guo-5'-P. The virtual absence of monomer and lack of observed di- and trinucleotides in the product mixture do not appear related to the low recovery of oligo(G). That low recovery is undoubtedly due to extensive aggregation of oligomers which impedes dissolution and fractionation but which would not affect lower members of the homologous series.[14d] Rather, the very high incorporation of monomer and absence of the lowest members of the homologous series must reflect a characteristic of the synthesis itself. The high solubility of the blocked monomer in anhydrous pyridine has been noted, and it seems probable that the short blocked oligomers synthesized are also readily soluble in the reaction phase even in the form of trimetaphosphate esters.[30] This high reactant solubility (both monomer and growing polymer) would contribute significantly to the observed high incorporation of monomer, as might the slightly elevated temperature used, which would be expected to increase the solubility of the growing polymer in the reaction phase and improve the kinetics of each of the reaction steps.[10b,30] The ratio of monomer to terminator as well as their relative reactivities must also influence the average size as well as the size distribution of the oligomers.

The low recovery of polymerized oligo(G) from the ion-exchange column is the most serious unresolved problem in this work. Recoveries through the deblocking steps were good, but some aggregation and precipitation of oligo(G) probably occurred even in the 7 *M* urea solvent during the fractionation at 25°, resulting in incomplete elution. Although no procedures have been developed for preventing such aggregation, for disaggregating the oligo(G), or for maintaining the unaggregated polynucleotide in aqueous solution for extended periods either in this work or that of others,[14a,b,d,15a,b,31,32] it seems likely that the fractionation might have been performed more successfully at elevated temperature or with a water-miscible organic solvent.

Of the three classes of side product in nucleotide polymerizations,[10a] P^1,P^2-di(polynucleotidyl) pyrophosphates, 5'-*C*-pyridinium nucleotides, and macrocyclic polynucleotides, the elimination of the first two was planned. Any pyrophosphates were removed by acetic anhydride–pyridine treatment,[13] and C_5' was blocked by phosphoester. The absence of the analogous 3'-*C*-pyridinium-Guo-5'-P has been noted. However, the absence of macrocyclic oligo(G) was unexpected since these occur in all nucleotide polymerizations.[4,10a,17] A more rapid incorporation of monofunctional 3'-terminator into growing polymer may account for their absence, though steric hindrance to backside attack of the activated growing chain on its own 3'-OH must also be considered. Molecular models suggest such hindrance from the adjacent THP group to backside (hemiacetal side) attack without impeding intermolecular phosphodiester synthesis with a reactant approaching the 3'-OH frontally. This possible hindering effect gives added importance to the use of the THP blocking group in polyribonucleotide syntheses.

The demonstrated synthesis of oligo(G) suggests that the overall approach used can have general applicability to polyribonucleotide synthesis. The preparation of blocked monomer can be accomplished in good yield[6] and is applicable to adenosine- and uridine-5'-P as well. The methods developed for conversion of 2'-*O*-THP-Guo-5'-P derivatives to pyridinium salts

(26) Reference 22, p 32.

(27) (a) P. O. P. Ts'o, N. S. Kondo, M. P. Schweizer, and S. P. Hollis, *Biochemistry*, **8**, 997 (1969); (b) N. S. Kondo, H. M. Holmes, L. M. Stempel, and P. O. P. Ts'o, *ibid.*, **9**, 3479 (1970).

(28) We thank Dr. A. M. Michelson for his kind gift of $(Gp)_{7\text{-}14}$ synthesized by the nucleoside 2',3'-cyclic phosphate anhydride method.[19] The dp was determined as the ratio total P/P_i following exhaustive treatment of the $(Gp)_{7\text{-}14}$ with *E. coli* alkaline phosphatase.

(29) A. M. Michelson, *J. Chem. Soc.*, 1371 (1959).

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

(31) M. Gellert, M. N. Lipsett, and D. R. Davies, *Proc. Nat. Acad. Sci. U. S.*, **48**, 2013 (1962).

(32) F. Pochon and A. M. Michelson, *Proc. Nat. Acad. Sci. U. S.*, **53** 1425 (1965).

solve a major problem in using these blocked nucleotides in DCC-activated polymerizations. Arylsulfonyl chloride activation, not requiring pyridinium-based protonation of DCC, would be even simpler, only requiring displacement of a weak reactive base such as NH_3 by a strong unreacting base such as a tertiary amine. The sequential removal of blocking groups, each under very mild conditions, confers a further advantage to our synthetic approach. Base hydrolysis of the *N*-benzoyl substituent with the THP group in place prevents any product depolymerization, and the weakly acidic conditions established for hydrolysis of the THP group from oligo(G) should not endanger product structure. The high incorporation of blocked monomer and absence of side products ought to apply to other nucleotide polymerizations and may be advantageous for synthesis of nonrepeating oligoribonucleotides by specific coupling reactions. Finally, some of the methods for characterizing oligo(G), particularly the thin layer ion-exchange chromatography systems, periodate end group analysis, and nmr spectroscopy, should be valuable for studying other oligonucleotides.

Experimental Section

Materials. *N*-Bz-2′-*O*-THP-5′-P was synthesized as previously described,[6] as was *N*-Bz-2′,3′-di-*O*-THP-Guo-5′-P.[9] Guanosine, Guo-5′-P, Guo-5′-diphosphate, and Guo-5′-triphosphate were from Calbiochem; spectroquality pyridine,[33] ammonium bicarbonate, triethylamine, tri-*n*-butylamine, and acetic anhydride from Matheson Coleman and Bell; Dowex 50W, P-cellulose, CM-cellulose, and DEAE-cellulose[34] from Bio-Rad; Amberlite IRC-50, urea (reagent grade) and sodium metaperiodate (Certified reagent) from Fisher; DCC from Aldrich; cellulose and DEAE-cellulose for thin layer chromatography from Machery Nagel and Co.; snake venom phosphodiesterase and *E. coli* alkaline phosphatase (BAPC grade) from Worthington Biochemicals. Triethylammonium bicarbonate was prepared by the method of Smith, *et al.*[11a]

General Methods. Ultraviolet spectra were determined using a Cary 14 recording spectrophotometer; routine absorbances with a Zeiss PMQII spectrophotometer.

Orthophosphate was determined according to Lowry, *et al.*,[35] but modified to a final volume of 0.250 ml and sensitive to 0.1 nmol of P_i ($A_{820} = 0.010$). Total P was determined as P_i using this colorimetric method after wet ashing the sample with H_2SO_4–$HClO_4$[35] and hydrolysis of polyphosphates. All microphosphorus analyses were run in quadruplicate. Both standard P_i and standard Ado-5′-P were carried through the entire procedure as controls for the wet ashing and polyphosphate hydrolysis steps. Extinction coefficients per mole of P (ϵ_P) were determined by direct analysis of diluted spectrophotometric samples for total P corrected for any P_i present.

Triethylammonium bicarbonate and NH_4HCO_3 used in ion-exchange chromatography were removed by repeated concentration–dilution cycles in a rotary evaporator at <20° with 0.1–2 Torr pressure.

For paper chromatography, samples containing 0.1–0.25 μmol of nucleotide were spotted on Whatman No. 1 paper and developed in the descending mode at 22°. Nucleotides were visualized with ultraviolet light, or by phosphoester color development.[36] R_f values are given in Table III.

Thin layer partition chromatography was carried out on 20 × 20 cm glass plates spread with cellulose.[37] Samples spotted usually contained 30–60 nmol of nucleotide P, though as little as 10 nmol of a given substance could be visualized on cellulose and 1 nmol on fluorescent cellulose. Spotted plates were developed by the ascending mode until the solvent front migrated 16–18 cm. The plates were dried at 22°. R_f values are given in Table IV.

Table III. Paper Chromatography of Guanosine Nucleotides

Substance[a]	R_f[c] in solvent[b] A	B	C
Guanosine	0.42	0.57	0.40
Guo-3′(2′)-P	0.33	0.07	0.45
Guo-5′-P	0.31	0.07	0.23
2′-*O*-THP-Guo-5′-P		0.28	0.56
N-Bz-Guo-5′-P		0.21	0.37
N-Bz-2′-*O*-THP-Guo-5′-P		0.54	0.68
N-Bz-2′,3′-di-*O*-THP-Guo-5′-P		0.62	
Oligo(*N*-Bz-2′-*O*-THP-G) product mixture	*d*	0.0→0.31	
Oligo(G) product mixture	0.0→0.26		
$(pG)_4$	0.24[e]		
$(pG)_5$	0.22[e]		
$(pG)_6$	0.19[e]		

[a] Bz is for benzoyl. [b] A is 55:10:35 1-propanol–16 *N* NH_4OH–H_2O; B is 5:2 2-propanol–0.5 *M* NH_4OAc, pH 6.0; C is 6:3:1 absolute ethanol–2% (w/v) H_3BO_3–16 *N* NH_4OH. [c] R_f values were determined on Whatman No. 40 paper. [d] No spots visible with as much as 0.3 μmol of P (see text). [e] R_f refers to front of spots all of which tailed to the origin.

Table IV. Thin Layer Partition Chromatography of Guanosine Nucleotides

Substance[a]	R_f in solvent[b] A	B	C[c]
Guanosine	0.65	0.44	0.73
Guo-3′(2′)-P	0.44	0.38	0.33
Guo-5′-P	0.38	0.07	
Guo-5′-diphosphate	0.34		0.21
Guo-5′-triphosphate			0.17
N-Bz-2′-*O*-THP-Guo-5′-P		0.75	
2′-*O*-THP-Guo-5′-P		0.56	

[a] Bz is for benzoyl. [b] A is 55:10:35 1-propanol–16 *N* NH_4OH–H_2O; B is 6:3:1 absolute ethanol–2% (w/v) H_3BO_3–16 *N* NH_4OH; C is 5:1:1:3 pyridine–isoamyl alcohol–16 *N* NH_4OH–H_2O. [c] Usual visualization by ultraviolet quenching not possible with this solvent due to residual pyridine. Nucleotide spots were visualized by irradiation (253.7 nm) of plate for 5 min, which gave light brown spots in visible light and white spots in a darker field with ultraviolet light. This visualization is as sensitive as usual ultraviolet quenching.

Quantitative Analysis of Thin Layer Ion-Exchange Chromatograms. DEAE-cellulose for thin layer chromatography was fragmented further by treating for 1 min at high speed in a Waring Blendor (1 g of dry DEAE-cellulose/9 ml of H_2O), and the plates were spread. Before use, DEAE-cellulose plates were developed in either 0.05 *M* triethylammonium bicarbonate, pH 7.0, or in 0.5 *M* LiCl to convert them to the desired salt. The bicarbonate plates were dried, and excess buffer was evaporated in a vacuum oven at 60° (10 Torr) for 60 min. The LiCl plates were dipped three times into fresh distilled water, air dried, and then developed in the particular LiCl solvent to be used, again rinsed three times with water, and dried in a vacuum oven at 60° for 60 min just before chromatography.

Standards and samples (20–70 nmol of P) were spotted; the plates were equilibrated with solvent and then developed and dried at 22° as described for partition thin layer chromatography. Development times were 45–60 min for simple salt solutions and 4–5 hr for solvents containing 7 *M* urea. In several systems, sequential development with a series of eluents was used.[36] With triethylammonium bicarbonate, the plate was air dried and immediately developed with the next solvent; with LiCl in 7 *M* urea–0.01 *M* NH_4OAc, removal of urea and salts was necessary at each stage. To do this, the plate was soaked three times, 15 min each, in a shallow pan containing fresh absolute methanol. Triethylammonium

(33) The pyridine was distilled in the presence of BaO with the still protected from atmospheric moisture and stored with CaH_2.

(34) The Bio-Rad DEAE-cellulose used in column chromatography in this work frequently gave double peaks for a pure substance as fraction 4 (Figure 1).

(35) O. H. Lowry, N. R. Roberts, K. Y. Leiner, M. Wu, and A.L. Farr, *J. Biol. Chem.*, 207, 1 (1954).

(36) R. S. Bandurski and B. Axelrod, *J. Biol. Chem.*, **193**, 405 (1951).

(37) K. Randerath, "Thin-Layer Chromatography," Academic Press, New York, N. Y., 1963, pp 21–53.

bicarbonate was removed by heating in a vacuum oven 1–4 hr at 60°.

For analysis of thin layer chromatograms, rectangles circumscribing spots of nucleotide material were scored with a razor blade and the matted DEAE-cellulose was rolled using forceps. Identically sized blank areas at the same R_f were also removed. The unknown and blank samples were transferred to 2 ml of medium fritted disk Buchner funnels and mixed with 2.00 ml of 0.5 *M* LiCl or 0.5 *M* triethylammonium bicarbonate, pH 7.0, depending on the developing solvent. After 1 min the solutions were filtered with suction and the eluates brought to 3.0 ml and analyzed spectrophotometrically or for phosphorus.

This procedure was checked with standards of Guo, Guo-3′(2′)-P, Guo-5′-diphosphate, and Guo-5′-triphosphate. Recovery of guanosine nucleotides was quantitative, 94–100%, but that of guanosine was incomplete, 60–85%. The relative migrations of some guanosine nucleotides and polynucleotides are presented in Table V.

Table V. Thin Layer Ion-Exchange Chromatography of Guanosine Nucleotides

Substance	R_M[a] in solvent system[b] A	B[c]	C	D	E
Guanosine			2.1	2.1	2.1
Guo-5′-P	1.0	1.0			
Guo-3′(2′),5′-diphosphate			0.42	0.57	0.42
Guo-3′(2′)-P			1.0	1.0	1.0
Guo-5′-diphosphate			0.67	0.83	0.66
Guo-5′-triphosphate			0.62		0.29
$(pG)_4$	0.73	0.49		0.61	
$(pG)_5$	0.71	0.40		0.56	
$(pG)_6$	0.69			0.52	

[a] The reference substance (R_M = 1.0) is Guo-5′-P *or* Guo-3′(2′)-P. [b] A is 0.08 *M* LiCl in 7 *M* urea–0.01 *M* NH_4OAc, pH 4.5; B is, in sequence, 0.01 *M*–0.04 *M*–0.06 *M* LiCl in 7 *M* urea–0.01 *M* NH_4OAc, pH 4.5; C is 0.15 *M* triethylammonium bicarbonate, pH 7.0; D is 0.20 *M* triethylammonium bicarbonate, pH 7.0. Solvent system E is, in sequence, 0.075 *M*–0.175 *M*–0.200 *M* triethylammonium bicarbonate, pH 7.0. DEAE-cellulose was used in all separations. R_M's were reproducible, but absolute migration of a substance varied considerably and was particularly dependent on the batch of DEAE-cellulose. [c] Methanol washing was done between each of the sequential LiCl eluents to remove urea, LiCl, and NH_4OAc.

Determination of Pyridinium Cation. An aliquot of an anhydrous pyridine solution of the presumed pyridinium nucleotide containing ~200 nmol of P was lyophilized. The residue was repeatedly flushed with air by alternate evacuation and readmission of air to remove traces of pyridine. The residue was dissolved in 1 ml of 0.001 *N* HCl and the solution applied to a 1 × 1 cm column of DEAE-cellulose (Cl^-). The initial eluate plus 5 ml of 0.001 *N* HCl wash were collected in a graduated tube and diluted with an equal volume of 0.02 *N* HCl. The solution was analyzed spectrophotometrically, using ϵ_{256} 4.92 × 10^3 found for pyridine in 0.01 *N* HCl. The nucleotide bound to the anion exchanger was then eluted with 0.2 *M* triethylammonium bicarbonate, pH 7.0, the eluate freed of buffer, and the residue dissolved in 5.0 ml of H_2O and analyzed.

Pyridinium *N*-Bz-2′-*O*-THP-Guo-5′-P. An aqueous solution of triethylammonium *N*-Bz-2′-O-THP-Guo-5′-P (153 μmol) was dried (rotary evaporator) and dissolved in 50 ml of 50% aqueous pyridine. The solution at 4° was applied to a 4 × 4 cm column of pyridinium Dowex 50W in 50% aqueous pyridine at 4°, and the column was washed with 130 ml of aqueous pyridine. Suction was used to speed column flow. It is estimated that >95% of the nucleotide was removed from the Dowex 50W in <40 sec. The combined filtrate and wash was immediately chilled to −40° and lyophilized, and the residue was dissolved in 20 ml of anhydrous pyridine and lyophilized, and this coevaporation was repeated four times. The anhydrous pyridinium salt was dissolved in 16 ml of anhydrous pyridine and stored at −20°. Paper chromatography of 0.25 μmol (Table III) showed *N*-Bz-2′-*O*-THP-Guo-5′-P and a trace, ~1%, of *N*-Bz-Guo-5′-P (the latter shown to form during chromatography[9]). The ratio of pyridinium–nucleotide was 2:1.

Pyridinium *N*-Bz-2′,3′-di-*O*-THP-Guo-5′-P. Triethylammonium *N*-Bz-2′,3′-di-O-THP-Guo-5′-P (26 μmol) in 5 ml of 50% aqueous pyridine was converted to the pyridinium salt as for monomer using a 1 × 4 cm column of pyridinium Dowex 50W at 4°, and wash solution totalling 40 ml. Nucleotide was recovered and stored as for monomer. Paper chromatography of 0.25 μmol (Table III) showed a trace (~3%) with an R_f similar to monomer, presumably *N*-Bz-2′(3′)-mono-*O*-THP-Guo-5′-P.

Polymerization. The anhydrous pyridine stock solutions of monomer and 3′-terminator were analyzed at 290 nm, where neither pyridine nor pyridinium ion absorbs (ϵ_{290} 15.5 × 10^3 for *N*-BzGuo derivatives[6]), and for total P. Monomer (80 μmol) was lyophilized in the reaction vessel and an aliquot (20 μmol) of the 3′-terminator added; the mixture was lyophilized and coevaporated three times with 0.5 ml of anhydrous pyridine by lyophilization. The anhydrous gum was dissolved in <4 min in 0.1 ml of anhydrous pyridine, and 0.1 ml of 2.5 *M* DCC in anhydrous pyridine[38] was added, giving a polymerization medium 0.5 *M* in nucleotide and 1.25 *M* in DCC. The reaction mixture was turbid in 5 min, contained a gelatinous white precipitate in 10 min, and was viscous in 15 min, at which time the flask was covered with foil and placed on a shaker at 40°. After 46 hr the dark red-brown solution phase (atop DCU crystals and the white gelatinous precipitate) was immobile, so 0.05 ml of 2.5 *M* DCC and 0.05 ml of anhydrous pyridine were added, resulting in a flowing liquid phase. After 80 hr, 0.05 ml more of 2.5 *M* DCC was added. After 135 hr at 40°, the reaction was stopped by adding 0.2 ml of pyridine and 0.25 ml of H_2O.

After 16 hr at 4° and 2 hr at 22°, the reaction mixture was filtered and the residue (DCU, devoid of P) was washed with H_2O and with pyridine. The combined filtrate and washes was lyophilized, and the residue was coevaporated by lyophilization four times each with 5 ml of anhydrous pyridine. The anhydrous solid was dissolved in 2 ml of anhydrous pyridine, 0.05 ml of tri-*n*-butylamine[39] and 0.25 ml of acetic anhydride were added, and the solution was incubated in the dark at 22° for 84 hr, in which time a white crystalline solid was deposited.[40] The mixture was then cooled to 0°, mixed with 6 ml of ice–water, and lyophilized for 24 hr.

The resulting syrup plus solid was mixed with 4 ml of 20% aqueous pyridine but much solid remained. The solvent was lyophilized and coevaporated five times with 20% aqueous pyridine to remove pyridinium acetate and finally with 50% aqueous pyridine. The residue had an oily coating (tri-*n*-butylamine) which was removed by dissolving in 33% aqueous pyridine and extracting this solution three times, each with 15 ml of ether. The aqueous phase was lyophilized, the dry gum was mixed with 10 ml of 50% aqueous pyridine, residual DCU was removed by filtration, and the filtrate plus washes were lyophilized. The residue dissolved readily in 10 ml of H_2O, but extensive precipitation of the oligo(*N*-Bz-2′-*O*-THP-G) occurred within 16 hr. This mixture was dried on a rotary evaporator (without foaming); the residue redissolved in 10 ml of H_2O, but after 16 hr at 4° precipitation recurred. The precipitate dissolved in 1 min at 100°. After the solution cooled to 22°, aliquots were chromatographed on paper (Table III) and analyzed for total phosphorus.

The yield of P was 88 μmol (88%). In chromatographic solvent B, nearly all the pink fluorescing material (*N*-Bz-Guo chromophore[6]) had an R_f less than the monomer and no *N*-Bz-2′-O-THP-Guo 3′,5′-cyclic phosphate was detected. But in solvent A, no quenching or fluorescence was observed. Possibly, these properties are balanced at alkaline pH.

Removal of Blocking Groups. For debenzoylation, the oligomer product in H_2O (79 μmol of P, mostly precipitated) was diluted to 20 ml in 0.05 *N* NH_4OH (pH 11.1, glass electrode), brought to 60°,

(38) DCC occupies considerable volume in anhydrous pyridine solutions. It is essential to make up such concentrated DCC solutions in volumetric glassware in order to avoid addition of less than the desired amounts of the activating agent.

(39) This tertiary base was used by Khorana, *et al.*,[13] in order to solubilize polymeric products for reaction with acetic anhydride. Since the pyridinium salts of the product oligo(G)s in this work appear to be completely dissolved in the volume of anhydrous pyridine used and since there is no need to prevent acetylation of functional groups, all such being blocked in the products, the tri-*n*-butylamine could be omitted.

(40) Though no characterization was attempted, these crystals appeared to be DCU resulting from previous incomplete hydration of excess DCC used in the polymerization. The possible presence of a small amount of DCC in the acetic anhydride reaction would not complicate the reaction because of the large excess of acetic anhydride used and because tri-*n*-butylamine would prevent any reaction beyond acyl exchange.[10b]

whereupon it dissolved readily, and incubated for 80 hr at 60°. This hydrolysate was then dried on a rotary evaporator and co-evaporated twice with 80 ml of H_2O. Foaming, which could be a difficulty during such evaporations, was minimized by letting the partially concentrated sample stand 1–2 hr at 22° before continuing evaporation so that oligonucleotide aggregates could precipitate.

The residue was then suspended in 80 ml of 0.01 *M* NH_4OAc, pH 4.6, and incubated at 75° for 70 hr[41] to hydrolyze the THP ethers; the precipitate dissolved in the first 10 min. The hydrolysate was cooled to 22° and concentrated on a rotary evaporator to about 10 ml, and then the temperature was raised to 45° to accelerate decomposition of NH_4OAc.

The dry residue containing unblocked oligo(G) was extracted with 20 ml of ether; the residue from the ether, identified spectrally as benzoic acid, weighed 9 mg, close to the 9.65 mg expected from 79 μmol of blocked oligomer.

Fractionation of Oligo(G). The oligo(G) residue was suspended in 80 ml of 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2, and brought to 75°. Dissolution was incomplete after 5 min and rapid precipitation occurred on cooling to 22°. On reheating to 56°, all fresh precipitate, but not that insoluble at 75°, seemed to redissolve. With the mixture kept at 56°, 5-ml aliquots of the solution phase were placed atop a 2 × 36 cm column of DEAE-cellulose (HCO_3^-) at 22° and allowed to permeate the column before adding the next aliquot. This procedure allowed binding of the soluble oligo(G) to the exchanger without visible precipitation. When all of the product solution was sorbed, the flask was rinsed with 40 ml of 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2 at 56°, and the rinses were added to the column; additional dissolution of insoluble material was slight.

The column was washed with 100 ml of 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2, eluting peak 1 (Figure 1). Elution at 1–2 ml/min was with a 4-l. 0.0 → 0.5 *M* linear gradient of NaCl in 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2, followed by a 2-l. 0.5 → 1 *M* linear NaCl gradient in 7 *M* urea–0.01 *M* NH_4HCO_3, pH 8.2. No material absorbing at 260 nm was eluted beyond 0.24 *M* NaCl. However, 0.02 *N* NaOH did elute the residual phosphate, presumably in the form of digested nucleotide. Fractions were pooled as indicated in Figure 1 and diluted with H_2O so that Cl^- was <0.02 *M*, and each peak was passed through a 1 × 2 cm concentration column of DEAE-cellulose (Cl^-). Each column was washed with 100 ml of H_2O (no A_{260} was eluted), at which point eluates were Cl^- and urea free. It was then converted to the HCO_3^- form with 100 ml of 0.01 *M* NH_4HCO_3, pH 8.2, which was sufficient to make the eluates Cl^- free again (no A_{260} material eluted). Elution with less than 100 ml of 0.5 *M* NH_4HCO_3, pH 8.2, brought off the fractionated oligomer, which was freed of the vaporizable buffer.

Fraction 4 gave a double peak[34] so it was rechromatographed under different conditions. The fraction (12.7 μmol of P) was dissolved in 10 ml of 7 *M* urea and passed through a 1 × 12 cm column of DEAE-cellulose (HCO_3^-), which was eluted with a 2-l. 0.0 → 0.125 *M* linear gradient of triethylammonium bicarbonte, pH 7.5 in 7 *M* urea; 20-ml fractions were collected at 1 ml/min. Two peaks were again observed, but the first at 0.01 *M* HCO_3^- was only 3% of the total and was discarded. The second peak at 0.036 *M* HCO_3^- was concentrated and made urea free using a 1 × 5 cm DEAE-cellulose column eluted with 80 ml of 0.25 *M* triethylammonium bicarbonate, pH 7.5, and vaporizable buffer was removed.

The oligo(G) fractions, free of salts and urea, were dissolved in H_2O and characterized. Recoveries are given in Figure 1 and spectra in Figure 2.

Ion-Exchange Chromatography of Oligo(G) Fraction 4 at pH 4.5. The rechromatographed fraction 4 was dissolved in 100 ml of 7 *M* urea–0.01 *M* NH_4OAc, pH 4.5, and applied to a 1 × 10 cm DEAE-cellulose (Cl^-) column that had been equilibrated with 0.5 *M* LiCl–0.01 *M* NH_4OAc, pH 4.5, and then washed with H_2O until Cl^- free. Elution with a 1-l. 0.0 → 0.15 *M* linear gradient of LiCl in 7 *M* urea–0.01 *M* NH_4OAc, pH 4.5 (2 ml/min, 20-ml fractions), brought off a single peak sharply between 0.023 and 0.025 *M* LiCl. The peak fractions were pooled, diluted with 10 volumes of H_2O, and made urea and Cl^- free using a 1 × 5 cm DEAE-cellulose concentration column eluted with triethylammonium bicarbonate that was later removed by vaporization. Further analyses of fraction 4 were made on this material.

Alkaline Hydrolysis of Oligo(G) Fraction 4. An aliquot (112 nmol of P) in 0.1 ml of 0.3 *N* NaOH was incubated 29 hr at 40°, then neutralized with 10 mg of dry Dowex 50W H^+. The mixture was centrifuged 5 min at 10,000*g*, and the supernatant plus three 0.2-ml H_2O washes of the resin were evaporated. The residue was dissolved in 0.100 ml of H_2O, and a 0.050-ml aliquot was analyzed on a DEAE-cellulose thin layer using solvent E (Table V). Three spots were found and quantitated, Guo, Guo-3′(2′)-P, and Guo-3′(2′),5′-diphosphate. From the organic P spotted (total P and P_i analysis of another aliquot) and the quantity of Guo-3′(2′),5′-diphosphate, the ratio of P/end group was 4.7.

End Group Analysis by Periodate Reduction.[19] Spectrophotometer cells were filled to a final volume of 1.00 ml as follows: a, distilled H_2O (blank); b, 5–20 nmol of oligo(G) in H_2O; c, 100 nmol of standardized $NaIO_4$ in H_2O; d, oligo(G) as in b + $NaIO_4$ as in c. The $NaIO_4$ in 10 μl was added rapidly to both c and d at t_0 and its reduction followed by the absorbance (A) decrease at λ_{min} for oligo(G) (Figure 2) until reduction was complete (when $A_d - (A_b + A_c)$ became constant). Then

$$\text{nmoles of } IO_4^- \text{ reduced} = [(A_b + A_c - A_d)/A_{c_{t_0}}][\text{total nmol of } IO_4^-]$$

The IO_4^- reduced corresponds to the amount of *cis-vic*-glycol in the oligomer; and dp is organic P/IO_4^- reduced. Organic P is directly determined during periodate oxidation from A_{260} of cell b using the measured ϵ_P for the oligo(G) fraction. The periodate end group analyses are presented in Table I.

Snake Venom Phosphodiesterase Digestion. Mixtures containing 20 nmol of P of an oligo(G) fraction, 20 nmol of $Mg(OAc)_2$, 0.4 μmol of Tris acetate, pH 8.8, and 160 units of enzyme in a total volume of 0.035 ml were incubated 120 min at 37°. Reaction was stopped (5 min at 100°), the samples were centrifuged, and 30-μl aliquots were analyzed on a fluorescent cellulose thin layer developed with solvent B (Table IV). The only substance present in digests of $(pG)_4$, $(pG)_5$, and $(pG)_6$ was Guo-5′-P. No Guo-3′-P, Guo, or 2′-O-THP-Guo-5′-P was observed nor was pink fluorescence characteristic of the *N*-Bz-Guo chromophore. The same result was obtained with 10 units of enzyme hydrolyzing 100 nmol of $(pG)_4$ P.

E. coli **Alkaline Phosphatase Digestion.** Incubation of $(pG)_4$ with enzyme even over long periods at 37° with as much as 10 μg of enzyme/25 nmol of oligomer yielded only traces of P_i (<0.5 nmol). Ion exchange thin layer chromatography of the digests showed only the oligomer. Control experiments with Guo-5′-P gave Guo quantitatively.

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(41) These conditions are 32 half-lives of the removal of THP from 2′-*O*-THP-Guo-5′-P *at pH 5.0*. Experiments at pH 4.6 were not done, but it is estimated that 70 hr at 75° at this pH would be about 70 half-lives.