The Preparation of Deoxyguanosine Oligomers on an Insoluble Polymer Support

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The syntheses of deoxyguanosine-containing oligonucleotides, deoxyguanylyldeoxyguanylyldeoxyguanosine (d-GpGpG), deoxyguanylyldeoxyguanylyldeoxyguanylyldeoxyguanosine (d-GpGpGpG) and deoxyguanylyldeoxyguanylyldeoxyguanylylthymidine (d-GpGpGpT) have been described. Those nucleotidic materials were synthetized on an insoluble polymer support; the polymer was a copolymer of styrene, *p*-vinylbenzoic acid, and divinylbenzene. The blocking group used at the *N*-position of deoxyguanosine was di-*p*-methoxytrityl.

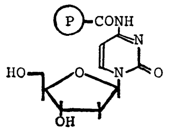
Since Letsinger and his co-worker[1] used a polymer support technique to synthetize an oligonucleotide, many similar investigations have been made.[2-9] The most striking characteristic of the polymer support technique is the simplicity of its treatment and reaction-controlling of the intermediary products of nucleotidic materials, as in the Merrifield method[10] used in the synthesis of oligopeptide. However, in polymer support techniques used to synthetize a polypeptide with a higher molecular weight at a higher degree of condensation reaction, there is a limitation which results from a failure of sequence in the synthetized polypeptide, which can not be isolated from the desired product.[11] That is, in the polymer support techniques in polypeptide and polynucleotide syntheses, by-products resulting from an incompleteness of condensation steps accumulate until there is a cleavage of the product from the polymer support. Still, this technique is useful for the synthesis of a oligonucleotide unless the separation and isolation of the desired oligonulceotide is impossible. Table 1 shows the results of several syntheses of oligonucleotides by the polymer support technique. These procedures give higher yields than would a conventional homogeneous method.

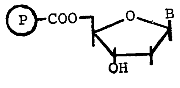
In a preceding paper,[9] we described the synthesis of deoxyguanylyldeoxyguanosine on an insoluble polymer support; on it the 5′-OH of deoxyguanosine was bound as an anchor group. In this paper, the application of this method to the syntheses of trinucleotide, tetranucleotides, and their derivatives will be described. That is, deoxyguanylyldeoxyguanylyldeoxyguanosine (d-GpGpG), deoxyguanylyldeoxyguanylyldeoxyguanyl-

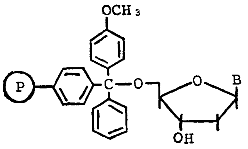
TABLE 1. SUMMARY OF OLIGONUCLEOTIDE SYNTHESES BY POLYMER SUPPORT TECHNIQUES[a]

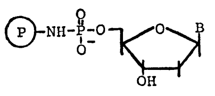
$\textcircled{P}$—COCl				$\textcircled{P}-\phi-\underset{\phi}{\overset{\overset{OCH_3}{\phi}}{C}}-Cl$		$\textcircled{P}$—NH_2
St-DVB[3] 6′-NH_2	St-DVB[9,10] 5′-OH	AN-AA[12] 5′-OH	DMBD-VBA 5′-OH	St[6] 5′-OH	St[7] 5′-OH	St[8] 5′-Phosphate
d(CT) 77% (55)	(TT) 82% (80)	(TT) 72%	(TT) 68%	(TT) 54%	(TT) 96%	p(TT) 40%
d(CTT) 34% (18)	d(TA) 70% (64)			(TTT) 38%	d(TC) 88%	p(TTT) 5%
d(CTTT) 15% (8)	d(TC) 72% (60)			(TTTT) 37%	d(TG) 94%	
d(CTA) 26%	(TTT) 56% (40)			d(ATT) 30%	(TTT) 84%	
	d(GG) 60% (48)					
	d(GGG) 22% (7)					
	d(GGGG) 7%					
	d(GGGT) 18%					

Shape of Anchorage


6′NH_2[3]


5′-OH[1,4,8]


5′-OH[6,7]


5′-Phosphate[8]

% shows overall yield　( ) shows overall yield by conventional homogeneous methods.

a) A new polymer support technique using a polymer containing 2-pyridineethanol has been published during contribution: W. Freist and F. Cramer, *Angew. Chem.* (*Intl. Ed.*), **9**, 368 (1970).

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2) R. L. Letsinger, M. J. Kornet, V. Mahadevan, and D. M. Jerina, *ibid.*, **86**, 5163 (1964).

3) R. L. Letsinger and V. Mahadevan, *ibid.*, **87**, 5326 (1965); **88**, 5319 (1966).

4) F. Cramer, R. Helbig, H. Hettler, K. H. Scheit, and H. Siegler, *Angew. Chem.*, **78**, 640 (1966).

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9) T. Shimidzu and R. L. Letsinger, *J. Org. Chem.*, **33**, 708 (1968).

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12) T. Shimidzu, *J. Syn. Org. Chem., Jap.*, **28**, 581 (1970).

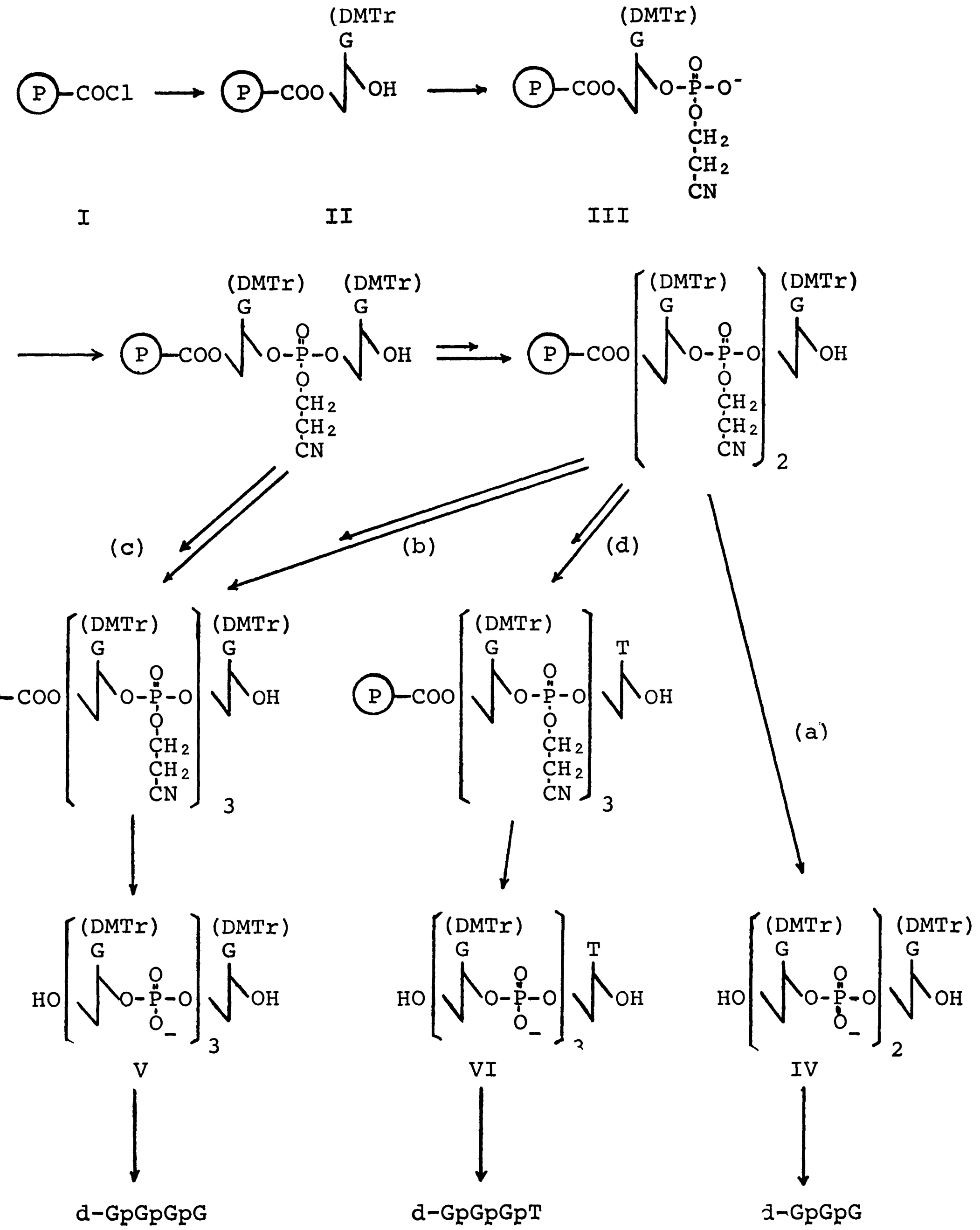

Fig. 1. Synthetic path of oligonucleotides.[13]

yldeoxyguanosine (d-GpGpGpG), deoxyguanylyldeoxyguanylyldeoxyguanylylthymidine (d-GpGpGpT), and their *N'*-di-*p*-methoxytrityl-substituted derivatives are synthetized. As the blocking group at the *N*-position[13] of deoxyguanosine, di-*p*-methoxytrityl (DMTr) is used.

These nucleotides were synthetized by the phosphodiester route on insoluble polymer supports like those described in the preceding paper.[9] The course of the synthesis is shown in Fig. 1.

The polymer supports used are tabulated in Table 2. The selectivity in the position of the anchorage of the hydroxy group in *N*-di-*p*-methoxytrityl deoxyguanosine[14] ((DMTr)d-G) depends on the crosslinkage of the polymer. A lower degree of crosslinkage gave a low selectivity of the 5'-0 anchorage, although the reac-

TABLE 2. COMPOSITION OF POLYMER SUPPORTS

	I	II	III	IV
Styrene	57.5	57.5	57.5	50.0 mmol
p-Divinylbenzene	0.040	0.064	0.012	0.063 mmol
p-Vinylbenzoic acid	6.36	6.90	6.35	12.5 mmol
% COCl	10	11	10	20
% Crosslinkage	0.06	0.1	0.02	0.1

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[13] For convenience the di-*p*-methoxytrityl group is represented on the amino group, though the exact position it occupies in the guanine ring has not been established with certainty: W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2105 (1959).

[14] H. Schaller, G. Weimann, B. Lerch, and H. G. Khorana, *J. Amer. Chem. Soc.*, **85**, 3821 (1963).

Table 3. Additions of di-p-methoxytrityldeoxyguanosine on polymer supports

	Ⓟ-COCl (g)	Nucleoside (g)	Pyridine (m*l*)	Reaction time (hr)	Nucleoside anchored (mmol/g·pol.)	(g)	Selectivity (5′O-linkage) (%)
I	1.0	1.28	25	48	0.120	0.23	
	1.0	1.28	40	48	0.129	0.25	100
	1.0	1.28	40	72	0.118	0.22	
II	6.0	4.56	50	12	0.410	0.11	
	6.0	4.00	50	24	0.855	0.23	100
	5.0	3.42	40	48	1.395	0.44	
III	1.0	1.28	40	48	0.180	0.34	85
IV	1.0	2.00	40	48	0.340	0.30	95

Table 4. Overall yield of d-GpGpG

Polymer Support	I	IV
Overall yield	22%	12%

tion rate was faster. When the COCl content in the polymer support reaches 20%, the yield of the oligonucleotide decreases. These results are tabulated in Tables 3 and 4.

The synthetized *N*-substituted oligonucleotides were finally separated using a Sephadex LH-20 column. When gel filtration with Sephadex LH-20, for example, is used, the limited separation capacity of the column must be considered. It can be seen from Fig. 2 that the upper limit of the molecular size of water-insoluble nucleotidic material which can be applied to the Sephadex LH-20 column is between five and six in the degree of condensation. In general, the separation and purification of oligonucleotide using an ion-exchange cellulose or an ion-exchange gel column is easier than that using a gelfiltration column when the oligonucleotide is soluble in water.

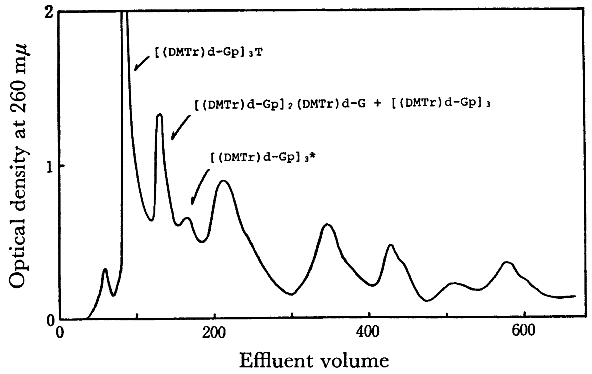

Fig. 2. Isolation of [(DMTr)d-Gp]$_3$T, VI, by sephadex LH 20 column.

*) (DMTr) is partly cloven.

The overall yields of these nucleotides were 22% (for d-GpGpG), 7% (for d-GpGpGpG *via* (b)), 17.5% (for d-GpGpGpG *via* (c)), and 18% (for d-GpGpGpT).

Experimental

Polymer Support, p-COCl. As is shown in Table 2, definnite amounts of freshly-distilled styrene, *p*-divinylbenzene, and *p*-vinylbenzoic acid[15] were placed, with a few seeds of the popcorn polymer and 0.5 mg of benzoyl peroxide, in an Erlenmeyer flask which had been well purged with nitrogen. Polymerization and chlorination were performed by the method described in the preceding paper.[9]

Di-p-methoxytrityldeoxyguanosine. Prepared by the procedure of Schaller *et al.*,[14] mp 167—168°C.

Paperchromatography was performed by the descending technique using Toyo Roshi No. 53 paper. The solvents were: A, isopropyl alcohol - concentrated ammonia - water (7:1:2); C, ethyl alcohol - 1M ammonium acetate at pH 7.35 (7:3). The phosphate buffer solution used in the electrophoreses was a mixture of 1/15M KH_2PO_4 and 1/15M Na_2HPO_4 (1:17).

The selectivity in the position of the anchorage of the hydroxy group in (DMTr)d-G was determined after the first phosphorylation of the anchored (DMTr)d-G. The selectivity (5′-0 linkage) was defined as [d-Gp/(d-Gp+pd-G)]×100.

N-*Di-p-methoxytrityldeoxyguanosine Trimer (IV).* *N*-Di-*p*-methoxytrityldeoxyguanosine (1.2 g) was treated with 1.0 g of the polymer support (I) in 20 m*l* of dry pyridine for 48 hr. Then, 10 m*l* of methanol was added to esterify any remaining acid chloride groups, and the mixture was stirred for 12 hr. Then, the polymer was collected by filtration and washed completely with methanol in an Soxhlet extractor until no residual *N*-di-*p*-metoxytrityldeoxyguanosine was found in the extract. The polymer was then dried under a vacuum over phosphorus pentoxide. The weight of the polymer was 1.170 g, from which value the amount of the *N*-di-*p*-methoxytrityldeoxyguanosine bound to the polymer support was estimated to be about 0.170 g.

The polymer-deoxyguanosine derivative (II) was added to a mixture of *β*-cyanoethylphosphate[16,17] (1 mmol) and mesitylenesulfonyl chloride[18] (2 mmol) in 20 m*l* of dry pyridine, and the mixture was stirred for 50 hr. Then, the solids were separated and kept at 50° with a 50% aqueous solution of pyridine for 30—40 min in order to hydrolyze a condensed phosphate. Finally, the solids, consisting of the polymer (III), were washed with methanol and dried under a vacuum over phosphorus pentoxide.

Prior to the addition of the second nucleosidic material, the activation of the terminal phosphate was achieved with mesitylenesulfonyl chloride. The polymer (III) was suspended in 20 m*l* of dry pyridine, after which a 2 mmol portion of mesitylenesulfonyl chloride was added. The reaction mixture was stirred for 24 hr. Then a solid was allowed to settle and the supernatant liquid was withdrawn. The solid was washed three times with dry pyridine to remove the major portion of mesitylenesulfonyl chloride, moisture being

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[15] J. R. Leebrick and H. E. Ramsden, *J. Org. Chem.*, **23**, 935 (1958).

[16] P. T. Gilham and G. M. Tener, *Chem. Ind.* (London), 542 (1959).

[17] G. M. Tener, *J. Amer. Chem. Soc.*, **83**, 159 (1961).

[18] C. H. Wang and S. G. Cohen, *ibid.*, **79**, 1924 (1957).

kept off meanwhile. Then 1.0 g of *N*-di-*p*-methoxytrityldeoxyguanosine was added to the solid along with 20 m*l* of dry pyridine. The reaction was carried out under stirring for 3 days. Then, 20 m*l* of methanol were added. After 3 hr's stirring, the mixture was filtered, and the solid was washed with pyridine, methanol, and ether successively.

With the 1.0 g portion of the solid, such phosphorylation and the addition of nucleosidic material were repeated using appropriate amounts of the reagents.

After the addition of the third nucleosidic material, *N*-di-*p*-methoxytrityldeoxyguanosine, 0.2 g of the resulting solid was treated with a mixture of 1 m*l* of 2N NaOH, 2 m*l* of ethanol, and 2 m*l* of dioxane for 12 hr in order to cleave a nucleotide from the polymer support. The polymer was collected by filtration and washed with ethanol several times. The alkaline filtrate, together with ethanol washes of the polymer, was neutralized with Dowex 50 resin (pyridinium form). After the removal of the Dowex resin, the addition of ethanol to the solution and evaporation were repeated 2 times; the resulting material was made up with 1 m*l* of an ethanol solution. The solution was placed in a Sephadex LH-20 column (1 cm × 70 cm) and eluted with 90% ethanol at the rate of 0.5 m*l*/min. After a small peak, the major peak (160—220 m*l*) appeared. The third peak partly overlapped the second. The major elution part, the second peak, was concentrated and was then placed in the column again. A major peak (168—260 m*l*) was found just after a small peak (158—167 m*l*). The elution of the major peak was concentrated and kept in a refrigerator after a small amount of ether had been added. A pale yellow precipitate was collected and dried under reduced pressure over phosphorus pentoxide. A soft powdery material was thus obtained (13 mg). This material was homogeneous in the solvent A (R_f 0.75—78). The ultraviolet characteristics in an ethanol solution were: λ_{max}, 278 mμ (55,000), 263 mμ (56,200), 237 mμ (78,400); λ_{min}, 274 mμ (51,700), 252 mμ (53,400), 225 mμ (70,900).

Found: C, 60.50; H, 5.01; N, 11.29%. Calcd for $C_{93}H_{89}O_{22}N_{15}P_2$: C, 61.01; H, 4.90; N, 11.48%; for $C_{93}H_{91}O_{23}N_{15}P_2$ (monohydrate): C, 60.42; H, 4.96; N, 11.36%; for $C_{93}H_{93}O_{24}N_{15}P_2$ (dihydrate): C, 59.84; H, 5.02; N, 11.26%.

Deoxyguanylyldeoxyguanylyldeoxyguanosine (d-GpGpG). From 0.2 g of the solid, the nucleotidic materials were cloven as has been described above. The neutralized solution was made up to 1 m*l*. Then 3 m*l* of 80% acetic acid was added to the solution, and the mixture was kept for 5 hr. The acetic acid was evaporated under reduced pressure at room temperature. Then 20 m*l* of dilute ammonium hydroxide was added, the mixture was centrifuged, and the supernatant was concentrated to 5 m*l*. The supernatant solution was then adjusted to pH 9.5 with a very small amount of acetic acid. The supernatant was applied to the top of a DEAE-cellulose (carbonate form) column (2 cm × 20 cm). The column was washed with 0.02M NH_4HCO_3 until no ultraviolet-absorbing material was eluted. Further elution was carried out using a linear gradient of ammonium bicarbonate. The mixing vessel and the reservoir contained 1 *l* of 0.02M salt and an equal volume of 0.3M salt. The main peak ranged from the elution volume of 700 to that of 1120 m*l*. The total optical density was 240 O.D. The elution was repeated to dryness by adding water, and finally lyophilized.

Thus, 11.2 mg of the desired product, d-GpGpG, was obtained. The material was homogeneous in electrophoresis at pH 8.0 (1/15M phosphate buffer). The electrophoretic mobility, compared with that of deoxyguanosine 5′-phosphate, was 0.15. The ultraviolet absorption characteristics in water were: λ_{max}, 255 mμ (23,900); λ_{min}, 224 mμ (10,100); $\lambda_{shoulder}$, 268 mμ. R_f in the solvent A was 0.01. A venom phosphodiesterase preparation[19] was added to a small amount of the product, and the mixture was incubated at 37° for 12 hr. When paper electrophoresis was then carried out at pH 8.0, three spots were obtained: mobility 0 (2.4 O.D.), mobility 0.15 (0.1 O.D.), and mobility 1.0 (5.1 O.D.). Those spots corresponded to deoxyguanosine, an undegraded material, and deoxyguanosine 5′-phosphate. The optical densities were determined from the differences between the absorbances of the eluted solutions of the spots and those of the elutions of the appropriate blanks. From these results, the material was identified as deoxyguanylyldeoxyguanylyldeoxyguanosine, d-GpGpG.

N-*Di*-p-*methoxytrityldeoxyguanosine Tetramer (V) and (VI).* The residual polymer was divided in half. Each polymer was phosphorylated with an appropriate amount of β-cyanoethylphosphate and mesitylenesulfonyl chloride, as has been described above. Then the phosphorylated polymers were activated, and *N*-di-*p*-methoxytrityldeoxylguanosine and thymidine (in appropriate amounts) respectively were added. On the other hand, 0.15 g of the solid that contained (*N*-di-*p*-methoxytrityldeoxyguanosine dimer was phosphorylated and then activated with appropriate amount of the reagents. Then, 0.07 g of *N*-di-*p*-methoxytrityldeoxyguanylyl-*N*-di-*p*-methoxytrityl)deoxyguanosine was added (route (c)). The procedures of the cleaving of the nucleotidic materials and purifications were the same as above.

The material (V) (*via* (b)) appeared in the elution of 95—130 m*l* after a small peak of an ultraviolet-absorbing material when the Sephadex LH-20 column and 95% ethanol were used just as above. Also, the material (V) (*via* (c)) appeared in the elution of 97—143 m*l* in another run. The material (VI) appeared in the elution of 81—121 m*l* (Fig. 2). When these materials were precipitated in the way mentioned above and dried, we obtained 16 mg of the nucleotidic material (V) (*via* (b)), 20 mg of the nucleotidic material (V) (*via* (c)), and 36 mg of the nucleotidic material (VI). Each material was dissolved homogeneously in the solvent A; the R_fs were 0.54—0.56 for the nucleotidic material (V) and 0.48—0.51 for the nucleotidic material(VI), respectively. The ultraviolet characteristics in ethanol solutions of the material(V) were: λ_{max}, 278 mμ (73,800), 264 mμ (74,100), 238 mμ (105,000); λ_{min}, 274 mμ (68,400), 253 mμ (71,200), 226 mμ (93,100): and of the material(VI), λ_{max}, 277 mμ (60,000), 260 mμ (61,200), 232 mμ (83,000); λ_{min}, 270 mμ (59,200), 250 mμ (59,000).

Found: C, 60.00; H, 4.91; N, 11.20%. Calcd for the material(V) (*via* (b)): $C_{124}H_{117}O_{30}N_{20}P_3$: C, 60.54; H, 4.80; N, 11.39%: for $C_{124}H_{121}O_{32}N_{20}P_3$ (dihydrate): C, 59.66; H, 4.89; N, 11.22: for $C_{124}H_{123}O_{33}N_{20}P_3$ (trihydrate): C, 59.23; H, 4.93; N, 11.14%.

Found: C, 57.01; H, 4.91; N, 10.83%. Calcd for the material(VI): $C_{103}H_{102}O_{29}N_{17}P_3$: C, 57.97; H, 4.78; N, 11.16%; for $C_{103}H_{106}O_{31}N_{17}P_3$ (dihydrate): C, 57.01; H, 4.88; N, 10.97%; for $C_{103}H_{108}O_{32}N_{17}P_3$ (trihydrate): C, 56.54; H, 4.93; N, 10.88%.

A comparison of the syntheses of the *N*-di-*p*-methoxytrityldeoxyguanosine tetramer shows that the conversion of each additional reaction was 40—50%, regardless of the length of the additional nucleosidic or nucleotidic materials.

The residual unreacted portions in the addition reaction were found to be nucleotidic materials which lacked the desired sequences, such as [(DMTr)d-Gp]$_2$(DMTr)d-G, [(DMTr)d-Gp]$_3$, and [(DMTr)d-Gp]$_2$T. They were ob-

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[19] 500 units of the phosphodiesterase in 2.5 m*l* of 0.3M Tris buffer at pH 8.85.

tained in considerable amounts, as is shown in Fig. 2. Those materials were identified and determined after the cleavage of the respective blocking groups, as will be described below.

Deoxyguanylyldeoxyguanylyldeoxyguanylyldeoxyguanosine (d-GpGpGpG). Eight mg of the nucleotidic material(V) (*via* (c)) were treated with 1 m*l* of 80% acetic acid for 5 hr at room temperature. The acetic acid was then evaporated in a vacuum. To the resulting materials, 10 m*l* of dilute ammonium hydroxide was added; the mixture was then centrifuged, and the supernatant was concentrated to 3 m*l*. The supernatant solution was made up to 5 m*l* with 0.2 M ammonium bicarbonate. The solution was then placed in a DEAE-cellulose column, just has been described above. The column was washed with 0.02 M NH_4HCO_3 until no further ultraviolet-absorbing material was eluted. Then, further elution was carried out using the linear gradient of ammonium bicarbonate described above. The major peak (the 1060—1280 m*l*-portion) was collected and lyophilized. The d-GpGpGpG thus obtained (4.1 mg) was homogeneous in paper electrophoresis at pH 8 (the relative mobility was 0.09 for d-pG=1.0).

The ultraviolet absorption characteristics in water were: λ_{max}, 257 mμ (18,000); λ_{min}, 224 mμ (12,500); $\lambda_{shoulder}$, 268 mμ; in 1 M NaCl were: λ_{max}, 256 mμ (26,400); λ_{min}, 224 mμ (12,300) $\lambda_{shoulder}$, 267 mμ.

A portion of the material was incubated at 37° for 12 hr with 0.1 m*l* of the venom phosphodiesterase preparation. Three spots were obtained on chromatography in the solvent C; the R_fs were 0—0.04, 0.35 and 0.69, corresponding to an undegraded material (d-GpGpGpG or d-GpGpG), deoxyguanosine 5′-phosphate, and deoxyguanosine, respectively. The optical density units found on eluting these spots were 0.2, 6.7, and 2.1, respectively. Those results show that the material was d-GpGpGpG and that 98% of the tetranucleotide was degraded; d-pG/d-G=3.2.

Deoxyguanylyldeoxyguanylyldeoxyguanylylthymidine (d-GpGpGpT). Ten mg of the nucleotidic material(VI) was trated with acetic acid by the procedure described in the preceding section. The major peak (1100—1290 m*l* portion) was collected and lyophilized. Thus, d-GpGpGpT was obtained (5.7 mg); it was homogeneous in paper electrophoresis at pH 8 (the relative mobility was 0.11—0.12 for d-pG=1.0). The ultraviolet absorption characteristics in water were: λ_{max}, 263 mμ (30,000); λ_{min}, 230 mμ (18,900); $\lambda_{shoulder}$, 240 mμ: in 1 M NaCl were: λ_{max}, 261 mμ (29,200); λ_{min}, 229 mμ (18,700); $\lambda_{shoulder}$, 238 mμ.

The enzymatic hydrolysis by the venom phosphodiesterase yielded 1.0 optical density unit of deoxyguanosine, 2.1 optical density units of thymidine 5′-phosphate, 0.8_5 optical density unit of thimidine 5′-phosphate, and 0.1 optical density unit of deoxyguanylyldeoxyguanosine; *i.e.*, 79% of d-GpGpGpT was hydrolyzed (neglecting the hypochromic effect of degraded nucleotides). Also, the enzymatic degradation by spleen phosphodiesterase was achieved. In this case, 2.0 optical density units of deoxyguanosine 3′-phosphate, 0.6 optical density unit of thymidine, and 0.1_5 optical density unit of undegraded material were yielded. These results confirmed that the resulting tetranucleotide was d-GpGpGpT.

Isolation of By-products, d-GpGpG, d-GpGpGp, and d-GpGpT. The elutions of the peaks (120—155 m*l*, 155—185 m*l*, and 185—300 m*l*) shown in Fig. 2 were concentrated and treated with 80% acetic acid to cleave the blocking group; then they were placed in a DEAE-cellulose column in a way similar to that described above.

From the first portion, two major nucleotidic materials (elutions at 0.120—1.130 M NH_4HCO_3 (39 optical density units) and at 0.135—0.165 M NH_4HCO_3 (18 optical density units)) were obtained. From the second portion a single major nucleotidic material (elution at 0.130—0.155 M NH_4HCO_3 (20 optical density units)), and form the third portion, another single nucleotidic material (elution at 0.125—0.135 M NH_4HCO_3 (112 optical density units)) from three ultravioletabsorbing materials, were obtained. These materials were identified as d-GpGpG, d-GpGpGp, d-GpGpGp, and d-GpGpT, respectively by means of enzymatic hydrolyses and electrophoreses.

From the result that d-GpGpGp was found in both the first and the second portions in the Sephadex LH-20 column separation, it might be concluded that a partial cleaving of the blocking group takes place in the course of the synthesis. The greater quantity of d-GpGpT comparing with those of d-GpGpG and d-GpGpGp is believed to be due to one of two routes in the synthesis; one is the route skipping over the first coupling step, and the other is that skipping over the second coupling step.