From eq. 4 and 5, the radioactivity of the $G\text{-}1,6\text{-}P_2$ at equilibrium, *i.e.*, $[G\text{-}1,6\text{-}P_2]_e$, may be calculated from the measured radioactivity of the added substrate and of the phospho-enzyme at equilibrium, and $(EP)_e/(EP)_i$ may then be represented as

$$(EP)_e/(EP)_i = [EP]_e/\{[EP]_e + 0.5[G\text{-}1,6\text{-}P_2]_e\} = 1/F_1 \quad (6)$$

Since $(EP)_e = [EP]_e/k_e$, the concentration of active phospho-enzyme initially present is given by eq. 7, where k_e is the specific activity of the exchangable phosphate at equilibrium and will be evaluated below.

$$(EP)_i = F_1[EP]_e/k_e \quad (7)$$

Values of F_1 calculated from eq. 6 are given in Table I as the equilibrium correction factor.

Isotope Dilution Correction Factor.—If the initial specific activity of added substrate is represented by k_i, the following equation may be written

$$k_e = k_i(G\text{-}6\text{-}P)_i/\{(EP)_i + (G\text{-}6\text{-}P)_i\} \quad (8)$$

After the appropriate manipulations this becomes

$$k_e = k_i\{[T] - [EP]_e - 0.5[G\text{-}1,6\text{-}P_2]\}/[T] = k_i/F_2 \quad (9)$$

Values of F_2 from measured values of $[T]$ and $[EP]_e$ and calculated values of $[G\text{-}1,6\text{-}P_2]$ are given in Table I as the isotope dilution correction factor.

Over-all Correction.—From eq. 7 and 9, $(EP)_i = F_1F_2[EP]_e/k_i$. From Table I it may be seen that the product F_1F_2 is reasonably constant, in spite of variations in both F_1 and F_2. Thus to a good approximation $(EP)_i = k[EP]_e$. It might also be observed that the F_1F_2 product will be reasonably constant no matter what value is chosen for the equilibrium of eq. 2, and hence the validity of the present procedure is dependent only on the direct measurement of $[EP]_e$, since the ratio of $(EP)_i$ at any photoöxidation time to $(EP)_i$ at a reaction time of zero is the quantity actually under consideration. Thus if $X = \{[EP]_e + \frac{1}{2}[G\text{-}1,6\text{-}P_2]\}$, $F_1F_2 = X[T]/\{[T] - X\}$, which means that F_1F_2 is essentially constant over narrow ranges of X.

Studies on Polynucleotides. XVIII.[1] Experiments on the Polymerization of Mononucleotides. The Synthesis and Characterization of Deoxyguanosine Oligonucleotides[2]

BY R. K. RALPH, W. J. CONNORS, H. SCHALLER AND H. G. KHORANA

In attempts to prepare deoxyguanosine oligonucleotides by polymerization of suitably protected deoxyguanosine-5' phosphate, the following derivatives of the nucleotide were prepared: $N,O^{3'}$-diacetyl, N-acetyl, N-benzoyl, N-naphthoyl, N-di-p-methoxytrityl, $O^{3'}$-di-p-methoxytrityl and $N,O^{3'}$-bis-di-p-methoxytrityl. Only the di-p-methoxytrityl derivatives proved soluble in anhydrous pyridine, the medium used in all the previous work, but the polymerization was unsatisfactory. In search for alternative solvent systems, thymidine-5' phosphate could be polymerized well in a mixture of pyridine and dimethylformamide in the presence of pyridinium Dowex-50 ion exchange resin. Using this procedure, N-acetyldeoxyguanosine-5 phosphate gave the expected homologous oligonucleotides which were separated on a DEAE–cellulose column and characterized before the removal of the N-acetyl protecting group from the guanine ring. Deoxyguanosine oligonucleotides obtained after removal of the N-acetyl-protecting group formed very large molecular weight aggregates having highly ordered secondary structure. This property of aggregation complicated the problem of characterization of the oligonucleotides by the standard chemical and enzymic methods.

In several previous papers from this Laboratory the polymerization of deoxyribomononucleotides to form homologous series of polynucleotides has been reported.[3-5] The method of polymerization was first developed for thymidine-5' phosphate.[4] Because of the insolubility of other deoxyribonucleotides in dry pyridine, the only medium found satisfactory so far, and, of the reactivity of the amino groups, methods were developed for the selective protection of the latter groups in deoxycytidine aud deoxyadenosine nucleotides.[5] The resulting protected nucleotides served as suitable starting materials for the polymerization procedure and the protecting groups were removed under mild conditions before separation and characterization of the polymeric products.[5] The extension of this work to the polymerization of a protected deoxyguanosine-5' phosphate did not prove straightforward and a modification of the polymerization procedure has now been developed. Furthermore, the characterization of the synthetic oligonucleotides by the chemical and enzymic methods used previously proved difficult until the discovery was made that the guanosine oligonucleotides were capable of forming large molecular weight aggregates with highly ordered secondary structure.[6] The present paper contains a detailed report of our experiments on the synthesis of these oligonucleotides, and, in particular, their isolation and characterization as the N-acetyldeoxyguanosine derivatives which are devoid of the property of aggregation and, therefore, are amenable to analysis by the methods developed previously.[4,5]

Acetylation of pyridinium deoxyguanosine-5 phosphate with a mixture of acetic anhydride and pyridine gave quantitatively $N,O^{3'}$-diacetyldeoxyguanosine-5' phosphate[7] (I). It is interesting to note that the

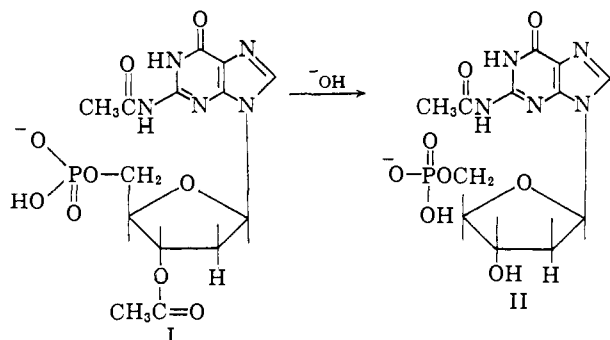(1) Studies on Polynucleotides. XVII: G. Weimann and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 4329 (1962).

(2) This work has been supported by grants from the National Cancer Institute of the National Institutes of Health, the National Science Foundation, Washington, D. C.; and the Life Insurance Medical Research Fund, New York, N. Y.

(3) 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, Chapter 5.

(4) (a) G. M. Tener, H. G. Khorana, R. Markham and E. H. Pol, *J. Am. Chem. Soc.*, **80**, 6223 (1958); (b) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **83**, 675 (1961); (c) H. G. Khorana, J. P. Vizsolyi and R. K. Ralph, *ibid.*, **84**, 414 (1962).

(5) (a) H. G. Khorana, A. F. Turner and J. P. Vizsolyi, *ibid.*, **83**, 686 (1961); (b) R. K. Ralph and H. G. Khorana, *ibid.*, **83**, 2926 (1961).

(6) R. K. Ralph, W. J. Connors and H. G. Khorana, *ibid.*, **84**, 2265 (1962).

(7) The acetyl group in the ring is tentatively placed on the N-2-amino group. It is possible that it is on the 3-position (partial structure of the nucleotide III). It is improbable that the acetyl group is on O^6 or N-1-posi-

III
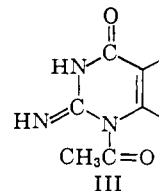
tion, because spectrophotometrically an ionization around pH 10–11 can be demonstrated and this probably is due to the loss of a proton from N-1 position. The presence of an acetyl group at this portion of the molecule would preclude this ionization. The alternative that the acetyl group is on N-1 and that the ionization is due to the loss of a proton from the guanidine system involving the 2- and 3-position of the pyrimidine ring is considered unlikely.

acetylation of the amino group[7] in the nucleotide was much faster than that in deoxyguanosine[8] itself, and it is probable that in the nucleotide the acetylation is assisted by the intramolecular transfer of the acetyl group from the acetyl phosphate mixed anhydride. N-Acetyldeoxyguanosine-5' phosphate (II) was obtained quantitatively from I by selective O-deacetylation in alkali[9]; II was practically insoluble in dry pyridine, the medium used satisfactorily in earlier polymerization studies, and, therefore, other protected derivatives containing more lipophilic organic groups were tried. These included N-benzoyl-, N-naphthoyl- and N-di-p-methoxytrityldeoxyguanosine-5' phosphates. Of these only the last-mentioned gave a homogeneous anhydrous pyridine solution of adequate concentration. However, polymerization of this derivative under the standard conditions[4,5] was unsatisfactory, there being recovered 55% of the unreacted mononucleotide, 8% of the di-

nucleotide and only about 12% of higher oligonucleotides. Subsequent to these experiments, it was recognized that the size of the organic group in the nucleotide portion has a marked detrimental effect on the internucleotide bond synthesis[1,10] and the experiments with the above-protected derivatives were not pursued further.

While the use of dicyclohexylcarbodiimide (DCC) as the polymerizing agent does not permit the use of strong bases such as trialkylamines,[3,11] which could aid in solubilization, reagents such as p-toluenesulfonyl chloride bring about activation merely by anhydride exchange process and some experiments were carried out with the latter reagent using the long chain amine, notably tri-n-dodecylamine, salts of N-acetyldeoxyguanosine-5' phosphate. Although some polymerization did occur, the elution pattern from a DEAE–cellulose column was rather complex, there being a series of unidentified nucleotidic peaks, in addition to those expected.[12]

Further attempts were directed to a re-examination of the polymerization conditions using DCC as the condensing agent. In earlier work,[13] other polar solvents such as dimethylformamide and dimethyl sulfoxide had given poor results in experiments on the synthesis of thymidyl-(3' → 5')-thymidine from a protected nucleotide and a nucleoside and, especially, in the case of dimethylformamide the result was ascribed to the formation of inhibitory strong base

from the decomposition of the solvent. In the present work too, the polymerization of thymidine-5' phosphate failed to proceed in a mixture of anhydrous dimethylformamide and pyridine, the major product being P^1,P^2-dithymidine-5' pyrophosphate. When, however, pyridinium Dowex-50 ion exchange resin was included in the reaction mixture, the polymerization of the above nucleotide proceeded normally. The pyridinium resin apparently served to substitute pyridinium cations for any alkylammonium cations in the medium and, thus, to provide the proton concentration necessary for the carbodiimide reaction.[3,10,11] These conditions were used with reasonable success in the polymerization of N-acetyldeoxyguanosine-5' phosphate here described.

In the first experiments, the mixture of products obtained by polymerization of N-acetyldeoxyguanosine-5' phosphate was treated with concentrated ammonia to remove the N-acetyl groups and the resulting products were separated on a DEAE–cellulose column. Although from the pattern of elution it was clear that homologous oligonucleotides, up to about octanucleotide, were present, only the mononucleotide and the dinucleotide (5'-O-phosphoryldeoxyguanylyl-(3' → 5')-deoxyguanosine, d-pGpG) could be positively characterized. Thus the dinucleotide gave, after dephosphorylation with bacterial phosphomonoesterase, deoxyguanylyl-(3' → 5')-deoxyguanosine (d-GpG) identical with a sample prepared by an unambiguous procedure.[10b] The dephosphorylated product, d-GpG, was attacked by spleen phosphodiesterase[14] to give deoxyguanosine-3'phosphate and deoxyguanosine. Even in the paper chromatography of the dinucleotide (d-pGpG), the tendency of the compound to stick to the origin on paper chromatograms was noted.[15] With the oligonucleotides higher than the dinucleotide no solvent system could be found in which the spots would move from the origin.[15] The oligonucleotides could not be rechromatographed reproducibly as sharp peaks on DEAE–cellulose columns (carbonate or chloride form) even when high salt concentration (up to 1 M) was used. A variable part of the nucleotidic material could be eluted subsequently with sodium hydroxide, a behavior reminiscent of large molecular weight nucleic acids.[16] Repeated attempts to degrade the tri- and tetranucleotide peaks with the snake venom phosphodiesterase[17] caused degradation to deoxyguanosine-5' phosphate only to the extent of 10–20%. The action of the bacterial alkaline phosphomonoesterase, as determined by the release of inorganic phosphate, was very sluggish on the trinucleotide and the tetranucleotide was essentially resistant.

All the above properties indicated some type of hydrogen-bonded interactions[18] and examination in the ultracentrifuge and a study of the temperature–ultraviolet absorbance curves confirmed the presence of large molecular weight aggregates with highly ordered structures.[6,19,20]

(8) H. Schaller, G. Weimann, B. Lerch and H. G. Khorana, J. Am. Chem. Soc., in press.

(9) Of the N-acetyl groups in the cytosine, adenine and guanine ring of the corresponding nucleosides, that in the guanine ring is the most stable to alkaline conditions and the preferential removal of the O-acetyl group in this case presents no difficulty.

(10) H. Schaller and H. G. Khorana, J. Am. Chem. Soc., in press.

(11) M. Smith, J. G. Moffatt and H. G. Khorana, ibid., 82, 6204 (1958).

(12) The result is similar to that obtained previously in a study of thymidine-5' phosphate polymerization with p-toluenesulfonyl chloride.[4c]

(13) Unpublished work of P. T. Gilham, carried out in 1957. The addition of pyridine hydrochloride to the reaction mixture in dimethylformamide did cause improvement in the yield, indicating that the activation of the nucleotide was probably being inhibited by dimethylamine resulting from the decomposition of the solvent.

(14) (a) R. J. Hilmoe, J. Biol. Chem., 235, 2117 (1960); (b) W. E. Razzell and H. G. Khorana, ibid., 236, 1144 (1961).

(15) In the case of the dinucleotide, but not of the higher oligonucleotides, the phenomenon of sticking to the origin could be prevented by letting the chromatographic solvent travel over the spot before it dried.

(16) See, e.g., D. A. Goldthwait and J. L. Starr, J. Biol. Chem., 235, 2025 (1960).

(17) W. E. Razzell and H. G. Khorana, ibid., 234, 2105 (1959).

(18) Of all the evidence thus far the resistance to venom phosphodiesterase was the most strongly suggestive of complexed multistranded structure. For example, double-stranded molecules are extremely slowly attacked (W. E. Razzell and H. G. Khorana, unpublished observations).

(19) Further physicochemical studies which are being carried out in collaboration with Dr. R. M. Bock and co-workers will be reported subsequently.

(20) Findings by a number of groups of workers concerning guanine polynucleotide chemistry and enzymology are explained by the conclusions reported here. The failure hitherto recorded in obtaining extensive poly-

In subsequent work the products obtained by polymerization of N-acetyldeoxyguanosine-5' phosphate were directly chromatographed on a DEAE–cellulose (bicarbonate form) column in the cold and the isolation procedure was modified so as to avoid any loss of the N-acetyl groups from the oligonucleotides. The pattern of elution from a small (0.1 mmole) chromatographic run is shown in Fig. 1, the concentration of the different peaks being given in Table I. The N-acetyl derivatives were found to be completely devoid of the property of aggregation discussed above. Such oligonucleotides behaved normally on paper chromatograms (R_f's in Table II) and the linear members (general structures IV) were susceptible to the action of bacterial phosphomonoesterase.[21] The series of oligonucleotides (V) thus obtained could be degraded completely by the splenic phosphodiesterase to N-acetyldeoxyguanosine-3' phosphate and N,O³'-diacetyldeoxyguanosine (Table III). Furthermore, crude snake venom (*Crotalus adamanteus*), used at a high concentration, degraded the oligonucleotides to, as expected, N-acetyldeoxyguanosine and N,O³'-diacetyldeoxyguanosine-5' phosphate.[22]

Fig. 1.—Chromatography of N-acetyldeoxyguanosine oligonucleotides on a DEAE–cellulose (bicarbonate) column. For details see text.

increasing contamination at that stage with side products.

IV, R = N-acetylguanine; R' = $-\overset{\overset{\displaystyle O}{\|}}{\underset{\underset{\displaystyle O^-}{|}}{P}}-OH$ VI, R = N-acetyl-guanine

V, R = N-acetylguanine;   R' = H

TABLE I

CHROMATOGRAPHY OF POLYMERIC PRODUCTS FROM
N-ACETYLDEOXYGUANOSINE-5' PHOSPHATE

Peak no.	Optical density units at 260 mμ	Composition remarks
1	5	N-Alkylpyridinium nucleotide
2	12	Unidentified
3	16	Unidentified (N-acetyldeoxyguanosine spectrum)
4	23	Unidentified (N-acetyldeoxyguanosine spectrum)
5	121	N-Acetyldeoxyguanosine-5' phosphate
6	22	Unidentified
8	131	N-Acetyldeoxyguanosine dinucleotide
9	31	Unidentified
10	83	N-Acetyldeoxyguanosine cyclic trinucleotide
11	80	N-Acetyldeoxyguanosine trinucleotide
14	44	N-Acetyldeoxyguanosine tetranucleotide
15	42	N-Acetyldeoxyguanosine pentanucleotide
16 1 M eluate	228	Higher polynucleotides (see below)

Although in the run shown in Fig. 1 all the nucleotidic material after the pentanucleotide (peak 15, Fig. 1) was collected together with strong eluent, the same polymerization mixture has been fractionated in other runs up to the heptanucleotide. There is, however,

guanylate synthesis by polynucleotide phosphorylase-catalyzed polymerization of guanosine-5' diphosphate may simply be due to the removal from the medium of the low amounts of oligonucleotides, that may initially be formed, by such multistranded aggregate formation and consequent lack of priming material. Similarly, the apparent inability of the deoxy-polyguanylate [prepared by strand separation of the enzymatically synthesized deoxy-polyguanylate–deoxypolycytidylate polymer [C. M. Redding, J. Josse and A. Kornberg, *J. Biol. Chem.*, **237**, 2869 (1962)] to serve as a primer (template) for RNA-polymerase catalyzed polymerization of cytidine triphosphate [M. Chamberlain and P. Berg, *Federation Proc.*, **21**, 385 (1962)] may simply be because the deoxypolyguanylate itself exists in a strongly hydrogen-bonded multistranded structure.

(21) The complete conversion of the original oligonucleotide to a single faster band corresponding to the oligonucleotide lacking the terminal phosphate, proved, as before,[4,5] that the oligonucleotides were pure.

(22) Degradation by purified venom diesterase was slow and exhaustive attempts to drive the degradations to completion have not been made. Two reasons may be advanced for the sluggishness. First, the presence of 3'-O-acetyl group at the terminus will cause retardation of the rate [W. E. Razzell and H. G. Khorana, *J. Biol. Chem.*, **234**, 2114 (1959)]. Second, it looks probable that the presence of N-acetyl on the guanine ring also retards the reaction. Using massive amounts of the diesterase, as was done with the crude venom, probably the degradations would go to completion.
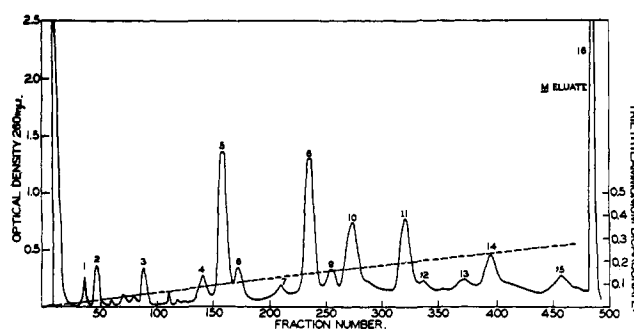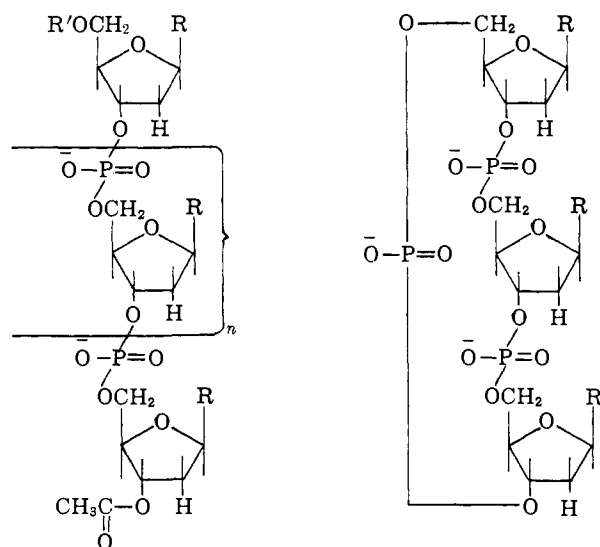
TABLE II

PAPER CHROMATOGRAPHY OF DIFFERENT COMPOUNDS

Compound	Solvent A	B	C
Deoxyguanosine-5' phosphate (d-pG)			0.39
N-Acetyldeoxyguanosine	0.53		.77
Deoxyguanosine	.44		.70
N,O³'-Diacetyldeoxyguanosine-5' phosphate			.61
N-Acetyldeoxyguanosine-5' phosphate			.49
Oligonucleotides with 5'-phosphomonoester groups			
N-Acetyl-d-pGpG(3'-O-acetyl)	0.33		0.35
N-Acetyl-d-pGpGpG(3'-O-acetyl)	.23		.17
N-Acetyl-d-pGpGpGpG(3'-O-acetyl)	.152		.09
N-Acetyl-d-pGpGpGpGpG(3'-O-acetyl)	.11		.05
Oligonucleotides without 5'-phosphomonoester groups			
d-GpG	0.08		
N-Acetyl d-GpG(3'-O-acetyl)	.024		0.67
N-Acetyl d-GpGpG(3'-O-acetyl)			.47
N-Acetyl d-GpGpGpG(3'-O-acetyl)			.32
N-Acetyl d-GpGpGpGpG(3'-O-acetyl)			.20
Cyclic oligonucleotide			
N-Acetyl cyclic trinucleotide		0.36	0.40
N,O³'-Diacetyldeoxyguanosine	0.66	.43	.81

TABLE III

RESULTS OF DEGRADATION OF N-ACETYLDEOXYGUANOSINE OLIGONUCLEOTIDES LACKING TERMINAL PHOSPHATE GROUPS BY SPLEEN PHOSPHODIESTERASE

From peak no. (Fig. 1)	Compound	N-Acetyldeoxyguanosine-3' phosphate units (total optical density units at 260 mμ)	N,O^3-Diacetyldeoxyguanosine + N-acetyldeoxyguanosine (total optical density 260 mμ)	Found	Theory
				Ratio nucleotide/ nucleoside	
8	Dinucleotide (V, $n = 0$)	1.6	1.6	1	1
11	Trinucleotide (V, $n = 1$)	5.5	2.7	2.04	2
14	Tetranucleotide (V, $n = 2$)	3.0	0.99	3.04	3
15	Pentanucleotide (V, $n = 3$)	3.08	0.776	3.97	4

Peak 10 (Fig. 1), a major peak, corresponded, presumably, to the cyclic trinucleotide VI. This conclusion was drawn from its resistance to crude venom, to the phosphomonoesterase and from electrophoretic mobility and position of elution. The minor peaks (1–4), were not investigated, but presumably they correspond to the minor products in this region obtained earlier.[4,5] The general pattern is thus similar to those obtained in earlier work[4,5] and, in particular, the formation of the cyclic trinucleotide (peak 10) as a major product is analogous to the findings in the deoxyadenosine (also a purine nucleoside) series.[5]

For physicochemical studies,[19] it thus is best to isolate and characterize the deoxyguanosine oligonucleotides as the N-acetyl derivatives and, subsequently, to treat the purified individual members with concentrated ammonia to remove the N-acetyl groups.

Experimental

Pyridinium N,O$^{3'}$-Diacetyldeoxyguanosine-5' Phosphate.— The commercially available disodium salt of deoxyguanosine-5' phosphate was converted to the pyridinium salt by passage through a suitable column of pyridinium Dowex-50 ion exchange resin. The total aqueous pyridine solution of the nucleotide was lyophilized to give a fine white powder. The pyridinium salt (2.14 g.) was suspended in a mixture of pyridine (50 ml.) and acetic anhydride (20 ml.) and the mixture shaken in the dark at room temperature. A clear solution usually resulted in 1 to 2 hr. and the shaking was continued for about 4 hr. longer. The solution was then cooled in an ice-bath and an excess of methyl alcohol (25 ml.) was added. The clear solution was allowed to stand at room temp. for 1 hr. and then evaporated to a gum under reduced pressure. The gum was taken up in water and the solution extracted repeatedly with ether to remove the bulk of pyridinium acetate. The resulting aqueous solution was then evaporated at below 30°. The residue was again dissolved in water and the evaporation repeated. The gum was again dissolved in about 100 ml. of water and the solution lyophilized. Pyridinium N,O$^{3'}$-diacetyldeoxyguanosine-5' phosphate was thus obtained as a fine white powder (2.56 g.). It was homogeneous on paper chromatography in solvent C. The spectral characteristics were: λ_{max} 259 mμ with a broad shoulder at 273–280 mμ, λ_{min} 226 mμ, in water. The ϵ_{max} (259 mμ) for N-acetyldeoxyguanosine-5' phosphate as determined by conversion of a known amount of the compound to the parent deoxyguanosine-5' phosphate by treatment with concd. ammonia was 16,700 (259 mμ). In 0.1 N sodium hydroxide, λ_{max} was 263 mμ, λ_{min} 239 mμ, ϵ_{max} (263 mμ) 14,250.

Pyridinium N-Acetyldeoxyguanosine-5' Phosphate.— Pyridinium N,3'-O-diacetyl-deoxyguanosine-5' phosphate (2.16 g.) was treated with 100 ml. of 1 N sodium hydroxide for 2 min. at 0°. An excess of pyridinium Dowex-50 ion exchange resin was then added to remove the sodium ions and the resulting aqueous pyridine solution and the resin added on top of a column (25 cm. × 2 cm.) of fresh pyridinium Dowex-50 resin. The column was washed thoroughly with aqueous pyridine and the total eluate and washings containing the nucleotide were partly evaporated in vacuo at below 30°. The resulting solution of pyridinium N-acetyldeoxyguanosine-5' phosphate containing some pyridine was lyophilized. The yield of the fine white powder was 1.85 g. This product was homogeneous on paper chromatography in solvent C. It was stored in a tightly sealed bottle in a desiccator at −15°. Treatment of the compound with concd. ammonium hydroxide for 2 days at room temp. caused complete conversion to deoxyguanosine-5' phosphate.

In 80% acetic acid at room temp., N-acetyldeoxyguanosine-5' phosphate was stable up to 4 hr.; with longer time a new ultraviolet absorbing material, presumably N-acetylguanine, appeared. No cleavage of the acetyl group prior to glycosyl bond cleavage could be detected.

Pyridinium N-Benzoyldeoxyguanosine-5' Phosphate.[23]— Pyridinium deoxyguanosine-5' phosphate was prepared from the ammonium salt (365 mg., 1 mmole) by treatment with pyridinium Dowex-50 ion exchange resin. The solution of the pyridinium salt was lyophilized and the resulting fine powder was treated in the dark with a mixture of pyridine (20 ml.) and benzoyl chloride (1.25 ml., 10 mmoles) for 2 hr. at room temp. The light brown solution was then treated at 4° with a mixture of water (100 ml.) and chloroform (100 ml.) and the chloroform layer separated. After a water wash, the solvent was removed completely in vacuo and the resulting gum was dissolved in 20 ml. of pyridine. Water (10 ml.) and then sodium hydroxide (30 ml. of 2 N) was added and the clear solution which soon resulted was kept at room temp. for 10 min. An excess of pyridinium Dowex-50 ion exchange resin was then added and the total solution and resin transferred to a column (10 × 2.5 cm.) of the same resin in the pyridinium form. The total eluate of the nucleotide material was evaporated in vacuo (20°) to a small volume when much benzoic acid crystallized. Extraction of the benzoic acid in the cold with ether left behind an aqueous solution with pH 4. Paper chromatography in solvent C at this stage showed complete absence of benzoic acid and the presence of only one spot corresponding to N-benzoyldeoxyguanosine-5' phosphate. The aqueous solution was treated with 5 ml. of pyridine and then evaporated to a gum at low temperature. The product was stored in the cold as its solution in pyridine. The ultraviolet absorption characteristics of the product after paper chromatography in solvent C and elution with water were: λ_{max} 291, 262, a shoulder at 241 mμ; λ_{min} 276 and 223 mμ.

When the above product was chromatographed on a DEAE-cellulose (acetate form) column using a linear gradient of triethylammonium acetate as described previously,[5a] a single peak of ultraviolet-absorbing material was obtained.

Pyridinium N-naphthoyldeoxyguanosine-5' phosphate was prepared essentially by the method used for the preparation of N-benzoyldeoxyguanosine-5' phosphate. For 1 mmole of pyridinium deoxyguanosine-5' phosphate 3.8 g. of β-naphthoyl chloride was used, the reaction being carried out in 20 ml. of dry pyridine for 1 hr. at 20° in the dark (a clear solution was obtained). After the work-up, pyridinium β-naphthoyldeoxyguanosine-5' phosphate was obtained as a fine white powder (lyophilization) (594 mg.). The product was homogeneous on paper chromatography in solvent D, giving a strongly fluorescent spot under ultraviolet light; λ_{max} 245 and 300 mμ in phosphate buffer pH 7.1. The product was insoluble in dry pyridine.

N-Di-p-methoxytrityldeoxyguanosine-5' Phosphate.— A suspension of pyridinium deoxyguanosine-5' phosphate (1.85 g., 4.3 mmoles) in dry pyridine (20 ml.) was treated with di-p-methoxytrityl chloride (1.62 g., 4.79 mmoles) and the sealed reaction mixture kept at room temp. At different intervals aliquots were removed and examined by paper chromatography in solvent A. After 1.25 hr. when all the nucleotide had dissolved, about 50% reaction had occurred. The products detected on paper chromatograms in order of increasing R_f were: starting material, N-di-p-methoxytrityldeoxyguanosine-5' phosphate (R_f 0.49) and N,O$^{3'}$-bis-di-p-methoxytrityldeoxyguanosine-5' phosphate (R_f 0.69). After a 7-hr. reaction period, 18% of unreacted starting material was present, N-di-p-methoxytrityldeoxyguanosine-5' phosphate amounted to 74% while the bis-trityl nucleotide was 8%. After an 8-hr. reaction period, methyl alcohol (1 ml.) was added and the reaction mixture evacuated to remove the solvent at low temperature. The residual oil was dissolved in 10 ml. of solvent A and the solution reevaporated. When 5 ml. of solvent A was re-added to the residue unreacted ammonium deoxyguanosine-5' phosphate largely precipitated (5,600 optical density units at 252 mμ) and was removed by centrifugation. One-half of the supernatant solution was applied to the top of a cellulose column (120 cm. × 2.5 cm.) previously equilibrated with solvent A. Elution was carried out with the same solvent at a flow rate of 0.6 ml./min., 10-min. fractions being collected. Fractions 53–60 contained some non-nucleotidic material and bis-di-p-methoxy-

(23) Cf. the preparation of N-benzoylguanosine-5' phosphate: M. Smith, G. I. Drummond and H. G. Khorana, J. Am. Chem. Soc., 83, 608 (1961).

trityldeoxyguanosine-5' phosphate. Fractions 61–70 also contained the preceding compound as well as a little of the mono-di-p-methoxytrityldeoxyguanosine-5' phosphate, while the bulk of the latter compound was in fractions 71–109 (25,500 optical density units at 270 mμ). Some deoxyguanosine-5' phosphate (1300 optical density units at 252 mμ) appeared in the last peak, fractions 165–190. The pooled fractions containing pure N-di-p-methoxytrityldeoxyguanosine-5' phosphate were evaporated with the addition of pyridine and a little ammonium hydroxide and the aqueous ammoniacal solution finally obtained was lyophilized (1.104 g.). This preparation had an optical density of 21.1 units at 270 mμ/mg. The yield was determined by treatment of an aliquot (containing 10.67 mg.) from a stock solution with 80% acetic acid and following the concentration of deoxyguanosine-5' phosphate at 252 mμ. Deoxyguanosine-5' phosphate thus formed was 17.5 optical density units/mg. of the above preparation. The yield of the above preparation thus was 2.67 mmoles, 252 mμ, from the total of 4.3 mmoles of starting material. The ultraviolet absorption characteristics were: λ_{max} 276 mμ (ϵ_{max} 16,600), λ_{min} 270 mμ (ϵ_{max} 16,000), shoulder 260 mμ, λ_{max} 234 mμ (ϵ_{max} 25,500), λ_{min} 225 mμ (ϵ_{max} 24,500). The ratio of the extinction at the two maxima 234 and 276 mμ was 1.54, this figure being the same as obtained for N-di-p-methoxytrityldeoxyguanosine.[24]

Treatment of a sample with 80% acetic acid at room temp. for 4 hr. caused the complete removal of the N-di-p-methoxytrityl group, the sole product being deoxyguanosine-5' phosphate.[25]

3'-O-Di-p-methoxytrityldeoxyguanosine-5' Phosphate.—A sample was prepared by the reaction of pyridinium N-acetyldeoxyguanosine-5' phosphate with 2 molar equivalents of di-p-methoxytrityl chloride for 2 days at room temp. followed by treatment with concd. ammonium hydroxide for 10 hr. at room temp. The product (R_f 0.49 in solvent A) was isolated by chromatography on Whatman 3 MM paper in solvent A; λ_{max} 235 mμ (ϵ 23,000), λ_{min} 223 mμ (ϵ 18,500), there being a shoulder at 270 mμ (ϵ 10,300).

N,3'-O-Bis-(di-p-methoxytrityl)-deoxyguanosine-5' Phosphate. —A mixture of dry pyridinium deoxyguanosine-5' phosphate (301 mg.) and di-p-methoxytrityl chloride (728 mg.) in 2 ml. of dry pyridine was kept at room temp. for 30 hr. Paper chromatography in solvent A at this stage showed complete conversion to the N,O$^{3'}$-bis-di-p-methoxytrityl compound. Methyl alcohol was added to stop the reaction and the solution evaporated *in vacuo* after the addition of concd. ammonia. After extraction of the solution of the product in concd. ammonia with ether, the solution was re-evaporated and the residue dissolved in solvent A (2 ml.). Chromatography on a cellulose column as described above for the N-di-p-methoxytrityl compound yielded the pure product in fractions 60–65. The compound was isolated as described above, the yield being 73% (9,450 optical density units at 270 mμ); λ_{max} 276, 262 and 234 mμ; ϵ_{276} mμ 19,000, ϵ_{234} mμ 46,000; λ_{min} 270, 259 and 224 mμ; ϵ_{270} mμ 18,400, ϵ_{224} mμ 43,500.

Attempted Polymerization of Thymidine-5' Phosphate in Dimethylformamide.—Pyridinium thymidine-5' phosphate (1 mmole) was dissolved in a mixture of anhydrous pyridine (0.5 ml.) and dimethylformamide (0.4 ml., freshly distilled *in vacuo* over phosphorous pentoxide). A solution of DCC (412 mg., 2 mmoles) in pyridine (0.1 ml.) was then added and the sealed clear solution which soon deposited crystals was kept at room temp. for 1 day. A further amount of DCC (206 mg.) was added and the mixture shaken for 4 days longer. The reaction mixture was then worked up and treated with sodium hydroxide as described in the previous papers.[4] The products were analyzed by chromatography on a DEAE–cellulose (carbonate form) column by the standard method. The main product was P^1,P^2-dithymidine-5' pyrophosphate and it was characterized by paper chromatography paper electrophoresis and by virtue of its hydrolysis in hot 0.1 N hydrochloric acid[4a] (2 hr.) to thymidine-5' phosphate. When in the above experiment N,N-dimethylacetamide was used in place of dimethylformamide, the result was similar.

Polymerization of Thymidine-5' Phosphate in Dimethylformamide in the Presence of Pyridinium Dowex-50 Ion Exchange Resin.—Pyridinium thymidine-5' phosphate (0.25 mmole) was rendered anhydrous by repeated evaporation of its solution in dry pyridine. The residue was dissolved in freshly distilled dimethylformamide (0.5 ml.) and the solution treated with DCC (200 mg.). Dry pyridinium Dowex-50 ion exchange resin (1 g.) was then added and the resulting mixture was shaken in the dark at room temp. Examination of an aliquot removed after 1 hr. by paper chromatography in solvent C showed the main product to be P^1,P^2-dithymidine-5' pyrophosphate. After 24 hr., paper chromatography in solvent C showed the presence of a number of

polymeric products[26] traveling slower than thymidine-5' phosphate. The over-all pattern resembled the polymerizations carried out in dry pyridine alone.

Polymerization of N-Protected Doxyguanosine-5' Phosphate and the Isolation of Deoxyguanosine Oligonucleotides. (a) By Using p-Toluenesulfonyl Chloride.—To pyridinium N-acetyldeoxyguanosine-5' phosphate (0.7 mmole) in a mixture of methyl alcohol and pyridine was added tri-n-dodecylamine (1.4 mmoles, 0.684 ml.) and the solution evaporated to a gum. Separately, the same salt of N,O$^{3'}$-diacetyldeoxyguanosine-5' phosphate was prepared from 0.3 mmole of the nucleotide and 0.6 mmole (0.36 ml.) of tri-n-dodecylamine. The two protected nucleotides were mixed and the mixture rendered anhydrous with dry pyridine. Finally, dry pyridine (1 ml.) was added followed by p-toluenesulfonyl chloride (0.3 g., about 1.6 mmoles). The mixture was shaken vigorously first by hand and then mechanically for 5 days in the dark. Water (2.5 ml.) was then added and then 5 ml. of 1 N sodium hydroxide. After 4 hr. at room temp., alkali was removed by passage of the total solution through a Dowex-50 (pyridinium) ion exchange resin column and the total effluent and washings were evaporated. The residue was kept in concd. ammonia (50 ml.) for 70 hr. at room temp. After removal of ammonia, the products were chromatographed on a DEAE-cellulose (bicarbonate form) column (30 cm. × 4 cm. dia.). The elution and isolation of the peaks was as described previously[4,6] except that the linear salt gradient consisted of 8 l. of water in the mixing vessel and an equal volume of 0.6 M triethylammonium bicarbonate (pH 7.5) in the reservoir. About twelve clearly separated peaks were thus obtained. Rechromatography of several of the major peaks was carried out at pH 5 using DEAE-cellulose in the chloride form.[5] The only compound that was isolated pure was the dinucleotide (d-pGpG) and it was present in peak 6.

In a repeat of the above experiment the polymeric mixture was treated with acetic anhydride in pyridine as described previously.[27] Subsequent chromatography showed only a limited extent of polymerization, the yield of oligonucleotide peaks falling off rapidly after the dinucleotide peak. In addition to the desired oligonucleotide peaks, there were present minor peaks of unidentified products (cf. the earlier results on polymerization using p-toluenesulfonyl chloride).[4c]

(b) Polymerization of N-Di-p-methoxytrityldeoxyguanosine-5' Phosphate Using DCC.—Ammonium N-di-p-methoxytrityldeoxyguanosine-5' phosphate (800 mg.) was converted to the pyridinium salt in the cold by using pyridinium Dowex-50 ion exchange resin and maintaining an excess of pyridine. The total solution was evaporated and the residue rendered anhydrous by repeated evaporation from pyridine (chromatography in solvent A showed that no loss of the N-di-p-methoxytrityl group occurred during this treatment). Dry pyridinium Dowex-50 ion exchange resin (50 mg.) was added and the dry pyridine solution finally evaporated to the point that about 1 ml. of the solvent remained (the net weight of the total reaction mixture was 1.8 g.); DCC (450 mg.) was added and the sealed reaction mixture shaken vigorously. Within the first half-hour the reaction mixture turned partly solid; this was shaken in the dark for 7 days. Aqueous pyridine (4.5 ml. of 90% pyridine) was added and the mixture shaken for 2.5 hr. when a completely clear solution resulted. The total solution was divided into two parts. A 1-ml. aliquot was treated with 0.2 ml. of tri-n-hexylamine, and after removal of the resin and the urea and extraction with petroleum ether the polymeric mixture was rendered anhydrous by repeated evaporation with pyridine (4 × 2 ml.). Finally the dried residue was kept in 2 ml. of pyridine and 0.4 ml. of acetic anhydride. After 3 days at room temp., the mixture was worked up in the standard way and analyzed by chromatography on a DEAE-cellulose column (25 cm. × 2.5 cm. dia.). After pooling of the appropriate fractions and spectrophotometric estimation, the composition of the polymeric mixture was found to be: mononucleotide, 55%; dinucleotide, 8.1%; higher oligonucleotides, about 12%, the side products being neglected.

The bulk of the reaction products obtained above (after treatment with aqueous pyridine) was rendered anhydrous with dry pyridine and dimethylformamide and was retreated in a concentrated solution (total weight of reaction mixture 1.43 g.) with an excess of DCC (added in portions, total amount 800 mg.). After work-up and analysis by column chromatography, the results were found to be not significantly different from those obtained above on the first aliquot.

Polymerization of N-Acetyldeoxyguanosine-5' Phosphate.—Pyridinium N-acetyldeoxyguanosine-5' phosphate (470 mg., 1

(24) H. Schaller, G. Weimann, B. Lerch and H. G. Khorana, *J. Am. Chem. Soc.*, in press.

(25) The glycosyl bond in the nucleoside deoxyguanosine is probably more labile than that in the corresponding 5' phosphate. Only a maximum period of 2 hr. at room temp. is safe for treatment of the nucleoside, N-di-p-methoxytrityldeoxyguanosine, with 80% acetic acid.

(26) Since this work was done, polymerization of thymidine-5' phosphate in dimethylformamide has been carried out in the presence of pyridinium Dowex-50 ion exchange resin with a view to large scale (8 mmoles) preparation of thymidine polynucleotides. The results were similar to those published previously.

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

mmole) was rendered anhydrous by repeated evaporation of dry pyridine from its suspension in the solvent. The residue was taken up in freshly distilled dimethylformamide (2 ml.) and dry pyridinium Dowex-50 ion exchange resin (1 g.) was added. The solvent was evaporated *in vacuo*, using an oil-pump. The resulting gummy mixture was treated with dry pyridine and the solvent re-evaporated *in vacuo*. The process of addition of pyridine and evaporation was repeated three times, dry air being introduced into the system after every evaporation. Dimethylformamide (1 ml.) was added and when all the nucleotide had gone into solution, DCC (520 mg., 2.5 mmoles) was added and the sealed reaction mixture shaken vigorously for about 15 min. It was then shaken in the dark for a total of 4 days. Aqueous pyridine (10 ml. of 50%) was added and the mixture kept for a further 2 hr. The solution was filtered from the insoluble resin and dicyclohexylurea, the latter being thoroughly washed with aqueous pyridine (50%) and water. After concentration *in vacuo*, the mixture was extracted with ether to remove excess of DCC. The clear solution was treated with more pyridine (over-all proportion of pyridine in the aqueous solution more than 50%) and evaporated. The residue of polymeric mixture was rendered anhydrous by repeated evaporation of pyridine. To the dry residue was added dry pyridine (25 ml.), acetic anhydride (5 ml.) and tri-*n*-hexylamine (0.68 ml., 2 mmoles). A practically clear solution resulted in 2–4 hr. and the sealed flask was kept in the dark for 3 days at room temp. Water (20 ml.) was then added under cooling and the clear yellow to brown solution kept for 1 hr. at room temp. It was then evaporated to a gum *in vacuo* at below 30° bath temp. On the addition of ether (20 ml.) a semisolid separated while the ether layer was cloudy. After cooling in an ice-bath the ether layer, which was now clear, was poured off. The residue was again washed with 10 ml. of ether and then made up to 10 ml. in 10% aqueous pyridine. Aliquots from this solution were used for column chromatography.

Isolation of N-Acetyldeoxyguanosine Oligonucleotides.—A 1-ml. aliquot (corresponding to 0.1 mmole of starting mononucleotide) was applied to the top of a DEAE–cellulose (carbonate form) column (25 cm. × 2.4 cm. dia.) in a 2° room. After a water wash (10 ml.), elution was carried out with a linear gradient of triethylammonium bicarbonate (pH 7.5) with the mixing vessel containing 4 l. of water and the reservoir 4 l. of 0.4 M triethylammonium bicarbonate. A flow rate of about 1–1.2 ml./min. was maintained, 12–14-ml. fractions being collected. The elution pattern is shown in Fig. 1. Soon after each peak appeared, the appropriate fractions were pooled, the ultraviolet absorption at 259 mμ was determined and a 2–5-fold excess (calculated on the basis of total triethylammonium bicarbonate expected to be present) of pyridinium Dowex-50 ion exchange resin[28] was added to replace all the triethylamine by pyridine. The pooled fractions containing the suspended resin were swirled in the cold for 10–15 min. and then poured into an empty glass column and the liquid passed slowly through the column. The resin was washed thoroughly with water and the combined effluent was evaporated under reduced pressure, maintaining an excess of pyridine during evaporation. The total concentrate from each peak was made up to 2 ml. with 10% pyridine and stored frozen. The percentage recoveries of nucleotide material in different peaks are in Table I.

General Methods.—Paper chromatography was performed by the descending technique using Whatman No. 1 or 40 paper. The solvents used were: solvent A, isopropyl alcohol–concd. ammonia–water (7:1:2); solvent B, *n*-propyl alcohol–concd. ammonia–water (55:10:35); solvent C, ethyl alcohol–1 M ammonium acetate (pH 7.5) (7:3, v./v.); solvent D, *n*-butyl alcohol–acetic acid–water (5:2:3, v./v.).

Paper electrophoresis was performed using either an apparatus similar to that of Markham and Smith[29] or a commercially available apparatus of similar design, but capable of giving 4000 volts

across an 18-inch length of paper strip. The buffers used were: phosphate 0.03 M, pH 7.1; 1 M acetic acid adjusted to pH 2.7 and 0.05 M ammonium formate, pH 3.5. The R_f's of different oligonucleotides are in Table II.

Enzymic degradations used bacterial alkaline phosphomonoesterase,[30] spleen phosphodiesterase,[14] the crude snake venom (*Crotalus adamanteus*), as well as a purified phosphodiesterase preparation from it.[17] The standard procedures used were as follows. (a) **Alkaline Phosphomonesterase.**—Solutions of the oligonucleotides containing 0.2–0.5 μmole of the nucleotide were lyophilized. The residue was taken up in 0.05 ml. of water + 0.01 ml. of 0.5 M trishydroxyethylaminomethane buffer (pH 8). The enzyme preparation (a gift from Mr. J. Schwartz of The Rockefeller Institute, concentration of protein about 2 mg./ml. 0.005 ml.) was added and the mixtures incubated from 2–4 hr. at 37°. Complete removal of the terminal phosphomonoester group occurred under these conditions, no diester bond cleavage being detected.

(b) **Spleen Phosphodiesterase.**—The preparation described previously[14b] was used. It was standardized by its rate of degradation of thymidylyl-(3'→5')-thymidine. Appropriate solutions (0.3–1 μmole of the mononucleotide) of the oligonucleotides were lyophilized and the residues dissolved in 0.05 ml. of water + 0.01 ml. of 1 M ammonium acetate (pH 5.9) buffer. Ethylenediamine tetraacetate (0.01 ml. of 0.1 M) and the enzyme preparation (0.04 ml.) were added. Incubation at 37° for 4 hr. caused complete cleavage to the mononucleotide and the terminal nucleoside. Such a degradation was performed on the homologous series of 3'-O-acetyl-(N-acetyl)-deoxyguanosineoligonucleotides (general structure III) which had been prepared by incubation of the peaks 8, 11, 14 and 15 (Fig. 1; di- to pentanucleotide, respectively) with phosphomonoesterase, subsequent chromatography in solvent C, elution, passage of the eluates through columns of pyridinium Dowex-50 ion exchange resin and lyophilization to remove pyridinium acetate. The total digestion mixture from the spleen phosphodiesterase was then applied to Whatman No. 40 paper, with adequate space for blanks. Chromatography was performed either in solvent A or B for about 18 hr. The nucleotide and nucleoside (mostly N,O$^{3'}$-diacetylguanosine but also a small amount of N-acetylguanosine) spots were determined spectrophotometrically after soaking in water for at least 24 hr. The results are in Table III.

(c) **Crude Venom.**—The incubation was in a total volume of 0.1 ml. and the mixture contained the oligonucleotide (0.2–0.5 μmole of the parent nucleotide), 0.01 ml. of 2 M ammonium carbonate, 0.01 ml. of a lyophilized crude venom (*Crotalus adamanteus*) solution (20 mg./ml. in 0.05 M TRIS pH 8 buffer). Incubation was for 2–4 hr. at 37°.

(d) **Venom Phosphodiesterase.**—Solutions of the oligonucleotides containing about 5–10 optical density units (260 mμ) were lyophilized. The residue was taken up in 0.05 ml. of water and 0.01 ml. of 2 M ammonium carbonate. Venom diesterase preparation (0.04 ml.) was added and the mixture incubated at 37° for about 6 hr. Under identical conditions thymidine tetranucleotide (pTpTpTpT) was completely degraded to thymidine-5' phosphate (chromatography in solvent B). Similarly, thymidylyl-(3'→5')-thymidine (10 optical density units at 267 mμ) was completely degraded to thymidine-5' phosphate and thymidine in about 1 hr.

With the deoxyguanosine oligonucleotides the results were: deoxyguanylyl-(3'→5')-deoxyguanosine[10b] prepared in a stepwise synthesis procedure or by dephosphorylation of the dinucleotide (d-pGpG) was completely degraded in 9 hr. In contrast, the di-, tri and tetranucleotides (d-pGpG, d-pGpGpG and d-pGpGpGpG), prepared by separation of the N-deacetylated polymeric mixture on a DEAE–cellulose column, were attacked only to a very small extent (5–20%), the new product being deoxyguanosine-5'-phosphate, as judged by paper chromatography in solvent B.

In one experiment, the tetranucleotide (d-pGpGpGpG) was rendered alkaline with 1 N sodium hydroxide, the solution neutralized with 1 N hydrochloric acid and then incubated with the phosphodiesterase under standard conditions. The result was similar to those obtained above.

(28) It is important to wash the resin thoroughly (for 12–24 hr.) in a column with aqueous pyridine (5%) and then water, just before use. Otherwise there is the danger that low molecular weight polymers released from the resin, which on paper chromatograms fluoresce under ultraviolet light, may contaminate the nucleotidic products.

(29) R. Markham and J. D. Smith, *Biochem. J.*, **52**, 552 (1952).

(30) A. Garen and C. Levinthal, *Biochem. Biophys. Acta*, **38**, 470 (1960).