bubbled through a tube containing a solution of 3.2 g. (0.02 mole) of bromine in 40 ml. of methylene chloride, followed by sweeping the gas remaining in the reaction flask through the same solution with a nitrogen stream. The bromine solution was washed with 10% aqueous sodium thiosulfate to remove the excess bromine, then with saturated salt solution, dried over anhydrous potassium carbonate, and fractionally distilled to yield 2.0 g. (53.4% based on the sulfonium salt) of ethylene dibromide, b.p. 130–132° (lit. 131.6°). The infrared spectrum was identical with that of an authentic sample.

Attempted Reactions of Dimethylsulfonium Methylide. Attempts were made to effect reaction of the sulfonium ylide with desoxybenzoin, *trans*-stilbene, and diphenylacetylene using the general procedure. In each case starting material was recovered almost quantitatively.

Synthesis of N-Benzoyl-2'-O-tetrahydropyranylguanosine-5'-phosphate, an Intermediate in the Chemical Synthesis of Polyriboguanylic Acid[1]

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The synthesis of N-benzoyl-2'-O-tetrahydropyranylguanosine-5'-phosphate, a suitable monomer for chemical synthesis of 5'-phosphomonoester-terminated polyriboguanylic acid, has been achieved. Starting with guanosine-5'-phosphate, N-benzoylguanosine-3',5'-cyclic phosphate was synthesized in two steps by known reactions. Acid-catalyzed reaction of this cyclic phosphate with dihydropyran gave, quantitatively, N-benzoyl-2'-O-tetrahydropyranylguanosine-3',5'-cyclic phosphate which was then specifically hydrolyzed with a partially purified enzyme to give, in high yield, N-benzoyl-2'-O-tetrahydropyranylguanosine-5'-phosphate. Yields of the pure final product, based on starting guanosine-5'-phosphate, were near 50%. Synthesis of 2'-O-tetrahydropyranylguanosine-5'-phosphate by a similar route has also been accomplished. Procedures have been devised for removal of the benzoyl and tetrahydropyranyl protecting groups from these substituted guanosine nucleotides under milder conditions than anticipated. Details of these synthetic reactions and of ancillary studies of protecting group stabilities and the properties of the phosphodiesterase employed are presented.

The chemical polymerization of ribonucleotides has been under investigation for a number of years in several laboratories.[3] The mixed anhydride approach of Michelson[4] and the ethyl polyphosphate method of Schramm, *et al.*[5] bypass the problem of protecting groups, but, consequently, preclude exclusive formation of $C_{3'} \rightarrow C_{5'}$[6] phosphodiester linkages in the polymeric product. On the other hand, Khorana and co-workers, in particular, and others as well, have studied extensively the preparation of specifically blocked ribonucleotides which might be polymerized by the carbodiimide method in order to obtain $C_{3'} \rightarrow C_{5'}$ phosphodiester linkages exclusively.[7] Reaction conditions and many other problems of carbodiimide polymerizations have been worked out for deoxynucleotides, in which case sugar protecting groups are not required.[3b] However, a comparable range of polyribonucleotides has not been synthesized, primarily because of the difficult accessibility of the appropriately blocked intermediates.

A bifunctional intermediate containing both a phosphomonoester group and a hydroxyl group is required for such polymerization. For exclusive $C_{3'} \rightarrow C_{5'}$ phosphodiester linkages in the polymer, the phosphate may be esterified either at the 3'- or the 5'-hydroxyl of a ribonucleoside. The 2'-hydroxyl must be blocked in order to prevent formation of a nucleoside-2',3'-cyclic phosphate when the 3'-hydroxyl is esterified,[8] and mixed $C_{2'} \rightarrow C_{5'}$ and $C_{3'} \rightarrow C_{5'}$ phosphodiester linkages when the 5'-hydroxyl is esterified.[8] Polymers of the type $(Up)_n$[7a] and $(Ap)_n$[9] have been synthesized utilizing

(1) This work was supported by grants from the National Institutes of Health.

(2) Established Investigator of the American Heart Association during part of this investigation.

(3) For extensive reviews of this field, see (a) A. M. Michelson, "The Chemistry of Nucleosides and Nucleotides," Adademic Press Inc. (Ltd.), London, 1963, pp. 400–443; (b) 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, pp. 93–125.

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

(5) G. Schramm, H. Grotsch, and W. Pollman, *Angew. Chem.*, 74, 53 (1962).

(6) The abbreviations and nomenclature used to describe internucleotide linkage are those proposed by Khorana (see ref. 3b, pp. 93–95). In naming derivatives of guanosine, the abbreviation Guo is used (see IUPAC, Tentative Rules, Abbreviations and Symbols for Chemical Names of Special Interest in Biological Chemistry, *J. Biol. Chem.*, 237, 1381 (1962), paragraph 5.4). Phosphoesters are referred to by the symbol P, and cyclic phosphate as CP. Other abbreviations include THP for tetrahydropyranyl substituents; DCC for N,N'-dicyclohexylcarbodiimide; DMSO for dimethyl sulfoxide; DEAE for diethylaminoethyl; and Tris for trishydroxymethylaminomethane.

(7) Examples include: (a) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *J. Am. Chem. Soc.*, 85, 1989 (1963); (b) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *ibid.*, 84, 430 (1962); (c) D. H. Rammler and H. G. Khorana, *ibid.*, 84, 3112 (1962); (d) J. Smrt and F. Sorm, *Collection Czech. Chem. Commun.*, 27, 73 (1962); (e) F. Cramer and K. H. Scheit, *Angew. Chem.*, 74, 717 (1962).

(8) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, 80, 6204 (1958).

(9) Y. Lapidot and H. G. Khorana, *ibid.*, 85, 3857 (1963).

the bifunctional 2'-O-acetylnucleoside-3'-phosphates, and the method should yield other polynucleotides terminated with 3'-phosphomonoester groups. However, it was desired in the present work to obtain the polyguanylic acids whose over-all structure most nearly resembles naturally occurring polynucleotides. Therefore, the alternative approach based on protected guanosine-5'-phosphate (Guo-5'-P)[6] was undertaken.

General Considerations. While blocking of the 2'-hydroxyl of Guo-5'-P is an obvious necessity, the value of blocking the guanosine amino function is less clear. Guanosine is the least reactive of the amino nucleosides for phosphoramidate formation[10] using 2-cyanoethyl phosphate and N,N'-dicyclohexylcarbodiimide (DCC),[6] so this side reaction might not be serious. However, it was found, confirming the work of Smith, *et al.*,[11] that guanosine nucleotides are virtually insoluble in many organic solvents. Thus, yields of protected intermediates are low unless the Guo-5'-P is initially acylated.

Acyl, tri-*p*-methoxytrityl, and tetrahydropyranyl (THP)[6] protecting groups have been described for blocking the 2'-hydroxyl of ribonucleotides.[7] Acyl groups cannot, however, be used for specific blocking of the 2'-hydroxyl in 5'-nucleotides since these groups migrate readily between the 2'- and 3'-hydroxyls of ribose derivatives[12] in the presence of either acid or base. Use of the acid-labile tri-*p*-methoxytrityl protecting group might be feasible, but such a large group would be expected to hinder the polymerization of the bifunctional nucleotide.[13] The THP group has been employed successfully to protect hydroxyl groups of nucleotides that have served as intermediates in dinucleotide synthesis. Tetrahydropyranyl ethers are not known to migrate in 1,2-diol systems, and they are easily hydrolyzed under mild acidic conditions. Thus, dihydropyran appears to be the agent of choice for blocking the 2'-hydroxyl of 5'-nucleotide monomers in polymerization reactions.

The THP group must be selectively placed at the 2'-position. That this could be accomplished by selective removal of a THP group from the 3'-position in 2',3'-di-O-THP-Guo-5'-P seems unlikely because of the close chemical reactivity of the two comparable secondary hydroxyl functions involved. An alternative approach is to first block the 3'-hydroxyl with a group that can be removed under conditions where the subsequently formed 2'-O-THP derivative is stable. Smith and Khorana[14] used such a reaction sequence in the synthesis of 2'-O-THP-uridine-3'- and -5'-phosphates using the reaction sequence I → III → V + VI (Chart I, R = R' = uracil, R'' = tetrahydropyranyl). In view of the fact that the 5'-nucleotide was desired in the present study, another means of nucleoside-3',5'-CP hydrolysis than the NaOH[14] or Ba(OH)$_2$[11] procedures, which yield only about 15% of the 5'-nucleotide, was required. Detailed study of the alkaline hydrolysis of N-benzoyl-2'-O-tetrahydropyranylguanosine-3',5'-cyclic phosphate (N-benzoyl-2'-O-THP-Guo-3',5'-CP)

and 2'-O-tetrahydropyranylguanosine-3',5'-cyclic phosphate (2'-O-THP-Guo-3',5'-CP) showed, in addition to the low yield of the 5'-nucleotide relative to Guo-3'-P, that the 2'-O-THP group is simultaneously removed thereby making this approach inapplicable to guanosine derivatives. Instead, a specific enzymic hydrolysis of the protected 3'.5'-cyclic phosphate was developed that gives good yields of the protected 5'-nucleotide and is applicable on a preparative scale.

Thus, the principal sequence of reactions used in this work includes: (1) initial benzoylation of Guo-5'-P; (2) reaction of this protected nucleotide with DCC in very dilute anhydrous pyridine solution giving N-benzoyl-Guo-3',5'-CP; (3) acid-catalyzed reaction of the cyclic phosphate with dihydropyran yielding N-benzoyl-2'-O-THP-Guo-3',5'-CP; (4) enzymic hydrolysis of the latter to the desired bifunctional 5'-nucleotide, N-benzoyl-2'-O-THP-Guo-5'-P (Chart I, I → II → III → IV → VII, R = guanine, R' = N-benzoylguanine). In addition, the complete reaction sequence I → III → IV → VII, R = R' = guanine, has also been carried out.

Guanosine-3',5'-cyclic Phosphate. Attempts to synthesize Guo-3',5'-CP directly from pyridinium or triethylammonium Guo-5'-P by reaction with DCC in very dilute pyridine solution[11] were largely failures. After fractionation of the reaction mixture by preparative paper chromatography, isolated yields of 5–10% were obtained. The poor yields were due to the very low solubility of Guo-5'-P in pyridine; recovery of total nucleotide from reaction mixtures was never more than 15% of the starting material, which remained in the form of a gum that did not dissolve in pyridine. Somewhat greater success was obtained by dispersing the pyridinium Guo-5'-P[15] in anhydrous pyridine using 200 μ glass beads[16] and adding the suspension, dropwise, to a refluxing solution of DCC in anhydrous pyridine. Further mechanical modification of this reaction would be expected to increase the yield above the 25% obtained, but such modifications were not pursued due to the simpler cyclization reaction of N-benzoyl-Guo-5'-P described by Smith, *et al.*[11]

N-Benzoylguanosine-3',5'-cyclic Phosphate. This substance was synthesized by the method of Smith, *et al.*[11] These workers did not isolate the N-benzoyl-Guo-3',5'-CP, but first removed the benzoyl group. Since the N-benzoyl derivative was desired in this work, the hydrolysis step was omitted. Unreacted N-benzoyl-Guo-5'-P was separated from the corresponding 3',5'-CP on DEAE cellulose (HCO$_3^-$). The product and starting material had identical ultraviolet absorption spectra, indicating no change in the N-benzoylguanine chromophore. On paper chromatography in three solvent systems the product appeared homogeneous and migrated faster than the starting material, as would be expected of a substance with a lower charge. Similarly, on paper electrophoresis at pH 8.5, the product had a lower mobility than the starting material. After alkaline hydrolysis the product spectrum changed to that of guanosine, R_f became identical with that of Guo-3',5'-CP, and mobility on paper electrophoresis was only slightly changed. From these

(10) G. M. Tener, *J. Am. Chem. Soc.*, **83**, 159 (1961).
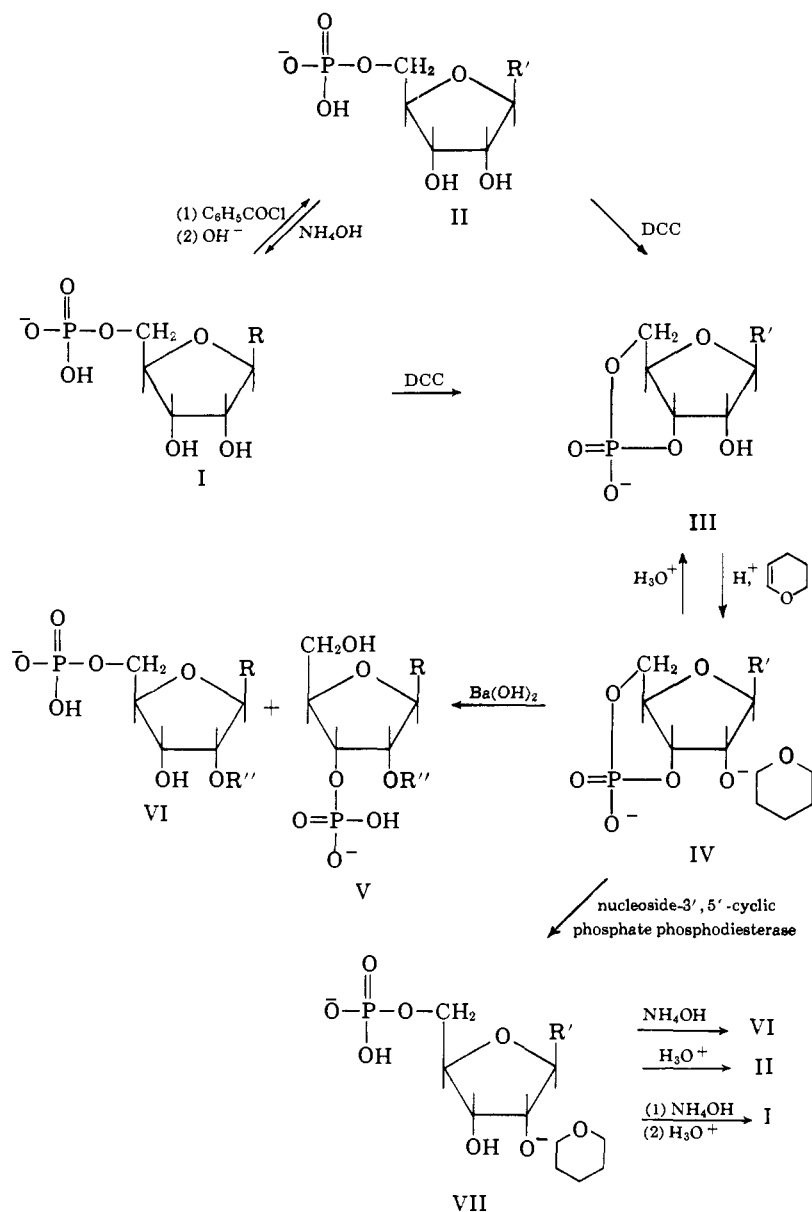(11) M. Smith, G. I. Drummond, and H. G. Khorana, *ibid.*, **83**, 698 (1961).
(12) R. K. Ness and H. G. Fletcher, *ibid.*, **78**, 4710 (1956).
(13) G. Weimann and H. G. Khorana, *ibid.*, **84**, 4329 (1962).
(14) M. Smith and H. G. Khorana, *ibid.*, **81**, 2911 (1959).

Chart I

$$O{-}P{-}O{-}CH_2 \quad O \quad R'$$

II

(1) C₆H₅COCl
(2) OH⁻ / NH₄OH

DCC

$$O{-}P{-}O{-}CH_2 \quad O \quad R$$
OH

OH OH

I

DCC →

III

O=P—O

H₃O⁺ | H⁺

$$O{-}P{-}O{-}CH_2 \quad O \quad R$$
OH

CH₂OH O R

+

Ba(OH)₂ ←

IV

VI

O=P—OH

V

nucleoside-3',5'-cyclic
phosphate phosphodiesterase

$$O{-}P{-}O{-}CH_2 \quad O \quad R'$$
OH

NH₄OH → VI
H₃O⁺ → II
(1) NH₄OH → I
(2) H₃O⁺

OH O⁻

VII

facts the isolated product was deduced to be pure and assigned the structure N-benzoyl-Guo-3',5'-CP.

No precise structural proof for N-benzoyl-Guo-5'-P was presented by Smith, et al.,[11] nor attempted in this work. No change in the chromophore occurs during the cyclization reaction, so conclusions concerning the location of the benzoyl group in either II or III (R' = N-benzoylguanine) apply to both substances. The fact that both II and III migrate as anions on paper electrophoresis at pH 8.5 and 3.5 eliminates N^7 as the point of substitution even though this nitrogen appears to be the strongest base in N-9-substituted guanines,[17] reacting most readily with dimethyl sulfate and nitrogen mustard alkylating agents.[18] The ultraviolet spectral changes occurring on acidification of N-benzoyl-Guo-3',5'-CP (see Figure 1) are consistent with the lack of substitution at N-7 since the molecule can be protonated, probably at N-7, as suggested by Miles, et al.[17] A large spectral change is also found on titration of the nucleotide to pH 12, indicating that the keto–enol tautomerism associated with the $-HN^1{-}C^6{=}O$ is possible and, therefore, the N-1 is probably not the point of benzoyl substitution. However, similar considerations might be used to explain both acid and base spectral changes assuming benzoyl substitution on either N-2 or N-3 (VIII and IX, respectively). Of course, many resonance structures could be drawn for each of these and for their tautomers. The present evidence is insufficient to determine the point of benzoyl substitution, but it should be pointed out that only with the N-2 benzoylation could resonance stabilization of the amide linkage in basic solution be invoked as in the tautomer VIIIc (cf. ref. 19).

Synthesis of N-benzoyl-Guo-3',5'-CP was carried out several times in the 50–500 μmole range, with isolated yields of 50–70%. When the reaction was scaled up to 10 mmoles, using a proportionate increase in all reagents including the solvent, the isolated yield

(17) H. T. Miles, F. B. Howard, and J. Frazier, Science, 142, 1458 (1963).
(18) P. Brookes and P. D. Lawley, J. Chem. Soc., 3923 (1961).

(19) E. A. Peterson and H. A. Sober, J. Am. Chem. Soc., 78, 751 (1956); (b) R. K. Ralph, W. J. Conners, H. Schaller, and H. G. Khorana, ibid., 85, 1983 (1963).

Chart II

VIIIa   VIII

VIIIc   VIIIb

IXa   IX

IXb
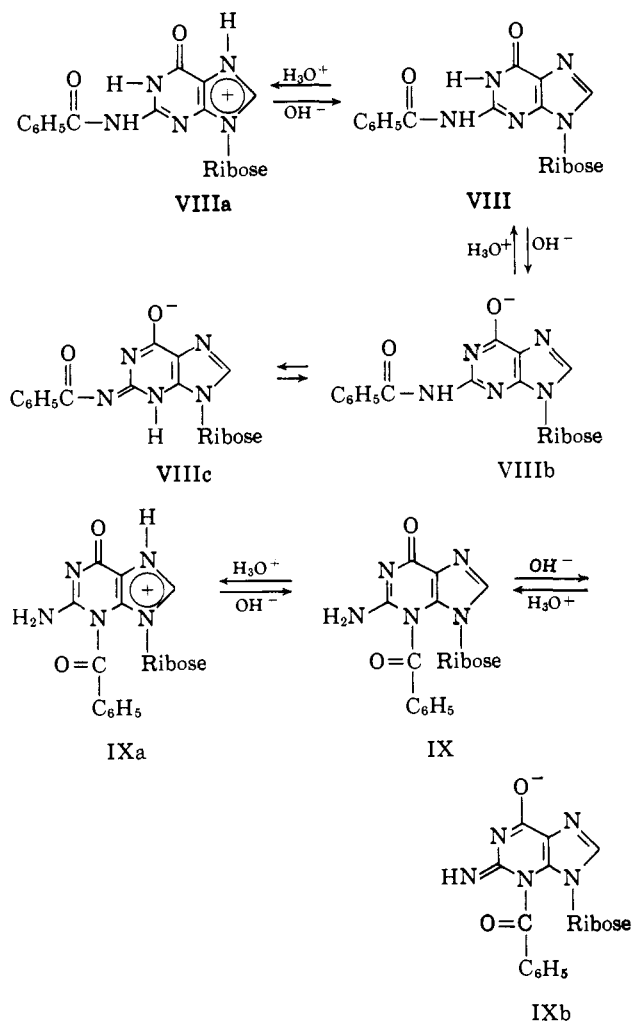
was only 43%. The amount of N-benzoyl-Guo-5'-P recovered, about 25%, was comparable with the smaller syntheses. Additional N-benzoylguanosine nucleotide material was eluted in a number of fractions from DEAE (HCO_3^-) after the starting material, and showed low R_f values on paper chromatography in solvent C which is consistent with their being poly(N-benzoyl)guanylic acids (presumably containing mixed $C_{2'} \rightarrow C_{5'}$ and $C_{3'} \rightarrow C_{5'}$ phosphodiester linkages). The reaction time was not proportionately increased in the 10 mmole synthesis and probably the concentration of N-benzoyl-Guo-5'-P, added dropwise, became too high for exclusive intramolecular cyclization so some polymer was also formed.[20]

2'-O-Tetrahydropyranylguanosine-3',5'-cyclic Phosphate. Pure Guo-3',5'-CP was treated with a large excess of dihydropyran in dimethyl sulfoxide (DMSO[6]) in the presence of a slight excess of anhydrous hydrogen

(20) Inter- or intramolecular phosphodiester bond formation may be expected at elevated temperatures despite the presence of the strong base, 4-morpholine-N,N'-dicyclohexylcarboxamidine, if monomeric metaphosphate[21] or trimetaphosphate[13] esters of N-benzoylguanosine are the *ultimate* phosphorylating agents under these conditions rather than the 1,2-adduct with DCC.[8] The latter would not be expected to react with weakly nucleophilic hydroxyl groups in the presence of dianions unless such a reaction were sterically favored as in the synthesis of nucleoside-2',3'-cyclic phosphates. The elevated temperature is presumably necessary to overcome the kinetic barrier to metaphosphate production which is reflected in the quantitative formation of symmetrical pyrophosphates at lower temperatures.[8]

(21) A. R. Todd, *Proc. Natl. Acad. Sci. U. S.*, **45**, 1389 (1959).

chloride to synthesize 2'-O-THP-Guo-3',5'-CP. Chromatography of the product mixture indicated incomplete reaction when a twofold excess of acid over nucleotide was used, but all starting material was converted to the desired product when a fourfold excess of hydrogen chloride was used. The work-up, involving neutralization and passage of the reaction mixture through lithium Dowex-50, converts all the hydrogen chloride to LiCl, but contamination of the protected nucleotide with this salt does not affect the next step in the sequence (Chart I, IV → VII). Thus, the enzymic hydrolysis rates were the same for the nucleotide when contaminated by LiCl or freed of it by paper or ion-exchange chromatography.

N-Benzoyl-2'-O-tetrahydropyranylguanosine-3',5'-cyclic Phosphate. This substance was synthesized just as the 2'-O-THP-Guo-3',5'-CP by acid-catalyzed reaction of N-benzoyl-Guo-3',5'-CP with dihydropyran. Yields were usually quantitative, though use of a large excess of NH_4OH in neutralizing the acid catalyst, particularly when the DMSO was allowed to absorb atmospheric moisture, led to some hydrolysis of the benzoyl group. On the other hand, precise neutralization of the hydrogen chloride gave sufficient acidity, presumably through hydrolysis of NH_4Cl, to hydrolyze the THP group. Such hydrolysis was particularly marked when larger reaction mixtures were exposed to room temperature for long periods during removal of DMSO *in vacuo*. Both groups were stable, however, if an excess of NH_4OH was used, followed immediately by quick freezing and lyophilization, to prevent wetting of the mixture by water vapor. The LiCl, which contaminates the nucleotide after the product mixture is passed through lithium Dowex-50 and dried, could be removed, with a decrease in recovered yield from 100% to 80%, by washing the dry residue with small volumes of water, since the lithium salt of the nucleotide is not soluble in water above a concentration of 0.003 M.[22] Another method suitable for processing both large- and small-scale syntheses of N-benzoyl-2'-O-THP-Guo-3',5'-CP involves ion-exchange fractionation of the aqueous DMSO nucleotide solution containing NH_4Cl, on DEAE cellulose (Cl^-). Though cumbersome, this ion-exchange chromatographic step is the most satisfactory method investigated for preparing the protected nucleotide in pure form free of salt and DMSO. However, except for purposes of characterization, it is not necessary to remove these impurities.

Alkaline Hydrolysis of Protected Guanosine-3',5'-cyclic Phosphates. The finding that the THP group is quantitatively hydrolyzed simultaneously with cyclic phosphate hydrolysis when 2'-O-THP-Guo-3',5'-CP, or the corresponding N-benzoyl derivative, is heated with 0.4 N $Ba(OH)_2$[11] prompted a further search for conditions where the THP group would be stable while the cyclic phosphate is completely hydrolyzed. This search had three goals: (1) to find the mildest conditions where the cyclic phosphate would be hydrolyzed; (2) to determine the conditions where the THP group first becomes unstable; and (3) to investigate the

(22) Unpublished experiments. It should be kept in mind that aqueous solutions of nucleoside-3',5'-*cyclic* phosphates, in contrast to the uncyclized nucleotides, are unbuffered, assuming the pH of the water, generally 4-5. This is sufficiently acid to hydrolyze the THP derivatives at an appreciable rate. Hence, such solutions must be handled rapidly at low temperature.

effect of variation of the cation on THP and cyclic phosphate stabilities.

The rate of hydrolysis of Guo-3',5'-CP at 100° at various concentrations of Ba(OH)₂ from pH 8 to 12 was studied by following the release of the Guo-3'- and -5'-phosphates on paper chromatograms. No hydrolysis was detectable, below pH 12, up to 48 hr. at 100°. At pH 12.2, Guo-3'-P was just detectable (about 2% of total nucleotide) after 2.5 hr. at 100° and its quantity increased very slowly to about 20% at 48 hr. Under the same conditions, with 2'-O-THP-Guo-3',5'-CP, about 15% of the protected nucleotide was converted to Guo-3',5'-CP in 24 hr., at which point Guo-3'-P appeared at about 4% of the total nucleotide. Other bases were studied briefly and it was found that NaOH, LiOH, and NH₄OH at pH above about 12.5 hydrolyze the 2'-O-THP group from guanosine nucleotides at elevated temperature, but these bases, under the same conditions, do not hydrolyze the cyclic phosphate group. Thus, the desired conditions for chemical hydrolysis of the cyclic phosphate where the 2'-O-THP group is stable were not found.

Hydrolysis of the THP group of guanosine nucleotides under alkaline conditions was surprising in view of the reported stability at high pH of these substituents on other nucleotides.[7b,23] However, alkaline hydrolysis of THP ethers is not without precedent since Amir, et al.,[24] have reported that hydrolysis of O-THP-N-benzylsulfonylserine is even more rapid, in 0.01 N NaOH, than that of 2'-O-THP-guanosine derivatives. The reasons for the differing stability of 2'-O-THP ethers of nucleosides are not clear, though the differing reactivities of the respective guanosine and uridine ethers suggest a neighboring group effect possibly involving the N-2 amino function of the guanine. In any case, this work indicates that generalizations concerning the stability of 2'-O-THP-nucleoside derivatives in strong base are not warranted and that the stability of specific derivatives deserves careful scrutiny before they are used for other synthetic reactions.

Enzymic Hydrolysis of Guanosine-3',5'-cyclic Phosphate Derivatives. Enzymes which catalyze the hydrolysis of adenosine-3',5'-cyclic phosphate have been partially purified from several sources and shown to produce adenosine-5'-phosphate exclusively.[25-27] In addition to adenosine-3',5'-CP, Drummond and Perrott-Yee[26] showed that the nucleoside-3',5'-CP phosphodiesterase from rabbit brain also hydrolyzes guanosine- and uridine-3',5'-CP, though cytidine-3',5'-CP is not attacked. The rabbit brain enzyme was isolated by a modification of the procedure of Drummond and Perrott-Yee.[26] Tests for activity toward N-benzoyl-Guo-3',5'-CP, 2'-O-THP-Guo-3',5'-CP, and N-benzoyl-2'-O-THP-Guo-3',5'-CP, in addition to Guo-3',5'-CP and adenosine-3',5'-CP, were all positive on the basis of orthophosphate produced when the rabbit brain nucleoside-3',5'-CP phosphodiesterase was cou-

pled with *E. coli* alkaline phosphatase.[28] Isolation of Guo-5'-P as the exclusive nucleotide after removal of protecting groups of enzymic hydrolysates (by chromatography in a solvent[29] separating 3'- and 5'-nucleotides) demonstrated that the specificity of the hydrolysis by this enzyme is not altered by the different protecting groups. Clearly, the specificity of the enzymic hydrolysis is of great advantage in this synthetic work since yields of the desired 5'-nucleotide are markedly increased and problems of separating the desired product from the 3'-nucleotide produced by Ba(OH)₂ hydrolysis are eliminated.

In control experiments without *E. coli* alkaline phosphatase, a slow release of orthophosphate was noted. This is due to a contaminating enzyme tentatively identified as a 5'-nucleotidase. Thus, incubation of the rabbit brain enzyme with adenosine- or Guo-5'-phosphates resulted in production of equimolar amounts of orthophosphate and the corresponding nucleoside, but no reaction occurred when adenosine- or Guo-3'-(or -2'-) phosphates were used as substrate. The specific activities of these two rabbit brain enzymes toward several substrates are given in Table I.

Table I. Nucleoside-3',5'-cyclic Phosphate Phosphodiesterase and 5'-Nucleotidase Activities from Rabbit Brain[a]

| Substrate | Specific Activity[b] | |
	Phospho-diesterase	5'-Nucleo-tidase
Adenosine-3',5'-CP	6.53	1.67
Guanosine-3',5'-CP	0.97	0.75
N-Benzoyl-Guo-3',5'-CP	1.06	0.60
2'-O-THP-Guo-3',5'-CP	0.11	<0.04
N-Benzoyl-2'-O-THP-Guo-3',5'-CP	0.17	<0.04

[a] Assay mixtures contained 0.1 μmole of MgCl₂, 5 μmoles of Tris-HCl, pH 7.80, 0.1 μmole of substrate, and enzymes in a total volume of 0.100 ml. For phosphodiesterase assays, 10 μg. of *E. coli* alkaline phosphatase was added, in addition to the rabbit brain cyclic phosphate phosphodiesterase, at zero time. Alkaline phosphatase was omitted from 5'-nucleotidase assays which were otherwise identical; incubation at 38°. Reaction was stopped by cooling in ice and addition of 0.025 ml of 1.25 N HClO₄. Orthophosphate was then determined on aliquots of the supernatants using a modification of the method of O. H. Lowry and J. A. Lopez, *J. Biol. Chem.*, 162, 421 (1946). [b] Specific activity = μmoles of PO₄⁻³ produced/mg. of protein/hr.

Some additional properties of the rabbit brain nucleoside-3',5'-cyclic phosphate phosphodiesterase have been determined. The assay employed, which involves coupling the phosphodiesterase with *E. coli* alkaline phosphatase, made it possible to extend the pH range over which the properties of the phosphodiesterase could be examined, and also takes advantage of the lack of specificity of the indicator enzyme. Neither the pH optimum of the phosphodiesterase, 7.8, nor the specific activity is significantly affected by the buffer; Tris hydrochloride, NH₄HCO₃, and triethylammonium bicarbonate gave the same results. The pH optimum of the 5'-nucleotidase is 7.5. The optimal Mg²⁺ concentration, 0.8 mM, reported by Drummond and Perrott-Yee[26] was confirmed where the substrate concentration is less than this, but Mg²⁺

(23) B. Iselin and R. Schwyzer, *Helv. Chim. Acta*, 39, 57 (1956).
(24) S. M. Amir, M. Z. Atassi, S. A. Barker, J. S. Brimacombe, and M. C. Keith, *Nature*, 192, 1069 (1961).
(25) (a) E. W. Sutherland and T. W. Rall, *J. Biol. Chem.*, 232, 1077 (1958); (b) R. W. Butcher and E. W. Sutherland, *ibid.*, 237, 1244 (1962).
(26) G. I. Drummond and S. Perrott-Yee, *ibid.*, 236, 1126 (1961). We are grateful to Dr. Drummond for information on this work, prior to publication.
(27) K. G. Nair, *Federation Proc.*, 21, 84 (1962).

(28) A. Garen and C. Levinthal, *Biochim. Biophys. Acta*, 38, 470 (1960).
(29) D. B. Straus and E. Goldwasser, *J. Biol. Chem.*, 236, 849 (1961).

should be equal to substrate when the latter is at higher concentration. Imidazole, reported to stimulate nucleoside-3',5'-cyclic phosphate phosphodiesterase isolated from beef[25b] and dog heart,[27] slightly inhibited the rabbit brain enzyme.

Attempts to purify the rabbit brain phosphodiesterase further and particularly to remove the 5'-nucleotidase activity did not meet with success.[30] Acetone fractionation, ethanol fractionation, reprecipitation with ammonium sulfate at different pH, and shaking with $CHCl_3$ all resulted in large losses in phosphodiesterase activity with little or no increase in specific activity or in the ratio of phosphodiesterase to 5'-nucleotidase activities. In fact, the two activities were completely separated by shaking with $CHCl_3$, which denatures the phosphodiesterase but has no effect on the nucleotidase. The phosphodiesterase was also rapidly and irreversibly denatured, even at low temperatures, below pH 6. Because of this acid lability, the published purification procedure[26] was modified to include buffered media (pH 7.5) at all times, giving increased recovery of phosphodiesterase activity as well as higher specific activities.

Failure to free the phosphodiesterase of 5'-nucleotidase prompted investigation of possible inhibitors of the latter enzyme. Orthophosphate, which inhibits several phosphomonoesterases, was without effect. However, no 5'-nucleotidase activity could be detected when excess adenosine or guanosine was added to the assay system; uridine had no such effect. Under the standard assay (Table I), a tenfold excess of adenosine relative to protected Guo-3',5'-CP completely inhibits the 5'-nucleotidase, up to 75% hydrolysis of the substrate. The rate of further hydrolysis of cyclic phosphate then becomes very slow, precluding a quantitative yield of the protected Guo-5'-P when the phosphodiesterase is used for large-scale hydrolysis of N-benzoyl-2'-O-THP-Guo-3',5'-CP; but the unreacted cyclic phosphate can be isolated and recycled. In all large-scale hydrolyses, about 5–10% of the total nucleotide has been recovered without the benzoyl substituent even though great care was taken during work-up to maintain both protecting groups. This matter has not been thoroughly investigated, but it seems possible that the crude rabbit brain enzyme contains an amidase which hydrolyzes N-benzoylguanosine nucleotides to the appropriate guanosine nucleotides.

Because of the incomplete hydrolysis of the cyclic phosphate and the removal of benzoyl groups, four nucleotides occurred in the enzymic digests of N-benzoyl-2'-O-THP-Guo-3',5'-CP: starting material,

2'-O-THP-Guo-3',5'-CP, N-benzoyl-2'-O-THP-Guo-5'-P, and 2'-O-THP-Guo-5'-P. This mixture can be fractionated on DEAE cellulose (HCO_3^-) using a discontinuous triethylammonium bicarbonate gradient.

The desired end product of the four-step synthesis, N-benzoyl-2'-O-THP-Guo-5'-P, isolated in yields ranging from 45 to 70% and chromatographically homogeneous in two solvent systems, was quantitatively converted to 2'-O-THP-Guo-5'-P and N-benzoyl-Guo-5'-P after hydrolysis with NH_4OH or HOAc, respectively, and was quantitatively converted to Guo-5'-P when hydrolyzed with both acid and base. The protected 5'-nucleotide did not reduce periodate,[31] unless previously hydrolyzed with HOAc to remove the tetrahydropyranyl group. This result demonstrates that the 2'-hydroxyl group remains blocked after the cyclic phosphate is hydrolyzed.

Stability of Protecting Groups. The suitability of protecting groups on monomers for polynucleotide synthesis depends on their stability throughout a large number of chemical operations and, also, on their facile and selective removal under conditions which do not otherwise affect the structure of the desired polymeric end product. Inevitably, a compromise must be made between these conflicting ideals of stability and lability.

In aqueous solutions, the benzoyl group of N-benzoylguanosine derivatives is stable between pH 3 and 9 for several hours at temperatures up to 100°. At neutral pH, the benzoyl group is stable for many months at 4° and probably indefinitely in solution at −20° or when stored dry. In the reactions employed in the present work, hydrolysis of the benzoyl group from any of the N-benzoylguanosine derivatives can occur during removal of triethylammonium bicarbonate from chromatographic effluents and during paper chromatography with basic solvents such as solvent A (*cf.* ref. 11). In these circumstances, where triethylammonium bicarbonate hydrolyzes and the triethylamine and CO_2 are removed *in vacuo*, CO_2 is removed more rapidly so that the pH of the concentrated solutions rises to near 10. Benzoyl group hydrolysis can be avoided, however, if the temperature of the solution being concentrated is maintained below 22°. It can also be reduced during chromatography to about 5% by minimizing the time of equilibration of the spotted chromatogram with solvent. The occurrence of such hydrolysis may be recognized by the presence of nucleotide over a diffuse area located between the R_f values of the protected and unprotected nucleotides. This problem was avoided by using neutral or acidic solvents. Quantitative hydrolysis of the benzoyl group was routinely carried out by heating the N-benzoylguanosine derivative for 2 hr. at 100° in 2 N NH_4OH. Milder conditions could also be used. Thus, in 0.05 N NH_4OH, benzoyl hydrolysis is complete in less than 21 hr. at 55° and, at pH 9.95, in about six days at room temperature.[32]

The 2'-O-THP derivatives of guanosine are very sensitive to acid and, in fact, are readily hydrolyzed

(31) J. S. Dixon and D. Lipkin, *Anal. Chem.*, **26**, 1092 (1954).
(32) The hydrolysis was followed spectrophotometrically by observing the change in the 290 mμ/260 mμ absorbance ratio which is 0.92 for the N-benzoylguanosine derivatives and 0.28 for guanosine derivatives[33] at neutral pH. N-Benzoyl-Guo-3',5'-CP was used in these studies.
(33) W. Cohn, *Methods Enzymol.*, **3**, 740 (1957).

below pH 7. In the synthesis, isolation, and handling of N-benzoyl-2'-O-THP-Guo-3',5'-CP and N-benzoyl-2'-O-THP-Guo-5'-P, rapid neutralization, low temperatures, and removal of water were necessary to avoid rapid hydrolysis of the THP ether. Even chromatography in solvent B sometimes resulted in small amounts of THP group hydrolysis which did not, however, occur in solvent A. Preferably the THP derivative should be synthesized and characterized just prior to use, and, if necessary, stored as dry solid or in anhydrous solution at the lowest possible temperatures. With such precautions it has been possible to maintain the THP group without detectable hydrolysis.

Though the extreme acid lability of the THP ethers complicates use of these derivatives, the quantitative removal of this protecting group can be carried out under extremely mild conditions. Previously, quantitative THP hydrolysis has been carried out using 50–80% aqueous acetic acid (nominal pH 1.5–2.2, 22°) for a few hours.[7b-e] However, it is possible to achieve quantitative hydrolysis of all the tetrahydropyranyl derivatives used in the present work by heating aqueous solutions of the nucleotides in 0.01 N acetic acid (pH 3.7, 22°) for 2 hr. at 100°. At room temperature in 0.01 N acetic acid, N-benzoyl-2'-O-THP-Guo-5'-P is quantitatively converted to N-benzoyl-Guo-5'-P with a half-life of about 14 hr., and 2'-O-THP-Guo-5'-P, in 0.1 M pyrophosphate–0.2 M NaCl, pH 5.0 (20°), was hydrolyzed to Guo-5'-P with half-lives of 138 and 68 min. at 75 and 95°, respectively.[34] It is clear that the quantitative removal of the THP protecting group from guanosine nucleotides can be accomplished under conditions much less severe than those used previously for other THP nucleotides. These findings suggest the value of more detailed investigation of the conditions necessary for tetrahydropyranyl ether hydrolysis in other nucleotides and in polynucleotides.

Obviously, with the doubly protected guanosine nucleotides, as required for polyriboguanylic acid synthesis, still greater restriction in conditions is necessary in order to avoid protecting group hydrolysis. The tolerable pH range is very limited, 7–9. Outside this range, low temperature and rapid handling are required. Of course, the more stringent restrictions necessary for maintaining the tetrahydropyranyl group suffice for preventing the hydrolysis of either protecting group.

General Conclusions. This investigation demonstrates the feasibility of preparation of protected 5'-ribonucleotides suitable for polymerization. The yield of N-benzoyl-2'-O-THP-Guo-5'-P between 30 and 50% based on starting guanosine-5'-phosphate is reasonably high and probably could be increased by improvements in the cyclization reaction and with further purification of the cyclic phosphate phosphodiesterase. There is every reason to believe that the reaction sequence used to obtain the protected Guo-5'-phosphate could be adapted to the preparation of the other protected ribonucleotides with the possible exception of the cytidine derivative since the cyclic phosphate

phosphodiesterase does not hydrolyze cytidine-3',5'-cyclic phosphate. The problems associated with the instability of the two protecting groups used have been largely overcome and preliminary investigations show that the N-benzoyl and the 2'-O-tetrahydropyranyl groups may be removed from the guanosine nucleotides much more easily than previously suspected. The ease of removal of these protecting groups should prove an asset in polynucleotide synthesis.

Experimental

Materials. Guanosine-5'-phosphate was obtained from the California Corporation for Biochemical Research. Pyridine, both reagent and Spectroquality[35] grades, was dried and stored over calcium hydride. Dimethyl sulfoxide and dihydropyran were distilled, immediately before use, in the absence of atmospheric moisture. Dioxane was dried over metallic sodium before saturation with anhydrous hydrogen chloride; the acidity of the resulting solution was determined by titration with standard NaOH. Quick frozen, stripped rabbit brains were obtained from Pel-Freez Biologicals Inc., Rogers, Ark., and the adenosine-3',5' cyclicphosphate used in routine assays of the nucleoside-3',5'-cyclic phosphate phosphodiesterase isolated from these brains was obtained from Schwarz Bioresearch, Inc. Triethylammonium bicarbonate buffer, pH 7.5, was prepared by the method of Smith, *et al.*[7b] The DEAE cellulose (0.9 mequiv./g., Bio-Rad Laboratories) was prepared for use by the method of Peterson and Sober.[19a] All other reagents were of the highest grade obtainable commercially and were used without further purification.

General Methods. Paper chromatography was used to assess purity and as a means of characterization. Solutions containing 0.1–0.25 μmole[36] of nucleotide were spotted, with appropriate standards, on Whatman No. 1 paper, and the chromatograms were developed by the descending technique. Nucleotides were visualized by use of ultraviolet light, whereby 5 mμmoles of guanosine and 2–3 mμmoles of N-benzoylguanosine derivatives could be distinguished. Solvent systems used include (composition in terms of volume for volume): solvent A,[8] 7:1:2 2-propanol–concentrated NH$_4$OH–H$_2$O; solvent B, 5:2 2-propanol–0.5 M NH$_4$OAc, pH 6.0; solvent C,[8] 5:2:3 1-butanol–glacial HOAc–H$_2$O; solvent D,[37] 80:2:18 saturated aqueous (NH$_4$)$_2$SO$_4$–2-propanol–1 M NaOAc; solvent E,[29] 6:3:1 absolute ethanol–2% (w./v.) H$_3$BO$_3$–concentrated NH$_4$OH. R_f values are presented in Table II.

Paper electrophoresis was carried out using a water-cooled, flat bed apparatus[38] with a 110-cm. distance between electrodes, for 90–120 min. at field strengths of approximately 40 v./cm., with 0.05 M NH$_4$OAc buffer, pH 8.5; the mobilities of various substances relative to Guo-5'-P are also listed in Table II.

(34) The 2'-O-THP-guanosine nucleotide was incubated under the stated conditions with NaIO$_4$ and the changes in absorbance at 225 mμ were followed.[31] Precisely 2 equiv. of periodate were reduced for each equivalent of nucleotide present, indicating that the hydrolysis product, tetrahydropyranyl alcohol or its breakdown products such as 5-hydroxy-pentanal,[23] also reduces periodate.

(35) Matheson Coleman and Bell, East Rutherford, N. J.

(36) The use of much larger quantities of nucleotide in chromatography has been recommended as the most sensitive method of estimating purity.[7b] While this is so for detecting impurities with R_f *higher* than that of the major substance, impurities of *lower* R_f will be obscured by the very large amount of the major substance. Tailing is particularly prevalent with guanosine derivatives due to their poor solubility.

(37) R. Markham and J. D. Smith, *Biochem. J.,* 49, 401 (1951).

(38) Savant Instruments, Inc., Hicksville, N. Y.

Table II. R_f Values and Relative Mobilities of Some Guanosine Derivatives

	R_f Solvent					$R_M{}^a$
Substance	A	B	C	D	E	pH 8.5
Guanosine	0.26	0.57		0.28	0.40	0.12
Guo-3'-P	0.08	0.07		0.39	0.45	1.00
Guo-5'-P	0.03	0.07	0.07	0.45	0.23	1.00
Guo-3',5'-CP	0.26	0.43	0.22	0.15	0.58	0.65
2'-O-THP-Guo-3',5'-CP	0.56	0.62				
2'-O-THP-Guo-5'-P	0.16	0.28		0.35	0.56	0.88
N-Benzoyl-Guo-5'-P[b]	0.15	0.21	0.46	c	0.37	0.91
N-Benzoyl-Guo-3',-5'-CP[b]	0.44	0.59	0.60	c		0.54
N-Benzoyl-2'-O-THP-Guo-3',5'-CP[b]	0.68	0.76		c		0.52
N-Benzoyl-2'-O-THP-Guo-5'-P[b]	0.34	0.54		c	0.68	0.92
N-Benzoyl-2'-O-THP-guanosine[b]		0.87		c		

[a] See text for conditions and other details. Guo-5'-P is the reference substance. [b] Some debenzoylation occurs during chromatography in alkaline solvents. N-Benzoylguanosine derivatives appear as pink fluorescing spots in quantities less than 0.1 μmole but as white fluorescing rings around a pink center when larger quantities are used. [c] N-Benzoylguanosine derivatives do not migrate in solvent D.

Ultraviolet absorption spectra were determined with a Cary 14 recording spectrophotometer. Other absorbance measurements were made with a Zeiss PMQII spectrophotometer. Spectrophotometric assays requiring constant temperature were performed in a Beckman DU spectrophotometer equipped with thermospacers.

Orthophosphate was determined by the method of Lowry and Lopez[39] modified for a total volume of 1.5 ml. and sensitive to 3 mμmoles of orthophosphate. Total phosphorus was determined by first wet ashing either with H_2SO_4–H_2O_2[40] or H_2SO_4–$HClO_4$,[41] followed by dilute acid hydrolysis of polyphosphates, and then orthophosphate determination either with the Lowry–Lopez[39] procedure or the more sensitive method of Lowry, et al.,[41] similar to the procedure of Chen, et al.[42]

Guanosine-5'-phosphate derivatives devoid of 2'-O blocking groups were also determined by periodate oxidation.[31] The extent of hydrolysis of the THP group from appropriate derivatives was similarly determined (in a total volume of 1.00. ml. using 0.100 μmole of $NaIO_4$ and 10–40 mμmoles of nucleotide).

In ion-exchange chromatography for isolation purposes, ammonium or triethylammonium bicarbonate buffers were used for elution with discontinuous or linear[43] concentration gradients. Salt was removed by evaporating the solutions in rotary evaporators with repeated dilution-concentration cycles. Bath temperatures were kept below 22° and evaporated solvent was collected in a 0° trap, under 0.1 torr pressure.

(39) See Table I, footnote a.
(40) G. A. LePage in "Manometric Techniques," W. W. Umbreit, R. H. Burris, and J. F. Stauffer, Ed., 3rd Ed., Burgess Publishing Co., Minneapolis, Minn., 1957, p. 273.
(41) O. H. Lowry, N. R. Roberts, K. Y. Leiner, M. Wu, and A. L. Farr, J. Biol. Chem., 207, 1 (1954).
(42) P. S. Chen, Jr., T. Y. Toribara, and H. Warner, Anal. Chem., 28, 1756 (1956).
(43) (a) C. W. Parr, Biochem. J., 56, xxvii (1954); (b) R. M. Bock and N. S. Ling, Anal. Chem., 26, 1543 (1954).

Hydrolytic derivatization for structure determination of the protected guanosine nucleotides was performed on 0.1–0.5 μmole quantities. A nucleotide derivative in 2 N NH_4OH or 0.01 N HOAc at 10^{-3} M nucleotide concentration was heated for 2 hr. at 100°. A third sample of the nucleotide was sequentially hydrolyzed similarly with NH_4OH and, after lyophilization, with HOAc. The three hydrolysates were lyophilized, dissolved in H_2O, and 0.1-μmole quantities were examined by paper chromatography in at least two solvents.

No attempt was made to obtain crystalline materials, nor were elemental analyses performed. Chromatographic analysis, suitable derivatization, and determination of the ratio of chromophore to phosphorus were considered adequate means for characterization and assessment of purity of these guanosine derivatives.

Guanosine-3',5'-cyclic Phosphate. An aqueous solution of pyridinium Guo-5'-P (100 μmoles) was taken to dryness and the residue was coevaporated five times with anhydrous pyridine. The resulting gum was shaken with 75 ml. of anhydrous pyridine and 2 g. of Superbrite glass beads, 200 μ in diameter.[16] The dispersion of Guo-5'-P was transferred to a separatory funnel, protected from the atmosphere with silica gel, and then added, dropwise, to 500 μmoles of DCC in 125 ml. of anhydrous pyridine under reflux over a 48-hr. period. After 16 hr., some aggregation of Guo-5'-P was observed in the separatory funnel; this aggregated material was not added to the reaction mixture.

After 175 hr. under reflux, the reaction mixture was cooled, mixed with 50 ml. of H_2O, and left standing 2 hr. at room temperature. After removing the solvent in a rotary evaporator, the residue was mixed with 50 ml. of H_2O and taken to dryness, this coevaporation being repeated three times. The residue was then extracted five times with 1-ml. portions of H_2O. The pooled filtrates were extracted five times with ether, the latter being discarded. An aliquot of the aqueous extract chromatographed on paper in solvent A revealed two spots, in approximately equal quantities, one corresponding to Guo-3',5'-CP and the other to starting material. Poor resolution of this mixture on Dowex-1 ($HCO_3{}^-$) precluded accurate determination of the two components; however, the yield of Guo-3',5'-CP was estimated as 25%, based on starting Guo-5'-P. The total nucleotide recovered was about 50%, the rest was lost due to aggregation. After hydrolysis of the pure Guo-3',5'-CP product with $Ba(OH)_2$,[11] chromatography in solvent A showed the presence of both Guo-3'- and Guo-5'-phosphates; the former was predominant.

N-Benzoylguanosine-5'-phosphate. This compound was synthesized by the method of Smith, et al.[11] Quantitative yields were obtained routinely. The dry pyridinium salt was stored over P_2O_5 in vacuo. The absorption spectrum of the ammonium salt was identical with that given in Figure 1 for the corresponding 3',5'-cyclic phosphate. Using ϵ_{260} of 17.1 $\times$ 10^3,[11] molar ratios of N-benzoylguanosine:ribose (periodate analysis):phosphorus were 1.00:1.00:0.98. The product moved as a single substance in solvents B and C. After hydrolysis with 2 N NH_4OH, Guo-5'-P was the exclusive product.
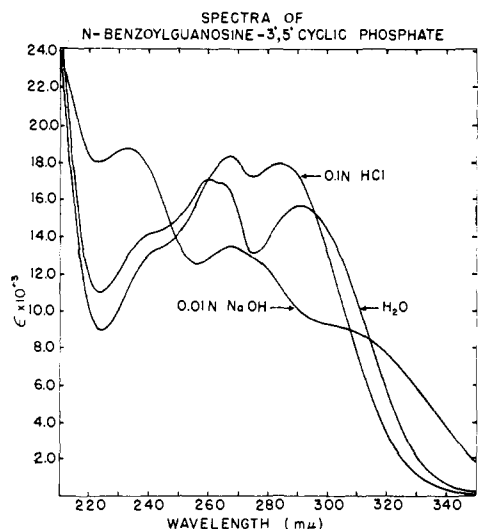
SPECTRA OF
N-BENZOYLGUANOSINE-3',5' CYCLIC PHOSPHATE

Figure 1. Absorption spectra of triethylammonium N-benzoyl-guanosine-3',5' cyclic phosphate in 0.1 N HCl, H_2O, and 0.01 N NaOH. The spectrum in alkali was stable for 30 min. at room temperature; see text for discussion. In 0.1 N HCl: λ_{max} (mμ) 242 (shoulder), 267, and 284; λ_{min} (mμ) 223 and 275; A_{250}/A_{260} = 0.84, A_{280}/A_{260} = 1.01, and A_{290}/A_{260} = 0.97. In H_2O: λ_{max} (mμ) 240 (shoulder), 259, 266 (shoulder), and 290; λ_{min} (mμ) 224 and 275; A_{250}/A_{260} = 0.88, A_{280}/A_{260} = 0.81, and A_{290}/A_{260} = 0.92. In 0.01 N NaOH: λ_{max} (mμ) 233 and 268; λ_{min} (mμ) 223 and 256; A_{250}/A_{260} = 1.05, A_{280}/A_{260} = 0.94, and A_{290}/A_{260} = 0.77.

N-Benzoylguanosine-3',5'-cyclic Phosphate. Anhydrous pyridinium N-benzoyl-Guo-5'-P (10 mmoles) was dissolved in 1000 ml. of anhydrous pyridine and 2.84 g. of 4-morpholine-N,N'-dicyclohexylcarboxami-dine[44] (9.7 mmoles) was added. The solution was placed in a separatory funnel protected from the atmosphere with silica gel and run, dropwise, into 1000 ml. of anhydrous pyridine containing 8.24 g. of DCC (40 mmoles) under reflux (reflux condenser also protected with silica gel) over a period of 3 hr. The reaction mixture was kept under reflux for an additional hour after the last N-benzoyl-Guo-5'-P had been added. The bright yellow solution was cooled and taken nearly to dryness on a rotary evaporator. The residue was washed thoroughly with 3000 ml. of water and the mixture was filtered to remove N,N'-dicyclohexylurea. The filtrate was concentrated to about 300 ml., after which a yellow gum started to separate. This mixture was brought to pH 7.5 by addition of triethylamine. Paper chromatography (solvent A) of this mixture revealed the major product migrating with a higher R_f than that of the starting material. This product was converted to Guo-3',5'-CP upon hydrolysis with NH$_4$OH.

This mixture was passed through a 4 × 40 cm. column of DEAE cellulose (HCO$_3^-$), and the column was washed with about 10 l. of H_2O (the wash was yellow in color). Elution with an 8-l. linear gradient of triethylammonium bicarbonate buffer, pH 7.5, 0.005–0.04 M, brought off two small ultraviolet-absorbing peaks and finally the beginning of a large peak, the desired product. Elution of the latter was completed with 8 l. of 0.06 M triethylammonium bicarbonate, pH 7.5. The starting material was then eluted with 0.15 M triethylammonium bicarbonate, pH 7.5. Successive elution with 0.2, 0.3, 0.4, 0.6, and 1.0 M triethylam-

(44) J. G. Moffatt and H. G. Khorana, *J. Am. Chem. Soc.*, **83**, 663 (1961).

monium bicarbonate, pH 7.5, then brought off at least one small ultraviolet-absorbing peak with each.

The two main peaks were separately pooled and buffer removed from these, and samples of the other peaks as well, in the usual way. Paper chromatography in solvents A, B, and C served for identification of the two main peaks, and showed them to be pure. More-over, hydrolysis of the desired product with NH$_4$OH yielded Guo-3',5'-CP quantitatively. The nucleotidic material eluted with higher buffer concentrations migrated behind N-benzoyl-Guo-5'-P, with successively lower R_f in solvent C. The ion-exchange and paper chromatographic properties of these fractions suggest that they are poly(N-benzoyl)guanylic acids, with mixed C$_{2'}$→C$_{5'}$ and C$_{3'}$→C$_{5'}$ phosphodiester linkages, but their further identification was not undertaken.

Quantitative analysis of the desired product by absorption spectroscopy (see Figure 1) and analysis for total phosphorus showed a recovered yield of 4.3 mmoles (43%), based on an ϵ_{260} of 17.1 × 10^3, and a ratio of N-benzoyl-Guo to total phosphorus of 1.00: 0.98. Periodate was not reduced. Paper electrophoresis also showed the product to be a monoanion at both pH 8.5 and 3.5, consistent with the expected behavior of N-benzoyl-Guo-3',5'-CP.

2'-O-Tetrahydropyranylguanosine-3',5'-cyclic Phosphate. Pyridinium Guo-3',5'-CP (30 μmoles) was taken to dryness on a rotary evaporator and rendered anhydrous by repeated coevaporation with anhydrous pyridine. This gum was mixed with 3 ml. of DMSO, 3 ml. of dihydropyran, and 120 μmoles of anhydrous HCl in dioxane; most of the solid dissolved in a few minutes, and the remainder within 2 hr. with shaking at room temperature. The reaction mixture was incubated for 19 hr. at 40°; 400 μmoles of NH$_4$OH were added to stop the reaction, and the mixture was concentrated for 2 hr. on a rotary evaporator. About 2 ml. of dihydropyran were thereby distilled. On adding 10 ml. of H_2O to the solution a turbid mixture resulted, which was passed through a 3 × 1 cm. column of lithium Dowex-50. The resin was washed with H_2O until the eluate was clear, about 30 ml. being required. The aqueous eluate plus wash solution was extracted eight times with 2.5-ml. volumes of ether, and the extracts were discarded. The aqueous solution was taken nearly to dryness on a rotary evaporator and then placed under high vacuum. After 5 hr., a thick orange sirup remained which was washed twice with 10-ml. volumes of ether, which were discarded. Part of the resulting yellow-orange gum dissolved in 10 ml. of CHCl$_3$, and the remainder in 6 ml. of methanol. Paper chromatography of the CHCl$_3$ and methanol extracts with solvent A revealed a common ultraviolet-absorbing spot with R_f 0.65 as the exclusive nucleotide component. An aliquot of the combined extracts treated with 80% aqueous acetic acid for 64 hr. at room temperature gave Guo-3',5'-CP quantitatively.

N-Benzoyl-2'-O-tetrahydropyranylguanosine-3',5'-cyclic Phosphate. (a) Lithium Salt Contaminated with LiCl. Anhydrous triethylammonium N-benzoyl-Guo-3',5'-CP (100 μmoles) was mixed with 4 ml. each of DMSO and dihydropyran and then 475 μmoles of anhydrous HCl in dioxane. The resulting colorless,

homogeneous reaction mixture was incubated at 40° for 21 hr. The yellow solution was cooled on ice and 520 μmoles of NH₄OH was added. Lyophilization over 24 hr. gave a brown gum which was dissolved in 100 ml. of 7:3 methanol–water and mixed with 5 mequiv. of lithium Dowex-50 suspended in 10 ml. of methanol. The resin was filtered and washed with 7:3 methanol–water, and the filtrate and washes were pooled and concentrated to about 20 ml. on a rotary evaporator, giving a turbid solution which was extracted twice with 10-ml. volumes of ether. The aqueous layer was taken to dryness and the residue was dissolved in 10 ml. of absolute methanol and stored at −20°.

Chromatography in solvents A and B showed the product to be homogeneous. Hydrolysis with NH₄OH gave 2'-O-THP-Guo-3',5'-CP exclusively; with HOAc gave N-benzoyl-Guo-3',5'-CP exclusively; and with both NH₄OH and HOAc gave Guo-3',5'-CP quantitatively. The absorption spectrum of the product was identical with that of the starting material above 250 mμ. Using the ϵ_{260} of the latter, the yield of N-benzoyl-2'-O-THP-Guo-3',5'-CP was 104 μmoles (100%).

(b) *Triethylammonium Salt.* N-Benzoyl-Guo-3',5'-CP (1 mmole) was treated with dihydropyran as in (a). After stopping the reaction with NH₄OH and distillation of the excess dihydropyran, the impure product, in DMSO, was diluted to 1000 ml. with water and passed through a 2 × 20 cm. column of DEAE cellulose (Cl⁻), which was then washed with 1000 ml. of water. Elution with 0.02 *M* triethylammonium bicarbonate, pH 7.5, brought off some nucleotide (1%) (which was discarded) before the column was completely converted to the bicarbonate form. The bulk of the product was eluted Cl⁻ free with 0.02 *M* buffer (0.1 *M* buffer speeded elution of this peak). Peak fractions were pooled and buffer removed.

Chromatography of the product in solvents A and B revealed the presence of N-benzoyl-2'-O-THP-Guo-3',5'-CP contaminated with some 2'-O-THP-Guo-3',5'-CP.[45] Hydrolyses with NH₄OH, HOAc, and both gave the derivatives expected from these two compounds. Spectrophotometric analysis also showed that benzoyl hydrolysis had occurred since the A_{290}/A_{260} ratio was less than expected for the N-benzoyl-Guo chromophore. Using the absorbances at 260 and 290 mμ and the known molar extinction coefficients of the two chromophores at the two wavelengths, it was possible to calculate the amount of each nucleotide. The analysis showed that recovery of N-benzoyl-2'-O-THP-Guo-3',5'-CP was 731 μmoles (73%) and 2'-O-THP-Guo-3',5'-CP was 173 μmoles (17%), giving a total recovered yield of 2'-O-tetrahydropyranyl nucleotide of 90%.

Passage of this mixture through a 4 × 5 cm. column of lithium Dowex-50 converted these nucleotides to lithium salts. The effluent and washes were taken to dryness, and the resulting yellow gum dissolved in methanol and stored at −20°.

Enzymic Hydrolysis of N-Benzoyl-2'-O-tetrahydro-

pyranylguanosine-3',5'-cyclic Phosphate. (a) *Preparation of Rabbit Brain Nucleoside-3',5'-cyclic Phosphate Phosphodiesterase.* Frozen, stripped rabbit brains (434 g.) were thawed at 0–4°, which temperature was maintained throughout. The brains were mixed with 870 ml. of 0.25 *M* sucrose–0.05 *M* Tris, pH 7.8, and 100 mg. of Antifoam 60,[46] and homogenized in a blender[47] for 30 sec. at low speed and 60 sec. at high speed. The resulting homogenate was centrifuged 20 min. at 20,000 × g, and the supernatant was carefully decanted. The residue was washed with a volume of homogenization medium equal to the supernatant volume. To the combined supernatant and wash in an ice bath was added 170 g. of (NH₄)₂SO₄ over 30 min. and enough 1.5 *N* NH₄OH to maintain a pH of 8.05. After 5 min. further stirring, the mixture was centrifuged 40 min. at 11,000 × g. To the decanted supernatant was added 234 g. of (NH₄)₂SO₄ at 0° over 25 min., the pH again maintained at 8.05. The mixture was allowed to stand 2 hr. and centrifuged as before. The supernatant was discarded, and the residue (20–50% (NH₄)₂SO₄ fraction) was mixed with 450 ml. of 0.02 *M* Tris, pH 7.8, and clarified by high-speed centrifugation. The enzyme was dialyzed against three 3-l. changes of 0.02 *M* Tris, pH 7.8, over 36 hr. The dialyzed enzyme, which appeared as a turbid, but stable, suspension was stored at 4°, with little loss of activity over several months. Over-all purification represents a 4.5-fold increase in specific activity (μmoles of orthophosphate released/mg. of protein/hr.). This preparation yielded 4.1 g. of protein (specific activity = 11.5 with adenosine-3',5'-CP substrate) which represents about 50% recovery of initial activity.

(b) *Assay of Nucleoside-3',5'-cyclic Phosphate Phosphodiesterase.* The substrate (0.1 μmole), dry or in aqueous solution, was mixed with 5 μmoles of Tris hydrochloride, pH 7.8, 0.1 μmole of MgCl₂, H₂O, and enzymes in a final volume of 0.100 ml. In addition to the phosphodiesterase being assayed, 10 μg. of highly purified *E. coli* alkaline phosphatase (Worthington Biochemicals, Freehold, N. J.) was added to appropriate samples as an indicator enzyme. Controls lacking substrate, both enzymes, or only alkaline phosphatase were run routinely. For each substrate, incubation time at 38° was adjusted to yield 0.04–0.06 μmole of orthophosphate in the presence of alkaline phosphatase. This approach was taken because the time course of orthophosphate release from 3',5'-CP substrates is not linear, but rather continually decreases, even though orthophosphate release is a linear function of the amount of phosphodiesterase used for identical incubation times. After incubation, samples were cooled to 0°, mixed with 0.025 ml. of 1.25 *N* HClO₄, incubated at 0° for 10 min., and centrifuged to remove protein. Orthophosphate was determined on 0.100-ml. aliquots (Lowry and Lopez; see Table I, ref. *a*). Specific activity of the phosphodiesterase was calculated as described. Enzyme protein was determined by the method of Lowry, *et al.*[48]

(c) *Assay and Inhibition of 5'-Nucleotidase Activity.* Two assays were used for measuring the 5'-nucleo-

(45) The debenzoylated contaminant probably was formed immediately after neutralization of the reaction mixture with excess NH₄OH. In this synthesis, the reaction mixture was not cooled prior to NH₄OH addition and, for technical reasons, was distilled near room temperature rather than lyophilized. In more recent experiments ether extraction of the reaction mixture at 4° has been used with some success to remove the dihydropyran.

(46) General Electric Co., Schenectady, N. Y.
(47) John Oster Manufacturing Co., Milwaukee, Wis.
(48) O. H. Lowry, N. J. Rosebrough, A. L. Farr, and R. J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).

tidase activity contaminating the phosphodiesterase. The first, similar to the phosphodiesterase assay except that Tris, pH 7.50, is the buffer and adenosine- or Guo-5'-P is the substrate, measures the nucleotidase directly, while the second assay is the phosphodiesterase control without alkaline phosphatase, and therefore measures nucleotidase activity under the very conditions in which the phosphodiesterase is used for synthetic purposes. Though less precise, the second assay affords more relevant information for the purposes to which the brain enzyme is used in this investigation. Consequently, the second assay was used to evaluate nucleotidase inhibitors while the first was utilized to ascertain the specificity of the contaminating nucleotidase. Adenosine and guanosine were found to be effective inhibitors of the 5'-nucleotidase when added to the phosphodiesterase control at the 1-μmole level (tenfold excess over substrate). Under these conditions, no 5'-nucleotidase activity was detected up to 75% hydrolysis of N-benzoyl-2'-O-THP-Guo-3',5'-CP. When the phosphodiesterase was raised above 0.2 mg. of protein in the standard assay, with 1 μmole of adenosine, 5'-nucleotidase activity was noted at somewhat lower levels of cyclic phosphate substrate hydrolysis. Because the ratio of phosphodiesterase to nucleotidase was found to vary in different enzyme preparations, assessment of the nucleoside inhibition was required for each.

(d) *Preparation of N-Benzoyl-2'-O-tetrahydropyranylguanosine-5'-phosphate.* A solution containing 150 μmoles of lithium N-benzoyl-2'-O-THP-Guo-3',5'-CP, 128 μmoles of MgCl$_2$, 1 mmole of Tris, pH 7.9, 1.5 mmoles of adenosine, and 115 ml. of H$_2$O[49] was preincubated 30 min. at 38°. Then, 30 ml. of rabbit brain phosphodiesterase (20–50% (NH$_4$)$_2$SO$_4$ fraction, specific activity = 11.5 *vs.* adenosine-3',5'-CP, containing 217 mg. of protein) was added and incubation continued. At regular intervals aliquots of the enzymic digest were taken for assay of orthophosphate directly (nucleotidase activity) and after alkaline phosphatase action (phosphodiesterase activity), and for paper electrophoresis. Because some nucleotidase activity first appeared at 410 min., the reaction was terminated 20 min. later by cooling to 0°. At 410 min., the orthophosphate analyses indicated the presence of 102 μmoles of N-benzoyl-2'-O-THP-Guo-5'-P, 10 μmoles of N-benzoyl-2'-O-THP-guanosine, and, by difference, 38 μmoles of starting material. Paper electrophoresis at pH 8.5 confirmed these findings.

The reaction mixture at 0° was titrated to pH 4.5 with 1 N HOAc to precipitate protein, *rapidly* transferred to precooled centrifuge tubes, and centrifuged 5 min.

at 15,000 $\times$ g at 0°. The supernatant was decanted into a cold flask and rapidly titrated with *1 N* NH$_4$OH to pH 8.5. The precipitate was washed twice with 100-ml. volumes of cold 0.02 *M* NH$_4$OAc buffer, pH 4.2, and the washes were added to the initial supernatant which necessitated further pH adjustment with NH$_4$OH, this time to 7.5.

The product mixture was diluted to 2000 ml. with H$_2$O, and passed through a 2 $\times$ 20 cm. column of DEAE cellulose (Cl$^-$), followed by 100 ml. of H$_2$O, 100 ml. of 0.01 *M* triethylammonium bicarbonate, and 400 ml. of 0.025 *M* buffer, in sequence, at which point the eluate was free of chloride and showed an A_{260} <0.05, indicating that the nucleosides were completely eluted and the column was in the bicarbonate form. Elution with 0.06 *M* triethylammonium bicarbonate gave two poorly resolved peaks in the next 1000 ml. which from A_{290}/A_{260} ratios were shown to contain guanosine and N-benzoylguanosine chromophores. Elution with 0.10 *M* triethylammonium bicarbonate gave a final peak in 1000 ml. with absorbance ratios of the N benzoylguanosine chromophore. Elution with more concentrated buffer did not bring off any other nucleotidic material.

Paper chromatography with solvent A identified the components of each peak. In addition to adenosine as the main component, traces of N-benzoyl-2'-O-THP-Guo-3',5'-CP and the corresponding 5'-phosphate were found in the initial eluate. The two poorly resolved peaks contained 2'-O-THP-Guo-3',5'-CP, 2'-O-THP-Guo-5'-P, and N-benzoyl-2'-O-THP-Guo-3',5'-CP, eluted in that order.[50] The peak eluted with 0.10 *M* triethylammonium bicarbonate showed only a single component in solvents A and B. Hydrolytic derivatization with HOAc, NH$_4$OH, or both, followed by chromatography with solvents A, B, and E, confirmed the above identifications and also showed that the constituent of the chromatographically homogeneous peak was N-benzoyl-2'-O-THP-Guo-5'-P. The latter product did not reduce periodate, except at acid pH, in which case IO$_4^-$ was slowly reduced, corresponding to the hydrolysis of the THP group. The spectrum of N-benzoyl-2'-O-THP-Guo-5'-P (250–350 mμ) was identical with that of the 3',5'-cyclic phosphate starting material and other N-benzoylguanosine derivatives. Based on an ϵ_{260} of 17.1 $\times$ 10^3, the yield of pure N-benzoyl-2'-O-THP-Guo-5'-P was 75.8 μmoles (50%), and the ratio of N-benzoylguanosine chromophore to total phosphorus was 0.98.

(49) This mixture was similar to enzyme assay mixtures except for lower concentrations of buffer and MgCl$_2$, which simplifies the ion-exchange fractionation of the enzymic digest.

(50) The retardation, on anion exchangers, afforded by the N-benzoyl substitutent on guanosine is particularly striking here since a dianion is eluted before a benzoylated monoanion.