Analyses. Aliquots (100 μl which corresponded to about 10^4 dpm in the experiments with labeled thymine dimer) of the solutions were spotted on Whatman No. 1 paper and chromatographed (descending) with 1-butanol–water–acetic acid (80:30:12, v/v/v).[48] Under these conditions the R_f for thymine dimer is 0.25 and that for thymine is 0.59. Thymine appears as a dark spot when the chromatogram is viewed under ultraviolet light. Thymine dimer can be visually detected after irradiating the dried chromatogram with 2537-Å light which converts dimer to monomer. There was no interference from any of the sensitizers used. The radioactive areas were determined by means of a Vanguard Autoscanner Model 880 (scan speed 12 in./hr, time constant *D*, counts full scale 300, slit width 0.25 cm). The areas under the tracings were measured with a planimeter.

(48) K. Smith, *Photochem. Photobiol.*, **2**, 503 (1963).

A second chromatographic system employing Whatman No. 1 paper and 2-propanol–concentrated HCl–water (68:15.5:16.5, v/v/v) was also employed. Under these conditions the R_f for thymine dimer is 0.50 and for thymine 0.80.

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Studies on Polynucleotides. LI.[1] Syntheses of the 64 Possible Ribotrinucleotides Derived from the Four Major Ribomononucleotides[2]

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Contribution from the Institute for Enzyme Research of the University of Wisconsin, Madison, Wisconsin. *Received September 2, 1965*

Abstract: The chemical syntheses of all of the possible ribotrinucleotides derivable from the four major mononucleotides have been accomplished using the general approach illustrated in Chart I for the synthesis of guanylyl(3′→5′)cytidylyl(3′→5′)adenosine I).

Methods for the specific synthesis of ribopolynucleotides containing $C_{3'}$–$C_{5'}$ interribonucleotidic linkages have formed the subject of an extended study in this laboratory,[3] and the procedures which have emerged have been applied successfully to (a) the stepwise synthesis of a variety of dinucleotides, of several trinucleotides, and of a tetranucleotide and (b) the polymerization of suitably protected ribomononucleotides to yield a series of homologous oligonucleotides.[3,4] The recent demonstration by Nirenberg and Leder[5] that ribotrinucleotides can stimulate the binding of specific aminoacyl-t-RNA[6] to ribosomes has provided a new approach to the deciphering of nucleotide sequences within the trinucleotides which serve as coding units for different amino acids. This discovery and our own work on the genetic code, which has been reviewed elsewhere,[7,8] have stimulated us to focus on the preparation of ribotrinucleotides and in the present paper we report on the stepwise synthesis of all of the possible ribotrinucleotides[9] derived from the four common mononucleotides by general and satisfactory methods.[10] Results of the extensive tests made with these trinucleotides on the stimulation of the binding of the different aminoacyl-t-RNA's[6] to ribosomes have been described separately.[11] Recently, a number of other laboratories have also reported on the preparation of ribotrinucleotides by alternative methods. Thus, Bernfield and Nirenberg,[12] Leder, Singer, and Brimacombe,[13] Thatch and Doty,[14] and Gilham and co-workers[15]

(1) Paper L in this series is by R. D. Wells, E. Ohtsuka, and H. G. Khorana, *J. Mol. Biol.*, **14**, 221 (1965).

(2) This work has been supported by grants from the National Science Foundation (Grant No. GB-3342), the National Cancer Institute of the National Institutes of Health, U. S. Public Health Service (Grant No. CA-05178), the Life Insurance Medical Research Fund (Grant No. G-62-54), and Wisconsin Alumini Research Foundation.

(3) (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *J. Am. Chem. Soc.*, **84**, 430 (1962); (b) D. H. Rammler and H. G. Khorana, *ibid.*, **84**, 3112 (1962); (c) D. H. Rammler, Y. Lapidot, and H. G. Khorana, *ibid.*, **85**, 1989 (1963); (d) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3852 (1963); (e) Y. Lapidot and H. G. Khorana, *ibid.*, **85**, 3857 (1963); (f) C. Coutsogeorgopoulos and H. G. Khorana, *ibid.*, **86**, 2926 (1964); (g) R. Lohrmann and H. G. Khorana, *ibid.*, **86**, 4188 (1964); (h) D. Söll and H. G. Khorana, *ibid.*, **87**, 350 (1965); (i) D. Söll and H. G. Khorana, *ibid.*, **87**, 360 (1965).

(4) R. Lohrmann and H. G. Khorana, Abstracts, 145th National Meeting of the American Chemical Society, New York, N. Y., 1963, p 37C.

(5) M. W. Nirenberg and P. Leder, *Science*, **145**, 1399 (1964).

(6) t-RNA is abbreviation for transfer or amino acid acceptor ribonucleic acid.

(7) H. G. Khorana, *Federation Proc.*, **24**, 1473 (1965).

(8) H. G. Khorana, T. M. Jacob, M. W. Moon, S. A. Narang, and E. Ohtsuka, *J. Am. Chem. Soc.*, **87**, 2954 (1965).

(9) The compounds prepared in this paper lack phosphomonoester groups and therefore on the basis of phosphorus content are not strictly "trimers" of mononucleotides. Traditionally they would perhaps be more appropriately described as trinucleoside diphosphates. The latter designation, however, is more ambiguous and less likely to convey the main structural feature of biological interest which is that all these compounds contain three nucleosides in repeating $C_{3'}$–$C_{5'}$ internucleotidic linkage. The general name trinucleotide is therefore retained for the present series of compounds.

(10) A brief report of this work has been made in ref 7.

(11) D. Söll, E. Ohtsuka, D. S. Jones, R. Lohrmann, H. Hayatsu, S. Nishimura, and H. G. Khorana, *Proc. Natl. Acad. Sci. U. S.*, **54**, 1378 (1965).

(12) M. Bernfield and M. W. Nirenberg, *Science*, **147**, 479 (1965).

(13) P. Leder, M. F. Singer, and R. L. C. Brimacombe, *Biochemistry*, **4**, 1561 (1965).

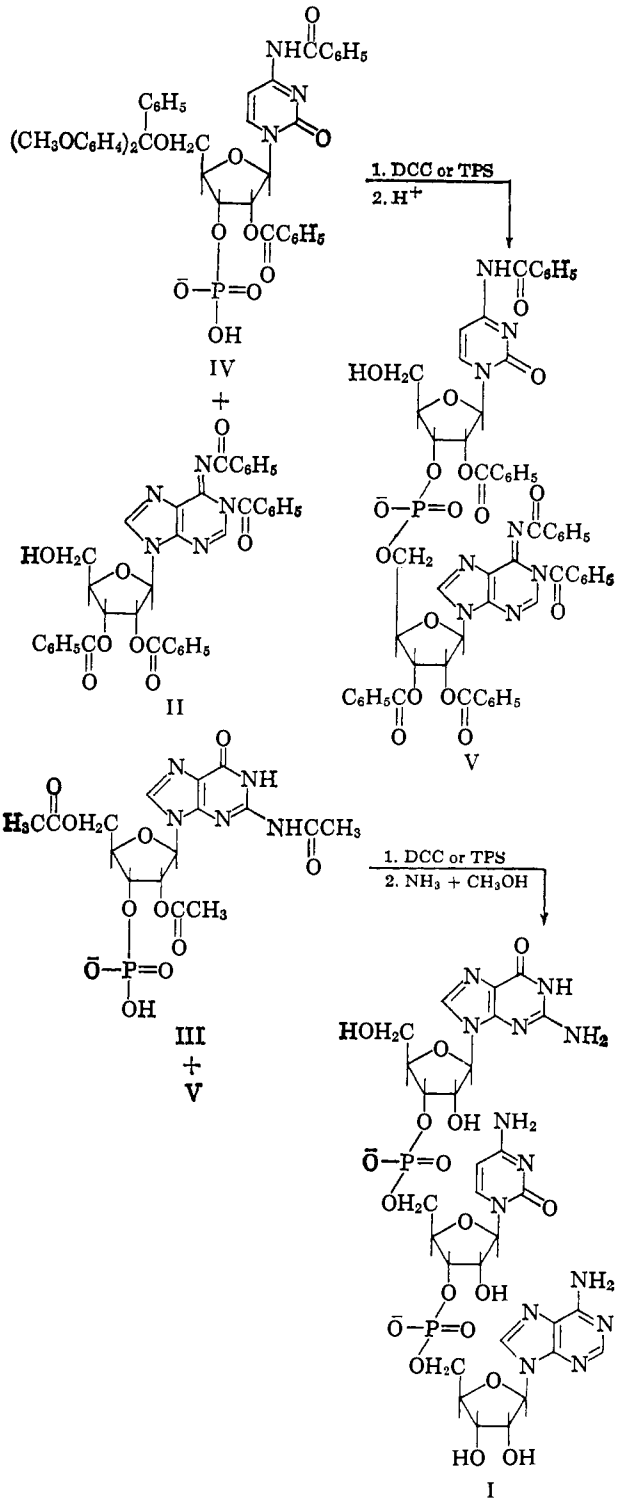
(14) R. Thatch and P. Doty, *Science*, **147**, 1310 (1965); **148**, 632 (1965).

(15) J. C. Lee, N. W. Y. Ho, and P. T. Gilham, *Biochim. Biophys. Acta*, **95**, 503 (1965).

have used enzymatic methods for the synthesis of a large number of oligonucleotides, while Smrt and Cramer and their co-workers[16,17] have reported on the chemical synthesis of a few tri- and higher oligonucleotides.

As concluded in earlier papers,[3] the most satisfactory approach to the specific synthesis of the $C_{3'}$–$C_{5'}$ interribonucleotidic linkage is that which involves the condensation of a protected ribonucleoside 3′-phosphate with a protected ribonucleoside carrying a free 5′-hydroxyl group. The most suitable protecting groups for the 2′-hydroxyl groups in ribonucleoside 3′-phosphates are the alkali-labile acetyl or benzoyl groups. From previous work, acyl groups also have proved to be satisfactory for the protection of the amino groups on the heterocyclic rings. For the stepwise synthesis of oligonucleotides larger than the dinucleotides, the 5′-hydroxyl group in the mononucleotide component is usually protected by a *p*-methoxytrityl (MMT) or a di-*p*-methoxytrityl (DMT) group. These considerations lead to the general approach, which is illustrated in Chart I for the synthesis of the trinucleotide, guanylyl-(3′→5′)cytidylyl(3′→5′)adenosine (I). This approach has been used throughout in the present work. It is seen that the synthesis of all of the possible 64 ribotrinucleotides containing the four standard mononucleotides requires three sets of the following protected derivatives: (1) four protected ribonucleosides bearing only the 5′-hydroxyl group free, such as N,N′,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (II) (HO-A^{Bz_2}-$(OBz)_2$);[18] (2) four "fully" acylated ribonucleoside 3′-phosphates such as N^2,O,$^{2'}O^{5'}$-triacetylguanosine 3′-phosphate (III) (AcO-G^{Ac}-OAc-p);[18] and (3) four protected nucleotides of the type IV, which serve as the "middle" components in the trinucleotides and therefore must bear a protecting group on the 5′-hydroxyl position such that it can be removed selectively without damage to other protecting groups (see Chart I). Methods for the preparation of the required protected ribonucleosides and the fully acylated ribonucleoside 3′-phosphates are available from previous work.[3] While compounds of the type IV corresponding to uridine and adenosine nucleotides have also been described previously, the preparation of this class of protected derivatives has now been studied further for all of the mononucleotides.

Chart I. Steps in the Synthesis of the Trinucleotide, Guanylyl(3′→5′)cytidylyl(3′→5′)adenosine (I)



Protected Ribonucleoside 3′-Phosphates. Pyridinium uridine 3′-phosphate on reaction with mono- or di-*p*-methoxytrityl chloride in pyridine is converted in high yield to the 5′-O-methoxytrityl derivatives[3c,d,f] and, similarly, adenosine 3′-phosphate on tritylation in dry pyridine affords the 5′-O-monomethoxytrityl derivative in satisfactory yield (ref 3e and present work). An

(16) S. Chladek and J. Smrt, *Collection Czech. Chem. Commun.*, **29**, 214 (1964); J. Smrt, *ibid.*, **29**, 2049 (1964); J. Smrt and F. Sorm, *ibid.*, **29**, 2971 (1964).

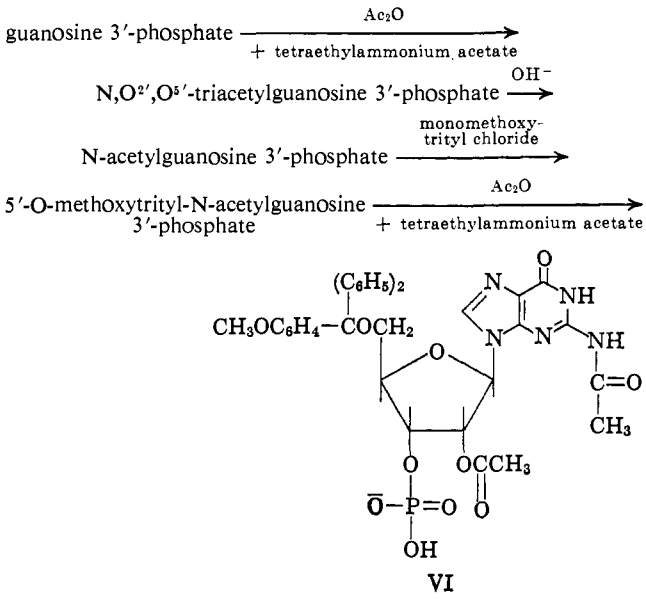
(17) F. Cramer and S. Rittner, *Tetrahedron Letters*, 107 (1964); K. H. Scheib and F. Cramer, *ibid.*, 3765 (1964); F. Cramer, H. J. Rhaese, S. Rittner, and K. H. Scheib, *Ann. Chem.*, **683**, 199 (1965).

(18) For convenience, especially in presentation of Tables I, II, and IV, the system of abbreviations developed previously for protected deoxyribonucleotides and oligonucleotides has been used and extended further for abbreviated presentation of protected ribonucleosides, ribonucleotides, and protected diribonucleoside phosphates (Table I). The protecting groups on the 5′-hydroxyl groups of mononucleotides are to the left of the standard nucleoside initials G, A, C, and U. The presence of the free 5′-hydroxyl group in nucleosides or protected dinucleoside phosphates is indicated by adding HO to the left of the nucleoside initial. The protecting groups on the heterocyclic rings of the nucleosides are designated by the abbreviations Ac or Bz added as superscripts to the nucleoside letters. The protecting groups on the 2′-hydroxyl group of nucleotides or 2′- and 3′-hydroxyl groups of ribonucleosides are shown by adding the abbreviations OAc or OBz to the right of the nucleoside initials. The letter p separated by hyphens indicates the phosphodiester linkage between the 3′-hydroxyl group of the nucleosides, which appear to the left, and the 5′-hydroxyl group of the nucleosides, which appear to the right of the letter p. Thus, taking the protected derivatives, II–V of Chart I, N,N′,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (II) carrying a free 5′-hydroxyl group is abbreviated to HO-A^{Bz_2}-$(OBz)_2$. The fully acylated, N,$O^{2'}O^{5'}$-triacetylguanosine 3′-phosphate (III) is abbreviated to AcO-G^{Ac}-OAc-p. 5′-O-Dimethoxytrityl-N,$O^{2'}$-dibenzoylcytidine 3′-phosphate (IV) is abbreviated to DMT-C^{Bz}-OBz-p. N,$O^{2'}$-Dibenzoylcytidylyl(3′→5′)-N,N′,$O^{2'}$,$O^{3'}$-tetrabenzoyladenosine (V) is abbreviated to HO-C^{Bz}-OBz-p-A^{Bz_2}-$(OBz)_2$.

alternative procedure described previously for the preparation of protected adenosine 3′-phosphate derivatives involved the prior preparation of N-benzoyladenosine 3′-phosphate followed by tritylation of the 5′-hydroxyl group with mono-*p*-methoxytrityl chloride.[3e]

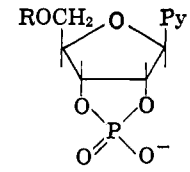
In the present work, with a view to avoiding concomitant tritylation of the amino group on the cytosine ring, a new procedure was developed for the tritylation of the 5′-hydroxyl group in cytidine 3′-phosphate. This involved the reaction of the nucleotide with the methoxytrityl chloride in anhydrous dimethylformamide in the presence of a restricted amount of pyridine, conditions under which the amino group is presumably protonated. 5′-O-Monomethoxytrityl and 5′-O-dimethoxytrityl derivatives of cytidine 3′-phosphate thus were obtained in good yield. The same method also was applied to an alternative preparation of 5′-O-dimethoxytrityladenosine 3′-phosphate, a simple extraction procedure being used for the isolation of the required product. Application of the above procedure for tritylation to guanosine 3′-phosphate did not give selective substitution at the 5′-hydroxyl group, some N-tritylation being detected. The procedure used for the preparation of the protected derivative VI is shown in Chart II.

Chart II. Synthesis of
N,O$^{2'}$-Diacetyl-5′-O-methoxytritylguanosine 3′-Phosphate



VI

A simpler and efficient procedure was developed for the preparation of the 5′-O-monomethoxytrityl and dimethoxytrityl derivatives of pyrimidine nucleoside 3′-phosphates. The large-scale preparation of pure uridine and cytidine 3′-phosphates entails the cyclization of the commercially available mixtures of 2′- and 3′-phosphates to the corresponding 2′,3′-cyclic phosphates followed by pancreatic ribonuclease catalyzed hydrolysis to the 3′-phosphates. The direct reaction of the pyrimidine 2′,3′-cyclic phosphates with mono- or di-*p*-methoxytrityl chloride followed by a simple extraction procedure has now been found to give compounds of the type VII in excellent yield. On incubation with pancreatic ribonuclease in aqueous dimethylformamide, these derivatives were quantitatively converted to the corresponding 3′-phosphates.



VII, Py = uracil or cytosine;
R = monomethoxytrityl or
dimethoxytrityl

Procedures are described in the Experimental Section for the acylations of the 2′-hydroxyl groups and of the heterocyclic amino groups present in the 5′-O-methoxytrityl ribonucleoside 3′-phosphates. Because of the relative lability of the 2′-O-acetyl group, the more stable benzoyl group was preferred. Although the benzoylation reactions in general required 2 or more days for completion, no harm was detected so far as the sensitive 5′-O-di-*p*-methoxytrityl group was concerned. In the benzoylation of 5′-O-dimethoxytrityladenosine 3′-phosphate to form the N,O$^{2'}$-dibenzoyl derivative, the protected nucleotide first was treated with benzoic anhydride and tetraethylammonium benzoate to benzoylate the 2′-hydroxyl group and the benzoylation of the amino group was expedited by subsequent treatment with benzoyl chloride in pyridine.

Protected Dinucleoside Phosphates. Condensations of the protected nucleosides bearing 5′-hydroxyl groups with protected mononucleotides of the type IV were performed by the general methods described in the Experimental Section using dicyclohexylcarbodiimide (DCC) or triisopropylbenzenesulfonyl chloride (TPS)[19] as the condensing agents. An excess (100–200%) of the protected nucleoside was used and this ensured practically quantitative conversion of the protected nucleotide to the protected dinucleoside phosphate. After a short acidic treatment to selectively remove the methoxytrityl groups, the reaction products (of the type V) were chromatographed on triethylammonium–ethyl (TEAE)–cellulose[20] columns using buffered triethylammonium acetate in 95% ethyl alcohol as the eluent. After pooling of the appropriate fractions and concentration, the products were recovered by precipitation from a mixture of ether and pentane, a solvent mixture in which triethylammonium acetate is soluble.

The 16 protected dinucleoside phosphates necessary as intermediates in the synthesis of all of the required trinucleotides thus were prepared and are listed in Table I, which also gives the reacting components and the reaction conditions used in the individual condensations. While analyses of aliquots of the reaction mixtures after complete removal of the protecting groups showed practically quantitative yields throughout, the yields of the protected dinucleoside phosphates isolated after chromatography and precipitation were, in general, much lower than theoretical. (The isolated yields are listed in Table I.) It is clear that losses occurred during the precipitation of the heavily protected dinucleoside phosphates and also during the pooling of the fractions from the columns which was carried out so as to avoid any contamination from the starting materials or any side products. An addi-

(19) R. Lohrmann and H. G. Khorana, *J. Am. Chem. Soc.*, **88**, 829 (1966).

(20) This anion exchanger appears to be preferable to DEAE–cellulose for isolation of protected ribooligonucleotides containing labile 2′-O-acyl groups.

Table I. Synthesis of Protected Dinucleoside Phosphates

Protected dinucleoside phosphate[a]	Starting materials[a] Nucleotide	Amount, mmole	Nucleoside	Amount, mmole	Condensing agent, mmoles	Reaction time, hr	Isolated yield, %
HO-A^{Bz}-OBz-p-A^{Bz_2}-$(OBz)_2$	MMT-A^{Bz}-OBz-p	0.12	HO-A^{Bz_2}-$(OBz)_2$	0.30	DCC, 1.25	72	63
HO-C^{Bz}-OBz-p-A^{Bz_2}-$(OBz)_2$	DMT-C^{Bz}-OBz-p	1.0	HO-A^{Bz_2}-$(OBz)_2$	2.5	TPS, 2.18	8	64
HO-C^{Ac}-OAc-p-A^{Bz_2}-$(OBz)_2$	DMT-C^{Ac}-OAc-p	0.30	HO-A^{Bz_2}-$(OBz)_2$	0.9	TPS, 0.69	5.5	38
HO-G^{Ac}-OAc-p-A^{Bz_2}-$(OBz)_2$	MMT-G^{Ac}-OAc-p	0.24	HO-A^{Bz_2}-$(OBz)_2$	0.48	DCC, 2.4	72	28
HO-U-OBz-p-A^{Bz_2}-$(OBz)_2$	MMT-U-OBz-p	0.25	HO-A^{Bz_2}-$(OBz)_2$	0.50	DCC, 2.5	48	35
HO-A^{Bz}-OBz-p-C^{Bz}-$(OBz)_2$	DMT-A^{Bz}-OBz-p	0.26	HO-C^{Bz}-$(OBz)_2$	0.65	DCC, 2.5	96	37
HO-C^{Bz}-OBz-p-C^{Bz}-$(OBz)_2$	DMT-C^{Bz}-OBz-p	0.30	HO-C^{Bz}-$(OBz)_2$	0.75	DCC, 3.0	96	34
HO-G^{Ac}-OAc-p-C^{Bz}-$(OBz)_2$	MMT-G^{Ac}-OAc-p	0.30	HO-C^{Bz}-$(OBz)_2$	0.75	DCC, 3.0	96	66
HO-U-OAc-p-C^{Bz}-$(OBz)_2$	DMT-U-OAc-p	0.31	HO-C^{Bz}-$(OBz)_2$	0.75	DCC, 3.5	96	33
HO-A^{Bz}-OBz-p-G^{Ac}-$(OAc)_2$	MMT-A^{Bz}-OB_2-p	0.1	HO-G^{Ac}-$(OAc)_2$	0.2	DCC, 0.5	96	82
HO-C^{Bz}-OBz-p-G^{Ac}-$(OAc)_2$	DMT-C^{Bz}-OBz-p	0.2	HO-G^{Ac}-$(OAc)_2$	0.6	DCC, 1.8	216	40
HO-G^{Ac}-OAc-p-G^{Ac}-$(OAc)_2$	MMT-G^{Ac}-OAc-p	0.1	HO-G^{Ac}-$(OAc)_2$	0.25	DCC, 1.0	96	50
HO-U-OBz-p-G^{Ac}-$(OAc)_2$	MMT-U-OBz-p	0.16	HO-G^{Ac}-$(OAc)_2$	0.50	DCC, 1.5	96	57
HO-A^{Bz}-OBz-p-U-$(OBz)_2$	DMT-A^{Bz}-OBz-p	0.3	HO-U-$(OBz)_2$	1.0	TPS, 0.72	8	69
HO-C^{Ac}-OAc-p-U-$(OBz)_2$	DMT-C^{Ac}-OAc-p	0.3	HO-U-$(OBz)_2$	0.82	TPS, 0.67	6	62
HO-G^{Ac}-OAc-p-U-$(OBz)_2$	MMT-G^{Ac}-OAc-p	0.33	HO-U-$(OBz)_2$	0.75	TPS, 0.68	6	63
HO-U-OAc-p-U^{Bz}-$(OBz)_2$	DMT-U-OAc-p	0.33	HO-U^{Bz}-$(OBz)_2$	0.9	TPS, 0.73	7	65

[a] See ref 18.

tional factor is that recoveries of the protected nucleotidic materials from chromatographic columns usually are not quantitative.

That the protected dinucleoside phosphates listed in Table I contained exclusively C_3'–C_5' internucleotidic linkage was confirmed by removal of the protecting groups and subsequent check of complete susceptibility toward an appropriate nuclease. The most sensitive protecting group in the protected dinucleoside phosphates, however, is the 2′-O-acetyl group, and its partial loss could cause detectable randomization of the internucleotidic linkage during the subsequent condensation step to form the trinucleotide. The crucial test of purity of compounds of the type V, therefore, lay in checking for the complete retention of the 2′-O-acyl group. This did not prove feasible by paper chromatographic methods because of the high R_f values of this class of compounds.[21] The complete retention of the 2′-O-acyl groups in the individual preparations of protected dinucleoside phosphates was in fact shown by going through the next step involving condensation with a fully acylated ribonucleoside 3′-phosphate (*e.g.*, III + V → I in Chart I). The trinucleotide obtained after removal of the protecting groups was checked for susceptibility toward an appropriate nuclease or phosphodiesterase. If the product was completely degraded to the expected mononucleotides and the nucleoside,[22] the protected dinucleoside phosphate used in the synthesis was concluded to be pure. In early experiments, during chromatography of some of the protected dinucleoside phosphates, 5–10% loss of the 2′-O-acetyl group was observed. Careful control of the pH value of the eluent, the use of low temperature during column chromatography, and, in particular, the use of the more stable benzoyl group for protection of the 2′-hydroxyl group completely eliminated the above danger.

Extensive characterization, with respect to purity, of the fully protected dinucleoside phosphates as well as of the products obtained after removal of the protecting groups, is given in Table IV.

The Synthesis of Trinucleotides. Condensations of the protected dinucleoside phosphates (*e.g.*, V) with fully acylated ribonucleoside 3′-phosphates (*e.g.*, III) were again carried out in dry pyridine using either DCC or TPS as the condensing agents, two- to threefold excess of the mononucleotidic component being used. After appropriate work-up inclusive of an ammoniacal treatment, the general method for the isolation of the trinucleotides involved high-voltage paper electrophoresis followed by further purification by paper chromatography. The conditions for the individual condensations between every one of the protected dinucleoside phosphates and all of the acylated mononucleotides are listed in Table II, together with the yields of the expected products. It is seen that all of the reactions could be carried out successfully on a rather small scale and that the yields of the products were high in most cases. Only in the case of compounds containing guanosine was there a frequent tendency for the yields to be lower and it is possible that side reactions occur with the guanine ring.[23]

Characterization of all of the isolated trinucleotides was accomplished by paper chromatography (two solvents) and by paper electrophoresis (at two pH values) and the data are recorded in Table V. For establishing exclusive presence of C_3'–C_5' internucleotidic linkages in the trinucleotides, it was necessary only to test selected members since as discussed above the only step, which could introduce a possibility for randomization of the internucleotidic linkage during the trinucleotide condensation step, was the chromatographic isolation of the protected dinucleoside phosphates of the type V. Therefore, for enzymic degradation, trinucleotides were selected so as to check out all of the protected dinucleoside phosphates used in the syntheses listed in Table II. Adequate amounts (5–10 OD_{260} units) of the trinucleotides were subjected to the action of the nucleases as shown below: GpGpG,

(21) Earlier, in the case of the protected uridylyl(3′→5′)uridine,[3d] it was possible to check for the loss of the 2′-O-acetyl group by a chromatographic solvent. However, the presence of bulkier and more numerous protecting groups in the various protected dinucleoside phosphates did not permit the use of simple chromatographic analysis.

(22) No undegraded dinucleoside phosphate should remain during this enzymic degradation.

(23) This is an interpretation which is also supported by work in the deoxyribopolynucleotide field. *Cf.* S. A. Narang, T. M. Jacob, and H. G. Khorana, *J. Am. Chem. Soc.*, 87, 2988 (1965), and unpublished work of Dr. Jacob in this laboratory.

Table II. Synthesis of Trinucleoside Diphosphates (XpYpZ)

Trinucleoside diphosphate	Starting materials: Dinucleoside phosphate[a]	Amount, μmoles	Mononucleotide[a]	Amount, μmoles	Condensing agent, μmoles	Reaction time, hr	Yield, %
ApApA	HO–A^{Bz}–OBz–p–A^{Bz}–(OBz)$_2$	5	BzO–A^{Bz}–OBz–p	15	DCC, 200	60	75
CpApA	HO–A^{Bz}–OBz–p–A^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	40	DCC, 300	72	88
GpApA	HO–A^{Bz}–OBz–p–A^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	40	DCC, 300	72	55
UpApA	HO–A^{Bz}–OBz–p–A^{Bz}–(OBz)$_2$	10	AcO–U–OAc–p	40	DCC, 300	72	88
ApCpA	HO–C^{Bz}–OBz–p–A^{Bz}–(OBz)$_2$	10	BzO–A^{Bz}–OBz–p	30	TPS, 47	10.5	57
CpCpA	HO–C^{Ac}–OAc–p–A^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	TPS, 58	8	54
GpCpA	HO–C^{Bz}–OBz–p–A^{Bz}–(OBz)$_2$	10	Ac–OG^{Ac}–OAc–p	30	TPS, 50	12	55
UpCpA	HO–C^{Bz}–OBz–p–A^{Bz}–(OBz)$_2$	10	AcO–U–OAc–p	30	TPS, 47	10.5	63
ApGpA	HO–G^{Ac}–OAc–p–A^{Bz}–(OBz)$_2$	10	BzO–A^{Bz}–OBz–p	30	DCC, 300	72	31
CpGpA	HO–G^{Ac}–OAc–p–A^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	40	DCC, 300	72	75
GpGpA	HO–G^{Ac}–OAc–p–A^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	40	DCC, 300	72	68
UpGpA	HO–G^{Ac}–OAc–p–A^{Bz}–(OBz)$_2$	10	AcO–U–OAc–p	40	DCC, 300	72	83
ApUpA	HO–U–OAc–p–A^{Bz}–(OBz)$_2$	10	BzO–A^{Bz}–OBz–p	40	DCC, 300	72	71
CpUpA	HO–U–OAc–p–A^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	40	DCC, 300	72	92
GpUpA	HO–U–OAc–p–A^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	40	DCC, 300	72	82
UpUpA	HO–U–OAc–p–A^{Bz}–(OBz)$_2$	10	AcO–U^{Ac}–OAc–p	40	DCC, 300	72	91
ApApC	HO–A^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	DCC, 300	72	55
CpApC	HO–A^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	DCC, 300	72	81
GpApC	HO–A^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 60	7	73
UpApC	HO–A^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–U–OAc–p	30	DCC, 300	72	80
ApCpC	HO–C^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	DCC, 300	72	60
CpCpC	HO–C^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	DCC, 300	72	90
GpCpC	HO–C^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 60	7	50
UpCpC	HO–C^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$	10	AcO–U–OAc–p	30	DCC, 300	72	86
ApGpC	HO–G^{Ac}–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	DCC, 300	72	68
CpGpC	HO–G^{Ac}–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	DCC, 300	72	83
GpGpC	HO–G^{Ac}–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 60	7	58
UpGpC	HO–G^{Ac}–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–U–OAc–p	30	DCC, 300	72	83
ApUpC	HO–U–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	DCC, 300	72	65
CpUpC	HO–U–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	DCC, 300	72	90
GpUpC	HO–U–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 60	7	76
UpUpC	HO–U–OAc–p–C^{Bz}–(OBz)$_2$	10	AcO–U–OAc–p	30	DCC, 300	72	88
ApApG	HO–A^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	8	BzO–A^{Bz}–OBz–p	24	DCC, 300	120	30
CpApG	HO–A^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	5	AcO–C^{Ac}–OAc–p	25	DCC, 210	96	56
GpApG	HO–A^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	5	AcO–G^{Ac}–OAc–p	25	DCC, 200	144	65
UpApG	HO–A^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	6	AcO–U–OAc–p	28	DCC, 250	96	66
ApCpG	HO–C^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	8	BzO–A^{Bz}–OBz–p	32	DCC, 400	96	89
CpCpG	HO–C^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	10	AcO–C^{Ac}–OAc–p	32	DCC, 300	72	85
GpCpG	HO–C^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	7	AcO–G^{Ac}–OAc–p	28	DCC, 250	96	60
UpCpG	HO–C^{Bz}–OBz–p–G^{Ac}–(OAc)$_2$	7	AcO–U–OAc–p	28	DCC, 250	72	85
ApGpG	HO–G^{Ac}–OAc–p–G^{Ac}–(OAc)$_2$	7	BzO–A^{Bz}–OBz–p	27	DCC, 250	96	92
CpGpG	HO–G^{Ac}–OAc–p–G^{Ac}–(OAc)$_2$	6.5	AcO–C^{Ac}–OAc–p	21	DCC, 250	96	71
GpGpG	HO–G^{Ac}–OAc–p–G^{Ac}–(OAc)$_2$	7	AcO–G^{Ac}–OAc–p	28	DCC, 250	96	62
UpGpG	HO–G^{Ac}–OAc–p–G^{Ac}–(OAc)$_2$	7	AcO–U–OAc–p	28	DCC, 250	120	85
ApUpG	HO–U–OBz–p–G^{Ac}–(OAc)$_2$	9	BzO–A^{Bz}–OBz–p	27	DCC, 300	72	78
CpUpG	HO–U–OBz–p–G^{Ac}–(OAc)$_2$	7	AcO–C^{Ac}–OAc–p	25	DCC, 225	144	49
GpUpG	HO–U–OBz–p–G^{Ac}–(OAc)$_2$	7	AcO–G^{Ac}–OAc–p	20	DCC, 250	96	67
UpUpG	HO–U–OBz–p–G^{Ac}–(OAc)$_2$	10	AcO–U–OAc–p	30	DCC, 400	96	74
ApApU	HO–A^{Bz}–OBz–p–U–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	TPS, 58	5	57
	HO–A^{Bz}–OBz–p–U–(OBz)$_2$	10	BzO–A^{Bz}–OBz–p	30	DCC, 390	144	72
CpApU	HO–A^{Bz}–OBz–p–U–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	TPS, 58	5	54
GpApU	HO–A^{Bz}–OBz–p–U–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 58	5	32
UpApU	HO–A^{Bz}–OBz–p–U–(OBz)$_2$	10	AcO–U–OAc–p	30	TPS, 58	5	77
ApCpU	HO–C^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	TPS, 58	5	73
CpCpU	HO–C^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	TPS, 58	8.5	72
	HO–C^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	DCC, 390	160	77
GpCpU	HO–C^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 58	10	64
	HO–C^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	DCC, 390	160	78
UpCpU	HO–C^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–U–OAc–p	30	TPS, 47	12	67
ApGpU	HO–G^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	TPS, 58	4	77
	HO–G^{Ac}–OAc–p–U–(OBz)$_2$	10	BzO–A^{Bz}–OBz–p	30	TPS, 50	10	73
CpGpU	HO–G^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	TPS, 58	5	68
GpGpU	HO–G^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 50	12	61
UpGpU	HO–G^{Ac}–OAc–p–U–(OBz)$_2$	10	AcO–U–OAc–p	30	TPS, 58	5	69
ApUpU	HO–U–OAc–p–U^{Bz}–(OBz)$_2$	10	AcO–A^{Ac}–OAc–p	30	TPS, 58	8	73
CpUpU	HO–U–OAc–p–U^{Bz}–(OBz)$_2$	10	AcO–C^{Ac}–OAc–p	30	TPS, 58	15	79
GpUpU	HO–U–OAc–p–U^{Bz}–(OBz)$_2$	10	AcO–G^{Ac}–OAc–p	30	TPS, 50	12	63
UpUpU	HO–U–OAc–p–U^{Bz}–(OBz)$_2$	20	AcO–U–OAc–p	60	TPS, 120	9	70

[a] See ref 18.

ApGpA, GpGpA, GpGpC (T_1-ribonuclease), ApApG, GpApA, ApApC, ApUpU, ApApU, UpGpU (spleen phosphodiesterase), and UpUpG, CpCpG, CpUpA, CpUpC, UpCpC, CpUpU, CpCpU, CpCpA (pancreatic

Table III. Paper Chromatography of Mononucleotide Derivatives

	Solvent A	Solvent B	Solvent C
Uridine 3′-phosphate	0.08	0.30	0.30
Cytidine 2′,3′-cyclic phosphate	0.30	0.51	0.47
Uridine 2′,3′-cyclic phosphate	0.25	0.56	0.34
N-Acetylguanosine 3′-phosphate	0.056	0.31	
5′-O-Dimethoxytritylcytidine 2′,-3′-cyclic phosphate	0.78		
5′-O-Monomethoxytrityluridine 2′,3′-cyclic phosphate	0.81		
5′-O-Dimethoxytrityluridine 2′,-3′-cyclic phosphate	0.83		
5′-O-Dimethoxytrityladenosine 3′-phosphate	0.55		
5′-O-Monomethoxytritylcytidine 3′-phosphate	0.49		
5′-O-Dimethoxytritylcytidine 3′-phosphate	0.47		
5′-O-Monomethoxytrityl-N-acetylguanosine 3′-phosphate	0.44	0.64	
5′-O-Monomethoxytrityluridine 3′-phosphate	0.45		
5′-O-Dimethoxytrityluridine 3′-phosphate	0.48		
5′-O-Monomethoxytrityl-N,O$^{2'}$-dibenzoyladenosine 3′-phosphate		0.75	
5′-O-Dimethoxytrityl-N,O$^{2'}$-dibenzoyladenosine 3′-phosphate	0.75	0.75	
5′-O-Monomethoxytrityl-N,O$^{2'}$-diacetylcytidine 3′-phosphate		0.74	
5′-O-Dimethoxytrityl-N,O$^{2'}$-diacetylcytidine 3′-phosphate		0.76	
5′-O-Dimethoxytrityl-N,O$^{2'}$-dibenzoylcytidine 3′-phosphate	0.69	0.80	
5′-O-Monomethoxytrityl-N,O$^{2'}$-diacetylguanosine 3′-phosphate		0.70	
5′-O-Monomethoxytrityl-2′-O-benzoyluridine 3′-phosphate	0.60	0.82	
5′-O-Dimethoxytrityl-2′-O-acetyluridine 3′-phosphate		0.75	
5′-O-Dimethoxytrityl-2′-O-benzoyluridine 3′-phosphate	0.62	0.84	

ribonuclease). Complete degradation in the expected manner was obtained in all these cases and therefore the exclusive presence of the C_3'–C_5' internucleotidic linkages in all of the synthetic trinucleotides is assured.

Experimental Section

General Methods and Materials. Reagent grade pyridine was distilled and dried over calcium hydride or molecular sieve beads (4A) (Linde Co.) for several weeks. All evaporations were carried out using a rotary evaporator under reduced pressure. For condensations involving DCC, solutions were rendered anhydrous by repeated evaporation of added pyridine to pyridine solutions of the compounds using the vacuum from an oil pump and a Dry Ice–acetone trap.

Paper chromatography was performed using the descending technique on Whatman No. 1 paper except where noted otherwise. The solvent systems used were: solvent A, 2-propanol–concentrated ammonia–water (7:1:2, v/v); solvent B, ethanol–1 *M* ammonium acetate, pH 7.5, (7:3, v/v); solvent C, isobutyric acid–concentrated ammonia–water, pH 3.7, (66:1:33, v/v); solvent D, 1-propanol–concentrated ammonia–water (55:10:35, v/v); solvent E, 1-butanol–30% acetic acid (2:1, v/v). The R_f values of the different compounds are listed in Tables III, IV, and V. Paper electrophoresis for analytical purposes was performed in a high-voltage apparatus in which the paper was immersed in a high-boiling petroleum fraction (Varsol). The buffers used were: buffer I, phosphate buffer, pH 7.1, 0.03 *M*; buffer II, ammonium formate buffer, pH 2.7, 0.05 *M*. The paper electrophoretic mobilities of different compounds are included in Tables IV and V.

The trityl-containing compounds were made visible on paper chromatograms after spraying the chromatograms with the perchloric acid spray and warming in an oven. The compounds containing mono-*p*-methoxytrityl group appeared yellowish orange; those containing di-*p*-methoxytrityl group appeared orange-red.

Enzymic degradations of the synthetic products with pancreatic ribonuclease, spleen phosphodiesterase, and T_1-ribonuclease were carried out as described in earlier papers.[3a,c,24]

The abbreviation OD_{260} refers to the extinction of a nucleotidic solution at neutral pH at 260 mμ in 1 ml of solution using a 1-cm light path quartz cell.

Protected Nucleosides. 2′,3′-Di-O-benzoyluridine, N,O$^{2'}$,O$^{3'}$-tribenzoyluridine, N,N′,O$^{2'}$,O$^{3'}$-tetrabenzoyladenosine, N,O$^{2'}$,O$^{3'}$-tribenzoylcytidine, and N,O$^{2'}$,O$^{3'}$-triacetylguanosine were prepared as described previously.[3g] The last-mentioned compound was also prepared with the omission of the purification step involving silicic acid chromatography.[3g] Starting from 0.575 g of 5′-O-monomethoxytritylguanosine, 0.370 g (89%) of N,O$^{2'}$,O$^{3'}$-triacetylguanosine was obtained. Paper chromatography in solvent E showed a single nucleosidic product, a very small amount of methoxytritanol being present.

Fully Acylated Ribonucleoside 3′-Phosphates. Pyridinium N,O$^{2'}$-O$^{5'}$-triacetyladenosine 3′-phosphate, pyridinium N,O$^{2'}$,O$^{5'}$-tribenzoyladenosine 3′-phosphate, pyridinium N,O$^{2'}$,O$^{5'}$-triacetylcytidine 3′-phosphate, pyridinium 2′,5′-di-O-acetyluridine 3′-phosphate, and pyridinium N,O$^{2'}$,O$^{5'}$-triacetylguanosine 3′-phosphate were prepared as described previously.[3d,e,g] In the preparation of anhydrous mixtures of tetraethylammonium acetate or benzoate and of the mononucleotides for acetylation and for benzoylation last traces of pyridine were removed by evaporation of added dry toluene to the mixtures. After this treatment no odor of pyridine could be detected.

Uridine 2′,3′-Cyclic Phosphate. Dicyclohexylguanidinium uridine 2′,3′-phosphate was prepared according to procedures described previously.[3g,25] However, in some cases the 4-morpholine-N,N′-dicyclohexylcarboxamidinium salt of uridine 2′,3′-cyclic phosphate was used. The preparation is described as follows. A solution of pyridinium uridine 2′(3′)-phosphate (0.5 mmole), 4-morpholine-N,N′-dicyclohexylcarboxamidine[26] (1.25 mmoles), and DCC (500 mg) in dry pyridine (3 ml) was heated at 90–100° (bath temperature) for 3.5 hr. Examination of an aliquot with paper electrophoresis showed the reaction to be complete. Pyridine was evaporated and the residue was treated with water (2 ml). After extraction with ether (two 10-ml portions), the aqueous layer was filtered and evaporated, pyridine being added frequently. Subsequent precipitation from a pyridine–ether mixture gave 285 mg (98%) of 4-morpholine-N,N′-dicyclohexylcarboxamidinium uridine 2′,3′-cyclic phosphate as a colorless powder. Paper electrophoresis (17 OD_{260} units) showed this product to be homogeneous. The same experiment has been repeated on a much larger scale (3 mmoles) with similar result.

Ammonium 5′-O-Dimethoxytrityladenosine 3′-Phosphate. A mixture of anhydrous pyridinium adenosine 3′-phosphate (436 mg) (lyophilized and then dried over phosphorus pentoxide under vacuum) and anhydrous dimethylformamide (8 ml) was chilled to −20° and dimethoxytrityl chloride (1.05 g) then was added. The mixture was allowed to warm up to room temperature (30 min) under stirring. To the clear solution was added a solution of pyridine (0.10 ml) in dimethylformamide (2 ml) in four portions over a period of 40 min. After a total of 75 min, concentrated ammonium hydroxide (10 ml) in ethanol (50 ml) was added with external cooling. Examination of an aliquot by paper chromatography in solvent A showed three nucleotidic compounds: unreacted adenosine 3′-phosphate (1%), 5′-O-dimethoxytrityladenosine 3′-phosphate (69%), and bisdimethoxytrityladenosine 3′-phosphate (30%). The reaction mixture was evaporated under reduced pressure in the presence of an excess of pyridine. To the concentrated pyridine solution was added an equal volume of 1% aqueous ammonium hydroxide followed by 20% aqueous pyridine (about 30 ml). The total mixture was now extracted several times (three to five times) with ethyl acetate until the paper chromatographic (solvent A) examination of an aliquot of the aqueous layer showed it to be free of bisdimethoxytrityladenosine 3′-phosphate. The aqueous layer then was saturated with sodium sulfate and extracted with *n*-butyl alcohol (two 100-ml portions). The aqueous layer was checked by paper chromatography and shown to be essentially free of the desired product. The combined butanol

(24) S. Nishimura, T. M. Jacob, and H. G. Khorana, *Proc. Natl. Acad. Sci. U. S.*, **51**, 1494 (1964).

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

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Table IV. Paper Electrophoresis and Paper Chromatography of Dinucleoside Phosphates

	R_m[a]		R_f				
	I	II	A	B	C	D	E
HO–C^{Bz}–OBz–p–A^{Bz_2}–(OBz)$_2$				0.88			0.90
HO–C^{Ac}–OAc–p–A^{Bz_2}–(OBz)$_2$				0.88			0.83
HO–A^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$			0.86				0.86
HO–C^{Bz}–OBz–p–C^{Bz}–(OBz)$_2$			0.88				0.88
HO–G^{Ac}–OAc–p–C^{Bz}–(OBz)$_2$			0.70				0.69
HO–U–OAc–p–C^{Bz}–(OBz)$_2$			0.79				0.72
HO–A^{Bz}–OBz–p–U–(OBz)$_2$				0.85			0.81
HO–C^{Ac}–OAc–p–U–(OBz)$_2$				0.87			0.84
HO–G^{Ac}–OAc–p–U–(OBz)$_2$				0.78			0.52
HO–U–OAc–p–U^{Bz}–(OBz)$_2$				0.86			0.71
Up[b]	1.0	1.0		1.0	1.0	1.0	
U	0.0	0.0					
ApA	0.27	−0.26		1.14	2.64		
CpA	0.37	−0.42					
GpA	0.30	−0.01		1.04	1.75		
UpA	0.35	0.09		1.50	1.71		
ApC	0.29	−0.48					
CpC	0.34	−0.60					
GpC	0.32	−0.10					
UpC	0.35	0.0					
ApG	0.32	0.04		1.01	1.68	1.00	
CpG	0.38	−0.05		1.10	1.24	0.88	
GpG	0.27	0.30		0.82	0.78	0.75	
UpG	0.39	0.53		1.34	0.83	0.92	
ApU	0.36	0.11					
CpU	0.40	0.0					
GpU	0.38	0.52					
UpU	0.41	0.75					

[a] The negative sign implies migration in the opposite direction (toward cathode). [b] Of the following dinucleoside phosphates the R_f's relative to Up are given.

extract was clarified by the addition of a small amount of sodium sulfate, filtered, and evaporated in the presence of an excess of pyridine. The residual concentrated pyridine solution was added dropwise to a large excess of ether with stirring. The white precipitate was collected by centrifugation, washed with dry ether, and dried over phosphorus pentoxide under vacuum. The yield of the product, which was pure chromatographically, was 400 mg.

Dicyclohexylguanidinium 5′-O-Dimethoxytritylcytidine 2′,3′-Cyclic Phosphate. A solution of dry dicyclohexylguanidinium cytidine 2′,3′-cyclic phosphate (9.3 g) and dimethoxytrityl chloride (14 g) in anhydrous dimethylformamide (140 ml) was stirred for 30 min at room temperature with exclusion of moisture. To the solution then was added dropwise an anhydrous mixture of pyridine (1.4 ml) in dimethylformamide (10 ml) within 15 min. After the reaction mixture had been stirred for another 60 min, an ice-cold solution of concentrated aqueous ammonium hydroxide (10 ml) in ethanol (90 ml) was added and the mixture immediately evaporated *in vacuo* in order to remove excess of ammonia and ethanol. Subsequently, 1% aqueous ammonia was added until the mixture became cloudy, and the mixture then was extracted twice with ether to remove dimethoxytritanol. The aqueous layer was then saturated with sodium sulfate and repeatedly extracted with *n*-butyl alcohol (total volume, 1.5 l.). The organic solvent extract was clarified by addition of some sodium sulfate, filtered, and evaporated *in vacuo* at 20° in the presence of an excess of pyridine. The residual syrup was dissolved in a small amount of dry pyridine and the solution was added dropwise into an excess of ether–pentane (3:2, v/v) under stirring. The resulting white precipitate was collected by centrifugation, washed with ether–pentane, and dried over phosphorus pentoxide *in vacuo*. The yield was 12.4 g (85% calculated as dicyclohexylguanidinium salt) of chromatographically pure product (solvent A).

5′-O-Dimethoxytritylcytidine 3′-Phosphate. Method A. A solution of dicyclohexylguanidinium 5′-O-dimethoxytritylcytidine 2′,3′-cyclic phosphate (2 g) and ammonium acetate (400 mg) in 30% aqueous dimethylformamide (80 ml) was prepared and the pH of the solution was adjusted immediately to 7.5 with dilute aqueous ammonia. Crystalline pancreatic ribonuclease (20 mg) was added and the solution was incubated at 37°. The pH of the solution, which was stirred, was kept at pH 7.5–7.6 by the use of a pH titrator which automatically added 2 *N* ammonium hydroxide. After 8 hr, the ring opening of the cyclic phosphate was complete as shown by paper electrophoresis at pH 7.1. Furthermore the examination of an aliquot by paper chromatography in solvent A showed that less than 1% of the product was detritylated. To prevent any detritylation during the following work-up 10% aqueous tetraethylammonium hydroxide (7 ml) was added to the reaction mixture. After saturation of the solution with sodium sulfate, the mixture was extracted repeatedly with *n*-butyl alcohol (total volume 400 ml) until the aqueous phase contained only traces of the dimethoxytrityl product (analysis by paper chromatography). The *n*-butyl alcohol extract was clarified by addition of anhydrous sodium sulfate, filtered, and evaporated *in vacuo* (10^{-1} mm) at about 10° with frequent additions of pyridine. The anhydrous residue was taken up in pyridine and the solution was added dropwise to an excess of ether–pentane under stirring. The resulting white precipitate was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide under a vacuum. Paper chromatography in solvent A showed that the product was completely free of any detritylated material. The dry product was generally stored *in vacuo* over phosphorus pentoxide for periods of several months without detectable decomposition. Prior to acylation, the product was passed in ethanol–water–pyridine (50:45:5, v/v) through a column of pyridinium Dowex-50 X2 ion-exchange resin (200–400 mesh) in order to remove pancreatic RNase which still contaminated the above product. The total eluate was immediately used for the benzoylation reaction.

Method B. Anhydrous cytidine 3′-phosphate (3.4 g), prepared by lyophilization of the ammonium salt followed by drying over phosphorus pentoxide, was stirred with dimethoxytrityl chloride (12 g) in freshly distilled anhydrous dimethylformamide (200 ml). When a clear solution resulted an anhydrous mixture of pyridine (1.75 ml) in dimethylformamide (10 ml) was added in four portions every 30 min under exclusion of moisture. After a total of 4 hr a mixture of concentrated ammonium hydroxide (10 ml) in ethanol (90 ml) was added under cooling. Ethanol then was removed *in vacuo* and the mixture was diluted with 1% aqueous ammonium hydroxide (formation of an emulsion). After two extractions with ether, the aqueous layer then was evaporated under reduced pressure with frequent additions of pyridine. To the residual concentrated solution aqueous methanol was added and the solution was placed on a column (105 × 5 cm) of DEAE-cellulose (carbonate form). Elution was carried out with a linear gradient of triethylammonium bicarbonate (pH 7.5), the mixing vessel containing 0.02 *M* buffer (14 l) and the reservoir 0.5 *M* buffer in 20% ethanol (14 l), the flow rate being 2.5 ml/min. Pyridine appeared in the first peak and the

Table V. Paper Electrophoresis and Paper Chromatography of Trinucleotides

	R_m[a]		R_f	
	I	II	B	C
Up	1.00	1.00	1.00	1.00
U	0.0	0.0	2.50	1.65
ApApA	0.53	−0.10	0.40	2.22
CpApA	0.52	−0.19	0.52	2.36
GpApA	0.46	0.10	0.37	1.68
UpApA	0.51	0.19	0.60	1.77
ApCpA	0.55	−0.18	0.43	2.13
CpCpA	0.59	−0.34	0.58	1.73
GpCpA	0.56	0.04	0.38	1.26
UpCpA	0.64	0.11	0.57	1.36
ApGpA	0.43	0.10	0.34	1.85
CpGpA	0.53	0.06	0.41	1.40
GpGpA	0.48	0.34	0.30	0.91
UpGpA	0.53	0.53	0.53	0.91
ApUpA	0.49	0.23	0.60	1.94
CpUpA	0.56	0.11	0.66	1.46
GpUpA	0.51	0.50	0.46	0.95
UpUpA	0.57	0.62	0.79	1.02
ApApC	0.51	−0.21	0.43	2.13
CpApC	0.58	−0.31	0.52	1.76
GpApC	0.54	0.10	0.36	1.20
UpApC	0.59	0.10	0.59	1.33
ApCpC	0.55	−0.29	0.44	1.83
CpCpC	0.59	−0.27	0.58	1.45
GpCpC	0.56	−0.03	0.42	0.90
UpCpC	0.61	0.0	0.67	1.00
ApGpC	0.49	0.09	0.36	1.30
CpGpC	0.56	−0.03	0.42	0.93
GpGpC	0.48	0.24	0.31	0.57
UpGpC	0.56	0.48	0.50	0.57
ApUpC	0.57	0.10	0.56	1.47
CpUpC	0.61	0.0	0.67	1.00
GpUpC	0.55	0.45	0.50	0.57
UpUpC	0.59	0.62	0.86	0.70
ApApG	0.47	0.08	0.32	1.69
CpApG	0.53	−0.02	0.43	1.16
GpApG	0.52	0.35	0.34	0.75
UpApG	0.54	0.45	0.53	0.84
ApCpG	0.52	0.03	0.36	1.31
CpCpG	0.58	−0.02	0.54	0.90
GpCpG	0.56	0.33	0.37	0.48
UpCpG	0.59	0.40	0.53	0.57
ApGpG	0.48	0.27	0.27	0.86
CpGpG	0.56	0.29	0.44	0.56
GpGpG	0.52	0.56	0.29	0.25
UpGpG	0.54	0.59	0.43	0.33
ApUpG	0.52	0.47	0.42	0.92
CpUpG	0.58	0.44	0.61	0.60
GpUpG	0.56	0.72	0.41	0.31
UpUpG	0.59	0.88	0.63	0.38
ApApU	0.54	0.11	0.46	1.61
CpApU	0.60	0.06	0.57	1.20
GpApU	0.51	0.50	0.46	0.97
UpApU	0.60	0.64	0.81	0.93
ApCpU	0.62	0.08	0.50	1.45
CpCpU	0.64	0.11	0.61	0.95
GpCpU	0.62	0.50	0.46	0.58
UpCpU	0.63	0.57	0.79	0.61
ApGpU	0.51	0.51	0.46	0.94
CpGpU	0.60	0.42	0.57	0.63
GpGpU	0.54	0.69	0.36	0.36
UpGpU	0.59	0.95	0.71	0.36
ApUpU	0.60	0.67	0.71	1.03
CpUpU	0.63	0.60	0.82	0.61
GpUpU	0.59	0.98	0.68	0.36
UpUpU	0.64	1.15	1.03	0.42

[a] The negative sign implies migration in the opposite direction (toward cathode).

second peak (at 0.1 *M* salt concentration) contained unreacted nucleotide (5900 OD_{272} units). It was followed by a major peak (beginning at about 0.17 *M* salt concentration) which contained the desired product. The appropriate fractions containing this product were pooled (68,000 OD_{272} units) and after addition of 10% aqueous tetraethylammonium hydroxide (2.1 equiv with respect to the nucleotide) were evaporated under reduced pressure (10^{-1} mm) with frequent additions of pyridine. The product was precipitated with ether in the usual way, collected by centrifugation, washed with ether, and dried over phosphorus pentoxide under vacuum. The yield of the tetraethylammonium salt was 6 g (68%).

Ammonium 5′-O-Monomethoxytritylcytidine 3′-Phosphate. A mixture of anhydrous cytidine 3′-phosphate (1 g, prepared by lyophilization of aqueous pyridinium salt solution and drying over phosphorus pentoxide under vacuum) and monomethoxytrityl chloride (3.55 g) in anhydrous dimethylformamide (75 ml) was stirred for 90 min at room temperature. To the resulting clear solution was added dropwise in six portions (every 30 min) an anhydrous mixture of pyridine (1.2 ml) in dimethylformamide (6 ml). The mixture was stirred for a total of 24 hr at room temperature and subsequently poured into an excess of anhydrous ether under stirring, in order to remove unreacted monomethoxytrityl chloride. The solvent mixture was poured off and the gummy precipitate which formed immediately was treated with anhydrous ethanolic ammonia followed by an excess of anhydrous ether. Trituration afforded a powder which was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide (1.92 g). Examination by paper chromatography in solvent A showed besides the main product (67%) some unreacted material and traces of a bistrityl derivative. 5′-O-Monomethoxytritylcytidine 3′-phosphate was purified by chromatography on a DEAE–cellulose (carbonate) column (90 × 4 cm) with a linear gradient of triethylammonium bicarbonate using 4 l. of 0.01 *M* buffer in the mixing vessel and an equal volume of 0.5 *M* buffer in 15% ethanol in the reservoir. The first peak contained unreacted cytidine 3′-phosphate and the desired product appeared in the large second peak (at about 0.15 − 0.2 *M* salt concentration). The fractions of this peak were pooled and evaporated in the presence of frequently added pyridine under reduced pressure. The residual salt-free gum was dissolved in dilute ammonium hydroxide (0.5%), lyophilized (yield 1 g), and stored under vacuum in the dry state over phosphorus pentoxide.

Dicyclohexylguanidinium 5′-O-Dimethoxytrityluridine 2′,3′-Cyclic Phosphate. Dry dicyclohexylguanidinium uridine 2′,3′-cyclic phosphate (3.7 g) and dimethoxytrityl chloride (6 g) were dissolved in anhydrous pyridine (50 ml) and the solution was kept sealed for 2.5 hr at room temperature. A mixture of concentrated aqueous ammonium hydroxide (5 ml) in ethanol (30 ml) then was added under cooling. Examination of an aliquot by paper chromatography in solvent A showed that tritylation was quantitative. The mixture was diluted with water (250 ml) and the resulting white emulsion was extracted twice with ether to remove the dimethoxytritanol. The aqueous layer was further extracted (three 200-ml portions) with a mixture of ethyl acetate–*n*-butyl alcohol (2:1, v/v). The combined organic layer was clarified with a small amount of sodium sulfate and evaporated *in vacuo* in the presence of an excess of pyridine. The gummy residue was dissolved in a small amount of pyridine and the solution was added dropwise into an excess of ether under stirring. The white precipitate was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide *in vacuo*. The yield of dicyclohexylguanidinium 5′-O-dimethoxytrityluridine 2′,3′-cyclic phosphate was 4.6 g. Paper chromatography in solvent A showed it to be homogeneous.

Dicyclohexylguanidinium 5′-O-Monomethoxytrityluridine 2′,3′-Cyclic Phosphate. A solution of dry dicyclohexylguanidinium uridine 2′,3′-cyclic phosphate (8.75 g) and monomethoxytrityl chloride (15 g) in anhydrous pyridine (75 ml) was shaken for 15 hr in the dark at room temperature. A mixture of concentrated aqueous ammonium hydroxide (10 ml) in ethanol (30 ml) then was added with cooling and the solution was poured into ice–water (about 500 ml). This was extracted twice with ether (two 250-ml portions) and the aqueous layer was extracted further three times with *n*-butyl alcohol–ethyl acetate (1:2, v/v) (three 400-ml portions). Further work-up was as described above for the corresponding dimethoxytrityl derivative. The yield of the chromatographically pure dicyclohexylguanidinium 5′-O-monomethoxytrityluridine 2′,3′-cyclic phosphate was 10.34 g.

Pyridinium 5′-O-Dimethoxytrityluridine 3′-Phosphate. A solution of dicyclohexylguanidinium 5′-O-dimethoxytrityluridine 2′,3′-cyclic phosphate (3.5 g) and ammonium acetate (2 g) in 30% aqueous dimethylformamide was incubated (37°) with pancreatic RNase (30 mg) for 24 hr under stirring. The pH of the solution was maintained between 7.5 and 7.6 by automatic addition of 10 *M* ammonium hydroxide from a microsyringe. The reaction mixture

was then diluted with 1% aqueous ammonia until it developed turbidity, and it was extracted with ethyl acetate (two 250-ml portions) to remove traces of the unchanged starting material. Chromatography of an aliquot in solvent A showed that 5′-O-dimethoxytrityluridine 3′-phosphate was the only dimethoxytrityl-containing product in the aqueous solution, but in addition there was a small amount (about 8%) of uridine 3′-phosphate. The aqueous solution was saturated with sodium sulfate and then extracted with *n*-butyl alcohol (four 250-ml portions). The combined organic layer was clarified by addition of a small amount of sodium sulfate and after filtration the solution was evaporated *in vacuo* in the presence of added pyridine. The residual concentrated pyridine solution (about 30 ml) was added dropwise to a large excess of anhydrous ether with vigorous stirring. The resulting white precipitate was collected by centrifugation, washed with ether, and finally dried over phosphorus pentoxide under vacuum. The product (2.6 g) was chromatographically pure but was usually contaminated with some ammonium acetate and pancreatic RNase. While this product was conveniently stored as such, before further use the compound was passed through a column of pyridinium Dowex-50 X2 resin as its solution in a mixture of pyridine–methanol–water (2:5:3, v/v). The effluent and the washings were collected in a flask containing pyridine and the total solution was evaporated *in vacuo* at low temperature with frequent additions of pyridine. Pyridinium 5′-O-dimethoxytrityluridine 3′-phosphate was precipitated in the usual way from anhydrous ether and briefly dried over phosphorus pentoxide under vacuum. The yield of the pyridinium salt was 2.3 g (77%).

Pyridinium 5′-O-Monomethoxytrityluridine 3′-Phosphate. To dicyclohexylguanidinium 5′-O-monomethoxytrityluridine 2′,3′-cyclic phosphate (10.3 g) dissolved in dimethylformamide (170 ml) an aqueous ammonium acetate solution (5 g in 360 ml) was added under stirring. After warming the solution to 37°, the pH was adjusted to 7.1 with ammonium hydroxide, and pancreatic RNase (155 mg) dissolved in a small amount of water was added. The stirred incubation mixture (37°) was diluted with more water (about 50 ml) until the solution just developed turbidity. The pH was kept at 7.1–7.2 by the automatic addition of 7 *M* ammonium hydroxide from a microsyringe. After 24 hr the reaction was virtually complete. To remove traces of undigested material the mixture was extracted with ethyl acetate. The aqueous phase was concentrated under reduced pressure with frequent additions of pyridine to a smaller volume and the concentrated solution was passed through a column of pyridinium Dowex-50 X2 ion-exchange resin. The column was washed with aqueous ethanol (30%) containing 5% pyridine. To the effluent and washings was added an excess of pyridine and the solution evaporated *in vacuo* with frequent additions of pyridine. The final anhydrous and concentrated pyridine solution was added dropwise to a large excess of ether and the resulting precipitate was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide under vacuum. The yield of the pyridinium salt was 8.1 g (93%).

Pyridinium N-Acetylguanosine 3′-Phosphate. Pyridinium $N,O^{2'},O^{5'}$-triacetylguanosine 3′-phosphate (1.37 g) was dissolved in cold 1 *N* sodium hydroxide (50 ml) and the solution kept for 5 min at 0°. A sufficient amount of pyridinium Dowex-50 X8 ion-exchange resin was added under stirring and cooling to rapidly remove the sodium ions. After removal of the resin the solution was evaporated *in vacuo* as usual in the presence of pyridine. The final anhydrous residue was dissolved in dimethylformamide and the expected compound precipitated from an excess of anhydrous ether. The resulting white precipitate was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide (1.01 g; 87%).

Triethylammonium 5′-O-Monomethoxytrityl-N-acetylguanosine 3′-Phosphate. To an anhydrous solution of pyridinium N-acetylguanosine 3′-phosphate (484 mg) and monomethoxytrityl chloride (950 mg) in dimethylformamide (20 ml) a mixture of anhydrous pyridine (0.32 ml) and dimethylformamide (2 ml) was added in four portions every half-hour under stirring. After a total of 5 hr 9 *M* ammonium hydroxide (5 ml) was added while cooling and the excess of ammonia was removed immediately by evaporating the solution at reduced pressure (10 mm). Spectrophotometric analysis after paper chromatography of an aliquot in solvent A, showed a trace of unreacted starting material, some bismonomethoxytrityl-N-acetylguanosine 3′-phosphate, and the major product 5′-O-monomethoxytrityl-N-acetylguanosine 3′-phosphate (82%). The reaction mixture was evaporated in the presence of added pyridine until a concentrated anhydrous pyridine solution resulted. This was added dropwise under vigorous stirring into anhydrous ether. The white precipitate was collected by centrifugation and chromatographed at 4° on a DEAE-cellulose (carbonate) column[27] (47 × 4 cm) with a linear gradient of triethylammonium bicarbonate (pH 7.5). The mixing vessel contained 0.01 *M* buffer (3 l.) and the reservoir 0.4 *M* buffer containing 15% ethanol (3 l.), the flow rate being 1.2 ml/min. Pyridine appeared in the first peak and was followed by unreacted N-acetylguanosine 3′-phosphate. The major peak contained 5′-O-monomethoxytrityl-N-acetylguanosine 3′-phosphate. A subsequent peak contained 2′,5′-bis-O-monomethoxytrityl-N-acetylguanosine 3′-phosphate. Fractions of the major peak were pooled and evaporated at 10° under reduced pressure with frequent addition of pyridine during the evaporation. The evaporation in the presence of pyridine was repeated several times to ensure complete removal of triethylammonium bicarbonate. The final anhydrous pyridine solution (10 ml) was added dropwise into a large excess of anhydrous ether under vigorous stirring. The white precipitate was collected by centrifugation, washed with ether, and dried over phosphorus pentoxide. The yield was 600 mg.

Pyridinium 5′-O-Monomethoxytrityl-2′-O-benzoyluridine 3′-Phosphate and Pyridinium 5′-O-Dimethoxytrityl-2′-O-benzoyluridine 3′-Phosphate. These two compounds were prepared either by the procedure described previously[3i] for the 5′-O-dimethoxytrityl derivative or by a modified procedure which is described below for the 5′-O-monomethoxytrityl derivative. Pyridinium 5′-O-monomethoxytrityluridine 3′-phosphate (0.37 mmole)[28] and tetraethylammonium benzoate (4 mmoles) together were rendered anhydrous in the usual way, the last traces of pyridine being removed by evaporation of added toluene. Benzoic anhydride (8 mmoles) was added and after warming to effect a clear solution, the sealed reaction mixture was kept at room temperature for 3 days. Aqueous pyridine (10 ml of 50%) was added and the solution was extracted with pentane (two 10-ml portions) and then with chloroform (20 and 10 ml). The chloroform layer was washed with water (10 ml) and evaporated with added pyridine. The residual gum was dissolved in 3 ml of dry pyridine and the solution was added dropwise to a mixture of ether (300 ml) and pentane (200 ml). The precipitate was washed with pentane and treated with acetic anhydride (2 ml) in pyridine (4 ml). After 20 hr, methanol (2 ml) was added with cooling and the solution was kept for 15 min at room temperature. Aqueous pyridine (50 ml of 25%) was added and the solution was passed through a column of pyridinium Dowex-50 X2 (1.2 × 20 cm). The total effluent and washings (with 20% aqueous pyridine) were kept for 2 hr at room temperature and then were evaporated with added pyridine. The anhydrous residue was precipitated in the usual way with pyridine (1 ml) and ether (300 ml). The precipitate was washed with ether and used immediately for the next step. The yield was estimated spectrophotometrically and found to be 0.25 mmole, 68%.

In cases where traces of the detritylated product were detected on paper chromatography (solvent A) after removal of the benzoyl group, the required product was purified by extraction into ethyl acetate from 20% aqueous pyridine. The solvent was evaporated again in the constant presence of added pyridine and the product was reprecipitated from pyridine–ether–pentane mixture.

Pyridinium 5′-O-Monomethoxytrityl-N,$O^{2'}$-diacetylcytidine 3′-Phosphate and Pyridinium 5′-O-Dimethoxytrityl-N,$O^{2'}$-diacetylcytidine 3′-Phosphate. The two compounds were prepared by the procedure described below for the monomethoxytrityl derivative. An anhydrous mixture of pyridinium 5′-O-monomethoxytrityl-cytidine 3′-phosphate (0.5 mmole) and tetraethylammonium acetate (5 mmoles) was treated with acetic anhydride (0.5 ml) for 12 hr in the dark. A mixture (10 ml) of methanol–pyridine (4:1, v/v) then was added and after an additional 10 min at room temperature, the mixture was evaporated under reduced pressure. Methanol–pyridine–water (3:1:1, v/v, 50 ml) was added to the residue, and the solution was passed through a column (22 × 1.4 cm) of pyridinium Dowex-50 X2 ion-exchange resin. The effluent and the washings were kept for a total period of 2 hr at room temperature. (Paper electrophoresis (pH 7.1) of an aliquot showed complete breakdown of the acetyl-phosphate mixed anhydride.) The solution was evaporated in the usual way with pyridine. The residual anhydrous concentrated solution in pyridine. The residual

(27) In earlier experiments in which the column chromatography was done at room temperature, the product was partially N-deacetylated during that procedure.

(28) The same procedure has been carried out on a much larger scale, starting with about 8 g of pyridinium 5′-O-monomethoxytrityluridine 3′-phosphate.

anhydrous concentrated solution in pyridine was added dropwise to an excess of ether–pentane. The resulting white precipitate was collected by centrifugation, washed with ether, and kept dry over magnesium perchlorate in the cold. The yield was 320 mg (84%).

Pyridinium 5′-O-Dimethoxytrityl-N,O²′-dibenzoylcytidine 3′-Phosphate. An anhydrous mixture of tetraethylammonium 5′-O-dimethoxytritylcytidine 3′-phosphate (4.8 mmoles) and tetraethylammonium benzoate (48 mmoles) was treated with benzoic anhydride (100 mmoles) and the reactants were warmed briefly until a homogeneous syrup resulted, which was shaken for 3 days in the dark at room temperature. Subsequently, pyridine was added and the mixture was poured into ice–water under vigorous stirring. The resulting white emulsion was extracted twice with pentane, then twice with chloroform. The chloroform solution containing all the dimethoxytrityl derivatives was clarified with a small amount of sodium sulfate and then evaporated *in vacuo*. The residual gum was dissolved in pyridine and the solution was added dropwise to an excess of ether–pentane (3:2) under stirring. The resulting white precipitate was collected, washed with pentane–ether, and dried over phosphorus pentoxide *in vacuo*. This product was treated overnight with pyridine–acetic anhydride (2:1, v/v, 30 ml) in the dark and an excess of anhydrous methanol in pyridine (1:1, v/v) was then added under efficient cooling. After removal of methyl acetate and methanol *in vacuo*, the residue was dissolved in a mixture of pyridine–ethanol–water (3:5:2, v/v) and the solution was passed slowly through a column of pyridinium Dowex-50 X2 resin (200–400 mesh). The effluent and washings were kept at room temperature for 2 hr and were then evaporated *in vacuo* in the presence of frequently added pyridine. The residual gum was rendered anhydrous by repeated evaporation of pyridine and finally the concentrated pyridine solution was added dropwise to a vigorously stirred mixture of ether–pentane (3:2, v/v). The white precipitate was collected by centrifugation, washed with ether–pentane, and dried over phosphorus pentoxide *in vacuo*. The yield (3.35 mmoles) as determined spectrophotometrically was 70%.

Pyridinium 5′-O-Monomethoxytrityl-N,O²′-dibenzoyladenosine 3′-Phosphate. The procedure used for the benzoylation of pyridinium 5′-O-monomethoxytrityladenosine 3′-phosphate was very similar to that described previously for the preparation of N,O²′,O⁵′-tribenzoyladenosine 3′-phosphate from adenosine 3′-phosphate.[3e] The yield of the required product was 62%.

Pyridinium 5′-O-Dimethoxytrityl-N,O²′-dibenzoyladenosine 3′-Phosphate. An anhydrous mixture of ammonium 5′-O-dimethoxytrityladenosine 3′-phosphate (1 mmole) and tetraethylammonium benzoate (10 mmoles) was treated with benzoic anhydride (20 mmoles) and the mixture shaken for 2 days at room temperature in the dark. A mixture of benzoyl chloride (20 mmoles) and anhydrous pyridine (20 ml) then was added and the mixture was kept for an additional 20 hr in the dark. The reaction mixture was subsequently poured onto ice, and the mixture was extracted twice with pentane and then with chloroform (twice). The chloroform extract was dried over sodium sulfate and evaporated *in vacuo* in the presence of added pyridine. The residual concentrated pyridine solution was added dropwise to an excess of ether–pentane (1:1, v/v) with vigorous stirring. The resulting white precipitate was collected by centrifugation and dried *in vacuo* over phosphorus pentoxide. The dry powder was now treated with acetic anhydride (10 ml) in anhydrous pyridine (20 ml) for about 10 hr. A mixture of anhydrous methanol (20 ml) and pyridine (20 ml) then was added under cooling. After 10 min the mixture was partly evaporated *in vacuo* to remove the methyl acetate. A mixture (50 ml) of pyridine, ethanol, and water (3:5:2, v/v) then was added and the solution was passed through a pyridinium Dowex-50 X8 ion-exchange resin column. The effluent and washings were kept for 2 hr and were then evaporated under reduced pressure with frequent additions of pyridine. The compound was isolated in the usual way by precipitating it with ether–pentane and dried *in vacuo* over phosphorus pentoxide. The yield was 0.65 mmole (65%).

Pyridinium 5′-O-Monomethoxytrityl-N,O²′-diacetylguanosine 3′-Phosphate. An anhydrous mixture of triethylammonium 5′-O-monomethoxytrityl-N-acetylguanosine 3′-phosphate (415 mg), tetraethylammonium acetate (5.6 mmoles), and acetic anhydride (0.7 ml) was shaken in the dark for 5 hr.[29] Pyridine–methanol (4:1, v/v, 15 ml) then was added and, after 15 min, the mixture was evaporated. Subsequently, a mixture (50 ml) of methanol–water–pyridine (1:3:1, v/v) was added and after 45 min, the solution was passed slowly through a column of pyridinium Dowex-50 X8 resin (22 × 1.4 cm). The effluent and washings were kept for a total period of 2 hr. The solution was evaporated at 10° under reduced pressure in the presence of added pyridine. The residual anhydrous pyridine solution was added dropwise into anhydrous ether under vigorous stirring. The white precipitate which formed was collected by centrifugation, washed with ether, dried, and kept as the pyridinium salt over silica gel and potassium hydroxide in the cold (390 mg, 89%).

General Procedure for the Synthesis of Protected Dinucleoside Phosphates. A. Using DCC. A mixture of the protected mononucleotide (0.1–0.4 mmole), the protected nucleoside (2–3 equiv with respect to the nucleotide), and dry pyridinium Dowex-50 ion resin (0.3–0.8 g) was rendered anhydrous by repeated evaporation of added dry pyridine. Anhydrous pyridine (0.5–3 ml) and DCC (about 10 equiv as based on the nucleotide) were added to the residue and the resulting mixture was kept at room temperature in the dark for 3–6 days. Before work-up of the total reaction mixture, an aliquot was removed and treated with 50% aqueous pyridine (4–8 hr), then successively with aqueous acetic acid and methanolic ammonium hydroxide to remove the protecting groups, and the products were examined by paper electrophoresis at pH 7.1. In most cases, the yields of the condensation products were excellent and very little of the unreacted mononucleotidic component was detected. For isolation of the protected dinucleoside phosphate, aqueous pyridine (2–10 ml) was added and the excess of DCC was extracted with pentane. The aqueous pyridine solution was kept for 4–16 hr at room temperature and the ion-exchange resin and the insoluble dicyclohexylurea were removed by filtration. The filtrate was evaporated to dryness and the residue was dissolved in 80% acetic acid (3–50 ml) and the acidic solution was kept for 15 min (for removal of the dimethoxytrityl group) or for 3–4 hr (for removal of the monomethoxytrityl group) at room temperature. The solvent was removed under vacuum and the residue was taken up in chloroform or ethanol (15–30 ml) and put on top of a TEAE–cellulose (acetate) column (about 3 × 33 cm), previously equilibrated with 95% ethanol. Elution was carried out using a linear gradient of triethylammonium acetate; the mixing vessel contained 95% ethanol (1–2 l) and the reservoir contained 0.1 *M* triethylamine and 0.2 *M* acetic acid in 95% ethanol (1–2 l.), the flow rate being 1–2 ml/min. Only the center fractions of the main peak were pooled and evaporated with added pyridine. The resulting anhydrous solution was added dropwise to an excess of ether–pentane mixture. The precipitate was collected by centrifugation, washed, and dried *in vacuo* over phosphorus pentoxide. For conversion to the pyridinium salt, it was dissolved in aqueous pyridine and passed through a small column of pyridinium Dowex-50 X2 ion-exchange resin, and the effluent and washings were lyophilized. The white, fluffy powder was stored in a desiccator over magnesium perchlorate. The yields of the protected dinucleoside phosphates prepared and isolated after column chromatography are given in Table I.

B. Using TPS. To an anhydrous pyridine solution (2 ml) of a protected mononucleotide (0.3 mmole) and a protected nucleoside (about 3 equiv with respect to the nucleotide) was added in the dry-box TPS (about 2 equiv relative to the mononucleotide). The mixture was kept for 6–8 hr at room temperature and was then treated with 50% aqueous pyridine (3 ml) under cooling. After 30 min at room temperature the solvent was evaporated and the removal of the trityl protecting group and the subsequent work-up were carried out as described above for DCC reactions.

General Procedure for the Synthesis of Trinucleoside Diphosphates. A. Using DCC. A mixture of the protected dinucleoside phosphate (5–10 μmoles), the fully acylated ribonucleoside 3′-phosphate (3–4 equiv with respect to the protected dinucleoside phosphate), and dry pyridinium Dowex-50 resin (50–150 mg) was rendered anhydrous by repeated evaporation of dry pyridine. Anhydrous pyridine (0.2–0.5 ml) and DCC (about 10 equiv based on the amount of mononucleotide) were added to the residue and the resulting mixture was kept at room temperature in the dark for 3–7 days. Then an equal amount of water was added and the excess of DCC was removed by extraction with cyclohexane. The aqueous pyridine solution was kept at room temperature for 16 hr. The resin and the insoluble dicyclohexylurea were removed by filtration and the filtrate was evaporated to dryness and the residue treated in a well-stoppered flask with cold 15 *M* methanolic ammonia (2–5 ml). The duration of ammonia treatment varied between 8 and 20 hr at room temperature depending upon the protecting groups on the heterocyclic rings.[3g] Thus for oligonucleotides containing only N-benzoyladenine the time was 10 hr, for those containing N-

(29) When the starting nucleotide contained partially N-deacetylated material, the acetic anhydride treatment was extended to 24 hr.

benzoylcytosine the time was 8 hr, but for those containing N^2-acetylguanine 20 hr was given. Subsequently, after evaporation of the solvent, the residue was dissolved in aqueous pyridine and subjected to paper electrophoresis (12–14 v/cm) on Whatman No. 3 MM or 31 paper in 0.05 *M* triethylammonium bicarbonate buffer (pH 7.5) at 2°. Five major ultraviolet light absorbing bands were observed. In order of decreasing mobility, these generally corresponded to: mononucleotide (band 1), nucleoside 2′,3′-cyclic phosphate (band 2), trinucleotide (band 3), unreacted dinucleoside phosphate (band 4), and benzamide (band 5) at the origin. The desired product, trinucleotide (band 3) was not clearly resolved from band 2. After cutting out the band and eluting the nucleotidic material with water containing a little ammonium hydroxide, further purification was accomplished by rechromatography on a strip of Whatman No. 40 paper in solvent A or D. The trinucleotide band (slower travelling) was eluted and the material stored frozen. The yield, which was estimated spectrophotometrically, was based on the dinucleoside phosphate recovered unchanged.

B. Using TPS. To an anhydrous pyridine solution (0.5 ml) containing the protected dinucleoside phosphate (10 μmoles) and the fully acylated mononucleotide (3 equiv with respect to the dinucleoside phosphate) was added in the drybox TPS (1.5–2 equiv with respect to the mononucleotide) and the sealed reaction mixture kept for 4–12 hr at room temperature. An equal volume of water then was added under cooling and the mixture kept at room temperature for 12–24 hr. After evaporating to dryness, the subsequent work-up including treatment with methanolic ammonia was as described in the preceding procedure.

The details of the individual syntheses leading to the various trinucleotides are given in Table II.

Studies on Polynucleotides. LII.[1] The Use of 2,4,6-Triisopropylbenzenesulfonyl Chloride for the Synthesis of Internucleotide Bonds[2]

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Abstract: Detailed studies of the $C_{3'}$–$C_{5'}$ interribonucleotide bond formation using mesitylenesulfonyl chloride and 2,4,6-triisopropylbenzenesulfonyl chloride are reported. While both reagents are efficient, the latter reagent shows, as expected, a much reduced rate of sulfonylation of the 5′-hydroxyl group of nucleosides. Syntheses of a large number of dinucleoside phosphates in high yield were accomplished using triisopropylbenzenesulfonyl chloride. The use of the reagent in the stepwise synthes's of ribotrinucleotides is given in the accompanying paper.[1]

Dicyclohexylcarbodiimide and arylsulfonyl chlorides previously have been shown to be the most satisfactory reagents for the synthesis of internucleotide bonds.[3] The characteristic features of the latter class of reagents are the great rapidity of reaction and the fact that nucleotide salts of strongly basic tertiary amines can be used.[3–5] The previous studies have, however, been all in the deoxyribonucleotide field where the synthetic work utilizes the condensation of a deoxyribonucleoside 5′-phosphate with the 3′-hydroxyl group of another nucleoside or oligonucleotide component. In the ribooligonucleotide field, where the most satisfactory approach involves the condensation of a protected ribonucleoside 3′-phosphate with the 5′-hydroxyl group of a second component,[1] the reagent used almost exclusively so far has been dicyclohexylcarbodiimide,[6,7] and no systematic studies of the utility of arylsulfonyl chlorides have been reported. In undertaking such studies which are the subject of the present paper, a further consideration was the possibility of facile sulfonylation of the primary hydroxyl group of the nucleosides in contrast with the slow and rather insignificant sulfonylation of the 3′-hydroxyl group in deoxyribonucleosides.[3] In fact, Michelson[8] had already noted that under certain conditions sulfonylation of the 5′-hydroxyl group could be a major reaction in attempted activation of nucleoside 2′,3′-cyclic phosphates for polynucleotide synthesis. It therefore was considered desirable to investigate the use of a sulfonyl chloride more hindered than mesitylenesulfonyl chloride,[3–5] which has been used successfully previously in the deoxyribopolynucleotide field. It should be added that the *ortho*-methyl groups in arylsulfonyl chlorides have been shown to offer only minor steric hindrance in sulfonylation reactions[9,10] and therefore we have investigated, in particular, the use of 2,4,6-triisopropylbenzenesulfonyl chloride[11] (TPS) for the synthesis of interribonucleotidic linkage.

(1) Paper LI is by R. Lohrmann, D. Söll, H. Hayatsu, E. Ohtsuka, and H. G. Khorana, *J. Am. Chem. Soc.*, **88**, 819 (1966).

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(3) T. M. Jacob and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 1630 (1964). References to earlier relevant papers are contained in this paper.

(4) T. M. Jacob and H. G. Khorana, *ibid.*, **87** 2971 (1965).

(5) S. A. Narang and H. G. Khorana, *ibid.*, **87**, 2981 (1965).

(6) S. Chladek and J. Smrt, *Collection Czech. Chem. Commun.*, **29**, 214 (1964), and J. Smrt, *ibid.*, **29**, 2049 (1964), have reported on the use of *p*-toluenesulfonyl chloride and dicyclohexylcarbodiimide in the synthesis of dinucleoside phosphates. No detailed study of the arylsulfonyl chloride as phosphorylating agent was reported, however.

(7) The statement is restricted to the approach for the synthesis of interribonucleotide bonds which relies on the activation of phosphomonoester groups. The use of sulfonyl chlorides for the activation of ribonucleoside 2′,3′-cyclic phosphates has been described by R. Letters and A. M. Michelson, *J. Chem. Soc.*, 71 (1962).

(8) A. M. Michelson, *ibid.*, 979 (1962).

(9) W. S. Johnson, J. C. Collins, Jr., R. Pappo, M. B. Rubin, P. J. Krupp, W. F. Johns, J. E. Pike, and W. Bartmann, *ibid.*, **85**, 1409 (1963).

(10) J. F. Bunnett and J. Y. Bassett, Jr., *J. Am. Chem. Soc.*, **81**, 2104 (1959), have shown that in the nucleophilic attack on arylsulfonate sulfur, *o*-methyl groups show only minor effect.

(11) A. Newton, *ibid.*, **65**, 2439 (1943).