Studies on Polynucleotides. XXXV.[1] The Specific Synthesis of $C_{3'}$-$C_{5'}$-Linked Ribooligonucleotides. VIII.[2] The Synthesis of Ribodinucleotides Bearing 3'-Phosphomonoester Groups[3]

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Two approaches were investigated for the synthesis of ribodinucleotides bearing 3'-phosphomonoester groups. The first involved the condensation of a fully protected ribonucleoside 3'-phosphate, e.g., N,O[2'],O[5']-triacetyladenosine 3'-phosphate, with another protected nucleotide bearing a free 5'-hydroxyl group, e.g., 2'-O-acetyluridine 3'-phosphate. The yield of the desired dinucleotides was 16–20% in this approach. The second approach, which gave much the more satisfactory results, involved the condensation of fully protected purine or pyrimidine ribonucleoside 3'-phosphates with uridine-2',3'-cyclic phosphate prepared, in situ, from uridine 2'(or 3')-phosphate. After a work-up including several, but straightforward, steps, the dinucleotides were isolated by anion exchange chromatography. Cytidylyl-(3'→5')-uridine 3'-phosphate, adenosine-(3'→5')-uridine 3'-phosphate, and inosinyl-(3'→5')-uridine 3'-phosphate were thus obtained in yields of 53, 47, and 44%, respectively, using stoichiometric amounts of the two mononucleotide components.

Introduction

Short-chain ribopolynucleotides of known sequence are of interest in the study of the amino acid code by the *in vitro* amino acid incorporation system.[5] One relatively rapid method for the synthesis of specific ribopolynucleotides may be expected to be the polymerization of preformed di- and triribonucleotides. The chemical methods for the envisaged polymerization reaction[6] will require, in rather large quantities, appropriately protected derivatives of the di- and trinucleotides. The present and an accompanying paper[7] report on a systematic investigation of the chemical synthesis of ribodinucleotides bearing the desired 3'-phosphate end groups. The alternative means of obtaining this class of compounds, namely by degradation of the ribonucleic acids with ribonucleases of defined specificity,[8] was considered at the outset of this investigation. However, for the quantities of the materials in question, the approach appeared to be no less demanding and, furthermore, the chemical studies were undertaken with the hope of advancing the synthetic chemistry of the ribooligonucleotides.

The approach which has emerged as the most attractive for the specific synthesis of the $C_{3'}$-$C_{5'}$ interribonucleotidic linkage utilizes as the key intermediates ribonucleoside 3'-phosphates appropriately protected in the 2'-hydroxyl position.[2c–f] For the synthesis of ribooligonucleotides containing only one type of nucleotide, 2'-O-acetyluridine 3'-phosphate and N-benzoyl-2'-O-acetyladenosine 3'-phosphate have been prepared and their polymerization has afforded, respectively, uridine and adenosine oligonucleotides. Extension of the same principles to the polymerization of preformed ribodinucleotides would require compounds of type I. The starting materials for these protected derivatives, therefore, would be ribodinucleotides bearing 3'-phosphomonoester groups. Three approaches designated (a), (b), and (c) in the general formulae of Chart I were investigated. For clarity of presentation of the chemistry involved approaches (a) and (b) form the subject of the present paper, while work on approach (c) is described in the following paper.[7] A brief report of part of this work has already appeared.[9] Recently, using the tetrahydropyranyl group for the protection of the 2'-hydroxyl group in ribonucleoside 3'-phosphates,[2a–c] Smrt and Sorm[10] have also described the synthesis of several pyrimidine ribodinucleotides.[11]

Approach (a). Previously, the synthesis of deoxyribodinucleotides bearing 5'-phosphomonoester groups by condensation of a N,O[3']-diacyldeoxyribonucleoside 5'-phosphate with another deoxyribonucleoside 5'-phosphate (bearing a free 3'-hydroxyl group) has been described.[12] Analogously, the first experiments in the

(1) Paper XXXIV: R. Lohrmann and H. G. Khorana, *J. Am. Chem. Soc.*, **86**, 4188 (1964).

(2) Previous papers which deal directly with this topic: (a) M. Smith, D. H. Rammler, I. H. Goldberg, and H. G. Khorana, *ibid.*, **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) ref. 1.

(3) This work has been supported in part by Public Health Service Research Grant No. CA-05178 from the National Cancer Institute, the National Science Foundation Grant No. GB-976, and Life Insurance Medical Research Grant No. G-62-54.

(4) Holder of a Wisconsin Alumni Research Fund Postdoctoral Fellowship.

(5) For a recent summary of the total work on the amino acid code see F. H. C. Crick in "Progress in Nucleic Acid Research," Vol. I, J. N. Davidson and W. E. Cohn, Ed., Academic Press Inc., New York, N. Y., 1963, p. 163.

(6) Michelson [*J. Chem. Soc.*, 3655 (1959)] studied briefly the polymerization of a few di- and triribonucleotides without providing any protection of the 2'-hydroxyl groups in them. The products, although not adequately characterized, clearly contained random $C_{2'}$-$C_{5'}$, and $C_{3'}$-$C_{5'}$-internucleotidic linkages.

(7) D. Söll and H. G. Khorana, *J. Am. Chem. Soc.*, **87**, 360 (1965).

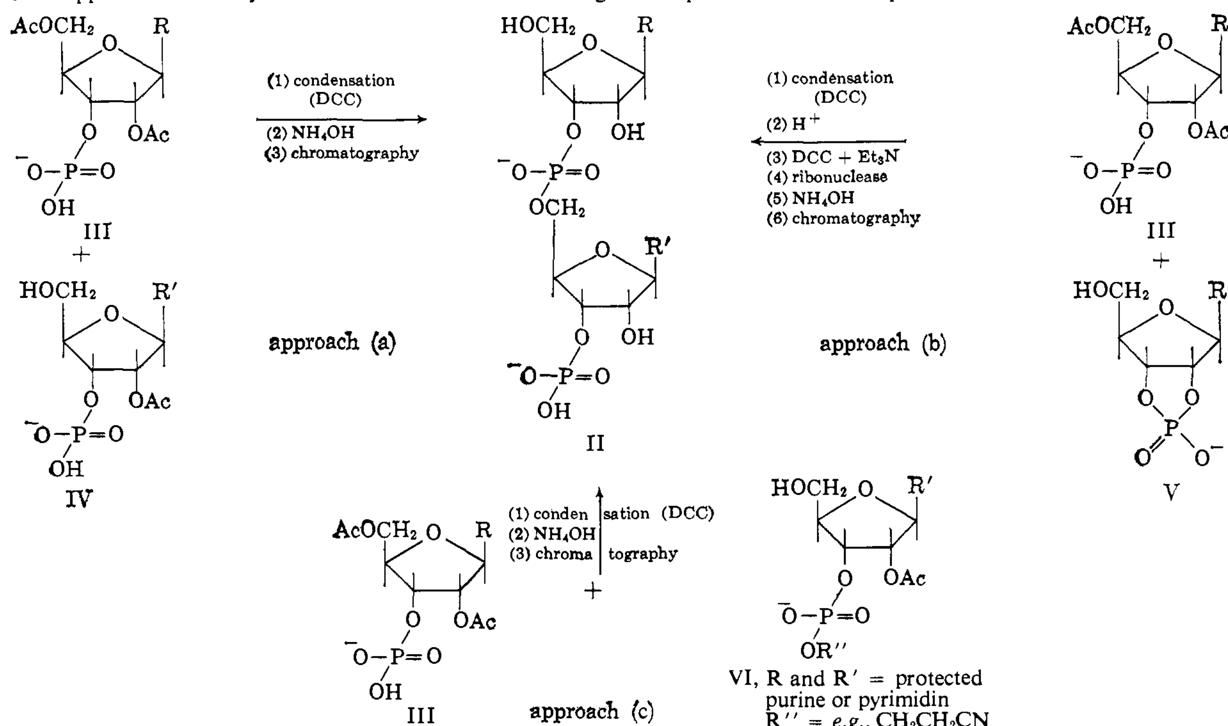
(8) H. G. Khorana in "The Enzymes," Vol. V, P. D. Boyer, H. A. Lardy, and K. Myrback, Ed., Academic Press Inc., New York, N. Y., 1961, p. 79.

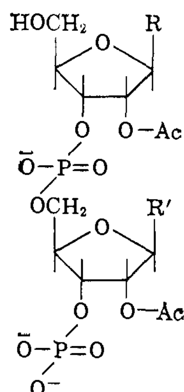
(9) D. Söll and H. G. Khorana, *Angew. Chem.*, **76**, 435 (1964); *Angew. Chem., Intern. Ed. Engl.*, **3**, 374 (1964).

(10) J. Smrt and F. Sorm, *Collection Czech. Chem. Commun.*, **28**, 2415 (1963).

(11) Other aspects of this work,[10] namely, the use of the methoxytrityl group for the protection of the 5'-hydroxyl group and the use of DCC as the condensing agent, are similar to the current work in this laboratory.[2]

Chart I. Approaches to the Synthesis of Ribodinucleotides Bearing 3′-Phosphomonoester Groups





I, R and R′, = protected purine or pyrimidine
Ac = an acyl group

present work were directed to the synthesis of ribodinucleotides (II) by the condensation of, *e.g.*, N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate with another protected ribonucleoside 3′-phosphate bearing a free 5′-hydroxyl group, *e.g.*, 2′-O-acetyluridine 3′-phosphate. The condensing agents tried were dicyclohexylcarbodiimide (DCC) and mesitylenesulfonyl chloride, the solvents investigated for the reaction being anhydrous pyridine and dimethylformamide. Before removal of the protecting groups from the reaction products, an acetic anhydride–pyridine treatment[13] was given in order to cleave the residual pyrophosphate bonds which linked the oligonucleotides through the terminal 3′-phosphomonoester groups. The products were separated by chromatography on DEAE-cellulose anion exchanger columns and subsequently characterized by a combination of paper chromatography (Table I), paper electrophoresis (Table II), ultraviolet absorption spectra, and appropriate enzymatic degradation. The results of a typical run are given in Figure 1[14] and Table III, the desired dinucleotide, adenylyl-(3′→5′)-uridine 3′-phosphate, being present in peak IX.

While the concomitant formation of uridine homopolymers and of some side products was to be expected, the yield of the mixed dinucleotide, adenylyl-(3′→5′)-uridine 3′-phosphate, did not exceed about 20%[15] even when a rather high proportion (stoichiometric with 2′-O-acetyluridine 3′-phosphate) of N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate was used. DCC gave the better results in this approach, mesitylenesulfonyl chloride gave only a little (under 5%) of the desired dinucleotide, the major product being uridine-3′,5′-cyclic phosphate.

The extent of the intramolecular reactions leading to the undesired side products (*e.g.*, nucleoside-3′,5′-cyclic phosphates, 5′-C-pyridinium nucleotides, and derivatives) is evidently much greater when nucleoside 3′-phosphates rather than nucleoside 5′-phosphates are used as the starting materials.[2f,16,17] The results (Table III) of the present work further supported this conclusion. Indeed, rather large amounts of 5′-C-pyridinium uridine 3′-phosphate and uridine-3′,5′-cyclic phosphate were found as products. An experiment carried out, in an attempt to throw some light on the formation of the 5′-C-pyridinium compounds, is worthy of note. Authentic uridine-3′,5′-cyclic phosphate[18] was acetylated in

(12) H. G. Khorana, A. F. Turner, and J. P. Vizsolyi, *J. Am. Chem. Soc.*, **83**, 687 (1961).

(13) H. G. Khorana and J. P. Vizsolyi, *ibid.*, **81**, 4660 (1959); H. G. Khorana, J. P. Vizsolyi, and R. K. Ralph, *ibid.*, **84**, 414 (1962).

(14) Whereas chromatography in the standard way separated the dinucleotides adenylyluridine 3′-phosphate and uridylyluridine 3′-phosphate, chromatography in the presence of 7 *M* urea [R. V. Tomlinson and G. M. Tener, *Biochemistry*, 2, 697 (1963)] gave the dinucleotides as a single peak. Subsequent chromatography on a DEAE–cellulose column without urea resolved the two compounds, uridylyluridine 3′-phosphate emerging first.

(15) Including the yield of the dinucleotide present as adenylyl-(3′→5′)-uridine-2′,3′-cyclic phosphate (peak VII of Figure 1).

(16) A. F. Turner and H. G. Khorana, *J. Am. Chem. Soc.*, **81**, 4651 (1959).

(17) G. Weimann and H. G. Khorana, *ibid.*, **84**, 419 (1962).

(18) M. Smith, G. I. Drummond, and H. G. Khorana, *ibid.*, **83**, 698 (1961).

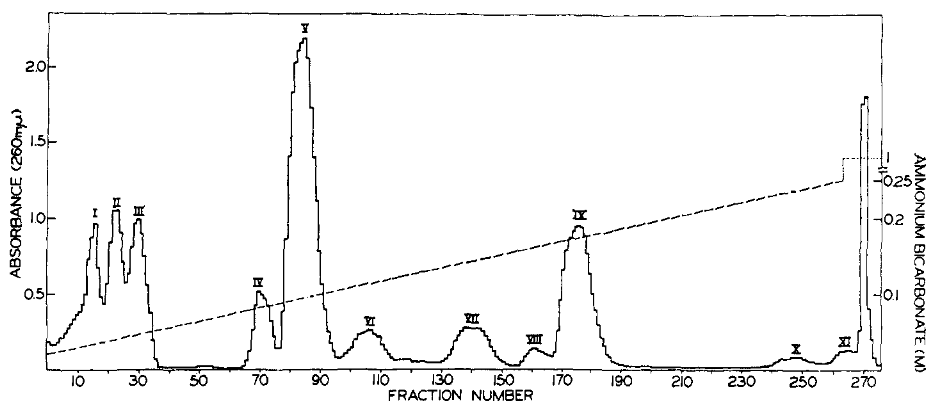


Figure 1. Chromatography of products formed by the condensation of N,O2',O5'-triacetyladenosine 3'-phosphate and 2'-O-acetyluridine 3'-phosphate (expt. II) on a DEAE–cellulose (carbonate) column. For details of the gradient see Experimental. The characterization of the peaks is given in Table III.

Table I. Paper Chromatography of Nucleotides and Derivatives

Compound	R_f Solvent A	Solvent B	Solvent C
Uridine 3'-phosphate	0.09	0.30	0.35
Cytidine 3'-phosphate	0.10	0.27	0.36
Adenosine 3'-phosphate	0.10	0.21	0.38
Inosine 3'-phosphate	0.063	0.25	0.34
Uridine-2',3'-cyclic phosphate	0.26	0.58	0.54
Uridine-3',5'-cyclic phosphate	0.26	0.58	0.54
Adenosine-2',3'-cyclic phosphate	0.39	0.50	0.61
5'-O-Dimethoxytrityluridine 3'-phosphate	0.49		
Bis-2',5'-O-dimethoxytrityluridine 3'-phosphate	0.68		
N,O2',O5'-Triacetyladenosine 3'-phosphate		0:67	
N,O2',O5'-Triacetyladenosine 3'-acetyl phosphate		0.78	
2',5'-Di-O-acetylinosine 3'-phosphate		0.58	
N,O2',O5'-Triacetylcytidine 3'-phosphate		0.70	
Adenylyl-(3'→5')-uridine	0.17	0.40	
Methyl adenosine 3'-phosphate	0.40	0.58	
2'-O-Acetyluridine-3',5'-cyclic phosphate		0.75	
N-(5'-C-Pyridinium-2'-O-acetyluridylyl)-N,N'-dicyclohexylurea		0.91	
N-(5'-C-Pyridinium-uridylyl)-N,N'-dicyclohexylurea		0.88	
5'-C-Pyridinium-uridine 3'-phosphate	0.05	0.26	0.37
N-(Uridylyl)-N,N'-dicyclohexylurea	0.80	0.90	0.90
N-(Adenylyl)-N,N'-dicyclohexylurea	0.80	0.85	0.90
Cytidylyl-(3'→5')-uridine-(2')3'-phosphoryl-N,N'-dicyclohexylurea	0.53	0.78	0.81
Adenylyl-(3'→5')-uridine-2',3'-cyclic phosphate	0.11	0.27	0.38
Uridine cyclic dinucleotide	0.11	0.50	0.41
Uridylyl-(3'→5')-uridine 3'-phosphate	0.24[a]		0.30
Cytidylyl-(3'→5')-uridine 3'-phosphate	0.36[a]	0.49[a]	0.29
Adenylyl-(3'→5')-uridine 3'-phosphate	0.34[a]	0.44[a]	0.30
Inosinyl-(3'→5')-uridine 3'-phosphate	0.18[a]	0.39[a]	0.28
Cytidylyl-(3'→5')-uridylyl-(2'→5')-uridine 3'-phosphate	0.12[a]	0.24[a]	0.23

[a] R_f relative to that of disodium uridine 3'-phosphate.

Table II. Paper Electrophoretic Mobilities of Nucleotides and Derivatives

Compound	Mobility pH 7.1[a]	pH 2.7[a]
Uridine 3'-phosphate	1.00	1.00
Adenosine 3'-phosphate		0
Uridine-2',3'-cyclic phosphate	0.63	1.00
Uridine-3',5'-cyclic phosphate	0.63	1.00
Adenosine-2',3'-cyclic phosphate	0.61	0
Cytidine-2',3'-cyclic phosphate	0.63	0
Inosine-2',3'-cyclic phosphate	0.63	
2'-O-Acetyluridine 3'-phosphate	0.87	
2'-O-Acetyluridine 3'-acetyl phosphate	0.50	
N,O2',O5'-Triacetyladenosine 3'-phosphate	0.68	
N,O2',O5'-Triacetyladenosine 3'-acetyl phosphate	0.51	
2',5'-Di-O-acetylinosine 3'-phosphate	0.86	
N,O2',O5-Triacetylcytidine 3'-phosphate	0.81	
Adenylyl-(3'→5')-uridine	0.37	0
Methyl adenosine 3'-phosphate	0.55	
2'-O-Acetyluridine-3',5'-cyclic phosphate	0.57	
N-(5'-C-Pyridinium-2'-O-acetyluridylyl)-N,N'-dicyclohexylurea	0	
N-(5'-C-Pyridinium-uridylyl)-N,N'-dicyclohexylurea	0	
5'-C-pyridinium-uridine 3'-phosphate	0.43	
5'-C-pyridinium-uridine	−1.07[b]	
N-(Uridylyl)-N,N'-dicyclohexylurea	0.42	0.77
N-(Adenylyl)-N,N'-dicyclohexylurea	0.28	
Cytidylyl-(3'→5')-uridine-2'(3')-phosphoryl-N,N'-dicyclohexylurea	0.60	0.61
Adenylyl-(3'→5')-uridine-2',3'-cyclic phosphate	0.70	
Uridine cyclic dinucleotide	0.78	
Uridylyl-(3'→5')-uridine 3'-phosphate	1.03	
Cytidylyl-(3'→5')-uridine 3'-phosphate	1.03	0.72
Adenylyl-(3'→5')-uridine 3'-phosphate	0.94	0.73
Inosinyl-(3'→5')-uridine 3'-phosphate	1.02	1.17
Cytidylyl-(3'→5')-uridylyl-(2'→5')-uridine 3'-phosphate	1.03	

[a] The mobilities under these columns for the compounds derived from different nucleotides are relative to those of the parent ribonucleoside 3'-phosphate except for the di- and trinucleotides and their derivatives, where the mobilities are all relative to that of uridine 3'-phosphate. [b] The negative sign implies migration in the opposite direction (toward the cathode).

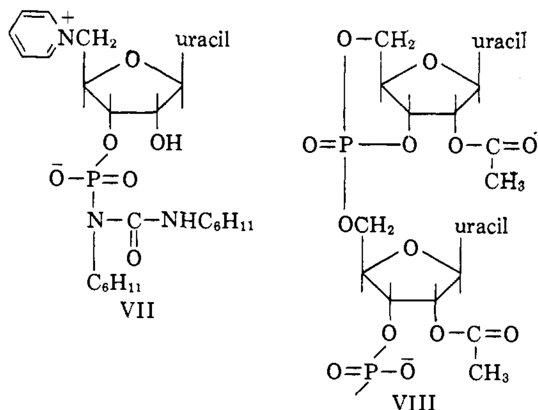
the 2'-hydroxyl position and the acetylated derivative treated with DCC in anhydrous pyridine for a period of up to three weeks. After a work-up including an ammoniacal treatment to remove the acetyl group, paper chromatography showed the slow formation (43% in three weeks) of a new product which is concluded (see Experimental) to be VII. While the formation of this

phosphorylurea is interesting, its slow rate of formation and the fact that it was present only in a small amount in the reaction products[19] (Table III) show that, in the polymerization experiments, the formation of a phosphorylurea from the initially formed uridine-3′,5′-cyclic phosphate is not the major route to the formation of the C5′-pyridinium compounds. The major reaction responsible for the formation of the C5′-pyridinium compounds appears to be the initial formation of a cyclic tertiary phosphate ester (partial structure VIII) by reaction of the terminal internucleotidic linkage with the free 5′-hydroxyl group. This conclusion is also consistent with the experiments reported earlier on the reaction of thymidylyl-(3′→5′)-3′-O-acetylthymidine with DCC.[17]

Table III. Analysis of Products Formed by Condensation of Pyridinium N,O2′,O5′-Triacetyladenosine 3′-Phosphate and 2′-O-Acetyluridine 3′-Phosphate[a]

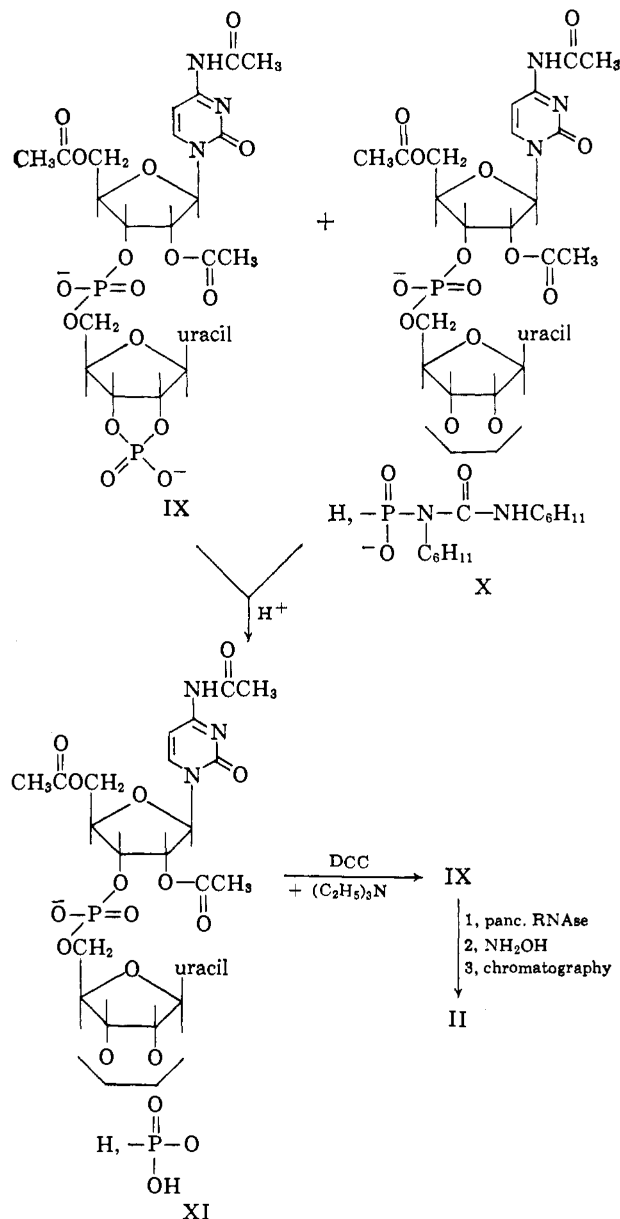
Compound	No. of peak from column	% yield[b] in expt. I	II	III	IV
5′-C-Pyridinium uridine 3′-phosphate	I	6.9	4.1		6.6
N-(Uridylyl)-N,N′-dicyclohexylurea	I		2.9		
N-(Adenylyl)-N,N′-dicyclohexylurea	I		0.5		
Uridine-3′,5′-cyclic phosphate	II	4.9	7.9	16.8	33.1
Adenosine-2′,3′-cyclic phosphate	III	6.9	9.3	13.4	
Uridine 3′-phosphate	IV	11.3	6.3	11.2	15.0
Uridine cyclic dinucleotide					7.4
Adenosine 3′-phosphate	V	41.8	26.7	28.5	
Uridylyl-(3′→5′)-uridine-2′,3′-cyclic phosphate	VI		6.3		
Adenylyl-(3′→5′)-uridine-2′,3′-cyclic phosphate	VII		5.3	3.2	
Uridylyl-(3′→5′)-uridine 3′-phosphate	VIII	2.9	2.2	4.4	10.3
Adenylyl-(3′→5′)-uridine 3′-phosphate	IX	14.8	16.6	12.6	5.5
Higher oligonucleotides	X–XI	10.6	12.0	9.8	22.0

[a] For method of column chromatography see text. Typical elution patterns were as in Figure 1. [b] % yields in experiments I–III are calculated in molar concentrations of the total nucleotidic material eluted. In experiment IV % yields are based on the total amount of uridine nucleotides eluted. Any hypochromicity in di- and higher oligonucleotides is ignored.



(19) The ammoniacal treatment given during the work-up would not cause decomposition of the phosphorylureas [C. A. Dekker and H. G. Khorana, *J. Am. Chem. Soc.*, 76, 3522 (1954)].

Approach (b). This approach involves the use of the nucleotide component, which will carry the 3′-phosphomonoester group in the desired dinucleotide, as the 2′,3′-cyclic phosphate (V). This cyclic diester was expected to serve as a "protected" derivative and as the source of the terminal 3′-phosphomonoester group by specific enzymatic ring opening at a later step. In the condensations studied, a mixture of uridine 2′- and 3′-phosphates was, in fact, used as the starting material, an initial reaction with the activating agent being expected to give the 2′,3′-cyclic phosphate (V) quantitatively.[20] Thus, stoichiometric amounts of pyridinium N,O2′,O5′-triacetylcytidine 3′-phosphate (III, R = N-acetylcytosine) and pyridinium uridine 2′(or 3′)-phosphate were brought into reaction with DCC in anhydrous pyridine. The initial dinucleotide derivatives were expected to be IX and X[19] and, therefore, a mild,



but adequate, acidic treatment was given to hydrolyze the phosphorylureas (X) to the protected dinucleotide bearing 2′(or 3′)-phosphomonoester groups (XI). The

(20) H. G. Khorana, G. M. Tener, R. S. Wright, and J. G. Moffatt, *ibid.*, 79, 430 (1957).

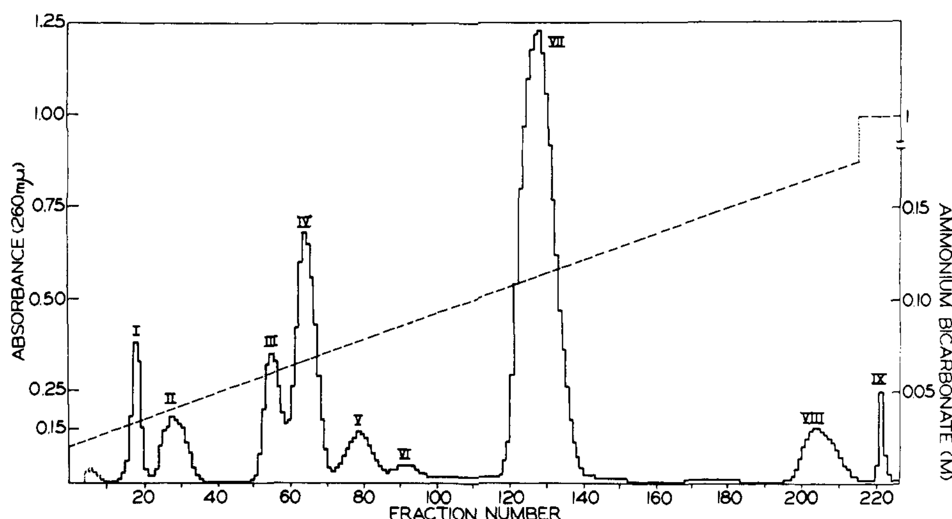


Figure 2. Chromatography of products formed by the condensation of N,$O^{2'}$,$O^{5'}$-triacetylcytidine 3'-phosphate and uridine (3')2'-phosphate on a DEAE–cellulose (carbonate) column. For details of the gradient see Experimental. The characterization of the peaks is given in Table IV.

latter was treated with DCC in the presence of triethylamine[21] to convert specifically the phosphomonoester groups to the 2',3'-cyclic phosphate (product IX).[22] The total product was now treated with the pancreatic ribonuclease to form the protected dinucleotide with the 3'-phosphomonoester group (XI, 3'-phosphate isomer). After *complete* removal of the enzyme, an ammoniacal treatment was given to remove the protecting groups and the products were separated by chromatography on a DEAE–cellulose (carbonate) column (Figure 2). As shown in Table IV, the yield of cytidylyl-(3'→5')-uridine 3'-phosphate was 53%. The product was characterized carefully for its complete susceptibility to the pancreatic ribonuclease. It should be added that a condensation experiment carried out using mesitylenesulfonyl chloride as the reagent gave only a trace (less than 1%) of the desired dinucleotide. Most of the uridine nucleotide had apparently undergone extensive self-condensation to form uridine oligonucleotides.[6]

Table IV. Column Chromatography of Products from the Condensation of N, $O^{2'}$,$O^{5'}$-Triacetylcytidine 3'-Phosphate and Uridine 2'(3')-Phosphate[a]

Peak	Fractions	O.D.$_{260}$	% of total nucleotidic material	Identification
I	14–21	12	5.8	Cytidine-2',3'-cyclic phosphate
II	23–33	13		Uridine-2',3'-cyclic phosphate + uridylyldicyclohexylurea
III	51–57	17	8.3	Cytidine 3'-phosphate
IV	59–69	41	14.7	Uridine 3'-phosphate
V	72–85	11	4.6	Cytidylyl-(3'→5')-uridine-2'(3')-phosphoryl-N,N'-dicyclohexylurea[b]
VI	87–95	3		Unidentified
VII	119–135	129	52.7	Cytidylyl-(3'→5')-uridine 3'-phosphate
VIII	197–214	15		Cytidylyl-(3'→5')-uridylyl-(2'→5')-uridine 3'-phosphate
IX	1 molar fraction	4		Higher oligonucleotides

[a] For details of chromatography, see text. The elution pattern is in Figure 2. [b] Degradation with the pancreatic ribonuclease gave cytidine 3'-phosphate and uridylyl-N,N'-dicyclohexylurea in the ratio 1:1.

By an analogous method, adenylyl-(3'→5')-uridine 3'-phosphate was prepared in 47% yield (Figure 3, Table V) from stoichiometric amounts of the two nucleotidic components. However, because of the resistance of the internucleotidic linkage to pancreatic ribonuclease, it was unnecessary to remove the enzyme from the reaction mixture during work-up. Inosinyl-(3'→5')-uridine 3'-phosphate was prepared in 44% yield (Table VI) by an analogous method except that it was neces-

Table V. Chromatographic Analysis of Products from the Condensation of N,$O^{2'}$,$O^{5'}$-Triacetyladenosine 3'-Phosphate and Uridine 2'(3')-Phosphate[a]

Peak	Fractions	O.D.$_{260}$	% of total nucleotidic material	Identification
I	18–35	17	5.3	Adenosine-2',3'-cyclic phosphate
II	65–74	17	7.5	Uridine 3'-phosphate
III	76–84	8		Uridylyl-(3'→5')-uridylyl-N,N'-dicyclohexylurea
IV	86–97	17	5.3	Adenosine 3'-phosphate
V	99–110	17	6.3	Adenylyl-(3'→5')-uridylyl-N,N'-dicyclohexylurea
VI	122–131	5		Unidentified
VII	135–149	20	8.8	Uridylyl-(2'→5')-uridine 3'-phosphate
VIII	162–183	126	46.8	Adenylyl-(3'→5')-uridine 3'-phosphate
IX	1 molar fraction	43		Higher oligonucleotides

[a] For details see text. The elution pattern is in Figure 3.

(21) M. Smith, J. G. Moffatt, and H. G. Khorana, *J. Am. Chem. Soc.*, **80**, 6204 (1958); H. G. Khorana, *ibid.*, **81**, 4657 (1959).
(22) It is important to ensure that the cyclization reaction proceeds *quantitatively*, because any residual dinucleotide bearing the 2'-phosphomonoester group would contaminate the dinucleotide bearing the 3'-phosphate group obtained on enzymatic unidirectional ring opening of the terminal cyclic phosphate.

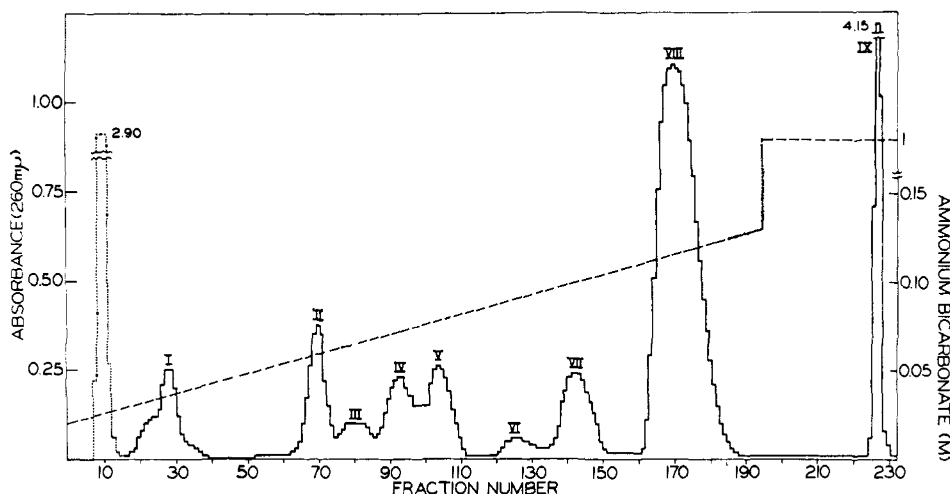


Figure 3. Chromatography of products formed by the condensation of N,O[2']',O[5']'-triacetyladenosine 3'-phosphate and uridine (3')2'-phosphate on a DEAE–cellulose (carbonate) column. For details of the gradient see Experimental. The characterization of the peaks is given in Table V.

sary to use dimethylformamide for the condensation reaction, pyridinium 2',5'-di-O-acetylinosine 3'-phosphate being insoluble in dry pyridine.

Table VI. Products from the Condensation of 2',5'-Di-O-acetylinosine 3'-Phosphate and Uridine 2'(3')-Phosphate[a]

Peak	Fractions	$O.D._{260}$	% of total nucleotidic material	Identification
I	15–22	2		Unidentified
II	37–42	4		Unidentified
III	44–52	29	12.4	Inosine-2',3'-cyclic phosphate
IV	55–68	68	20.6	Uridine 3'-phosphate
V	94–108	35	15.1	Inosine 3'-phosphate
VI	126–137	3		Unidentified
VII	152–168	2		Uridylyl-(2'→5')-uridine-3'-phosphate
VIII	173–203	123	44.4	Inosinyl-(3'→5')-uridine 3'-phosphate
IX	1 molar fraction	11		Higher oligonucleotides

[a] For details of column chromatography see text.

The approach just described is the most satisfactory of those investigated (Chart I). The starting materials used are readily accessible in quantity and the yields using stoichiometric amounts of the two components are satisfactory. The approach is general for the synthesis of any ribodinucleotide of type II, because the danger of internucleotide bond cleavage by the ribonuclease in question can be overcome by the presence of 2'-O-acetyl group adjacent to the internucleotide bond. Although, in the present work, only pancreatic ribonuclease was used for the unidirectional ring opening of the terminal pyrimidine 2',3'-cyclic phosphate, other known enzymes[8] (*e.g.*, Takadiastase ribonuclease-I, leaf ribonuclease) would permit analogous reactions when dinucleotides with terminal purine nucleoside 3'-phosphate groups are desired.

Experimental

General Methods and Materials. Reagent grade pyridine was distilled and dried over calcium hydride or molecular sieve beads (4XA)[23] 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, 1-propanol–concentrated ammonia–water (55:10:35, v./v.); solvent D, ethanol–1 *M* ammonium acetate, pH 3.8 (7:3, v./v.). The R_f values of different compounds are listed in Table I.

The nucleotidic compounds were detected by viewing under an ultraviolet lamp. The dimethoxytrityl-containing compounds were made visible on paper chromatograms by spraying the paper with 10% perchloric acid.

Paper electrophoresis was performed in a high voltage (4000 v.) apparatus in which the paper was immersed in a high-boiling petroleum fraction (Varsol). The buffers used were: phosphate buffer, pH 7.1, 0.03 *M*; ammonium formate buffer, pH 2.7, 0.05 *M*. The paper electrophoretic mobilities of different compounds are listed in Table II.

Enzymatic degradations of the synthetic products with pancreatic ribonuclease,[2a] *E. coli* alkaline phosphomonoesterase, *Lactobacillus acidophilus* R-26 phosphodiesterase, and spleen phosphodiesterase were carried out as described earlier.[2a,c,d]

Disodium uridine 3'-phosphate was prepared by fractional crystallization of the commercially available crystalline disodium uridine 2'(3')-phosphate from aqueous ethanol. Pure adenosine 3'-phosphoric acid was obtained commercially. Inosine 3'-phosphate was prepared by the method of Promel and Khorana.[24] Cytidine 3'-phosphate was prepared by the ribonuclease-catalyzed ring opening[1] of cytidine-2',3'-cyclic

(23) Linde Co., Division of Union Carbide.
(24) R. Promel and H. G. Khorana, unpublished work.

phosphate. N-(Uridylyl)-N,N′-dicyclohexylura and N-(adenylyl)-N,N′-dicyclohexylurea were prepared as previously described[19,21] for use as chromatographic markers. DEAE–cellulose Selectacel Type 40, Brown Co., Berlin, N. H., was converted to the carbonate form as described previously.[25]

The abbreviation O.D.$_{260}$ 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.

Pyridinium 5′-O-Dimethoxytrityluridine 3′-Phosphate. The procedure was similar to that described previously[2c] except that anhydrous pyridinium uridine 3′-phosphate was used in place of the disodium salt of the nucleotide. On the addition of dimethoxytrityl chloride a homogeneous solution resulted immediately, the reaction mixture being worked up after 50 min. at room temperature. After anion exchange chromatography, pure pyridinium 5′-O-dimethoxytrityluridine 3′-phosphate was obtained in 66% yield.

The ultraviolet absorption characteristics of this compound in aqueous solution at pH 7.5 were as follows: λ_{max} 233 mμ and a shoulder at 261 mμ.

Pyridinium 2′-O-Acetyluridine 3′-Phosphate. The procedure described previously for this compound started with 5′-O-monomethoxytrityluridine 3′-phosphate. The following procedure is similar except that the removal of the dimethoxytrityl group required shorter acidic treatment.

A mixture of pyridinium 5′-O-dimethoxytrityluridine 3′-phosphate (0.1 mmole) and tetraethylammonium acetate (1 mmole) was rendered anhydrous by repeated coevaporation from pyridine. During the last evaporation the suction under vacuum was continued until a viscous gum remained. Acetic anhydride (1 mmole) was added and the sealed reaction mixture was kept for 2 hr. at room temperature. The excess of acetic anhydride was then destroyed by the addition of methanol (2 ml.) and pyridine (2 ml.). After 15 min. at room temperature the solution was evaporated. The residue was taken up in 50% aqueous pyridine (10 ml.) and kept for 4 hr. After that time no acetyl–phosphate anhydride was present as determined by paper electrophoresis. The mixture was evaporated and the residual pyridine removed by coevaporation with water. The pyridine-free residue was dissolved in a mixture of ethanol (3 ml.) and water (1 ml.) and the solution shaken with Dowex-50 (H^+) resin (4 ml. suspended in 75% aqueous ethanol) for 15 min. at room temperature. The resin was removed by filtration and washed with pyridine. The combined filtrate and washings were evaporated and the residue taken up in dry pyridine (5 ml.). This solution was added dropwise to an excess of anhydrous ether (100 ml.) and the resulting precipitate was collected by centrifugation and washed with dry ether. The white powder (37 mg., 0.085 mmole) of pyridinium 2′-O-acetyluridine 3′-phosphate was stored in a desiccator over potassium hydroxide.

Pyridinium 2′,5′-Di-O-acetylinosine 3′-Phosphate. Pyridinium inosine 3′-phosphate (213 mg., 0.5 mmole) was dissolved in aqueous tetraethylammonium acetate (5 ml. of a 1 *M* solution). The mixture was rendered anhydrous by repeated coevaporaton with dry pyridine. During the last evaporation the suction under vacuum was continued until a viscous gum remained. Acetic anhydride (0.75 ml., 7.5 mmole) was added and the sealed reaction mixture was kept, with occasional shaking, for 2 hr. at 40°. Methanol (0.75 ml.) and pyridine (0.75 ml.) was then added and after 15 min. at room temperature the solution was evaporated. The residue was dissolved in 5% aqueous pyridine (10 ml.) and passed through a Dowex-50 (pyridinium) column (2 × 17 cm.). The column was washed with two bed volumes of 5% aqueous pyridine. The total eluate and washings were kept at room temperature for 2 hr. during which time the acetyl–phosphate anhydride had completely broken down (analysis by paper electrophoresis). The solution was evaporated to dryness and the residue was dissolved in anhydrous dimethylformamide (4 ml.). The solution was added dropwise to an excess of ethyl acetate (50 ml.) and the resulting precipitate was collected by centrifugation and washed with dry ether. The precipitate was dissolved in water (30 ml.), the solution was passed through a column of pyridinium Dowex-50 ion-exchange resin, and the eluate was lyophilized to remove last traces of pyridinium acetate. The yield of pyridinium 2′,5′-di-O-acetylinosine 3′-phosphate was 250 mg. (0.48 mmole). The powder was stored over potassium hydroxide pellets in a vacuum desiccator.

Methyl Adenosine 3′-Phosphate. The synthesis of this product was undertaken during the early experiments on the acetylation of pyridinium adenosine 3′-phosphate to N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate. The characterization of the methyl ester provided further confirmation of the purity of the acetyl derivative.

Methanol (1 ml.) was added to a dry pyridine solution (0.5 ml.) of pyridinium N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate (5 mg., 0.01 mmole) and the total solution was then treated with DCC (100 mg., 0.5 mmole). After 24 hr. at room temperature, pyridine (1 ml.) and water (1 ml.) were added and the mixture was extracted with cyclohexane (three 1-ml portions). The aqueous pyridine solution was kept at room temperature for 16 hr., then concentrated to 1 ml. and treated with an equal volume of concentrated ammonium hydroxide for 6.5 hr. at room temperature. The mixture was evaporated, the residue was taken up in a little water, and the insoluble dicyclohexylurea was removed by filtration. The filtrate was chromatographed on Whatman 40 paper using solvent A. Methyl adenosine 3′-phosphate was the sole product.

Adenylyl-(3′→5′)-uridine. An anhydrous pyridine solution (0.3 ml.) of pyridinium N,O$^{2'}$,O$^{5'}$-triacetyladenosine 3′-phosphate (5 mg., 0.01 mmole), and N,O$^{2'}$-,O$^{3'}$-tribenzoyluridine[1] (9 mg., 0.016 mmole) was treated with dry pyridinium Dowex-50 resin (50 mg.) and DCC (20 mg., about 0.1 mmole). The mixture was kept sealed for two days at room temperature, more DCC (30 mg., 0.15 mmole) was added, and the mixture was kept further for 4 days at room temperature. Water (0.3 ml.) was then added and the excess of DCC was extracted with cyclohexane (three 0.5-ml. portions). The aqueous pyridine solution was kept at room temperature overnight and then treated with an equal volume of concentrated ammonium hydroxide for 8 hr. at room temperature. Ammonia was then removed by evaporation, the residue was dissolved in water, and the insoluble dicyclohexylurea was removed by filtration.

(25) H. G. Khorana and W. J. Connors, *Biochem. Prepn.*, in press.

The total solution was chromatographed on Whatman 3 MM paper using solvent A. The yield of adenylyl-(3′→5′)-uridine was 91% as determined spectrophotometrically. Incubation with the *L. acidophilus* phosphodiesterase under the standard conditions showed complete degradation to adenosine 3′-phosphate and uridine (chromatography in solvent A).

The Synthesis of Adenylyl-(3′→5′)-uridine 3′-Phosphate by Condensation of Pyridinium N,O2′,O5′-Triacetyladenosine 3′-Phosphate and 2′-O-Acetyluridine 3′-Phosphate. Experiment I. To an anhydrous mixture of pyridinium N,O2′,O5′-triacetyladenosine 3′-phosphate (275 mg., 0.5 mmole), pyridinium 2′-O-acetyluridine 3′-phosphate (220 mg., 0.5 mmole), and pyridinium Dowex-50 ion-exchange resin (230 mg.) in dry pyridine (2 ml.) was added DCC (400 mg., 1.9 mmoles). The solution which was clear except for the ion-exchange resin was concentrated under vacuum to reduce the volume of the solvent to about one-half. The sealed mixture was then shaken for 4 days at room temperature in the dark. Subsequently, water (1 ml.) was added, the excess of DCC extracted with cyclohexane (three 1-ml. portions), and the insoluble dicyclohexylurea removed by filtration. The aqueous pyridine solution was kept for 1.5 hr. at room temperature and evaporated, and the residue was rendered anhydrous by repeated evaporation of added dry pyridine. The residue was dissolved in dry pyridine (10 ml.), the solution treated with acetic anhydride (3 ml.) and triethylamine (0.2 ml.), and the mixture kept in the dark for 3 days at room temperature. Methanol (3 ml.) was then added and, after 15 min. at room temperature, the excess of methanol and methyl acetate was removed by evaporation. The mixture was treated with an equal volume of concentrated ammonium hydroxide for 7 hr. at room temperature. Subsequently, ammonia was removed by evaporation and the residue was made up with water to 60 ml. and an aliquot of this stock solution was analyzed by column chromatography as described below.

Experiment II. To an anhydrous pyridine solution (2 ml.) of pyridinium N,O2′,O5′-triacetyladenosine 3′-phosphate (140 mg., 0.25 mmole) and pyridinium 2′-O-acetyluridine 3′-phosphate (110 mg., 0.25 mmole) was added dry pyridinium Dowex-50 ion-exchange resin (150 mg.) followed by DCC (250 mg.). Most of the solvent (0.3 ml. left as determined by difference in weight) was removed under vacuum when a gum separated. The total mixture was shaken in the dark for 5.5 days at room temperature and then worked up as described above for experiment I.

Experiment III. The amounts of the two nucleotidic components were the same as in experiment II. The mixture of the two reactants and pyridinium Dowex-50 resin (450 mg.) was rendered anhydrous by repeated evaporation under vacuum of added pyridine. The residue was taken up in 2 ml. of freshly distilled dimethylformamide and DCC (250 mg., 1.23 mmole) was added. The solution, which was clear except for the insoluble resin, was evaporated under vacuum to remove most of the solvent (0.27 ml. left as determined by difference in weight). The residual mixture, which deposited a gum, was kept sealed in the dark for 20 days at room temperature and then worked up as described under experiment I. An aliquot was analyzed by column chromatography as described below.

Experiment IV. A mixture of pyridinium N,O2′,O5′-triacetyladenosine 3′-phosphate (55 mg., 0.1 mmole) and dry pyridinium Dowex-50 resin (150 mg.) was rendered anhydrous by several evaporations of added dry pyridine. The residue was taken up in dry pyridine (2 ml.) and DCC (180 mg., 0.87 mmole) was added. To the resulting clear solution was then added an anhydrous pyridine solution (1.5 ml.) of pyridinium 2′-O-acetyluridine 3′-phosphate (220 mg., 0.5 mmole). Most of the solvent was removed immediately under vacuum (only 0.5 ml. of the solvent left). A solid was deposited on the walls of the flask which was kept sealed in the dark for 5.5 days at room temperature. Water (0.5 ml.) and pyridine (1.5 ml.) were added and the subsequent work-up was as in experiment I above.

Experiment V, Using Mesitylenesulfonyl Chloride. To an anhydrous pyridine solution (1 ml.) of pyridinium N,O2′,O5′-triacetyladenosine 3′-phosphate (140 mg., 0.25 mmole) was added mesitylenesulfonyl chloride (55 mg., 0.25 mmole) in a drybox (relative humidity less than 10%). After 30 min. an anhydrous pyridine solution (1 ml.) of pyridinium 2′-O-acetyluridine 3′-phosphate (110 mg., 0.25 mmole) was added from a pressure equalizing dropping funnel. The sealed reaction mixture was kept for 11 hr. at room temperature. Water (0.5 ml.) was added to destroy the excess of the sulfonyl chloride and the aqueous pyridine solution was kept for 10 hr. at room temperature. The mixture was treated with an equal volume of concentrated ammonium hydroxide for 4 hr. at room temperature and the ammonia was then removed by evaporation. Analysis of the mixture showed that only a small amount of the desired dinucleotide was formed (less than 5%), the main products being adenosine 3′- and uridine 3′-phosphates and the corresponding cyclic phosphates.

Analysis and Identification of Products of Experiments I–IV. An aliquot of about 500 $O.D._{260}$ of each of the stock solutions was chromatographed on a DEAE–cellulose (carbonate) column (2 × 22 cm.). The column was washed with two bed volumes of water and the elution was carried out with a linear gradient of ammonium bicarbonate. The mixing vessel contained 1 l. of 0.02 *M* ammonium bicarbonate and the reservoir an equal volume of 0.25 *M* ammonium bicarbonate. Fractions of 7.5 ml. were collected at 5-min. intervals. Figure 1 shows a typical elution pattern. The distribution of the ultraviolet absorbing material in the different peaks and the identification of the different peaks is given in Table III. The various pooled fractions were repeatedly lyophilized to remove ammonium bicarbonate.

For identification, the components of each peak were examined by paper chromatography, paper electrophoresis, ultraviolet absorption spectrum, and enzymatic degradation. The R_f values and electrophoretic mobilities are summarized in Tables I and II. Adenylyl-(3′→5′)-uridine 3′-phosphate was degraded with bacterial phosphomonoesterase to adenylyl-(3′→5′)-uridine, which was identical on paper chromatography and paper electrophoresis with the synthetic sample described separately here. Also, the dinucleotide was completely degraded by the *L. acidophilus* phosphodiesterase to adenosine 3′-phosphate and uridine 3′-phosphate.

Cytidylyl-(3′→5′)-uridine 3′-Phosphate. A mixture of pyridinium N,O2′,O5′-triacetylcytidine 3′-phosphate[1]

(54 mg., 0.1 mmole), pyridinium uridine 3′(2′)-phosphate (from 33 mg., 0.1 mole, of free acid), and dry Dowex-50 (pyridinium) resin (150 mg.) was rendered anhydrous by several evaporations of added dry pyridine. The residual gum was taken up in dry pyridine (2 ml.) and the solution was treated with DCC (300 mg., 1.46 mmole). After thorough mixing some pyridine (about 0.5 ml.) was removed under vacuum. The resulting clear solution containing the resin suspension was shaken for 5 days at room temperature in the dark. Water (2 ml.) was then added and the excess of DCC extracted with pentane (three 3-ml. portions). The insoluble dicyclohexylurea was removed by filtration, the filtrate was made up with 50% aqueous pyridine to 5 ml., and this stock solution was kept frozen.

One-fourth of the above solution was completely freed from pyridine by coevaporation with water. The residue was dissolved in 50% aqueous ethanol (6 ml.) and shaken with Dowex-50 (H^+) resin (3.0 g.)[26] for 2.5 hr. at room temperature. The resin was then removed by filtration and washed with pyridine. The total filtrate was evaporated and the residue rendered anhydrous by evaporation of added dry pyridine. The residual gum was taken up in dry pyridine (2 ml.), triethylamine (0.05 ml.) and then DCC (50 mg.) were added, and the mixture was kept for 24 hr. at room temperature. The reaction was terminated by adding water (2 ml.) and the excess of DCC was extracted with pentane (three 2-ml. portions). The aqueous pyridine solution was completely freed from pyridine by coevaporation with water. The residue was suspended in imidazole–hydrochloric acid buffer (1.5 ml. of 0.1 *M*, pH 7.0) and incubated with pancreatic ribonuclease (750 μg.) for 3 hr. at 37°. The enzyme was removed by passing the solution through a column (20 × 1 cm.) of pyridinium Dowex-50 ion-exchange resin (2% cross-linked, 200–400 mesh) and the nucleotidic material was eluted with two bed volumes of 1% aqueous pyridine (adjusted to pH 6.5). The eluate was concentrated to 3 ml. and treated with an equal volume of concentrated ammonium hydroxide for 30 min. at room temperature. Subsequently, the ammonia was removed by evaporation, water (20 ml.) was added to the residue, and the resulting solution placed on a DEAE–cellulose (carbonate) column (22 × 2 cm.). Elution was carried out with a linear salt gradient, the mixing vessel containing 1.5 l. of 0.02 *M* ammonium bicarbonate and the reservoir an equal volume of 0.25 *M* ammonium bicarbonate. Fractions of 9.5 ml. were collected at 5-min. intervals. The elution pattern is shown in Figure 2 and the distribution of the nucleotidic materials in the different peaks and the identification of the peaks are shown in Table IV. The yield of the desired dinucleotide recovered by lyophilization of fractions 120–135 (peak VII of Table IV) was 52.7%. The product was homogeneous by paper chromatography and paper electrophoresis. Degradation with the pancreatic ribonuclease at 10 $O.D._{260}$ level followed by chromatography of the entire mixture showed complete degradation to cytidine 3′- and uridine 3′-phosphates.

Attempted Synthesis of Cytidylyl-(3′→5′)-uridine 3′-Phosphate Using Mesitylenesulfonyl Chloride. An anhydrous pyridine solution (0.5 ml.) of mesitylenesulfonyl chloride (90 mg., 0.4 mmole) was added to an anhydrous solution (in 1.5 ml. pyridine) of pyridinium $N,O^{2'},O^{5'}$-triacetylcytidine 3′-phosphate (54 mg., 0.1 mmole) and uridine 2′(or 3′)-phosphate (48 mg. of free acid, 0.15 mmole). The sealed mixture was kept for 4 hr. at room temperature and then treated with 1 ml. of water. The aqueous pyridine solution was evaporated and the residue dried by evaporation of added pyridine. The gum was then dissolved in pyridine (2 ml.) and the solution was treated with triethylamine (0.1 ml.) and DCC (103 mg., 0.5 mmole). After 30 min. at room temperature, the reaction was terminated by the addition of water (2 ml.) and the excess of DCC extracted with pentane (three 3-ml. portions). After removal of the insoluble dicyclohexylurea, the subsequent steps (treatment with the pancreatic ribonuclease and with ammonia) were as described in the preceding experiment. Analysis of the products by column chromatography on DEAE–cellulose, by paper chromatography and paper electrophoresis at pH 2.7, showed little (less than 1%) formation of the mixed oligonucleotides but extensive formation of the uridine oligonucleotides.

Adenylyl-(3′→5′)-uridine 3′-Phosphate. A. Using Stoichiometric Amounts of the Two Components. An anhydrous pyridine (2 ml.) mixture of pyridinium $N,O^{2'}$, $O^{5'}$-triacetyladenosine 3′-phosphate (55 mg., 0.1 mmole), uridine 2′(or 3′)-phosphate (0.1 mmole), and dry pyridinium Dowex-50 ion-exchange resin (150 mg.) was treated with DCC (300 mg., 1.46 mmole) and after thorough mixing about 0.5 ml. of the solvent was removed under vacuum. The reaction mixture was shaken for 5 days at room temperature in the dark. Water (2 ml.) was then added, the excess of DCC extracted with pentane (three 3-ml. portions), and the insoluble dicyclohexylurea removed by filtration. The filtrate was made up with 50% aqueous pyridine to 5 ml. and this stock solution was kept frozen.

One-fourth of the above stock solution was completely freed from pyridine by coevaporation with water. The white residue was dissolved in 50% aqueous ethanol (6 ml.) and shaken with Dowex-50 (H^+) resin (3.0 g.)[26] for 2.5 hr. at room temperature. Paper chromatography at this stage showed almost complete breakdown of the fast-traveling phosphorylureas. The resin was removed by filtration and washed with pyridine. The total filtrate was evaporated and the residue was rendered anhydrous by coevaporation from dry pyridine. The gum was taken up in dry pyridine (2 ml.) and the solution treated with triethylamine (0.05 ml.) and DCC (50 mg., 0.25 mmole). After 24 hr. at room temperature, the reaction was terminated by adding water (2 ml.) and the excess of DCC was extracted with pentane (three 2-ml. portions). The aqueous pyridine solution was evaporated and the residual pyridine was completely removed by coevaporation with water. The residue was taken up in Tris buffer (1.5 ml. of 0.3 *M*, pH 7.5) and incubated with pancreatic ribonuclease (750 μg.) for 3 hr. at 37°. Then, the mixture was treated with an equal volume of concentrated ammonium hydroxide for 4.5 hr. at room temperature. Subsequently, the ammonia was removed by evaporation, water (20 ml.) was added, and the resulting solution was placed on a DEAE–cellulose (carbonate) column (25 × 2 cm.).

(26) The resin was prepared fresh in the acid form and washed thoroughly on a Buchner funnel with 50% aqueous ethanol and sucked dry for about 0.5 hr.

Elution was carried out with a linear salt gradient, the mixing vessel containing 1.5 l. of 0.02 M ammonium bicarbonate and the reservoir an equal volume of 0.25 M ammonium bicarbonate. Fractions of 8.5 ml. were collected at 5-min. intervals. The elution pattern is shown in Figure 3, and the distribution of the nucleotidic material in the different peaks and their identification are in Table V. Fractions 163–180 were pooled and lyophilized. The desired product (46.8%) was homogeneous (R_f's in Table I and II) and was completely degraded (10 $O.D._{260}$ used for chromatographic spot) to adenosine 3′-phosphate and uridine 3′-phosphate by the *L. acidophilus* phosphodiesterase.

B. Using Excess of One Compound. The above experiment was repeated exactly except that the amount of pyridinium $N,O^{2'},O^{5'}$-triacetyladenosine 3′-phosphate used was 0.2 mmole in place of 0.1 mmole. The yield of adenylyl-(3′→5′)-uridine 3′-phosphate was 48% as based on the uridylic acid component used (0.1 mmole).

Inosinyl-(3′→5′)-Uridine 3′-Phosphate. An anhydrous mixture of pyridinium 2′,5′-di-O-acetylinosine 3′-phosphate (51 mg., 0.1 mmole), uridine 3′(2′)-phosphate (33 mg., 0.1 mmole of free acid), and dry pyridinium Dowex-50 resin (1 g.) in freshly distilled dry dimethylformamide (2 ml.) was treated with DCC (300 mg., 1.46 mmole). After thorough mixing, some (about 0.5 ml.) of the solvent was removed under vacuum and the resulting mixture with all the reactants in clear solution was shaken for 5 days at room temperature in the dark. Water (2 ml.) was then added, the excess of DCC extracted with pentane (three 3-ml. portions), and the insoluble dicyclohexylurea removed by filtration. The filtrate was made up with 50% aqueous pyridine to 10 ml. and this stock solution kept frozen.

One-fourth of the above stock solution was taken through the steps exactly as described above for a similar portion in the preparation of adenylyl-(3′→5′)-uridine 3′-phosphate. However, the duration of the ammoniacal treatment was 0.5 hr. in place of 4.5 hr. The results of anion-exchange chromatography are given in Table VI. Fractions 173–203 (peak VIII of Table VI) were lyophilized and gave pure inosinyl-(3′→5′)-uridine 3′-phosphate. Incubation of 10 $O.D._{260}$ of this product with the spleen phosphodiesterase followed by paper chromatography of the entire mixture in solvent A showed complete degradation.

The Reaction of Pyridinium 2′-O-Acetyluridine-3′,5′-cyclic Phosphate with DCC. A solution of triethylammonium uridine-3′,5′-cyclic phosphate[18] (20 mg., 0.05 mmole) in water (1 ml.) was passed through a small Dowex-50 (H^+) column. The eluate was evaporated and the residue was rendered anhydrous by repeated coevaporation with dry pyridine. The residual gum was dissolved in dry pyridine (1 ml.) and acetic anhydride (0.1 ml., 1 mmole) added. After 2 hr. at room temperature the excess of acetic anhydride was destroyed with methanol (0.2 ml.). The mixture was then passed through a small Dowex-50 (H^+) column and the acidic eluate quickly frozen and lyophilized. The resulting white powder of 2′-O-acetyluridine-3′,5′-cyclic phosphoric acid was taken up in pyridine, dry Dowex-50 (pyridinium) resin (150 mg.) added, and the solution evaporated repeatedly (addition of pyridine) to remove any moisture. DCC (40 mg., 0.2 mmole) was added and the sealed reaction mixture was kept at room temperature. Aliquots of 0.01 ml. were taken out at different intervals, kept in 50% aqueous pyridine for 1 hr., and analyzed by paper chromatography (a) in solvent B, and (b) in solvent A after an ammoniacal treatment to remove the acetyl group. In addition to the unchanged starting material, the formation of a new ultraviolet-absorbing spot (R_f's in Tables I and II) (structure VII) was noted. The properties of this material were as follows: (1) It had zero mobility on paper electrophoresis at neutral pH values. (2) Its mobility on paper chromatograms in solvent A was high (Table I). (3) Treatment of an aqueous solution with sodium borohydride caused a change in the ultraviolet absorption spectrum, the ratio of absorption at 280 mμ/260 mμ changing from 0.21 to 0.36. The latter ratio corresponds to that of uridine. (4) Treatment with 0.01 N hydrochloric acid[19] gave 5′-C-pyridinium uridine 2′(3′)-phosphate, which after dephosphorylation with the bacterial alkaline phosphatase gave 5′-C-pyridinium-uridine (cationic in nature as evidenced by movement toward cathode on paper electrophoresis). This product also gave a positive *cis*-diol reaction (periodate–benzidine test).[27]

(27) M. Viscontini, D. Hoch, and P. Karrer, *Helv. Chim. Acta*, **38**, 642 (1955).